

## TOR Complex 2 Controls Gene Silencing, Telomere Length Maintenance, and Survival under DNA-Damaging Conditions<sup>∇†</sup>

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**The Target Of Rapamycin (TOR) kinase belongs to the highly conserved eukaryotic family of phosphatidylinositol-3-kinase-related kinases (PIKKs). TOR proteins are found at the core of two distinct evolutionarily conserved complexes, TORC1 and TORC2. Disruption of TORC1 or TORC2 results in characteristically dissimilar phenotypes. TORC1 is a major cell growth regulator, while the cellular roles of TORC2 are not well understood. In the fission yeast *Schizosaccharomyces pombe*, Tor1 is a component of the TORC2 complex, which is particularly required during starvation and various stress conditions. Our genome-wide gene expression analysis of  $\Delta tor1$  mutants indicates an extensive similarity with chromatin structure mutants. Consistently, TORC2 regulates several chromatin-mediated functions, including gene silencing, telomere length maintenance, and tolerance to DNA damage. These novel cellular roles of TORC2 are rapamycin insensitive. Cells lacking Tor1 are highly sensitive to the DNA-damaging drugs hydroxyurea (HU) and methyl methanesulfonate, similar to mutants of the checkpoint kinase Rad3 (ATR). Unlike Rad3, Tor1 is not required for the cell cycle arrest in the presence of damaged DNA. Instead, Tor1 becomes essential for dephosphorylation and reactivation of the cyclin-dependent kinase Cdc2, thus allowing reentry into mitosis following recovery from DNA replication arrest. Taken together, our data highlight critical roles for TORC2 in chromatin metabolism and in promoting mitotic entry, most notably after recovery from DNA-damaging conditions. These data place TOR proteins in line with other PIKK members, such as ATM and ATR, as guardians of genome stability.**

The TOR protein kinase is a major cell growth regulator that links cellular growth with cell divisions (18, 42, 64, 65). TOR is an atypical protein kinase conserved from yeast to humans that was isolated as the target of the immunosuppressive and anticancer drug rapamycin (28). TOR proteins can be found in two distinct complexes, known as TORC1 and TORC2 (27, 64). These complexes mediate their distinct cellular functions via phosphorylation and activation of different sets of AGC-like kinases, including mammalian p70S6K, downstream of TORC1, and AKT/protein kinase B (PKB) downstream of TORC2 (18). TORC1 in mammals contains mTOR (Tor1 or Tor2 in *Saccharomyces cerevisiae*; Tor2 in *Schizosaccharomyces pombe*) and the Raptor protein (Kog1 in *S. cerevisiae*; Mip1 in *S. pombe*). TORC1 in many different eukaryotes plays a central role in the control of growth (mass accumulation) in response to external stimuli, particularly nutrient availability. Disruption of TORC1, either by mutating its components or by rapamycin treatment, can lead to a starvation-like phenotype (64). The cellular roles of TORC2, on the other hand, are less well defined. TORC2 in mammals contains mTOR (Tor2 in *S. cerevisiae*; Tor1 in *S.*

*pombe*) together with Rictor (Avo3 in *S. cerevisiae*; Ste20 in *S. pombe*) and mSin1 (Avo1 in *S. cerevisiae*; Sin1 in *S. pombe*). TORC2 plays a role in regulating the actin cytoskeleton and cell wall integrity pathway in *S. cerevisiae* (3, 15, 27), a function that is at least partially conserved in human cells (17, 47).

Fission yeast contains two TOR homologues, Tor1 and Tor2 (59), which form the TORC2 and TORC1 complexes, respectively (14, 32). Disruption  $tor2^+$  (TORC1) mimics nitrogen starvation responses (1, 14, 32, 56, 57, 62), while disruption of  $tor1^+$  (TORC2) results in pleiotropic defects, including elongated cells, sensitivity to osmotic and oxidative stress, inability to execute developmental processes in response to nutrient depletion, and a decrease in amino acid uptake (16, 22, 59). Tor1 regulates cell survival under stress conditions and starvation responses via the AGC protein kinase Gad8, a putative homologue of mammalian AKT/PKB (16).

In budding yeast and mammalian cells, TORC1 mediates the rapamycin-sensitive signaling branch while TORC2 is far less sensitive to inhibition by this drug (27, 48). Curiously, rapamycin does not inhibit growth of *S. pombe* cells but partially inhibits sexual development and amino acid uptake (60–62). Inhibition of amino acid uptake is likely a result of inhibiting Tor1 (61, 62). Accordingly, a  $tor1$  rapamycin-defective allele ( $tor1^{S1834E}$ ) confers rapamycin resistance to strains that are dependent on amino acid uptake for their growth (61). Yet rapamycin also induces a response similar to that for a shift from rich to poor nitrogen conditions, an effect that may involve inhibition of both Tor1 and Tor2 (41).

While other members of the phosphatidylinositol-3-kinase-

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related kinase (PIKK) family of proteins, such as ATM and ATR, have been shown to play central roles in the DNA damage response, little is known about roles that TOR proteins might play in such processes. Recently it was shown that the rapamycin-sensitive TORC1 complex participates in regulating cell survival under DNA-damaging conditions (24, 42, 49). Currently, no such role has been attributed to TORC2.

Here we show that Tor1 (TORC2) is critical for cell survival under DNA-damaging conditions, gene silencing at heterochromatic regions, and telomere length maintenance and for regulation of cell cycle progression. Since the TOR complexes are highly conserved in evolution, this novel TORC2 function may also be conserved in other organisms.

#### MATERIALS AND METHODS

**Yeast techniques.** *S. pombe* strains are described in Table S1 in the supplemental material. All experiments were performed by using standard genetic and molecular yeast techniques as described in reference 35. Growth medium was prepared as described previously (59). Rapamycin (R0395; Sigma) was used at a final concentration of 100 ng/ml. For cell killing assays, HU (H8627; Sigma) or methyl methanesulfonate (MMS) (129925; Sigma) was added at appropriate concentrations. UV irradiation was performed using a UV Stratilinker 1800 crosslinker (Stratagene). Cells were visualized using a Nikon eclipse E600 fluorescence microscope and photographed using a Nikon digital camera (DXM1200) and the ACT1 software. Cell length was determined at septation and measured using Scion Image software. For fluorescence-activated cell sorting (FACS) analysis, nuclei were isolated as described previously (10), stained with propidium iodide, and analyzed by a Becton Dickinson FACSsort cell sorter. Data were analyzed by Cell Quest software for Macintosh.

**Telomere gels.** DNA was isolated from logarithmically growing cells, digested with EcoRI, and subjected to Southern blotting (33). A DNA probe corresponding to the telomere repeats was generated from pIRT2-TELO29 (33).

**RNA and protein manipulations.** RNA for microarray hybridization and Northern blots was prepared using the hot phenol method. Northern blot analysis was carried out as described previously (62). Gene-specific probes were labeled with [ $\alpha$ -<sup>32</sup>P]dCTP using the Random Primer DNA labeling kit (20-101-25A; Biological Industries). Transcripts were quantified using Gelquant software. For Western blot analysis, 50 ml of logarithmically growing cells were harvested, resuspended in protein extraction buffer (20% glycerol, 20 mM HEPES [pH 7.9], 50 mM NH<sub>2</sub>SO<sub>4</sub>, 5 mM EDTA [pH 8.0]) in the presence of protease inhibitor and broken with glass beads. Immunoblotting was performed as described previously (59).

**Microarray experiments and data evaluation.** We used DNA microarrays displaying probes for >99% of all known and predicted genes of *S. pombe* spotted in duplicate onto glass slides. RNA extraction, hybridization, and initial data processing and normalization were performed as described previously (29). Three independent biological experiments were performed, including a dye swap. The data were visualized and analyzed using the GeneSpring software program (Agilent). The significance of overlaps between different gene lists was calculated in GeneSpring using a standard Fisher exact test, and *P* values were adjusted with a Bonferroni multiple testing correction. Cutoff values of 1.5-fold change in all biological repeats were used. Gene annotations were downloaded from *S. pombe* GeneDB (<http://www.genedb.org/genedb/pombe/>). The data can be obtained from the ArrayExpress account at [www.ebi.ac.uk/aerep/login](http://www.ebi.ac.uk/aerep/login), accession number E-MTAB-39.

