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The *Saccharomyces cerevisiae* protein Stm1p facilitates ribosome preservation during quiescence

Natalya Van Dyke, Ekkawit Chanchorn, Michael W. Van Dyke *

Department of Chemistry and Physics, Western Carolina University, 111 Memorial Drive, Cullowhee, NC 28723, USA

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ABSTRACT

Once cells exhaust nutrients from their environment, they enter an alternative resting state known as quiescence, whereby proliferation ceases and essential nutrients are obtained through internal stores and through the catabolism of existing macromolecules and organelles. One example of this is ribophagy, the degradation of ribosomes through the process of autophagy. However, some ribosomes need to be preserved for an anticipated recovery from nutrient deprivation. We found that the ribosome-associated protein Stm1p greatly increases the quantity of 80S ribosomes present in quiescent yeast cells and that these ribosomes facilitate increased protein synthesis rates once nutrients are restored. These findings suggest that Stm1p can act as a ribosome preservation factor under conditions of nutrient deprivation and restoration.

1. Introduction

Stationary phase occurs when liquid cultures of yeast are grown to saturation and available carbon sources, both fermentable and nonfermentable, are exhausted [1,2]. At this point yeast cells enter a state of quiescence, whereby they are able to survive extended periods of time without added nutrients [3]. While a considerable amount of information is known regarding the mechanisms and proteins involved in entry into, survival within, and exit from quiescence, our understanding of this process is as yet incomplete.

Entry into quiescence initiates autophagy, the engulfment of cytoplasmic components into lipid vesicles for delivery to the vacuole for degradation [4,5]. One of the major cytoplasmic components to be degraded is ribosomes, the macromolecular ribonucleoprotein complexes responsible for protein synthesis [6]. Autophagic degradation of ribosomes or ribophagy provides multiple benefits for the quiescent cell, preventing the synthesis of superfluous proteins and liberating copious quantities of amino acids and nucleotides [7]. However, not all ribosomes need be consumed by ribophagy, especially if the goal of quiescence is to survive until growth can once again be sustained. Thus yeast lacking sufficient ribosomes would be at a grievous disadvantage once environmental changes have occurred and carbon sources are once again abundant.

Stm1p is a *Saccharomyces cerevisiae* protein that is associated as a 1:1 complex with 80S ribosomes and polysomes isolated from logarithmically growing cultures [8,9]. Stm1p modulates the association of the eEF3 translation elongation factor with 80S ribosomes, which is necessary for optimal protein synthesis [10]. While *STM1* is not an essential gene under optimal growth conditions, *STM1* null strains have been observed to exhibit overall reduced rates of vegetative growth, a reduced growth phenotype when galactose is the sole carbon source, inoxitol auxotrophy, and increased sensitivity to inhibitors of TOR signaling [10–14]. Notably, TOR signaling is an important pathway controlling cellular responses to nutrient levels, including entry of cells into quiescence [2,15,16]. In addition, we have found that *stm1Δ* yeast strains were highly sensitive to the TOR-specific inhibitor rapamycin, demonstrated reduced viability following nitrogen deprivation and replenishment, and that these effects coincided with reduced protein synthesis capabilities [8]. Taken together, these findings suggested that Stm1p plays an important role in translation, especially under nutrient stress conditions.

To better understand the mechanisms by which Stm1p affects ribosome function under nutrient stress conditions, we explored the effects quiescence on *stm1Δ* yeast. Compared to isogenic wild type strains, *stm1Δ* yeast possessed reduced ribosome levels during quiescence and exhibited lower protein synthesis capabilities upon exit from quiescence. These data suggest that Stm1p plays a direct role as a ribosome preservation factor both during quiescence and following its recovery, stabilizing ribosomes and preventing their degradation, thereby allowing the efficient resumption of protein synthesis once nutrients are again plentiful. We hypothesize that yeast possessing Stm1p would have a survival and growth advantage under conditions of nutrient deprivation and recovery commonly experienced in the wild.

* Corresponding author. Fax: +1 828 227 7393.
E-mail address: mvandyke@email.wcu.edu (M.W. Van Dyke).

