Hyperactivation of the yeast DNA damage checkpoint by *TEL1* and *DDC2* overexpression

**Michela Clerici, Vera Paciotti,
Veronica Baldo, Michele Romano,
Giovanna Lucchini and Maria Pia Longhese[1]**

Dipartimento di Biotecnologie e Bioscienze, Università degli Studi di
Milano-Bicocca, 20126 Milan, Italy

[1]Corresponding author
e-mail: mariapia.longhese@unimib.it

M.Clerici and V.Paciotti contributed equally to this work

The evolutionarily conserved yeast Mec1 and Tel1
protein kinases, as well as the Mec1-interacting
protein Ddc2, are involved in the DNA damage check-
point response. We show that regulation of Tel1 and
Ddc2–Mec1 activities is important to modulate both
activation and termination of checkpoint-mediated
cell cycle arrest. In fact, overproduction of either Tel1
or Ddc2 causes a prolonged cell cycle arrest and cell
death in response to DNA damage, impairing the
ability of cells to recover from checkpoint activation.
This cell cycle arrest is independent of Mec1 in
UV-irradiated Tel1-overproducing cells, while it is
strictly Mec1 dependent in similarly treated *DDC2*-
overexpressing cells. The Rad53 checkpoint kinase is
instead required in both cases for cell cycle arrest,
which correlates with its enhanced and persistent
phosphorylation, suggesting that unscheduled Rad53
phosphorylation might prevent cells from re-entering
the cell cycle after checkpoint activation. In addition,
Tel1 overproduction results in transient nuclear div-
ision arrest and concomitant Rad53 phosphorylation
in the absence of exogenous DNA damage independ-
ently of Mec1 and Ddc1.
Keywords: checkpoints/Ddc2/DNA damage/Mec1/Tel1

Introduction

Eukaryotic cells respond to DNA damage and replication
blocks by delaying cell cycle progression through a
surveillance mechanism known as the DNA damage
checkpoint, thus providing the time to restore the correct
DNA structure before entering the next cell cycle phase.
Failure of DNA repair or checkpoint controls can lead to
cell lethality, mutations, genome instability and ultimately
cancer (reviewed in Zhou and Elledge, 2000).

In *Saccharomyces cerevisiae*, the DNA damage check-
point is a signal transduction cascade involving the
evolutionarily conserved protein kinases Mec1, Rad53
and Chk1 (reviewed in Zhou and Elledge, 2000). Rad53
and Chk1 are both phosphorylated in a Mec1-dependent
manner (Sanchez *et al.*, 1996; Sun *et al.*, 1996). While
Rad53 is required for a proper response to DNA damage in
all the cell cycle phases, Chk1 contributes to the activation

of the G_2/M checkpoint in a Rad53-independent manner
(Sanchez *et al.*, 1996, 1999). Mec1 belongs to the
phosphatidylinositol-3-kinase motif family, which also
includes *S.cerevisiae* Tel1 (Greenwell *et al.*, 1995;
Morrow *et al.*, 1995), *Schizosaccharomyces pombe* Rad3
(Bentley *et al.*, 1996), *Drosophila melanogaster* Mei-41
(Hari *et al.*, 1995), human ATM (Savitsky *et al.*, 1995) and
ATR (Bentley *et al.*, 1996). Mec1 physically interacts with
Ddc2, which undergoes Mec1-dependent phosphorylation
both *in vivo* and *in vitro* (Paciotti *et al.*, 2000; Rouse and
Jackson, 2000; Wakayama *et al.*, 2001). The finding that
the DNA damage-induced Mec1-dependent Ddc2 phos-
phorylation does not require other known checkpoint
factors suggests that Mec1, as well as *S.pombe* Rad3 and
human ATM, may be involved in sensing DNA damage
(Paciotti *et al.*, 2000; reviewed in O'Connell *et al.*, 2000;
Zhou and Elledge, 2000). Additional checkpoint proteins
essential for DNA damage-induced cell arrest are Ddc1,
Rad17, Mec3, Rad24 and Rad9 (Weinert and Hartwell,
1988; Siede *et al.*, 1993; Weinert *et al.*, 1994; Longhese
et al., 1996, 1997; Paulovich *et al.*, 1997). Ddc1, Mec3 and
Rad17, like their *S.pombe* and human homologs, physic-
ally interact with each other (Kostrub *et al.*, 1998; Paciotti
et al., 1998; Kondo *et al.*, 1999; St Onge *et al.*, 1999;
Caspari *et al.*, 2000) and are structurally related to the
proliferating cell nuclear antigen (PCNA) clamp (Thelen
et al., 1999), which tethers DNA replication proteins to the
replicating DNA (Waga and Stillman, 1998). Moreover,
Rad24 is homologous to, and interacts with, subunits of the
PCNA clamp loader, replication factor C (RF-C) (Griffiths
et al., 1995; Lydall and Weinert, 1997; Green *et al.*, 2000).
Based on these similarities and genetic and biochemical
evidence, Ddc1, Rad17, Mec3 and Rad24 are proposed to
work together with Mec1 in sensing damaged DNA
molecules and modulating Mec1 activity (reviewed in
Longhese *et al.*, 1998; Weinert, 1998; Lowndes and
Murguia, 2000). The DNA damage sensing functions
are then linked with downstream effectors by Mec1-
dependent phosphorylation of Rad9. In fact, Rad9 phos-
phorylation triggers its interaction with Rad53 and con-
sequent release of active Rad53 kinase (Emili, 1998; Sun
et al., 1998; Vialard *et al.*, 1998; Gilbert *et al.*, 2001), thus
indicating that Mec1 can regulate both sensing and
transducing checkpoint signals.

In addition to their involvement in the checkpoint
responses, *DDC2*, *MEC1* and *RAD53* are essential for cell
viability, and their essential function, but not the check-
point functions, can be bypassed by increasing the
intracellular concentrations of deoxyribonucleotides
(dNTPs), either by overexpression of *RNR* genes encoding
ribonucleotide reductase (Desany *et al.*, 1998) or by
deletion of the *SML1* gene (Zhao *et al.*, 1998), which
negatively affects the dNTP pool (Chabes *et al.*, 1999;
Zhao *et al.*, 2001).

The Mec1 homolog Tel1 is a protein kinase (Mallory and Petes, 2000) primarily required for telomere length maintenance (Greenwell *et al*., 1995; Morrow *et al*., 1995). Several data indicate that it also has a role in the cellular response to DNA damage, which becomes evident in the absence of Mec1. In fact, *TEL1* deletion increases the sensitivity of *mec1* mutant cells to DNA-damaging agents (Ritchie *et al*., 1999), and high levels of Tel1 can suppress both cell lethality and hypersensitivity to DNA-damaging agents of *mec1Δ* cells, suggesting that Tel1 and Mec1 may have partially overlapping functions (Sanchez *et al*., 1996). Moreover, Tel1 has recently been implicated, together with the Mre11 complex, in a DNA damage checkpoint pathway that responds primarily to double strand breaks and parallels the Mec1 pathway, leading to Rad9 phosphorylation and interaction with Rad53 (D'Amours and Jackson, 2001; Grenon *et al*., 2001; Usui *et al*., 2001).

Once the checkpoint is activated by DNA damage, some mechanism must exist to allow cells to resume cell cycle progression when the aberrant DNA structures have been removed. In fact, the inability of cells to recover from checkpoint activation once the damage is repaired would result in cell death in the presence of genotoxic agents. While our knowledge about the mechanisms underlying checkpoint activation is continuously increasing, the processes involved in recovery from DNA damage-induced cell cycle arrest are still completely unknown.

Here we show that regulation of Tel1 and Ddc2–Mec1 activities is important to modulate both the activation of checkpoint-mediated cell cycle arrest and the subsequent recovery. In fact, although by different processes, both *TEL1* and *DDC2* overexpression cause prolonged cell cycle arrest and cell death after DNA damage, and this correlates with high and persistent levels of Rad53 phosphorylation. In addition, Tel1 overproduction results in Rad53 phosphorylation and nuclear division delay in the absence of exogenous DNA damage, suggesting that Tel1, when present in high levels, might activate the checkpoint even in the absence of external checkpoint signals.

Results

TEL1 overexpression causes prolonged nuclear division delay and phosphorylation of Rad53 in the absence of exogenous DNA damage

The finding that *TEL1* overexpression can suppress hypersensitivity of *mec1Δ* cells to genotoxic agents (Sanchez *et al*., 1996; see also Figure 3D) indicates that high levels of Tel1 can partially substitute for Mec1 in the response to DNA damage. Furthermore, Tel1 has been implicated together with the Mre11 complex in triggering Rad53 activation and its interaction with Rad9 in response to DNA damage independently of Mec1 (Usui *et al*., 2001). To gain insights into the function of Tel1 and its involvement in checkpoints, we first analyzed cell cycle progression in the absence of DNA damage of cells carrying the galactose-inducible *GAL1–TEL1* fusion at the *TEL1* locus. As shown in Figure 1A and B, when cell cultures were arrested in G_1 with α-factor in the presence of galactose and released from the block under galactose-

induced conditions, *GAL1–TEL1* cells progressed through S phase only slightly more slowly than wild type, but then they surprisingly underwent a prolonged arrest with 2C DNA content and undivided nuclei, while nuclear division and cell cycle progression occurred normally in wild-type cells under the same conditions. Similarly, when cell cultures arrested in G_2 with nocodazole in the presence of galactose were released from the nocodazole block under galactose-induced conditions, *GAL1–TEL1* cells were still arrested with undivided nuclei 240 min after release, while wild-type cells underwent nuclear division within 90 min (Figure 1D). Thus, overexpression of *TEL1* causes a G_2/M cell cycle arrest in the absence of exogenous DNA damage, suggesting that high levels of Tel1 might lead to activation of a checkpoint pathway. The Tel1-induced cell cycle arrest was transient, since both *GAL1–TEL1* and *GAL1–TEL1-HA3* cell cultures underwent nuclear division, and resumed budding and DNA replication with wild-type kinetics 12–14 h after a shift to galactose-containing medium, although the Tel1-HA3 level remained unchanged throughout the experiments (data not shown). Moreover, when exponentially growing *GAL1–TEL1* cell cultures were shifted to galactose-containing medium for 4 h (when nearly all cells had grown to the dumb-bell shape characteristic of G_2/M-arrested cells) and then plated on galactose-containing plates, 70% of cells produced microcolonies with >4 cells or buds within 24 h. Consistent with this observation, Tel1-overproducing cells also showed a slow growth phenotype in the absence of genotoxic agents (Figures 3D, 4C and 5C).

