TORC1 inactivation promotes APC/C-dependent mitotic slippage in yeast and human cells

Highlights

Yeast TORC1 inhibition promotes Net1 degradation and Cdc14 release

Yeast TORC1 inhibition invokes mitotic slippage in an APC/C-Cdh1-dependent manner

Human mTORC1 inhibition also elicits mitotic slippage
TORC1 inactivation promotes APC/C-dependent mitotic slippage in yeast and human cells

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SUMMARY

Unsatisfied kinetochore-microtubule attachment activates the spindle assembly checkpoint to inhibit the metaphase-anaphase transition. However, some cells eventually override mitotic arrest by mitotic slippage. Here, we show that inactivation of TORC1 kinase elicits mitotic slippage in budding yeast and human cells. Yeast mitotic slippage was accompanied with aberrant aspects, such as degradation of the nucleolar protein Net1, release of phosphatase Cdc14, and anaphase-promoting complex/cyclosome (APC/C)-Cdh1-dependent degradation of securin and cyclin B in metaphase. This mitotic slippage caused chromosome instability. In human cells, mammalian TORC1 (mTORC1) inactivation also invoked mitotic slippage, indicating that TORC1 inactivation-induced mitotic slippage is conserved from yeast to mammalian cells. However, the invoked mitotic slippage in human cells was not dependent on APC/C-Cdh1. This study revealed an unexpected involvement of TORC1 in mitosis and provides information on undesirable side effects of the use of TORC1 inhibitors as immunosuppressants and anti-tumor drugs.

INTRODUCTION

Accurate chromosome segregation is essential for cell proliferation and genome stability in all organisms. Errors in chromosome segregation result in aneuploidy, which is manifested in genetic disorders and cancer. The spindle assembly checkpoint (SAC) ensures faithful chromosome segregation during cell division (Bharadwaj and Yu, 2004; Musacchio and Salmon, 2007). In the presence of unsatisfied kinetochore-microtubule attachments, the SAC inhibits anaphase onset by inhibition of the ubiquitin ligase (E3) Cdc20-associated anaphase-promoting complex/cyclosome (APC/C). The APC/C recruits various checkpoint proteins to unattached kinetochores, and checkpoint proteins Mad2, BubR1 (Mad3 in yeast), and Bub3 bind to and inhibit APC/C-Cdc20 (Kulukian et al., 2009). Once proper kinetochore-microtubule attachments are established, the SAC is deactivated and APC/C-Cdc20 becomes active and mediates degradation of the separase inhibitor securin. Liberated separase cleaves the cohesin subunit Scc1/Mcd1/Rad21, allowing sister chromatid separation (anaphase onset) (Peters et al., 2008).

Microtubule poisons are of clinical importance in the successful treatment of a variety of human cancers, because they activate the SAC and induce mitotic arrest, which eventually leads to apoptotic cell death (Rieder and Maiato, 2004). However, in the course of prolonged treatment with such drugs, some cells escape from mitosis, resulting in aneuploid cells (Minn et al., 1996; Rieder and Maiato, 2004). This phenomenon is called mitotic slippage, and it is responsible for the failure to efficiently block tumor progression. Mitotic slippage depends on progressive degradation of cyclin B, although the SAC is active, indicating that mitotic slippage occurs through the overriding of activated SAC signaling (Brito and Rieder, 2006; Gascoigne and Taylor, 2008). Mitotic exit occurs only once cyclin B-Cdk1 activity has decreased below a critical threshold required to maintain a mitotic state (Brito and Rieder, 2006). In addition to cyclin B, other mitotic APC/C substrates, including securin, are also degraded during mitotic slippage. Mitotic slippage occurs in a manner dependent on APC/C (Brito and Rieder, 2006; Gascoigne and Taylor, 2008; Lee et al., 2010). It is likely that mitotic slippage in human cells is mainly driven by APC/C-Cdc20 resulting from incomplete repression of the SAC (Brito and Rieder, 2006; Collin et al., 2013; Dick and Gerlich, 2013; Huang et al., 2009).
Cdc20 is activated at metaphase-anaphase transition, whereas the Cdc20 homolog Cdh1 is normally activated in late mitosis. In the budding yeast Saccharomyces cerevisiae, APC/C-Cdh1 is activated at telophase (Schwab et al., 1997; Visintin et al., 1997). The switch from APC/C-Cdc20 to APC/C-Cdh1 is regulated by multiple mechanisms (Simanis, 2003; Stegmeier and Amon, 2004; Sullivan and Morgan, 2007; Tan et al., 2005). Cyclin B-Cdk1 (Cdc28 in yeast) phosphorylates and inhibits Cdh1 until anaphase, but the cyclin-dependent kinase (CDK)-antagonizing phosphatase Cdc14 dephosphorylates and activates Cdh1 upon telophase onset. Recently, we found that Cdh1 is partially active and mediates securin degradation even in SAC-active metaphase cells of budding yeast (Nagai and Ushimaru, 2014). These results indicated that Cdh1 has a potential to oppose the SAC and promote anaphase transition. Indeed, we reported Cdh1-mediated mitotic slippage: if Cdh1 is further activated in metaphase by loss of Bub2, an inhibitor of the mitotic exit network (MEN) pathway, APC/C-Cdh1 aberrantly mediates securin degradation at metaphase with the SAC active (Toda et al., 2012). However, it is still unknown in what kind of physiological (stress) conditions Cdh1-mediated mitotic slippage is promoted in wild-type strains.

Target of rapamycin complex 1 (TORC1) kinase is a central controller of cell growth and proliferation in response to nutrient availability. TORC1 controls diverse cellular events, including transcription, translation, and autophagy (Loewith and Hall, 2011; Zoncu et al., 2011). Mammalian TORC1 (mTORC1) is crucially involved in diabetes, cancer, and aging (Cornu et al., 2013; Shimobayashi and Hall, 2014; Zoncu et al., 2011). Of note, treatment with rapamycin, a specific TORC1 inhibitor, induces chromosome instability in yeast and mammalian cells (Bonatti et al., 1998; Choi et al., 2000). This indicates that TORC1 is critical for accurate genome transmission, although its molecular mechanism remains elusive. Here we show that nutrient starvation and TORC1 inhibition antagonize CDK and promote Cdh1-mediated mitotic slippage and chromosome instability via an aberrant pathway in yeast cells. Furthermore, mammalian TORC1 (mTORC1) inactivation provoked mitotic slippage in a different manner in human cells. This study uncovered an unconventional conserved role of TORC1 as a regulator of mitotic progression.

RESULTS
TORC1 inactivation overrides SAC-mediated metaphase arrest
Because rapamycin induces chromosome instability (Bonatti et al., 1998; Choi et al., 2000), we suspected that TORC1 inactivation compromises SAC function. Here, we tested this idea using budding yeast. Securin/Pds1 degradation was repressed by the SAC in nocodazole-treated metaphase cells (Figure 1A, -Rap), whereas rapamycin facilitated securin degradation (Figure 1A, +Rap). Furthermore, rapamycin evoked cleavage of Scc1, a kleisin subunit of cohesin, and sister chromatid separation (Figures 1B and 1C and also see Figure 2F, WT). These findings indicated that TORC1 inactivation overrode SAC-mediated metaphase arrest. Because microtubule formation was impaired by nocodazole, rapamycin-induced sister chromatid separation was not remarkable compared with nocodazole removal-induced one, despite Scc1 cleavage in these conditions (see Figure 3C, WT + Rap and WT-Noc).

We assessed whether rapamycin-mediated TORC1 inactivation overcame metaphase arrest in different SAC-active conditions. Cells of the temperature-sensitive SCC1 mutant scc1-73 are defective in tension generation between sister kinetochores and arrest in metaphase with the SAC active at restrictive temperatures (Severin et al., 2001). Securin accumulated in scc1-73 cells at 37°C, but securin degradation was induced by rapamycin treatment (Figure S1A). Overexpression of the SAC kinase Mps1 activates the SAC, leading to metaphase arrest (Hardwick et al., 1996). Rapamycin promoted chromosome segregation in MPS1 overexpression-mediated metaphase arrest (Figure S1B). Thus, TORC1 inactivation overcame various types of SAC-mediated metaphase arrest.