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2. Materials and methods

2.1. Yeast strains and growth conditions

Yeast strains used in this study included K699 (*MAT*a *ade2–1 can1–100 his3–11,–15 leu2–3,–112 ssd1-d trp1–1 ura3*), BY4741 (*MAT*a *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and their respective *STM1* null mutants, *stm1Δ* and 4107 [8]. In addition the proteasome-deficient strain AVL78 (*MAT*a *leu2 trp1 ura3–52 prb prc pep4–3*), transfected with either the high copy control plasmid pBG1805 or its derivative YLR150W containing the *STM1* gene, were used in studies requiring galactose-inducible Stm1p overexpression [10]. Yeast strains K699 and BY4741 and their isogenic *stm1Δ* counterparts were routinely propagated in YPD medium (1% yeast extract, 2% peptone and 1% dextrose) at 30 °C with 250 rpm agitation. Yeast strain AVL78 transformed with either pBG1805 or YLR150W was propagated in synthetic complete (SC) medium lacking uracil, to allow maintenance of these plasmids. The primary carbon source in SC medium was glucose (2%) by default unless substituted with galactose (2%), where indicated, for protein induction experiments.

For quiescent yeast experiments, once cultures reached stationary phase as determined by no increase in absorbance at 600 nm, cultures were maintained for an additional 96 h at 30 °C and 250 rpm agitation before cells were harvested. For quiescence release experiments, cells were diluted to an OD_{600} = 0.2 with YPD medium containing 2% glucose final concentration before harvesting.

2.2. Rapamycin sensitivity assays

Yeast cultures were diluted in YPD or SC (−ura) to an optical density at 600 nm (OD_{600}) of 0.4. Ten microliters of these cultures and 10-fold serial dilutions were spotted onto agar plates containing either YPD or SC (−ura, +gal) medium supplemented with the TOR inhibitor rapamycin (1 or 2 ng/ml), as indicated. Plates were incubated at 30 °C for 3 days and colony growth determined by visual inspection.

2.3. Yeast extract preparation and ribosome fractionation

Yeast cultures were chilled on ice immediately before harvesting by centrifugation (3000×*g* for 5 min at 4 °C). Pelleted yeast cells were washed with ice-cold lysis buffer (50 mM Tris–HCl [pH 7.5], 100 mM NaCl, 7 mM $MgCl_2$, 1% Triton X-100, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin and 2.5 μg/ml antipain) and then resuspended in 0.5 ml of lysis buffer together with a quarter-volume of acid-washed 0.5-mm Glasperlen glass beads (B. Braun Biotech, Allentown, PA). Cells were disrupted by vortexing for 20 s and then cooling on ice for 30 s, for a total of ten cycles. Unbroken cells and large debris were removed by low-speed centrifugation (800*g* for 10 min at 4 °C), thereby yielding yeast whole-cell extract.

For polyribosome analysis, five OD_{260} units (~150 μl) of whole-cell extracts were layered onto a 12-ml linear sucrose gradient (10–40%) containing 50 mM Tris–acetate (pH 7.0), 50 mM NH_4Cl, 3 mM $MgCl_2$ and 1 mM dithiothreitol. These gradients were centrifuged in an SW-40 rotor (Beckman Coulter) at 38,500 rpm for 3.5 h, and 0.4-ml fractions of the gradients were recovered using an Auto Densi-Flow IIC gradient fractionator (Labconco, Kansas City, MO). During fraction collection, the OD_{254} was recorded using a UA-5 absorbance/fluorescence detector (Isco, Lincoln, NE).

2.4. Immunoblotting

Protein samples from whole cell yeast extracts or sucrose gradient fractions were resuspended in Laemmli sample buffer, denatured by heating to 95 °C for 5 min and loaded onto SDS-12% polyacrylamide gels using standard methods. After electrophoretic separation, the proteins were electroblotted onto Hybond-C Extra nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). Membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline containing 0.2% Triton X-100 and then probed with 1:5000 dilutions of anti-Stm1p rabbit polyclonal antibodies [17], anti-L3 mouse monoclonal antibody [18], or anti-β actin mouse monoclonal antibody (Abcam) followed by 1:6000 dilutions of sheep anti-rabbit or anti-mouse IgG horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences). Antibodies were visualized using a SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology, Rockford, IL) following the manufacturer's instructions.