Since activation of the known DNA damage checkpoint pathways leads to phosphorylation of Rad53, which results in changes in Rad53 electrophoretic mobility (Sanchez *et al*., 1996; Sun *et al*., 1996), we used western blot analysis to study the Rad53 phosphorylation pattern during the above experiments. Rad53 phosphorylation was clearly detectable in galactose-induced *GAL1–TEL1* cells released either from the G_1 or the G_2 block in the absence of DNA damage, but not in wild-type cells lacking the *GAL1–TEL1* fusion under the same conditions (Figure 1C and E). It is worth noting that Rad53 phosphorylation was detectable in *GAL1–TEL1* cells both when they were released from the G_2 block under galactose-induced conditions (Figure 1E) and when they were kept in G_2 by nocodazole treatment after galactose addition (Figure 1F), indicating that high levels of Tel1 induce Rad53 phosphorylation independently of cell cycle progression. Interestingly, the effects of *TEL1* overexpression on cell cycle progression were partially counteracted by increased levels of wild-type Mec1 expressed from a *GAL1–MEC1* fusion integrated at the *URA3* chromosomal locus. In fact, as shown in Figure 1B and D, when galactose-induced α-factor or nocodazole-arrested cells were released into the cell cycle in galactose-containing media, nuclear division occurred faster in *GAL1–TEL1 GAL1–MEC1* than in *GAL1–TEL1* cells, although with slower kinetics compared with wild-type cells. Moreover, galactose-induced *GAL1–TEL1 GAL1–MEC1* cells released from the G_1 or G_2 blocks contained a reduced amount of Rad53 phosphorylated forms compared with similarly treated *GAL1–TEL1* cells (Figure 1C and E), while overexpression of *MEC1* did not affect cell cycle progression or
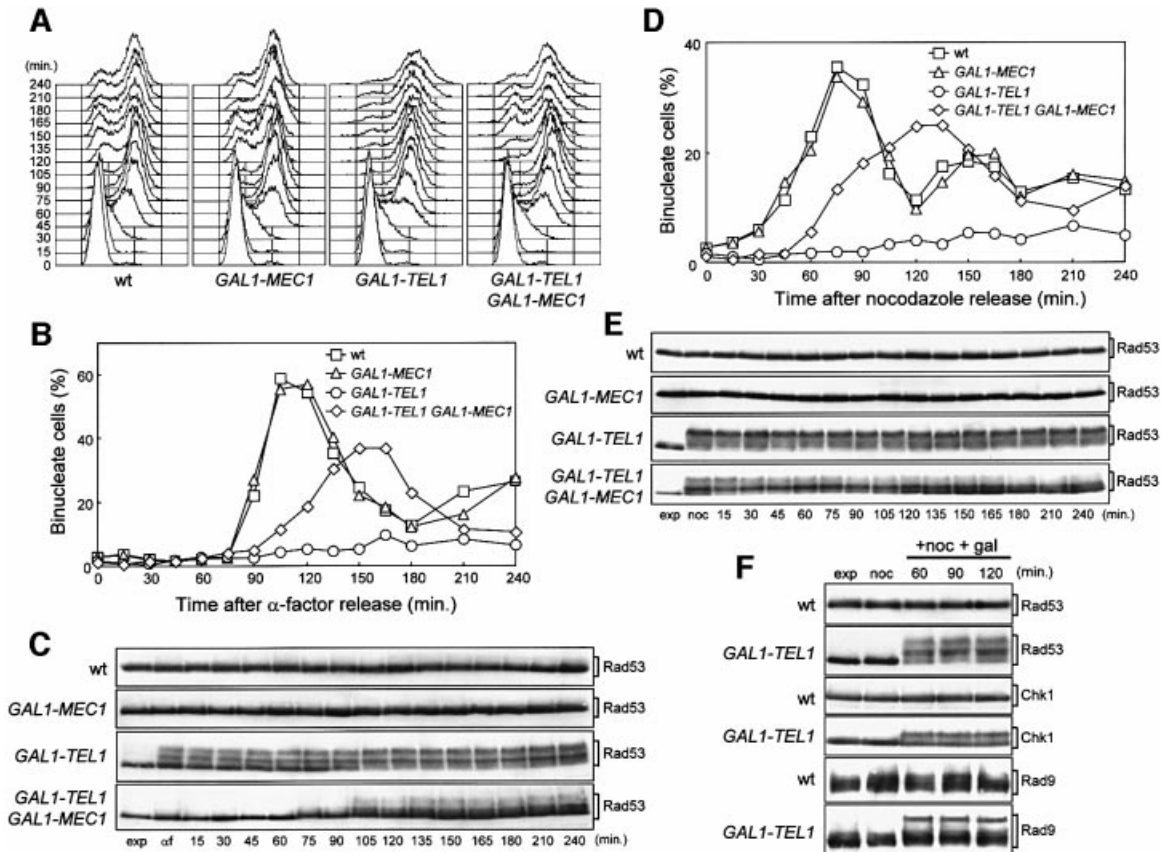
Fig. 1. Effects of *TEL1* overexpression during an unperturbed cell cycle. Strains were as follows: wild type (K699), *GAL1–MEC1* (YLL516), *GAL1–TEL1* (DMP3539/10D) and *GAL1–TEL1 GAL1–MEC1* (DMP3539/9D). (A–C) Cell cultures, growing logarithmically in YEP-raf, were synchronized in G_1 with α-factor in the presence of galactose (2 h). Release from α-factor block was performed at time zero (C, αf) by transferring cell cultures to fresh YEP-raf-gal medium. Samples were taken at the times indicated after release into the cell cycle to determine the DNA content by fluorescence-activated cell sorter (FACS) analysis (**A**), the kinetics of nuclear division by direct visualization by propidium iodide staining (**B**) and the amount and phosphorylation of Rad53 by western blotting of protein extracts with anti-Rad53 antibodies (**C**). (D and E) Cell cultures, growing logarithmically in YEP-raf, were synchronized in G_2 with nocodazole in the presence of galactose (2 h). Release from nocodazole block was performed at time zero (E, noc) by transferring cell cultures to fresh YEP-raf-gal medium. Samples were taken at the times indicated after release from nocodazole to determine the kinetics of nuclear division (**D**) and Rad53 levels and phosphorylation (**E**) as described in (B) and (C), respectively. (**F**) Cell cultures of wild-type (DMP3598/21D) and *GAL1–TEL1* (DMP3598/6A) strains were arrested with nocodazole in YEP-raf medium (noc) and resuspended in YEP-raf-gal medium containing 15 μg/ml nocodazole (+noc +gal). Protein extracts were prepared from samples withdrawn at the times indicated and analyzed by western blotting. The slowly migrating protein species specifically reacting with anti-Rad53, anti-MYC (Chk1) and anti-Rad9 antibodies represent phosphorylated forms of the corresponding proteins (Sanchez *et al.*, 1996; Vialard *et al.*, 1998, 1999). exp, exponentially growing cells.

Rad53 phosphorylation *per se*. This suggests that an excess of Tel1 might compete with Mec1 for interactions with downstream effectors in the absence of DNA damage, thus causing unscheduled phosphorylation events and cell cycle arrest.

The above results prompted us to ask whether high levels of Tel1 might induce phosphorylation of checkpoint proteins other than Rad53, such as Ddc1, Ddc2, Chk1 and Rad9, which are all known to undergo Mec1-dependent phosphorylation resulting in electrophoretic mobility shift of the corresponding protein species (Emili, 1998; Paciotti *et al.*, 1998, 2000; Sun *et al.*, 1998; Vialard *et al.*, 1998; Sanchez *et al.*, 1999). Significant amounts of phosphorylated Chk1 and Rad9 could be detected in nocodazole-arrested *GAL1–TEL1* cell cultures after addition of galactose (Figure 1F), while Ddc1 and Ddc2 phosphorylations were not influenced by Tel1 overproduction (data not shown), indicating that high levels of Tel1 specifically induce phosphorylation of Rad53, Rad9 and Chk1 in the absence of exogenous DNA damage.

Tel1 overproduction affects the cell response to DNA damage

Since overexpression of *TEL1* causes G_2/M cell cycle arrest independently of exogenous DNA damage, we investigated its effects in the presence of genotoxic insults. Galactose-induced *GAL1–TEL1* cells were more sensitive to hydroxyurea (HU), methyl methanesulfonate (MMS) and UV than isogenic wild-type strains not carrying the *GAL1–TEL1* fusion (Figure 2A). This hypersensitivity can be partially suppressed by increasing the level of wild-type Mec1, since *GAL1–TEL1* cells concomitantly expressing a *GAL1–MEC1* fusion showed lower sensitivity to HU, MMS and UV than *GAL1–TEL1* cells (Figure 2A), indicating that high levels of Tel1 might cause the observed phenotypes by competing with physiological amounts of Mec1.

Moreover, cell cycle progression after UV irradiation was dramatically altered by high levels of Tel1 (Figure 2B). In fact, when galactose-induced cell cultures were arrested in G_1, UV irradiated and then released from
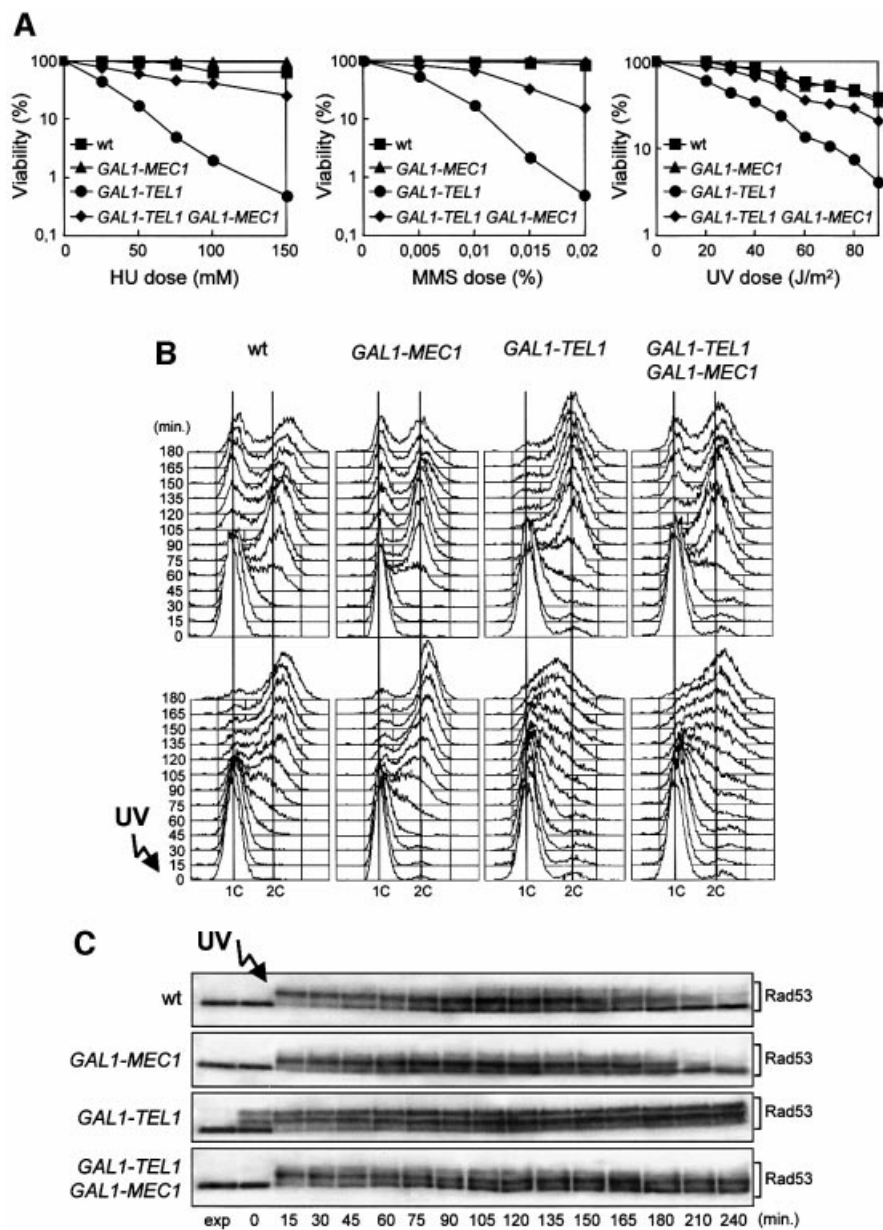
Fig. 2. Response to DNA damage of *TEL1*-overexpressing cells. Strains were as follows: wild type (K699), *GAL1–MEC1* (YLL516), *GAL1–TEL1* (DMP3539/10D) and *GAL1–TEL1 GAL1–MEC1* (DMP3539/9D). (**A**) Dose–response killing curves were determined by plating serial dilutions of YEP-raf exponentially growing cell cultures on YEP-raf-gal plates with or without MMS or HU at the concentrations indicated. One set of YEP-raf-gal plates was exposed at the UV doses indicated. Plates were incubated at 25°C and colony-forming units were counted after 3 days. (B and C) Cell cultures growing logarithmically in YEP-raf were synchronized in G_1 with α-factor in the presence of galactose (2 h). Cells were released from the α-factor block at time zero in YEP-raf-gal, or were UV irradiated (40 J/m^2) prior to release in YEP-raf-gal. Samples of untreated and UV-irradiated cultures were taken at the times indicated after α-factor release to analyze the DNA content by FACS (**B**) and to determine the level and phosphorylation of Rad53 by western blot analysis of protein extracts from the UV-treated cell cultures with anti-Rad53 antibodies (**C**). exp, exponentially growing cells.

the G_1 block under galactose-induced conditions, *GAL1–TEL1* cells dramatically slowed down entry and progression through S phase compared with similarly treated wild-type cells, which completed DNA replication 120 min after UV irradiation (Figure 2B). This correlated with the persistence of high levels of phosphorylated Rad53 in UV-treated *GAL1–TEL1* cells even 240 min after release into the cell cycle, while Rad53 phosphorylation began to decrease after 180 min in wild-type cells under the same conditions (Figure 2C).

