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TOR complex 2 controls gene silencing, telomere length maintenance and survival under DNA damaging conditions

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Abstract

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 evolutionary 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 Δ*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-methane sulfonate (MMS), 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 de-phosphorylation and re-activation of the cyclin-dependent kinase Cdc2, thus allowing re-entry 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.

Introduction

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 man 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 the mammalian p70S6K downstream of TORC1 and AKT/protein kinase B (PKB) downstream to TORC2 (18). TORC1 in mammalians 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 mammalian 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 cell morphology,

sensitivity to osmotic and oxidative stress, inability to execute developmental processes in response to nutrient depletion and 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 the mammalian AKT/protein kinase B (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 a shift from rich to poor nitrogen conditions, an effect that may involve both inhibition of Tor1 and Tor2 (41).

While other members of the 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 has been 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, as well as for regulation of cell cycle progression. As the TOR complexes are highly conserved in evolution, this novel TORC2 function may also be conserved in other organisms.

Results

tor1 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*$^+$ (Δ*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 Δ*tor1* compared to wild type cells (Supplementary Fig. 1 and Fig. 2 and data submitted at ArrayExpress 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*, Δ*clr3 clr6-1* (12) or Δ*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 *wtf* elements, and several non-coding RNA telomeric duplications, suggesting that the Δ*tor1* mutation leads to a de-repression of gene transcription mediated by heterochromatin. Accordingly, upregulated genes were significantly clustered at subtelomeric regions compared to a random distribution (P <0.05). We verified, using Northern blot analysis that genes that are upregulated in *clr3* or *clr6-1* (12) are also upregulated in Δ*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*$^+$, inserted at the mating type locus (2). We found that loss of Tor1 relieved the

repression of *ade6*$^{+}$ 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 Δ*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 Δ*tor1* mutants, we noted several transporters, including *str1*$^{+}$, encoding a component of the iron-sidephore system. The transcription of *str1*$^{+}$ 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 sensitivity. Indeed, the *clr6-1* Δ*clr3* double mutant was highly sensitive to 1M KCl (12), showing similar osmotic sensitivity to that observed in Δ*tor1* mutants (59). Thus, Δ*tor1* mutants share with HDAC mutants their gene expression pattern, de-repression of genes at heterochromatic regions, as well as 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 Δ*tor1,* Δ*ste20* or Δ*gad8* mutants were elongated by ~150 bp compared to wild type, similar to the elongation observed in *clr6-1* mutants. In contrast, wild type cells grown in the presence of 100 ng/ml rapamycin did not affect the length of telomeres (Fig. 1E). We conclude that TORC2-Gad8 regulates telomere length in a rapamycin

insensitive manner. Overexpression of Gad8 did not suppress telomere over-elongation in Δ*tor1* mutants (data not shown). However, since Gad8 is a substrate for phosphorylation by Tor1, it is likely to be poorly active in Δ*tor1* mutants.

DNA checkpoint proteins play a central role in telomere maintenance. Mutants 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 in its clamp loader Rad17, result in short telomeres (38). We found that the length of telomeres of the double mutant Δ*tor1* Δ*rad3* or Δ*tor1* Δ*rad17* are as short as single Δ*rad3* or Δ*rad17* mutants, respectively. Thus, Tor1 may induce telomere over-elongation 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*$^+$ in either Δ*chk1* or Δ*cds1* backgrounds resulted in a similar telomere elongation as in single Δ*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 over-elongation in Δ*tor1* mutants did not require the presence of Tel1 (Fig. 1E). We thus suggest that 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 in either 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 Δ*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 Δ*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*$^+$ or *tsc2*$^+$ did not markedly affect HU sensitivity (Fig. 2B and data not shown). Thus, it appears that mutations in TORC2 but not TORC1 are sensitive to HU. Overexpression of *gad8*$^+$ partially suppressed the HU sensitivity of Δ*tor1* mutants, further suggesting that Tor1 acts via Gad8 in tolerating replication stress (Fig. 2C).

We also found that Δ*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 the 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. 3 A, B). Combining the Δ*tor1* mutation with Δ*rad3* or Δ*rad17* did not result in further sensitivity to the DNA damaging conditions (Fig. 3A, B). Thus, the function of Tor1 in 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 Δ*tor1* mutants to HU was further augmented when combined with either loss of function of *cds1*$^+$ or its specific

mediator *mrc1*$^{+}$, 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 cells lacking Chk1, the main effector of the DNA damage response pathway (Fig. 3B). Yet, the Δ*tor1* mutation showed additive effects with Δ*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 Δ*tor1* cells to DNA replication stress induced by HU

A non-synchronized wild type population of fission yeast cells mainly contains G2 cells. Addition of HU to such a population results in the doubling in cell number, as 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 cells with an elongated cell morphology (8, 9).

