

**METHODS TO INCREASE PROTEIN PRODUCTION YIELD OF A YEAST AND
HUMAN CELL-FREE LYSATE**

CROSS-REFERENCE TO RELATED APPLICATIONS

- 5 [0001] This application claims the benefit of priority of Singapore application No. 10202400339T filed on 6 February 2024, the contents of it being hereby incorporated by reference in its entirety for all purposes.

FIELD OF THE INVENTION

- 10 [0002] The invention relates to a method of increasing protein expression. Specifically, the invention relates to a method of increasing protein expression in a cell-free protein synthesis (CFPS) system supplemented with one or more compounds which increase protein expression.

BACKGROUND OF THE INVENTION

- 15 [0003] Cell-free protein synthesis (CFPS) is a technology that enables production of proteins of interest without the use of living cells. In a simple biochemical reaction, a CFPS reaction generates the desired proteins in just hours compared to traditional *in vivo* protein expression approaches that take several days or longer. Eukaryotic CFPS systems in general are advantageous for synthesis of proteins with higher complexity and post-translational
20 modifications. Moreover, yeast CFPS was shown to be suitable for synthesis of virus and virus-like particles.

- [0004] However, cost and productivity continue to be significant barriers to widespread adoption of the CFPS technology. Hence, there is a growing interest in identifying means to increase the protein yield of CFPS systems, particularly those derived from eukaryotic cells,
25 which are capable of post-translational modifications often required for therapeutic proteins. Therefore, there is a need to provide a method of increasing protein expression in a CFPS system that overcomes, or at least ameliorates, one or more of the disadvantages described above.

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SUMMARY

- [0005] In one aspect, provided herein is a method of increasing expression of a protein in a cell-free protein synthesis (CFPS) system, wherein the CFPS system comprises a cell lysate, a reaction mixture, and a nucleic acid template that encodes the protein, the method comprising:
- 5 i) supplementing the CFPS system with one or more compounds which increase expression of the protein; and ii) incubating the CFPS system and the one or more compounds which increase expression of the protein under conditions that allow expression of the protein, wherein the expression of the protein is increased compared to a CFPS system expressing the same protein that is not supplemented with the one or more compounds.
- 10 [0006] In another aspect, provided herein is a protein obtained by the method as described herein.
- [0007] In another aspect, provided herein is a cell-free protein synthesis (CFPS) system supplemented with a combination of cerdulatinib and nafarelin.

DEFINITIONS

- 15 [0008] As used herein, the term “heterologous protein” refers to a protein that does not naturally exist in the organism from which the cell lysate of the CFPS system is generated. Heterologous protein is expressed when a gene, encoding for said heterologous protein, is expressed in a lysate derived from a cell type that does not endogenously express said
- 20 heterologous protein.
- [0009] As used herein, the term “endogenous protein” refers to a protein that naturally exists within the organism from which the cell lysate of the CFPS system is generated. Endogenous protein is encoded by the organism’s own genetic material.
- [0010] As used herein, the term “cell-free protein synthesis” or “CFPS” refers to a protein
- 25 expression approach that enables synthesis of a protein *in vitro*, without the use of living cells. The term “cell-free protein synthesis” is used interchangeably with the term “cell-free gene expression”. The CFPS system requires isolating the molecular components for transcription and translation from living cells by preparing a cell lysate comprising cytoplasmic lysate that is free of membranes. The lysate is then supplemented with a reaction mixture containing a
- 30 mixture of components needed to initiate protein synthesis (i.e., amino acids, nucleotides and salts) that is used to transcribe and translate a supplied nucleic acid template (e.g., plasmid

DNA, linear DNA or mRNA). As such, a CFPS system would comprise at least the following components: i) cell lysate; ii) reaction mixture; and iii) nucleic acid template.

[0011] As used herein, the term “cell lysate” refers to a cytoplasmic lysate that has been stripped of the cell membrane and genomic DNA, and contains genetic material, ribosomes, 5 organelles and other cytosolic material.

[0012] As used herein, the term “reaction mixture” in the context of a CFPS system refers to a mixture of components needed to initiate protein synthesis. This mixture of components includes amino acids, nucleotides and salts that are required for transcription and translation of the nucleic acid template.

10 [0013] As used herein, the term “ATP consumption” refers to the process in which adenosine triphosphate (ATP), an energy-carrying molecule that fuels cellular functions for living cells, is hydrolyzed into adenosine diphosphate (ADP) and inorganic phosphate, thus releasing energy to perform cellular functions.

[0014] As used herein, the term “afatinib” is the abbreviated form of the compound “afatinib 15 dimaleate” and the terms are used interchangeably.

[0015] As used herein, the term “nafarelin” is the abbreviated form of the compound “nafarelin acetate” and the terms are used interchangeably.

[0016] As used herein, the term “protein target” refers to a specific protein that is the specific binding partner or substrate of a drug, compound, protein or molecular agent. In the 20 case of a protein target of another protein (such as an enzyme, receptor or signaling molecule), the protein directly binds to the protein target, or regulates the activity or function of the protein target. For example, a protein target of a tyrosine kinase inhibitor typically is a tyrosine kinase or a downstream effector of a tyrosine kinase signaling pathway, that is inhibited by the tyrosine kinase inhibitor. The tyrosine kinase inhibitor thus blocks the activity of the tyrosine kinase or 25 the downstream effector of the tyrosine kinase signaling pathway.