In agreement with the observed suppression of *GAL1–TEL1* cell hypersensitivity to DNA-damaging agents by *GAL1–MEC1*, overexpression of *MEC1* was able to reduce both the DNA damage-induced slowing down of S-phase progression and the concomitant Rad53 phosphorylation in Tel1-overproducing cells. In fact, galactose-induced UV-irradiated *GAL1–TEL1 GAL1–MEC1* cells completed DNA replication and showed a reduced amount of phosphorylated Rad53 after release into the cell cycle (Figure 2B and C).
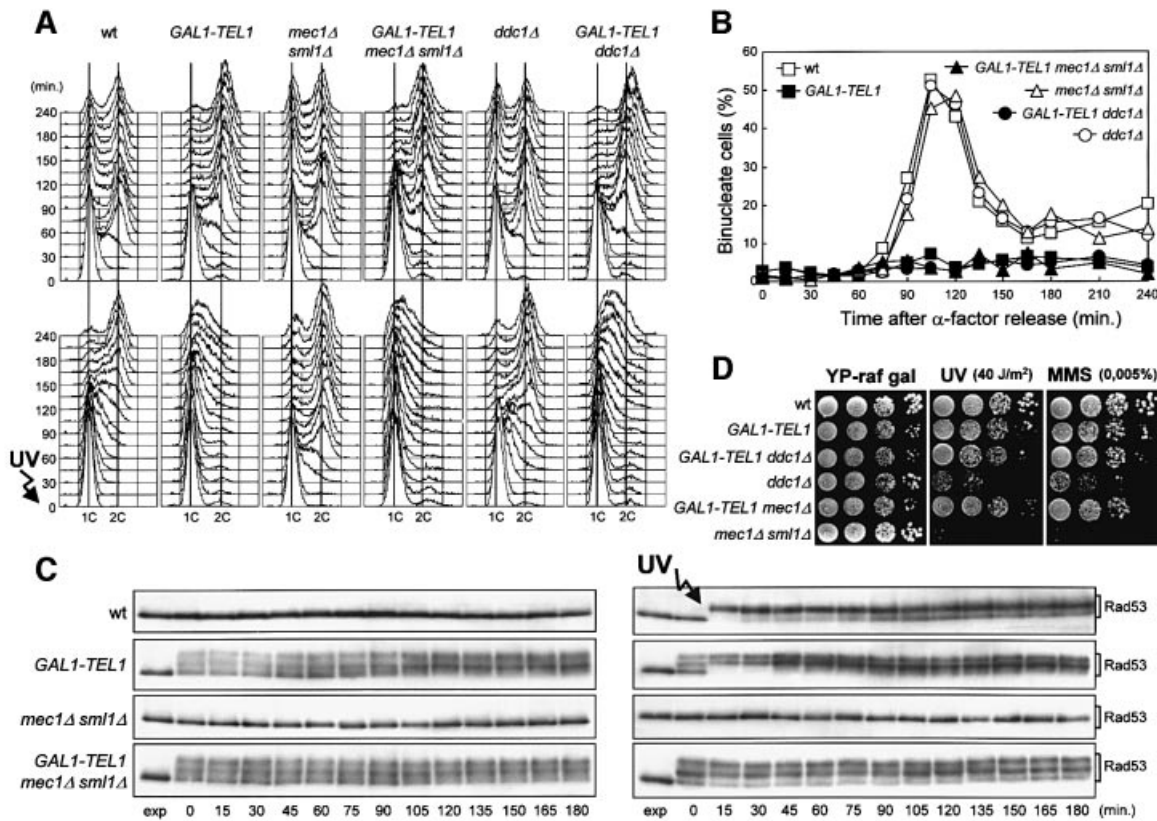
Fig. 3. Effects of *TEL1* overexpression in the absence of Mec1 or Ddc1. Strains were as follows: wild type (K699), *GAL1–TEL1* (DMP3539/10D), *mec1Δ sml1Δ* (YLL490), *GAL1–TEL1 mec1Δ sml1Δ* (DMP3562/2A), *ddc1Δ* (YLL244) and *GAL1–TEL1 ddc1Δ* (DMP3575/4A). (A–C) Cell cultures growing logarithmically in YEP-raf were synchronized in G$_1$ with α-factor in the presence of galactose (2 h) and released from the α-factor block at time zero in YEP-raf-gal, or were UV irradiated (40 J/m^2) prior to release in YEP-raf-gal. Samples were collected at the times indicated after α-factor release to analyze the DNA content of untreated (top) and UV-treated (bottom) cell cultures by FACS (**A**), to score the untreated cell cultures for the percentage of binucleate cells by propidium iodide staining (**B**) and to analyze protein extracts from the untreated (left) and UV-treated (right) cell cultures by western blotting using anti-Rad53 antibodies (**C**). exp, exponentially growing cells. (**D**) Serial dilutions of YEP-raf exponentially growing cell cultures were spotted on YEP-raf-gal plates with or without MMS at the concentration indicated. One YEP-raf-gal plate was UV irradiated (UV).

Delay of cell cycle progression and Rad53 phosphorylation caused by high levels of Tel1 do not require Mec1 and Ddc1

The data presented above indicate that an excess of Tel1 kinase causes Rad53, Chk1 and Rad9 phosphorylation, which may be responsible for the G$_2$/M cell cycle arrest in the absence of exogenous insults and for the dramatic slowing down of cell cycle progression in response to DNA damage. Since the G$_2$/M cell cycle arrest caused by Tel1 overproduction can be partially suppressed by increasing Mec1 levels, and an excess of Tel1 can suppress the hypersensitivity to genotoxic agents of *mec1Δ* cells, Mec1 should not be required for the Tel1-dependent cell cycle arrest. Indeed, neither the G$_2$/M cell cycle arrest nor the Rad53 phosphorylation caused by high levels of Tel1 requires Mec1. In fact, when cells were arrested in G$_1$ in the presence of galactose and then released from the block under galactose-induced conditions, *GAL1–TEL1 mec1Δ sml1Δ* as well as *GAL1–TEL1* cells were still arrested with 2C DNA content and undivided nuclei 240 min after release into the cell cycle, whereas both *mec1Δ sml1Δ* and wild-type cells progressed through the cell cycle with indistinguishable kinetics (Figure 3A, top and B). Moreover, phosphorylated Rad53 was clearly detectable in *GAL1–TEL1 mec1Δ*

sml1Δ cells throughout the experiment, while it was completely absent in wild-type and *mec1Δ sml1Δ* cells under the same conditions (Figure 3C, left).

Furthermore, overexpression of *TEL1* is epistatic to deletion of *MEC1* with respect to hypersensitivity to genotoxic agents (Sanchez *et al.*, 1996; Figure 3D), Rad53 phosphorylation (Figure 3C) and cell cycle progression after DNA damage (Figure 3A, bottom). In fact, when G$_1$-arrested cells were UV irradiated and then released into the cell cycle under galactose-induced conditions, *mec1Δ sml1Δ* cells completed DNA replication in 45–60 min, while both *GAL1–TEL1* and *GAL1–TEL1 mec1Δ sml1Δ* cell cultures showed a dramatic slowing down of entry and progression through S phase even compared with wild-type cells, which completed DNA replication after 180 min (Figure 3A, bottom). The DNA damage-induced slowing down of cell cycle progression paralleled the presence of phosphorylated Rad53 in UV-irradiated *GAL1–TEL1* and *GAL1–TEL1 mec1Δ sml1Δ* cells throughout the experiment, while Rad53 phosphorylation was completely absent in similarly treated *mec1Δ sml1Δ* cells (Figure 3C, right).

Since high levels of Tel1 induce a Rad53-dependent cell cycle arrest in the absence of exogenous DNA damage, we asked whether checkpoint proteins thought to be acting at
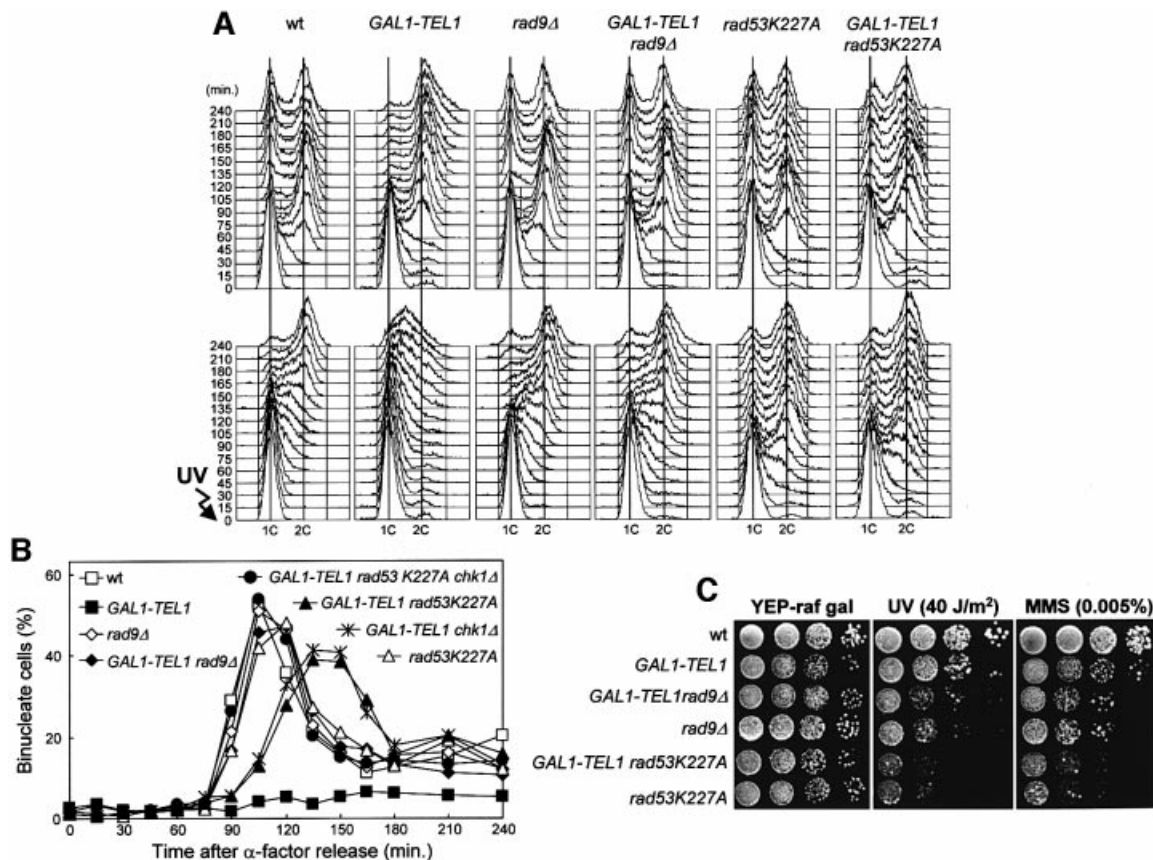
Fig. 4. Cell cycle delay caused by high levels of Tel1 involves Rad53, Rad9 and Chk1. Strains were as follows: wild type (K699), *GAL1–TEL1* (DMP3539/10D), *rad9Δ* (YLL157), *GAL1–TEL1 rad9Δ* (DMP3575/6B), *rad53K227A* (DMP3479/2A), *GAL1–TEL1 rad53K227A* (DMP3479/2B), *GAL1–TEL1 chk1Δ* (DMP3611/6D) and *GAL1–TEL1 rad53K227A chk1Δ* (DMP3611/3B). (A and B) Cell cultures growing logarithmically in YEP-raf were synchronized in G₁ with α-factor in the presence of galactose (2 h) and released from the α-factor block at time zero in YEP-raf-gal or were UV irradiated (40 J/m²) prior to release in YEP-raf-gal. Samples were collected at the times indicated after α-factor release to analyze the DNA content of untreated (top) and UV-treated (bottom) cell cultures by FACS (A) and to score the untreated cell cultures for the percentage of binucleate cells by propidium iodide staining (B). (C) Serial dilutions of YEP-raf exponentially growing cell cultures were spotted on YEP-raf-gal plates with or without MMS. One YEP-raf-gal plate was UV irradiated (UV).

early steps of DNA damage recognition, such as Ddc1, were required for the Tel1-dependent arrest. As shown in Figure 3A and B, when *GAL1–TEL1 ddc1Δ* cells were arrested in G₁ in galactose and then released from the G₁ block in galactose-containing medium, they arrested with 2C DNA content and undivided nuclei, while *ddc1Δ* cells underwent DNA replication and nuclear division with wild-type kinetics under the same conditions. Moreover, when the same cultures were UV irradiated before release from the G₁ block under galactose-induced conditions, *GAL1–TEL1 ddc1Δ* cells showed a dramatic slowing down of cell cycle progression in response to DNA damage, similar to that observed for *GAL1–TEL1* cells, while similarly treated *ddc1Δ* cells progressed through S phase faster than wild type (Figure 3A, bottom). Therefore, Ddc1 is not required for the cell cycle progression delay caused by high levels of Tel1. Accordingly, Tel1 overproduction is also epistatic to *ddc1Δ* with respect to hypersensitivity to genotoxic agents, since galactose-induced *GAL1–TEL1 ddc1Δ* cells were as sensitive as *GAL1–TEL1* cells and less sensitive than the otherwise isogenic *ddc1Δ* strains to both UV and MMS (Figure 3D).

