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RESEARCH ARTICLE

Growth conditions inducing G1 cell cycle arrest enhance lipid production in the oleaginous yeast *Lipomyces starkeyi*

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ABSTRACT

Lipid droplets are cytoplasmic organelles that store lipids for energy and membrane synthesis. The oleaginous yeast *Lipomyces starkeyi* is one of the most promising lipid producers and has attracted attention as a biofuel source. It is known that the expansion of lipid droplets is enhanced under nutrient-poor conditions. Therefore, we prepared a novel nitrogen-depleted medium (N medium) in which to culture *L. starkeyi* cells. Lipid accumulation was rapidly induced, and this was reversed by the addition of ammonium. In this condition, cell proliferation stopped, and cells with giant lipid droplets were arrested in G1 phase. We investigated whether cell cycle arrest at a specific phase is required for lipid accumulation. Lipid accumulation was repressed in hydroxyurea-synchronized S phase cells and was increased in nocodazole-arrested G2/M phase cells. Moreover, the enrichment of G1 phase cells seen upon rapamycin treatment induced massive lipid accumulation. From these results, we conclude that *L. starkeyi* cells store lipids from G2/M phase and then arrest cell proliferation in the subsequent G1 phase, where lipid accumulation is enhanced. Cell cycle control is an attractive approach for biofuel production.

KEY WORDS: Cell cycle, Lipid, Oleaginous yeast, Rapamycin, Biofuel

INTRODUCTION

Lipids play important roles in energy homeostasis, membrane synthesis and the formation of lipophilic molecules. To avoid lipotoxicity, cells convert excess lipids into neutral lipids, mainly triacylglycerol (TAG), which are stored in lipid droplets (Schepers and Behl, 2021). When lipids from lipid droplets are not available, various disease states, such as type 2 diabetes, inflammation and cancer are induced (Krahmer et al., 2013). Previous studies on lipid droplets in cancer cells suggest that an increase in lipid droplet number is associated with a high proliferation rate (Accioly et al., 2008). In epithelial cells, leptin-triggered activation of the mechanistic/mammalian target of rapamycin (mTOR) promotes the accumulation of intracellular lipids and their entry into the cell cycle (Fazolini et al., 2015). In addition, caveolin-1-deficient mice show impaired liver regeneration after hepatectomy, which is caused by cell cycle arrest of hepatocytes due to their inability to efficiently accumulate lipid droplets (Fernández et al., 2006). These results imply that lipid droplets are involved in cell proliferation by regulating the cell cycle.

The molecular and regulatory mechanisms for lipid droplet formation are highly conserved from yeast to mammals (Martin and Parton, 2006). Yeast studies have shown a direct link between lipid homeostasis and the cell cycle. In *Saccharomyces cerevisiae*, lipids stored in droplets are mobilized when quiescent G0 phase cells resume cell division (Kurat et al., 2006). A subsequent study showed that the cell cycle-regulatory kinase Cdc28 is involved in the activation of Tgl4 lipase by phosphorylation and TAG degradation (Kurat et al., 2009). Moreover, it has been shown that lipolysis occurs during meiosis II, and lipid droplet-deficient cells form abnormal spore membranes and inviable spores (Hsu et al., 2017). Mobilization of TAG provides the fatty acids to maintain the synthesis of phospholipids for new membranes, including bud formation and the prospore membrane (Hsu et al., 2017; Rajakumari et al., 2010). In *Schizosaccharomyces pombe*, a fatty acid synthase mutant strain shows a defect in nuclear division, indicating that lipogenesis is required for the completion of mitosis (Saitoh et al., 1996). These studies indicate that cell cycle progression is dependent on the accurate control of lipid homeostasis.

For biofuel production by oleaginous microorganisms, the storage of abundant lipids into droplets is very beneficial. Compared to traditional fuels, microbial-derived biofuel has the advantage of increased sustainability. Therefore, effective lipid accumulation in oleaginous microorganisms has been widely studied (Athenaki et al., 2018). *Lipomyces starkeyi* is a particularly useful host for efficient biofuel production among oleaginous yeasts, because it has a strong lipid production capacity and accumulates giant lipid droplets consisting of 65–85% of its dry cell weight (Angerbauer et al., 2008; Juanssilfero et al., 2018). Owing to its industrial relevance, there have been several attempts to improve lipid production efficiency in *L. starkeyi* by optimizing culture conditions, for instance temperature, aeration, pH, carbon source and nitrogen concentrations (McNeil and Stuart, 2018; Takaku et al., 2020). In particular, the molar ratio of carbon to nitrogen (C:N ratio) in the medium affects lipid production, and detailed analyses indicate that when the C:N ratio is elevated, the accumulation of lipids is increased (Angerbauer et al., 2008; Calvey et al., 2016; Salunke et al., 2015). Furthermore, high lipid yields are obtained when *L. starkeyi* cells are cultured in a glucose solution that contains no nitrogen source (Lin et al., 2011), indicating that the amount of nitrogen is an important factor in media used for lipid production. The correlation between environmental parameters and lipid amounts are well studied; however, the regulation of *L. starkeyi* lipid metabolism upon cell proliferation and/or the effect of the cell cycle has not been studied.

In this study, we report that a novel nitrogen-depleted medium (N medium) induced cell division arrest and led to rapid expansion

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of lipid droplets. When *L. starkeyi* cells were grown in N medium, the cells contained giant lipid droplets and were arrested in G1 phase of the cell cycle. To ascertain whether cell cycle arrest is necessary for lipid accumulation, we measured the amounts of lipids in cells arrested in each cell cycle stage. Lipid accumulation occurred outside of S phase and was upregulated in G1 phase cells upon rapamycin-mediated arrest. Thus, our results strongly imply that the cell cycle and the TOR pathway provide new approaches for improving industrial lipid production.