Clustering along chromosomes of genes with induced expression in  $\Delta tor1$  cells was analyzed using an in-house Perl script which compares clustered genes to a random distribution (31). *P* values were adjusted for multiple testing using the Benjamini-Hochberg false discovery rate.

#### RESULTS

***tor1*<sup>+</sup> deletion leads to upregulation of repeated elements and subtelomeric genes.** In order to uncover the underlying mechanism for the pleiotropic defects in cells lacking *tor1*<sup>+</sup> ( $\Delta tor1$ ), we performed genome-wide gene expression profiling. This analysis revealed that in growing cells, 117 and 48 genes

were at least 1.5-fold upregulated or downregulated, respectively, in  $\Delta tor1$  cells compared to results for wild-type cells (see Fig. S1 and S2 in the supplemental material and data submitted at ArrayExpress [[www.ebi.ac.uk/aerep/login](http://www.ebi.ac.uk/aerep/login)]). Comparison of these transcriptional profiles with profiles of other mutants showed an extensive overlap among upregulated genes with either *clr6-1*,  $\Delta clr3 clr6-1$  (12), or  $\Delta rsc58$  mutants (34) (Fig. 1A). Clr3 and Clr6 are histone deacetylases (HDACs), while Rsc58 is part of the conserved RSC complex, a member of the SWI/SNF chromatin-remodeling family. Genes that are upregulated in the absence of Tor1 include repeated genes, such as the *wf* elements, and several noncoding RNA telomeric duplications, suggesting that the  $\Delta tor1$  mutation leads to a derepression of gene transcription mediated by heterochromatin. Accordingly, upregulated genes were significantly clustered at subtelomeric regions compared to a random distribution (*P* < 0.05). Using Northern blot analysis, we verified that genes that are upregulated in *clr3* or *clr6-1* cells (12) are also upregulated in  $\Delta tor1$  mutants (Fig. 1B). One of these genes, C186.05c, is located close (~30 kbp) to the telomeric region (12).

*S. pombe* contains heterochromatin in centromeric and telomeric regions and at the mating type locus. Since our microarray experiments suggested upregulation of genes at heterochromatic regions, we examined the expression of a reporter gene, *ade6*<sup>+</sup>, inserted at the mating type locus (2). We found that a loss of Tor1 relieved the repression of *ade6*<sup>+</sup> inserted at the mating type locus (Fig. 1C), further supporting a role for Tor1 in chromatin-mediated gene silencing.

Inhibition of HDACs caused hyperacetylation at centromeres and defective chromosome segregation (52). Accordingly, *clr6-1* mutants exhibit sensitivity to thiabendazole (TBZ), a drug that destabilizes microtubules and thus aggravates chromosome loss in strains with compromised centromeres (52). Similarly, we found that  $\Delta tor1$  mutants are highly sensitive to TBZ (Fig. 1D), raising the possibility that *tor1* mutants are also defective in accurate chromosome segregation.

Among the genes that were downregulated in  $\Delta tor1$  mutants, we noted several transporters, including *str1*<sup>+</sup>, encoding a component of the iron-siderophore system. The transcription of *str1*<sup>+</sup> is also downregulated in *clr1*, *clr3*, and *clr4* mutants (12). The findings that transporters and stress-responsive genes are aberrantly expressed in *clr* mutants led Hansen et al. (12) to examine the sensitivity of *clr* mutants to osmotic stress. Indeed, the *clr6-1*  $\Delta clr3$  double mutant was highly sensitive to 1 M KCl (12), showing an osmotic sensitivity similar to that observed in  $\Delta tor1$  mutants (59). Thus,  $\Delta tor1$  mutants share with HDAC mutants their gene expression pattern, derepression of genes at heterochromatic regions, and sensitivity to TBZ and KCl.

**Tor1 is required for telomere length maintenance.** Mutations in chromatin modifiers can affect telomere length (12). Thus, we examined telomere length in different TORC2 mutants. We found that telomeres of  $\Delta tor1$ ,  $\Delta ste20$ , or  $\Delta gad8$  mutants were elongated by ~150 bp compared to those of the wild type, similar to the elongation observed in *clr6-1* mutants. In contrast, for wild-type cells grown in the presence of 100 ng/ml rapamycin, the length of telomeres was not affected (Fig. 1E). We conclude that TORC2-Gad8 regulates telomere length in a rapamycin-insensitive manner. Overexpression of