FACS analysis of Δ*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 towards a content of 1.5N DNA is observed at 4-5 hours in HU in Δ*tor1* nuclei. The meaning of this drift is not clear. However, since Δ*tor1* cells maintain full viability following incubation of 4-5 hours in HU (see below), we suggest that this "drift" reflects changes in the structure or size of Δ*tor1* nuclei rather than the inability of Δ*tor1* cells to properly arrest in G1.

Consistent with the slower kinetics in which Δ*tor1* nuclei accumulated with 1N DNA content in response to HU, we detected a slower and reduced accumulation of Cdc10/MBF-dependent S phase-specific transcripts [e.g., *cdt2*$^+$ and *cdc18*$^+$; (63)] in Δ*tor1* mutants compared to wild type cells (Fig. 4B). This finding could either reflect a defect in cell cycle progression in Δ*tor1* mutants or a more direct defect in activating the transcriptional response to HU (5-7, 46).

Although Δ*tor1* cells arrested with nuclei of 1N DNA content and induced S phase specific transcripts, these cells did not show elongation in cell size in response to HU (Fig. 4C). Staining of the cells with DAPI and calcofluor, in order to view nuclei and septa, respectively, revealed that exposure of Δ*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 septa (Fig. 4 C, E), as previously shown (8). The septated HU-arrested Δ*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, Δ*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 Δ*liz1* mutants, addition of pantothenate to the medium did not rescue the HU sensitivity of Δ*tor1* mutants (data not shown), thus the aberrant response to HU in Δ*tor1* mutants occurs via a distinct mechanism. Importantly, however, our observation that Δ*tor1* cells are highly sensitive to MMS suggests that Δ*tor1* cells have a general defect in coping with DNA damage, rather than a specific defect concerning the response to HU.

The viability of Δ*tor1* mutants in response to short exposure to HU is in sharp contrast to the rapid drop in viability observed in checkpoint-deficient Δ*rad3* or Δ*cds1* mutants (4). As previously described, Δ*rad3* mutants do not elongate but continue to divide in the presence of unreplicated 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 double mutant Δ*tor1* Δ*rad3* to HU was similar to that of single Δ*tor1* mutants, and very few cells with a 'cut' phenotype were observed (Fig. 4C). Consistently, the Δ*tor1* mutation partially rescued the lethality of Δ*rad3* mutants in response to acute exposure to HU (Fig. 4D). Δ*cds1* mutants do not show the lethal 'cut' phenotype in the presence of HU, yet they die rapidly in HU (4). The Δ*tor1* Δ*cds1* double mutants displayed phenotypes similar to single Δ*tor1* mutants (Fig. 4E, F) and, like the interaction with Δ*rad3* mutants, Δ*tor1* partially rescued the rapid loss of viability of Δ*cds1* mutants in response to HU (Fig. 4D). Notably, Δ*tor1* only rescued the lethality of Δ*rad3* or Δ*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 Δ*cds1* or Δ*rad3* is postponed due to slow progression during the first mitosis, before cells halts in early S phase. However, when cells eventually enter S phase, the Δ*tor1* mutation cannot rescue the lethal events that occur in Δ*cds1* or Δ*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 Δ*tor1* is synthetic lethal with the temperature sensitive mutation in *cdc25-22* mutation (Fig. 5A). Cdc25 is a phosphatase that activates Cdc2, the cyclin-dependent kinase (CDK) that controls

mitotic entry (12). Overexpression of Gad8 partially rescued the synthetic lethality between Δ*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 Δ*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 Δ*tor1* mutants and the double mutant strain Δ*tor1 cdc2-Y15F* looked indistinguishable from the single *cdc2-Y15F* mutant (Fig. 5C). Thus, it appears that Tor1 controls entrance into mitosis via regulating the status of Cdc2 phosphorylation.

Introduction of the Δ*tor1* mutation into the genetic background of Δ*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 Δ*tor1* mutation with the *wee1-50* mutation resulted in a slight elongation of the 'wee' (very short) phenotype (Table 1, Fig.5E). Similarly, Δ*wee1* Δ*tor1* double mutant were slightly more elongated compared with single Δ*wee1* mutants (our unpublished observation). Cells lacking Wee1 show a G1 delay, since they are 'born' at a cell size shorter than the threshold required for the G1-S transition (see in (36). Our FACS analysis indicated that double mutant *wee1-50* Δ*tor1* cells are also delayed in G1, albeit slightly less so compared to single *wee1-50* mutants (Fig. 5E). In addition, we also found that the elongated morphology of Δ*tor1* cells was highly augmented when combined 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 elongated morphology conferred by the Δ*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 Δ*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 as 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 MAPK Spc1/Sty1 (equivalent to p38 in mammalian cells). Moreover, deletion of *spc1*$^+$/*sty1*$^+$ or its downstream effector *wis1*$^+$ resulted in a highly similar set of genetic interactions with cell cycle mutants as recorded by us for Δ*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 has also been demonstrated that Tor1 acts upstream of Spc1/Sty1 (41). Combining Δ*tor1* with Δ*spc1/sty1* resulted in an intermediate cell-size elongation compared to single Δ*tor1* or Δ*spc1/sty1* mutants, in concert with the possibility that Tor1 and Spc1/Sty1 act in the same pathway (Table 1, and also see our 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 Δ*tor1* cells in the presence of 12 mM HU for 3.5 hours before release into fresh medium that does not contain HU. Following treatment with HU, both wild type and Δ*tor1* cells arrested with highly phosphorylated Cdc2 on Tyr15 (Fig. 6A). In wild type cells, de-phosphorylation of Cdc2 occurred at 100 minutes following release from HU, consistent with previous studies (43), while in Δ*tor1* cells, Cdc2 remained phosphorylated on Tyr15 for at least 200 minutes following release from HU (Fig. 6A). We conclude that Tor1 is required for activation of Cdc2 by Tyr15 de-phosphorylation following recovery from HU treatment.