RESULTS

A nitrogen-depleted novel medium effectively induces lipid accumulation

Nitrogen depletion is an approach for improving lipid production in oleaginous yeasts *Yarrowia lipolytica* and *Rhodosporidium toruloides* (Seip et al., 2013; Yang et al., 2016b; Zhu et al., 2012). It has also been reported that in *L. starkeyi* cells, lipid droplet formation is accelerated under nutrient-poor conditions (Duan and Okamoto, 2021; Juanssilfero et al., 2018). Additionally, under nitrogen starvation, yeast cells execute meiosis and subsequent sporulation. TAG lipolysis is required for sporulation (Hsu et al., 2017; Liu et al., 2020), and therefore an increase in lipids is observed before sporulation (Liu et al., 2020). Accordingly, based on the *L. starkeyi* sporulation medium (AF medium; sucrose 2.85 g/l, aspartic acid 230 mg/l, glutamic acid 510 mg/l, KH_2PO_4 245 mg/l, $MgSO_4{\cdot}7H_2O$ 660 mg/ml, $FeCl_3{\cdot}6H_2O$ 1.7 mg/l, $MnSO_4{\cdot}6H_2O$ 0.51 mg/l, $ZnSO_4{\cdot}7H_2O$ 4.5 mg/l, KOH 410 mg/ml, pH 6.5) (Kurtzman, 2011; Takayama, 2021; Watanabe et al., 1997), we prepared a novel nitrogen-depleted medium [N medium; 0.3% sucrose, 0.025% KH_2PO_4, 0.066% $MgSO_4{\cdot}7H_2O$, and trace elements solution ($FeCl_3{\cdot}6H_2O$ 1.7 mg/l, $MnSO_4{\cdot}6H_2O$ 0.51 mg/l, $ZnSO_4{\cdot}7H_2O$ 4.5 mg/l)] by removing its nitrogen source. To ascertain whether lipid accumulation was induced by culture in N medium, we observed lipid droplet formation. *L. starkeyi* cells precultured in YPD medium (2% D-glucose, 1% yeast extract, 2% hipolypeptone) were transferred to N medium (0 h), and aliquots from every 24 h of culture were collected. The lipid droplets in cells from each time point were stained with BODIPY™ 493/503 and observed by fluorescence microscopy (Fig. 1A). Lipid droplets, which were almost absent at 0 h, became appreciably visible in cells at 24 h. Thereafter, lipid droplets expanded with time. To validate the lipid production efficiency using N medium, lipid droplet formation was compared to S medium [5% D-glucose, 0.5% $(NH_4)_2SO_4$, 0.1% KH_2PO_4, 0.01% NaCl, 0.1% yeast extract, 0.05% $MgSO_4{\cdot}7H_2O$, 0.01% $CaCl_2{\cdot}2H_2O$], which is known as a lipid-induction medium (Takayama, 2021; Yamazaki et al., 2019). Unlike in N medium, at 24 h, the cells contained one or a few small lipid droplets. After 48 h, lipid droplets became gradually larger (Fig. 1B). Since lipid droplets contain mainly TAG in *L. starkeyi* cells (Kamineni and Shaw, 2020), we measured the amount of TAG induced upon culture in these media (Fig. 1C). Consistent with the lipid droplet observations, the onset of TAG accumulation was earlier in N medium than in S medium. Specifically, it took 48 h for TAG accumulation to start increasing in S medium (1.0±0.3 mg/10^8 cells; mean±s.d.), whereas it took only 24 h or less in N medium (1.6±0.6 mg/10^8 cells) to reach the equivalent to that in S medium after a 72-h cultivation (1.5±0.5 mg/10^8 cells). The cellular TAG level reached 2.4±0.7 mg/10^8 cells at 48 h in N medium, whereas it took 98 h for cells in S medium to produce a similar amount of TAG (2.2±0.6 mg/10^8 cells). These results indicate that TAG accumulation in *L. starkeyi* cells cultured in N medium is faster than that in S medium.

Lipid accumulation by N medium is repressed by addition of ammonium

The preference of N medium for lipid production suggests that the depletion of nitrogen triggers and/or accelerates lipid production. To clarify whether lipid accumulation is an effect of nitrogen depletion, cells were cultured in N medium containing different nitrogen sources, and the amounts of TAG were measured. When cells were cultured in N medium containing 0.5% ammonium sulfate, which corresponds to S medium, lipid accumulation was significantly inhibited [Fig. 2, $(NH_4)_2SO_4$]. This inhibitory effect was also observed with ammonium chloride (Fig. 2, NH_4Cl), suggesting that the presence of an ammonium source prevents lipid accumulation. Next, to examine the effect of other nutrient components on lipid production, the carbon source of N medium was changed from sucrose to glucose. When the medium contained with 0.3% glucose, the lipid amounts changed little compared to what was seen with N medium (Fig. 2, 0.3% Glu). However, when the glucose concentration was increased up to 5%, the lipid amounts were decreased at 72 h, but increased to a level comparable to that in regular N medium containing 0.3% sucrose at 96 h, suggesting that TAG accumulates faster under low glucose conditions (Fig. 2, 5% Glu). Consistent with this, at earlier time points (24 and 48 h), the levels of accumulated TAG were higher in 0.3% glucose N medium than in 5% glucose N medium; at 24 and 48 h, the TAG amounts were 1.0±0.3 mg/10^8 cells and 1.8±0.2 mg/10^8 cells in 0.3% glucose N medium, whereas they were 0.6±0.1 mg/10^8 cells and 1.3±0.3 mg/10^8 cells in 5% glucose N medium (mean±s.d.). Moreover, when trace elements were depleted, lipid accumulation was almost halved (Fig. 2, −Vitamin). Collectively, the suitable components of N medium include 0.3% sucrose or glucose and trace elements, and nitrogen deficiency plays a key role in lipid accumulation.