[0017] As used herein, the term “associated” in the context of a protein with a biological pathway refers to a protein that has a functional involvement in the biological processes of the pathway. For example, a protein that is associated with a pathway is one that plays a role in the pathway (such as contributing to the activation or regulation of the pathway), or one that 30 physically interacts with proteins, enzymes or molecular complexes of the pathway. The term “associated” in the context of a process refers to a biological process. For example, a biological

process may include a process that plays a role in protein expression (such as transcription or translation etc.).

[0018] As used herein, the term “about”, is used in the context of, but not limited to, concentrations of components and percentages of compounds, typically refers to +/- 10% of the stated value, to +/- 9% of the stated value, to +/- 8% of the stated value, to +/- 7% of the stated value, to +/- 6% of the stated value, to +/- 5% of the stated value, +/- 4% of the stated value, more typically +/- 3% of the stated value, more typically, +/- 2% of the stated value, even more typically +/- 1% of the stated value, and even more typically +/- 0.5% of the stated value. Throughout this disclosure, certain embodiments may be disclosed in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the disclosed ranges. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

BRIEF DESCRIPTION OF THE DRAWINGS

[0019] The invention will be better understood with reference to the detailed description when considered in conjunction with the non-limiting examples and the accompanying drawings, in which:

[0020] A yeast lysate CFPS phenotypic screening workflow was utilized to identify compounds that increase protein expression yield and **Fig. 1** depicts a schematic of the drug screen workflow. Drugs (100 mM, n=1443) were incubated with yeast extract for 15 minutes prior to addition of nLuc DNA template and other essential components (amino acids, nucleotides, salts) to initiate expression. nLuc produced was measured after 2.5h incubation.

[0021] **Fig. 2** depicts the results from a drug screen with collection of 1443 FDA-approved small molecule agonists. CFPS reactions were screened in 96-well format taking average RLU from two reads to assay nano-luciferase production. CFPS yield was normalised against the average of at least three no-drug control groups to determine fold change. The lines indicate 50% threshold for identification of drug hits that increase (dashed line) or decrease (dotted line)

CFPS activity. (Second half of Plate 16 and entire Plate 17 drugs were dissolved in water, while the rest contained 10% DMSO.

[0022] The yeast lysate CFPS phenotypic screening workflow was utilized to identify compounds that increase heterologous protein expression yield and **Fig. 3** displays repeat experiments of selected drug candidates showing fold increase in nLuc production. $n=3 \pm \text{SD}$. Significance measured using two-tailed Student's t-test.

[0023] **Fig. 4** depicts the results from a counter-screen assay in which the effect of identified positive hits on nLuc activity was assessed by adding compounds into CFPS reactions upon completion of nLuc synthesis. $n=3 \pm \text{SD}$.

10 [0024] **Fig. 5** displays the CFPS inhibitors identified in screen. Selected drug candidates were validated in the second-round screen. Positive hits (Figure 3) and inhibitors with significant fold change reduction in CFPS yields are shown. $n=3 \pm \text{SD}$.

[0025] **Fig. 6** depicts the results from a counter-screen assay in which the effect of identified inhibitors on nLuc activity was assessed by adding compounds into CFPS reactions upon completion of nLuc synthesis. $n=3 \pm \text{SD}$.

[0026] **Fig. 7** depicts the optimization of drug incubation conditions. **Fig. 7A** shows the effect of CFPS temperature and drug pre-incubation on the final nLuc yield. Reaction mix containing nLuc template was added to yeast extract fraction either directly with 100 μM bosutinib/vehicle or after 15 min pre-incubation of the extract. Extract pre-incubation and subsequent CFPS were performed either at 22°C or 30°C. $n=2 \pm \text{SD}$. $*p < 0.05$; $**p < 0.01$; $***p < 0.005$ (two-tailed Student's t-test). **Fig. 7B** displays the nLuc expression in yeast lysate measured over indicated drug concentrations. $n=3 \pm \text{SD}$. In **Fig. 7C and 7D**, Bosutinib, cerdulatinib, nafarelin acetate and dasatinib were added to CFPS reactions at their optimum concentrations individually or in indicated combinations. Drug effect was assessed on synthesis of nLuc (**Fig. 7C**) and p53 (**Fig. 7D**) by means of luminescence and western blot densitometry analysis respectively. All readings were normalised to DMSO-treated controls. $n=3 \pm \text{SD}$.

25 [0027] **Fig. 8** shows the characterization of bosutinib effect on yeast CFPS. In **Fig 8A**, nLuc synthesis in CFPS reactions treated with 0 μM and 100 μM bosutinib was monitored at varying time intervals from 0-5 h. $n=2 \pm \text{SD}$. **Fig. 8B** shows the corresponding ATP levels measured from nLuc-synthesizing CFPS reactions, NTC controls (-/+ indicating addition of bosutinib) and CFPS reactions without creatine phosphate secondary energy regeneration system.

