

A novel *chk1*-dependent G1/M checkpoint in fission yeast

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Summary

Fission yeast cells with a temperature-sensitive Orp1 protein, a component of the origin recognition complex, cannot perform DNA replication at the restrictive temperature. Seventy percent of *orp1-4* cells arrest with a 1C DNA content, whereas 30% proceed to mitosis ('cut'). The arrest depends upon the checkpoint Rad proteins and, surprisingly, the Chk1 protein, which is thought to act only from late S phase. The arrested cells maintain a 1C DNA content, as judged by flow cytometry, and the early origin *ars3001* has not been initiated, as judged by 2D gel analysis.

We show that in G1-arrested *orp1-4* cells, Wee1 phosphorylates and inactivates Cdc2. Activation of Chk1 occurs earlier than Cdc2 phosphorylation, indicating a novel role for Chk1, namely to induce and/or maintain Cdc2 phosphorylation upon checkpoint activation in G1. We also show that commitment to cutting occurs already in early G1 phase.

Key words: Checkpoint, *S. pombe*, *orp1*, DNA replication, Mitosis

Introduction

The maintenance of genomic integrity is important for the survival of eukaryotic cells. Checkpoint mechanisms prevent cell cycle transitions until previous events have been completed or damaged DNA has been repaired. Checkpoint pathways and proteins are evolutionarily conserved from yeast to man, underlining their importance in maintaining genomic integrity. In fission yeast several checkpoint pathways monitor the status of the DNA and arrest the cell cycle in response to DNA damage or inhibition of DNA replication. The so-called checkpoint Rad proteins, Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1, are required for damage sensing and processing and are involved both in the DNA damage checkpoint pathway and in responding to replication arrest. There are two known effector kinases downstream of the checkpoint Rad proteins, Chk1 and Cds1. Chk1 is phosphorylated in response to DNA damage induced in late S or G2 in a *rad3*-dependent manner (Walworth et al., 1993; Walworth and Bernards, 1996; Martinho et al., 1998; Tanaka and Russell, 2001). Phosphorylation of Chk1 correlates with Chk1-dependent checkpoint activation, but the exact significance of the phosphorylation for either Chk1 kinase activity or for checkpoint regulation is as yet unclear. Cds1 is activated as a part of the intra-S checkpoint when the DNA is damaged during S phase or when DNA replication is inhibited by hydroxyurea or by a DNA polymerase defect (Murakami and Okayama, 1995; Lindsay et al., 1998). Activation of either kinase leads to inhibition of Cdc2 activity by maintaining the inhibitory phosphorylation of Tyr15 (O'Connell et al., 1997; Rhind et al., 1997; Rhind and Russell, 1998; Wan and Walworth, 2001; Liu et al., 2002). Tyr15 of Cdc2 is phosphorylated by Wee1 and Mik1, and dephosphorylated in preparation for mitosis by Cdc25 (reviewed by Berry and Gould, 1996). In the rest of the paper

we shall refer to Tyr15 phosphorylation when discussing phosphorylation of Cdc2p.

Much less is known about checkpoints in the G1 phase of the cell cycle. This is mostly because G1 in fission yeast is very short under standard laboratory growth conditions, rendering the investigation of the existence and mechanism of such a checkpoint(s) difficult. However, several cell cycle mutants arrest in the G1 phase of the cell cycle (i.e. before any DNA replication has occurred and without proceeding to mitosis). This arrest might be due to checkpoint activation, thereby providing a means to investigate G1 checkpoint(s) in fission yeast. We have used the *orp1-4* replication initiation mutant, which at the restrictive temperature either enters mitosis in the absence of DNA replication ('cut' phenotype) or arrests with a 1C DNA content without entering mitosis ('arrest' phenotype) (Grallert and Nurse, 1996). When the *orp1-4* mutant is incubated at the restrictive temperature, about 70% of the cells arrest, whereas 30% cut (Grallert and Nurse, 1996). In the complete absence of *orp1*⁺ function, in the *orp1Δ* mutant, all of the cells cut.

The *orp1* gene encodes one of the components of the fission yeast origin recognition complex (ORC). The ORC binds the replication origins throughout the cell cycle and serves as a landing pad for other proteins essential for the initiation of replication (reviewed by Leatherwood, 1998). One of these is the Cdc18 protein which, together with Cdt1 (Nishitani et al., 2000), is required for loading of the MCM proteins (minichromosome maintenance) onto the origins, thus forming the pre-replication complex (preRC).

In this work we have studied both the arrest and the cut phenotypes of the *orp1-4* mutant. We show that *orp1-4* cells arrest in G1 owing to a checkpoint that requires the checkpoint Rad proteins and Chk1, but not Cds1. Cdc2 is phosphorylated by Wee1 in G1 after activation of Chk1 and the arrest of *orp1-*

4 cells depends on this phosphorylation. We also analyse the cutting and show that commitment to cutting occurs before S phase.

Materials and Methods

Fission yeast strains and methods

All our strains are congenic with the *Schizosaccharomyces pombe* 972h⁻ strain. Strains used in this study are listed in Table 1. All basic growth and media conditions were as described (Moreno et al., 1991). The *rum1Δ* and *ste9Δ* strains have been described (Moreno et al., 1994; Yamaguchi et al., 1997; Kitamura et al., 1998; Edwards et al., 1999; Wright et al., 1998). Since they are sterile, the *orp1-4 rum1Δ* and *orp1-4 ste9Δ* strains were made by protoplast fusion (Sipiczki and Ferenczy, 1977). Experiments in liquid culture were performed using EMM medium, and exponential cultures were grown to a density of 2-4×10⁶ cells/ml at the start of each experiment.

2D gel analysis

Purified genomic DNA was digested with *Hind*III and *Kpn*I and analysed as described before (Muller et al., 2000). After blotting, the membrane was probed with a ³²P-labelled fragment within the 3kb *Hind*III-*Kpn*I fragment of the rDNA repeat of *S. pombe* (Sanchez et al., 1998).

Protein extracts and western blots

Protein extracts for western blotting were made by TCA extraction, as described previously (Caspari et al., 2000). For western blot analysis the following antibodies were used: anti-phosphotyrosine Cdc2 (Sigma C0228) at a dilution of 1:400; anti-pSTAIR against Cdc2 (Santa Cruz sc-53) at a dilution of 1:2000; anti-HA (Babco) at a dilution of 1:1000; anti-myc (PharMingen 9E10) at a dilution of 1:1000; and anti-α-tubulin (Sigma T5168) at a dilution of 1:20,000. The secondary antibodies were either HRP or AP conjugates, used at a dilution of 1/5000. Detection was performed using the enhanced chemiluminescence procedure (NEN ECL kit). Cdc2 and phosphorylated Cdc2 was measured using ECF detection (Amersham) and quantified with a phosphoimager and the Image Quant software. Chk1 and Wee1 phosphorylation was followed by Super Signal (Pierce) detection.

