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Energizing eukaryotic cell-free protein synthesis with glucose metabolism

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ABSTRACT

Eukaryotic cell-free protein synthesis (CFPS) is limited by the dependence on costly high-energy phosphate compounds and exogenous enzymes to power protein synthesis (*e.g.*, creatine phosphate and creatine kinase, CrP/CrK). Here, we report the ability to use glucose as a secondary energy substrate to regenerate ATP in a *Saccharomyces cerevisiae* crude extract CFPS platform. We observed synthesis of 3.64 ± 0.35 μg mL^{-1} active luciferase in batch reactions with 16 mM glucose and 25 mM phosphate, resulting in a 16% increase in relative protein yield (μg protein/$ reagents) compared to the CrP/CrK system. Our demonstration provides the foundation for development of cost-effective eukaryotic CFPS platforms.

1. Introduction

Cell-free protein synthesis (CFPS) is an emerging field that allows for the production of proteins without intact cells [1,2]. Crude cell lysates, or extracts, are employed instead. Supplying chemical energy (in the form of ATP) for the aminoacylation of tRNAs and peptide bond formation has been a grand challenge for CFPS development [1]. Historically, high-energy phosphate bond donors; such as phosphoenolpyruvate (PEP), creatine phosphate (CrP) (Fig. 1A), and acetyl phosphate have been used [1,3–7]. In these cases, ATP regeneration requires the addition of pyruvate kinase, creatine kinase, or acetate kinase, respectively, or the endogenous presence of these enzymes in the cell extract. Unfortunately, rapid production of phosphate from these high-energy compounds has been shown to be inhibitory to CFPS (*e.g.*, *Escherichia coli* [8] and yeast [9]). Furthermore, batch reactions using these secondary energy substrates typically provide only a brief burst of ATP. In addition, phosphorylated energy compounds are costly, which limits industrial applications [10–12]. To address these limitations, new cost-effective secondary energy regeneration systems are sought.

Within the last decade, the *E. coli* CFPS platform has been able to activate natural metabolism within the lysate to fuel highly active CFPS from non-phosphorylated energy substrates by replacing PEP with glucose [11–13]. Mainly enabled by advances from Swartz and colleagues, glucose drives CFPS with a much lower cost and generates more ATP per secondary energy substrate molecule [11–13]. For example, glucose has a 2:1 molar ratio of secondary energy metabolite to ATP, compared to 1:1 ratio for both CrP and PEP (Fig. 1A,B) [14]. As an extension of the pioneering works above, many groups have turned to use of slowly metabolized glucose polymers to fuel *E. coli* based CFPS, including starch [15], maltodextrin [16,17], and maltose [18].

While *E. coli* based CFPS systems have been developed from non-phosphorylated energy substrates, making possible many new applications in industrial biotechnology and rapid prototyping [19–26], most eukaryotic CFPS platforms have been limited to the

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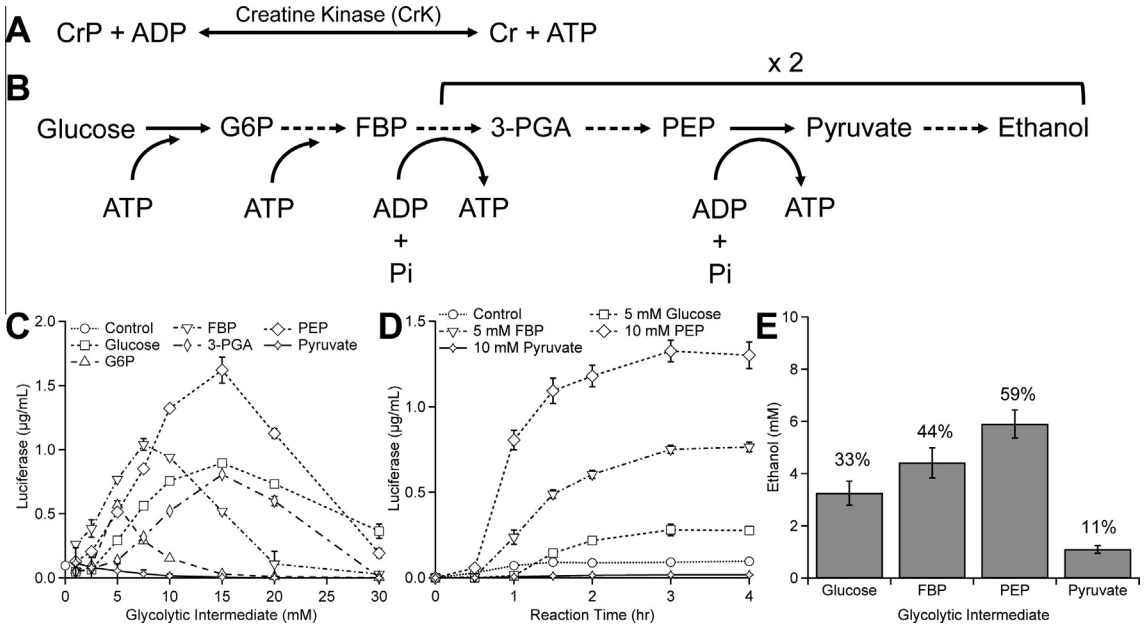