Discussion

Our data reveal novel and unexpected roles for TORC2 in regulating gene silencing, telomere length, and survival under DNA damaging conditions. These TORC2-dependent functions are rapamycin insensitive and thus could easily be overlooked in studies in mammalian cells, which are largely based on the use of rapamycin as a specific inhibitor of TOR. Global gene expression analysis in Δ*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, Δ*tor1* cells de-repressed gene expression at heterochromatic regions, exhibited elongated telomeres and were sensitive to osmotic stress, DNA damage and to the microtubules de-stabilizer TBZ.

In budding yeast, TORC1 regulates 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 Δ*tor1* cells have longer telomeres compared to wild type. It will clearly be important to determine whether TOR also affects telomere length in higher eukaryotes. Loss of Tor1 induced over-elongation of telomeres in the Δ*tel1*, Δ*chk1* or Δ*cds1* checkpoint mutants, but not in cells lacking the ATR-like kinase Rad3. Thus, 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 *clr6-1* (12) and loss of *set1*$^+$, encoding the histone H3-K4 methyltransferase in fission yeast (19), raising the possibility of a mechanistic link between chromatin structure defects and telomere elongation. TOR signaling pathway may provide a link between nutrient signaling and cellular processes that govern chromatin and telomere structures.

Like Δ*tor1* mutants, Δ*ste20* or Δ*sin1* mutants are also highly sensitive to TBZ, HU and show highly elongated telomeres (Fig. 1E and Fig. 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 Δ*tor1* cells partially rescued HU or TBZ sensitivity (Fig. 1D and Fig. 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*$^+$ did not suppress telomere over-elongation in Δ*tor1* 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*$^{+}$.

Unlike Clr6 and Clr3, Tor1 has also been strongly implicated in regulating cell cycle progression and response to nitrogen starvation [(22, 41, 59) and this manuscript]. In addition, Δ*tor1* cells are far more sensitive to DNA damaging conditions compared with the *clr6* or *clr3* mutant cells. For example, the growth of Δ*tor1* mutant cells is greatly inhibited at the concentrations of 2 mM HU or 0.003% MMS (Fig. 3 A, 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) and our unpublished data]. We speculate that the cell cycle defects observed in Δ*tor1* mutants contribute to its sensitivity to DNA damaging conditions.

How Tor1 (TORC2) may integrate its function in mitotic progression and 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 kinases in response to DNA damage or replication stress. Activated Chk1 or Cds1 inhibit 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 Δ*tor1* cells to HU or MMS is augmented when combined with Δ*cds1* or Δ*chk1*, 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 positive regulator of mitotic entry under normal growth conditions. Moreover, Tor1 is critical for de-phosphorylation of Cdc2 Tyr-15 upon recovery from HU treatment, thus promoting re-entry into mitosis and cellular proliferation. It has been reported that reducing the level of Tor1 induced entry into mitosis via regulating the Spc1/Sty1 pathway (41). Our results are consistent with a role of Tor1 in the same pathway as Spc1/Sty1, 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*$^{+}$, 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 revising this manuscript, it has been 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 entrance 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, both in mammalian (23) and in budding yeast cells (25). In growing fission yeast cells, Tor2 fused to GFP localizes to the cytoplasm and to the peri-nuclear 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 amongst 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.

Materials and Methods

Yeast techniques

S. pombe strains are described in Supplementary Table 1. All experiments were performed by standard genetic and molecular yeast techniques as described in (35). Growth medium was prepared as in (59). Rapamycin (R0395, Sigma) was used at a final concentration of 100 ng/ml**.** For cell killing assays, HU (H8627, Sigma) or MMS (129925, Sigma) were added at the indicated concentrations. UV irradiation was performed using UV Stratalinker 1800 (Stratagene). Cells were visualized using a Nikon eclipse E600 fluorescence microscope, 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 sorter (FACS) analysis, nuclei were isolated as previously described (10), stained with propidium iodide and analyzed by a Becton Dickinson FACSort. Data were analyzed by Cell Quest software for Macintosh.