Changes in cell morphology through lipid accumulation

L. starkeyi cells filled with a single large lipid droplet almost completely lacked bud formation (Fig. 1A,B; Starkey, 1946). To ascertain whether the excess lipid accumulation resulted in a no-bud phenotype, the temporal changes in cell proliferation and morphology were examined. When cells were inoculated in N medium at a concentration of 3×10^6 cells/ml (0 h), cell proliferation slowed down after 24 h and stalled at ∼9×10^6 cells/ml (Fig. 3A, white circles). When the initial cell concentration was reduced to 10^6 cells/ml, cell proliferation stalled at ∼3×10^6 cells/ml (Fig. 3A, white diamonds). Regardless of the initial cell concentration, cell division was arrested at three times the initial concentration. This phenomenon is consistent with the reported cell response when the nitrogen source is limited (Johnston et al., 1977). The proportion of no-bud cells had already increased to 93±6% at 24 h (Fig. 3B, white circles), which coincided with the time when giant lipid droplets were observed (Fig. 1A). The same experiment was performed in S medium. When cells were inoculated at a cell concentration of ∼10^6 cells/ml (0 h), cell proliferation slowed down after 48 h and arrested at a stationary concentration of ∼6×10^7 cells/ml (Fig. 3A, black circles). At 48 h, when lipid droplet expansion was observed (Fig. 1B), the percentage of no-bud cells was increased to 77±3% and remained at the same level after that (Fig. 3B, black circles). Regardless of the media, the dominance of no-bud cells was concurrent with the cell division arrest, and then lipid droplets were enlarged.

In budding yeast, the cell cycle can be estimated by the cell morphology, and no-bud cells indicate G1 phase (Calvert et al., 2008). To verify whether the no-bud cells with large lipid

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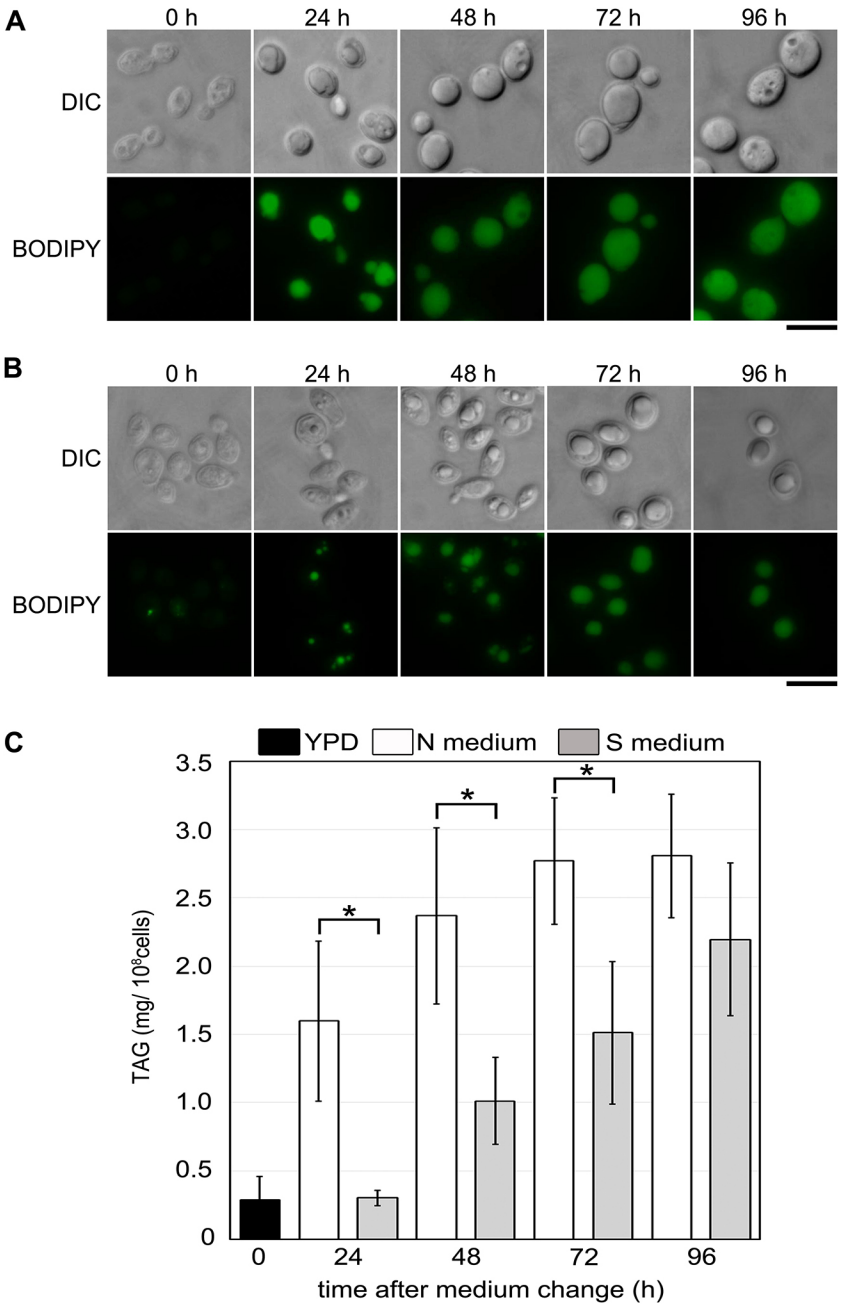

Fig. 1. Comparison of lipid accumulation in different media. *L. starkeyi* cells were inoculated in N medium (A) or S medium (B) and collected at the indicated times. Lipid droplets were stained with BODIPY. Scale bar: 10 µm. (C) Cells were grown in YPD medium and inoculated into N or S medium. The amount of TAG was measured as the value obtained using the TG E-test. White bars, N medium; gray bars, S medium; black bar, YPD medium (0 h). The error bars were calculated from six (YPD) or eight (S and N med.) independent cell cultures (mean±s.d.). *P<0.01 (compared with S medium; unpaired two-tailed Student's t-test).

droplets were also in G1 phase of the cell cycle, fluorescence-activated cell sorting (FACS) analysis was employed. *L. starkeyi* cells precultured in YPD medium were transferred into N medium. Aliquots were taken every 24 h after the medium change, and the DNA content of cells was measured (Fig. 3C). The DNA contents of asynchronous cells (0 h) showed 1C and 2C DNA amounts. Cells containing 1C DNA are in G1 phase, whereas those with 2C DNA are in G2/M phase. FACS analysis revealed that cells with 1C DNA became dominant 24 h after the transfer to N medium (Fig. 3C). However, in S medium, the 2C population remained at 24 h, and then converged to a 1C DNA content at 48 h (Fig. 3D). Thus, G1 phase cells accumulated concomitant with the no-bud phenotype, and G1 phase synchronization occurred earlier in N medium than in S medium. These results indicate that efficient lipid accumulation in N medium might be the result of the rapid induction of G1 phase.