2.5. Protein synthesis efficiency determination

[^{35}S] methionine incorporation was used to determined total protein synthesis efficiency. These assays were performed essentially as previously described [10]. For logarithmic phase experiments, yeast were grown to mid-logarithmic phase in methionine-free SC medium plus 2% glucose and then diluted to an OD_{600} = 0.2 in the same medium. For stationary phase experiments, yeast cells were first maintained in YPD medium for 96 h after achieving stationary phase, then cells were harvested by centrifugation (3000×g for 5 min at 4 °C) and pelleted yeast cells were washed twice in SC medium without methionine and glucose, then diluted to OD_{600} = 0.2 in the same medium. For radiolabeling, cells

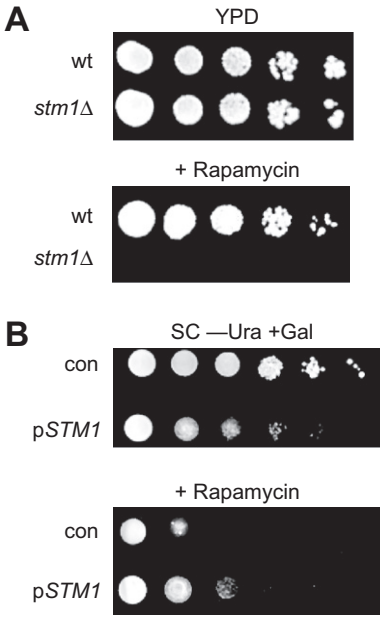

Fig. 1. Stm1p affects yeast sensitivity to the starvation mimic rapamycin. (A) Tenfold serial dilutions of wild type yeast strain K699 (wt) or its isogenic counterpart lacking Stm1p (*stm1Δ*) were plated on rich medium (YPD) agar containing 2 ng/ml rapamycin, as indicated. Colony growth after three days of incubation at 30 °C is shown. (B) Tenfold serial dilutions of proteasome-deficient yeast strain AVL78 transformed with either a galactose-inducible Stm1p expression plasmid (p*STM1*) or a control plasmid (con) were plated on synthetic complete medium agar lacking uracil and containing galactose (SC –Ura +Gal) with or without 1 ng/ml rapamycin as indicated. Colony growth after three days of incubation at 30 °C is shown.

were diluted to OD_{600} = 0.05, then 0.01 OD_{600} equivalents were removed and labeled for 7 min at 30 °C with 2 μCi [^{35}S] methionine in 130 μM total methionine final concentration. Afterwards, 200 μl aliquots were taken and added directly to 700 ml ice-cold 10% trichloroacetic acid (TCA). These were subsequently heated for 4 min at 95 °C, filtered through TCA-soaked glass fiber filters, washed twice with ice-cold 10% TCA and once with acetone, and air-dried. The TCA-precipitable radioactivity was determined by liquid scintillation counting and normalized against initial time point values to account for differential uptake of methionine label in logarithmic and recovering stationary phase yeast.

3. Results and discussion

3.1. Stm1p affects yeast sensitivity to the starvation mimic rapamycin

Rapamycin is a macrolide antibiotic that binds the peptidyl-prolyl cis–trans isomerase Fpr1p (FKBP) and inhibits the signaling activity of TOR complex 1 in yeast [19]. TOR complexes are responsible for cellular responses to nutrients, especially growth and cell cycle progression [16,20]. Inhibition of TOR complexes causes cellular changes, including repression of ribosomal RNA and protein gene expression, suppression of general protein synthesis, accumulation of storage carbohydrates, and initiation of autophagy characteristic of cells in stationary phase. Thus rapamycin acts as a starvation mimic, initiating a quiescence-like state in treated cells.

Previously we had found that *stm1Δ* yeast strains exhibited rapamycin hypersensitivity [8]. This phenotype was further validated with the wild type K699 strain using a serial dilution colony growth assay. An isogenic *stm1Δ* K699 mutant exhibited near complete loss of growth when plated onto YPD agar containing 2-ng/ml rapamycin (Fig. 1A). In addition, the proteasome-deficient yeast strain AVL78 exhibited increased resistance to rapamycin when Stm1p was overexpressed (Fig. 1B). It should be noted that Stm1p homeostasis is tightly regulated by ubiquitination and proteasomal degradation, thus necessitating the use of a proteasome-deficient strain to allow Stm1p overexpression [10]. Taken together, these data indicate that cellular Stm1p levels greatly affect cellular growth and proliferation under conditions that mimic starvation.

