Morphogenesis checkpoint kinase Swe1 is the executor of lipolysis-dependent cell-cycle progression

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Cell growth and division requires the precise duplication of cellular DNA content but also of membranes and organelles. Knowledge about the cell-cycle-dependent regulation of membrane and storage lipid homeostasis is only rudimentary. Previous work from our laboratory has shown that the breakdown of triacylglycerols (TGs) is regulated in a cell-cycle-dependent manner, by activation of the Tgl4 lipase by the major cyclin-dependent kinase Cdc28. The lipases Tgl3 and Tgl4 are required for efficient cell-cycle progression during the G1/S (Gap1/repllication phase) transition, at the onset of bud formation, and their absence leads to a cell-cycle delay. We now show that defective lipolysis activates the Swe1 morphogenesis checkpoint kinase that halts cell-cycle progression by phosphorylation of Cdc28 at tyrosine residue 19. Saturated long-chain fatty acids and phytosphingosine supplementation rescue the cell-cycle delay in the Tgl3/Tgl4 lipase-deficient strain, suggesting that Swe1 activity responds to imbalanced sphingolipid metabolism, in the absence of TG degradation. We propose a model by which TG-derived sphingolipids are required to activate the protein phosphatase 2A (PP2ACdc55) to attenuate Swe1 phosphorylation and its inhibitory effect on Cdc28 at the G1/S transition of the cell cycle.

Significance

Little information is currently available on how a cell coordinates the expansion of its membranes with growth and cell-cycle progression. Synthesis (lipogenesis) and degradation (lipolysis) of the major storage lipid triacylglycerol (TG) are regulated in yeast by the cyclin-dependent kinase Cdc28. TG breakdown provides precursors for the synthesis of sphingolipids, which in turn regulate cell-cycle progression upon exit from G0 phase of the cell cycle via the regulatory circuit comprised of Cdc55 phosphatase, Swe1 morphogenesis checkpoint kinase, and Cdc28 cyclin-dependent kinase. This finding unveils an unexpected function of the Swe1 morphogenesis checkpoint kinase in regulating lipolysis-dependent cell-cycle entry from G0.

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novo FA synthesis, to generate precursors for membrane lipids. As a consequence of defective lipolysis, entry of quiescent cells into vegetative growth is significantly delayed; thus, TG breakdown is particularly important for promoting exit from the stationary phase and entry into the gap1 (G1) phase of the cell cycle (4, 6, 19).

Progression through the cell cycle is regulated by specific checkpoint pathways that ensure completion of crucial events and execute a halt under nonconducive conditions. Checkpoint mechanisms slow down or arrest the cell cycle to enable cells to fix damage or to obtain the required metabolites before proceeding and are as such important for the integrity of cell division (20–22). According to this critical function in quality control, mutations in checkpoint genes in mammals have been linked to cancer predisposition and progression. The first discovered cell-cycle checkpoint in Schizosaccharomyces pombe that regulates entry into mitosis is executed by the Wee1 kinase (23, 24), which delays mitosis by phosphorylating and inhibiting cyclin-dependent kinase Cdk1 (25). Conversely, the phosphatase Cdc25 promotes entry into mitosis by removing the inhibitory phosphorylation of Cdk1 (26–28). The budding yeast orthologs of Wee1 and Cdc25 are called Swe1 and Mih1, and their key functions in regulating Cdk1 activity are highly conserved (29, 30). Swe1 phosphorylates Cdk1 (encoded by CDC28) in budding yeast) at the tyrosine 19 residue and inhibits its kinase activity (29, 31, 32); the Mih1 phosphatase removes this inhibitory phosphorylation initiating G2/M cell-cycle progression (26).

The Swe1 and Cdk1/Cdc28 kinases operate in an autoregulatory loop in which Swe1 is initially phosphorylated and activated by Cdk1/Cdc28 that is associated with mitotic cyclins; subsequently, activated Swe1 phosphorylates and inhibits Cdk1/Cdc28 (33). The initial phosphorylation of Swe1 is opposed by the protein phosphatase 2A (PP2A) with its catalytic subunits Pph21 or Pph22 and the regulatory subunit Cdc55 (PP2A-Cdc55), which sets a threshold, limiting the activation of Swe1 by Cdk1/Cdc28 in early mitosis (34, 35). Loss of the regulatory subunit Cdc55 leads to hyperactivation of Swe1 (35); after the initial phosphorylation of Swe1 in early mitosis, subsequent phosphorylation events trigger full hyperphosphorylation of Swe1 (33), which leads to its ubiquitin-mediated degradation (36, 37). Of note, regulation of Cdk1/Cdc28 by the G1 cyclin Cln2 plays an important role in actin cytoskeleton polarization and the localized delivery of secretory vesicles, which contribute membrane lipids to the developing bud, thus linking cell surface growth to the cell cycle (38).