Cds1 kinase activity

Cds1 was immunoprecipitated and Cds1 kinase activity was measured using MBP as substrate as described (Lindsay et al., 1998). Quantification of the kinase activity was performed using a phosphoimager and the ImageQuant software.

Flow cytometry and microscopy

About 10⁷ cells were spun down for each sample and fixed in 70% ethanol before storing at 4°C. Samples were processed for flow cytometry as described (Sazer and Sherwood, 1990), stained with Sytox Green (Molecular Probes S-7020, <http://pingu.salk.edu/fcm/protocols/ycc.html>) and analysed with a Becton-Dickinson FACSCalibur machine. 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear staining, and Calcofluor was used for cell wall and septum staining as described previously (Moreno et al., 1991).

Synchronous cells

Partially synchronous cells were selected from exponentially growing cultures by lactose gradient centrifugation on 10-30% gradients, as described (Carr et al., 1995).

Table 1. Strains used in this study

Strain	Source	Cutting at 36°C*
<i>orp1-4</i>	Own isolate	orp1
<i>orp1-4 cdc18-K46</i>	Own isolate	Synthetic
<i>cdc10-129</i>	P. Nurse	No
<i>orp1-4 cdc10-129</i>	This study	orp1
<i>rad3</i>	P. Nurse	No
<i>orp1-4 rad3</i>	This study	Synthetic
<i>rad1</i>	P. Nurse	No
<i>orp1-4 rad1</i>	This study	Synthetic
<i>rad17</i>	P. Nurse	No
<i>orp1-4 rad17</i>	This study	Synthetic
<i>rad26::ura4⁺ ura4-D18</i>	T. Wang	No
<i>orp1-4 rad26::ura4⁺ ura4-D18</i>	This study	Synthetic
<i>rad9-HA Kan^R ura4-D18 leu1-32 ade6-704</i>	T. Caspari	No
<i>orp1-4 rad9-HA Kan^R</i>	This study	orp1
<i>cdc17-K42</i>	P. Nurse	No
<i>cdc17-K42 rad9-HA Kan^R</i>	This study	No
<i>cds1::ura4⁺ ura4-D18</i>	H. Okayama	No
<i>orp1-4 cds1::ura4⁺ ura4-D18</i>	This study	orp1
<i>chk1::ura4⁺ ura4-D18</i>	P. Nurse	No
<i>orp1-4 chk1::ura4⁺ ura4-D18</i>	This study	Synthetic
<i>chk1::HA ade6-216 leu1-32</i>	N. Walworth	No
<i>orp1-4 chk1-HA ade6</i>	This study	orp1
<i>orp1-4 cdc18-K46 chk1-HA ade6</i>	This study	Synthetic
<i>mik1::ura4⁺ ura4-D18</i>	P. Nurse	No
<i>orp1-4 mik1::ura4⁺ ura4-D18</i>	This study	orp1
<i>wee1-50</i>	P. Nurse	No
<i>orp1-4 wee1-50</i>	This study	Synthetic
<i>mik1-myc-ade6⁺ ura4-D18 leu1-32 ade6-704</i>	A. Carr	No
<i>orp1-4 mik1-myc-ade6⁺ ade6-704 ura4-D18</i>	This study	orp1
<i>orp1::ura4⁺/orp1-4 ura4-D18/ura4-D18 h+/h-</i>	Own isolate	Synthetic
<i>rum1::ura4⁺ ura4-D18</i>	S. Moreno	No
<i>orp1-4 rum1::ura4⁺ ura4-D18</i>	This study	orp1
<i>ste9::ura4⁺ ura4-D18</i>	S. Moreno	No
<i>orp1-4 ste9::ura4⁺ ura4-D18</i>	This study	orp1
<i>wee1-HA leu1-32 ura4-D18</i>	M. O'Connell	No
<i>orp1-4 wee1-HA leu1-32 ura4-D18</i>	This study	orp1

*orp1, the indicated strain cuts to the same extent as *orp1-4*; synthetic, the cutting phenotype of the indicated double mutant is enhanced when compared with that of *orp1-4*.

Results

orp1-4 cells have not initiated DNA replication at the arrest point

Several pieces of evidence suggest that *orp1-4* cells arrest in G1 (Grallert and Nurse, 1996). However, it could not be excluded that they initiate DNA replication and arrest at an early stage, since flow cytometry analysis cannot distinguish cells in G1 from early S phase cells that have synthesised a small fraction of their DNA. In order to investigate where the cells arrest, we analysed replication initiation at the early replication origin *ars3001*, which is located in the rDNA repeats. Together with the K-repeat origin this is the first known origin to be replicated as the cells enter S phase (Kim and Huberman, 2001). In the positive control, which is *orp1-4* cells at the permissive temperature, the *ars3001* origin was both used as an origin, evidenced by a bubble-arc, and replicated passively, evidenced by a Y-arc (Fig. 1, top panel). These signals are due to a small percentage (less than 10%) of cells that are in S phase at any one time. On continued incubation at the restrictive temperature the bubble arc disappears and the Y-arc becomes much weaker and is hardly discernible after 3 hours. If all the cells had accumulated in