Nitrogen depletion causes TORC1 inactivation (Loewith and Hall, 2011; Zoncu et al., 2011). Securin degradation was similarly evoked by nitrogen starvation (Figure 1D). Overall, starvation and TORC1 inactivation overrode SAC-mediated metaphase arrest and evoked anaphase onset (Figure 1E).

APC/C-Cdh1 mediates TORC1 inactivation-induced anaphase onset
Securin degradation after rapamycin treatment suggested that APC/C might be activated. Indeed, rapamycin also induced degradation of another APC/C substrate Clb5 (cyclin B5) in nocodazole-arrested cells (Figure 2A). First, we assessed a possibility that rapamycin causes SAC deactivation and thereby APC/C-Cdc20 activation. However, the SAC proteins Bub1 and Mad2 were still localized on kinetochores after rapamycin treatment (Figures 2B–2D), indicating that the SAC is still active after rapamycin treatment.
Rapamycin-induced anaphase onset occurred slowly compared with nocodazole removal-induced anaphase onset: most securin molecules were degraded by 30 min after nocodazole removal (thereafter securin again increased in the next cell cycle after 60 min), whereas some securin molecules remained 30 min after rapamycin treatment (Figure 2E, WT). Important, securin degradation after nocodazole

**Figure 1.** TORC1 inactivation overrides SAC-mediated metaphase arrest
(A) Exponentially growing cells of strain SCU2755 (PDS1-3HA) were arrested in metaphase by nocodazole treatment for 3 h at 30°C. Thereafter, cells were treated with or without rapamycin for the indicated times. 3HA-tagged securin/Pds1 was detected by western blotting analysis using an anti-HA antibody. CDK was detected as a loading control.
(B) Cells of strain SCU1964 (SCC1-TAP) were treated as described in (A). Cleaved products of Scc1 (shown by an arrow) were detected in a long-exposure image using an anti-TAP antibody (PAP).
(C) Cells of strain SCU69 (CEN4-GFP), of which the vicinity of the centromere of chromosome IV was labeled with GFP, were arrested in metaphase as described in (A) and then incubated with or without rapamycin for 1 h. Images of cells, GFP and DAPI were captured using a microscope. Scale bar, 2.5 μm. Separated sister centromeres were counted and are expressed as percentages in Figure 2G, WT.
(D) Cells of strain SCU2755 (PDS1-3HA) were arrested in metaphase and transferred to nitrogen-free media containing nocodazole.
(E) Model of TORC inactivation-induced anaphase onset. Nutrient starvation sequentially causes the inactivation of TORC1, securin degradation, cohesin cleavage, and sister separation.

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Figure 2. APC/C-Cdh1 mediates TORC1 inactivation-induced anaphase onset

(A) Cells of strain SCU1712 (CLB5-TAP) were treated as described in Figure 1A. TAP-tagged cyclin B5 (Clb5) was detected by western blotting analysis using the anti-TAP antibody (PAP).

(B–D) Cells of strains SCU1485 (BUB1-GFP) and SCU1337 (MAD2-GFP) harboring plasmid pSCU1701 (pMTW1-RFP) were treated as described in Figure 1C. Bub1-GFP and Mad2-GFP signals were captured with Mtw1-RFP (a kinetochore marker) signals. Scale bar, 2.5 μm. Percentages of cells with Bub1-GFP or Mad2-GFP on the kinetochore were determined, and averages and error bars from two independent experiments are shown. Statistical analyses were carried out using the two-tailed Fisher’s exact test. ns, not statistically significant.

(E) Cells of strains SCU2755 (PDS1-3HA) and SCU2693 (PDS1-3HA cdc20-3) precultured at 25°C were arrested in metaphase by nocodazole treatment for 3 h and transferred to 37°C for 30 min. Thereafter, cells were treated with rapamycin (+Rap) or released into nocodazole-free media (-Noc) (Time 0). Cells of strain SCU2282 (PDS1-3HA cdh1Δ)
removal (SAC deactivation) was suppressed by a cdc20-3 mutation, whereas securin degradation after rapamycin addition was not suppressed by this mutation (Figure 2E). This demonstrated that TORC1 inactivation-induced anaphase onset was independent of Cdc20.

Recently, we have found that Cdh1 already has a partial activity in metaphase, and if Cdh1 is further activated in metaphase, APC/C-Cdh1 causes mitotic slippage with the SAC being active (Nagai and Ushimaru, 2014; Toda et al., 2012). We suspected that rapamycin evokes Cdh1-mediated mitotic slippage in metaphase cells arrested by the SAC. This was indeed the case: deletion of CDH1 completely repressed rapamycin-induced securin degradation and sister chromatid separation (Figures 2E and 2F). These findings demonstrated that TORC1 inactivation caused Cdh1-mediated mitotic slippage. We found that cdh1Δ cells were hypersensitive to rapamycin (Figure 2G). This indicated that Cdh1 is essential for cell cycle progression, in which TORC1 activity is repressed, and supported the interconnection between TORC1 and Cdh1. Collectively, TORC1 inactivation provoked aberrant APC/C-Cdh1-dependent anaphase onset (Figure 2H).

As for a normal anaphase-telophase transition, Cdh1 is dephosphorylated and relocated from the cytoplasm to the nucleus (Jaspersen et al., 1999). However, no significant dephosphorylation and relocation of Cdh1 was detectable after rapamycin treatment (data not shown). It was likely that TORC1 inactivation caused a partial, but not full, activation of Cdh1. This was consistent with the observation of the slow progression of metaphase-anaphase transition after rapamycin treatment.

Cdc14 mediates TORC1 inactivation-induced anaphase onset

Cdh1 is dephosphorylated and activated by Cdc14 at telophase onset in a normal cell cycle (Jaspersen et al., 1999; Visintin et al., 1998). We found previously that Cdc1-mediated mitotic slippage in bub2Δ cells was dependent on Cdc14 (Toda et al., 2012). We suspected that Cdc14 is also involved in the case of rapamycin-induced anaphase onset. In fact, rapamycin-induced, but not nocodazole removal-induced, securin degradation and sister chromatid separation were repressed in cdc14-1 mutant cells (Figures 3A–3C). Thus, Cdc14 mediated TORC1 inactivation-induced anaphase onset in SAC-active metaphase cells.

In a normal cell cycle, Cdc14 is released from the nucleolus into the nucleus and activated via the Cdc14 early release (FEAR) pathway in early anaphase, and Cdc14 further diffuses throughout the cells via the MEN pathway at telophase onset, and cytoplasmic Cdc14 effectively dephosphorylates Cdh1 localized in the cytoplasm (Bosl and Li, 2005; D’Amours and Amon, 2004). Rapamycin promoted Cdc14 diffusion from the nucleolus (Figures 3D and 3E). This indicated that Cdc14 is activated by TORC1 inactivation. In the case of nocodazole removal, Cdc14 diffused from the nucleolus throughout the cell, whereas in the case of rapamycin treatment, diffused Cdc14 was resident only in the nucleus (Figure S2A). Nuclear-localized Cdc14 could not efficiently dephosphorylate cytoplasmic Cdh1, consistent with the partial activation of Cdh1 after TORC1 inactivation. Consistently, Cdc14 function was critical for cell proliferation, when TORC1 activity was repressed: cdc14-1 cells were hypersensitive to rapamycin at permissive temperatures (Figure 3F). Here we used cells lacking autophagy-related Atg1 as a control: atg1Δ cells were also hypersensitive to rapamycin (Figure 3F), similar to atg7Δ cells (in preparation). Overall, these findings suggested that Cdc14 is activated by TORC1 inactivation, promoting anaphase onset via APC/C-Cdh1-mediated securin degradation (Figure 3G).