Despite its proposed role as a gap2 phase (G2) checkpoint regulator, we now show that Swe1 kinase is responsible for the G1/S (Gap1/replication phase) cell-cycle delay in mutants defective in TG lipolysis by phosphorylating Cdk1/Cdc28 at tyrosine 19. Deletion of Swe1 in the tgl3 tgl4 lipase mutant restores normal cell-cycle progression; similarly, supplementation of mutant cells with saturated FAs (myristic acid, palmitic acid) or a precursor of sphingolipid synthesis, phytosphingosine (PHS), suppress the cell-cycle delay in the lipase mutants. These data suggest that Swe1 is a lipid-regulated kinase that is activated in the absence of specific lipids, presumably sphingolipids, and halts G1/S transition by phosphorylating Cdk1/Cdc28 in lipase-deficient cells that exit from the G0 phase of the cell cycle.

Results and Discussion
Tgl3/Tgl4-Catalyzed Lipolysis Provides Precursors to Promote G1/S Cell-Cycle Progression. Previous work from our laboratory has demonstrated a requirement of lipolysis for efficient cell-cycle progression (6). Absence of the major TG lipases Tgl3 and Tgl4 extends the G1/S transition of the cell cycle by ~30 min in RediGrad-synchronized G0 cells (Fig. 1A). This cell-cycle delay is also reflected in the timing of the onset of bud formation (Fig. 1B), consistent with previous results (6). However, there was no general defect in cell growth during the log phase, and all mutants essentially reached the same cell density in the stationary phase (Fig. S1). Because the primary products of lipolysis are FAs, we next investigated whether their external administration would suppress the cell-cycle delay of tgl3 tgl4 mutants. This was indeed the case when either myristic acid (C14:0) or palmitic acid (C16:0) were supplied (Fig. 1A and B), whereas unsaturated FAs (C16:1 or C18:1) did not suppress the cell-cycle delay. This result is surprising, as C16:1 and C18:1 have no effect on cell-cycle progression of the tgl3 tgl4 mutant. Initiation of the S phase is indicated by arrows. Experiments were performed in biological triplicates. (B) The timing of bud formation was analyzed in synchronized G0 cells by microscopy. Initiation of bud formation (indicated by white arrowheads) occurs concomitantly with the G1/S phase cell-cycle transition under all conditions tested. (Scale bar, 10 μm.)

Fig. 1. Deletion of Tgl3 and Tgl4 results in a cell-cycle delay at G1/S. Cells were grown for 7 d in YPD medium, and quiescent (G0) cells were obtained by RediGrad centrifugation. After release into fresh glucose containing external media, cells were harvested at the indicated time points and analyzed by FACS and microscopy. (A) In the lipase-deficient tgl3 tgl4 mutant, entry into the S phase is delayed. Wild type entered the cell cycle 90 min after release, and the 2N peak in tgl3 tgl4 appears 30 min later, at 120 min. Supplementation of media with FAs, C14:0 and C16:0 (0.025% in 1% tergitol), or PHS (1 μM in 0.2% tergitol) rescues the cell-cycle delay of the tgl3 tgl4 mutant. The 2N peak in the tgl3 tgl4 mutant starts to appear 90 min after release, similar to wild type. The unsaturated FAs (C16:1 and C18:1) have no effect on cell-cycle progression of the tgl3 tgl4 mutant. Initiation of the S phase is indicated by arrows. Experiments were performed in biological triplicates. (B) The timing of bud formation was analyzed in synchronized G0 cells by microscopy. Initiation of bud formation (indicated by white arrowheads) occurs concomitantly with the G1/S phase cell-cycle transition under all conditions tested. (Scale bar, 10 μm.)
Absence of Inositol Extends the G1/S Cell-Cycle Delay of the \textit{tgl3 tgl4} Mutant. PI plays an essential role as a precursor for complex sphingolipids. Gaspar et al. previously showed that absence of lipolysis drastically attenuates the synthesis of PI upon administration of inositol to inositol-starved cells, suggesting that TG-derived metabolites provide precursors for PI production (18). In addition, we have previously reported that lack of inositol in standard cultivation media extends the cell cycle at the G2/M boundary (41), demonstrating that endogenous inositol production is limiting even in wild-type cells during logarithmic growth. Thus, we next investigated whether and to what extent inositol depletion affected cell-cycle progression in wild-type and lipase-deficient mutants (Fig. S3). RediGrad-synchronized G0 phase cells were released into inositol-free media, and cell-cycle progression over time was analyzed by FACS. In the absence of inositol, the \textit{tgl3 tgl4} mutant entered the cell cycle 150 min after release into fresh media, in contrast to wild type (minus inositol), which showed a 2N peak (postreplication) at 120 min (Fig. S3). The delay in the lipase mutant in the absence of inositol indicates additive effects of inositol starvation and defective lipolysis on cell-cycle progression, both of which contribute to sphingolipid synthesis.