Telomere gels

DNA was isolated from logarithmically growing cells, digested with *Eco*RI 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 (62). Gene-specific probes were labeled with [α-^{32}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, 20mM hepes pH7.9, 50mM NH_2SO_4, 5mM EDTA pH8.0) in the presence of protease inhibitor and broken with glass beads. Immunoblotting was performed as previously described (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 previously described (29). Three independent biological experiments were performed, including a dye swap. The data were visualized and analyzed using GeneSpring (Agilent). The significance of overlaps between different gene lists was calculated in GeneSpring using a standard Fisher's exact test, and *P* values were adjusted with a

Bonferroni multiple testing correction. Cut-off 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 ArrayExpress account at www.ebi.ac.uk/aerep/login..

Clustering along chromosomes of genes with induced expression in Δ*tor1* 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 Benjamini-Hochberg False Discovery Rate.

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Figure legends

Figure 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), Δ*tor1* and *clr6-1* mutants grown to mid-log in rich medium. Northern blots were probed with the indicated genes. (C) Tor1 promotes silencing at the mating type locus. Strains containing an *ade6*$^+$ cassette at the mating type locus were spotted onto the indicated plates. In an otherwise wild type background, the *ade6*$^+$ gene insertion produced a typical position variegation effect (PVE), as only a portion of the colonies are white (express the *ade6*$^+$ gene) while others are red due to decreased level of *ade6*$^+$ transcript and accumulation of a red pigment. Only white colonies are present in cells carrying the Δ*tor1* mutation. (D) Tor1 is required for tolerance to microtubule de-stabilizing 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 *Eco*RI, which in wild type cuts about 1 Kb from the terminus, and analyzed by Southern blotting. The resulting filter was probed with α^{32}P-labelled telomere repeat DNA.

Figure 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 onto

plates with or without the indicated amounts of HU. (C) Overexpression of *gad8*$^+$ partially rescues the lethal phenotype of Δ*tor1* on HU. *gad8*$^+$ is expressed from the thiamine (T) repressible *nmt1*$^+$ promoter from the plasmids pREP1, 41 and 81 that allow strong, moderate and weak expression, respectively. *tor1*$^+$ 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^2 with or without 100 ng/ml rapamycin (R).

Figure 3: Mutations in TORC2 confer sensitivity to DNA damaging conditions independent 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 Δ*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).

Figure 4: Tor1 is required for a normal response to hydroxyurea. (A) and (B) The response of *tor1* mutants to HU is delayed. Wild-type and Δ*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 shift to 12 mM HU. Northern blots were probed with *cdt2*$^+$ and *cdc18*$^+$ (MBF targets), and with *act1*$^+$ (loading control). (C) Loss of Tor1 rescues the mitotic catastrophe of Δ*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 Δ*rad3* or Δ*cds1* mutant strains is rescued by Δ*tor1*. Cells were grown to log phase, shifted to 12 mM HU for 6 h, and samples were taken every hour to determine cell viability by plating efficiency on rich medium. (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 fluorescent microscopy.

Figure 5: Tor1 positively regulates mitosis. (A) The Δ*tor1* mutation is synthetic lethal with *cdc25-22*. A diploid strain heterozygous for Δ*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. (D) The Δ*tor1* mutation reverses the suppression of *cdc25-22* by *wee1-50*. Cells from the indicated genotypes were streaked onto plates either at 28° or 35°C (left panel), and cells were visualized by light microscopy (right panel), bar length: 20μm. (E) The *wee1-50* mutation partially suppresses the elongated phenotype of cells lacking Tor1. Cells were grown to mid-log phase, photographed and subjected to FACS analysis.

Figure 6: Tor1 is required for activation of Cdc2 after release from HU arrest. (A) Wild type and Δ*tor1* cells were treated with 12mM HU for 3.5 h, washed and resuspended in fresh YE. 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 delay in mitotic entry. In parallel, Rad3, keeps Tor1 inactive till DNA replication is completed. Regulation of Tor1 activity is not essential to prevent premature entry into mitosis, but is required for re-entry upon recovery from checkpoint arrest.

Table 1

		tor1$^+$		Δ*tor1*	
	Temp. ^oC	*Mean (μm)	± Stdv	Mean (μm)	± Stdv
Wild type	30	15.1	0.9	17.5	2.3
cdc25-22	28	20.4	1.4	**>35 synthetic lethal**	
wee1-50	35	8.0	1.3	**8.9**	**1.7**
cdc2-1w	30	8.0	1.6	**11.2**	**1.8**
cdc2-3w	30	8.3	1.7	**12.6**	**2.3**
Δ*nim1*	30	15.1	1.5	**16.0**	**1.6**
Δ*cdr2*	30	17.2	1.6	**>30 synthetic sick**	
Δ*cdc25 cdc2-3w*	30	14.0	2/9	**21.1**	**1.8**
Δ*sty1*	30	24.3	2.9	**19.0**	**2.3**

*cell length at division (n=200).