[0028] **Fig. 9** depicts the effect of bosutinib on glucose-driven CFPS reactions. In **Fig. 9A**, CFPS reactions were set using either creatine phosphate or glucose as secondary energy source. Reactions were treated with 100 μ M bosutinib to assess drug effect on nLuc yield. $n=3 \pm \text{SD}$. In **Fig. 9B**, creatine phosphate and glucose-driven CFPS reactions were tested with indicated
5 amounts of nLuc plasmid template (10 - 40 nM). $n=3 \pm \text{SD}$.

[0029] **Fig. 10** shows the characterization of bosutinib effect on yeast CFPS. In **Fig 10A**, the productivity of the yeast extract from cells harvested at $\text{OD}_{600} = 1.2$ was assessed. CFPS reactions were treated with 100 μ M bosutinib and set with either creatine phosphate or glucose as secondary energy source. $n=3 \pm \text{SD}$. **Fig. 10B** shows the nLuc levels in glucose-driven
10 reactions monitored from 0-8 h. $n=2 \pm \text{SD}$. **Fig. 10C** shows the ATP levels in glucose-driven reactions monitored from 0-8 h. $n=2 \pm \text{SD}$.

[0030] **Fig. 11** depicts the drug effects on mammalian cell-based CFPS. Bosutinib and dasatinib monohydrate were pre-incubated with HeLa cell lysate fraction for 15 or 30 mins. Subsequent nLuc production was assessed with respect to DMSO-treated controls. $n \geq 3 \pm \text{SD}$.

15 [0031] **Fig. 12** shows the effect of bosutinib on proteins and pathways in CFPS identified by thermal proteomics. **Fig. 12A** shows the workflow for thermal proteomics on cell free protein system. Yeast extracts were treated with different doses of bosutinib (vehicle, 0.01nM, 0.1nM, 1nM, 100nM, 1 μ M, 10 μ M, 100 μ M and 1mM) and subjected to heat stress at 55°C. Proteins from the different conditions were processed and peptides were labelled using tandem
20 mass tags (TMT). Liquid chromatography coupled with tandem mass-spectrometry (LC-MS/MS) acquisition was performed to profile proteins based on dose response relationship. Data analysis was carried out to identify proteins and pathways affected by bosutinib treatment. **Fig. 12B** depicts the bar plot of the twenty proteins with greater than 1.2-fold increase in decreasing order of magnitude. SSB2 and SSA1 were identified as the top 2 proteins with mean
25 fold change of 1.37 and 1.33 respectively. **Fig. 12C** depicts individual loess curves fitted for drug dose against protein quantity measured by mass spectrometry for SSA1 and SSB2 respectively. **Fig. 12D** depicts protein interaction network for the top 20 hits, highlighting four chaperone proteins (SSB2, SSA1, SKP1, STI1) using STRING database (proteins are coloured in red based on mean fold change with the color intensity reflecting a higher mean fold-change).
30 **Fig. 12E** depicts the top 10 biological processes based on Gene Ontology (GO) enrichment analysis using 231 proteins with mean increase $\geq 10\%$.

[0032] **Fig. 13** shows the protein levels of SSA1 and SSB2 to remain stable over the course of a CFPS reaction. **Fig. 13A** shows proteomics profiling of CFPS over the course of a reaction at different time points (0, 10, 30, 60, 120min). Proteins were then subjected to sample processing, TMT labelling and LC-MS acquisition. **Fig. 13B and 13C** demonstrate the protein abundance of SSA1 and SSB2 to remain stable over the course of a CFPS reaction.

[0033] **Fig. 14** depicts the BY4743-WT and BY4743- Δ SSA1 growth curves. Growth of BY4743-WT and BY4743- Δ SSA1 was monitored for 12 h from starting $OD_{600} = 0.1$. $n=2 \pm SD$.

[0034] **Fig. 15** depicts chemoproteomic-guided strain engineering. **Fig. 15A** shows the activity of extracts from BY4743-WT and BY4743- Δ SSA1 strains (without drug treatment and with bosutinib, nafarelin acetate, cerdulatinib and dasatinib) were compared in nLuc expressing CFPS reactions set using glucose as the secondary energy source. $n=4 \pm SD$. p values determined by two-tailed Student's t -test. **Fig. 15B** presents the drug effects on nLuc synthesis in CFPS reactions using WT and Δ SSA1 extracts, compared by determining fold increase in yield with respect to corresponding drug-free controls. $n=4 \pm SD$. p values determined by two-tailed Student's t -test. **Fig. 15C and 15D** present CFPS reactions in which WT and Δ SSA1 extracts were set for Mdm2 protein synthesis. Protein yields were assessed using densitometry analysis of western blot image. Mdm2 yield in Δ SSA1 extract is normalised to that of WT extract (C top). Representative blot of Mdm2 synthesized by drug treated extracts is presented (C bottom). Fold increase in Mdm2 levels is calculated with respect to corresponding WT and Δ SSA1 controls (D). $n=2 \pm SD$. p values determined by two-tailed Student's t -test.

[0035] **Fig. 16** displays the identification of Bosutinib targets by MS-CETSA. The relative amounts of each indicated protein are plotted as a function of drug concentration. SSA2, SSA3 and SSB1 are stabilized in presence of drug.