3.2. Stm1p affects ribosome maintenance during quiescence

It is well established that yeast entering quiescence lose a substantial percentage of their ribosomes, either through ribophagy or by alternative mechanisms [6,21]. However, while protein synthesis may drop ~300-fold upon entry into quiescence [22], not all ribosomes are lost. Some are required to permit cap-independent translation of specific mRNAs during quiescence (e.g., Sno1 and Snz1) while others will be required upon quiescence exit to

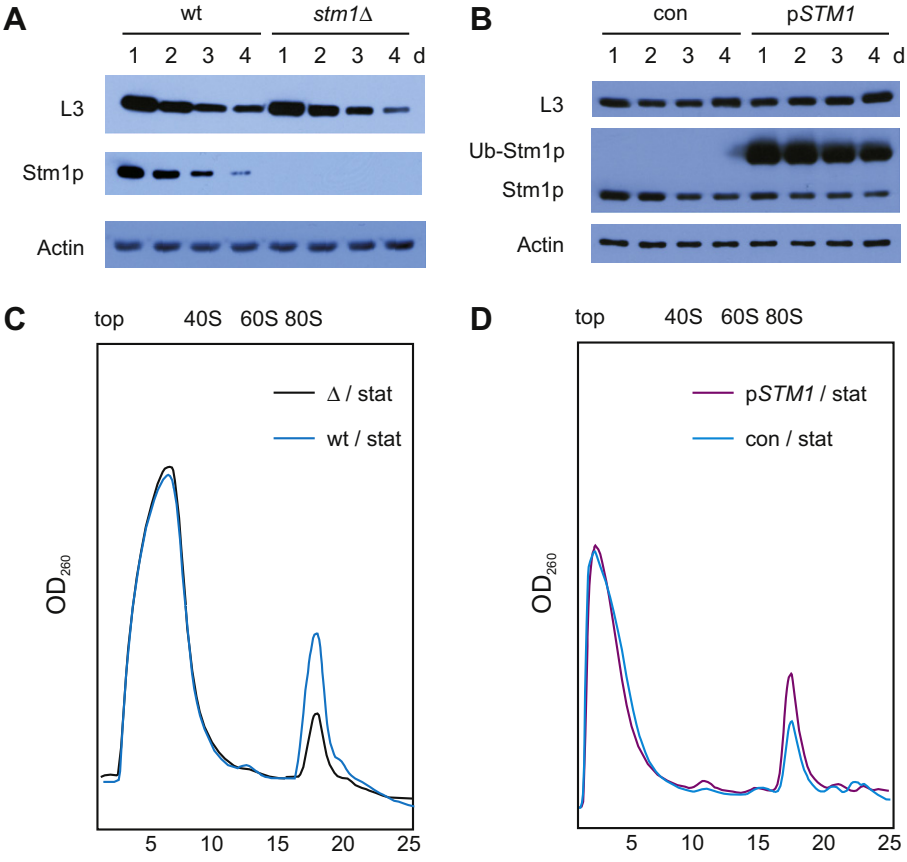

Fig. 2. Stm1p affects cellular ribosome levels during quiescence. (A) Isogenic yeast strains K699 (wt) or *stm1Δ* were cultured under stationary phase conditions for the durations indicated before harvesting and whole-cell extract preparation. Proteins from whole-cell extracts were separated by SDS–PAGE and analyzed by Western blotting using antibodies against ribosomal protein L3, Stm1p or actin, as indicated. In this experiment, actin served as a loading control. (B) As in (A) except the proteasome-deficient yeast strain AVL78 transformed with a high copy number control plasmid (con) or one expressing Stm1p (p*STM1*) was used instead. The slower mobility protein recognized by the anti-Stm1p antibodies has previously been identified as ubiquitinated Stm1p (Ub-Stm1p). (C) Shown are the UV absorbance traces from the sucrose gradient fractionation of wild type K699 yeast extract (wt, black line) and its isogenic counterpart lacking Stm1p (△, blue line) prepared with yeast cells cultured for two days in stationary phase. Locations of the 40S, 60S and 80S ribosomal species are indicated above; gradient faction numbers are indicated below. (D) As in (C) except extracts were made from yeast strain AVL78 transformed with either a control plasmid (con) or one expressing Stm1p (p*STM1*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

facilitate renewed growth upon nutrient replenishment [22]. As yet, the decision process by which certain ribosomes are preserved and the proteins involved is not known.