Cell length of double mutant cells are presented in bold numbers

Figure 1

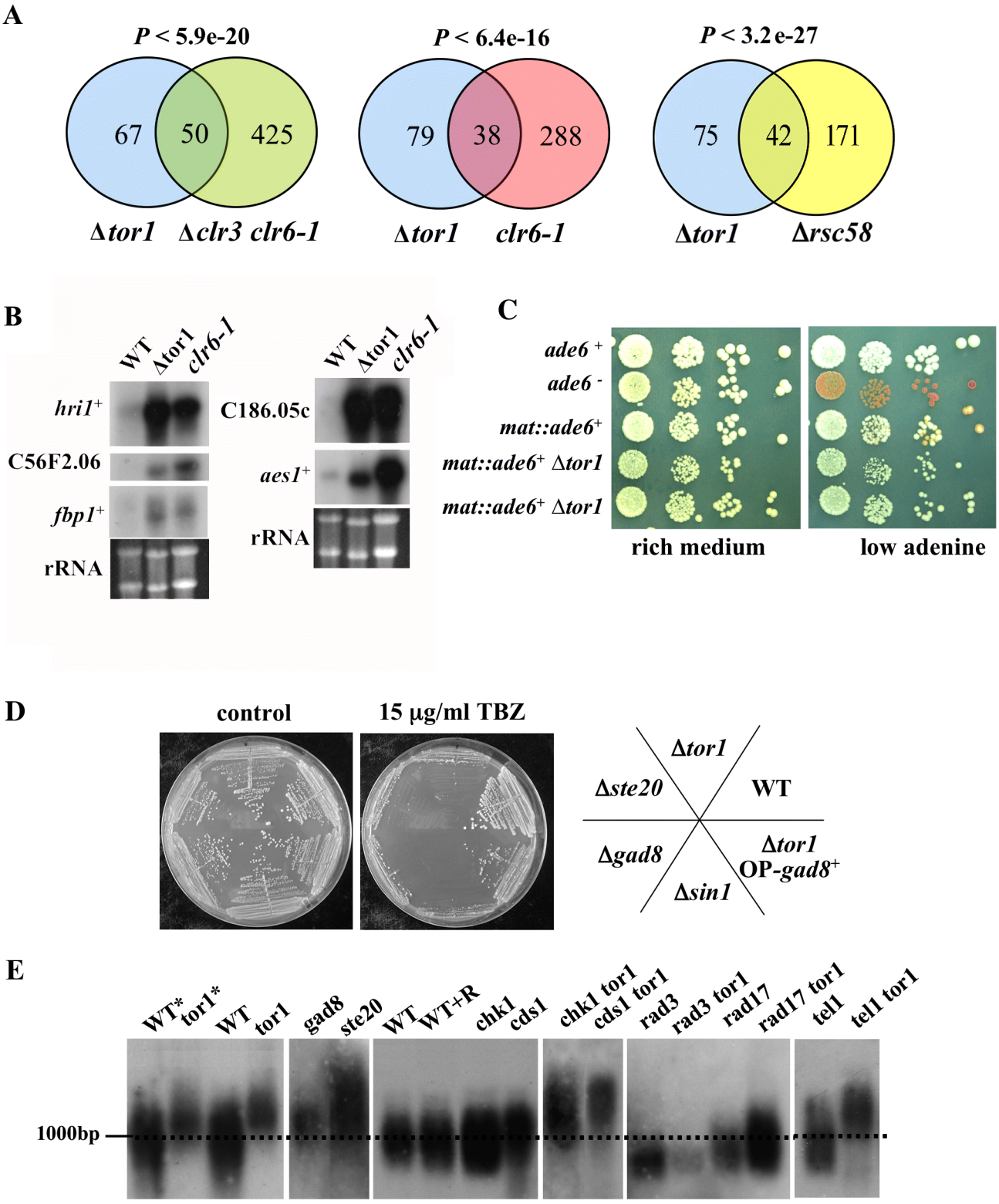

Figure 2

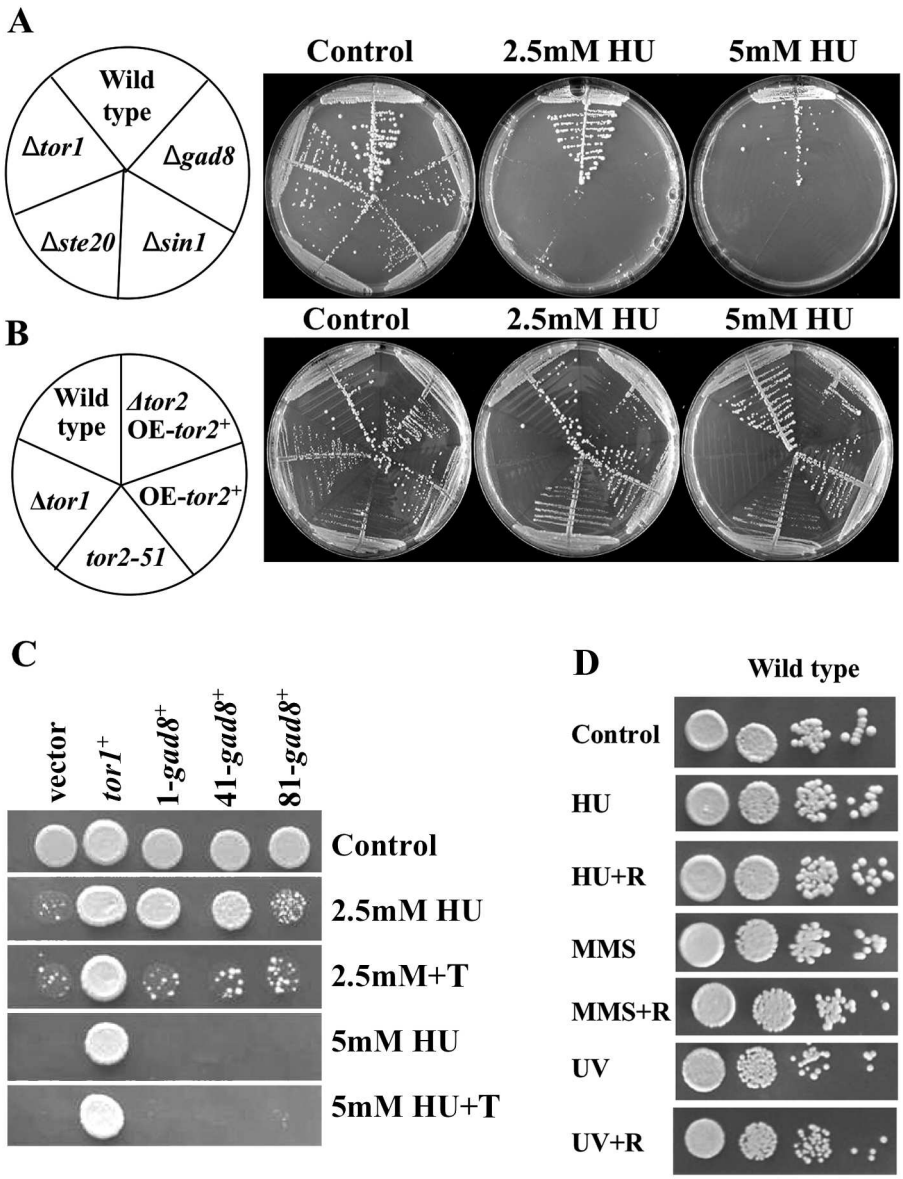