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DETAILED DESCRIPTION OF THE PRESENT INVENTION

[0036] In a first aspect the present invention refers to a method of increasing expression of a protein in a cell-free protein synthesis (CFPS) system, wherein the CFPS system comprises a cell lysate, a reaction mixture, and a nucleic acid template that encodes the protein, the method comprising: i) supplementing the CFPS system with one or more compounds which increase expression of the protein; and ii) incubating the CFPS system and the one or more compounds which increase expression of the protein under conditions that allow expression of

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the protein, wherein the expression of the protein is increased compared to a CFPS system expressing the same protein that is not supplemented with the one or more compounds.

[0037] In some examples, the nucleic acid template may be but is not limited to a mRNA, a linear DNA or a circular DNA.

5 [0038] In some examples, the circular DNA may be but is not limited to a plasmid or a DNA minicircle.

[0039] The CFPS system may be supplemented with one or more compounds which increase protein expression. In some examples, the number of compounds supplemented may be but is not limited to one, two, three, four, five, six, seven, eight, nine or ten compounds.

10 [0040] The one or more compounds which increase protein expression may be a kinase inhibitor, a gonadotropin-releasing hormone receptor agonist, an antibiotic, or combinations thereof.

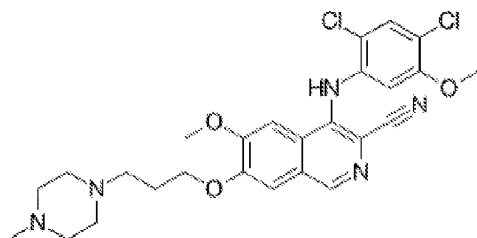
[0041] It will be appreciated by a person skilled in the art that when more than one compound is added or supplemented to the CFPS system, these can come from the same class
15 of compounds or from different classes of compounds. For example, the CFPS system may be supplemented by more than one kinase inhibitor. In another example, the CFPS system may be supplemented by more than one gonadotropin-releasing hormone receptor agonist. In another example, the CFPS system may be supplemented by more than one antibiotic. In another example, the CFPS system may be supplemented by one or more kinase inhibitor and one or
20 more gonadotropin-releasing hormone receptor agonist. In another example, the CFPS system may be supplemented by one or more kinase inhibitor and one or more antibiotic. In another example, the CFPS system may be supplemented by one or more gonadotropin-releasing hormone receptor agonist and one or more antibiotic. In another example, the CFPS system may be supplemented by one or more kinase inhibitor, one or more gonadotropin-releasing
25 hormone receptor agonist, and one or more antibiotic. It would be understood that these are non-exhaustive examples of combinations of compounds that may be used to supplement the CFPS system.

[0042] In some examples, the kinase inhibitor includes but is not limited to an ATP-competitive kinase inhibitor or an allosteric kinase inhibitor. In further examples, the ATP-
30 competitive kinase inhibitor or allosteric kinase inhibitor may be but is not limited to a tyrosine kinase inhibitor or a serine/threonine kinase inhibitor.

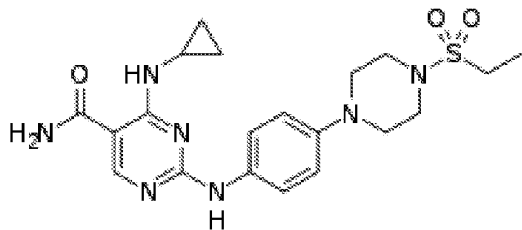
[0043] Examples of a serine/threonine kinase inhibitor include but is not limited to Adenosine Monophosphate-Activated Protein Kinase (AMPK) inhibitor, Phosphoinositide 3-Kinase (PI3K) inhibitor, Mitogen-Activated Protein Kinase (MAPK) inhibitor.

[0044] In some examples, the ATP-competitive kinase inhibitor is a tyrosine kinase inhibitor, and the tyrosine kinase inhibitor may be but is not limited to bosutinib, cerdulatinib, dasatinib, neratinib, afatinib or combinations thereof.

[0045] The chemical structure of bosutinib is:

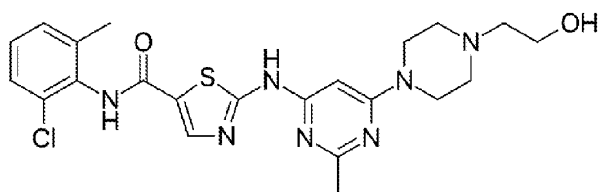


[0046] The chemical structure of cerdulatinib is:

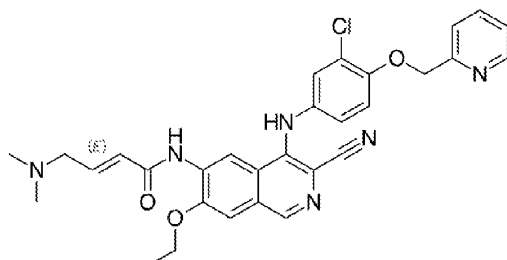


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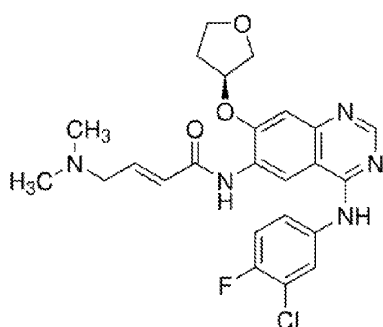
[0047] The chemical structure of dasatinib is:



[0048] The chemical structure of neratinib is:

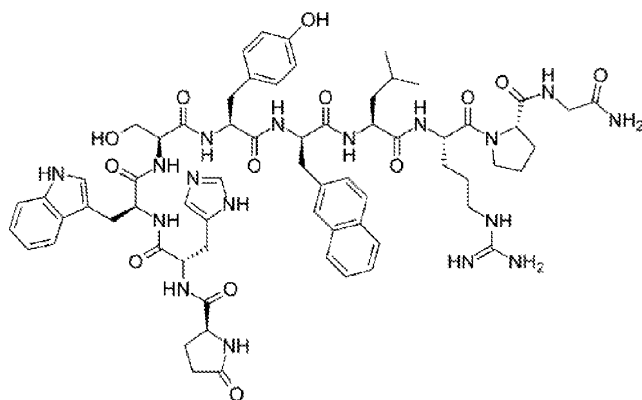


[0049] The chemical structure of afatinib is:



[0050] In some examples, the gonadotropin-releasing hormone receptor agonist may be nafarelin.

[0051] The chemical structure of nafarelin is:



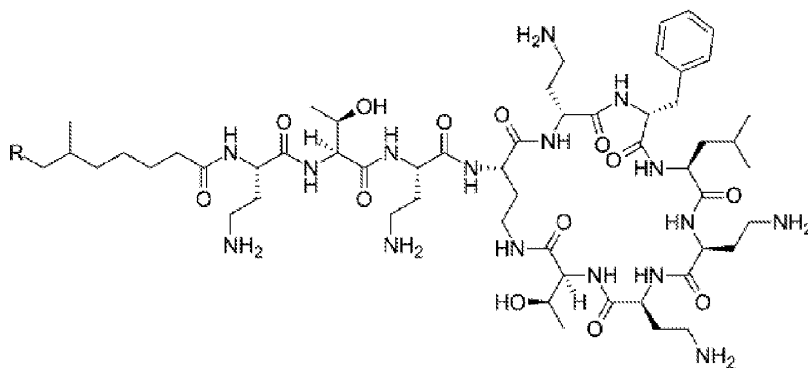
[0052] In some examples, the one or more compounds are kinase inhibitor, gonadotropin-releasing hormone receptor agonist molecule or combinations thereof which increase protein expression in a CFPS system by decreasing ATP consumption in metabolic processes not

required for protein expression in the CFPS system. It would be understood that the metabolic processes refer to processes that utilize ATP but do not result in or significantly contribute to protein expression in a CFPS system. Examples include proteostasis, gluconeogenesis, fatty acid, lipid, and nitrogen metabolism. The one or more compounds may block other metabolic processes that occur in the cell lysate, decreasing ATP consumption in the metabolic processes and channeling more ATP into expression of protein of interest. In another example, the one or more compounds may also block enzymes that inhibit expression of protein of interest.

[0053] In some examples, the antibiotic may target gram negative bacteria or gram positive bacteria.

10 [0054] In one example, the antibiotic targeting gram negative bacteria is polymyxin B.

[0055] The chemical structure of polymyxin B is:



[0056] In one example, the CFPS system is supplemented by bosutinib only. In another example, the CFPS system is supplemented by cerdulatinib only. In another example, the CFPS system is supplemented by dasatinib only. In another example, the CFPS system is supplemented by neratinib only. In another example, the CFPS system is supplemented by afatinib only. In another example, the CFPS system is supplemented by nafarelin only. In another example, the CFPS system is supplemented by polymyxin B only.

[0057] In another example, the one or more compounds used to supplement the CFPS system are bosutinib and cerdulatinib. In another example, the one or more compounds used to supplement the CFPS system are bosutinib and dasatinib. In another example, the one or more compounds used to supplement the CFPS system are bosutinib and neratinib. In another example, the one or more compounds used to supplement the CFPS system are bosutinib and afatinib. In another example, the one or more compounds used to supplement the CFPS system

are bosutinib and nafarelin. In another example, the one or more compounds used to supplement the CFPS system are cerdulatinib and dasatinib. In another example, the one or more compounds used to supplement the CFPS system are cerdulatinib and neratinib. In another example, the one or more compounds used to supplement the CFPS system are cerdulatinib and afatinib. In another example, the one or more compounds used to supplement the CFPS system are cerdulatinib and nafarelin. In another example, the one or more compounds used to supplement the CFPS system are dasatinib and neratinib. In another example, the one or more compounds used to supplement the CFPS system are dasatinib and afatinib. In another example, the one or more compounds used to supplement the CFPS system are dasatinib and nafarelin. In another example, the one or more compounds used to supplement the CFPS system are neratinib and afatinib. In another example, the one or more compounds used to supplement the CFPS system are neratinib and nafarelin. In another example, the one or more compounds used to supplement the CFPS system are afatinib and nafarelin. In another example, the one or more compounds used to supplement the CFPS system are polymyxin B and bosutinib. In another example, the one or more compounds used to supplement the CFPS system are polymyxin B and dasatinib. In another example, the one or more compounds used to supplement the CFPS system are polymyxin B and cerdulatinib. In another example, the one or more compounds used to supplement the CFPS system are polymyxin B and neratinib. In another example, the one or more compounds used to supplement the CFPS system are polymyxin B and afatinib. In another example, the one or more compounds used to supplement the CFPS system are polymyxin B and nafarelin. It would be understood that these are non-exhaustive examples of combinations compounds that may be used to supplement the CFPS system. It would also be understood that other combinations of compounds including but not limited to three, four, five, six, seven, eight, nine, ten compounds are possible.