Lipolysis-Defective Mutants Accumulate Saturated and Unsaturated TG Species. Because the cell-cycle defect of \textit{tgl3 tgl4} mutants was suppressed by supplementation with C14:0 and C16:0, we next investigated whether these specific FAs are indeed derived from TG breakdown during cell-cycle progression. Synchronized cells were harvested and total lipids were extracted and analyzed by ultra high performance liquid chromatography–electrospray ionization–mass spectrometry (UPLC–ESI–MS) over a period of 2 h (Fig. 2 and Fig. S4). In wild-type cells, TG species containing saturated FAs are present only in minor amounts (Fig. 2A and Fig. S4A). Throughout the cell cycle, TG molecular species containing saturated FAs steadily increased over time, whereas the major unsaturated species (containing three unsaturated FAs) declined, as expected, in the presence of \textit{tgl3} and \textit{tgl4} lipases. This observation supports the notion that FA de novo synthesis contributes to the cellular lipid content during the cell cycle and that a minor fraction of the newly generated (saturated) FAs indeed feed into the TG pool. In wild-type cells, TG levels decreased by 30%; this decline was due to active lipolysis rather than simple dilution by cell division, which did not take place during the observed time window. The observed prevalent turnover of unsaturated TG species is consistent with the described in vitro specificity of the yeast TG lipases (12, 42). As expected and consistent with previous observations (4, 12, 42), \textit{tgl3 tgl4} mutants had about threefold elevated TG levels (Fig. 2B and Fig. S4B); notably, despite the absence of the major lipases, TG levels significantly declined by \textasciitilde 10% during the first hour after entry into the cell cycle, indicating that additional TG lipases/acyltransferases may be active during this growth phase. Subsequently, TG levels increased again until the cell cycle was completed, which again reflects endogenous FA synthesis and deposition into TG, in the absence of lipolysis. \textit{Tgl3} and \textit{Tgl4} lipases appear to have a strong substrate preference for TG molecular species harboring three unsaturated FAs, which remain unaltered in the lipase mutants during the initial cell duplication (4, 12, 42). Most notably, however, fully saturated TG molecular species, including those harboring C14:0 and C16:0 acylenes (TG 42:0, TG 48:0), were substantially increased in \textit{tgl3 tgl4} mutants during the initial cell cycle and that a minor fraction of the newly generated (saturated) TG levels decreased by 30%; this decline was due to active lipolysis after entry into the cell cycle, indicating that additional TG lipases/transacylases may be active during this growth phase. Because maximum growth rates of \textit{tgl3 tgl4} mutants are identical to wild-type cells (Fig. S1), TG accumulation per se does not impair growth, but rather TG-derived sphingolipid precursors become limiting upon cell-cycle entry from the stationary phase, in the absence of lipases and insufficient endogenous FA synthesis.