Fig. 1. Glycolysis is active in yeast crude extract CFPS. (A) Schematic of creatine phosphate (CrP)/creatine kinase (CrK) energy regeneration system. (B) Proposed glycolytic energy regeneration system in yeast crude extracts. (C) To assess the possibility of using glycolytic intermediates to fuel CFPS, six glycolytic intermediates (FBP, PEP, glucose, 3-PGA, pyruvate, and G6P) were added as the sole secondary energy substrate to different yeast CFPS reactions in concentrations ranging from 0 mM to 30 mM and compared to a control containing no secondary energy substrate (circle). Of the non-phosphorylated secondary energy substrates assessed, glucose is the highest yielding for yeast CFPS. (D) Active luciferase is reported for time course reactions containing equivalent of 30 mM carbon for select glycolytic intermediates (*e.g.*, 5 mM glucose or 10 mM PEP) and (E) HPLC analysis of ethanol production after 4-h incubation for reactions performed in panel D. The numbers above each column denote the percentage of theoretical conversion of each secondary energy substrate to ethanol. Values shown are means with error bars representing the standard deviation of at least three independent experiments.

use of high-energy phosphate secondary energy substrates. This includes, for example, a yeast-based CFPS system we developed that leverages creatine phosphate and creatine phosphokinase (CrP/CrK) to power protein synthesis [4,9,27,28]. Here, we sought to assess the possibility to activate glycolysis in crude yeast cell extracts to regenerate cofactors and energy to provide the support system necessary to fuel highly active protein synthesis. The ability to use glucose to fuel CFPS is not only important for CFPS applications, but also can expand the impact of cell-free synthetic biology by joining a rapidly growing number of reports highlighting the ability to co-activate multiple biochemical systems in an integrated cell-free platform [10,11,13,16,18,29–33]. We demonstrate that it is indeed possible to power yeast CFPS reactions with glucose, as well as other glycolytic intermediates and non-phosphorylated energy sources, and have reached synthesis yields of 1.05 ± 0.12 μg mL^{-1} active luciferase with 16 mM glucose. After demonstrating synthesis of luciferase from glucose as the sole secondary energy substrate, we optimized our glucose energy system with the addition of cyclic AMP (cAMP) and exogenous phosphate, reaching batch yields of 3.64 ± 0.35 μg mL^{-1} active luciferase. To the best of our knowledge, our work is the first example of powering a eukaryotic CFPS reaction from the native glycolytic pathway. This opens the way to development of cost-effective eukaryotic CFPS platforms from multiple host organisms for a variety of applications.

2. Materials and methods

Yeast extract preparation, CFPS reactions, and luciferase quantification were performed as previously described [4,9,27], with the exception that the energy regeneration system (CrP/CrK) was replaced with glycolytic intermediates. The concentration of magnesium glutamate added to CFPS reactions was optimized for each extract, as CFPS yields are known to be sensitive to magnesium [4] (*e.g.*, Supplemental Fig. 1A). We tested glucose, glucose-6-phosphate (G6P), 3-phosphoglyceric acid (3-PGA), phosphoenolpyruvate (PEP), fructose-1,6-bisphosphate (FBP), and pyruvate in concentrations ranging from 0 to 30 mM. We also tested CFPS reactions containing 0–25 mM glucose in combination with the CrP/CrK energy regeneration system. When denoted, 0.15 mM cAMP and phosphate (in the form of potassium phosphate, pH 7.4) were included in the reaction mixture. Reaction conditions can be found in Supplemental Table 1. HPLC analysis of ethanol was performed as previously described [27]. Nucleotide analysis was performed as previously described [9] except the gradient for buffer B was adjusted to: 0 min, 0%; 10 min, 30%; 50 min, 80%; 55 min, 100%; 60 min, end.