Effect of cell cycle arrest on lipid accumulation

Given that the accumulation of cells containing 1C DNA coincides with the timing of the increase of no-bud cells (Fig. 3B,C), this strongly indicates that *L. starkeyi* no-bud cells filled with lipid droplets are aligned with the G1 phase of the cell cycle. From these results, however, it is not clear whether TAG accumulation is promoted by cell division arrest at specifically the G1 phase or any other cell cycle phase. First, to test whether TAG can accumulate in phases other than G1, the cells were arrested at S and G2/M phases using hydroxyurea (HU) and nocodazole, respectively (Madeira et al., 2019). *L. starkeyi* cells were treated with drugs for 3 h in YPD medium, then the synchronized cells were cultured for 18 h in N medium with drugs (Fig. 4A). FACS analysis confirmed that HU- or nocodazole-treated cells were still synchronized in S or G2/M phase after 18 h (Fig. 4B). In HU-arrested cells, only a small amount of TAG accumulated (0.19±0.07 mg/10^8 cells; mean±s.d.), which is

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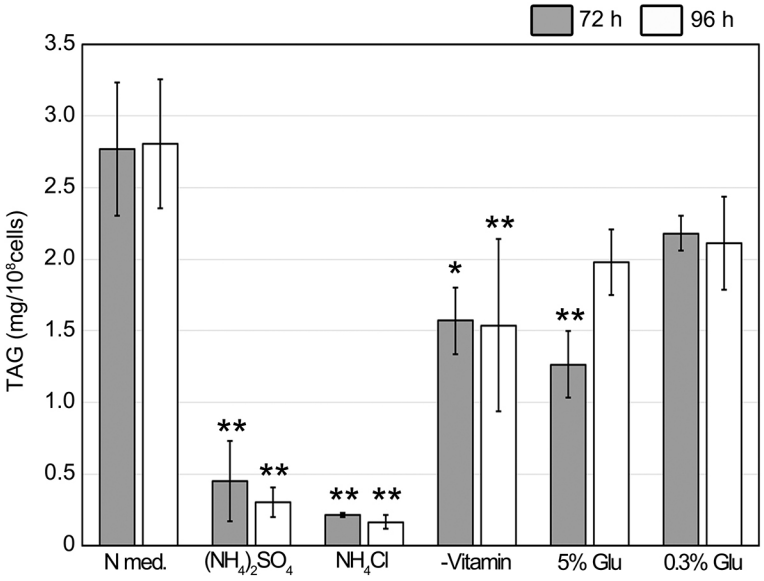

Fig. 2. Lipid accumulation is repressed by nitrogen addition. Cells were grown in YPD medium and inoculated into N medium with or without supplements. The cells cultured at 72 or 96 h were collected, and the amount of TAG was measured. Gray bars, 72 h; white bars, 96 h. The error bars were calculated from independent cell cultures (mean±s.d.) [n=16, 11, 4, 4, 3, 3 (N med. to 0.3% Glu)]. *P<0.01, **P<0.001 (for comparison with N medium at same time points; Dunnett's multiple comparison test).

the same amount as seen in YPD-grown cells (0.27±0.09 mg/10^8 cells) (Fig. 4C, HU versus YPD). In contrast, nocodazole-arrested cells showed accumulation of TAG (0.59±0.08 mg/10^8 cells), the amount of which was the same as that in asynchronous N medium-cultured cells (0.63±0.09 mg/10^8 cells) (Fig. 4C, Noc versus N med). TAG accumulation was detected in cells released from both drug treatments. Furthermore, the viability of cells treated with drugs for 18 h was ∼80% of that at 0 h, suggesting that these drugs had little effect on cell viability. These results indicate that TAG accumulation is not restricted to G1 phase; cells arrested in G2/M phase also accumulate lipids, whereas cells in S phase fail to accumulate them.

Next, we examined the possibility that pre-synchronization at G1 phase before the medium change might increase the amount of TAG accumulation. In *S. cerevisiae*, rapamycin treatment leads to G1 arrest (Zinzalla et al., 2007); however, in *L. starkeyi*, the addition of rapamycin alone failed to cause synchronization at G1 phase. To overcome this problem, the cells were first arrested at G2/M phase with nocodazole, transferred to fresh YPD medium, and cultivated for 30 min, during which time the cells resumed cell cycle progression toward the next G1 phase, and then rapamycin was added to the medium (Fig. 4A). In this preparation, the *L. starkeyi* cells were synchronized at G1 phase (Fig. 4B, Rapa. 0 h). The G1-synchronized cells were cultured in N medium with rapamycin for 18 h, and the amounts of TAG were significantly increased (0.90±0.22 mg/10^8 cells) compared to asynchronous N medium-cultured cells (Fig. 4C, Rapa versus N med.). This result shows that cells arrested at G1 phase effectively induce TAG accumulation. Moreover, the increase of TAG was also observed when the G1 phase cells were cultured without rapamycin (0.89±0.18 mg/10^8 cells) (Fig. 4C, Rapa & Release versus N med), indicating that the amount of TAG can be enhanced by synchronizing cells in G1 phase before transfer to N medium. Taken together, not only G1 phase but also G2/M phase cells accumulate TAG, and the accumulation is enhanced by G1 phase dominance.