The Cell-Cycle Delay in the \textit{tgl3 tgl4} Mutant Is Executed by the Checkpoint Kinase \textit{Swe1}. As cells progress through the cell cycle, various checkpoints are activated during defined stages of cell division. Because lack of lipolysis delayed cell-cycle progression at the onset of bud formation, which is associated with actin polarization and morphology alterations, we hypothesized that the morphogenesis checkpoint kinase \textit{Swe1} (25, 30, 43) might play a role in executing this delay. In addition to its role in establishing actin polarity and functioning as a cell-cycle checkpoint (22, 29, 32), \textit{Swe1} was also identified in a large-scale study to be connected to sphingolipid metabolism by its genetic interaction with \textit{LCB1}, encoding the catalytic subunit of SPT (44, 45). A related sphingolipid-associated checkpoint function of \textit{Swe1} was also reported in the context of inositol sphingolipase C (Iscl), which catalyzes the formation of bioactive ceramides from complex sphingolipids (46, 47). Based on these observations, \textit{Swe1} appeared as a likely candidate for regulating cell-cycle progression in response to altered lipolysis and the subsequent sphingolipid deficiencies. Indeed, additional deletion of \textit{SWE1} in the \textit{tgl3 tgl4} mutant background suppressed the G1/S phase delay, and the \textit{tgl3 tgl4 swe1} triple mutant progressed through the cell cycle like wild type (Fig. 3A). These data therefore confirm that \textit{Swe1} kinase is the checkpoint that senses the lack of lipolysis and delays the cell cycle in the absence of saturated FA or long-chain sphingoid base supply. Previous studies have identified tyrrosine 19 of the cyclin-dependent kinase Cdk1/Cdc28 as the specific phosphorylation site through which \textit{Swe1} regulates cell-cycle progression.
progression (22). As expected, and similar to a Swe1 deletion, point mutation of Cdc28 also suppressed the cell-cycle delay in TGL3 TGL4-deficient cells (Fig. 3A). Bud emergence in the tgl3 tgl4 swe1 and tgl3 tgl4 cdc28F mutants was restored to wild type as well (Fig. 3B). Because Swe1 is established as a regulator of entry into mitosis (22, 48), we next tested conditions under which Swe1 would also display a function in G1, as suggested by its negative regulatory role in tgl3 tgl4 mutants. SWE1 overexpression in vegetatively growing wild type induced a morphology defect—that is, mononucleate mother cells and nucleus-free elongated daughter cells—and a G2 arrest, consistent with published data (22, 30, 33, 49) (Fig. 3C). In contrast, SWE1 overexpression in tgl3 tgl4 mutants, however, induced a similar morphology defect but a G1 arrest; both phenotypes were not observed by additional mutation of the Swe1 phosphorylation site in Cdc28Y19. From these experiments we conclude that Swe1 indeed becomes activated in G1 in the absence of lipolysis, supporting a previously unidentified function of this checkpoint kinase in cell-cycle regulation.

To further confirm the unexpected role of Swe1 in G1, we next characterized the appearance of cyclins and the phosphorylation status of Cdc28 in strains lacking tgl3 tgl4 lipases under various lipid supplementation conditions (Fig. 4 and Fig. S5). In the absence of lipolysis in tgl3 tgl4 mutants, Cdk1/Cdc28 is phosphorylated already at 60 min after release of G0 cells into the cell cycle, before the onset of bud formation; this is in marked contrast to wild-type cells, which display Cdc28 phosphorylation only after 120 min, at the G2/M transition (Fig. 4A). Cdc28 phosphorylation in tgl3 tgl4 mutant cells indeed responds to the presence of lipid precursors, palmitic acid, or long-chain base (PHS) in a cell-cycle-dependent manner. Consistent with our hypothesis, Cdc28 phosphorylation in the tgl3 tgl4 mutant supplied with C160 or with PHS followed kinetics similar to wild-type cells (Fig. 4B). This further supports the notion that the cell-cycle delay in the absence of lipolysis is due to defective sphingolipid synthesis.

The cell-cycle delay of the tgl3 tgl4 mutant is also reflected in the delayed appearance of the G1 cyclin Cln2 (50, 51), S-phase cyclin Clb5 (52, 53), and the G2 cyclin Clb2 (54, 55) (Fig. S4D). Additional deletion of SWE1, mutation of the Swe1-dependent phosphorylation site of Cdc28Y19, or supplementation of lipid precursors restored wild-type cell-cycle entry and progression. These data confirm a role of Swe1 in executing the delay in G0 exit in the absence of TG breakdown.

Swe1 Does Not Directly Regulate Lipolysis. Because deletion of SWE1 in the lipase-deficient mutant suppressed the cell-cycle delay, we next investigated whether Swe1 directly affected lipid homeostasis and restored lipolysis by an unknown mechanism, in the absence of the major TG lipases Tgl3 and Tgl4. This, however, was not the case (Fig. 2C and Fig. S4C): Additional deletion of SWE1 in the lipase-deficient strain did not significantly reduce but rather slightly increased TG content per OD600 unit, excluding the possibility that absence of the kinase bypasses the cellular requirement for Tgl3 and Tgl4 by activation of alternative TG lipases.

The Role of PP2A CDC55 Phosphatase in Regulating the Cell-Cycle Delay in tgl3 tgl4 Mutant. Swe1 and Cdc28 kinases function in a positive feedback loop at G2/M (33) and are regulated by two phosphatases, PP2A CDC55 and Mih1. Cdc55 was described to be active during early mitosis and regulates removal of the activating phosphorylation of Swe1, thus preventing the inactivation of Cdc28; these combined activities allow accumulation of a threshold level of active Cdc28. A cdc55 mutation causes hyperphosphorylation of Swe1, indicating that PP2A CDC55 is the primary phosphatase that acts on Swe1 during early mitosis (34). Of note, sphingolipids have previously been reported to be effectors of PP2A CDC55 function (56, 57), supporting a role in regulating Swe1 activity. To further assess the role of Swe1 in regulating cell-cycle progression at the G1/S boundary, we next investigated the impact of the phosphatase PP2A CDC55. Deletion of CDC55 delayed entry into the cell cycle by 30 min compared with wild type, indicating a role of Cdc55 also in G1/S (Fig. 5 and Fig. S6). Deletion of CDC55 in the tgl3 tgl4 background extended this delay further by ~60 min, indicating add-}