3. Results

We sought to fuel yeast CFPS by activating glycolysis and central metabolism with non-phosphorylated energy substrates. We expect this metabolism to be active given the fact that Eduard Büchner discovered in 1897 that yeast extract could convert sugar to ethanol and carbon dioxide [34]. Initially, we screened for the ability of six different glycolytic intermediates to fuel combined transcription and translation in 15 μL batch CFPS reactions for 4 h at 21 °C (Fig. 1C). The six intermediates included fructose 1,6-bisphosphate (FBP), phosphoenolpyruvate (PEP), glucose, 3-phosphglyceric acid (3-PGA), pyruvate, and glucose 6-phosphate (G6P) at concentrations ranging from 0 to 30 mM. The CFPS reaction was programmed to synthesize luciferase as a model reporter protein and combined transcription and translation was enabled by the use of the Ω cap-independent translation initiation leader sequence [28]. Strikingly, our results demonstrated that it is indeed possible to activate yeast CFPS reactions from glycolytic intermediates upstream of pyruvate, reaching 1.04 ± 0.45 and 1.62 ± 0.10 μg mL^{-1} when powering the reaction with FBP and PEP, respectively. Of the six glycolytic intermediates, only pyruvate was unable to function as a secondary energy source

(Fig. 1C). The inability of pyruvate to power CFPS was expected due to the lack of ATP regenerating power of pyruvate alone in fermentation metabolic processes.

In order to more carefully understand the system dynamics, we subsequently performed time course CFPS reactions with the three highest-yielding intermediates (FBP, glucose, and PEP). This revealed that the choice of glycolytic intermediate impacted the rate of protein synthesis but not the reaction duration; in all cases protein synthesis had terminated after 4 h (Fig. 1D). Negative control reactions performed with pyruvate or no secondary energy substrate produced little to no luciferase (Fig. 1D). The carbon from the glycolytic intermediates is expected to produce ethanol through fermentation, as shown in previous works [34,35]. Thus, we measured ethanol production to confirm glycolysis was active for each carbon source. As expected, we found that ethanol is synthesized when glucose, FBP, and PEP are used to power protein synthesis (Fig. 1E). Ethanol is also produced in the presence of pyruvate, but no protein is synthesized due to limited ATP availability as described above (Fig. 1E).

With the goal of increasing protein synthesis yields, we next tested a dual system, in which glucose is used in combination with CrP/CrK. Previously, such a system was demonstrated by Kim et al. to enhance yields in an *E. coli* CFPS platform [36]. Unexpectedly, we found that the addition of glucose to the CrP/CrK system severely inhibits CFPS, with 10 mM glucose addition resulting in an 89% reduction in protein synthesis (Fig. 2A). We reasoned that this could result from a decrease in pH, as seen previously in *E. coli* CFPS platforms powered by glucose, or a toxicity effect from ethanol accumulation [10]. However, we observed no change in pH during the course of the reaction (Fig. 2B), and showed that ethanol is not toxic in our reactions at concentrations of up to 25 mM (Fig. 2C), which far exceeded the expected ethanol produced (Fig. 1E). Historically, non-productive energy consumption has been identified as one of the primary reasons for early termination of CFPS. Thus, we used quantitative HPLC analysis to track the ATP pool over time. Nucleotide analysis revealed that the decrease in protein synthesis yields when glucose is added to the reaction is due to rapid ATP consumption. For example, in the presence of 25 mM glucose, ATP is fully consumed within the first 15 min of reaction (Fig. 2D), constraining the ability to produce protein.