TORC1 inactivation evokes Net1 degradation

Cdc14 is sequestered in the nucleolus by the nucleolar protein Net1 until metaphase, but when in early anaphase Net1 is phosphorylated, and Cdc14 is released from Net1 (Azzam et al., 2004; Shou et al., 2007).
Figure 3. Cdc14 mediates TORC1 inactivation-induced anaphase onset

(A) Cells of strain SCU3250 (PDS1-3HA cdc14-1) were treated as described in Figure 2E. 3HA-tagged securin Pds1 was detected by western blotting analysis using the anti-HA antibody.

(B and C) Cells of strains SCU69 (CEN4-GFP) and SCU3204 (CEN4-GFP cdc14-1) preincubated at 25°C were arrested in metaphase by nocodazole treatment for 3 h and then transferred to 37°C for 30 min. Thereafter, the cells were further incubated with or without rapamycin for 1 h. Scale bar, 2.5 μm. Percentages of cells with separated sister chromatids are shown in (C). For the control, cells were observed 30 min after nocodazole removal. Averages and error bars from two independent experiments are shown. Statistical analyses were carried out using the two-tailed Fisher’s exact test. *, p < 0.05; **, p < 0.0001.

(D and E) Cells of strain SCU1000 (CDC14-5GFP) were treated as described in Figure 1C. Scale bar, 2.5 μm. Percentages of cells with diffused Cdc14 were determined and averages and SD from three independent experiments are shown in (E).
Because TORC1 inactivation promotes Cdc14 release from the nucleolus, we suspected that TORC1 inactivation impacted on Net1. Surprisingly, Net1 was lost in nocodazole-treated metaphase cells after rapamycin treatment (Figure 4A). Nitrogen depletion similarly reduced Net1 levels (Figure 4B). We found that Net1 was unstable (Figure 4C). Of note, rapamycin accelerated Net1 degradation in metaphase cells (Figure 4D). These findings showed that TORC1 inactivation promoted Net1 degradation, causing Cdc14 release from Net1, namely, from the nucleolus. Net1 degradation is repressed by treatment with the proteasome inhibitor MG132, but not a deletion of autophagy-related ATG1 gene (Figures S3A and S3B). This indicated that Net1 is degraded by proteasome but not autophagy.

Because Cdc14 reverses CDK-mediated protein phosphorylation, deregulation of Cdc14 is lethal. In this context, a temperature-sensitive CDC14TAB6-1 mutant defective in the interaction of Cdc14 with Net1 was lethal at restrictive temperatures (Shou and Deshaies, 2002). CDC14TAB6-1 cells were hypersensitive to rapamycin even at permissive temperatures (Figure 4E). This supported the idea that TORC1 inactivation reduced the interaction between Net1 and Cdc14.

**Net1 degradation promotes Cdc14 release in SAC-mediated metaphase cells**

We wondered if Net1 degradation after rapamycin treatment is sufficient for Cdc14 release. To assess this idea, we determined whether a forced degradation of Net1 also caused Cdc14 release, like rapamycin treatment, using an auxin-induced degron (AID) system (Nishimura et al., 2009). Net1-mediated Cdc14 sequestration is critical for cell proliferation (Shou et al., 1999). Consistently, net1-aid cells were sick when Net1 degradation was induced by treatment with 1-naphthaleneacetic acid (NAA) (Figure 5A). After NAA treatment, Net1-aid protein was rapidly lost in asynchronously growing cells and nocodazole-treated metaphase cells (Figures 5B and 5C). This forced degradation of Net1 in metaphase caused Cdc14 diffusion from the nucleolus (Figure 5D). Of importance, Cdc14 diffused to the nucleus but not into the cytoplasm (Figure S2B), as seen with rapamycin treatment, indicating that AID-mediated Net1 degradation in metaphase cells might mimic rapamycin treatment. Furthermore, sister chromatid separation was accelerated under these conditions (Figure 5E), although no remarkable decrease in protein levels of securin was observed (data not shown). Collectively, we concluded that Net1 degradation was sufficient for Cdc14 release and mitotic slippage in SAC-active metaphase cells.

**TORC1 inactivation elicits mitotic exit**

TORC1 inactivation partially stimulated APC/C-Cdh1 activity. As for a normal mitotic exit, APC/C-Cdh1 mediates cyclin B2 (Clnb2) degradation, which triggers mitotic exit. When rapamycin was added to nocodazole-treated metaphase cells, cyclin B2 was also degraded, similar to securin and cyclin B5 (Figure 6A). Consistently, the appearance of G1 cells was significantly increased after rapamycin treatment (Figures 6B–6D). Similarly, nitrogen starvation promoted mitotic exit of nocodazole-treated cells (Figures 6B–6D). Thus, nutrient starvation and TORC1 inactivation promoted not only anaphase onset but also mitotic exit in SAC-active metaphase cells. Collectively, we propose a model for the molecular mechanisms of TORC1 inactivation-induced anaphase onset and mitotic exit: nutrient starvation → TORC1 inactivation → Net1 degradation → Cdc14 release → APC/C-Cdh1 activation → securin and cyclin B2 degradation → anaphase onset and mitotic exit (Figure 6E). Thus, when cells suffer from nutrient starvation, they undergo mitotic slippage to enter into G1 via this aberrant route.

It could be suspected that TORC1 inactivation-induced mitotic slippage might cause chromosome missegregation and reduction in cell viability, because TORC1 inactivation may forcibly promote sister separation despite the presence of insufficient microtubule-kinetochore connections. It was reported that longer-term treatments of rapamycin increased chromosome missegregation (Bonatti et al., 1998; Choi et al., 2000), whereas short-term treatment (1 h) of rapamycin did not significantly induce chromosome...
missegregation (Figure 6F). However, rapamycin treatment exacerbated chromosome instability in the presence of nocodazole (Figure 6F), and rapamycin remarkably reduced cell viability only in the co-presence of nocodazole. Thus, TORC1 inactivation exaggerated chromosome instability in conditions in which microtubule-kinetochore attachment was impaired.

mTORC1 inactivation elicits mitotic exit in human cells, which is not dependent on APC/C-Cdh1

We suspected that TORC1 inactivation-invoked mitotic slippage is conserved among eukaryotic cells. To address this question, we examined whether mammalian TORC1 (mTORC1) inactivation overrides SAC-mediated mitotic arrest in human cells. In human cells, it was reported that mitotic slippage occurs as a result of a decreased level of cyclin B below the threshold required to keep cells in mitosis, due to incomplete penetrance of the SAC (Brito and Rieder, 2006). We treated lung cancer-derived A549 cells with rapamycin, following nocodazole treatment (Figure 7A). After prolonged mitotic arrest, cells either die (mitotic cell death) or enter into the next cell cycle (mitotic slippage), which varies not only between different cell lines but also between cells among the same cell line (Gascoigne and Taylor, 2008). Whether cells undergo mitotic cell death or mitotic slippage is explained by the competing networks model, in which cell fate is determined by the competition between cyclin B degradation and pro-apoptotic signal accumulation (Huang et al., 2010; Topham and Taylor, 2013). We observed cells that were in interphase at the beginning of imaging and tracked them for 60 h in live cell imaging. Fates of these cells were judged by their morphology, which were classified into five categories: (1) stayed in interphase throughout the time course, (2) died in interphase, (3) mitotic cell death, (4) mitotic slippage, and (5) stayed in mitosis at the end of imaging (Figures 7B and 7C, Figure S4A). We validated mTORC1 inhibition by the suppression of p70S6K