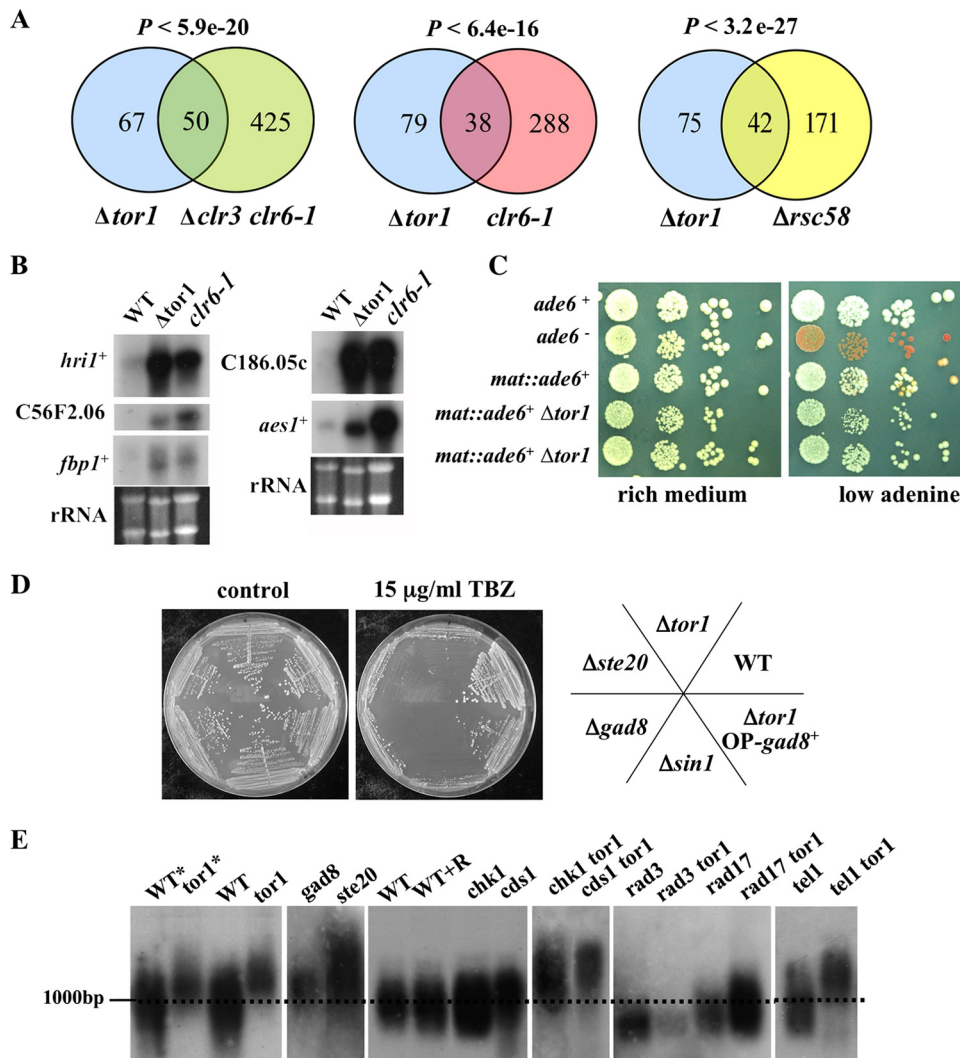


FIG. 1. Tor1 is required for gene silencing and maintenance of telomere length. (A) The set of genes upregulated by loss of Tor1 significantly overlaps with the set of genes upregulated in histone deacetylase mutants. The number of genes that were upregulated 1.5-fold in the indicated mutants is presented in Venn diagrams, along with corresponding *P* values. (B) Northern blot analysis. Total RNA was prepared from wild-type (WT),  $\Delta tor1$ , and  $clr6-1$  mutants grown to mid-log phase in rich medium. Northern blots were probed with the indicated genes. (C) Tor1 promotes silencing at the mating type locus. Strains containing an *ade6<sup>+</sup>* cassette at the mating type locus were spotted onto the indicated plates. In an otherwise wild-type background, the *ade6<sup>+</sup>* gene insertion produced a typical position variegation effect, since only a portion of the colonies is white (express the *ade6<sup>+</sup>* gene) while others are red due to a decreased level of *ade6<sup>+</sup>* transcript and accumulation of a red pigment. Only white colonies are present in cells carrying the  $\Delta tor1$  mutation. (D) Tor1 is required for tolerance to microtubule destabilizing agents. Cells were streaked on plates containing the indicated levels of TBZ. (E) Tor1 is required for the maintenance of telomere length regulation. DNA was extracted from cells grown in rich medium (or minimal medium; asterisk). When rapamycin was added (R), the cells were grown in the presence of 100 ng/ml rapamycin. Genomic DNA was digested with EcoRI, which in wild-type cuts about 1 kb from the terminus, and analyzed by Southern blotting. The resulting filter was probed with  $\alpha^{32}$ P-labeled telomere repeat DNA.

Gad8 did not suppress telomere overelongation in  $\Delta tor1$  mutants (data not shown). However, since Gad8 is a substrate for phosphorylation by Tor1, it is likely to be poorly active in  $\Delta tor1$  mutants.

DNA checkpoint proteins play a central role in telomere maintenance. Mutations in Rad3, the primary DNA damage checkpoint kinase, or in any of the subunits of the heterotrimeric checkpoint clamp complex Rad9-Rad1-Hus1 (9-1-1) or its clamp loader Rad17 result in short telomeres (38). We found that the telomeres of the  $\Delta tor1 \Delta rad3$  or  $\Delta tor1 \Delta rad17$  double mutant are as short as those of single  $\Delta rad3$  or  $\Delta rad17$

mutants, respectively. Thus, Tor1 may induce telomere overelongation via Rad3 and Rad17 (Fig. 1E). Chk1 and Cds1, the downstream effectors of Rad3 in the DNA damage and DNA replication checkpoints, respectively, play little or no role in regulating telomere length (38). Consistently, deletion of *tor1<sup>+</sup>* in either a  $\Delta chk1$  or  $\Delta cds1$  background resulted in a telomere elongation similar to that in single  $\Delta tor1$  mutants (Fig. 1E). We also tested for involvement of Tel1, a PIKK kinase similar to ATM that works together with Rad3 to regulate telomere length (38). Telomere overelongation in  $\Delta tor1$  mutants did not require the presence of Tel1 (Fig. 1E). We thus suggest that

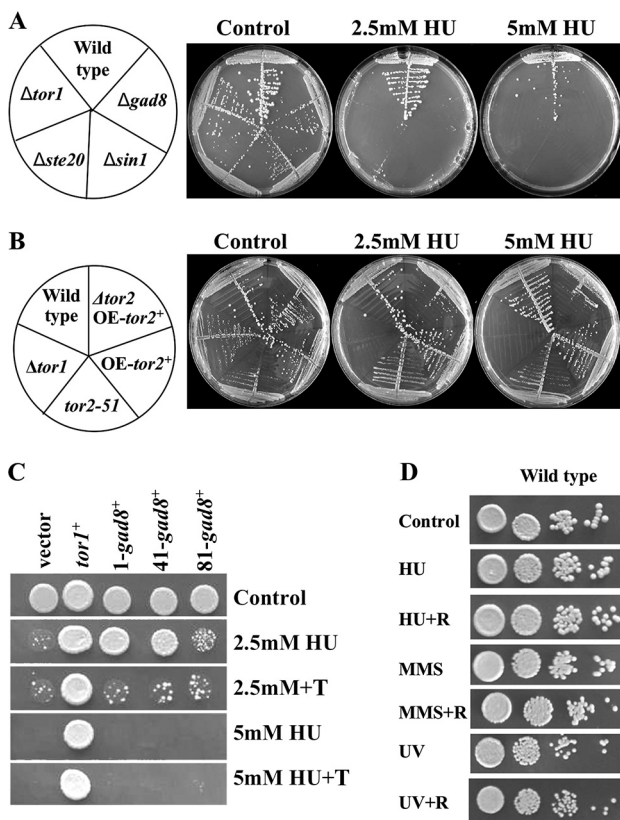


FIG. 2. Mutations in TORC2 but not TORC1 confer sensitivity to DNA replication stress in a rapamycin-independent manner. (A and B) TORC2 but not TORC1 components are required for HU tolerance. Strains were streaked on plates with or without the indicated amounts of HU. (C) Overexpression of *gad8*<sup>+</sup> partially rescues the lethal phenotype of  $\Delta$ *tor1* cells on HU. *gad8*<sup>+</sup> is expressed from the thiamine (T)-repressible *nmr1*<sup>+</sup> promoter from the plasmids pREP1, -41, and -81, which allow strong, moderate, and weak expression, respectively. *tor1*<sup>+</sup> is expressed from a plasmid under the regulation of its own promoter. (D) Rapamycin does not affect tolerance to DNA-damaging conditions. Serial dilutions of wild-type cells in the presence of 2.5 mM HU or 0.0025% MMS or UV irradiated at 75 J/m<sup>2</sup> with or without 100 ng/ml rapamycin (R).

Tor1 acts in a Rad3-dependent pathway to maintain proper telomere length and this function is independent of Tel1.

**TORC2 is required under DNA-damaging conditions.** Defects either in the RSC complex or in HDAC complexes can lead to sensitivity to DNA damage and replication stress conditions (26, 34, 39). We examined the sensitivity of  $\Delta$ *tor1* mutants to the drug hydroxyurea (HU), which halts DNA replication by inhibiting nucleotide synthesis from the ribonucleotide reductase (4). Deletion of each of the genes encoding specific TORC2 components, Tor1, Ste20, or Sin1, or the downstream effector Gad8 resulted in strong sensitivity to HU (Fig. 2A). HU sensitivity in  $\Delta$ *tor1* mutants has been observed previously (57), with no further analysis of the underlying mechanism.

In contrast, reduction of Tor2 (TORC1) activity, overexpression of Tor2, or deletion of *tsc1*<sup>+</sup> or *tsc2*<sup>+</sup> did not markedly affect HU sensitivity (Fig. 2B and data not shown). Thus, it appears that cells carrying mutations in TORC2 but not TORC1 are sensitive to HU. Overexpression of *gad8*<sup>+</sup> partially

suppressed the HU sensitivity of  $\Delta$ *tor1* mutants, further suggesting that Tor1 acts via Gad8 in tolerating replication stress (Fig. 2C).

We also found that  $\Delta$ *tor1* cells were strongly sensitive to the DNA-alkylating agent MMS (Fig. 3B) and slightly sensitive to UV irradiation (data not shown). Rapamycin did not affect sensitivity to these drugs (Fig. 2D), indicating that the functions of TORC2 under DNA-damaging conditions are rapamycin insensitive.