![Fig. 3.](https://example.com/fig3)
inactivation of Swe1 are dependent on sphingolipids. This is also consistent with a previous study that showed that the PP2A<sup>B55γ</sup>-Swe1 cascade is a downstream target of the sphingolipid, phytoceramide (59). We conclude that deletion of the regulatory subunit of PP2A<sup>B55γ</sup> makes the cells unresponsive to the availability of sphingolipids (SLs), which results in pertained activation of Swe1 and extension of the G1/S phase. In contrast, the Mih1 phosphatase that is required to remove the inhibitory phosphorylation of Cdc28 in the G2/M phase of the cell cycle (27, 28, 43) neither positively nor negatively affected the cell-cycle progression of the lipase-deficient mutant at the G1/S boundary (Fig. 5 and Fig. S6). Of note, Mih1 activity was previously implicated in regulating cell-cycle progression in response to altered secretion, which is also required for delivery of membrane lipids to the expanding plasma membrane in the growing bud. A block in secretion in a sect-4 temperature-sensitive mutant also triggers a Swe1-dependent checkpoint but during early mitosis (60). Thus, Swe1 may indeed function as a lipid-dependent checkpoint at multiple stages of the cell cycle; here, we show that it operates as a lipolysis-regulated checkpoint kinase at the G1/S transition of the cell cycle and that its activity is regulated by the PP2A<sup>B55γ</sup> phosphatase in a lipid-dependent manner.

**G0 to G1/S Lipid-Dependent Regulation of Cell-Cycle Progression: A Model.** Both plasma membrane and also intracellular membrane proliferation in growing cells demand net phospholipid synthesis, which is satisfied not only by de novo production of FAs but also by recycling of storage lipids—that is, TG. In addition, delivery of cell wall components through the secretory pathway relies on vesicular trafficking, linking cell-cycle progression to the regulation of key steps of lipid metabolism and membrane growth (6, 8, 38, 61, 62). In our experimental setup, we investigated the requirement of lipolysis to regulate recovery from quiescence and exit from G0 into a new cell cycle. Quiescence is important for survival under starvation conditions, and a hallmark of quiescent cells is their ability to synchronously enter the mitotic cycle (6, 63). G0 cells significantly differ in their physiology from vegetatively growing cells with respect to metabolism, protein synthesis, and membrane trafficking (64–67).

Mutants lacking the two major TG lipases Tgl3 and Tgl4 show a delayed entry of cells from G0 into the cell cycle (6) (Fig. 1), which can be rescued by supplementation with C14:0 or C16:0 FAs or PHS, which are all precursors for the synthesis of sphingolipids (Fig. 1). The specific lipid requirements upon entry into the growth cycle appear to be different from vegetatively growing cells, as none of the mutants display a significant growth defect during logarithmic growth (Fig. S1A and B). This is not unprecedented, as mutants lacking Gcs1, a regulator of the phospholipase D, Spo14 (68), or the FA activator Faal (69), are defective in exiting quiescence; neither spo14 nor faal1 mutants, however, display a significant vegetative growth phenotype, consistent with the notion that G0 reflects a metabolic state rather than a cell-cycle state (70, 71).

The cell-cycle delay of the tgl3 tgl4 mutants was efficiently suppressed by the additional deletion of SWE1; this kinase was previously described as a checkpoint that monitors actin polarization, which precedes bud formation (32, 70, 72). Because in our setup both DNA replication and bud formation are concomitantly delayed, the checkpoint role of Swe1 kinase is not unexpected. The delayed accumulation of late G1 cyclins (Clb5 and Clb2) and the G2 cyclin (Clb2) further demonstrates that absence of lipolysis results in a delayed onset of the G1/S transition (Fig. 4). This delay is clearly regulated by the activity of Swe1 that inhibits Cdc28; the nature of the Cdc28/cyclin complex that is inhibited or absent at the G0 exit in the absence of lipolysis remains to be determined (Fig. 4). We thus propose the following model for lipolysis-regulated cell-cycle progression (Fig. 6): When cells are released from the G0 phase into fresh glucose-containing media, Cdc28 phosphatases and activates