Figure 4. TORC1 inactivation evokes Net1 degradation

(A) Nocodazole-treated metaphase cells of strain SCU3260 (NET1-3HA) were treated with rapamycin or released into nocodazole-free media. 3HA-tagged Net1 was detected using the anti-HA antibody.
(B) Cells of strain SCU7048 (NET1-3HA) arrested in metaphase by nocodazole treatment were transferred to nitrogen-free media containing nocodazole. Pgk1 was detected as a loading control.
(C) Cells of the wild-type strain SCU893 harboring plasmid pSCU1819 (pGAL1-NET1-H6HAZZ) preincubated in a raffinose-based medium were supplemented with 0.01% galactose for 1 h for induction of NET1-H6HAZZ. Protein stabilization of Net1 was assessed after shutoff of NET1-H6HAZZ expression by glucose (2%) addition (time 0). Net1 levels were detected using the anti-ZZ antibody (PAP).
(D) Cells of the wild-type strain SCU893 harboring pSCU1819 (pGAL1-NET1-H6HAZZ) preincubated in raffinose-based medium were arrested in metaphase by nocodazole treatment for 3 h. Thereafter, cells were supplemented with 0.01% galactose for 1 h for induction of NET1-H6HAZZ. Protein stabilization of Net1 was assessed in the presence or absence of rapamycin after shutoff of NET1-H6HAZZ expression by glucose addition (time 0).
(E) Five-fold serial dilutions of cells were spotted in 1-μL drops onto YPAD plates with or without 5 ng/mL rapamycin. The plates were incubated at 30°C for 1 day for YPAD plates and 2 days for rapamycin-containing YPAD plates. The yeast strains SCU893 (wild type) and SCU1199 (CDC14TAB6-1) were used.
phosphorylation (Figure S4B). In mock-treated cells, 33.16 ± 1.33% (SE, n = 4) of cells exhibited mitotic slippage among cells that underwent mitotic cell death or mitotic slippage (Figures 7C and 7D, Mock -Rap). The duration of mitosis was comparable between cells that underwent mitotic cell death and mitotic slippage (Figure 7E, Mock -Rap). The majority of the cells that underwent mitotic slippage died or stayed in interphase after slippage, and only one cell entered mitosis again (Figure S4A, Mock -Rap). In the presence

Figure 5. Net1 degradation elicits Cdc14 release in SAC-mediated metaphase cells
(A) Five-fold serial dilutions of cells of strains SCU893 (NET1) and SCU3331 (net1-aid) were spotted in 1-μL drops onto YPAD plates with or without 1 mM 1-naphthaleneacetic acid (NAA). The plates were incubated at 30°C for 1 day.
(B) Cells of strain SCU3331 (net1-aid) were treated with 1 mM NAA. Net1 levels were followed by western blotting analysis using an anti-IAA17 antibody.
(C) Cells of strain SCU3331 (net1-aid) were arrested in metaphase by nocodazole treatment for 3 h and then Net1 degradation was induced by 1 mM NAA treatment (time 0).
(D) Cells of strain SCU3409 (CDC14-5GFP net1-aid) were arrested in metaphase by nocodazole treatment for 3 h and then Net1 degradation was induced by 1 mM NAA treatment (time 0). Scale bar, 2.5 μm. Percentages of cells with diffused Cdc14 were determined and averages and error bars from two independent experiments are shown. Statistical analyses were carried out using the two-tailed Fisher’s exact test. **, p <0.0001. 
(E) Cells of strains SCU69 (CEN4-GFP) and SCU6232 (net1-aid CEN4-GFP) were arrested in metaphase by nocodazole treatment for 3 h at 30°C and then incubated with or without NAA for 1 h. Scale bar, 2.5 μm. Separated sister centromeres were counted and are expressed as percentages. Averages and SDs from three independent experiments are shown. Statistical analyses were carried out using the two-way ANOVA with Bonferroni correction. *, p <0.01.
Figure 6. TORC1 inactivation elicits mitotic slippage

(A) Cells of strain SCU1709 (CLB2-TAP) were treated as described in Figure 1A. TAP-tagged cyclin B2 (Clb2) was detected by western blotting analysis using the anti-TAP antibody.

(B and C) Cells of the wild-type strain SCU893 were arrested in metaphase by nocodazole treatment for 3 h and then further cultured with or without (control) rapamycin, or in nitrogen-depleted media (SD-N) (time 0) for the indicated times. Scale bar, 2.5 μm. Percentages of G1 cells without buds were determined and averages and SDs from three independent experiments are shown in (C). p Values were calculated using the two-way ANOVA with Bonferroni correction. ***, p <0.0005.

(D) Cells of wild-type strain SCU893 were cultured as described in (B) and subjected to flow cytometric analysis. Accumulation of G1 cells after rapamycin treatment or nitrogen starvation was assessed.

(E) Model in which the inactivation of TORC1 elicits mitotic slippage.
mitotic slippage in the presence of MG132 (Figure S5F), corroborating that cyclin B degradation, which is kinetochore (Figures S5B and S5C) (Allan et al., 2020). Live cell imaging revealed that no cells underwent localization of Mad1 was also increased, in line with a recent finding that cyclin B1 scaffolds Mad1 at the although it is mediated by APC/C-Cdh1 in yeast cells but not in human cells.

data suggested that mTORC1 inactivation invoked mitotic slippage both in yeast and human cells, regardless of the underlying causes. To verify that this is due to inhibition of cyclin B degradation, we depleted cells (Figures 7B and 7C, Figures S4A and S4B, siCdh1 -Rap). These data confirmed that Cdc20 depletion eliminates mitotic slippage altogether, involving mitotic slippage and mitotic cell death.

To address the relationship between mTORC1 and APC/C-Cdh1 in human cells, we also observed Cdh1-depleted cells (Figures 7B and 7C, Figures S4A and S4B, siCdh1 -Rap). Cells that died in interphase increased significantly, which might be related to the role of APC/C-Cdh1 in the maintenance of G1 phase (Qiao et al., 2010). In contrast to the results in yeast, Cdh1 depletion rather increased mitotic slippage to 46.36 ± 2.79% (SE, n = 4), comparable with that in rapamycin-treated cells (Figures 7C and 7D siCdh1 -Rap). The duration of mitosis before mitotic slippage did not significantly shorten (Figure 7E, siCdh1 -Rap), suggesting that cyclin B degradation was not accelerated, similarly to rapamycin treatment. In contrast, the duration of mitotic arrest before cell death was markedly reduced in Cdh1-depleted cells (Figure 7E, siCdh1 -Rap). Furthermore, cells that underwent mitotic slippage died earlier in interphase after mitotic slippage compared with mock-treated cells (Figure 7F, siCdh1 -Rap). Therefore, the increase in the rate of mitotic slippage did not contribute to overall cell survival. Rapamycin treatment in Cdh1-depleted cells further reduced the fraction of cells that entered mitosis because of the increase in cells that stayed in interphase and did not result in further increase in the rate of mitotic slippage (52.77 ± 2.31% [SE, n = 4], Figures 7C and 7D, siCdh1 +Rap). The duration of mitotic arrest before mitotic cell death and duration of interphase after mitotic slippage were similarly reduced in Cdh1-depleted cells with or without rapamycin treatment (Figures 7E and 7F, siCdh1 -Rap vs siCdh1 +Rap). These data suggested that both mTORC1 inhibition and APC/C-Cdh1 depletion facilitate mitotic slippage, which is supposedly due to changes in the balance between mitotic slippage and cell death. However, Cdh1 depletion showed additional effects on the overall cell survival after mitotic arrest.