Figure 3

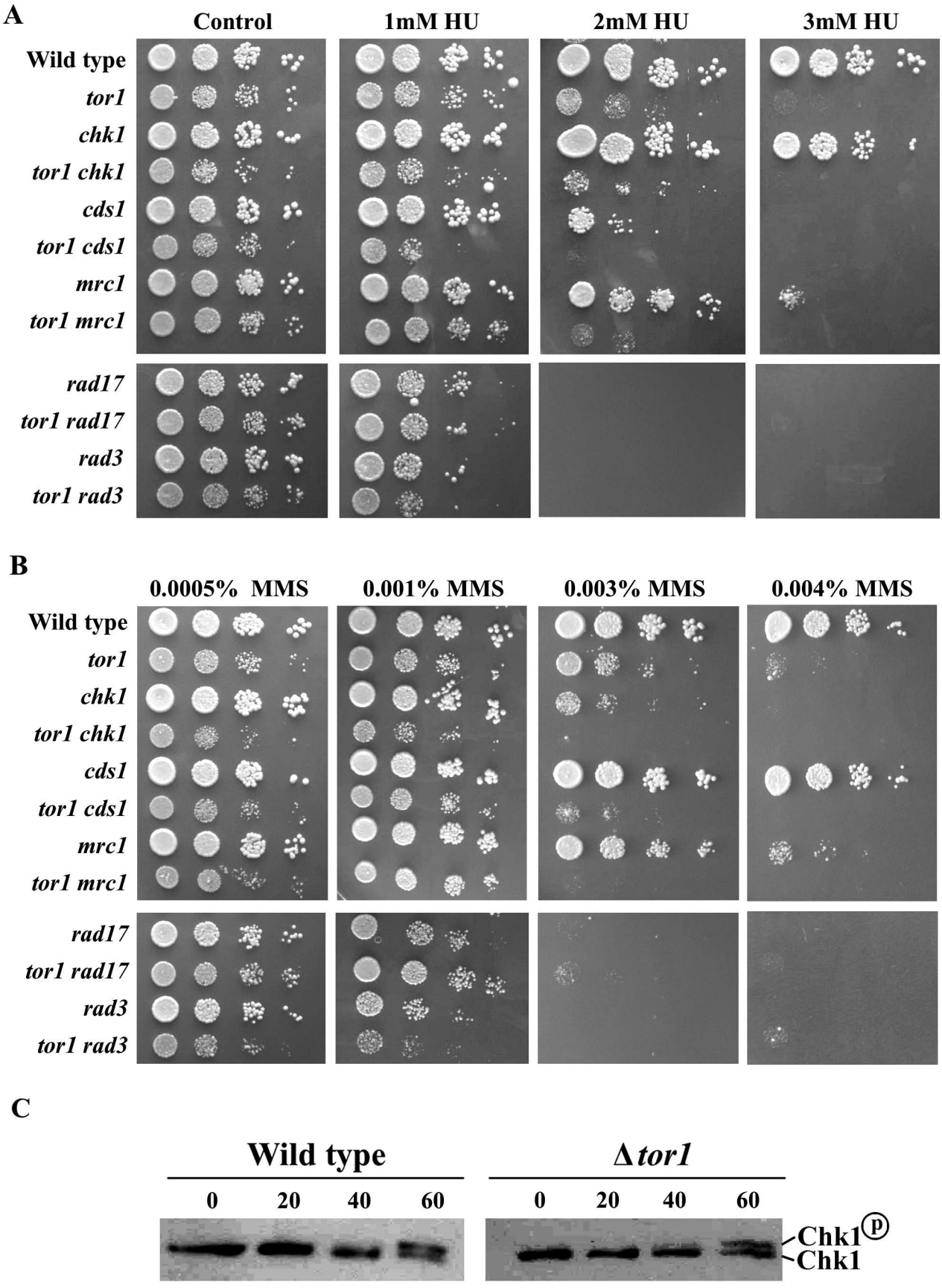

Figure 4