[0058] In another example, the CFPS system is supplemented by bosutinib, nafarelin, cerdulatinib and dasatinib.

[0059] In another example, the one or more compounds used to supplement the CFPS system are bosutinib and nafarelin or cerdulatinib and nafarelin.

[0060] The concentration of bosutinib may be about 6.25 μM to about 500 μM . For example, the concentration of bosutinib may be about 6.25 μM , about 12.5 μM , about 25 μM , about 50 μM , about 75 μM , about 100 μM , about 125 μM , about 150 μM , about 175 μM , about 200 μM , about 225 μM , about 250 μM , about 275 μM , about 300 μM , about 325 μM , about

350 μM , about 375 μM , about 400 μM , about 425 μM , about 450 μM , about 475 μM , about 500 μM . In a preferred example, the concentration of bosutinib is 100 μM or 150 μM .

[0061] The concentration of cerdulatinib may be about 12.5 μM to about 400 μM . For example, the concentration of cerdulatinib may be about 12.5 μM , about 25 μM , about 50 μM ,
5 about 75 μM , about 100 μM , about 125 μM , about 150 μM , about 175 μM , about 200 μM , about 225 μM , about 250 μM , about 275 μM , about 300 μM , about 325 μM , about 350 μM , about 375 μM , about 400 μM . In a preferred example, the concentration of cerdulatinib is 75 μM .

[0062] The concentration of dasatinib may be about 25 μM to about 800 μM . For example,
10 the concentration of dasatinib may be about 25 μM , about 50 μM , about 100 μM , about 200 μM , about 300 μM , about 400 μM , about 500 μM , about 600 μM , about 800 μM . In a preferred example, the concentration of dasatinib is 600 μM .

[0063] The concentration of neratinib may be about 0.5 μM to about 100 μM . For example, the concentration of neratinib may be about 0.5 μM , about 1 μM , about 2.5 μM , about 5 μM ,
15 about 10 μM , about 20 μM , about 40 μM , about 80 μM , about 100 μM . In a preferred example, the concentration of neratinib is 40 μM .

[0064] The concentration of afatinib may be about 25 μM to about 500 μM . For example, the concentration of afatinib may be about 25 μM , about 50 μM , about 75 μM , about 100 μM , about 125 μM , about 150 μM , about 175 μM , about 200 μM , about 225 μM , about 250 μM ,
20 about 275 μM , about 300 μM , about 325 μM , about 350 μM , about 375 μM , about 400 μM , about 425 μM , about 450 μM , about 475 μM , about 500 μM . In a preferred example, the concentration of afatinib is between 75 μM to 150 μM .

[0065] The concentration of nafarelin may be about 6.25 μM to about 500 μM . For example, the concentration of nafarelin may be about 6.25 μM , about 12.5 μM , about 25 μM , about 50
25 μM , about 75 μM , about 100 μM , about 125 μM , about 150 μM , about 175 μM , about 200 μM , about 225 μM , about 250 μM , about 275 μM , about 300 μM , about 325 μM , about 350 μM , about 375 μM , about 400 μM , about 425 μM , about 450 μM , about 475 μM , about 500 μM . In a preferred example, the concentration of nafarelin is between 100 μM to 200 μM .

[0066] The concentration of polymyxin B may be about 25 μM to about 250 μM . For example, the concentration of polymyxin B may be about 25 μM , about 50 μM , about 75 μM ,
30 about 100 μM , about 125 μM , about 150 μM , about 175 μM , about 200 μM , about 225 μM , about 250 μM . In a preferred example, the concentration of polymyxin B is 150 μM .

- [0067] The CFPS system in the method of the invention may in some examples be supplemented with an energy source.
- [0068] In some examples, the energy source supplemented to the CFPS system may be but is not limited to a carbohydrate, a lipid, a protein, an amino acid, a phosphorylated form of an amino acid, a phosphate salt or combinations thereof.
- [0069] In some examples, the phosphate salt may be sodium hexametaphosphate.
- [0070] In some examples, the carbohydrate may be a monosaccharide, disaccharide, a polysaccharide or combinations thereof.
- [0071] In some examples, the monosaccharide may be but is not limited to glucose, fructose, mannose or galactose, or combinations thereof.
- [0072] In some examples, glucose is metabolized, and the intermediates of glucose metabolism may be supplemented into the CFPS as an energy source, which may include but is not limited to glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-bisphosphate, acetyl-CoA, or combinations thereof.
- [0073] In some examples, the phosphorylated form of an amino acid may be but is not limited to creatine phosphate, guanosine triphosphate, cytidine triphosphate, inosine triphosphate or combinations thereof.
- [0074] In a preferred example, the energy source is glucose or creatine phosphate.
- [0075] In one example, the CFPS system is supplemented with creatine phosphate only.
- The CFPS system supplemented with creatine phosphate may be further provided with creatine kinase, the enzyme that catalyzes the transfer of phosphate group from creatine phosphate to ADP, forming ATP and creatine.
- [0076] The concentration of glucose may be about 10 mM to about 35 mM. For example, the concentration of glucose may be about 10 mM, about 11 mM, about 12 mM, about 13 mM, about 14 mM, about 15 mM, about 16 mM, about 17 mM, about 18 mM, about 19 mM, about 20 mM, about 21 mM, about 22 mM, about 23 mM, about 24 mM, about 25 mM, about 26 mM, about 27 mM, about 28 mM, about 29 mM, about 30 mM, about 31 mM, about 32 mM, about 33 mM, about 34 mM, about 35 mM. In a preferred example, the concentration of glucose is about 25 mM.
- [0077] The CFPS reaction supplemented with glucose may be carried out over a duration of up to 15 hours. For example, the CFPS reaction supplemented with glucose may be carried out for about 1 hour, about 1.5 hours, about 2 hours, about 2.5 hours, about 3 hours, about 3.5