We also observed cells depleted of Cdc20, which is crucial for mitotic exit. Consistent with the previous report (Huang et al., 2009), nearly all cells that entered mitosis died, and mitotic slippage was rarely seen (Figure 7C, siCdc20 -Rap). The situation was the same in the presence of rapamycin (Figure 7C, siCdc20 Rap), or when Cdh1 was simultaneously depleted (Figure 7C, siCdh1+siCdc20 -Rap and siCdh1+ siCdc20 + Rap). These data confirmed that Cdc20 depletion eliminates mitotic slippage altogether, regardless of the underlying causes. To verify that this is due to inhibition of cyclin B degradation, we observed nocodazole-treated cells in the presence of MG132, a proteasome inhibitor (Figure S5A). We confirmed that cyclin B level was increased upon MG132 treatment (Figures S5D and S5E). Kinetochore localization of Mad1 was also increased, in line with a recent finding that cyclin B1 scaffolds Mad1 at the kinetochore (Figures S5B and S5C) (Allan et al., 2020). Live cell imaging revealed that no cells underwent mitotic slippage in the presence of MG132 (Figure S5F), corroborating that cyclin B degradation, which is induced by APC/C-Cdc20 in physiological conditions, is required for mitotic slippage. Collectively, our data suggested that mTORC1 inactivation invoked mitotic slippage both in yeast and human cells, although it is mediated by APC/C-Cdh1 in yeast cells but not in human cells.
Figure 7. mTORC1 inactivation elicits mitotic slippage

(A) A schematic of the experimental procedure.

(B) Phase-contrast time-lapse imaging of nocodazole-treated A549 cells. An example representing each category of cell fate to classify cells in Figure S4A is shown. Numbers show elapsed time (hour:minute) relative to the start of imaging. Scale bar, 50 μm.

(C) Fate of nocodazole-treated A549 cells with or without rapamycin treatment after 60-h imaging. Proportion of cells classified into each category, obtained by averaging the results of four independent experiments shown in Figure S4A, is presented. Error bars represent standard errors.
DISCUSSION

Unconventional aspects of TORC1 inactivation-elicited mitotic slippage in yeast

Here, we showed that starvation-induced TORC1 inactivation promoted mitotic slippage in yeast cells. We proposed a model for its molecular mechanisms (Figure 6E). It should be noted that this TORC1 inactivation-specific pathway includes numerous unconventional aspects: Net1 degradation in metaphase, Cdc14 release in metaphase, and APC/C-Cdh1-mediated anaphase onset and mitotic exit.

APC/C-Cdc20 mediates anaphase onset in a normal cell cycle progression, whereas APC/C-Cdh1 mediates telophase onset (Schwab et al., 1997; Visintin et al., 1997). However, we recently found that APC/C-Cdh1 was partially active in nocodazole-treated metaphase cells (Nagai and Ushimaru, 2014). Furthermore, APC/C-Cdh1 mediated anaphase onset in Bub2-deficient cells (Toda et al., 2012). Bub2 is an inhibitory protein for the MEN pathway, and loss of Bub2 caused precocious APC/C-Cdh1 activation via Cdc14. TORC1 inactivation- and Bub2 deficiency-induced mitotic slippage have some similarities: (1) commitment of Cdc14, (2) APC/C-Cdh1-dependent securin degradation, and (3) slow metaphase-anaphase transition. Securin is degraded after ubiquitination mediated by APC/C-Cdc20 in anaphase and then APC/C-Cdh1 in telophase in a normal cell cycle progression (Nagai and Ushimaru, 2014; Toda et al., 2012)(Figure S6A). Namely, securin degradation is normally initiated by APC/C-Cdc20 at anaphase onset, but it was aberrantly mediated by APC/C-Cdh1 in specific conditions, including nutrient-starved conditions (Figures S6B and S6C). However, TORC1 inactivation- and Bub2 deficiency-induced mitotic slippage have a dissimilarity; Bub2 deficiency caused Cdc14 activation via the MEN pathway (Toda et al., 2012), whereas TORC1 inactivation caused Cdc14 activation via Net1 degradation (this study). We found that TORC1 inactivation induces proteasome-dependent Net1 degradation. Furthermore, after TORC1 inactivation, repression of protein synthesis by reduction in translation initiation and ribosome biogenesis, in addition to autophagic degradation of ribosomes (Beau et al., 2008; Loewith and Hall, 2011), promotes the reduction in Net1 protein levels.

APC/C-Cdc20 normally promotes anaphase onset, and thereafter APC/C-Cdh1 promotes telophase onset (Schwab et al., 1997; Visintin et al., 1997). This sequential activation of APC/C-Cdc20 and APC/C-Cdh1 is the heart of accurate mitosis (Toda et al., 2012). APC/C-Cdc20 recognizes the D-box of a relatively limited number of targets (the critical targets are securin and Clb5 in budding yeast) (Shirayama et al., 1999), whereas APC/C-Cdh1 recognizes various motifs (e.g., KEN box) on numerous targets, including substrates for APC/C-Cdc20 (for example, securin) (Manchado et al., 2010; Pines, 2006; van Leuken et al., 2008; Wasch et al., 2010)(Figure S6A). Therefore, when APC/C-Cdh1 is aberrantly activated in metaphase, APC/C-Cdh1 simultaneously triggers anaphase and telophase onset (Toda et al., 2012)(Figure S6C). In addition, APC/C-Cdh1-mediated anaphase onset caused chromosome missegregation, because anaphase progression occurred despite the presence of insufficient microtubule-kinetochore attachments (this study) (Toda et al., 2012).

Intercommunication between TORC1 and CDK is ambiguous. This study showed that TORC1 inactivation antagonized CDK through two different ways in yeast cells: APC/C-Cdh1-dependent degradation of Clb proteins (decrease in CDK activity itself) and activation of Cdc14 (increase in activity to reverse CDK/Clb-mediated phosphorylation). In other words, TORC1 guaranteed CDK action by repression of Cdc14 and Cdh1 in normal (nutrient-rich) conditions.

Cdc14 antagonizes the TORC1 signaling and is negatively regulated by TORC1

Protein phosphatase 2A (PP2A; Pph21 and Pph22) and the PP2A-related PP6 (Sit4) phosphatases are activated after TORC1 inactivation in budding yeast (Loewith and Hall, 2011; Zoncu et al., 2011). In nutrient-rich...
conditions, TORC1 promotes phosphorylation of Tap42, which is an inhibitor of PP2A and PP6 (Jiang and Broach, 1999). Upon TORC1 inactivation, Tap42 dissociates from PP2A and PP6, activating both phosphatases. Thus, PP2A and Sit4 are activated after the inactivation of TORC1 in a Tap42-dependent manner. A global kinase-phosphatase interaction network has suggested that Cdc14 is an antagonist of the TORC1 signaling (Breitkreutz et al., 2010), although the molecular connection between TORC1 and Cdc14 remained unclear. We showed here that Cdc14 is a TORC1-downstream phosphatase: Cdc14 was activated by TORC1 inactivation in a manner different from that in PP2A and PP6. Of note, Cdc14 dephosphorylates TORC1-mediated phosphorylation of the autophagy-related protein Atg13 after TORC1 inactivation (Yasmin et al., 2016). Collectively, Cdc14 antagonizes the TORC1 signaling and is negatively regulated by TORC1.

**Roles of Cdc14 as a stress-responsive protein phosphatase**

Cdc14 phosphatase reverses CDK-Clb (cyclin B)-dependent phosphorylation and is essential for mitotic exit in budding yeast (Queralt and Uhlmann, 2008; Stegmeier and Amon, 2004). We found that Cdc14 mediates TORC1 inactivation-induced mitotic slippage in budding yeast, probably through dephosphorylating Cdh1. The fission yeast homolog Clp1/Fpl1 is also involved in mitotic exit/cytokinesis (Clifford et al., 2008; Esteban et al., 2004; Wolfe and Gould, 2004). By contrast, the PP2A and PP1 phosphatases are responsible for mitotic exit in animal cells (Grallert et al., 2015; Mochida et al., 2009; Ohkura et al., 1989; Schmitz et al., 2010; Wu et al., 2009). Whether human homolog Cdc14B (hCdc14B) is involved in mitotic exit/cytokinesis is controversial (Dryden et al., 2003; Mocciaro et al., 2010; Rodier et al., 2008).