Cells lacking Tor1 are almost as sensitive to HU or MMS as mutants lacking the main checkpoint kinase Rad3 or mutants lacking the RFC-like protein Rad17 (Fig. 3A and B). Combining the  $\Delta$ *tor1* mutation with  $\Delta$ *rad3* or  $\Delta$ *rad17* did not result in further sensitivity to the DNA-damaging conditions (Fig. 3A and B). Thus, the function of Tor1 in the DNA damage response, as in telomere length control, may depend on the functions of Rad3 and Rad17. In fission yeast, the Rad3 kinase controls two checkpoint pathways: one responds to the DNA replication block, mainly through the Cds1 kinase (mammalian Chk2), while the other responds to DNA damage through activation of the Chk1 kinase (4). Cells lacking Tor1 exhibited HU sensitivity comparable to that of cells lacking Cds1, the main effector of the DNA replication stress response pathway (Fig. 3A). The sensitivity of  $\Delta$ *tor1* mutants to HU was further augmented in combination with loss of function of either *cds1*<sup>+</sup> or its specific mediator, *mrc1*<sup>+</sup>, encoding a Claspin homologue (50) (Fig. 3A). Thus, it appears that Tor1 acts in a Cds1-Mrc1-independent pathway.

Cells lacking Tor1 show sensitivity to MMS comparable to that of cells lacking Chk1, the main effector of the DNA damage response pathway (Fig. 3B). Yet the  $\Delta$ *tor1* mutation showed additive effects with  $\Delta$ *chk1* cells with respect to MMS sensitivity (Fig. 3A). Thus, Tor1 appears to act independently of Chk1. Consistently, Tor1 was not required for activation of Chk1 by phosphorylation in response to MMS treatment (Fig. 3C). Taken together, our genetic analysis is consistent with the possibility that Tor1 lies on the same pathway as Rad3 but acts independently of either Chk1 or Cds1 (see also below and our model in Fig. 6B).

**Aberrant response of  $\Delta$ *tor1* cells to DNA replication stress induced by HU.** A nonsynchronized wild-type population of fission yeast cells contains mainly G<sub>2</sub> cells. Addition of HU to such a population results in a doubling in cell number, since cells proceed through the first mitosis and then arrest in the subsequent S phase (8, 9). While HU induces a cell cycle arrest in wild-type cells, cellular growth continues, resulting in elongated cells (8, 9).

FACS analysis of  $\Delta$ *tor1* cells indicated that cells accumulated with 1N DNA content in response to HU, although with delayed kinetics compared to wild-type cells (Fig. 4A). Note that the FACS analysis presented in Fig. 4A is of isolated nuclei. A “drift” of the DNA content toward a content of 1.5N DNA is observed at 4 to 5 h in HU in  $\Delta$ *tor1* nuclei. The meaning of this drift is not clear. However, since  $\Delta$ *tor1* cells maintain full viability following incubation for 4 to 5 h in HU (see below), we suggest that this “drift” reflects changes in the structure or size of  $\Delta$ *tor1* nuclei rather than the inability of  $\Delta$ *tor1* cells to properly arrest in G<sub>1</sub>.

Consistent with the slower kinetics with which  $\Delta$ *tor1* nuclei accumulated with 1N DNA content in response to HU, we de-

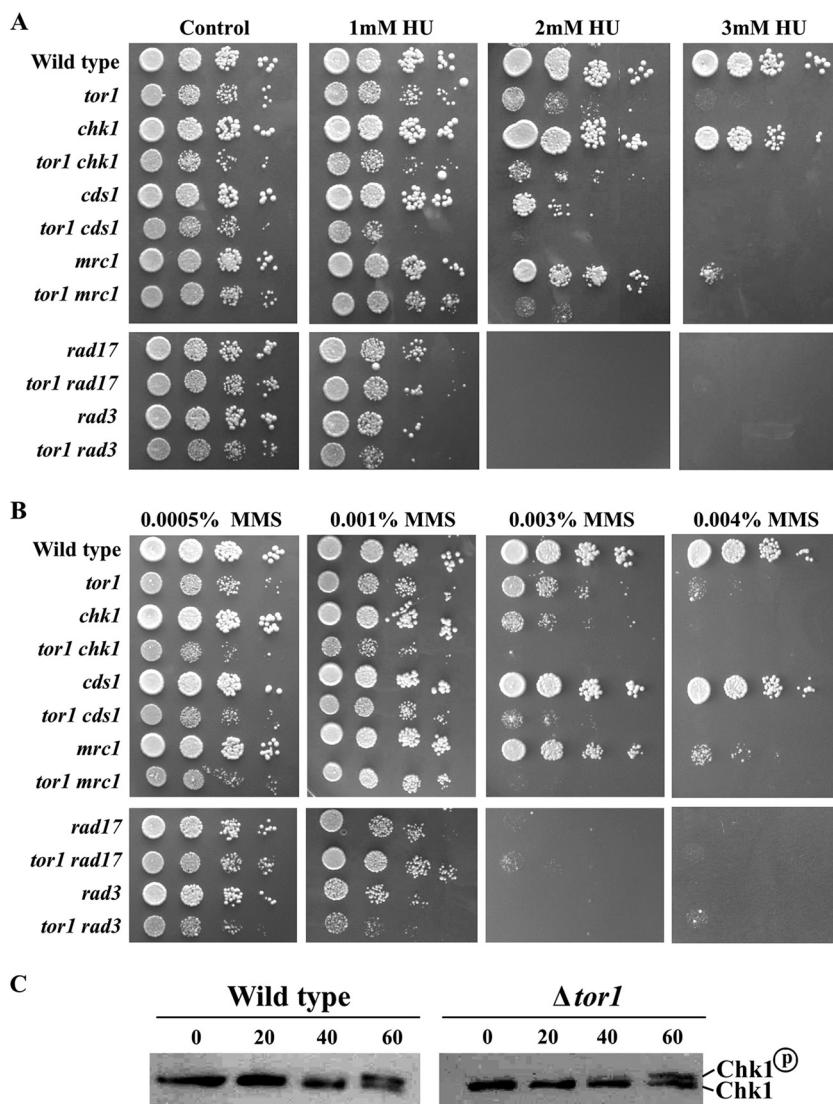


FIG. 3. Mutations in TORC2 confer sensitivity to DNA-damaging conditions independently of Cds1 or Chk1. (A and B) Tor1 functions independently of Chk1 or Cds1. Serial dilutions of mutant cells were plated with or without the indicated amounts of HU or MMS. (C) Tor1 is not required for phosphorylation of Chk1. Western blot analysis of HA-tagged Chk1. Wild-type or  $\Delta tor1$  cells containing HA-tagged Chk1 were grown to log phase. Protein was extracted from untreated cells or treated with 0.2% MMS for the indicated times (minutes).

tected a slow and reduced accumulation of Cdc10/MBF-dependent S-phase-specific transcripts (e.g., *cdt2<sup>+</sup>* and *cdc18<sup>+</sup>*) (63) in  $\Delta tor1$  mutants compared to results in wild-type cells (Fig. 4B). This finding could reflect either a defect in cell cycle progression in  $\Delta tor1$  mutants or a more direct defect in activating the transcriptional response to HU (5–7, 46).

Although  $\Delta tor1$  cells arrested with nuclei of 1N DNA content and showed induction of S-phase-specific transcripts, these cells did not show elongation in response to HU (Fig. 4C). Staining of the cells with 4',6'-diamidino-2-phenylindole (DAPI) and calcofluor in order to view nuclei and septa, respectively, revealed that exposure of  $\Delta tor1$  mutants to HU resulted in a ~40% increase in the number of septated cells. In contrast, addition of HU to wild-type cells resulted in a sharp reduction in the number of cells containing a septum (Fig. 4C and E), as shown previously (8). The septated HU-arrested

$\Delta tor1$  cells contained two condensed 1C nuclei (Fig. 4C) and maintained a high level of viability (Fig. 4D). These findings suggest that in response to HU,  $\Delta tor1$  cells are arrested with 1C nuclei content but cytokinesis of the previous cell cycle is delayed. A similar delay in septation in the presence of HU has been reported for mutants lacking Liz1, a pantothenate transporter (37, 53); this delay results from an indirect effect of HU on pantothenate biosynthesis (53). Unlike the case with  $\Delta liz1$  mutants, addition of pantothenate to the medium did not rescue the HU sensitivity of  $\Delta tor1$  mutants (data not shown); thus, the aberrant response to HU in  $\Delta tor1$  mutants occurs via a distinct mechanism. Importantly, however, our observation that  $\Delta tor1$  cells are highly sensitive to MMS suggests that  $\Delta tor1$  cells have a general defect in coping with DNA damage, rather than a specific defect concerning the response to HU.