Rad53, Rad9 and Chk1 are required for Tel1-dependent cell cycle arrest

Since high levels of Tel1 induce phosphorylation of Rad9, Chk1 and Rad53 in unperturbed conditions, and Rad9 and Rad53 have been implicated in a Tel1-dependent checkpoint (Usui *et al.*, 2001), we asked whether these proteins were required for the cell cycle delay caused by *TEL1* overexpression. We therefore analyzed cell cycle progression of *GAL1–TEL1* cells carrying the *rad9Δ*, *chk1Δ* or the kinase-defective *rad53K227A* alleles. Rad9 and Rad53 turned out to be necessary for the cell cycle delay caused by *TEL1* overexpression both in the presence and absence of DNA damage (Figure 4A and B). In fact, when galactose-induced G₁-arrested cells were released from the G₁ block in galactose-containing medium, the kinetics of entry and progression through S phase of *GAL1–TEL1 rad9Δ* and *GAL1–TEL1 rad53K227A* cells were comparable to those of *rad9Δ* and *rad53K227A* cells, respectively (Figure 4A, top). Furthermore, nuclear division occurred with wild-type kinetics in *GAL1–TEL1 rad9Δ* cells and with some delay in *GAL1–TEL1 rad53K227A* cells, while *GAL1–TEL1* cells under the same conditions were still arrested with undivided nuclei
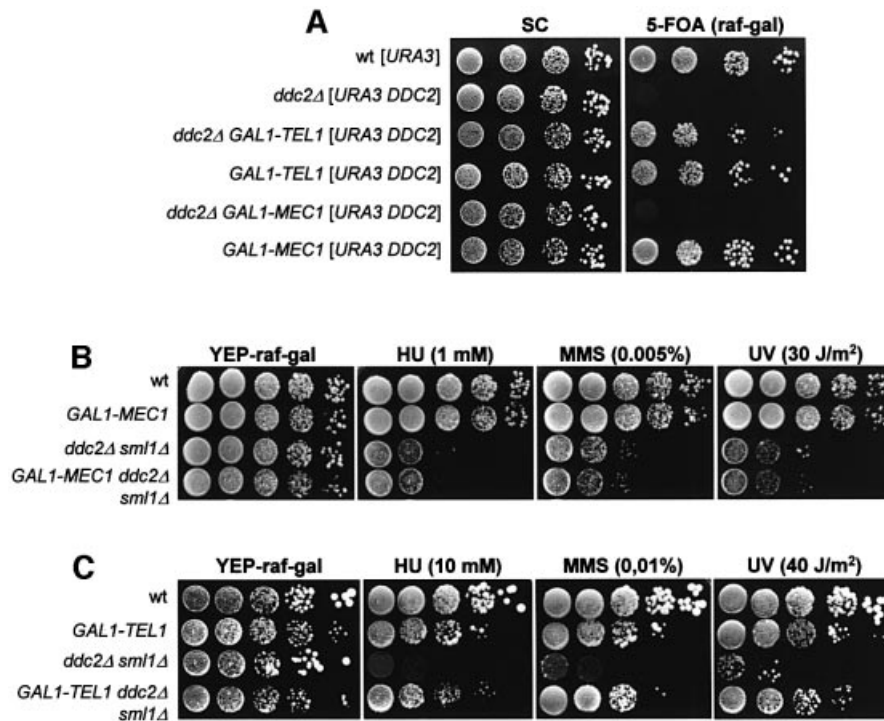
Fig. 5. Genetic interactions between *DDC2*, *MEC1* and *TEL1*. Strains were as follows: wild type [*URA3* YCplac33] (YLL827), *ddc2Δ* [*URA3 DDC2*] (YLL275), *ddc2Δ GAL1–TEL1* [*URA3 DDC2*] (DMP3475/3D), *GAL1–TEL1* [*URA3 DDC2*] (YLL943), *ddc2Δ* [*LEU2 GAL1–MEC1*] [*URA3 DDC2*] (YLL930), wild type [*LEU2 GAL1–MEC1*] [*URA3 DDC2*] (YLL944), *GAL1–MEC1* (YLL516), *ddc2Δ sml1Δ* (DMP2995/1B), *GAL1–MEC1 ddc2Δ sml1Δ* (DMP3532/8A), *GAL1–TEL1* (DMP3539/10D) and *GAL1–TEL1 ddc2Δ sml1Δ* (DMP3602/9C). Serial dilutions of YEP-raf exponentially growing cell cultures were spotted on Synthetic Complete (SC)-glucose and 5-Fluoro-orotic acid (5-FoA)-containing SC-raf-gal plates (**A**) or on YEP-raf-gal plates with or without MMS or HU at the concentrations indicated (**B** and **C**). One YEP-raf-gal plate was UV irradiated (UV).

after 240 min (Figure 4B). Similarly, when the same cultures were UV irradiated and then released from the G$_1$ block in galactose-containing medium, *GAL1–TEL1 rad9Δ* and *GAL1–TEL1 rad53K227A* cells replicated their DNA with kinetics indistinguishable from those of the *rad9Δ* and *rad53K227A* mutants, and much faster than *GAL1–TEL1* cells, which dramatically slowed down DNA replication even compared with a similarly treated wild-type strain (Figure 4A, bottom). In agreement with the observation that high levels of Tel1 were unable to suppress the checkpoint defects of *rad9Δ* and *rad53K227A* mutants, *TEL1* overexpression did not suppress the hypersensitivity to genotoxic agents of *rad9Δ* and *rad53K227A* cells (Figure 4C). In fact, UV and MMS sensitivities of galactose-induced *GAL1–TEL1 rad9Δ* and *GAL1–TEL1 rad53K227A* cells were indistinguishable from those of the otherwise isogenic *rad9Δ* and *rad53K227A* strains (Figure 4C).

Although the *rad53K227A* mutation completely abolished the dramatic slowing down of entry and progression through S phase of *GAL1–TEL1* cells after UV irradiation, *GAL1–TEL1 rad53K227A* cells still showed a delay of nuclear division in unperturbed conditions compared with the *rad53K227A* mutants (Figure 4B). This suggests that cell cycle arrest in G$_2$ caused by *TEL1* overexpression in the absence of exogenous insults might at least partially depend on proteins acting independently of Rad53. We therefore asked whether it might involve the Chk1 kinase, which is phosphorylated in Tel1-overproducing cells and is specifically required to prevent anaphase entry in *cdc13*

mutants at restrictive temperature, independently of Rad53 (Sanchez *et al.*, 1999). Indeed, when galactose-induced *GAL1–TEL1 chk1Δ* cells were released from G$_1$ arrest in galactose-containing media, they underwent nuclear division with kinetics comparable to that of *GAL1–TEL1 rad53K227A* cells, while *GAL1–TEL1 rad53K227A chk1Δ* cells divided nuclei with wild-type kinetics and therefore faster than either *GAL1–TEL1 rad53K227A* or *GAL1–TEL1 chk1Δ* cells under the same conditions (Figure 4B). This indicates that high levels of Tel1 require both Rad53 and Chk1 to arrest nuclear division completely in the absence of DNA damage.

TEL1, but not MEC1, overexpression suppresses cell lethality and hypersensitivity to genotoxic agents of ddc2Δ cells

Our data indicate that *TEL1* overexpression causes Mec1-independent unscheduled phosphorylation of Rad53 and hyperactivation of the checkpoint response, while cell cycle progression and Rad53 phosphorylation in both undamaged and UV-irradiated Mec1-overproducing cells are indistinguishable from wild type (Figures 1 and 2), suggesting that other proteins could be rate limiting for Mec1 activity. A likely candidate for this role could be Ddc2, since it physically interacts with Mec1 and may be required for Mec1 but not for Tel1 functions. As shown in Figure 5, this hypothesis is further strengthened by the observation that while *TEL1* overexpression can suppress cell lethality and hypersensitivity to genotoxic agents caused by deletion of *DDC2*, high levels of Mec1 can not
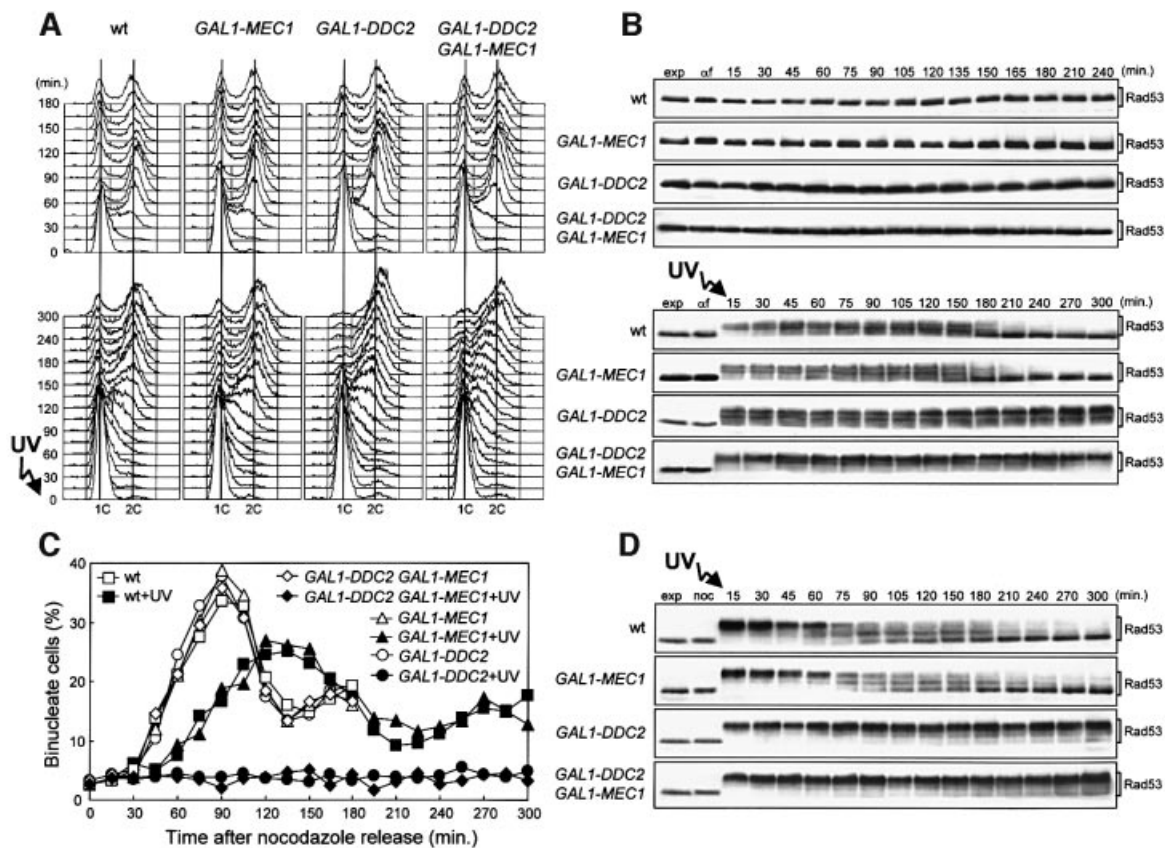
Fig. 6. *DDC2* overexpression leads to prolonged G₂/M cell cycle arrest after checkpoint activation. Strains were as follows: wild type [*URA3* YCplac33] (YLL827), wild type [*URA3 GAL1–MEC1*] (YLL826), *GAL1–DDC2* [*URA3* YCplac33] (YLL837) and *GAL1–DDC2* [*URA3 GAL1–MEC1*] (YLL836). (A and B) Cell cultures growing logarithmically in YEP-raf were synchronized with α-factor. Galactose was added 2.5 h before α-factor addition. α-factor-synchronized cells were released from the block at time zero [(B), αf] in YEP-raf-gal or were UV irradiated (40 J/m²) prior to release in YEP-raf-gal. Samples of untreated (top) and UV-treated (bottom) cell cultures were collected at the times indicated after α-factor release to analyze the DNA content by FACS (**A**) and protein extracts by western blotting, using anti-Rad53 antibodies (**B**). (C and D) Cell cultures growing logarithmically in YEP-raf were synchronized with nocodazole. Galactose was added 2 h before nocodazole addition. Nocodazole-synchronized cells were released from the block at time zero [(D), noc] in YEP-raf-gal or were UV irradiated (50 J/m²) prior to release in YEP-raf-gal. Samples of untreated and UV-treated cell cultures were collected at the times indicated after nocodazole release to score for the percentage of binucleate cells by propidium iodide staining (**C**) and to analyze protein extracts from the UV-treated cell cultures by western blotting, using anti-Rad53 antibodies (**D**). exp, exponentially growing cells. In all the experiments, samples were withdrawn from the UV-treated cultures at times zero and 120 min, and appropriate dilutions were plated on YEPD plates to score for colony-forming units (see text for the percentage of cell survival).