Aside from this, the Cdc14 family of phosphatases is also involved in DNA stress responses. Mammalian hCdc14B localizes to the nucleolus and is released from the nucleolus in response to DNA damage and dephosphorylates Cdh1 to regulate the DNA damage response (Bassermann et al., 2008; Kaiser et al., 2002; Mocciaro et al., 2010; Peddibhotla et al., 2011; Sudo et al., 2001). Fission yeast Clp1 is also released from the nucleolus to the nucleus in response to DNA replication stress in S phase and is required for the DNA damage responses (Diaz-Cuervo and Bueno, 2008). In budding yeast, a global PKI network analysis suggested that Cdc14 is involved in the DNA damage response (Breitkreutz et al., 2010). We showed herein that yeast Cdc14 promoted mitosis progression in response to starvation. In addition, Cdc14 is required for induction of autophagy (macroautophagy) and micronucleophagy in budding yeast (Kondo et al., 2018; Mostofa et al., 2021). Thus, Cdc14 is a key factor to respond to nutrient stress in budding yeast. In contrast to yeast, human cells undergo an open mitosis and disassemble both the nucleus and nucleolus in mitosis, and hCdc14B is diffused to the cytoplasm in metaphase. Therefore, even if hCdc14B responds to nutrient starvation in metaphase, its molecular mechanism should be completely different from that in budding yeast. Alternatively, it is likely that human Cdc14 is not involved in mitotic slippage.

**TORC1 switches nutrient- and starvation-specific mitotic routes toward G1**

TORC1 activity is critical for G1/S progression, and starved cells arrest at G1 phase eventually (Barbet et al., 1996; Zinalla et al., 2007). However, a mounting body of evidence shows that TORC1 regulates cell cycle progression other than G1 progression: TORC1 activity is required for proper S phase progression (DNA replication) and G2/M transition (Matsui et al., 2013; Nakashima et al., 2008; Tran et al., 2010; Yamamoto et al., 2018). Thus, TORC1 activity is required for various aspects of cell cycle phase progression and transition. However, because starved cells have to reach G1 phase, they should progress toward G1 phase albeit slowly.

If starved cells meet with microtubule problems in mitosis, what do cells do: stop at metaphase or override metaphase arrest? The present study demonstrated that starved yeast cells selected to override the SAC-mediated metaphase arrest to go toward G1 (Figures 6B–6D). Starvation-induced TORC1 inactivation opened the emergency exit toward G1. This might be an adaptive response to starvation, albeit at the cost of chromosome missegregation and death in some cells (Figures 6F and 6G). Because yeast cells in a population are clonal, some surviving cells may properly transmit their genome. Cells appeared to prefer to override the SAC, albeit with a risk, than the death of all clonal cells by eternal metaphase arrest. Many studies have been devoted to the analysis of cell cycle progression in laboratory conditions. However, in natural circumstances, organisms including yeast frequently suffer from nutrient starvation, and cells have to switch nutrient- and starvation-specific mitotic progress pathways in response to nutrient availability. We showed here that there is not just one route to lead to G1. In normal (nutrient-rich) conditions, TORC1 closes off the dangerous, starvation-specific route. Thus, TORC1 switches nutrient- (secure) and starvation-specific (dangerous) mitotic routes toward G1 in response to nutrient availability. This study suggested that a similar TORC1-regulated switching system also appears to be conserved...
in mammalian cells, although the biological benefits of the starvation-dependent risky route in multicellular organisms is unclear at present.

mTORC1 and mitotic slippage
We found that mTORC1 inhibition also elicits mitotic slippage in human cells. However, the increased mitotic slippage in human cells was not dependent on Cdh1, in contrast to that in yeast cells. Our data expand the idea that TORC1 plays a critical role in mitotic regulation not only in budding yeast but also in mammalian cells (Bonatti et al., 1998; Choi et al., 2000), although how mTORC1 inactivation promotes mitotic slippage in human cells is currently unclear. It has been revealed that mTORC1 is involved not only in G1 phase progression but also in various mitotic processes including G2/M transition, centrosome duplication, spindle assembly, and cytokinesis (Astrinidis et al., 2006; Platani et al., 2018; Ramírez-Valle et al., 2010). Related to these mitotic functions, a number of proteins related to TORC1 signaling are reported to reside at the mitotic apparatus such as centrosomes, spindle midzone, and midbody (Cuyàs et al., 2014). We observed the effect of rapamycin in A549 cells treated with nocodazole to mimic the conditions of yeast experiments. As spindle and midzone are not formed in the presence of nocodazole, the increased mitotic slippage by rapamycin treatment may not be related to the role of mTORC1 in spindle assembly and cytokinesis. Rapamycin treatment elicits premature anaphase onset in yeast cells by overriding active SAC signaling, whereas it facilitates mitotic slippage in human cells at similar timing with mitotic cell death after prolonged mitotic arrest, suggesting that mTORC1 plays a role in the balance between mitotic cell death and mitotic slippage, but not in the timing of anaphase onset.

Cdh1 targets securin in a manner dependent on D/KEN boxes, and our data in yeast cells are consistent with the previous reports that deregulation of Cdh1 in pre-anaphase results in premature securin degradation and sister-chromatid separation (Hagting et al., 2002; Jeganathan et al., 2005; Zur and Brandeis, 2001) (Figure S6C). On the other hand, we found that Cdh1 depletion rather increased the rate of mitotic slippage in human cells. It is plausible that mitotic slippage in human cells is mainly driven by APC/C-Cdc20 (Brito and Rieder, 2006; Huang et al., 2009), and one possibility is that Cdh1 competes with Cdc20 for the activation of APC/C, which may cause increased mitotic slippage upon Cdh1 depletion. Indeed, we found the competition between Cdh1 and Cdc20 in nocodazole-treated metaphase cells in yeast (Nagai and Ushimaru, 2014; Toda et al., 2012). Although both rapamycin treatment and Cdh1 depletion elicted mitotic slippage in human cells, durations of mitotic arrest before cell death and survival time after mitotic slippage were shorter in Cdh1-depleted cells compared with rapamycin-treated cells. They might be due to the link between Cdh1 and apoptosis, because it was reported that Cdh1 depletion enhances susceptibility to apoptosis in several occasions (Eguren et al., 2013; Liu et al., 2008). The increase in the rate of cells that died in interphase upon Cdh1 depletion may also be caused by the enhanced susceptibility to apoptosis. Overall, although Cdh1 depletion increased the rate of mitotic slippage in human cells, it did not contribute to enhanced cell survival.

Concluding remarks
Rapamycin treatment promotes chromosome instability in yeast and mammalian cells (Bonatti et al., 1998; Choi et al., 2000). This study dissected the molecular mechanism of TORC1 inactivation-induced chromosome instability. Rapamycin and other TORC1 inhibitors are used as anti-cancer drugs, in addition to being immunosuppressants (Benjamin et al., 2011; Vignot et al., 2005). However, these drugs might rather increase genome instability in healthy cells, causing tumor progression. We believe that the present results are useful for the anticipation of undesirable side effects of the use of TORC1 inhibitors. In addition, this study demonstrated that the TORC1-APC/C axis is a potent therapeutic target to prevent mitotic slippage in cancer cells in the presence of anti-tumor microtubule poisons.

Limitations of the study
This study demonstrated that the lack of Cdh1 repressed mitotic slippage after TORC1 inactivation in yeast cells, indicating that Cdh1 is required for mitotic slippage. However, we observed no detectable Cdh1 activation (dephosphorylation and relocation to the nucleus) after rapamycin treatment. Changes in Cdh1 features after rapamycin treatment remains to be investigated in the future.

STAR+ METHODS
Detailed methods are provided in the online version of this paper and include the following:
SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103675.