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**Fig. 4.** The cell-cycle delay mediated by Swe1-dependent phosphorylation of Cdc28 in the tgl3 tgl4 mutant occurs at G1. At the indicated time points, whole-cell extracts were prepared for Western blot analysis, using antibodies against phospho-Cdc28 (Tyr19) to probe Cdc28<sup>Y19</sup> phosphorylation, α-PSTAIRE to detect total levels of Cdc28, and α-GFP to detect Cln2-GFP, α-Cib5, and α-Cib2. (A) The cell-cycle delay in the tgl3 tgl4 mutant results from the inhibitory phosphorylation of Cdc28. In wild type, Cdc28<sup>Y19</sup> phosphorylation is detectable at G2/M, 120 min after release into fresh media. In contrast, in the tgl3 tgl4 mutant, phospho-Cdc28<sup>Y19</sup> is detectable already 60 min after release into fresh medium. Upon supplementation of PHS (1 μM in 0.2% tergitol) or C16:0 (0.025% in 1% tergitol) to the medium. Upon supplementation of PHS (1 μM in 0.2% tergitol) or C16:0 (0.025% in 1% tergitol) to the tgl3 tgl4 mutant, phospho-Cdc28<sup>Y19</sup> is detectable only 120 min after release, similar to wild type. (B) Low levels of Cln2-GFP are detectable in G0 cells and peak at the G1 transition, after 90 min in wild type. In the tgl3 tgl4 mutant, Cln2 levels peak after 120 min; wild-type appearance of Cln2 is restored in these mutants in the presence of C16:0 or PHS. (C) The appearance of Clb5 coincides with the initiation of DNA replication. S phase in the tgl3 tgl4 mutant is initiated 120 min after release; this is in contrast to wild-type, tgl3 tgl4 mutants supplemented with C16:0 or PHS, tgl3 tgl4 swe1 or tgl3 tgl4 cdc28<sup>Y19</sup> mutants, in which it is initiated already 90 min after release. (D) Clb2 marks the entry into mitosis. Clb2 is first detectable in synchronized wild type at 150 min, compared with the tgl3 tgl4 mutant in which it is first detected 180 min after release. The tgl3 tgl4 mutant supplemented with C16:0 or PHS, tgl3 tgl4 swe1, and tgl3 tgl4 cdc28<sup>Y19</sup> mutants behave like wild type. GAPDH was used as a loading control for B–D. Representative blots from three independent experiments are shown.
Deletion of the Swe1 phosphatase PP2Acdc55 affects cell-cycle progression in the lipase mutants. RediGrad synchronized G0 cells were released into fresh glucose-containing media. As indicated by arrows, the cdcc55 mutant strain enters the cell cycle at 120 min after release into fresh media. Deletion of CDC55 in the tgl3 tgl4 mutant further extended the initiation of the S phase, whereas the swe1 cdcc55 mutant behaved like wild type. The supplementation of PHS (1 μM in 0.2% tergitol) to the mutants lacking PP2Acdc55 did not affect cell-cycle progression. Deletion of MIH1 in the tgl3 tgl4 mutant has no influence on the cell-cycle progression. The experiments were independently repeated three times.

Materials and Methods

Strains and Growth Conditions. The Saccharomyces cerevisiae strains used in this study are listed in Table 1. Double mutants were constructed by standard genetic crosses and by targeted gene deletion by homologous recombination. cdcc55 deletion mutants were generated by replacing the ORF with a Nat⁵ cassette. Gene deletions (Euroscarf) were verified by colony PCR with the appropriate up-tag and down-tag primers. Strain RSY342 (MATa bar1 cdc28Y19F::TRP1 GAL1-SWE1myc::URA3) was a kind gift from Daniel Lew’s laboratory, Department of Pharmacology and Cancer Biology, Duke University Medical Center. The strain harbored the chromosomally integrated CLN2-mGFP fragment. Yeast cells were grown in YPD (extract, peptone, dextrose (glucose)) medium containing 1% yeast extract, 2% (wt/vol) peptone, 2% (wt/vol) glucose, or in yeast nitrogen base (YNB) minimal medium (MM) containing 0.67% yeast nitrogen base, 2% (wt/vol) glucose and the appropriate amino acids and nucleo bases; for experiments lacking inositol, threonine was omitted from the amino acid stock. Solid media had the same composition as the liquid media, but solidified with 2% (wt/vol) agar (74). Yeast strains carrying expression plasmids were maintained in uracil drop-out medium. YPD plates containing 200 mg/L G418 (Geneticin; Calbiochem) or 100 mg/L ClonNAT (nourseothricin; Sigma) were used to select for KanMX or Nat⁵ markers, respectively. Sporulation for constructing an N-terminal fusion with GST on an episomal plasmid, the SWE1 gene was amplified as a BamHI-NotI fragment by PCR, using genomic DNA as the template and primers 5′-GACTAGTTACAGCTTTGCTTTTG-GAAAGCTTACAGTAACC-3′ and 5′-GACTACCGGCGCTATATATATATATATATATATATATAAAAGGAGG-CGGAGCCGAGAATTGGTACCTTTTGCCATGCTCATG-3′ such that the Nat⁵ cassette was inserted downstream of the mutated CDC28 gene. This construct was chromosomally integrated into wild-type and tgl3 tgl4 mutants, and the transformed strains were selected for ClonNAT resistance; the mutation was confirmed by sequencing.