hours, about 4 hours, about 4.5 hours, about 5 hours, about 5.5 hours, about 6 hours, about 6.5 hours, about 7 hours, about 7.5 hours, about 8 hours, about 8.5 hours, about 9 hours, about 9.5 hours, about 10 hours, about 10.5 hours, about 11 hours, about 11.5 hours, about 12 hours, about 12.5 hours, about 13 hours, about 13.5 hours, about 14 hours, about 14.5 hours, about 15 hours. In a preferred example, the CFPS reaction supplemented with glucose is carried out for about 6 hours.

[0078] The concentration of creatine kinase may be about 0.20 mg/mL to about 0.40 mg/mL. For example, the concentration of creatine kinase may be about 0.20 mg/mL, 0.21 mg/mL, about 0.22 mg/mL, about 0.23 mg/mL, about 0.24 mg/mL, about 0.25 mg/mL, about 0.26 mg/mL, about 0.27 mg/mL, about 0.28 mg/mL, about 0.29 mg/mL, about 0.30 mg/mL, about 0.31 mg/mL, about 0.32 mg/mL, about 0.33 mg/mL, about 0.34 mg/mL, about 0.35 mg/mL, about 0.36 mg/mL, about 0.37 mg/mL, about 0.38 mg/mL, about 0.39 mg/mL, about 0.40 mg/mL. In a preferred example, the concentration of creatine kinase is about 0.27 mg/mL.

[0079] The concentration of creatine phosphate may be about 15 mM to about 50 mM. For example, the concentration of glucose may be about 15 mM, about 16 mM, about 16 mM, about 17 mM, about 18 mM, about 19 mM, about 20 mM, about 21 mM, about 22 mM, about 23 mM, about 24 mM, about 25 mM, about 26 mM, about 27 mM, about 28 mM, about 29 mM, about 30 mM, about 31 mM, about 32 mM, about 33 mM, about 34 mM, about 35 mM, about 36 mM, about 37 mM, about 38 mM, about 39 mM, about 40 mM, about 41 mM, about 42 mM, about 43 mM, about 44 mM, about 45 mM, about 46 mM, about 47 mM, about 48 mM, about 49 mM, about 50 mM. In a preferred example, the concentration of creatine phosphate is about 25 mM.

[0080] The CFPS reaction supplemented with creatine phosphate and creatine kinase may be carried out over a duration of up to 72 hours. For example, the CFPS reaction supplemented with creatine phosphate may be carried out for about 1 hour, about 1.5 hours, about 2 hours, about 2.5 hours, about 3 hours, about 3.5 hours, about 4 hours, about 4.5 hours, about 5 hours, about 10 hours, about 24 hours, about 48 hours, about 72 hours. In a preferred example, the CFPS reaction supplemented with creatine phosphate is carried out for about 2.5 hours.

[0081] The cell lysate of the CFPS system may be obtained from a eukaryotic cell or a prokaryotic cell.

[0082] In some examples, the prokaryotic cell may be but is not limited to a bacterial cell or an archaeal cell.

[0083] In some examples, the bacterial cell may be but is not limited to *Escherichia coli*, *Bacillus subtilis*, *Clostridium acetobutylicum* or *Lactobacillus casei*. It will generally be understood that any industrially available bacterial cell may be used in the present invention.

[0084] In some examples, the eukaryotic cell may be but is not limited to a yeast cell, animal cell, plant cell, fungal cell, protozoan cell or algal cells.

[0085] In some examples, the yeast cell may be but is not limited to *Saccharomyces cerevisiae*, *Pichia pastoris*, *Schizosaccharomyces pombe*, *Hanseniaspora uvarum* or *Candida sake*. It will generally be understood that any industrially available yeast cell may be used in the present invention.

[0086] In a preferred example, the yeast cell is *Saccharomyces cerevisiae*.

[0087] In some examples, the *Saccharomyces cerevisiae* may be but is not limited to a S288C, BY4743, W303, BY4741, BY4742, MBS or CEN.PK strain.

[0088] In some examples, the animal cell may be a mammalian cell, such as a human cell or cell line. The human cell may be but is not limited to Henrietta Lacks (HeLa) cell, Human embryonic kidney 293 (HEK293) cell, Rabbit reticulocyte or Human breast epithelial (MCF-10A and MCF-12A) cell. It will generally be understood that any commercially available human cell may be used in the present invention.