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AUTHOR CONTRIBUTIONS
T.U. conceived the project and designed the experiments. K.T. designed the experiments using human cells. C.Y., A.M., S. Miyazaki, M.N., S. Mase, K.I., M.N.T., T.T., S. Morshed, N.K., M.G.M., T.S., H.K., and K.O. performed the experiments and acquired, analyzed, and interpreted the data. M.A.R. provided technical support. T.U. drafted and revised the manuscript. K.T. drafted and revised parts as to experiments using human cells. M.N.T. and T.T. helped to write the revised manuscript. T.U. and K.T. are the guarantors of this work.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse monoclonal anti-HA (F7) | Santa Cruz | Cat#sc-7392; RRID:AB_2894930 |
| Peroxidase-anti-peroxidase soluble complex (PAP) | Sigma-Aldrich | Cat#P1291; RRID:AB_1079562 |
| Rabbit polyclonal anti-IAA17 | Gift from M. Kanemaki | N/A |
| Mouse monoclonal anti-CDK (PSTAIRE) | Santa Cruz | Cat#sc-53; RRID:AB_2074908 |
| Mouse monoclonal anti-Pgk1 | Thermo Fisher Scientific | Cat#A-6457; RRID:AB_221541 |
| Rabbit polyclonal anti-Cdh1 | Novus Biologicals | Cat#NB2-15840; RRID:AB_2895137 |
| Rabbit polyclonal anti-Cdc20 | Protein Tech Group | Cat#10252-1-AP; RRID:AB_2229016 |
| Rabbit polyclonal anti-phospho-p70 S6 kinase | Cell Signaling Technology | Cat#9205; RRID:AB_330944 |
| Rabbit polyclonal anti-p70 S6 kinase | Cell Signaling Technology | Cat#9202; RRID:AB_331676 |
| Mouse monoclonal anti-α-tubulin | Sigma | Cat#T6074; RRID:AB_477582 |
| Rabbit polyclonal anti-Mad1 | GeneTex | Cat#GTX109519; RRID:AB_1950847 |
| Mouse monoclonal anti-Cyclin B | BD Biosciences | Cat#610219; RRID:AB_397616 |
| Human polyclonal anti-centromere protein antibody | Antibodies Inc. | Cat#15-234; RRID:AB_2687472 |
| Goat polyclonal anti-Mouse IgG Alexa Fluor-488 | Thermo Fisher Scientific | Cat#A11029; RRID:AB_138404 |
| Goat polyclonal anti-Rabbit IgG Alexa Fluor-488 | Thermo Fisher Scientific | Cat#A11034; RRID:AB_2576217 |
| Goat polyclonal Anti-Human IgG Alexa Fluor-568 | Thermo Fisher Scientific | Cat#A21090; RRID:AB_1500627 |
| Bacterial and virus strains |        |            |
| JM109                | Takara  | Cat#9052  |
| Chemicals, peptides, and recombinant proteins |        |            |
| Rapamycin            | LC Laboratories | Cat#R5000; CAS:53123-88-9 |
| Nocodazole           | Sigma-Aldrich | Cat#M1404; CAS:31430-18-9 |
| 4′,6-diamidino-2-phenylindole (DAPI) | Dojin | Cat#D212; CAS:28718-90-3 |
| ProLong Gold         | Thermo Fisher Scientific | Cat#P36930 |
| MG132                | Sigma-Aldrich | Cat#4774790; CAS:133407-82-6 |
| Femtowtow Plus       | Michigan Diagnostics | Cat# 21008 |
| ECL prime Western Blotting Detection Reagents | Cytiva | Cat#GERPN2236 |
| Western BLot Quant HRP Substrate | Takara | Cat#77102 |
| RNase A              | Nippon Gene | Cat#318-06391 |
| Experimental models: Cell lines |        |            |
| Human: A549          | ATCC Cell Bank | Cat#CCL-18S; RRID:CVCL_0023 |
| Experimental models: Organisms/strains |        |            |
| S. cerevisiae: MATa his3-11,15 trp1-1 leu2-3,112 ura3-1 ade2-1 can1-1 | Lab stock | W303a |
| S. cerevisiae: W303a bar1::hisG | Lab stock | SCU893 (US356) |
| S. cerevisiae: MATa gal2 | Lab stock | S288C |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| S. cerevisiae: S288C MATa ade2 arg4 leu2-3,112 trp1-289, ura3-52 | (Gavin et al., 2006) | MGD353-13D |
| S. cerevisiae: S288C MATα ura3Δ0 leu2Δ0 his3Δ1 met15Δ0 | Lab stock | BY4741 |
| S. cerevisiae: SCU893 trp1::LacO::TRP1 his3::LacI-GFP::HIS3 | This study | SCU69 |
| S. cerevisiae: W303a scc1-73 | (Michaelis et al., 1997) | SCU244 |
| S. cerevisiae: W303a cdc14::CDC14-5×GFP::TRP1 | Gift from A. Toh-e | SCU1000 |
| S. cerevisiae: W303a cdc14-1 | Gift from A. Toh-e | SCU1001 |
| S. cerevisiae: W303a MATα CDC14ΔAB6-1 | (Shou and Deshaies, 2002) | SCU1199 |
| S. cerevisiae: W303a cdh1::kanR bar1 | (Ross and Cohen-Fix, 2003) | SCU1228 |
| S. cerevisiae: W303a + CflIII (CEN3.L.YPH278) URA3 SUP11 | (Warren et al., 2002) | SCU1237 |
| S. cerevisiae: SCU893 mad2Δ1:hisMX[pMAD2-GFP::URA3] | This study | SCU1337 |
| S. cerevisiae: BY4741 BUB1-GFP::HIS3MX | (Huh et al., 2003) | SCU1485 |
| S. cerevisiae: MGD353-13D CLB5-TAP::KIURA3 | (EUROSCARF) | SCU1712 |
| S. cerevisiae: MGD353-13D CLB2-TAP::KIURA3 | (EUROSCARF) | SCU1709 |
| S. cerevisiae: MGD353-13D SCC1-TAP::KIURA3 | (EUROSCARF) | SCU1964 |
| S. cerevisiae: W303a cdh1::kanR bar1 trp1::LacO::TRP1 his3::LacI-GFP::HIS3 | This study | SCU2114 |
| S. cerevisiae: W303a cdh1::kanR bar1 ura3::PDS1-HA3::URA3 | This study | SCU2282 |
| S. cerevisiae: SCU893 PDS1-3HA::URA3 cdc20-3::hisMX | This study | SCU2693 |
| S. cerevisiae: SCU893 PDS1-3HA::URA3 | This study | SCU2755 |
| S. cerevisiae: W303a scc1-73 PDS1-3HA::URA3 | This study | SCU2814 |
| S. cerevisiae: SCU893 trp1::LacO::TRP1 his3::LacI-GFP::HIS3 cdc14-1::hisMX | This study | SCU3204 |
| S. cerevisiae: W303a cdc14-1 PDS1-3HA::URA3 | This study | SCU3250 |
| S. cerevisiae: SCU893 NET1-HA3::LEU2 | This study | SCU3260 |
| S. cerevisiae: SCU893 ura3::ADH1 pr-osTIR1-9myc::URA3 NET1::AA17::kanMX | This study | SCU3331 |
| S. cerevisiae: SCU893 ura3::ADH1 pr-osTIR1-9myc::URA3 NET1::AA17::kanMX CDC14-5×GFP::TRP1 | This study | SCU3409 |
| S. cerevisiae: SCU893 atg1::kanMX | (Rahman et al., 2018) | SCU4067 |
| S. cerevisiae: SCU893 NET1::AA17::kanMX ura3::ADH1 pr-osTIR1-9myc9::URA3 trp1::LacO::TRP1 his3::LacI-GFP::HIS3 | This study | SCU6232 |
| S. cerevisiae: SCU893 NET1::AA17::LEU2 atg1::kanMX | This study | SCU6918 |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Takashi Ushimaru (ushimaru.takashi@shizuoka.ac.jp).

Materials availability
All unique materials generated in this study are available from the Lead Contact with a completed Material Transfer Agreement.

Data and code availability
This study did not generate datasets.