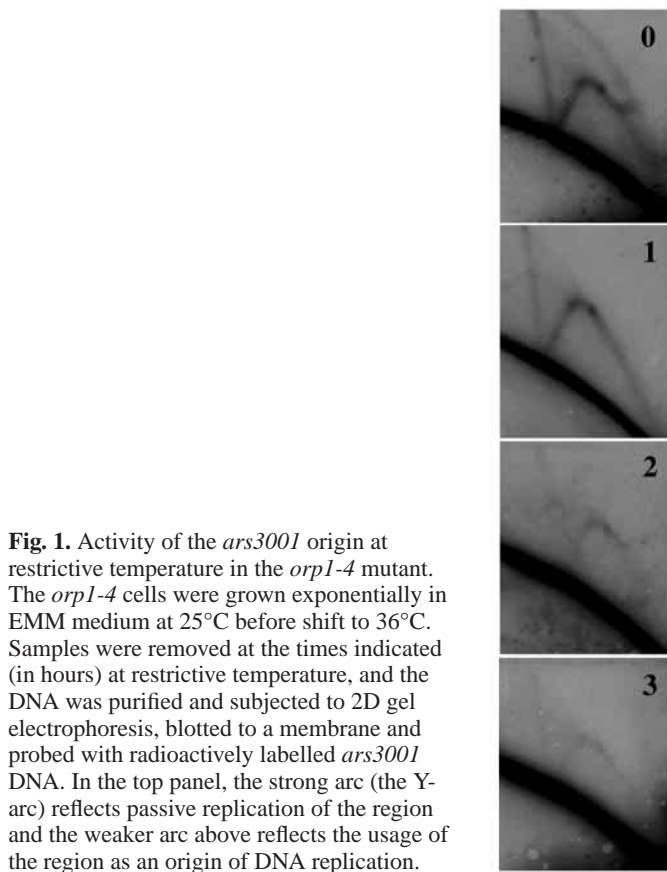


Fig. 1. Activity of the *ars3001* origin at restrictive temperature in the *orp1-4* mutant. The *orp1-4* cells were grown exponentially in EMM medium at 25°C before shift to 36°C. Samples were removed at the times indicated (in hours) at restrictive temperature, and the DNA was purified and subjected to 2D gel electrophoresis, blotted to a membrane and probed with radioactively labelled *ars3001* DNA. In the top panel, the strong arc (the Y-arc) reflects passive replication of the region and the weaker arc above reflects the usage of the region as an origin of DNA replication.

early S phase, the replication signals would be expected to increase rather than decrease. The strong decrease is evidence that *orp1-4* cells have not initiated DNA replication at the arrest point.

The G1 arrest of *orp1-4* cells is checkpoint dependent

About 70% of the *orp1-4* mutant cells arrest prior to DNA replication at the restrictive temperature. We combined *orp1-4* with a number of checkpoint mutations. Mutation of any of several checkpoint *rad* genes in *orp1-4* abolished the arrest (Fig. 2A), demonstrating that it is checkpoint dependent. Consistently, phosphorylation of Rad9, a marker of checkpoint activation (Caspari et al., 2000), was clearly induced in *orp1-4* cells (Fig. 2B). As a positive control, the extent of Rad9 phosphorylation was the same as that in a *cdc17^{ts}* mutant at the restrictive temperature (Fig. 2B), conditions that are known to require checkpoint activation for arrest. We conclude that arrest of the *orp1-4* mutant is checkpoint dependent.

The *orp1-4 rad* double mutants appear to enter mitosis (cut) earlier than the *orp1-4* single mutant (Fig. 2A). To verify that the double mutants cut earlier and not only more extensively, we selected synchronous *orp1-4*, *orp1-4 rad26* and *orp1-4 cdc18-K46* cells after lactose gradient centrifugation and followed cutting at the restrictive temperature. Both double mutants were found to cut significantly earlier than the single mutant (Fig. 2C). In these experiments, the level of cutting of the *orp1-4* mutant cells was less than shown above (Fig. 2A), because in the present experiment the cells were synchronized

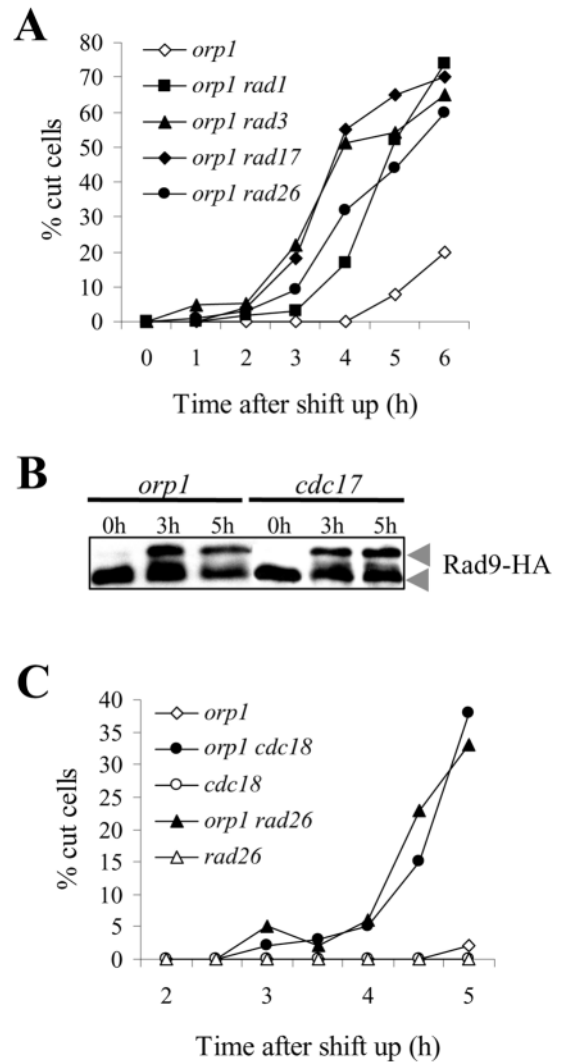


Fig. 2. The G1 arrest of *orp1-4* cells is checkpoint dependent. (A) Exponentially growing *orp1-4* and the indicated *orp1-4* checkpoint double mutant cells were shifted to the restrictive temperature of 36°C and cutting was monitored by DAPI staining. (B) *orp1-4 rad9-HA* and *cdc17 rad9-HA* cells were shifted to the restrictive temperature for the indicated times and the phosphorylation of Rad9-HA was monitored by SDS-PAGE and western blotting. (C) *orp1-4*, *cdc18-K46 orp1-4* and *orp1-4 rad26::ura4⁺* cells were synchronised by lactose gradient centrifugation in G2, and then shifted to the restrictive temperature. Septation index was determined by Calcofluor staining; cutting was measured by DAPI staining.

in early G2 phase and therefore reached the arrest point later than the asynchronous cells described in Fig. 2A.

Chk1, but not Cds1, is involved in the checkpoint response

In the DNA replication or the G2/M damage pathways the targets of the checkpoint Rad proteins are the effector kinases Chk1 and Cds1 (Walworth et al., 1993; Murakami and Okayama, 1995). Deletion of *cds1* did not affect entry into aberrant mitosis (cutting) in *orp1-4* (Fig. 3A), showing that