DISCUSSION

L. starkeyi is a remarkable microorganism that accumulates lipids, and since it has many industrial advantages, various methods for producing excess lipids have been reported (McNeil and Stuart, 2018; Takaku et al., 2020). In particular, nitrogen limitation can improve lipid production in oleaginous microorganisms (Athenaki et al., 2018), and *L. starkeyi* cells have accelerated lipid droplet formation under nutrient-poor conditions (Duan and Okamoto, 2021; Juanssilfero et al., 2018). Therefore, we prepared a novel nitrogen-depleted medium (N medium). Lipid accumulation was faster in N medium than in S medium (Fig. 1), which is typically used to analyze lipid production (Takayama, 2021; Yamazaki et al., 2019). We also showed that the addition of ammonium compounds into N medium significantly inhibited lipid accumulation (Fig. 2). Focusing on the amount of nitrogen, previous studies have evaluated medium components by the molar ratio of carbon to nitrogen (C:N ratio). Cells cultured at an initial C:N ratio of 72:1 have a 55% lipid yield (Calvey et al., 2016), and detailed analysis has indicated that when the C:N ratio is elevated, the lipid accumulation is increased (Angerbauer et al., 2008). In this study, when 0.5% ammonium sulfate was added into N medium, the C:N ratio was 6:5. Under this condition, the abundance of nitrogen is so high that it might not induce lipid accumulation. In addition, when the amount of ammonium sulfate added into N medium was reduced to 120:1, lipid accumulation was increased (data not shown). Furthermore, it has been reported that a high lipid yield is obtained when cells are cultured in a glucose-only solution (Lin et al., 2011). Therefore, nitrogen deficiency plays an important role in lipid accumulation in *L. starkeyi* cells.

Budding yeast studies have shown a direct link between lipid homeostasis and the cell cycle-regulatory kinase Cdc28 (Chauhan et al., 2015; Kurat et al., 2006). Human disease studies have also demonstrated a positive correlation between lipid droplets and cell division (Cruz et al., 2019; Fernández et al., 2006; Laplante and Sabatini, 2009). Thus, there are reports in other species of the regulation of lipid metabolism by cell cycle progression, although this had not been studied in *L. starkeyi*. We therefore investigated the relationship between the cell cycle and lipid accumulation. When *L. starkeyi* cells were cultured in N medium, cell division was rapidly blocked (Fig. 3A), followed by enlargement of the lipid droplets (Fig. 1A). The cells with giant lipid droplets were in G1 phase (Fig. 3B,C). Furthermore, we investigated specifically where TAG accumulation was promoted by cell cycle arrest, and found that it was induced in G2/M phase but not in S phase cells (Fig. 4C). Importantly, cells arrested in G1 phase had enhanced lipid accumulation (Fig. 4C). These results indicate that *L. starkeyi*

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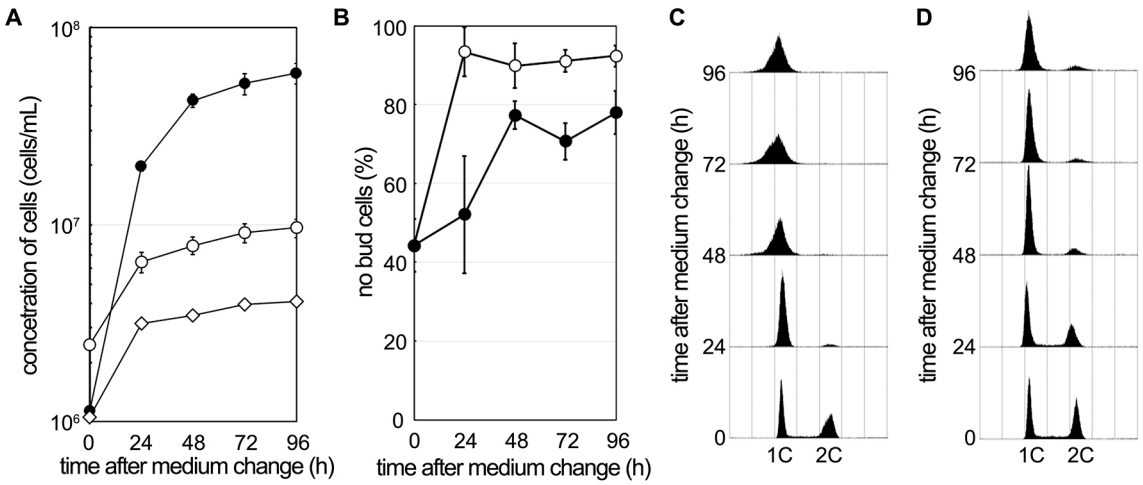

Fig. 3. Lipid-accumulating *L. starkeyi* cells were stalled in G1 phase of the cell cycle. *L. starkeyi* cells were inoculated in N or S medium (0 h) and collected at the indicated times. Cell concentration (A) and cell morphology (B) were measured. White circles, initial concentration is 3×10^6 cells/ml in N medium; white diamonds, initial concentration is 10^6 cells/ml in N medium; black circles, initial concentration is 10^6 cells/ml in S medium. (C,D) At the indicated time after medium change, cells were treated with SYTOX Green and the DNA content was measured. FACS analyses of cells grown in N (C) or S medium (D) are shown.

cells store lipids from G2/M phase and then arrest cell proliferation in the subsequent G1 phase, where lipid accumulation progresses further (Fig. 5).

Why is lipid accumulation not observed in S phase? Lipid accumulation is influenced by the balance between lipogenesis and lipolysis. The reason for the lack of lipid accumulation in S phase could be due to either no lipogenesis or the usage of lipids exceeding lipogenesis. Previous studies with a *S. cerevisiae* Tgl4 (a major TAG lipase)-deficient strain showed that lipolysis contributes to early bud formation at the G1/S boundary (Kurat et al., 2009). In addition, mobilization of TAG provides the fatty acids to maintain the synthesis of phospholipids for new membranes, including those used in bud formation (Rajakumari et al., 2010). When a budding yeast cell enters into S phase, the cell starts to bud, and the bud enlarges continually until it separates from the mother cell. Therefore, lipolysis of lipid droplets is an important intracellular pathway required to supply the components of biological membranes (Onal et al., 2017). Accordingly, lipolysis might be accelerated during S phase. In this study, HU, as an inhibitor of ribonucleotide reductase, was used to synchronize cells in S phase. HU mainly affects DNA replication, but not bud enlargement. Thereby, the HU-treated cells arrested in S phase with large buds (dumbbell shaped) (Kamimura et al., 1998). These results imply that even if lipogenesis occurred in S phase, lipids might not have accumulated in *L. starkeyi* cells because they were used as cell membrane components during bud growth.