EXPERIMENTAL MODEL AND SUBJECT DETAILS
All S. cerevisiae strains used in this study were derived from the W303a (MATa his3-11,15 trp1-1 leu2-3,112 ura3-1 ade2-1 can1-1) and S288C (MATa gal2) background strains. Human cell line A549 was used.
METHOD DETAILS

Yeast strains, plasmids, and media

*S. cerevisiae* strains and plasmids used are listed in the KEY RESOURCES TABLE. Gene disruption and tagging were conducted using a conventional one-step PCR-mediated method. Glucose-based YPAD (YPD containing 0.01% adenine) and synthetic minimal medium (SD) complemented with the appropriate nutrients for plasmid maintenance were prepared in a standard manner. SRGly was identical to SD except that it contained 2% raffinose and 3% glycerol instead of 2% glucose. For nitrogen depletion, exponentially growing cells cultured in SD medium were transferred to SD medium without ammonium sulphate or amino acids. For cell cycle arrest in metaphase, nocodazole (10 μg/mL) was added to the cell culture for 3 h.

Western blotting analysis

Proteins were extracted using a post-alkaline extraction method in accordance with the method of Kushnirov (Kushnirov, 2000). Briefly, exponentially growing cells (10 mL culture, OD₆₀₀ = 0.3–0.8) were treated with 200 μL of 0.1 M NaOH for 5 min, and then the cell pellet was collected by centrifugation. The cell pellet was treated with sample buffer (60 mM Tris-HCl (pH 6.8), 5% glycerol, 2% SDS, 4% 2-mercaptoethanol and 0.0025% bromophenol blue) at 95°C for 5 min. Crude extracts were cleared by centrifugation, and the supernatant was subjected to western blotting with specific antibodies for analysis. Chemiluminescence signals for horseradish peroxidase (HRP) were detected using an image analyzer (LAS3000mini or LAS4000-mini, Fuji, Tokyo, Japan). All western blotting experiments were done independently at least twice to confirm the reproducibility of the results.

To check the efficiency of RNAi in human cells, cells were lysed in TNE-N buffer (1% NP-40, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA), and were boiled for 10 min with 4xNuPAGE LDS sample buffer (Thermo Fisher Scientific). Proteins in cell lysate were separated using the NuPAGE SDS gel system (Thermo Fisher Scientific) and subjected to western blotting with specific antibodies for analysis.

Microscopic assay of yeast cells

Exponentially growing cells and rapamycin- or nitrogen starvation-treated cells were used for experiments. Cell, GFP, RFP, mCherry, and 4',6-diamidino-2-phenylindole (DAPI) images were captured using a Carl Zeiss Axio Imager M1 microscope with a cooled CCD camera (Carl Zeiss AxioCam MRm) or a Carl Zeiss Axio Observer 7 microscope with a cooled CCD camera (Carl Zeiss AxioCam 70Smono). For DAPI staining, cells were fixed with 70% ethanol briefly, washed with distilled water, and stained with 1 μg/mL DAPI briefly followed by washing. For observations of Mad1-GFP, Bub1-GFP, and Mtw1-RFP the cells were not fixed, because the GFP signals were weak. For statistical analyses, more than 100 cells were counted in each experiment. All experiments were performed at least twice independently to confirm the reproducibility of the results. Data are shown as means ± errors (or SD). Statistical analyses were carried out using the software GraphPad Prism (v. 9.1.1) followed by the two-tailed Fisher’s exact test or the two-way ANOVA with Bonferroni correction.

Chromosome instability (CIN) assay

This assay was performed as described previously (Warren et al., 2002). Briefly, strains containing a nonessential SUP11-marked mini-chromosome grown in selective media (SD-Ura) were treated with rapamycin (200 ng/mL) and/or nocodazole (10 μg/mL) for 1 h and then were plated at a density of 200 colonies per plate on medium including uracil (SD + Ura). Cells that had lost their mini-chromosomes turn red. Limiting adenine supplementation was used to facilitate red pigment development in ade2 cells. White colonies with red sectors among more than 100 cells were counted. All experiments were performed at least twice independently. Data are shown as means ± errors. Statistical analyses were carried out using the two-tailed Fisher’s exact test.

G1 cell proportion

Nocodazole-treated metaphase cells were treated with 200 ng/mL rapamycin for 4 h. More than 100 cells were examined for each sample, and the proportion of unbudded G1 cells to total cells was calculated. For flow cytometric analysis, cells were prepared according to the manufacturer’s instructions. Briefly, yeast cells (1 OD unit) grown in YPAD medium at 30°C until early log phase were treated with rapamycin or nitrogen starvation. Harvested cells were fixed in 70% ethanol, treated with 50 μg/mL RNase A at 50°C for 2 h, sonicated at amplitude 10 for 20 s using an Ultrasonic Processor S4000 (Misonix, Farmingdale, NY),
and stained with 2 μg/mL propidium iodide. DNA contents of propidium iodide-stained cells were analyzed using a CytoFLEX (Beckman Coulter) in accordance with the manufacturer’s instructions. Flow cytometric profiles were analyzed by CytExpert software.

Live cell imaging of human cells
A549 cells (ATCC) were grown at 37°C in a 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium (Life Technologies), supplemented with 10% fetal bovine serum and 20 mM HEPES, pH 7.0. For time-lapse imaging, cells were grown in glass chambers (Thermo). Cells were transfected with siRNA using Lipofectamine RNAiMAX reagent (Invitrogen) for 24 h, followed by synchronization with thymidine treatment (2 mM) for 24 h. Then cells were released from the thymidine block in the presence of nocodazole (2 μM). After 12 h, the medium was changed to pre-warmed Leibovitz’s L-15 medium (Life Technologies) supplemented with 20% fetal bovine serum and 20 mM HEPES, pH 7.0, in the presence of nocodazole (2 μM) with or without rapamycin (100 nM) and/or MG132 (20 μM). Recordings were made in a temperature-controlled incubator at 37°C. Z-series of three sections in 3 μm increments were captured every 15 min. Image stacks were projected. Images were collected with a BioStudio-T microscope (Nikon) using a ×4 objective lens or ZEISS Celldiscoverer 7 (Zeiss) using a ×20 objective lens. RNA oligonucleotides targeting Cdc20 (Kidokoro et al., 2008) and Cdh1 (Brummelkamp et al., 2002) were used. For control siRNA, Stealth RNAi siRNA negative control med GC duplex #2 (Invitrogen) was used.

Immunofluorescence analysis of human cells
Cells were grown on a glass coverslip and fixed with 3% paraformaldehyde in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.4) for 10 min at 37°C and permeabilized with 1% Triton X-100 in PBS for 5 min. Fixed cells were incubated for 1 h with the following primary antibodies: anti-Mad1, 1:2000; anti-Cyclin B, 1:2000; and anti-centromere (ACA), 1:2000. The samples were washed with PBS supplemented with 0.02% Triton X-100, and incubated with secondary antibodies coupled with Alexa Fluor–488/568 (1:3,000) for 1 h. Antibody incubations were performed in PBS supplemented with 0.02% Triton X-100. After final washes, cells were mounted with ProLong Gold. Z-image stacks were captured in 0.2-μm increments on an Olympus IX-71 inverted microscope controlled by DeltaVision softWoRx (Cytiva) using ×100 1.40 NA Plan Apochromat oil objective lens (Olympus). Deconvolution was performed when necessary using enhanced ratio algorithm, medium noise filtering, and 10 iterations per channel. Image stacks were projected and saved as Photoshop files.

QUANTIFICATION AND STATISTICAL ANALYSIS
Tests used to determine statistical significance include the two-tailed Fisher’s exact test (microscopic analyses in Figures 2D, 2F, 3C–3E and plate assays in Figures 6F and 6G); the two-way ANOVA with Bonferroni correction (microscopic analyses in Figures 5E and 6C); the Tukey’s multiple comparison test (microscopic analyses in Figures 7D and 7F); the Dunnett’s multiple comparison test (microscopic analyses in Figures SSC and SSE). Statistical Information for each experiment can be found in the figures and corresponding figure legend.