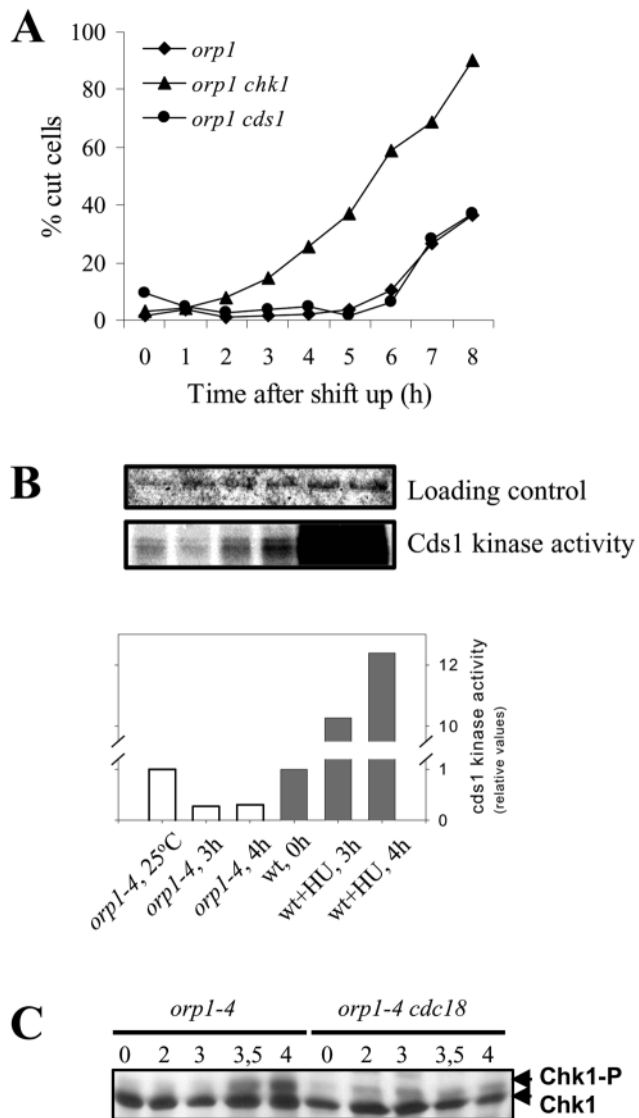


Fig. 3. Chk1, but not Cds1, is required to prevent cutting in *orp1-4*. (A) Exponentially growing *orp1-4*, *orp1-4 chk1Δ* and *orp1-4 cds1Δ* cells were shifted to the restrictive temperature for *orp1-4*. Cutting was monitored by DAPI staining. (B) *orp1-4* cells were shifted to the restrictive temperature for the indicated times and protein extracts were prepared. Cds1 was immunoprecipitated and Cds1 kinase activity was measured using MBP as substrate. Hydroxyurea-treated wild-type cells serve as positive controls. The graph shows quantification of the kinase activity corrected for loading. The immunoprecipitated IgG was used as a loading control. (C) *orp1-4 chk1-HA* and *orp1-4 cdc18-K46 chk1-HA* cells were shifted to the restrictive temperature for the indicated times. Total protein was prepared and Chk1 phosphorylation was investigated by SDS-PAGE and western blot analysis.

Cds1 is not required for the checkpoint. Consistently, Cds1 kinase activity was not induced in *orp1-4* upon temperature shift (Fig. 3B). This finding further supports the above conclusion that *orp1-4* cells do not arrest in S phase, since Cds1 is known to be required for checkpoints activated during S phase (Murakami and Nurse, 2001; Lindsay et al., 1998; Murakami and Okayama, 1995). Surprisingly, deletion of

chk1 abolished the checkpoint that ensures arrest of 70% of the *orp1-4* cells (Fig. 3A). When *orp1-4* cells were shifted to the restrictive temperature, Chk1 became phosphorylated (Fig. 3C), further suggesting that Chk1 is required to prevent cutting. In contrast, in *orp1-4 cdc18-K46*, where the vast majority of cells enter mitosis in the absence of DNA replication, phosphorylation of Chk1 did not increase upon shift-up (Fig. 3C), strongly suggesting that the phosphorylated Chk1 is indeed responsible for arresting *orp1-4* cells.

These results show that there is a checkpoint prior to DNA replication that is responsible for the arrest of 70% of *orp1-4* cells. This checkpoint involves the checkpoint Rad proteins and Chk1 but not Cds1.

The checkpoint is independent of Rum1 and Ste9

The major target of the S/M and the G2/M checkpoints is the Cdc2 kinase, which also plays an important role in the initiation of DNA replication. Since inhibiting Cdc2 is a likely target of the checkpoint activated in *orp1-4* cells, we investigated whether the known mechanisms of Cdc2 inhibition are required for the G1 arrest of 70% of *orp1-4* cells. Rum1 can inhibit the mitotic CDK, Cdc2-Cdc13, in vitro and is required for efficient proteolysis of Cdc13 (Moreno and Nurse, 1994; Correa-Bordes and Nurse, 1995; Davis and Smith, 2001; Correa-Bordes et al., 1997). Ste9 is required for efficient degradation of the mitotic cyclin Cdc13 during G1 arrest (Yamaguchi et al., 2000; Blanco et al., 2000; Guo and Dunphy, 2000). We constructed double mutants of *orp1-4* with *rum1Δ* and *ste9Δ* and compared cutting in the double mutants and the *orp1-4* single mutant. Both double mutants cut with the same timing and to the same extent as the *orp1-4* single mutant (data not shown). We conclude that Rum1 and Ste9 are not required for the arrest of *orp1-4* cells prior to DNA replication.

Cdc2 is phosphorylated by Wee1 in arrested *orp1-4* cells

In cycling cells, phosphorylation of Cdc2 is thought to start in late G1, close to the G1/S transition (Hayles and Nurse, 1995). The previously characterised S/M and DNA damage checkpoint pathways delay entry into mitosis by maintaining phosphorylation of Cdc2. When *orp1-4* cells are incubated at the restrictive temperature, phosphorylated Cdc2 can be detected (Grallert and Nurse, 1996). This could result from the activation of the Chk1-dependent checkpoint pathway (above), or Cdc2 could have been phosphorylated before checkpoint activation. We shifted synchronous *orp1-4* cells to the restrictive temperature and followed both the appearance of phosphorylated Cdc2 and the activation of the checkpoint, as marked by Chk1 phosphorylation. Chk1 and Cdc2 became phosphorylated 2.5 hours and 3.5 hours after shift-up, respectively (Fig. 4A,B), opening the possibility that Cdc2 phosphorylation in *orp1-4* is a result of activation of the checkpoint and of Chk1.

cdc2 mutations conferring a *wee* phenotype, *cdc2-1w* and *cdc2-3w*, make Cdc2 insensitive to phosphorylation by Wee1 and independent of dephosphorylation by Cdc25, respectively. When we combined *orp1* with the *cdc2-1w* and *cdc2-3w*