bypass the requirement for Ddc2. In fact, galactose induction of a *GAL1–MEC1* fusion did not allow *GAL1–MEC1 ddc2Δ* cells to lose the *URA3* centromeric plasmid carrying the wild-type *DDC2* allele and did not change the sensitivity to HU, MMS and UV of *ddc2Δ sml1Δ* cells (Figure 5A and B). Conversely, galactose-induced *GAL1–TEL1 ddc2Δ* cells were able to lose the plasmid-borne *DDC2* allele (Figure 5A), and *GAL1–TEL1 ddc2Δ sml1Δ* cells were less sensitive to HU, MMS and UV than otherwise isogenic *ddc2Δ sml1Δ* cells in galactose-containing medium (Figure 5C). Since both Mec1 and Tel1 are protein kinases (Mallory and Petes, 2000; Paciotti *et al.*, 2000), high levels of Tel1 may be able to overcome the lack of Ddc2 by replacing Mec1 activity, thus implying that Mec1 functions, but not Tel1 functions, are impaired in the absence of Ddc2.

DDC2 overexpression causes an irreversible G₂/M arrest after DNA damage

Since Ddc2 may be the rate-limiting factor for Mec1 activity, we analyzed cell cycle progression and Rad53 phosphorylation of cells overexpressing a *GAL1–DDC2*

fusion integrated in single copy at the *LEU2* locus. When galactose-induced cell cultures were blocked in G₁ or G₂ and then released in galactose-containing medium in the absence of DNA damage, kinetics of entry and progression through S phase (Figure 6A, top) and of nuclear division (Figure 6C) were similar in *GAL1–DDC2* and wild-type cells. Moreover, we could not detect Rad53 phosphorylated forms in *GAL1–DDC2* cells after release from G₁ (Figure 6B, top) or G₂ arrests (data not shown) under galactose-induced conditions. Therefore, unlike Tel1 overproduction, high levels of Ddc2 are not able to activate the checkpoint in the absence of DNA damage.

Since the Mec1–Ddc2 complex may be activated by exogenous DNA damage, we then analyzed the response to DNA damage of Ddc2-overproducing cells. Cells with very high levels of Ddc2 due to multiple *GAL1–DDC2* fusions integrated at the *LEU2* locus had previously been reported to have a slight defect in slowing down DNA replication after DNA damage (Paciotti *et al.*, 2000). Conversely, when galactose-induced cell cultures carrying a single copy of the *GAL1–DDC2* fusion integrated at the *LEU2* locus were blocked in G₁, UV irradiated, and

then released from the G_1 arrest in galactose-containing medium, the kinetics of entry and progression through S phase were similar to those observed in wild-type cells under the same conditions (Figure 6A, bottom). However, Rad53 phosphorylated forms began to disappear in wild-type and *GAL1–MEC1* cultures when DNA replication was completed (180 min after UV irradiation in G_1; Figure 6B, bottom) and viability remained high (70% survival). Conversely, UV-treated *GAL1–DDC2* cells, which dramatically lost viability (5% survival), still contained high amounts of phosphorylated Rad53 (Figure 6B, bottom) and were mostly arrested with a 2C DNA content (Figure 6A, bottom) and undivided nuclei (data not shown) 300 min after release into the cell cycle. Moreover, when galactose-induced cell cultures were arrested in G_2, UV irradiated and then released from the G_2 block in galactose-containing medium (Figure 6C and D), *GAL1–DDC2* cells lost viability (7% survival) and did not divide nuclei throughout the experiment (300 min), while wild-type cells were mostly viable (82% survival) and underwent nuclear division ~60 min after UV irradiation. Thus, cells containing high levels of Ddc2 appear to be impaired in undergoing the G_2/M transition after DNA damage in G_1 or G_2. This effect was probably due to the cells' inability to turn off the checkpoint response, since high amounts of phosphorylated Rad53 were present in *GAL1–DDC2* cells until the end of both experiments, while phosphorylated Rad53 was largely absent 210 min after UV irradiation in wild-type cells (Figure 6B, bottom and D).

The above data show that high levels of Ddc2 cause permanent Rad53 phosphorylation and inhibit nuclear division after DNA damage, while the same increase in the amount of Ddc2 is not sufficient to block DNA replication of a damaged template, raising the possibility that, despite Ddc2 high levels, the amount of Mec1 may be limiting under these conditions. This hypothesis is further supported by the finding that co-overexpression of *MEC1* and *DDC2* also impairs the ability of cells to replicate a damaged template (Figure 6A, bottom). In fact, galactose-induced *GAL1–MEC1 GAL1–DDC2* cells released from G_1 after UV irradiation entered and progressed through S phase much more slowly than wild-type and *GAL1–DDC2* cells under the same conditions. This suggests that while high levels of Ddc2 are sufficient to arrest cells at the G_2/M transition, it is necessary to increase the levels of both Mec1 and Ddc2 proteins to arrest DNA replication after DNA damage.

The DNA damage-induced cell cycle arrest caused by high levels of Ddc2 requires Mec1, Ddc1, Rad53 and Rad9

Since high levels of Tel1 can activate the G_2/M checkpoint in the absence of genotoxic agents, while overproduction of Ddc2 causes a permanent cell cycle arrest in G_2 only if DNA has undergone exogenous damage, we asked whether the DNA damage-induced cell cycle arrest mediated by *DDC2* overexpression may require proteins different from those involved in the Tel1-dependent arrest. If the DNA damage-induced arrest at the G_2/M transition in Ddc2-overproducing cells was due to increased Mec1 activity and dependent on the persistence of Rad53 phosphorylated forms, we expected both Mec1 and Rad53

to be absolutely required for this cell cycle arrest. Indeed, when cell cultures were arrested in G_2, UV irradiated, and then released from the G_2 block in galactose-containing medium, nuclear division occurred in *GAL1–DDC2 mec1Δ sml1Δ* and *GAL1–DDC2 rad53Δ sml1Δ* cells with kinetics indistinguishable from those of *mec1Δ sml1Δ* and *rad53Δ sml1Δ* mutants (Figure 7A and B). Moreover, Mec1 was also essential for Rad53 phosphorylation in *DDC2*-over-expressing cells, since we could not detect Rad53 phosphorylated forms in galactose-induced *GAL1–DDC2 mec1Δ sml1Δ* cells released from the G_2 arrest after UV irradiation, while they were present throughout the experiment in similarly treated *GAL1–DDC2* cells (Figure 7C).

As shown in Figure 7D, Ddc1 and Rad9 are also required for the DNA damage-induced G_2/M arrest caused by high levels of Ddc2. In fact, nuclear division in galactose-induced *GAL1–DDC2 ddc1Δ* and *GAL1–DDC2 rad9Δ* cells released from the G_2 arrest after UV irradiation occurred with kinetics comparable to those observed in similarly treated *rad9Δ* and *ddc1Δ* cells.

Discussion

The cells must be aware not only of damage, but also of when DNA repair is completed, in order to terminate the checkpoint response and resume cell cycle progression. A key unanswered question is how cells turn off the checkpoint after signals have been removed. It is possible that repair of DNA damage *per se* might restore normal cell cycle progression and/or active mechanisms might be required to shut off the checkpoint. For example, down-stream effectors might become insensitive to the kinase cascade, and/or the checkpoint pathway might be inactivated by dephosphorylating the Rad53 kinase itself. Furthermore, since Mec1 and its interacting factor Ddc2 are required to phosphorylate and activate Rad53, inactivation of the Mec1–Ddc2 complex may be critical to turn off Rad53 and subsequently the checkpoint response. Finally, the Mec1 homolog Tel1, which has been implicated in a Rad53-dependent DNA damage checkpoint pathway that parallels the canonical Mec1 pathway (Usui *et al.*, 2001), might also play a role in termination of the checkpoint response.

A possible way to approach this problem is to study whether and how overproduction of protein kinases and kinase-interacting factors acting upstream of Rad53 can affect cell cycle progression in the absence or in the presence of DNA damage. As discussed below, we found that the levels of Tel1, Mec1 and Ddc2 are important to regulate both activation of the checkpoint and resumption of cell cycle progression after DNA damage-induced cell cycle arrest.

Checkpoint activation in Tel1-overproducing cells

A major difference between the effects of Ddc2 and Tel1 overproduction is that high levels of Tel1 cause phosphorylation of Rad53 and G_2/M cell cycle arrest in the absence of exogenous DNA damage, while an excess of Ddc2 has the same effects only when DNA has suffered external insults. The Rad53-dependent cell cycle arrest caused by Tel1 overproduction under otherwise unperturbed conditions may be due to the presence of high levels
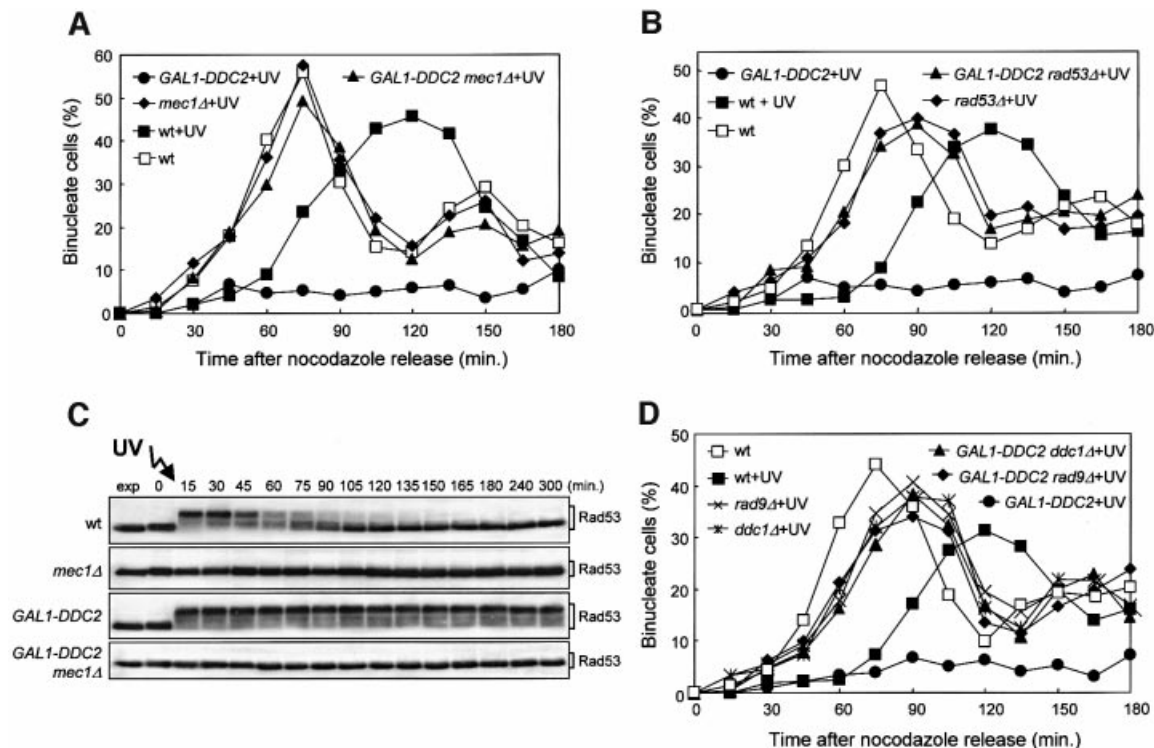
Fig. 7. Requirement of checkpoint genes for the Ddc2-dependent DNA damage-induced cell cycle arrest. Strains were as follows: wild type (K699), *GAL1–DDC2* (YLL279.2), *mec1Δ sml1Δ* (YLL490), *GAL1–DDC2 mec1Δ sml1Δ* (DMP3462/2B), *rad53Δ sml1Δ* (YLL509), *GAL1–DDC2 rad53Δ sml1Δ* (DMP3356/4A), *rad9Δ* (YLL157), *GAL1–DDC2 rad9Δ* (DMP3392/4A), *ddc1Δ* (YLL244) and *GAL1–DDC2 ddc1Δ* (DMP3463/10C). Cell cultures growing logarithmically in YEP-raf were synchronized with nocodazole. Galactose was added 2 h before nocodazole addition and nocodazole-synchronized cells were released from the block at time zero in YEP-raf-gal or were UV irradiated (50 J/m^2) prior to release in YEP-raf-gal. (**A**, **B** and **D**) Untreated and UV-treated cell cultures were scored at the times indicated for the percentage of binucleate cells by propidium iodide staining. (**C**) Protein extracts from the UV-treated cell cultures were analyzed by western blotting, using anti-Rad53 antibodies. exp, exponentially growing cells.