Given our prior observations on the importance of *STM1* in maintaining viability following nutrient deprivation [8], we investigated the effects of Stm1p absence and overexpression on ribosome species and ribosomal protein levels during stationary phase culturing. We found that ribosomal protein L3 levels decreased with duration of stationary culture (Fig. 2A). A similar decrease in Stm1p levels was observed, suggesting coordinate degradation of these proteins. Ribosomal protein L3 levels were also found to decrease to an approximately twofold greater extent in *stm1Δ* yeast cells than in wild type cells. These data suggest that Stm1p has a protective effect on ribosomal proteins in quiescent yeast cells. Similar effects were observed when we analyzed ribosome profiles from four-day quiescent yeast. Yeast containing Stm1p demonstrated over twofold higher levels of 80S ribosomes than their *stm1Δ* counterparts (Fig. 2C). These data suggest that Stm1p facilitates preservation of whole 80S ribosomes in quiescent cells.

Complementary experiments were performed with the yeast strain AVL78 overexpressing Stm1p. Notably, this strain is deficient in proteasome activity, which we have found necessary for maintaining elevated levels of Stm1p *in vivo* [10]. In this proteasome-deficient strain, ribosomal protein L3 levels are relatively unchanged during stationary phase culturing, suggesting a role for proteasome activity in their depletion (Fig. 2B). Degradation of Stm1p did occur in these cells, although at substantially reduced rates compared to proteasome-competent yeast. Overexpression of Stm1p had no apparent effect on L3 levels under these conditions. Notably, most overexpressed Stm1p was present as a high mobility species previously identified as ubiquitinated Stm1p. This