Given the inability to activate a dual energy regeneration system, we returned to the glucose-only system, and determined through an initial optimization that 16 mM glucose is the optimal substrate concentration (Fig. 3A). We subsequently carried out a series of additional optimization experiments to try to increase CFPS. We explored the effects of reaction temperature, magnesium glutamate concentration, potassium glutamate concentration, spermidine concentration and additives such as cyclic AMP (cAMP) (Supplemental Table 2). Despite a rigorous search, we only observed that addition of cAMP increased yields, suggesting that our original conditions for yeast CFPS captured a maximum. The addition of 0.15 mM cAMP increased our yields 1.5-fold, bringing our yields to approximately 1 $\mu g\,mL^{-1}$ (Supplemental Fig. 1B). The kinetics of protein synthesis follows an interesting trajectory when using glucose and cAMP. Specifically, protein synthesis is delayed when using glucose as the energy source (Fig. 3B), which we attribute to ATP availability. ATP is rapidly consumed in the first 30 min of the reaction, but more than 50% is regenerated after 90 min (Fig. 3C).

With the ability to fuel CFPS by glycolysis at hand, we next investigated the use of slowly metabolized carbon polymers to slow the initial consumption of ATP. We demonstrated that soluble starch can fuel CFPS, though at much lower yields than the glucose system, reaching only ~0.3 $\mu g\,mL^{-1}$ with 1.4% (w/v) starch

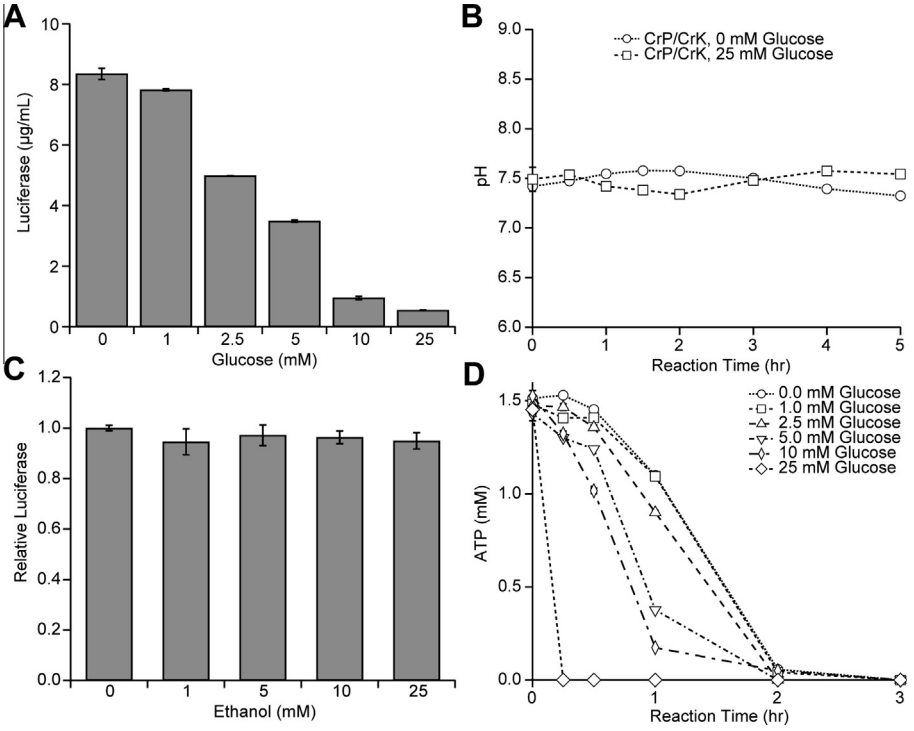

Fig. 2. Yeast CFPS CrP/CrK plus glucose dual system for energy regeneration does not improve CFPS yields. (A) 0 to 25 mM glucose was added to CFPS reactions containing 25 mM creatine phosphate (CrP) and 0.27 mg/mL creatine kinase (CrK). Increasing the starting glucose concentration decreases luciferase yields. (B) The pH of CFPS reactions containing 25 mM CrP, 0.27 mg/mL CrK, and either 0 mM or 25 mM glucose was measured at regular intervals. Reaction pH remains approximately constant over 5 h. (C) To assess possible ethanol inhibition, various concentrations of ethanol, ranging from 0 mM to 25 mM, were added to CFPS reactions. Active luciferase yields are reported relative to the 0 mM ethanol condition, showing that inhibition was not observed. (D) The concentration of ATP was measured at intervals during CFPS reactions including 25 mM CrP, 0.27 mg/mL CrK, and 0–25 mM glucose. ATP is rapidly depleted as the starting glucose concentration is increased. Values shown in A–C are means with error bars representing the standard deviation of at least three independent experiments. Data from panel D traces are individual measurements.