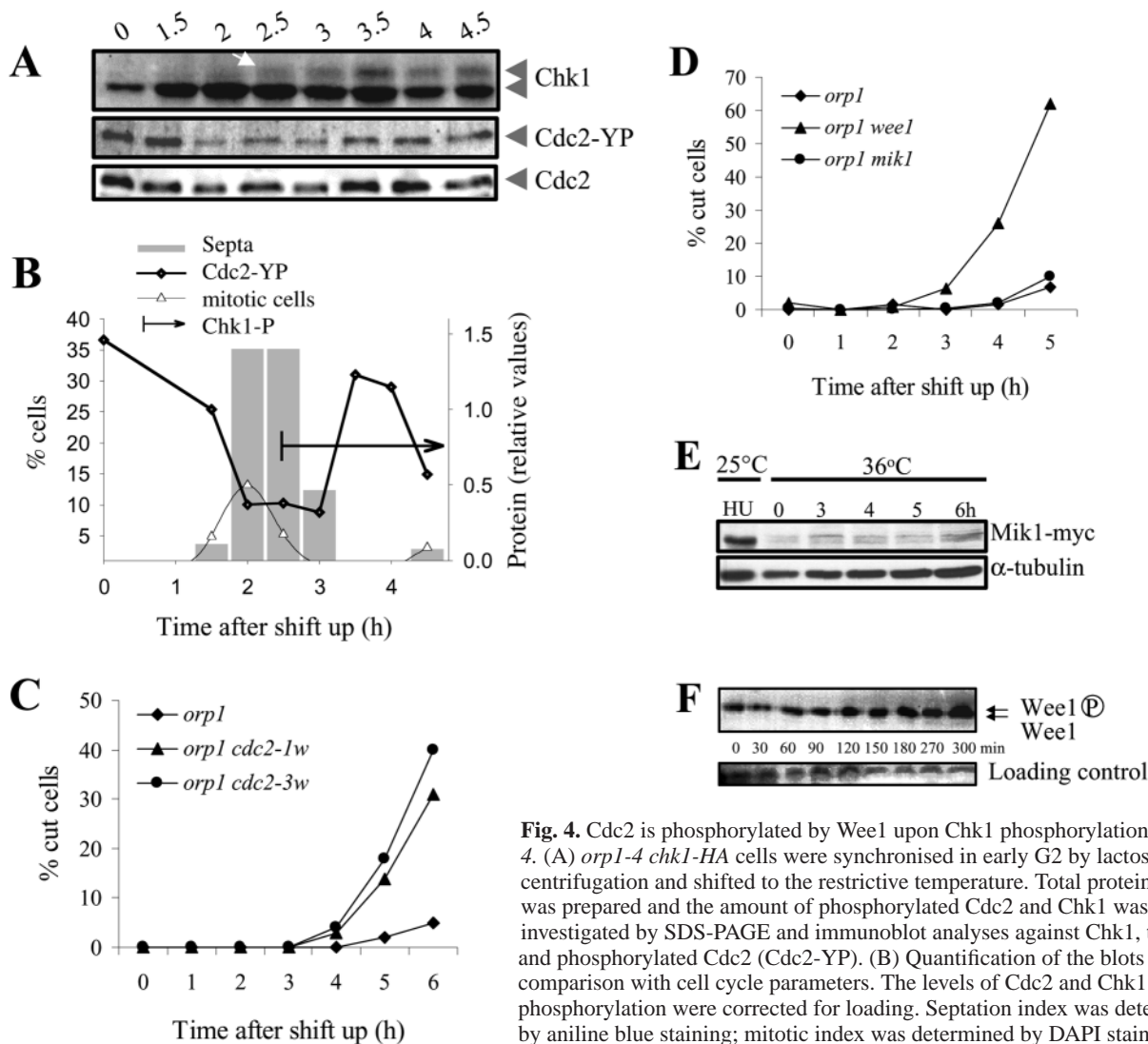


Fig. 4. Cdc2 is phosphorylated by Wee1 upon Chk1 phosphorylation in *orp1-4*. (A) *orp1-4 chk1-HA* cells were synchronised in early G2 by lactose gradient centrifugation and shifted to the restrictive temperature. Total protein extract was prepared and the amount of phosphorylated Cdc2 and Chk1 was investigated by SDS-PAGE and immunoblot analyses against Chk1, total Cdc2 and phosphorylated Cdc2 (Cdc2-Y-P). (B) Quantification of the blots (A) and comparison with cell cycle parameters. The levels of Cdc2 and Chk1 phosphorylation were corrected for loading. Septation index was determined by aniline blue staining; mitotic index was determined by DAPI staining. (C) Exponentially growing *orp1-4*, *orp1-4 cdc2-1w* and *orp1-4 cdc2-3w* cells

were shifted to the restrictive temperature for *orp1-4*. Cutting was monitored by DAPI staining. (D) Exponentially growing *orp1-4*, *orp1-4 mik1 Δ* and *orp1-4 weel-50* cells were shifted to the restrictive temperature for *orp1-4*. Cutting was monitored by DAPI staining. (E) The *orp1-4 mik1-myc* strain was shifted to the restrictive temperature for the indicated times. Total protein extract was prepared and the amount of mik1-myc was investigated by SDS-PAGE and western blot analysis. α -tubulin was measured as a loading control. (F) The *orp1-4 weel-HA* strain was shifted to the restrictive temperature for the indicated times. Total protein was extracted and the amount and phosphorylation state of Wee1 was investigated by SDS-PAGE and western blot analysis.

mutations, both double mutants were found to cut more than the *orp1-4* single mutant (Fig. 4C). This shows that phosphorylation of Cdc2 is important to keep the *orp1-4* cell from entering aberrant mitosis.

Phosphorylation of Cdc2 can be carried out by either of two kinases, Wee1 or Mik1 (reviewed by Berry and Gould, 1996). To investigate which kinase is responsible for phosphorylation of Cdc2 in arrested *orp1-4* cells, we constructed *orp1-4 weel-50* and *orp1-4 mik1 Δ* double mutants. Mutation of *weel*, but not of *mik1* exaggerated the cutting phenotype of *orp1-4* (Fig. 4D). Expression of *mik1* is cell cycle regulated and the protein is expressed in S phase. Activation of the intra-S phase and S/M checkpoints results in further induction of *mik1* in a *cds1*-dependent manner (Christensen et al., 2000; Baber-Furnari et al., 2000; Borgne and Nurse, 2000). Therefore, a low level of

expression of Mik1 in *orp1-4* cells at the restrictive temperature (Fig. 4E) is consistent with a lack of effect of a *mik1 Δ* mutation and is further evidence that the cells have not entered S phase.

When the G2/M damage checkpoint is activated, the Wee1 protein accumulates and is phosphorylated in a Chk1-dependent manner (Raleigh and O'Connell, 2001). We investigated whether a similar process takes place in the pre-replication checkpoint arresting *orp1-4* cells. When asynchronous *orp1-4* cells were arrested at the restrictive temperature an accumulation of Wee1 could be observed and at later times phosphorylated Wee1 was detected (Fig. 4F). We conclude that phosphorylation of Cdc2 in *orp1-4* cells is due to Wee1 activity prior to DNA replication and occurs after and possibly in response to checkpoint activation.