In this study, we found that TAG accumulated under G2/M phase arrest induced by nocodazole (Fig. 4); however, the lipid synthesis was not directly investigated. In other organisms, the relationship between lipid metabolism and cell division has been addressed using mutants or inhibitors of fatty acid synthase. In *S. cerevisiae*, TAG levels in the cells arrested by nocodazole are higher than in asynchronous log phase cells, and a fatty acid synthesis-deficient strain (*are1Δ are2Δ dga1Δ lro1Δ*) shows defects in cytokinesis when it is released from nocodazole-induced cell cycle arrest (Yang et al., 2016a). In *S. pombe*, a fatty acid synthetase mutant (*lsd1*) shows a defect in nuclear division, and cell mitosis rapidly decreases in the presence of cerulenin, which is a fatty acid synthesis inhibitor (Saitoh et al., 1996). Moreover, HeLa cells are arrested at G2/M phase by treatment with the fatty acid synthetase inhibitor C75, indicating that endogenous fatty acid synthesis is required to complete cellular division (Scaglia et al., 2014). These studies indicate that fatty acid synthesis is needed for mitosis. In contrast, in the dinoflagellate *Crypthecodinium cohnii*, cerulenin-treated cells exhibit cell cycle arrest at G1 phase but not G2/M phase; the authors speculated that the reason for this is that *C. cohnii* cell growth mainly occurs in G1, and that sufficient lipids might be synthesized during G2/M (Kwok and Wong, 2005). Thus, lipogenesis and lipid accumulation during G2/M phase are probably conserved across organisms as an important task to be performed before proper mitotic exit.

In *C. cohnii* cells, furthermore, the maximal increase in lipid content occurs in G1 phase, indicating that fatty acid synthesis continues during cell cycle arrest at the G1/S boundary (Kwok and Wong, 2005). A significant increase of TAG was also observed during G1 arrest in *L. starkeyi* cells induced by rapamycin treatment (Fig. 4). Rapamycin is a potent inhibitor of target of rapamycin complex 1 (TORC1). In mammalian cells, a single TOR kinase is associated with two distinct complexes, mechanistic/mammalian TORC1 and TORC2 (mTORC1 and mTORC2). In *S. cerevisiae*, two TOR kinases, Tor1p and Tor2p, have been identified. Both Tor1p and Tor2p can form TORC1, which positively regulates energy metabolism and cell growth, but only Tor2p is associated with TORC2, which is implicated in cytoskeleton organization (Loewith and Hall, 2011). Aside from this difference, these proteins are well conserved among organisms, and rapamycin inhibits mTORC1/TORC1 but not mTORC2/TORC2. In human cells, there is evidence that mTORC1 must be active to allow for the induction of lipogenesis genes, and lipid droplet formation is mTORC1 dependent. Therefore, lipid biosynthesis is inhibited by rapamycin (Laplante and Sabatini, 2009; Porstmann et al., 2008). Moreover, since rapamycin treatment leads to stimulated lipolysis, lipid mass is reduced (Chakrabarti et al., 2010). That is, lipid accumulation is repressed by rapamycin-mediated inhibition of mTORC1 in mammalian cells. In contrast, when *S. cerevisiae* cells are treated with rapamycin, TAG accumulation is induced in a TORC1-dependent manner (Madeira et al., 2015). In addition, TAG accumulation is also observed in G1 phase-arrested cells by α-factor or *swi4* mutations (Madeira et al., 2019). Given that cell division cycle mutants or pheromone-based methods for cell cycle

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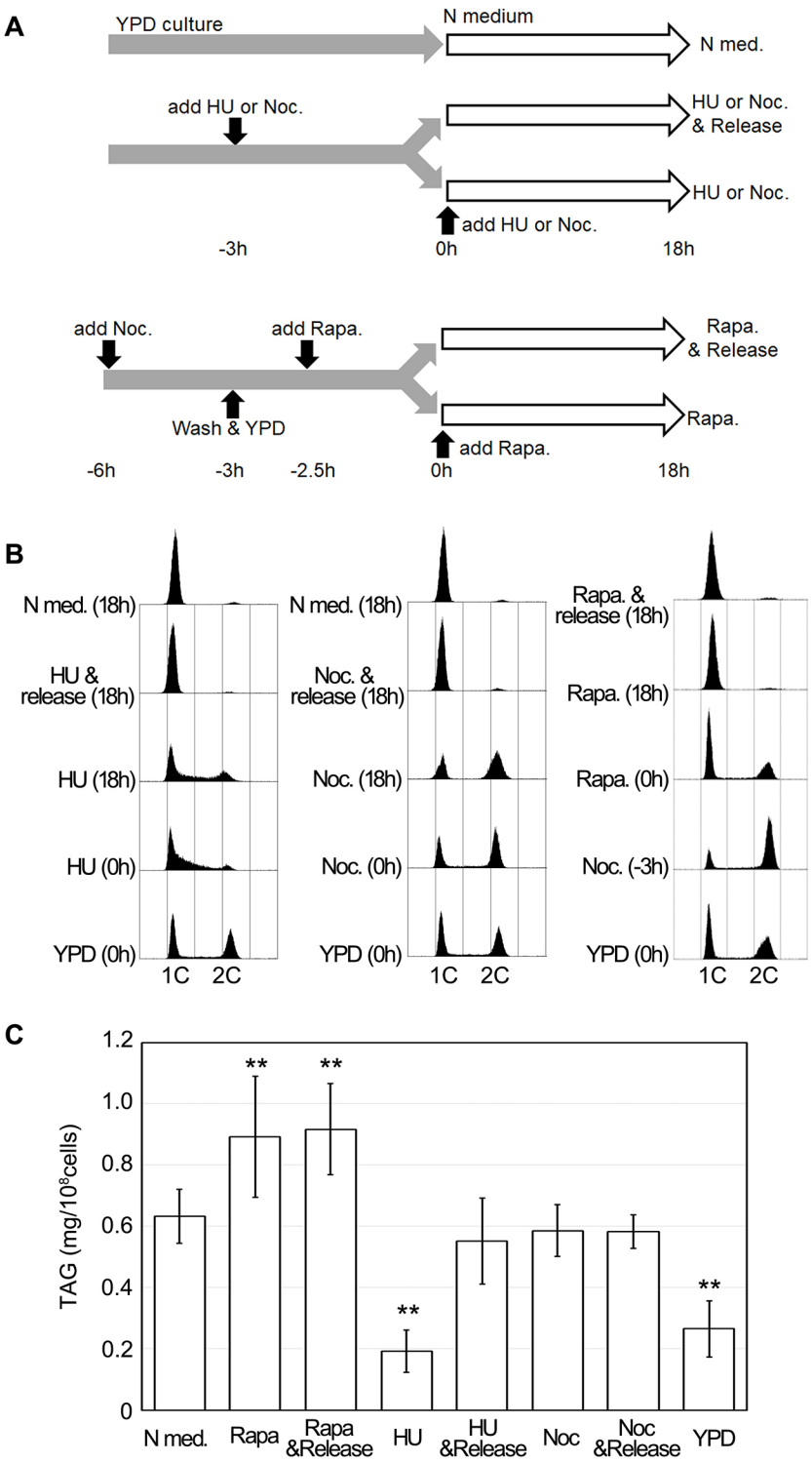