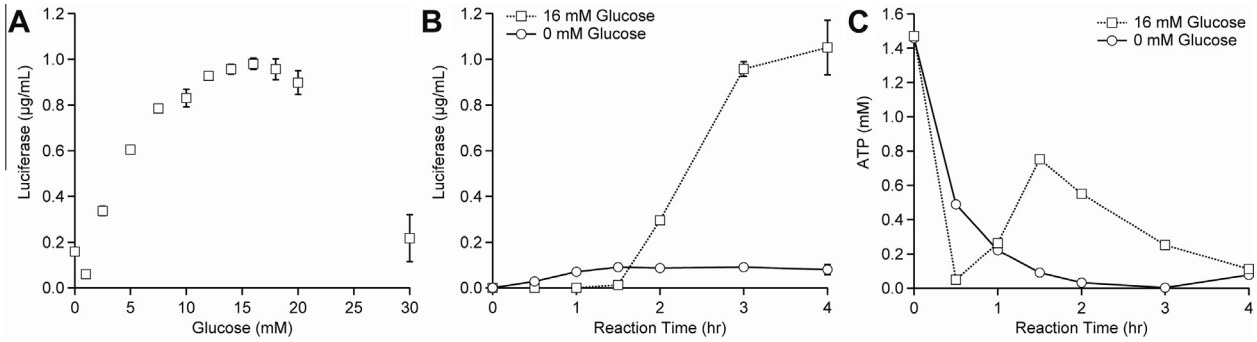

Fig. 3. Optimizing yeast CFPS reaction conditions with glucose as a secondary energy substrate. (A) The optimal starting concentration of glucose was determined via addition of 0–30 mM of glucose to CFPS reactions containing 0.15 mM cAMP. The optimum was observed at 16 mM glucose. (B) Luciferase and (C) ATP concentrations were measured at regular intervals over time in CFPS reactions containing 16 mM glucose or 0 mM glucose. Values shown are means with error bars representing the standard deviation of at least three independent experiments.

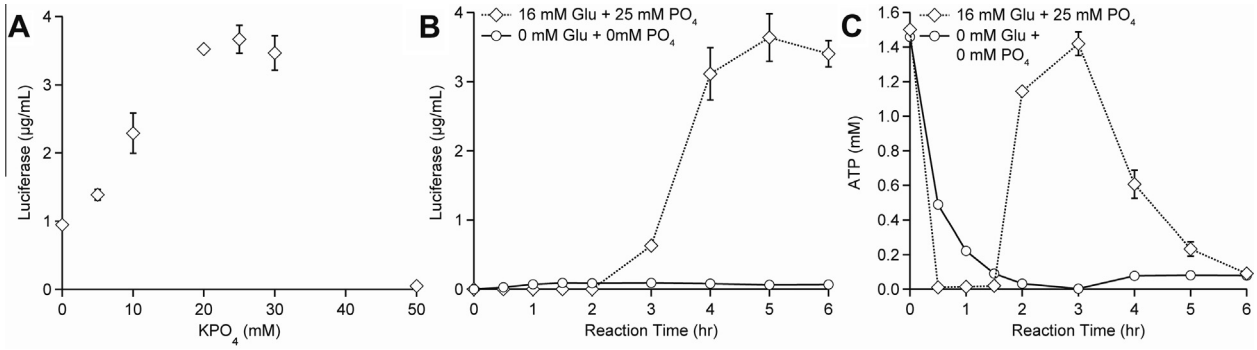

Fig. 4. CFPS reactions with glucose are phosphate-limited: increasing phosphate concentration increases protein yields and prolongs the CFPS reaction. (A) The optimal amount of exogenous phosphate was determined via addition of 0–50 mM of phosphate to CFPS reactions containing 16 mM glucose. The optimum was observed at 25 mM phosphate. (B) Luciferase and (C) ATP concentration were measured at regular intervals in CFPS reactions containing 16 mM glucose and 25 mM phosphate or 0 mM glucose and 0 mM phosphate. Values shown are means with error bars representing the standard deviation of at least three independent experiments.