of active Tel1 kinase resulting in unscheduled phosphorylation events, which in turn cause G$_2$/M transition delay. It is possible that an excess of Tel1 might lead to activation of a Rad53-dependent checkpoint by causing accumulation of DNA damaged molecules and impairment of repair capacity. However, the Mec1 kinase, which has been shown to play a primary role in activation of the checkpoint in response to different kinds of DNA alterations, is not required for the Tel1-dependent cell cycle arrest and Rad53 phosphorylation. Furthermore, Ddc1, which together with Rad24, Rad17 and Mec3 is thought to act at the beginning of the checkpoint pathway by recognizing changes in DNA structure and initiating the Mec1-dependent signal transduction cascade (reviewed in Longhese *et al.*, 1998), is neither phosphorylated nor required for checkpoint activation in Tel1-overproducing cells. This indicates that proteins presumably acting together with Mec1 in the DNA damage-sensing steps of the pathway are not required to support Tel1 functions. We therefore suggest that either overproduction of Tel1 *per se* might result in increased Tel1 kinase activity that could be sufficient to elicit unscheduled phosphorylation events primarily inhibiting the G$_2$/M transition, or high levels of Tel1 can be activated by endogenous DNA damage that is not sensed in the presence of physiological levels of this kinase. In any case, the Tel1-dependent cell cycle arrest requires both Rad53 and Chk1, which are known to cooperate in the activation of the G$_2$/M DNA damage

checkpoint (Sanchez *et al.*, 1999), and Rad9, whose phosphorylation and interaction with Rad53 are required for Rad53 activation (Emili, 1998; Sun *et al.*, 1998; Vialard *et al.*, 1998; Gilbert *et al.*, 2001). Furthermore, Rad9, Rad53 and Chk1 are phosphorylated in Tel1-overproducing cells in the absence of exogenous DNA damage, indicating a tight correlation between phosphorylation of these proteins and their requirement for the Tel1-dependent G$_2$/M arrest. Since it has recently been shown that Rad9 phosphorylation triggers Rad53 activation (Gilbert *et al.*, 2001), it is possible that unregulated levels of Tel1 result in hyperphosphorylation of Rad9, which in turn catalyzes Rad53 phosphorylation, resulting in ectopic checkpoint activation.

It is also worth noting that Tel1 overproduction suppresses the checkpoint defects and the hypersensitivity to DNA-damaging agents of *mec1Δ* cells. Therefore, increasing the levels of Tel1 uncovers its role in the checkpoint cascade both in the presence and absence of Mec1. In contrast to Mec1, Tel1 functions do not require Ddc2. In fact, *TEL1*, but not *MEC1*, overexpression can suppress hypersensitivity to genotoxic agents of *ddc2Δ* cells, implying that high levels of Tel1 can bypass the requirement for Ddc2. Moreover, the fact that Ddc2 can be co-immunoprecipitated with Mec1, while we were not able to co-immunoprecipitate Tel1 with Ddc2 even in the absence of Mec1 (our unpublished results), indicates that the Ddc2–Mec1 interaction is, in any case, preferential.

Interestingly, overexpression of *MEC1* can partially suppress the effect of Tel1 overproduction on cell cycle progression and greatly reduces the unscheduled Tel1-dependent Rad53 phosphorylation in the absence of exogenous DNA damage, suggesting that high levels of Tel1 might titrate out factors interacting with Mec1 also in the absence of external insults, thus leading to unregulated and signal-independent checkpoint activation.

DNA damage exacerbates all the phenotypes caused by *TEL1* overexpression in the absence of exogenous damage. In fact, Tel1-overproducing cells undergo a dramatic delay in S-phase progression and persistent Rad53 phosphorylation after DNA damage in G_1, suggesting that checkpoint activation by an excess of Tel1 in the absence of external insults is not maximal and can be increased by damaging the DNA. This increase is also thought to impair the ability of the cell to recover from the checkpoint-mediated arrest, since Tel1-overproducing cells are hypersensitive to genotoxic agents. Similarly to the results observed in the absence of exogenous DNA damage, Ddc1 and Mec1 are not required for the DNA damage-induced cell cycle arrest in Tel1-overproducing cells, which instead requires Rad53 and Rad9. Moreover, *TEL1* overexpression can partially suppress the hypersensitivity to genotoxic agents of *mec1* and *ddc1* mutants, while it has no effect on that of *rad53* and *rad9* mutants. Accordingly, recent data have implicated Tel1 together with the Mre11 complex in triggering Rad53 activation and its interaction with Rad9 in response to DNA damage independently of Mec1 and Rad24 (Usui *et al.*, 2001).

High amounts of Ddc2 impair the ability of the cell to recover from DNA damage-induced cell cycle arrest

Neither *MEC1* nor *DDC2* overexpression alters cell cycle progression or induces Rad53 phosphorylation in the absence of exogenous DNA damage, suggesting that activation by DNA structure signals might be essential for these proteins to function in the checkpoint. In contrast, Tel1 overproduction turns on the checkpoint response independently of external damage and presumed damage sensors, but requires other components of the pathway that function in the signal transduction cascade. Therefore, Tel1 escapes the normal rigorous regulation imposed upon Mec1 and Ddc2, which appear to be normally responsible for the checkpoint response. Moreover, UV-irradiated Ddc2-overproducing cells undergo a prolonged arrest at the G_2/M transition, and this correlates with persistent Rad53 phosphorylation and cell death. Since these effects of Ddc2 overproduction are seen only in the presence of Mec1, it is tempting to suggest that high levels of Mec1 *per se* do not cause any detectable phenotype under the same conditions because Ddc2 may be rate limiting for its activation. Moreover, the effects of Ddc2 overproduction are limited to the DNA damage checkpoint controlling the G_2/M transition. In fact, the same excess of Ddc2 causing DNA damage-induced arrest of nuclear division is not sufficient to stop S-phase progression after DNA damage in G_1, suggesting that the checkpoint responses in different cell cycle phases might have different requirements for Mec1–Ddc2 activity. This hypothesis is further supported by the finding that overexpression of *MEC1* in Ddc2-overproducing cells causes a further slowing down of S-phase progression after DNA damage in G_1 compared with wild-type cells. Thus, Mec1 activity becomes rate limiting for hyperactivation of the intra-S checkpoint despite the excess of Ddc2, while this does not appear to be the case for the G_2/M transition arrest. Accordingly, we previously showed that overproduction of Mec1 kinase-deficient variants has dominant-negative effects on S-phase entry and progression after DNA damage in G_1, but it does not abrogate the G_2/M checkpoint (Paciotti *et al.*, 2001). In addition, we described two hypomorphic *mec1* mutants that were completely defective in the G_1/S and intra-S DNA damage checkpoints, but they were able to activate the G_2/M checkpoint (Paciotti *et al.*, 2001). Altogether these data strengthen the hypoth-esis that the threshold level of Mec1 activity required to slow down replication of a damaged DNA template is higher than that required to block the G_2/M transition in the presence of damaged chromosomes.

The finding that both Ddc1 and Rad9 are required for the prolonged DNA damage-induced G_2/M arrest caused by *DDC2* overexpression indicates that this arrest involves the activation of the canonical DNA damage checkpoint pathway, and that the ability to turn it off is impaired in Ddc2-overproducing cells. The requirement of Ddc1 for the Ddc2-dependent, but not for the Tel1-dependent, cell cycle arrest indicates that Ddc1 is necessary to activate the Ddc2–Mec1 complex in the presence of DNA damage despite the high Ddc2 levels, thus specifically linking DNA damage sensing with Ddc2-mediated hyperactivation of the checkpoint pathway. The hypersensitivity to genotoxic agents of *DDC2*-overexpressing cells may then be the consequence of their inability to recover from the DNA damage-induced cell cycle arrest.

In summary, although with different features, overproduction of either Tel1 or Ddc2 results in prolonged delay of cell cycle progression, indicating that unbalancing the levels of checkpoint kinases or kinase-interacting factors may hyperactivate the checkpoint. In this view, other proteins important for dephosphorylating Mec1–Ddc2, Tel1 or Rad53 itself may then become rate limiting for recovery from the checkpoint-mediated cell cycle arrest in the presence of unregulated kinase levels. Finally, our finding that the cell cycle arrest caused by Ddc2 and Tel1 overproduction requires Rad53 and correlates with its enhanced and persistent phosphorylation strengthens the hypothesis that unscheduled Rad53 phosphorylation may prevent cells from re-entering the cell cycle after a checkpoint-mediated cell cycle arrest. A search for multicopy suppressors of the cell cycle arrest caused by Tel1 and Ddc2 overproduction may allow the identific-ation of proteins specifically required for termination of the checkpoint response.

Materials and methods

Plasmids, yeast strains and media
The genotypes of all the yeast strains used in this study are listed in Table I. All yeast strains were derivatives of W303 (*MAT*a or *MAT*α, *ade2-1, can1-100, trp1-1, leu2-3,112, his3-11,15, ura3*).