The viability of  $\Delta tor1$  mutants in response to a short expo-

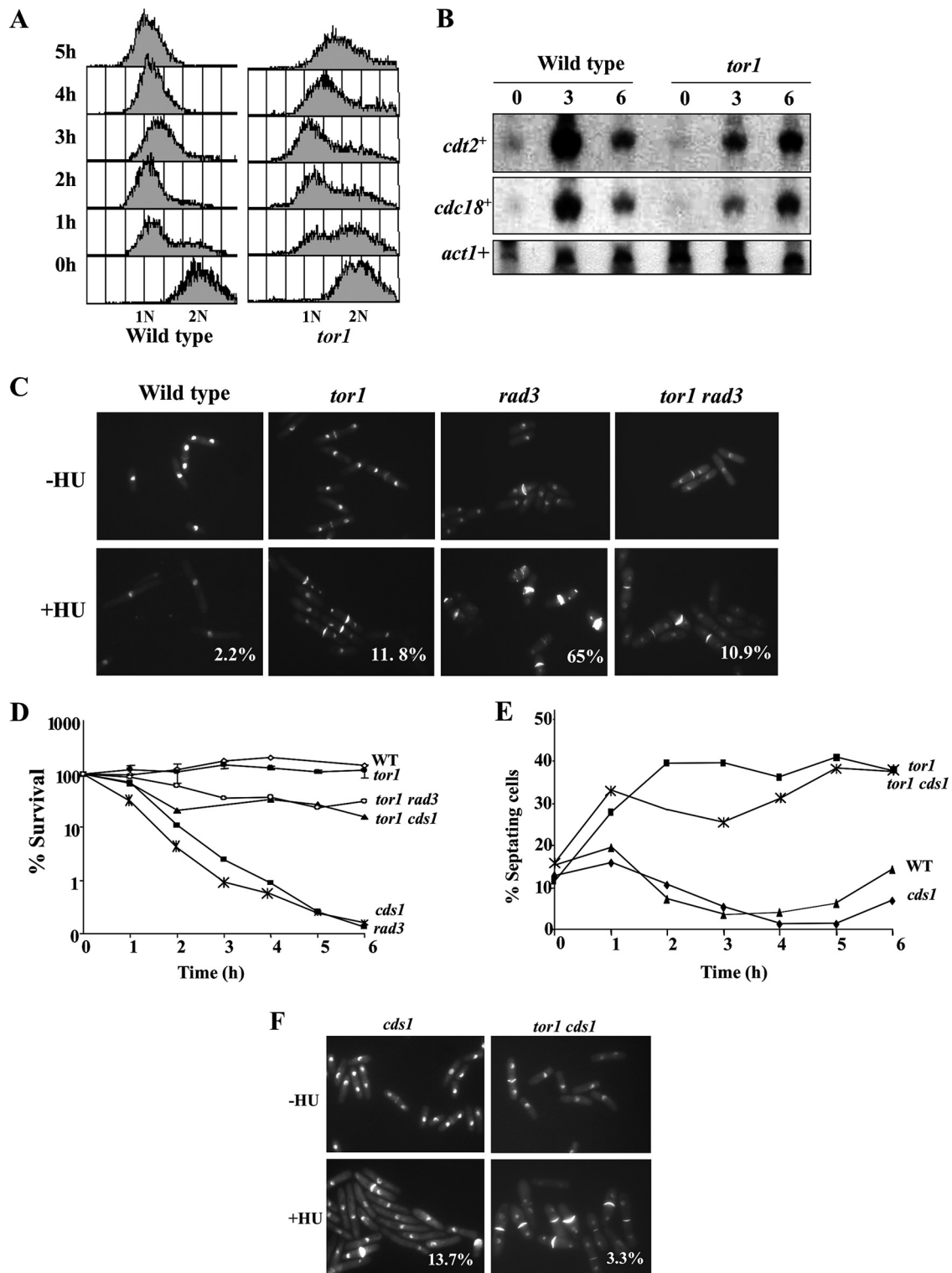


FIG. 4. Tor1 is required for a normal response to HU. (A and B) The response of *tor1* mutants to HU is delayed. Wild-type and  $\Delta$ *tor1* cells were grown to log phase and shifted to medium containing 12 mM HU. (A) Samples were taken every hour, and nuclei were isolated and subjected to FACS analysis. (B) Total RNA was prepared from samples taken at the indicated time points (hours) after a shift to 12 mM HU. Northern blots were probed with *cdt2*<sup>+</sup> and *cdc18*<sup>+</sup> (MBF targets) and with *act1*<sup>+</sup> (loading control). (C) Loss of Tor1 rescues the mitotic catastrophe of  $\Delta$ *rad3* mutants. Cells were incubated with or without 12 mM HU for 6 h at 30°C and then stained with DAPI and calcofluor to visualize nuclear DNA and septa, respectively. Percentages indicate abnormal mitosis, scoring for the “cut” phenotype in which the septum is formed despite the absence of chromosome replication. (D) The rapid loss of viability of the  $\Delta$ *rad3* or  $\Delta$ *cds1* mutant strain is rescued by  $\Delta$ *tor1*. Cells were grown to log phase and shifted to 12 mM HU for 6 h, and samples were taken every hour to determine cell viability by assessing plating efficiency on rich medium. WT, wild type. (E and F) Loss of Tor1 is epistatic over loss of Cds1. Strains were grown to log phase and shifted to 12 mM HU. The percentage of cells with septa was measured at the indicated times by staining with calcofluor and DAPI and visualized by fluorescence microscopy.

sure to HU is in sharp contrast to the rapid drop in viability observed in checkpoint-deficient  $\Delta rad3$  or  $\Delta cds1$  mutants (4). As previously described,  $\Delta rad3$  mutants do not elongate but continue to divide in the presence of unrepliated DNA, leading to a lethal phenotype known as “cut.” This phenotype is characterized by anucleate cells or cells with  $<1C$  DNA (8, 9) and can be observed by staining both nuclei and septa (Fig. 4C). The response of the  $\Delta tor1 \Delta rad3$  double mutant to HU was similar to that of single  $\Delta tor1$  mutants, and very few cells with a “cut” phenotype were observed (Fig. 4C). Consistently, the  $\Delta tor1$  mutation partially rescued the lethality of  $\Delta rad3$  mutants in response to acute exposure to HU (Fig. 4D).  $\Delta cds1$  mutants do not show the lethal “cut” phenotype in the presence of HU, yet they die rapidly in HU (4). The  $\Delta tor1 \Delta cds1$  double mutants displayed phenotypes similar to those of single  $\Delta tor1$  mutants (Fig. 4E and F), and like the interaction with  $\Delta rad3$  mutants,  $\Delta tor1$  partially rescued the rapid loss of viability of  $\Delta cds1$  mutants in response to HU (Fig. 4D). Notably,  $\Delta tor1$  rescued only the lethality of  $\Delta rad3$  or  $\Delta cds1$  upon short but not constant exposure to HU. We suggest that in the absence of Tor1, the death that occurs in the presence of HU in  $\Delta cds1$  or  $\Delta rad3$  cells is postponed due to slow progression during the first mitosis, before cells halt in early S phase. However, when cells eventually enter S phase, the  $\Delta tor1$  mutation cannot rescue the lethal events that occur in  $\Delta cds1$  or  $\Delta rad3$  mutants.