Fig. 4. Effect of cell cycle arrest on lipid accumulation. (A) Schematic diagrams of the synchronized cell culture. The detailed protocols are described in the Materials and Methods section. Cells cultured in N medium for 18 h were harvested, and the amounts of TAG were assayed. (B) The DNA content of cells was analyzed by FACS for the indicated culture protocol. (C) The amounts of TAG per 10^8 cells were measured. The error bars were calculated independent cell cultures (mean±s.d.) [*n*=16, 20, 10, 10, 6, 14, 10, 6 (N med. to YPD)]. ***P*<0.001 (for comparison with N medium; Dunnett's multiple comparison test).

synchronization are not yet established in *L. starkeyi*, we were not able to synchronize cells at G1 phase by methods other than using rapamycin in this study. Therefore, we could not distinguish whether lipid accumulation was due to cell arrest in G1 phase or to inhibition of the TORC1 pathway by rapamycin. TORC1 is known to regulate mitochondrial biogenesis, which provides the citric acid necessary for fatty acid synthesis, and thus it is important to clarify which action is responsible for the lipid accumulation in *L. starkeyi*. Regardless of the reason, this study strongly implies that the cell biology of *L. starkeyi*, including modification of the cell cycle and the TORC1 pathway, provide a new approach for improving industrial lipid production.

In mammals, adipocytes are specialized for the storage and retrieval of energy in the form of TAG in lipid droplets (Onal et al., 2017), and they constitute two types of adipose tissues, white and brown. Abnormalities in these tissues can cause obesity, which is the leading risk factor for type 2 diabetes. White adipose tissues store energy as TAG, whereas brown tissues dissipate energy for heat. Intriguingly, large unilocular lipid droplets found in the adipocytes of white adipose tissues structurally resemble those in

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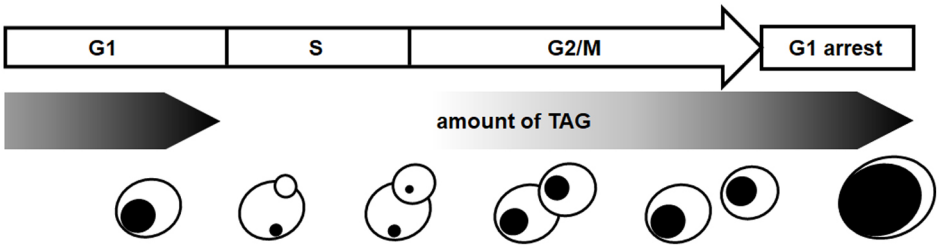

Fig. 5. Lipid accumulation and cell cycle progression. *L. starkeyi* cells store TAG in G2/M to G1 phase. When cells are arrested in G1 phase, TAG accumulation progresses. Lipid droplets in cells are represented as black circles.

L. starkeyi (Fig. 1A; Starkey, 1946), and small multilocular lipid droplets in adipocytes of brown adipose tissues are similar to those in *S. cerevisiae* (Yang et al., 2016a). The pathways of lipid metabolism are highly conserved from yeasts to humans, and thus these yeasts, *L. starkeyi* and *S. cerevisiae*, might be useful as genetically tractable model organisms to study the differentiation of adipocytes and obesity. The number of mitochondria and their metabolic activities are likely to be important factors determining the physiological roles of adipocytes, as brown adipocytes contain a higher number of mitochondria in which energy is characteristically produced from lipids via β-oxidation (Lee et al., 2019; Pu et al., 2021). A recent report showed that in *L. starkeyi*, mitochondria are degraded concomitantly with lipid droplet formation (Duan and Okamoto, 2021). In *S. cerevisiae*, mitochondrial morphology, which might reflect mitochondrial respiratory capacity, changes drastically depending on nutritional conditions (Chen et al., 2019; Duan and Okamoto, 2021). Thus, uncovering the mechanisms that regulate mitochondrial morphology and lipid metabolism in these yeasts might provide new insights into adipocyte differentiation and therapeutic intervention in obesity.