To obtain plasmid pML284.36, containing a *TEL1* DNA fragment spanning from the ATG (+1) to position +1476 fused to the *GAL1* promoter, the 1476 bp *TEL1* fragment was amplified by PCR using yeast genomic DNA as a template, the oligonucleotides PRP222 (5′-CGCGGATCCATATGGAGGATCATGGGATTGTAGAAACT-3′) and

Table I. *Saccharomyces cerevisiae* strains used in this study

Strain	Genotype	Reference/source
K699	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3*	Longhese *et al.* (1997)
K700	*MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3*	Longhese *et al.* (1997)
YLL157	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad9Δ::URA3*	Longhese *et al.* (1996)
YLL244	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ddc1Δ::KanMX4*	Longhese *et al.* (1997)
YLL275	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ddc2Δ::KanMX4* [pML94 *URA3 CEN4 DDC2*]	Paciotti *et al.* (2000)
YLL279.2	*MAT**a** ade2-1 can1-100 his3-11,15 trp1-1 ura3 leu2-3,112::GAL1–DDC2::LEU2*	this study
YLL490	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1Δ::HIS3 sml1Δ::KanMX4*	Longhese *et al.* (2000)
YLL516	*MAT**a** ade2-1 can1-100 his3-11,15 trp1-1 leu2-3 112 ura3::GAL1–MEC1::URA3*	Paciotti *et al.* (2001)
YLL509	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3 112 trp1-1 ura3 rad53Δ::HIS3 sml1Δ::KanMX4*	Longhese *et al.* (2000)
YLL826	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3* [pML225 *URA3 CEN4 GAL1–MEC1*]	this study
YLL827	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3* [YCplac33 *URA3 CEN4*]	this study
YLL836	*MAT**a** ade2-1 can1-100 his3-11,15 trp1-1 ura3 leu2-3,112::GAL1–DDC2::LEU2* [pML225 *URA3 CEN4 GAL1–MEC1*]	this study
YLL837	*MAT**a** ade2-1 can1-100 his3-11,15 trp1-1 ura3 leu2-3,112::GAL1–DDC2::LEU2* [YCplac33 *URA3 CEN4*]	this study
YLL848	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 TEL1-HA3::URA3::tel1*	this study
YLL930	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ddc2Δ::KanMX4* [pML94 *URA3 CEN4 DDC2*] [pML240 *LEU2 CEN4 GAL1–MEC1*]	this study
YLL932	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 GAL1–TEL1-HA3::LEU2::tel1*	this study
YLL943	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 GAL1–TEL1::LEU2::tel1* [pML94 *URA3 CEN4 DDC2*]	this study
YLL944	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3* [pML94 *URA3 CEN4 DDC2*] [pML240 *LEU2 CEN4 GAL1–MEC1*]	this study
DMP2995/1B	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ddc2Δ::KanMX4 sml1Δ::KanMX4*	Paciotti *et al.* (2000)
DMP3356/4A	*MAT**a** ade2-1 can1-100 his3-11,15 trp1-1 ura3 leu2-3,112::GAL1–DDC2::LEU2* *rad53Δ::HIS3 sml1Δ::KanMX4*	this study
DMP3392/4A	*MAT**a** ade2-1 can1-100 his3-11,15 trp1-1 ura3 leu2-3,112::GAL1–DDC2::LEU2 rad9Δ::URA3*	this study
DMP3462/2B	*MAT**a** ade2-1 can1-100 his3-11,15 trp1-1 ura3 leu2-3,112::GAL1–DDC2::LEU2 mec1Δ::HIS3* *sml1Δ::KanMX4*	this study
DMP3463/10C	*MAT**a** ade2-1 can1-100 his3-11,15 trp1-1 ura3 leu2-3,112::GAL1–DDC2::LEU2 ddc1Δ::KanMX4*	this study
DMP3475/3D	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ddc2Δ::KanMX4 GAL1–TEL1::LEU2::tel1* [pML94 *URA3 CEN4 DDC2*]	this study
DMP3479/2A	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53K227A::KanMX4*	this study
DMP3479/2B	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53K227A::KanMX4* *GAL1–TEL1::LEU2::tel1*	this study
DMP3532/8A	*MAT**a** ade2-1 can1-100 his3-11,15 trp1-1 leu2-3,112 ura3::GAL1–MEC1::URA3* *ddc2Δ::KanMX4 sml1Δ::KanMX4*	this study
DMP3539/9D	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3::GAL1–MEC1::URA3* *GAL1–TEL1::LEU2::tel1*	this study
DMP3539/10D	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 GAL1–TEL1::LEU2::tel1*	this study
DMP3562/2A	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 mec1Δ::HIS3 sml1Δ::KanMX4* *GAL1–TEL1::LEU2::tel1*	this study
DMP3575/4A	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 ddc1Δ::KanMX4 GAL1–TEL1::LEU2::tel1*	this study
DMP3575/6B	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad9Δ::URA3 GAL1–TEL1::LEU2::tel1*	this study
DMP3598/6A	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC2-HA3::URA3* *DDC1-HA2::LEU2::ddc1 CHK1-MYC18::HIS3 GAL1–TEL1::LEU2::tel1*	this study
DMP3598/21D	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 DDC2-HA3::URA3* *DDC1-HA2::LEU2::ddc1 CHK1-MYC18::HIS3*	this study
DMP3602/9C	*MAT**a** ade2-1 can1-100 his3-11,15 trp1-1 leu2-3,112 ura3 GAL1–TEL1::URA3::tel1* *ddc2Δ::KanMX4 sml1Δ::KanMX4*	this study
DMP3611/3B	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 rad53K227A::KanMX4* *chk1Δ::HIS3 GAL1–TEL1::LEU2::tel1*	this study
DMP3611/6D	*MAT**a** ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3 chk1Δ::HIS3 GAL1–TEL1::LEU2::tel1*	this study

Plasmids are indicated in square brackets.

PRP223 (5′-GCTCTAGAGTAAGCAAACTTTAAATAGGCGTGCCA-3′) as primers, and *Pfu* polymerase (Stratagene). The PCR amplification product was then cloned into the *Bam*HI–*Xba*I sites of plasmid pML95 (Longhese *et al.*, 1997) and controlled by DNA sequence analysis. To construct plasmid pML285, used to generate the *TEL1-HA3* allele, a *TEL1* fragment spanning from position –1000 to position +900 from the translation initiation codon was amplified by PCR using genomic DNA as template and oligonucleotides PRP213 (5′-GGAATTCAATCATA-CACGGCAAGCATA-3′) and PRP214 (5′-GGAATTCCTTAAGCGC-AGTAGAATGTA-3′) as primers, and then cloned into the *Eco*RI site of plasmid YIplac211 (Gietz and Sugino, 1988). A *Not*I restriction site was then introduced by PCR after the *TEL1* codon 2 and sequences encoding three tandem hemagglutinin (HA3) epitopes were inserted into it, thus

giving rise to plasmid pML285. Plasmid pML322, used to generate the *GAL1–TEL1-HA3* allele, was originated by inserting the 1012 bp *Hin*dIII *TEL1* fragment from pML285 into the *Hin*dIII site of plasmid pML284.

Strain YLL850.2, carrying one copy of the *GAL1–TEL1* fusion integrated at the *TEL1* locus, was obtained by transforming strain K699 with *Blp*I-digested plasmid pML284.36, and then crossed with strain DMP2760/1A (Longhese *et al.*, 2000) to obtain the meiotic segregants DMP3479/2A, DMP3479/2B and DMP3479/1D. Strains YLL848 and YLL932, carrying one copy of the *TEL1-HA3* or *GAL1–TEL1-HA3* alleles at the *TEL1* locus, were obtained by transforming strain K699 with *Mfe*I-digested plasmid pML285 and *Blp*I-digested plasmid pML322, respectively. The kinetics of cell cycle progression of these strains were

indistinguishable from those of strains K699 and DMP3539/10D, respectively, under both uninduced and galactose-induced conditions. To generate the *CHK1* chromosomal deletion, a *chk1Δ::HIS3* cassette was constructed by PCR using pFA6a-*HIS3* plasmid (Wach *et al*., 1994) as template, and oligonucleotides PRP190 (5′-TATCATAAGTTG-CTGTATATGGGCAGCACGTATTACTATGAGTCTCGTACGCTGC-AGGTCGAC-3′) and PRP191 (5′-TGTCTCCATTTTTTTCAGTTG-GGAATTAGGATAATATCCCTACAGATAGTATCGATGAATTCG-AGCTCG-3′) as primers, followed by transformation of strain K699 with the PCR product, giving rise to strain YLL736, where the 1540 bp of the *CHK1* coding region were replaced by the *Kluyveromyces lactis HIS3* gene. Strains DMP3611/3B and DMP3611/6D were meiotic segregants from a cross between strains YLL736 and DMP3479/1D. Strain YLL850.2 was also crossed with strain K700, and the derivative DMP3469/2B meiotic segregant was then crossed to strain YLL516 to obtain the DMP3539/9D and DMP3539/10D meiotic segregants. Strain DMP3469/2B was also crossed with strains YLL490 (Longhese *et al*., 2000), YLL244 (Longhese *et al*., 1997) and YLL157 (Longhese *et al*., 1996) to obtain the DMP3562/2A, DMP3575/4A and DMP3575/6B meiotic segregants, respectively. Strain YLL922, carrying the *CHK1-MYC18* allele at the *CHK1* chromosomal locus, was generated by the PCR one-step tagging method (Knop *et al*., 1999) using, respectively, plasmid 3746 (K.Nasmyth; IMP, Vienna) as the template and oligonucleotides PRP217 (5′-CTTTAGAATGGAGAAGATTGTTCA-AGAAAATTTCAACTATCTGTAGGGATATTATCCTAATTCCCA-ACTCCGGTTCTGCTGCTAG-3′) and PRP218 (5′-ATAAGTAGA-AAGAATTTTTTTTTTTTTTTGATCAGTGCATCTTAACCCTTCT-TTTGTCTCCATTTTTTCCTCGAGGCCAGAAGAC-3′) as primers. Since *CHK1* alterations do not cause obvious phenotypes, but impair Pds1 phosphorylation (Sanchez *et al*., 1999), we verified that DNA damage-induced Pds1 phosphorylation was unaffected in *CHK1-MYC18* cells. Strains DMP3598/6A and DMP3598/21D were derived from crossing strains YLL922 and DMP3548/3B, which was derived from a cross between strains DMP3198/1A (Paciotti *et al*., 2000) and DMP3469/2B.

Strain YLL279.2, carrying one copy of the *GAL1–DDC2* fusion integrated at the *LEU2* locus, was obtained by transforming strain K699 with *Cla*I-digested plasmid pML105 (Paciotti *et al*., 2000). Strains YLL826 and YLL827 were obtained by transforming strain K699 with plasmids pML225 (Longhese *et al*., 2000) and YCplac33 (Gietz and Sugino, 1988), respectively. Strains YLL836 and YLL837 were obtained by transforming strain YLL279.2 with plasmids pML225 and YCplac33, respectively. Strain YLL279.2 was also crossed with strains DMP2855/6B, DMP2946/2C, DMP2946/3A and DMP2854/2B to generate the DMP3356/4A, DMP3392/4A, DMP3463/10C and DMP3462/2B meiotic segregants, respectively. Strains DMP2855/6B, DMP2946/2C, DMP2946/3A and DMP2854/2B were, respectively, meiotic segregants from the crosses YLL509 (Longhese *et al*., 2000)/K700, YLL157/K700, YLL244/K700 and YLL490/K700. Strain DMP3475/3D was a meiotic segregant from a cross between strains YLL275 (Paciotti *et al*., 2000) and DMP3469/1A, which was derived from a cross between strains DMP3539/10D and K700. Strain YLL930 was obtained by transformation of strain YLL275 with plasmid pML240 (Longhese *et al*., 2000). Strain YLL943 was obtained by transformation of strain 3539/10D with plasmid pML94 (Paciotti *et al*., 2000). Strain YLL944 was obtained by transformation of strain K699 with plasmids pML240 (Longhese *et al*., 2000) and pML94 (Paciotti *et al*., 2000). Strain DMP3532/8A was a meiotic segregant from a cross of strains YLL516 and DMP2995/6C, which was derived from a cross between strains DMP2995/1B (Paciotti *et al*., 2000) and K700. Strain DMP3602/9C was derived from the cross between strains YLL2995/6C and DMP3539/10D.

The accuracy of all gene replacements and integrations was verified by Southern blot analysis or PCR. Standard yeast genetic techniques and media were according to Rose *et al*. (1990). Cells were grown in YEP medium (1% yeast extract, 2% bactopeptone, 50 mg/l adenine) supplemented with 2% glucose (YEPD) or 2% raffinose (YEP-raf) or 2% raffinose and 2% galactose (YEP-raf-gal). Transformants carrying the *KanMX4* cassette were selected on YEPD plates containing 400 μg/ml G418 (US Biological).

Evaluation of endogenous and galactose-induced Ddc2, Tel1 and Mec1 protein levels

To compare Ddc2, Tel1 and Mec1 protein levels expressed from the *GAL1* promoter under galactose-induced conditions to the endogenous levels of the same proteins, isogenic W303 derivative strains expressing tagged *DDC2-HA3* (Paciotti *et al*., 2000), *MEC1-HA9* (Paciotti *et al*., 2000) and *TEL1-HA3* alleles (Table I) either from their own promoters or

from the *GAL1* promoter were grown in raffinose-containing medium and then shifted to raf-gal-medium for 4 h. The same amounts of protein extracts prepared from the above cultures were loaded onto an SDS–polyacrylamide gel and subjected to western blot analysis with anti-HA antibodies. Films were then scanned and bands quantified with NIH Image. By this analysis, the levels of Mec1, Tel1 and Ddc2 produced under galactose-induced conditions by the strains carrying the *GAL1* constructs were ~20-, 20- and 25-fold higher, respectively, than the corresponding endogenous levels.