**Tor1 promotes mitotic entry via Cdc2.** Disruption of  $tor1^+$  generates moderately elongated cells, indicative of a delay in entry into mitosis (59). Accordingly, we found that  $\Delta tor1$  is synthetic lethal with the temperature-sensitive mutation in  $cdc25-22$  (Fig. 5A). Cdc25 is a phosphatase that activates Cdc2, the cyclin-dependent kinase that controls mitotic entry (12). Overexpression of Gad8 partially rescued the synthetic lethality between  $\Delta tor1$  and  $cdc25-22$  (Fig. 5B), suggesting that Tor1 affects entrance into mitosis via Gad8. This is also in concert with recent studies that reported lethality between  $\Delta gad8$  and  $cdc25-22$  (16), supporting a positive role for TORC2-Gad8 in regulating mitotic entry.

Two major antagonistic branches, the Cdc25- and Wee1-dependent pathways, regulate the status of Cdc2 phosphorylation on its tyrosine-15 residue (36). The  $cdc2-Y15F$  mutation, expressing an unphosphorylatable and constitutively active form of Cdc2 (11), completely reversed the elongated morphology of  $\Delta tor1$  mutants, and the  $\Delta tor1 cdc2-Y15F$  double mutant strain looked indistinguishable from the single  $cdc2-Y15F$  mutant (Fig. 5C). Thus, it appears that Tor1 controls entrance into mitosis by regulating the status of Cdc2 phosphorylation.

Introduction of the  $\Delta tor1$  mutation into the genetic background of  $\Delta cdc25 cdc2-3w$  cells resulted in cell cycle elongation (Table 1), indicating that Tor1 can regulate cell size in the absence of Cdc25. However, Tor1 is also capable of affecting cell size in the absence of Wee1. Combining the  $\Delta tor1$  mutation with the  $wee1-50$  mutation resulted in a slight elongation of the “wee” (very short) phenotype (Table 1; Fig. 5E). Similarly,  $\Delta wee1 \Delta tor1$  double mutants were slightly more elongated than single  $\Delta wee1$  mutants (our unpublished observation). Cells lacking Wee1 show a  $G_1$  delay, since they are “born” at a cell size shorter than the threshold required for the  $G_1$ -S transition (see reference 36). Our FACS analysis indi-

cated that double mutant  $wee1-50 \Delta tor1$  cells are also delayed in  $G_1$ , albeit slightly less so than single  $wee1-50$  mutants (Fig. 5E). In addition, we also found that the elongation of  $\Delta tor1$  cells was highly augmented in combination with deletion of  $cdr2^+$ , encoding a negative regulator of Wee1 (20) (Table 1), suggesting that Tor1 does not require Cdr2 for its cell cycle effect.

The elongation conferred by the  $\Delta tor1$  mutation was suppressed by two different activated alleles of  $cdc2$ ,  $cdc2-3w$  or  $cdc2-1w$  (Table 1), which are largely insensitive to Wee1 or Cdc25, respectively (45). This finding is consistent with the idea that Tor1 does not act solely via either the Wee1 or Cdc25 function.

The  $\Delta tor1$  mutation caused lethality when combined with the genetic background of  $wee1-50 cdc25-22$  and resulted in extreme cell size elongation at the restrictive temperature (Fig. 5D). The  $wee1-50 cdc25-22$  double mutant represents a genetic background in which the activity of Cdc2 is poorly regulated since both negative and positive effectors are missing. Another mutation which reverses the suppression of  $cdc25-22$  by the  $wee1-50$  mutation is the deletion of the stress-activated mitogen-activated protein kinase  $Spc1/Sty1$  (equivalent to p38 in mammalian cells). Moreover, deletion of  $spc1^+/sty1^+$  or its upstream effector  $wis1^+$  resulted in a set of genetic interactions with cell cycle mutants highly similar to that recorded by us for  $\Delta tor1$ , including synthetic lethality with the  $cdc25-22$  mutation (51, 58). It has previously been suggested that  $Spc1/Sty1$  regulates Polo kinase (Plo1) via its phosphorylation and localization to the spindle pole body, which in turn affects the balance between the activities of Wee1 and Cdc25 and determines mitotic progression (30, 40). It is possible that Tor1 also acts by affecting both Wee1 and Cdc25 (see our model, Fig. 6B). Recently it was also demonstrated that Tor1 acts upstream of  $Spc1/Sty1$  (41). Combining  $\Delta tor1$  with  $\Delta spc1/sty1$  resulted in an intermediate cell elongation compared to single  $\Delta tor1$  or  $\Delta spc1/sty1$  mutants, in concert with the possibility that Tor1 and  $Spc1/Sty1$  act in the same pathway (Table 1; also see Discussion).

**Tor1 is required for Cdc2 activation following recovery from HU-induced arrest.** In wild-type cells, Tyr15 phosphorylation on Cdc2 is required for the replication checkpoint arrest, and removal of the phosphate residue is critical to allow mitotic entry following recovery (43). We therefore examined the role of Tor1 in dephosphorylation of Cdc2-Tyr15 following release from HU arrest. To this aim, we incubated wild-type and  $\Delta tor1$  cells in the presence of 12 mM HU for 3.5 h before release into fresh medium that does not contain HU. Following treatment with HU, both wild-type and  $\Delta tor1$  cells arrested with highly phosphorylated Cdc2 on Tyr15 (Fig. 6A). In wild-type cells, dephosphorylation of Cdc2 occurred at 100 min following release from HU, consistent with results in previous studies (43), while in  $\Delta tor1$  cells, Cdc2 remained phosphorylated on Tyr15 for at least 200 min following release from HU (Fig. 6A). We conclude that Tor1 is required for activation of Cdc2 by Tyr15 dephosphorylation following recovery from HU treatment.

## DISCUSSION

Our data reveal novel and unexpected roles for TORC2 in regulating gene silencing, telomere length, and survival under