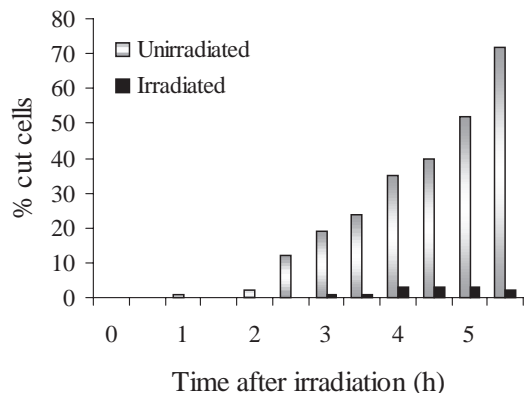


Fig. 5. Activating a putative damage checkpoint in G1 delays cutting in *orp1* Δ cells. An *orp1::ura4⁺/orp1-4 ura4-D18/ura4-D18 h⁺/h⁻* diploid was sporulated and the spores were germinated in the absence of uracil at the restrictive temperature for *orp1-4*. Half of the culture was irradiated 4 hours after inoculation and cutting was followed by DAPI staining in both cultures.

Activation of a damage checkpoint pathway in G1 prevents cutting in germinating *orp1* Δ spores

The above results suggest that there is a checkpoint prior to DNA replication that requires the checkpoint Rad proteins and Chk1 and acts via inducing and/or maintaining phosphorylation of Cdc2. In the *orp1-4* mutant this checkpoint might be activated by aberrant origin structures recognized as DNA damage (see Discussion). One prediction of this model is that if we inflict DNA damage in the absence of Orp1 and thereby induce this G1 checkpoint, cutting should be prevented. To test this hypothesis, we irradiated germinating *orp1* Δ spores derived from an *orp1-4/orp1* Δ diploid. These *orp1* Δ spores contain the temperature-sensitive Orp1 from the parental diploid and, when germinated at the restrictive temperature, all of them enter mitosis in the absence of DNA replication (Grallert and Nurse, 1996). The spores were irradiated with a UV dose (254 nm) that gave a 50% survival of wild-type cells. Irradiation of the germinating *orp1* Δ spores strongly reduced cutting (Fig. 5), suggesting that inflicting DNA damage in G1 in the absence of Orp1 can induce a checkpoint that inhibits the mitotic machinery.

orp1-4 cells become committed to cutting early in G1

Entry into aberrant mitosis (cutting) in *orp1-4* cells can be observed already 4 hours after a shift to the restrictive temperature, but only about 30% of the cells cut even after 6 hours (Grallert and Nurse, 1996). We argue that these cells enter mitosis from G1 (i.e. before the occurrence of any DNA replication). First, if cells leaked into S phase before cutting, it would be detectable by 2D gel analysis (Fig. 1), although synthesis of very short pieces of DNA would not be detected by this technique. Second, we examined whether *orp1-4* cells have to pass the *cdc10* arrest point before they become committed to cutting. Cdc10 is a transcription factor that activates transcription of a number of genes required for initiation of DNA replication (Aves et al., 1985; Tanaka et al., 1992; Lowndes et al., 1992). Therefore, temperature-sensitive *cdc10* mutants arrest at the restrictive temperature before any

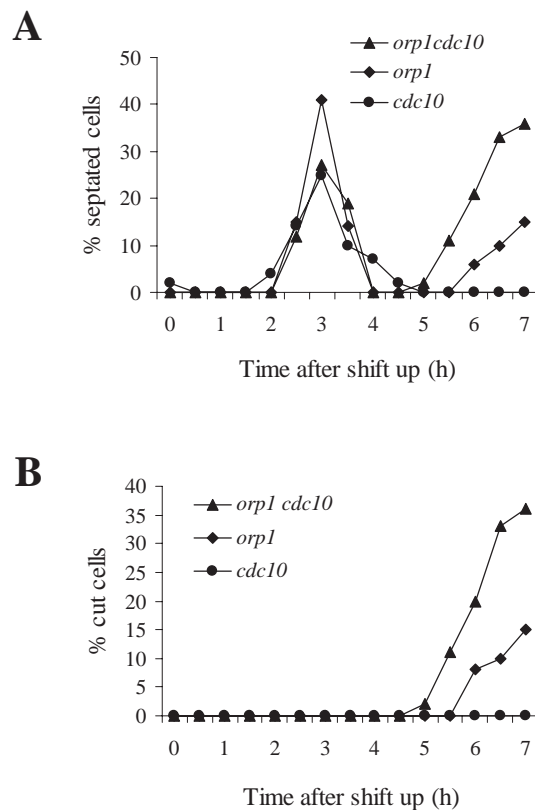


Fig. 6. Cutting in *orp1-4* is initiated early. *cdc10-129 orp1-4* and *orp1-4* cells were synchronised by lactose gradient centrifugation in early G2, and then shifted to the restrictive temperature. Septation index is given as a measure of synchrony and was determined by Calcofluor staining (A). Aberrant mitoses (cutting) were detected by DAPI staining (B).

DNA replication has occurred and with a 1C DNA content. If the *orp1-4* cells have to pass the *cdc10* arrest point to become committed to cutting, we would expect an *orp1-4 cdc10^{ts}* double mutant not to cut at the restrictive temperature. Even if the double mutant leaks through the *cdc10* arrest point, it would cut only after a delay compared with *orp1-4*. When synchronous *orp1-4* and *orp1-4 cdc10-129* cells were selected by lactose gradient centrifugation and shifted to the restrictive temperature, the double mutant was not delayed at cutting compared with the single *orp1-4* mutant (Fig. 6). On the contrary, the double mutant cut earlier than the single mutant. Asynchronous *orp1-4* and *orp1-4 cdc10-129* mutants cut to the same extent and with similar timing (data not shown). We conclude that commitment to cutting in *orp1-4* occurs before entry into S phase and not later than the *cdc10* arrest point.

Discussion

We have studied a G1 checkpoint by exploiting the *orp1-4* mutant, which has two alternative fates at the restrictive temperature: cell cycle arrest and cutting. The arrest is dependent upon the checkpoint Rad proteins, which demonstrates that the arrest is due to a checkpoint. Several lines of evidence argue that the *orp1-4* cells arrest in G1: they can mate from the arrest point, as shown previously (Grallert and

Nurse, 1996), they arrest before expression of *mik1*, and they have not initiated DNA replication, as shown by 2D gel analysis of the early replication origin *ars 3001*. Furthermore, a leakage into S phase should elicit the *cds1*-dependent intra-S and/or the S/M checkpoint pathway. We have shown that Cds1 is not activated in *orpl-4* cells (Fig. 3B).