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medium contained 0.25% yeast extract, 0.1% glucose, and 1% potassium acetate. Stocks of myristic acid (Fluka) and palmitic acid (Sigma) were prepared in 10% (v/vol) tertigol (Sigma) and supplemented to YNB MM at a final concentration of 0.025%. PHS (Avanti Lipids) stock was prepared in methanol and supplemented into YNB containing 0.2% tertigol at a final concentration of 1 μM. Expression of GST-SWE1 under the control of the CUP1 promoter was induced by the addition of 0.5 mM copper sulfate to the medium. Yeast strains were cultivated at 30 °C in a shaker incubator.

Synchronization of Cells by RediGrad Centrifugation. Cells were cultivated to the stationary phase for 7 d in YPD medium [1% yeast extract, 2% (wt/vol) peptone, 2% (wt/vol) dextrose], and quiescent G0 cells were harvested by centrifugation at 4500 × g for 15 min. Cells were resuspended in 1 mL of 50 mM Tris·HCl (pH 7.5) and carefully layered onto the formed gradient and centrifuged again at 40,000 × g for 15 min. Cells were pelleted and resuspended in PBS (pH 8.0). The DNA was stained with 1 mM SYTOX Green (Invitrogen, Inc.) and analyzed by Fluorescence-Activated Cell Sorting Analysis.

Lipid Analysis. Cells obtained by RediGrad centrifugation were inoculated into fresh synthetic complete YNB media and harvested at indicated time points, snap frozen in liquid nitrogen, and stored at −80 °C until lipid extraction was performed. An internal standard mix was added (78), and lipids were extracted according to Folch et al. after glass bead homogenization of cells (79). The lipid extracts were analyzed by UPLC–ESI–quadrupol time-of-flight (qTOF)–MS (78), and chromatograms and mass spectra were evaluated using Waters MassLynx and Lipid Data Analyzer Software (80).

Microscopy. RediGrad synchronized or logarithmically growing cells were harvested and resuspended in 100 μL of 4% paraformaldehyde/sucrose solution and incubated at room temperature for 15 min. The cells were then washed with 500 μL of K-Po4/sorbitol buffer (pH 7.5), resuspended in 100 μL of the same solution, and stored at 4 °C until further use. Before use, samples were briefly sonicated and imaging was performed using a Leica SP2 confocal microscope (Leica Microsystems, Inc.) using a 100× NA 1.4 oil objective and differential interference contrast optics.

Preparation of Yeast Whole-Cell Extracts and Immunoblotting. Total cell extracts were prepared as described by Baerends et al. (75). In brief, synchronized cells were released into fresh media, 0.1 OD600 units of cells were harvested at the indicated time points and processed as described above. Mouse anti-GFP antibody (Roche) was used for detection of Cln2-mGFP. Horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) and anti-mouse IgG (Pierce, Thermo Fisher Scientific) were the secondary antibodies, and ECL Western Blotting Substrate (Pierce) was used for detection.

Preparation of Yeast Whole-Cell Extracts and Immunoblotting. Total cell extracts were prepared as described by Baerends et al. (75). In brief, synchronized cells were released into fresh media, 0.1 OD600 units of cells were harvested at the indicated time points and incubated with 400 μL of 12.5% (wt/vol) trichloroacetic acid (TCA) at −80 °C for at least 30 min to allow protein precipitation. TCA was removed by centrifugation, and the cell pellets were washed twice with 80% (v/vol) ice-cold acetone, air-dried, and dissolved in 1% SDS/0.1% NaOH. We added 1× SDS loading buffer, and samples were boiled for 5 min before loading onto polyacrylamide gels. Proteins were resolved on 10% (v/vol) SDS polyacrylamide gels and blotted onto nitrocellulose membranes (Bio-Rad). Anti-rabbit phospho-cdc2 (Tyr15) antibody (Cell Signaling Technology) was used to detect phosphorylated Cdc28 and anti-human PSTAIRE antibody, which detects total Cdk1/Cdc28 (Santa Cruz Biotechnology), was used as a loading control. Anti-rabbit a-Clbs and anti-rabbit a-Clb2 antibodies were a kind gift from Adam Rudner, Ottawa Institute of Systems Biology, University of Ottawa, and were used as previously described (77). For Western blots of Cln2-mGFP, 10 OD600 units of cells were harvested at the indicated time points and probed as described above. Mouse anti-GFP antibody (Roche) was used for detection of Cln2-mGFP.