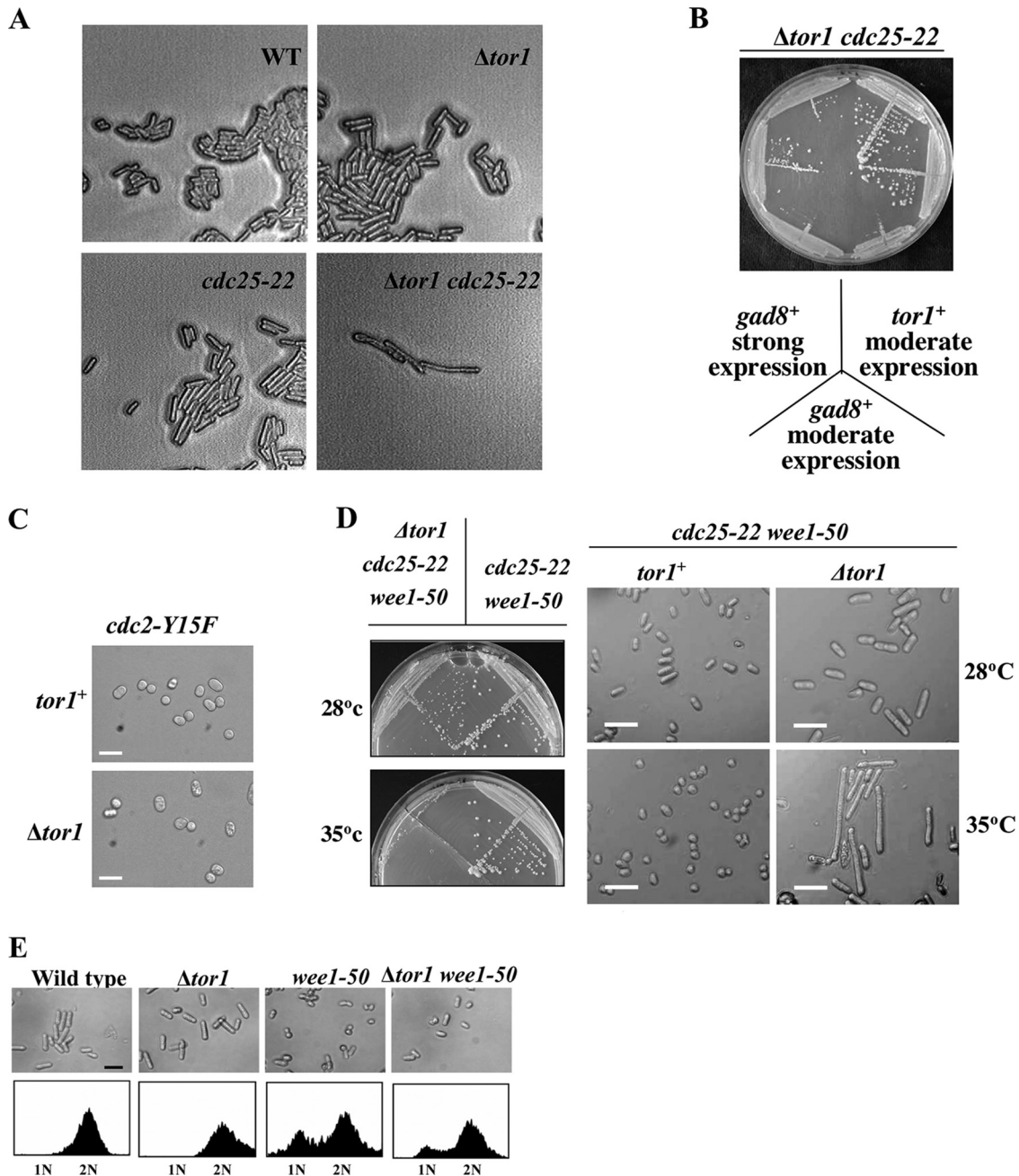


FIG. 5. Tor1 positively regulates mitosis. (A) The  $\Delta tor1$  mutation is synthetic lethal with  $cdc25-22$ . A diploid strain heterozygous for  $\Delta tor1$  and  $cdc25-22$  was subjected to meiosis and tetrad analysis. Plates were incubated at 25°C. (B) Overexpression of  $gad8^{+}$  rescues the synthetic lethality of  $tor1 cdc25-22$ . The same diploid strain as above was transformed with pIRT2- $tor1^{+}$ , pREP41- $gad8^{+}$  (moderate overexpression), and pREP1- $gad8^{+}$  (strong overexpression). Two double mutant spores containing each of the plasmids were isolated and streaked onto plates at 28°C (no viable spores were obtained with an empty vector). (C) The  $cdc2-Y15F$  mutation suppresses the elongated phenotype of cells lacking Tor1. Bars: 20  $\mu$ m. (D) The  $\Delta tor1$  mutation reverses the suppression of  $cdc25-22$  by  $wee1-50$ . Cells from the indicated genotypes were streaked onto plates either at 28°C or 35°C (left panel), and cells were visualized by light microscopy (right panel). Bars: 20  $\mu$ m. (E) The  $wee1-50$  mutation partially suppresses the elongated phenotype of cells lacking Tor1. Cells were grown to mid-log phase at 30°C, photographed, and subjected to FACS analysis.

DNA-damaging conditions. These TORC2-dependent functions are rapamycin insensitive and thus could easily be overlooked in studies of mammalian cells, which are largely based on the use of rapamycin as a specific inhibitor of TOR. Global gene expression analysis of  $\Delta tor1$  mutants revealed an extensive overlap with expression signatures in mutants in histone

deacetylase genes (*clr3* and *clr6*) or in the gene encoding the RSC58 subunit of the RSC complex. Like these chromatin structure mutants,  $\Delta tor1$  cells derepressed gene expression at heterochromatic regions, exhibited elongated telomeres, and were sensitive to osmotic stress, DNA damage, and the microtubule destabilizer TBZ. In budding yeast, TORC1 regulates



TABLE 1. Effects of mutations on cell size

| Genotype                      | Temp (°C) | Mean cell length ( $\mu\text{m}$ ) $\pm$ SD <sup>a</sup> |                                  |
|-------------------------------|-----------|--|----------------------------------|
|                               |           | <i>tor1</i> <sup>+</sup>                                 | $\Delta$ <i>tor1</i>             |
| Wild type                     | 30        | 15.1 $\pm$ 0.9   | 17.5 $\pm$ 2.3                   |
| <i>cdc25-22</i>               | 28        | 20.4 $\pm$ 1.4   | <b>&gt;35</b> (synthetic lethal) |
| <i>wee1-50</i>                | 35        | 8.0 $\pm$ 1.3  | <b>8.9 <math>\pm</math> 1.7</b>  |
| <i>cdc2-1w</i>                | 30        | 8.0 $\pm$ 1.6  | <b>11.2 <math>\pm</math> 1.8</b> |
| <i>cdc2-3w</i>                | 30        | 8.3 $\pm$ 1.7  | <b>12.6 <math>\pm</math> 2.3</b> |
| $\Delta$ <i>nim1</i>          | 30        | 15.1 $\pm$ 1.5   | <b>16.0 <math>\pm</math> 1.6</b> |
| $\Delta$ <i>cdr2</i>          | 30        | 17.2 $\pm$ 1.6   | <b>&gt;30</b> (synthetic sick)   |
| $\Delta$ <i>cdc25 cdc2-3w</i> | 30        | 14.0 $\pm$ 2/9   | <b>21.1 <math>\pm</math> 1.8</b> |
| $\Delta$ <i>sty1</i>          | 30        | 24.3 $\pm$ 2.9   | <b>19.0 <math>\pm</math> 2.3</b> |

<sup>a</sup> Cell length at division ( $n = 200$ ). Cell lengths of double or triple mutant cells are presented in bold.

the chromatin structure in a rapamycin-sensitive manner via Rpd3 (44, 55). Our data suggest that TORC2 may carry out a similar function, although the precise mechanism is yet to be determined.

A striking observation is that  $\Delta$ *tor1* cells have longer telomeres than wild-type cells. It will clearly be important to de-

termine whether TOR also affects telomere length in higher eukaryotes. Loss of Tor1 induced overelongation of telomeres in the  $\Delta$ *tel1*,  $\Delta$ *chk1*, or  $\Delta$ *cds1* checkpoint mutant but not in cells lacking the ATR-like kinase Rad3. Thus, this is although highly speculative at present, Tor1 and Rad3 may work in the same pathway, regulating telomeres in an antagonistic manner. Elongated telomeres have also been observed in several chromatin defect mutants, including the *clr6-1* mutant (12) and with loss of *set1*<sup>+</sup>, encoding the histone H3-K4 methyltransferase in fission yeast (19), raising the possibility of a mechanistic link between chromatin structure defects and telomere elongation. The TOR signaling pathway may provide a link between nutrient signaling and cellular processes that govern chromatin and telomere structures.

Like  $\Delta$ *tor1* mutants, the  $\Delta$ *ste20* or  $\Delta$ *sin1* mutant is also highly sensitive to TBZ and HU and shows highly elongated telomeres (Fig. 1E and 2A). Thus, it appears that TORC2 is the TOR complex required under DNA replication stress and for regulation of telomere length. Gad8 (equivalent to AKT/PKB1) acts downstream of Tor1 (TORC2) (16). Overexpression of Gad8 in the background of  $\Delta$ *tor1* cells partially rescued