It was shown earlier (Grallert and Nurse, 1996) that chromosomes prepared from arrested *orpl-4* cells do not enter the gel upon PFGE. This result suggests the presence of aberrant DNA structures but is also consistent with an early S phase arrest. Two-dimensional gel analysis of *ars3001* argues against the latter interpretation and leaves us to conclude that aberrant DNA structures are responsible for the arrest in *orpl-4* cells.

In synchronous *orpl-4* cells shifted to the restrictive temperature, Chk1 phosphorylation occurs before Cdc2 phosphorylation (Fig. 4A,B), leading us to speculate that activation of Chk1 brings about Cdc2 phosphorylation.

Of the two kinases that phosphorylate Cdc2, absence of Wee1 function abolishes the G1 arrest of *orpl-4* cells, strongly suggesting that Wee1 phosphorylates Cdc2 during G1 in the present checkpoint (Fig. 6). The following observations suggest that Wee1 is activated by Chk1. Deleting *chk1*⁺ in *orpl-4* cells has the same consequences as introducing a *wee1-50* mutation (this work). Furthermore, *wee1*⁻ cells are insensitive to the overexpression of Chk1, which can directly phosphorylate Wee1 in vitro (O'Connell et al., 1997) and activation of the G2/M damage checkpoint leads to accumulation and phosphorylation of the Wee1 protein (Raleigh and O'Connell, 2001). Accumulation and phosphorylation of Wee1 can also be observed in arrested *orpl-4* cells. Therefore, it is tempting to speculate that phosphorylation and/or an increase of Wee1 activity are contributing mechanisms to bring about and maintain a G1 arrest of *orpl-4* cells (Fig. 6). A direct way to examine this suggestion would be to measure Cdc2 phosphorylation in *orpl-4 chk1Δ* and *orpl-4 wee1* double mutants. However, these mutants start cutting less than 2 hours after shift-up to the restrictive temperature. Since Cdc2 becomes dephosphorylated as the cells go into mitosis and cut (Fig. 4B), it is not possible to determine whether the deletion of *chk1* or *wee1* results in lack of Cdc2 phosphorylation. In contrast to *wee1*, mutation of *mik1* has no effect on cutting in *orpl-4* cells, nor is *mik1* expression induced at the restrictive temperature. Thus, Mik1 is not a major target of the checkpoint induced in *orpl-4* cells.

Most temperature-sensitive replication initiation mutants arrest in G1 or inside S phase, whereas several deletion mutants proceed to aberrant mitosis without completing S phase (cutting). In general, the tighter the mutants are, the more cutting occurs. These observations have led to the model that the preRC complex, in addition to initiating DNA replication, provides a signal that S phase is in preparation and mediates inhibition of mitosis through activation of a checkpoint pathway. An alternative explanation is that a preRC-independent checkpoint is activated in G1. A third alternative is that the cells attempt to replicate

DNA in spite of compromised initiation, and that it is the S/M checkpoint pathway that prevents mitosis. The latter alternative has been excluded by the present experiments. We will discuss the two former alternatives below.

We suggest that the difference between the two populations of *orpl-4* cells (cutting versus arresting) is that the residual function of the Orp1-4 protein allows changing the origin structure in 70% of the cells to a structure that activates a checkpoint pathway (Fig. 7). Although the preRC does not assemble properly in *orpl-4* cells (Kearsey et al., 2000), some steps of preRC formation might be performed, at least on some origins. This incomplete preRC or the resulting origin structure might in turn activate the checkpoint (Fig. 7). The following observations support the idea that the arrested cells have proceeded further than cutting cells have, and that formation of a certain structure, possibly recognised as DNA damage, is responsible for the arrest. (1) The cutting cells become committed to cutting at a point before the *cdc10* arrest point (Fig. 6), while the arresting cells arrest at or later than the *cdc10* arrest point (Grallert and Nurse, 1996). (2) *orpl-4* cells rapidly lose viability upon shift to the restrictive temperature, before cells undergoing aberrant mitoses can be observed (data

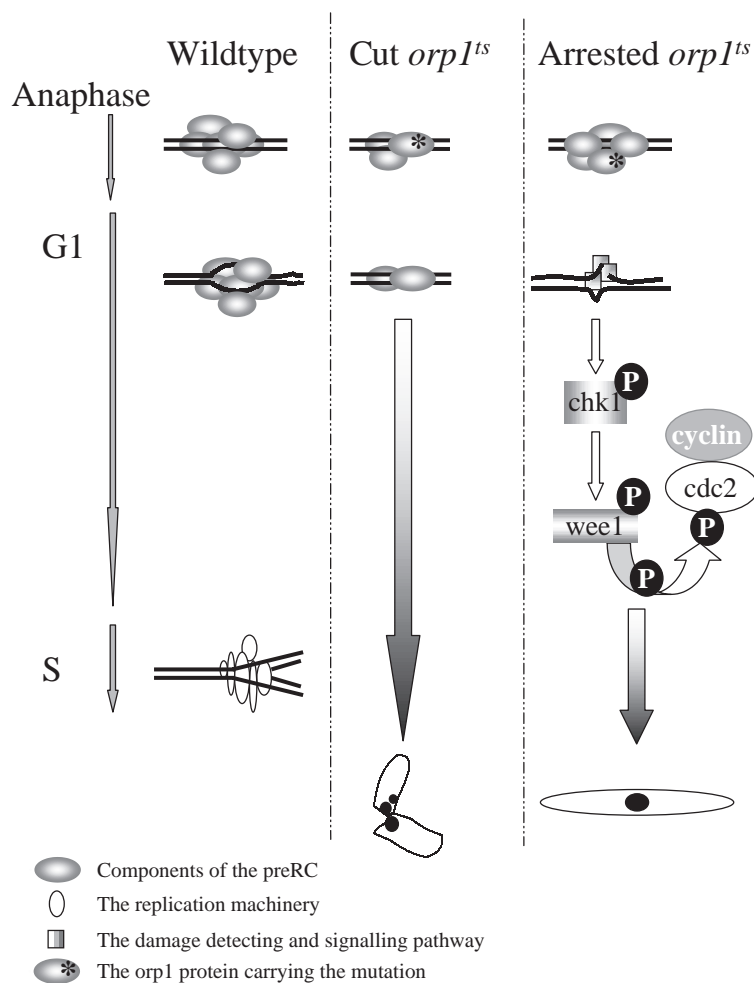


Fig. 7. Cutting and cell cycle arrest in *orpl-4* cells. The columns show the events taking place at a replication origin in wild-type, cutting and arrested *orpl-4* cells. See discussion for details. Damaged DNA is shown here as a strand break, but we have no evidence that this is indeed the case.

not shown). This argues for an irreversible formation of damage-like structures. (3) Chromosomes prepared from arrested *orp1-4* cells do not enter the gel upon PFGE (Grallert and Nurse, 1996), consistent with the presence of aberrant DNA structures. (4) Complete loss of Orp1 function or a combination of *orp1-4* with mutant alleles of genes encoding other preRC components leads to increased entry into mitosis in the absence of DNA replication relative to an *orp1-4* single mutation (Fig. 2C) (Grallert and Nurse, 1996), strongly suggesting that the former mutants are unable to modify the origin structure enough to activate a checkpoint. Consistently, no Chk1 phosphorylation was detected in *orp1-4 cdc18-K46* double mutant cells (Fig. 3C).