Other techniques

Synchronization experiments, total protein extract preparation and western blot analysis were performed as described in Paciotti *et al*. (2000).

References

Bentley,N.J., Holtzman,D.A., Flaggs,G., Keegan,K.S., Demaggio,A., Ford,J.C., Hoekstra,M. and Carr,A.M. (1996) The *Schizosaccharomyces pombe rad3* checkpoint gene. *EMBO J.*, **15**, 6641–6651.

Caspari,T., Dahlen,M., Kanter-Smoler,G., Lindsay,H.D., Hofman,K., Papadimitriou,N., Sunnerhagen,P. and Carr,A.M. (2000) Characterization of *Schizosaccharomyces pombe* Hus1: a PCNA-related protein that associates with Rad1 and Rad9. *Mol. Cell. Biol.*, **20**, 1254–1262.

Chabes,A., Domkin,V. and Thelander,L. (1999) Yeast Sml1, a protein inhibitor of ribonucleotide reductase. *J. Biol. Chem.*, **274**, 36679–36683.

D'Amours,D. and Jackson,S.P. (2001) The yeast Xrs2 complex functions in S phase checkpoint regulation. *Genes Dev.*, **15**, 2238–2249.

Desany,B.A., Alcasabas,A.A., Bachant,J.B. and Elledge,S.J. (1998) Recovery from DNA replicational stress is the essential function of the S-phase checkpoint pathway. *Genes Dev.*, **12**, 2956–2970.

Emili,A. (1998) *MEC1*-dependent phosphorylation of Rad9p in response to DNA damage. *Mol. Cell*, **2**, 183–189.

Gietz,R.D. and Sugino,A. (1988) New yeast–*Escherichia coli* shuttle vectors constructed with *in vitro* mutagenized yeast genes lacking six base-pair restriction sites. *Gene*, **74**, 527–534.

Gilbert,C.S., Green,C.M. and Lowndes,N.F. (2001) Budding yeast Rad9 is an ATP-dependent Rad53 activating machine. *Mol. Cell*, **8**, 129–136.

Green,C.M., Erdjument-Bromage,H., Tempst,P. and Lowndes,N.F. (2000) A novel Rad24 checkpoint protein complex closely related to replication factor C. *Curr. Biol.*, **10**, 39–42.

Greenwell,P.W., Kronmal,S.L., Porter,S.E., Gassenhuber,J., Obermaier,B. and Petes,T.D. (1995) *TEL1*, a gene involved in controlling telomere length in *S.cerevisiae*, is homologous to the human ataxia telangiectasia gene. *Cell*, **82**, 823–829.

Grenon,M., Gilbert,C.S. and Lowndes,N.F. (2001) Checkpoint activation in response to double-strand breaks requires the Mre11/Rad50/Xrs2 complex. *Nature Cell Biol.*, **3**, 844–847.

Griffiths,D.J., Barbet,N.C., McCready,S., Lehmann,A.R. and Carr,A.M. (1995) Fission yeast *RAD17*: a homologue of budding yeast *RAD24* that shares regions of sequence similarity with DNA polymerase accessory proteins. *EMBO J.*, **14**, 5812–5823.

Hari,K.L., Santerre,A., Sekelsky,J.J., McKim,K.S., Boyd,J.B. and Hawley,R.S. (1995) The mei-41 gene of *D.melanogaster* is a structural and functional homolog of the human ataxia telangiectasia gene. *Cell*, **82**, 815–821.

Knop,M., Siegers,K., Pereira,G., Zachariae,W., Winsor,B., Nasmyth,K. and Schiebel,E. (1999) Epitope tagging of yeast genes using a PCR-

based strategy: more tags and improved practical routines. *Yeast*, **15**, 963–972.

Kondo,T., Matsumoto,K. and Sugimoto,K. (1999) Role of a complex containing Rad17, Mec3 and Ddc1 in the yeast DNA damage checkpoint pathway. *Mol. Cell. Biol.*, **19**, 1136–1143.

Kostrub,C.F., Knudsen,K., Subramani,S. and Enoch,T. (1998) Hus1p, a conserved fission yeast checkpoint protein, interacts with Rad1p and is phosphorylated in response to DNA damage. *EMBO J.*, **17**, 2055–2066.

Longhese,M.P., Fraschini,R., Plevani,P. and Lucchini,G. (1996) Yeast *pip3/mec3* mutants fail to delay entry into S phase and to slow DNA replication in response to DNA damage and they define a functional link between Mec3 and DNA primase. *Mol. Cell. Biol.*, **16**, 3235–3244.

Longhese,M.P., Paciotti,V., Fraschini,R., Zaccarini,R., Plevani,P. and Lucchini,G. (1997) The novel DNA damage checkpoint protein Ddc1p is phosphorylated periodically during the cell cycle and in response to DNA damage in budding yeast. *EMBO J.*, **16**, 5216–5226.

Longhese,M.P., Foiani,M., Muzi Falconi,M., Lucchini,G. and Plevani,P. (1998) DNA damage checkpoint in budding yeast. *EMBO J.*, **17**, 5525–5528.

Longhese,M.P., Paciotti,V., Neecke,H. and Lucchini,G. (2000) Checkpoint proteins influence telomeric silencing and length maintenance in budding yeast. *Genetics*, **155**, 1577–1591.

Lowndes,N.F. and Murguia,J.R. (2000) Sensing and responding to DNA damage. *Curr. Opin. Genes Dev.*, **10**, 17–25.

Lydall,D. and Weinert,T. (1997) G_2/M checkpoint genes of *Saccharomyces cerevisiae*: further evidence for roles in DNA replication and/or repair. *Mol. Gen. Genet.*, **256**, 638–651.

Mallory,J.C. and Petes,T.D. (2000) Protein kinase activity of Tel1p and Mec1p, two *Saccharomyces cerevisiae* proteins related to the human ATM protein kinase. *Proc. Natl Acad. Sci. USA*, **97**, 13749–13754.

Morrow,D.M., Tagle,D.A., Shiloh,Y., Collins,F.S. and Hieter,P. (1995) *TEL1*, an *S.cerevisiae* homolog of the human gene mutated in ataxia telangiectasia, is functionally related to the yeast checkpoint gene *MEC1*. *Cell*, **82**, 831–840.

O'Connell,M.J., Walworth,N.C. and Carr,A.M. (2000) The G_2-phase DNA-damage checkpoint. *Trends Cell Biol.*, **10**, 296–303.

Paciotti,V., Lucchini,G., Plevani,P. and Longhese,M.P. (1998) Mec1p is essential for phosphorylation of the yeast DNA damage checkpoint protein Ddc1p, which physically interacts with Mec3p. *EMBO J.*, **17**, 101–111.

Paciotti,V., Clerici,M., Lucchini,G. and Longhese,M.P. (2000) The checkpoint protein Ddc2, functionally related to *S.pombe* Rad26, interacts with Mec1 and is regulated by Mec1-dependent phosphorylation in budding yeast. *Genes Dev.*, **14**, 2046–2059.

Paciotti,V., Clerici,M., Scotti,M., Lucchini,G. and Longhese,M.P. (2001) Characterization of *mec1* kinase-deficient mutants and of new hypomorphic *mec1* alleles impairing subsets of the DNA damage response pathway. *Mol. Cell. Biol.*, **21**, 3913–3925.

Paulovich,A.G., Margulies,R.U., Garvik,B.M. and Hartwell,L.H. (1997) *RAD9*, *RAD17* and *RAD24* are required for S phase regulation in *Saccharomyces cerevisiae* in response to DNA damage. *Genetics*, **145**, 45–62.

Ritchie,K.B., Mallory,J.C. and Petes,T.D. (1999) Interactions of *TLC1* (which encodes the RNA sub-unit of telomerase), *TEL1*, and *MEC1* in regulating telomere length in the yeast *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **19**, 6065–6075.

Rose,M.D., Winston,F. and Hieter,P. (1990) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Rouse,J. and Jackson,S.P. (2000) *LCD1*: an essential gene involved in checkpoint control and regulation of the *MEC1* signaling pathway in *Saccharomyces cerevisiae*. *EMBO J.*, **19**, 5801–5812.

Sanchez,Y., Desany,B.A., Jones,W.J., Liu,Q., Wang,B. and Elledge,S.J. (1996) Regulation of *RAD53* by the *ATM*-like kinases *MEC1* and *TEL1* in yeast cell cycle checkpoint pathways. *Science*, **271**, 357–360.

Sanchez,Y., Bachant,J., Wang,H., Hu,F.H., Liu,D., Tezlaff,M. and Elledge,S.J. (1999) Control of the DNA damage checkpoint by Chk1 and Rad53 protein kinases through distinct mechanisms. *Science*, **286**, 1166–1171.

Savitsky,K. *et al.* (1995) A single ataxia telangiectasia gene with a product similar to PI-3 kinase. *Science*, **286**, 1749–1753.

Siede,W., Friedberg,A.S. and Friedberg,E.C. (1993) *RAD9*-dependent G_1 arrest defines a second checkpoint for damaged DNA in the cell cycle of *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **90**, 7985–7989.

St Onge,R.P., Udell,C.M., Casselman,R. and Davey,S. (1999) The

human G_2 checkpoint control protein hRAD9 is a nuclear phosphoprotein that forms complexes with hRAD1 and hHUS1. *Mol. Biol. Cell*, **10**, 1985–1995.

Sun,Z., Fay,D.S., Marini,F., Foiani,M. and Stern,D.F. (1996) Spk1/Rad53 is regulated by Mec1-dependent protein phosphorylation in DNA replication and damage checkpoint pathways. *Genes Dev.*, **10**, 395–406.

Sun,Z., Hsiao,J., Fay,D.S. and Stern,D.F. (1998) Rad53 FHA domain associated with phosphorylated Rad9 in the DNA damage checkpoint. *Science*, **281**, 272–274.

Thelen,M.P., Venclovas,C. and Fidelis,K. (1999) A sliding clamp model for the Rad1 family of cell cycle checkpoint proteins. *Cell*, **96**, 769–770.

Usui,T., Ogawa,H. and Petrini,J. (2001) A DNA damage response pathway controlled by Tel1 and the Mre11 complex. *Mol. Cell*, **7**, 1255–1266.

Vialard,J.E., Gilbert,C.S., Green,C.M. and Lowndes,N.F. (1998) The budding yeast Rad9 checkpoint protein is subjected to Mec1/Tel1-dependent hyperphosphorylation and interacts with Rad53 after DNA damage. *EMBO J.*, **17**, 5679–5688.

Wach,A., Brachat,A., Pohlmann,R. and Philippsen,P. (1994) New heterologous modules for classical or PCR-based gene disruption in *Saccharomyces cerevisiae*. *Yeast*, **10**, 1793–1808.

Waga,S. and Stillman,B. (1998) The DNA replication forks in eukaryotic cells. *Annu. Rev. Biochem.*, **67**, 721–751.

Wakayama,T., Kondo,T., Ando,S., Matsumoto,K. and Sugimoto,K. (2001) Pie1, a protein interacting with Mec1, controls cell growth and checkpoint responses in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **21**, 755–764.

Weinert,T. (1998) DNA damage and checkpoint pathways: molecular anatomy and interactions with repair. *Cell*, **94**, 555–558.

Weinert,T.A. and Hartwell,L.H. (1988) The *RAD9* gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. *Science*, **241**, 317–322.

Weinert,T.A., Kiser,G.L. and Hartwell,L.H. (1994) Mitotic checkpoint genes in budding yeast and the dependence of mitosis on DNA replication and repair. *Genes Dev.*, **8**, 652–665.

Zhao,X., Muller,E.G.D. and Rothstein,R. (1998) A suppressor of two essential checkpoint genes identifies a novel protein that negatively affects dNTP pools. *Mol. Cell*, **2**, 329–340.

Zhao,X., Chabes,A., Domkin,V., Thelander,L. and Rothstein,R. (2001) The ribonucleotide reductase inhibitor Sml1 is a new target of the Mec1/Rad53 kinase cascade during growth and in response to DNA damage. *EMBO J.*, **20**, 3544–3553.

Zhou,B.B. and Elledge,S.J. (2000) The DNA damage response: putting checkpoints in perspective. *Nature*, **408**, 433–439.