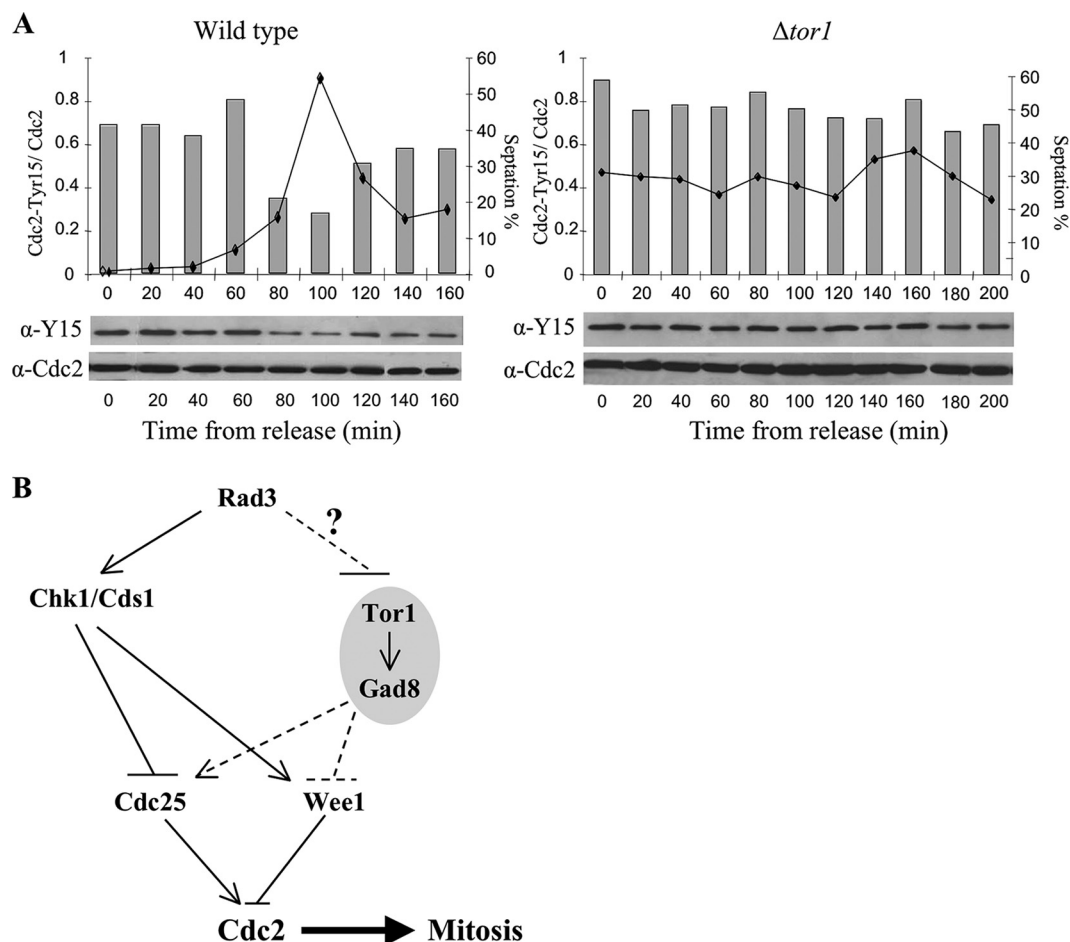


FIG. 6. Tor1 is required for activation of Cdc2 after release from HU arrest. (A) Wild-type and  $\Delta$ *tor1* cells were treated with 12 mM HU for 3.5 h, washed, and resuspended in fresh yeast extract. Samples from the indicated time points were taken for septation index measurement and Western blot analysis. (B) A working model. In response to DNA damage or DNA replication stress, Rad3 activates Chk1 or Cds1, respectively, leading to a delay in mitotic entry. In parallel, Rad3 keeps Tor1 inactive until DNA replication is completed. Regulation of Tor1 activity is not essential to prevent premature entry into mitosis but is required for reentry upon recovery from checkpoint arrest.

HU or TBZ sensitivity (Fig. 1D and 2C). Thus, most of the newly identified functions of Tor1 (TORC2) appear to be mediated via Gad8. Cells lacking Gad8 also exhibited elongated telomeres, yet overexpression of *gad8*<sup>+</sup> did not suppress telomere overelongation in *Δtor1* mutants (data not shown). It is likely that Gad8 is not fully activated in the absence of Tor1 and thus cannot fully rescue defects associated with disruption of *tor1*<sup>+</sup>.

Unlike Clr6 and Clr3, Tor1 has also been strongly implicated in regulating cell cycle progression and the response to nitrogen starvation (22, 41, 59; this work). In addition, *Δtor1* cells are far more sensitive to DNA-damaging conditions than the *clr6* or *clr3* mutant cells. For example, the growth of *Δtor1* mutant cells is greatly inhibited at the concentrations of 2 mM HU or 0.003% MMS (Fig. 3A and B). In contrast, the growth of *clr6-1* mutant cells is inhibited at the concentrations of 10 mM of HU or 0.01% of MMS (12; our unpublished data). We speculate that the cell cycle defects observed in *Δtor1* mutants contribute to their sensitivity to DNA-damaging conditions.

How may Tor1 (TORC2) integrate its function in mitotic progression and the response to DNA-damaging conditions? The Rad3 kinase is a major DNA damage sensor that regulates cell cycle progression via activation of the Chk1 or Cds1 kinase in response to DNA damage or replication stress. Activated Chk1 or Cds1 inhibits mitotic entry by regulating Cdc25 and Wee1/Mik1 activity (4). Our data suggest that Tor1 is not required for arresting mitotic entry in the presence of DNA damage or replication stress. Indeed, Tor1 is required for mitotic progression, a function that seems critical upon removal of HU. Thus, if Tor1 acts downstream of Rad3, it would be expected that Rad3 negatively regulates Tor1, keeping Tor1 inactive till DNA replication or repair is completed (Fig. 6B). However, the connection between Rad3 and Tor1 is yet to be determined. Our genetic data showing that the sensitivity of *Δtor1* cells to HU or MMS is augmented in combination with *Δcds1* or *Δchk1*, respectively, suggest that Tor1 acts independently of either Cds1 or Chk1. Consistently, Chk1 is normally phosphorylated in response to DNA damage in the absence of Tor1 (Fig. 3C). Yet, like Chk1/Cds1, Tor1 affects mitotic progression via regulation of the phosphorylation status of Cdc2 at the tyrosine-15 residues, possibly by controlling the balance between Cdc25 and Wee1 activity, as depicted in our working model (Fig. 6B).

Our data indicate that Tor1 acts as a positive regulator of mitotic entry under normal growth conditions. Moreover, Tor1 is critical for dephosphorylation of Cdc2 Tyr-15 upon recovery from HU treatment, thus promoting reentry into mitosis and cellular proliferation. It has been reported that reducing the level of Tor1 induced entry into mitosis via regulating the Spc1/StyI pathway (41). Our results are consistent with a role of Tor1 in the same pathway as Spc1/StyI but argue that Tor1 is a positive regulator of mitosis. This apparent discrepancy may be explained by the use of different *tor1* mutants in the two studies; while we used a complete disruption of *tor1*<sup>+</sup>, Petersen and Nurse (41) based their conclusions on cells expressing low levels of Tor1. Thus, the effect of Tor1 on mitotic entry may rely on its level of activity. Indeed, while we were revising the manuscript, it was reported that Tor1 can act as part of TORC1 in regulating entrance into mitosis (13). It is the inhibition of a Tor1-Mip1 (TORC1) complex that induces en-

trance into mitosis under poor nitrogen conditions (13). Thus, whether Tor1 acts as an inducer or inhibitor of mitosis may also rely on its partner proteins.

An intriguing question is how TORC2 may affect nuclear functions. TOR proteins seem to locate primarily in the cytoplasm (54) but have also been reported to shuttle into the nucleus in both mammalian (23) and budding yeast (25) cells. In growing fission yeast cells, Tor2 fused to green fluorescent protein localizes to the cytoplasm and to the perinuclear region, while no localization data exist for Tor1 (14). Thus, whether Tor1 affects nuclear functions directly or by controlling other regulators remains to be resolved in future experiments.

Finally, recent work (14, 21) demonstrated that Tel2, a fission yeast homologue of mammalian Clk2/Rad-2 required for the replication checkpoint, physically interacts with all PIKKs, suggesting a possible functional link among this family of proteins. Our study is consistent with this intriguing observation and argues that TORC2 is a regulator of survival under DNA damage conditions. Together, these findings place the TOR proteins alongside the other PIKKs, ATR and ATM, as regulators of nuclear processes and guardians of genome integrity and stability.

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