The checkpoint activated in *orp1-4* cells is clearly different from the intra-S checkpoint, since the former requires Chk1 but does not require Cds1 (this work) while the latter has the opposite requirement (Walworth et al., 1993; Lindsay et al., 1998).

We argue that complete lack of replication does not activate any checkpoint and therefore results in entry into mitosis in the absence of DNA replication. Induction of the checkpoint depends on an attempt to form a preRC. Formation of an incomplete preRC results in checkpoint induction, but this does not imply that the checkpoint is specifically activated by incomplete preRCs. Our finding that irradiating germinating *orp1Δ* spores prevents entry into mitosis points to the existence of a damage checkpoint activated in G1 that can inhibit the mitotic machinery. We favour the interpretation that this checkpoint is identical to the checkpoint activated in arresting *orp1-4* cells.

Studying germinating spores carrying a deletion of the *orp2* gene resulted in the conclusion that there is no checkpoint for a lack of DNA replication (Kiely et al., 2000), consistent with our interpretation. However, the *orp2^{ts}* mutant was arrested in S phase and therefore those experiments did not allow the detection of a checkpoint prior to DNA replication.

Combination of the *orp1-4* mutation with *cdc10-129* (Fig. 1B), *cdc18-K46* (Fig. 2C), checkpoint *rad* genes (Fig. 2A), or *chk1Δ* (Fig. 3A) makes the cells cut earlier and more extensively than the single *orp1-4* mutant. We suggest that the first two cut earlier and more because the arrest in *orp1* mutant cells is dependent on attempting preRC formation. To follow our line of reasoning above, more preRC formation will be achieved in the presence of Cdc18, which again will lead to more checkpoint arrest and less cutting. Consistently, it has been shown that Cdc18 overproduction can rescue the cutting phenotype of *orp1-4* (Grallert and Nurse, 1996). It has been shown (Greenwood et al., 1998) that overexpressing the C-terminus of Cdc18 in wild-type cells is sufficient for replication initiation and induction of a checkpoint inhibiting mitosis, but since these cells were shown to be arrested in S phase with between 1C and 2C DNA contents, the relevance for the present experiments is uncertain. The early-cut phenotype observed when *orp1-4* is combined with checkpoint *rad* mutations suggests that the checkpoint proteins might be physically present and active at the origin complex in *orp1-4* cells.

There are two contradictory reports on a G1 checkpoint in a *cdc10^{ts}* mutant. The Chk1 protein was found to be required (Carr et al., 1995) and not required (Hayles and Nurse, 1995) for arrest of *cdc10^{ts}* cells. In the two papers different

synchronisation methods were used, which might account for the discrepancy. Since Cdc2 is not phosphorylated in arrested *cdc10^{ts}* cells (Hayles and Nurse, 1995), it is unlikely that the checkpoint pathways arresting *cdc10^{ts}* and *orp1-4* cells are one and the same. Consistently, Rum1, which is required only in pre-START G1, is necessary for a *cdc10^{ts}* arrest (Moreno and Nurse, 1994), but not for arrest of *orp1-4* cells (this work). However, we cannot exclude the possibility that *cdc10^{ts}* cells leak into later parts of G1, although it was shown that they do not leak into S phase (Carr et al., 1995). If this is indeed the case, the Chk1-dependent checkpoint reported earlier (Carr et al., 1995) might be the same as the one arresting *orp1-4* cells.

The checkpoint activated in *orp1-4* cells appears to be similar to the G2/M damage checkpoint in that it requires Chk1 and the maintenance of Cdc2 phosphorylation. Also, failure of either checkpoint results in entry into mitosis. We have provided several pieces of evidence that *orp1-4* cells arrest in G1. Nevertheless, it is possible that the present G1/M checkpoint mechanism is using the same molecular signals and targets as the G2/M checkpoint. The phosphorylation state of Cdc2 and the levels and activities of the mitotic regulators Cdc13 and Cdc25 may be the same during a late G1 arrest as in a G2 arrest. In this context it is interesting to note that *orp1-4 cdc2-3w* cells are checkpoint deficient (Fig. 4C). This observation raises the possibility that, in addition to Wee1, Cdc25 might also be targeted by Chk1 in *orp1-4* cells, as in the G2/M checkpoint (Raleigh and O'Connell, 2001). In higher eukaryotes the biochemical differences during the G1 and G2 phases are more extensive, because of the divergent evolution of CDKs and their regulators. Therefore, failure of a G1 checkpoint cannot lead to premature entry into mitosis, but only to premature entry into S phase.

Checkpoint activation in G1 in higher eukaryotes depends upon phosphorylation and inhibition of CDKs, which is similar to the checkpoint activated in *orp1-4* cells. In human cells, CDK2 activity is rapidly inhibited in response to activation of the G1/S damage pathway, by destruction of CDC25A (Mailand et al., 2000). It is noteworthy that hChk1 phosphorylates hCdc25A in vitro (Sanchez et al., 1997) and destruction of Cdc25A is Chk1 dependent (Mailand et al., 2000). Less is known about the possible involvement of Wee1 or Mik1 in the DNA damage checkpoints in higher eukaryotes. *Xenopus* Chk1 phosphorylates Wee1 upon checkpoint activation, which leads to increased Wee1 kinase activity (Lee et al., 2001). In *Drosophila* the *rad3* homologue *mei-41* and the *chk1* homologue GRP are involved in the slowing of the early embryonic cycles, which indicates activation of a checkpoint once maternally provided replication functions become limiting (Fogarty et al., 1997; Sibon et al., 1999; Sibon et al., 1997; Zhou and Elledge, 2000; Rhind et al., 1997). Interestingly, mutation of *Wee1* has similar phenotypic consequences to mutation of *Grp* (Price et al., 2000) suggesting that this Grp-dependent checkpoint might target Wee1. The relative contributions of inhibiting the phosphatases and inducing the kinases might vary in different organisms, but the strategy of maintaining CDK phosphorylation upon checkpoint activation in G1 appears to be conserved.

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