MATERIALS AND METHODS

Yeast strains and media

L. starkeyi NBRC1289 (Takayama, 2021) was obtained from the National BioResource Project and was used in this study. The cells were cultured in YPD medium [2% D-glucose (Wako, Japan), 1% yeast extract (Oriental Yeast Co., Japan), 2% hipolypeptone (Nihon seiyaku, Japan)]. Lipid-inducing media were as follows: S medium [5% D-glucose, 0.5% $(NH_4)_2SO_4$ (Nacalai, Japan), 0.1% KH_2PO_4 (Nacalai), 0.01% NaCl (Kanto chemical, Japan), 0.1% yeast extract, 0.05% $MgSO_4{\cdot}7H_2O$ (Nacalai), 0.01% $CaCl_2{\cdot}2H_2O$ (Nacalai); Yamazaki et al., 2019); N medium {0.3% sucrose (Wako), 0.025% KH_2PO_4, 0.066% $MgSO_4{\cdot}7H_2O$, and trace elements solution [$FeCl_3{\cdot}6H_2O$ (Nacalai) 1.7 mg/l, $MnSO_4{\cdot}6H_2O$ (Nacalai) 0.51 mg/l, $ZnSO_4{\cdot}7H_2O$ (Nacalai) 4.5 mg/l]}. Solid media contained 2% agar (Sigma, A1296-500G). Cells were incubated at 26°C.

Lipid induction culture

Cells were harvested from YPD pre-culture by centrifugation (1750 ***g***, 3 min, 25°C) and washed three times with sterile water. Cells were inoculated at a density of 10^6 cells/ml or $3{\times}10^6$ cells/ml in medium and cultured at 26°C. Cell concentrations were determined by a particle counter, CDA-1000 (Sysmex, Japan), after 200-fold dilution with Cellpack (Sysmex) and brief sonication with a HandySonic (Power 4, Tomy Seiko, Japan).

Cell morphology analysis

Cells were collected by centrifugation (1750 ***g***, 3 min, 25°C) and then suspended in sterile water. The cells were sonicated using a HandySonic (Tomy Seiko, Power 4), and those with or without bud cells were counted using an Olympus CX41. At least 150 cells were counted.

BODIPY staining and microscopy analysis

Cells were stained with BODIPY as previously described (Takayama, 2021). The cells were collected by centrifugation (2300 ***g***, 3 min, 25°C) and then washed with phosphate-buffered saline (PBS; 137 mM NaCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl) twice. The cell pellets were suspended in 25 µl of 0.5 µg/ml BODIPY™ 493/503 (Invitrogen, USA) and incubated for 10 min at room temperature. After washing with PBS, the cells suspended in PBS were observed using a DM5500B digital microscope equipped with a 100× objective lens (N.A. 1.30) and a DFC 310FX digital CCD color camera (Leica Microsystems GmbH, Wetzlar, Germany).

Extraction and quantitation of lipids

The amounts of intracellular lipids were modified using the previously described procedure (Oguro et al., 2017; Yamazaki et al., 2019). $5{\times}10^7$ cells were harvested (1750 ***g***, 3 min, 25°C) and washed with distilled water. The cell pellets were incubated at 70°C for 5 min and stored at −80°C. The cells were suspended in 100 µl of 50% methanol and added to glass beads (0.5 mm diameter; BZ-04, AS ONE, Japan). The cells were broken by a FastPrep 24 (MP-Biomedicals, Japan) four times (30 s of homogenization and 5 min of rest) at 6.0 m/s. Then 100 µl of distilled water was added and the solution was mixed with a microtube mixer (TOMY, Tokyo, Japan) at top speed for 5 min. A hole was made in the tube containing the cell lysate and glass beads with a pin and put on a new 1.5 ml tube. The stack tubes were centrifuged (400 ***g***, 1 min, 25°C) to collect the cell lysate. The cell lysate was centrifuged again (2300 ***g***, 5 min, 25°C), and the supernatant was analyzed using a TG E-test (Wako) for lipids according to the manufacturer's instructions.

Synchronization of the cell cycle

Cells were synchronized in S or G2/M phase by the addition of 12 mM hydroxyurea (HU, Tokyo Chemical Industry, Tokyo, Japan) or 2.5 µg/ml nocodazole (Sigma, MO, USA) in YPD medium. After culturing for 3 h at 26°C, the cells were harvested by centrifugation (1750 ***g***, 3 min, 25°C) and washed three times with 10 ml of sterile distilled water. The washed cells were cultured in N medium with or without 12 mM HU or 2.5 µg/ml nocodazole. In G1 phase arrest, cells were first synchronized at G2/M phase in YPD medium containing 2.5 µg/ml nocodazole. After culturing for 3 h at 26°C, the cells were washed three times with sterile distilled water. The washed cells were cultured in YPD medium for 30 min to promote release from nocodazole arrest, and then rapamycin (Tokyo Chemical Industry) was added to a final concentration 0.2 µg/ml for G1 phase arrest, and cells were cultured for 2.5 h. The cells were washed three times with sterile distilled water and cultured in N medium with or without 0.2 µg/ml rapamycin. To calculate the cell viability, 300 cells were spread on YPD plates and incubated at 26°C. The viability was normalized by defining the number of colonies obtained prior to N medium culture.

Flow cytometric analysis

Flow cytometric analyses were performed as previously described (Takayama and Takahashi, 2007). The cultured cells were harvested and fixed in 70% ethanol at −20°C. The fixed cells were washed with 1 ml of 50 mM sodium citrate (pH 7.0) and subsequently suspended in 1 ml of 50 mM sodium citrate containing 0.1 mg RNase A (Sigma). After overnight incubation at 36°C to digest RNA, 50 µl of 20 mg/ml proteinase K (Merck, Germany) was added to the cell suspension, which was further incubated at 50°C for 2 h. After brief sonication, SYTOX Green (Thermo Fisher Scientific) was added for staining DNA at a final concentration of 1 µM. The stained cells were then analyzed by flow cytometry (FACSCalibur, Becton Dickinson, USA).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Methodology: Y.T.; Formal analysis: Y.T.; Investigation: Y.M., S.S., Y.T.; Data curation: Y.T.; Writing - original draft: S.S., Y.T.; Supervision: Y.T.; Project administration: Y.T.

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