(Supplemental Fig. 2A and B). Using starch did not reduce initial consumption of ATP, with only 0.2 mM left after 30 min of the reaction (Supplemental Fig. 2C). Our data suggest that ATP regeneration limits the use of starch when compared to glucose alone. Specifically, the regeneration of ATP when using starch is lower than with 16 mM glucose, leading to a lower protein yield. Supplying α-glucosidase and amyloglucosidase enzymes did not improve protein synthesis yields, suggesting the activity of our crude lysates is sufficient to metabolize starch (Supplemental Fig. 2D).

Although we demonstrated proof of principle with starch as an energy substrate, yields remained higher with the glucose energy regeneration system. Therefore, we returned to the glucose system to search for parameters that could increase the level of luciferase synthesized. Previously, Calhoun and Swartz showed that the use of non-phosphorylated energy substrates can result in phosphate limitation during energy regeneration. They observed that the addition of 10 mM inorganic phosphate provided a 3-fold increase in CFPS yields compared to their glucose-driven *E. coli* CFPS system alone [10]. Building off of this advance, we evaluated the addition of 0–50 mM inorganic phosphate in the form of potassium phosphate to our glucose-driven yeast CFPS system (Fig. 4A). With the addition of 25 mM inorganic phosphate, CFPS yields increased almost 3.5-fold, reaching 3.64 ± 0.35 μg mL^{-1} (Fig. 4A). Fig. 4B shows luciferase accumulation over time.

As reported for the glucose and starch systems, protein production appears to be linked to ATP availability, which can be described by Atkinson's adenylate energy charge (E.C.) calculation [37] (Supplemental Fig. 3A). *In vivo* studies have shown energy is limiting in systems with an E.C. less than 0.8 [38]. In reactions containing glucose and phosphate, we observed that ATP is rapidly consumed within the first 30 min of the reaction, but now almost 100% is regenerated after 3 h (Fig. 4C), enabling protein synthesis to extend to 5 h (Fig. 4B). The observed ATP regeneration coincides exactly with initiation of protein synthesis and the point at which E.C. rises above 0.8, between 2 and 3 h of the reaction (Supplemental Fig. 3B). Based on the adenylate energy charge calculations, we propose that this trend in ATP concentration is observed due to the activation of glucose metabolism. At the start of the reaction, ATP is consumed in the pay-in phase of glycolysis while glucose is metabolized. After all available glucose has been consumed, ATP is regenerated by glucose metabolism and accumulates until sufficient ATP is available for protein synthesis.

As compared to the glucose only system, ATP regeneration is improved in the glucose/phosphate system, resulting in prolonged availability of a high concentration of ATP, which manifests in higher protein synthesis yields. This is the longest reported batch yeast CFPS reaction to date, to the best of our knowledge. In follow-up experiments, we confirmed that the optimal concentrations of cAMP remained the same in the glucose/phosphate energy system as in the glucose system (Supplemental Fig. 4).

4. Conclusion

In summary, we have developed a new energy regeneration system for yeast CFPS that uses glucose and phosphate. This novel approach removes the need for an expensive phosphorylated secondary energy source and avoids inhibitory phosphate

accumulation. To our knowledge, this is the first time that a eukaryotic-based CFPS system has been powered by natural energy metabolism of a non-phosphorylated energy substrate. Although our yields do not exceed those previously reported with yeast extract and the CrP/CrK system [27], we have increased the relative protein yield (μg protein/$ reagents) by 16% with our novel glucose/phosphate system (Supplemental Fig. 5). Further optimization of this platform through host strain engineering, as has been done in *E. coli*-based systems [10,39], holds promise to result in a cost-effective eukaryotic CFPS platform for high throughput protein expression, synthetic biology, and proteomic and structural genomic applications. We anticipate that yeast CFPS will become a major player alongside other CFPS technologies in years to come.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.05.045.

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