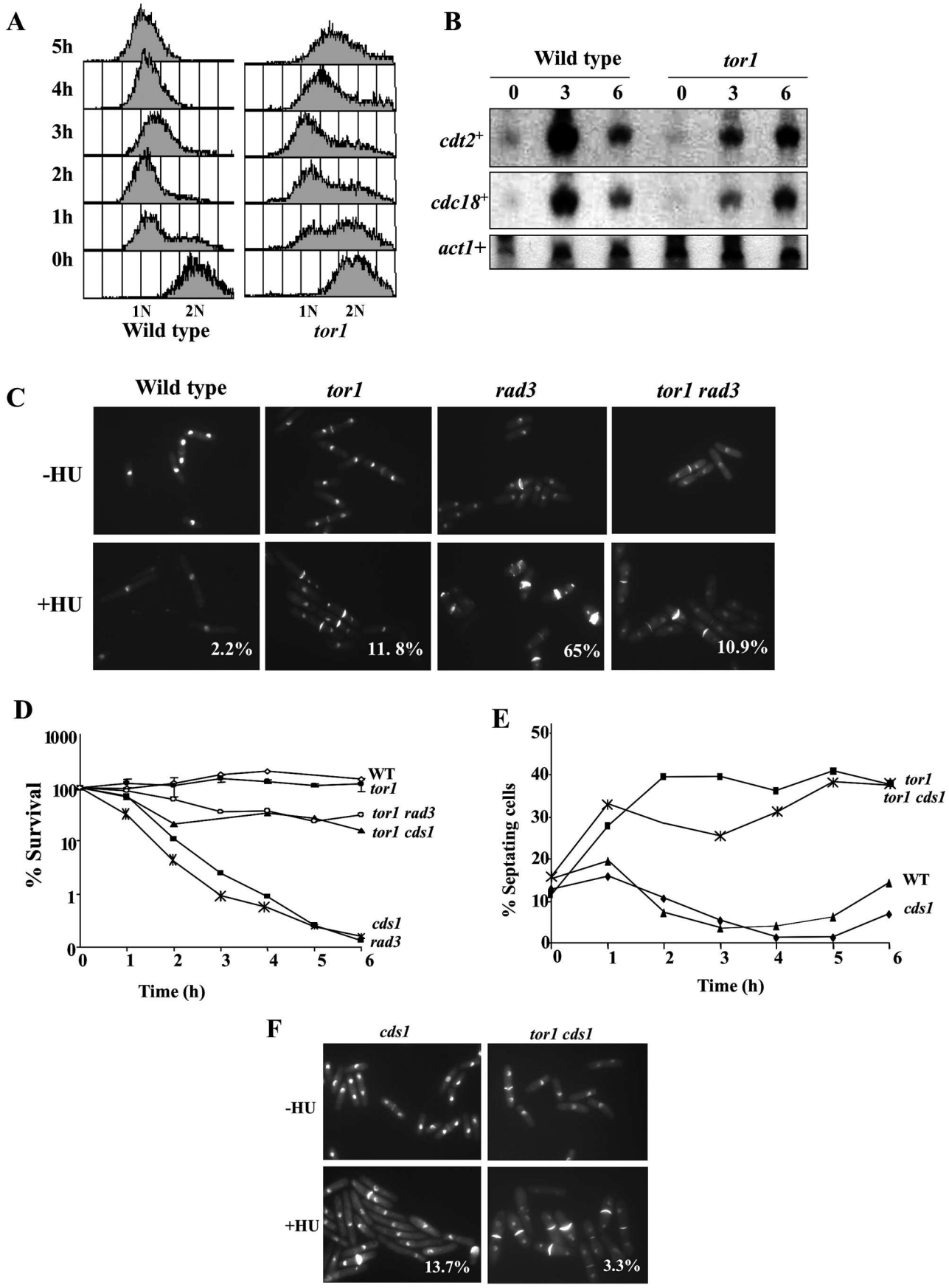

Figure 5

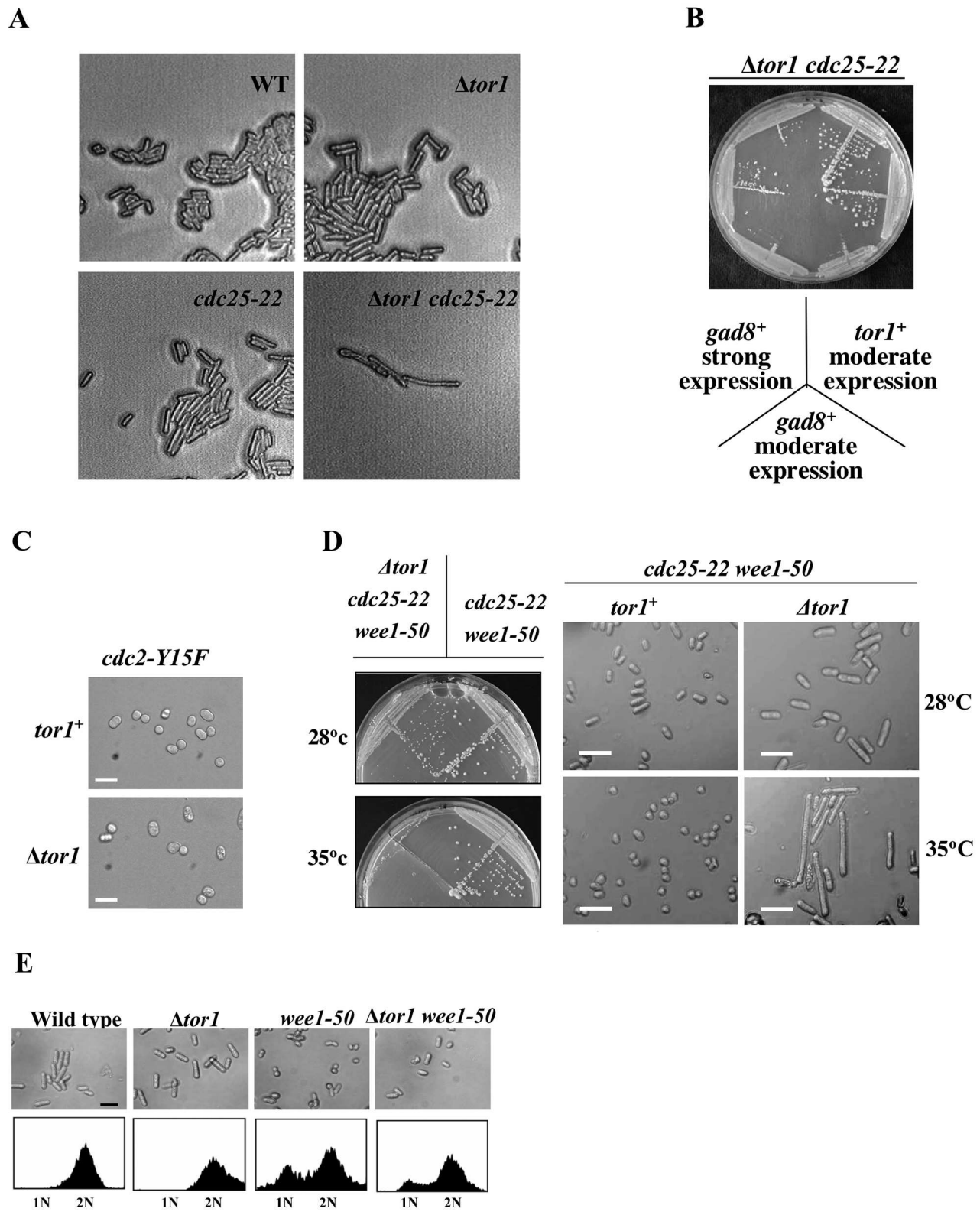

Figure 6