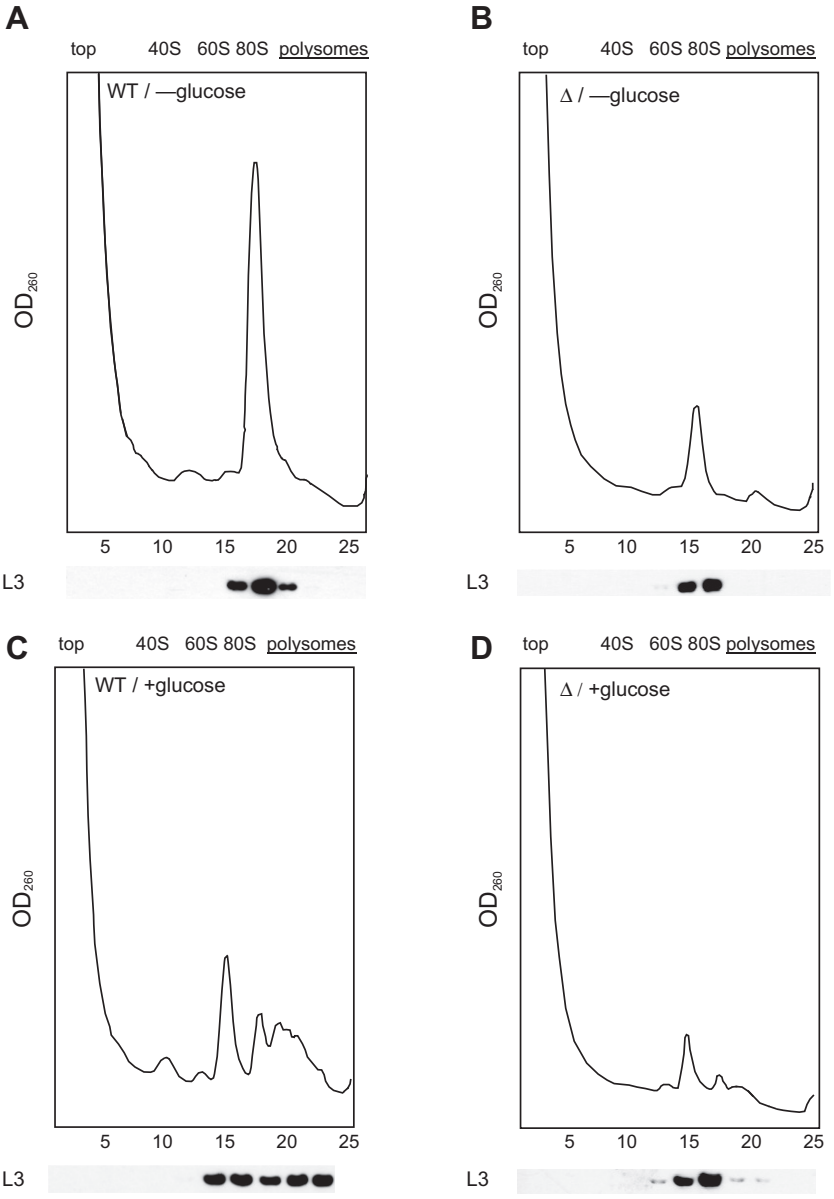

Fig. 3. Polysomes are less abundant in *stm1Δ* yeast following release from quiescence. Wild type K699 (WT) and *stm1Δ* (Δ) yeast strains were maintained in stationary phase for four days, after which they were treated for 30 min with or without 2% glucose to initiate exit from quiescence. Cell were harvested, whole-cell extracts prepared and resolved by ultracentrifugation through a 10–50% sucrose gradient. Shown are the UV absorbance traces from the sucrose gradient fractionation of yeast extracts from (A) stationary phase wild type K699 cells, (B) stationary phase *stm1Δ* cells, (C) previously stationary phase wild type K699 cells following glucose treatment, and (D) previously stationary phase *stm1Δ* cells following glucose treatment. Locations of the 40S, 60S and 80S ribosomal species and polysomes are indicated above; gradient faction numbers are indicated below. Also shown below are Western blots of ribosomal protein L3 present in each fraction.

protein has been found associated with 80S ribosomes, although its functional capabilities are not fully understood. However, ribosome profiles from two-day quiescent yeast indicated that yeast overexpressing Stm1p had increased quantities of 80S ribosome compared to yeast with wild type Stm1p levels, even in this proteasome-deficient strain (Fig. 2D). Taken together, these data support the contention that Stm1p preserves certain ribosomal species, including whole 80S ribosomes, in quiescent cells.

3.3. Stm1p affects polysome formation and protein synthesis following exit from quiescence

While Stm1p apparently preserves 80S ribosomes during quiescence, the question remains whether these ribosomes can be readily activated following exit from quiescence and whether this translates into increased rates of protein synthesis. Such could be quite advantageous under competitive circumstances, when the possibility of outgrowing other organisms would confer an advantage for a particular species' survival.

To address the role of Stm1p in preserving active ribosomes during quiescence, we investigated ribosome species distributions following nutrient reintroduction. Wild type K699 or its isogenic *stm1Δ* counterpart were maintained in stationary phase culture for four days before pelleting and resuspending in SC medium with or without 2% glucose as indicated. After 30 min in culture, yeast were harvested, whole cell extracts prepared, and their ribosome species resolved by 10–40% sucrose gradient ultracentrifugation and fractionation. The location of ribosomal protein L3 in these fractions was verified by Western blotting. We found that whole cell extracts from wild type yeast contained substantially higher levels of 80S ribosomes compared with extracts from isogenic *stm1Δ* strains, confirming prior observations (Fig. 3A and B). Note that polysomes (species containing multiple 80S ribosomes) were not apparent in either population, indicating that transcriptionally active ribosome assembly onto mRNAs does not occur in the absence of added nutrients. However, 30 min after glucose addition, evidence for substantial polysome formation is observed in extracts from wild type yeast and not in extracts from *stm1Δ* yeast (Fig. 3C and D). Taken together, these data strongly suggest that Stm1p preserves translationally competent ribosomes during quiescence and allows their efficient assembly onto mRNA following quiescence exit.