Fig. S1. Growth of strains used in this study. (A and B) Strains were precultured for 48 h in YPD and then transferred to MM media for analysis of growth (OD$_{600}$). No significant differences in growth rates compared with the wild type were observed for the mutants, except for an extension of the initial lag phase. (C) tgl3 tgl4 mutants are slightly more sensitive to the SPT inhibitor myriocin; as expected, SPT inhibition by myriocin can be suppressed by PHS but not by C16:0 supplementation.

Fig. S2. Lack of β-oxidation does not cause a cell-cycle delay. Synchronized pox1 and pox3/pot1 mutants, which are defective in β-oxidation, display wild-type–like cell-cycle progression. This excludes a role for lipolysis to provide precursors for β-oxidation/energy production upon exit from G0.
Fig. S3. Absence of inositol in the growth media extends the G1/S phase cell-cycle delay of the tgl3 tgl4 mutant dependent on Swe1. RediGrad synchronized G0 cells were released into fresh MM lacking inositol. Cells were harvested at the indicated time points, and the cell-cycle progression was followed over time by FACS analysis. In the presence of inositol (MM), wild type entered the G1/S phase of the cell cycle 90 min after release and the tgl3 tgl4 mutant displayed a 30 min delay (Fig. 1A). This delay is further extended by ~30 min in both wild type and the tgl3 tgl4 mutant when inositol is lacking in the media. This delay in the absence of inositol is mediated by the Swe1 kinase, as the swe1, tgl3 tgl4 swe1, and tgl3 tgl4 cdc28Y19F mutants do not show an inositol-dependent delay in cell-cycle progression (Fig. 3A). Initiation of DNA replication is indicated by arrows. Experiments were performed in biological triplicates.
Fig. S4. TG content in wild type, tgl3 tgl4, and tgl3 tgl4 swe1 mutants during the cell cycle. RediGrad synchronized G0 cells were released into fresh glucose-containing MM media, and 20 OD600 units were harvested at indicated time points for lipid analysis. (A) In wild type, total levels of TG decreased over time. The very minor fully saturated (N:0) TG species show a slight increase, the mono- (N:1) and diunsaturated (N:2) TG species remain mostly unchanged, and the triunsaturated (N:3) TG species are degraded as cells progress through the cell cycle, in the presence of Tgl3 Tgl4 lipases. (B and C) In the tgl3 tgl4 and tgl3 tgl4 swe1 mutants, total TG levels are about threefold higher compared with wild type; during the first 30 min into the cell cycle, some TG degradation was observed, indicating the presence of additional lipases/transacylases. Deletion of SWE1 does not activate additional lipases. Notably, saturated (N:0) TG species are about 2–10-fold higher in the lipase-deficient strains (depending on the molecular species) and increase up to fivefold during the cell cycle. The mono- (N:1), di- (N:2), and triunsaturated (N:3) TG species display minor fluctuations (~10%) in tgl3 tgl4 and tgl3 tgl4 swe1 mutants over the observed time course; the slightly increased relative TG content in the tgl3 tgl4 swe1 strain compared with tgl3 tgl4 mutants may be due to the different cell sizes that affect OD600 readings to which TG content was normalized. Error bars indicate SD of three independent experiments.
**Fig. S5.** The phospho-Cdc2 (Tyr15) antibody is specific for Cdc28\(^{Y19}\) phosphorylation. A control immunoblot was performed to confirm the specificity of the used Cdc2 (Tyr15) antibody. Phospho-Cdc28\(^{Y19}\) is not detectable in a strain lacking Swe1 or when the Swe1 kinase-specific tyrosin residue (Y19) on Cdc28 was mutated to phenylalanine.

**Fig. S6.** Budding is delayed in the tgl3 tgl4 cdc55 mutant. RediGrad synchronized G0 cells were released into fresh glucose-containing media, and cells were harvested at the indicated time points and analyzed for the initiation of budding by microscopy. The delays in budding and G1/S phase transition occurred concomitantly (Fig. 5). The tgl3 tgl4 cdc55 mutant started budding only ~180 min after release of G0 cells into fresh media, compared with cdc55 (120 min) and swe1 cdc55 (90 min), which started earlier. The supplementation of PHS did not influence the initiation of budding in the absence of Cdc55. (Scale bar, 10 μm.)