To determine whether efficient polysome assembly post quiescence exit equates to increased protein synthesis, [^{35}S] methionine incorporation assays were performed. Wild type K699 or isogenic *stm1Δ* yeast strains we maintained in stationary culture for four days, then cultured with or without glucose to initiate exit from quiescence and protein synthesis. We found that after an approximately 30 min lag following nutrient reintroduction, wild type yeast exhibited protein synthesis rates comparable to those observed in mid-logarithmic growth phase yeast (Fig. 4). Stm1p-deficient yeast demonstrated a considerably longer lag in protein synthesis and overall reduced rates throughout the time frame investigated (120 min). These data confirm that yeast possessing Stm1p can begin synthesizing proteins at normal (logarithmic growth) rates faster following exit from quiescence compared to Stm1p-deficient yeast. Such would be consistent with the preservation of larger quantities of ribosomes in Stm1p-containing cells, thereby allowing their rapid activation, association with mRNAs, and facile protein synthesis.

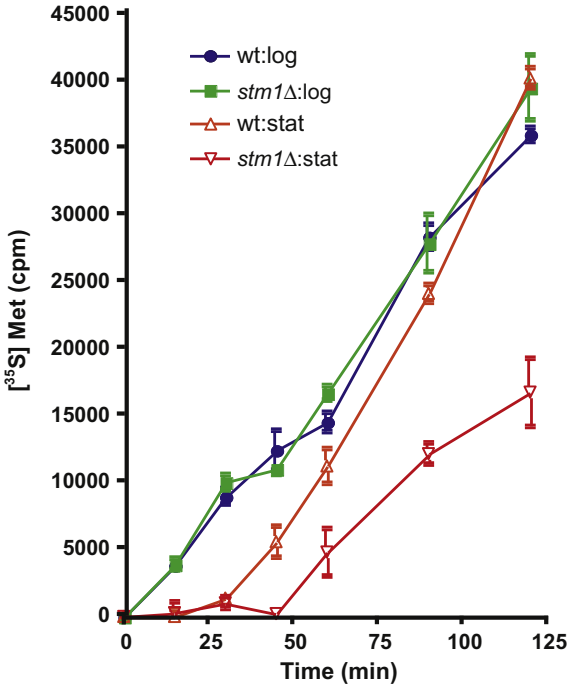

Fig. 4. Protein synthesis is reduced in *stm1Δ* yeast following release from quiescence. Wild type K699 (WT) and *stm1Δ* (Δ) yeast strains were either propagated in SC medium to early logarithmic phase (log) or maintained in stationary phase for four days (stat), then treated with 2% glucose. Afterwards aliquots were removed at the times indicated and processed for measuring protein synthesis by [^{35}S] methionine incorporation. TCA-precipitable radioactivity (cpm) was determined by scintillation counting. Data shown are the average of three independent experiments with margins of error indicated.

4. Discussion

Stm1p is a ubiquitous yeast protein quantitatively associated with 80S ribosomes and polysomes isolated from yeast whole cell extracts [8,9]. However, Stm1p is not an essential protein and yeast lacking Stm1p do not exhibit an obvious phenotype under optimal growth conditions [10,11]. Thus the true function for Stm1p may lie with cellular responses to certain environmental stresses. Previously we had found that Stm1p was essential for maximal viability under nitrogen deprivation conditions and modulates elongation factor Yef3 association with 80S ribosomes [8,10]. In the present report, we found that Stm1p facilitated ribosome preservation in quiescent yeast cells. These ribosomes were translationally competent, rapidly associating with mRNA and driving protein synthesis following nutrient reintroduction and exit from quiescence. These characteristics give Stm1p-containing yeast a definite growth advantage transitioning from stationary to logarithmic growth phases, circumstances that might be commonly experienced in wild type environments.

Stm1p is the first eukaryotic ribosome preservation factor to be described. Examples exist of prokaryotic-origin proteins that stabilize ribosomes under stress conditions (e.g., *E. coli* cold shock protein pY and plastid-specific ribosomal protein PSRP1) [23–25]. Interestingly, both of these proteins bind to the inter subunit region in 70S ribosomes, thereby stabilizing whole ribosome structure. Stm1p has also recently been identified to bind between subunits in 80S ribosomes and possibly accomplishes the same stabilization [9]. However, Stm1p shares little structural similarity with pY or PSRP1 and the degradation of ribosomes in prokaryotes or plastids follows entirely different pathways than present in eukaryotes. Ultimately the mechanisms by which ribosomes are degraded under stress conditions and the mechanisms by which proteins like Stm1p modulate these processes will be important biological questions to be answered.

Acknowledgment

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