[0089] The one or more genes in the eukaryotic cell or prokaryotic cell may be unmodified or modified. Modification of the one or more genes may include a genetic modification in the DNA sequence of the one or more genes and/or post-translation modification of the polypeptide translated from the one or more genes.

[0090] In some examples, genetic modification in the DNA sequence of the one or more genes may comprise deletion, mutation, truncation, translocation, substitution, insertion or combinations thereof. In some examples, post-translation modification of the polypeptide translated by one or more genes may comprise glycosylation, phosphorylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation.

[0091] In some examples, the one or more genes may be modified by deletion which may be but is not limited to a complete deletion of the one or more genes, a deletion of one or more DNA bases or a deletion of one or more exons.

- [0092] The one or more genes may in some examples encode a protein target of the one or more compounds which increase expression of the protein of interest. The protein target of the one or more compounds may be a cytosolic HSP70 chaperone protein. Examples of the cytosolic HSP70 chaperone protein include SSA1, SSB2, SSA3, SSA2 or SSB1. The protein target of the one or more compounds may be a protein target of the SSA1 or SSB2 protein. Examples of a protein target of the SSA1 or SSB2 protein include but are not limited to SKP1 or STI1. The protein target of the one or more compounds may be a proteasomal ubiquitin-independent protein. Examples of the proteasomal ubiquitin-independent protein include but are not limited to PRE1, PRE3-9 and PUP1-3. The protein target of the one or more compounds may be associated with a metabolic pathway. Examples of the metabolic pathway include dicarboxylic acid, glutamine, homoserine, hexose and gluconeogenesis metabolism. The protein target of the one or more compounds may be associated with a pathway that utilize ATP. Examples of components of a pathway that utilize ATP include proteasome complex, PCS60, THR1, PCK1 and PYC2.
- [0093] In one example, the modified gene in the eukaryotic cell is *SSA1* which encodes a SSA1 protein that has ATPase activity. In an example, there is a complete deletion of the *SSA1* gene. In another example the *SSA1* gene is mutated to render the expressed SSA1 protein catalytically inactive.
- [0094] In some examples, the method of the present invention may be used to increase expression of a heterologous protein or an endogenous protein.
- [0095] The heterologous protein or endogenous protein may be a eukaryotic protein or a prokaryotic protein.
- [0096] The eukaryotic protein or prokaryotic protein may in some examples be post-translationally modified.
- [0097] The post-translational modification on the eukaryotic protein or prokaryotic protein may be but is not limited to glycosylation, phosphorylation, ubiquitination, nitrosylation, methylation, acetylation, lipidation, or combinations thereof.
- [0098] In some examples, the protein of interest expressed may be but is not limited to nanoluciferase, p53, Mdm2, an antibody, a membrane protein, a protein incorporating unnatural amino acids or a virus-like particle.
- [0099] In one example, the CFPS system of the method of the invention comprises a cell lysate obtained from a *Saccharomyces cerevisiae* S288C strain, the one or more compounds

comprise cerdulatinib and nafarelin, and supplemented with creatine phosphate and creatine kinase.

[00100] In one example, the CFPS system of the method of the invention comprises a cell lysate obtained from a *Saccharomyces cerevisiae* S288C strain, the one or more compounds
5 comprise bosutinib and nafarelin, and supplemented with creatine phosphate and creatine kinase.

[00101] In one example, the CFPS system of the method of the invention comprises a cell lysate obtained from a *Saccharomyces cerevisiae* S288C strain and the one or more compounds comprise bosutinib, and supplemented with glucose.

10 [00102] In one example, the CFPS system of the method of the invention comprises a cell lysate obtained from a *Saccharomyces cerevisiae* S288C strain, and the one or more compounds comprise bosutinib, and supplemented with creatine phosphate and creatine kinase.

[00103] In one example, the CFPS system of the method of the invention comprises a cell lysate obtained from HeLa cells, the one or compounds comprises bosutinib, and supplemented
15 with creatine phosphate and creatine kinase.

[00104] In one example, the CFPS system of the method of the invention comprises a cell lysate obtained from HeLa cells, the one or compounds comprises bosutinib, and supplemented with glucose.

[00105] In one example, the CFPS system of the method of the invention comprises a cell
20 lysate obtained from HeLa cells, the one or more compounds comprises dasatinib, and supplemented with creatine phosphate and creatine kinase.

[00106] In one example, the CFPS system of the method of the invention comprises a cell lysate obtained from HeLa cells, the one or compounds comprises bosutinib, and supplemented with glucose.

25 [00107] In one example, the CFPS system of the method of the invention comprises a cell lysate obtained from a *Saccharomyces cerevisiae* BY4743 strain lacking *SSA1* gene, the one or more compounds comprises bosutinib, and supplemented with glucose.

[00108] In one example, the CFPS system of the method of the invention comprises a cell lysate obtained from a *Saccharomyces cerevisiae* BY4743 strain lacking *SSA1* gene, the one or
30 more compounds comprises nafarelin, and supplemented with glucose.

[00109] In one example, the CFPS system of the method of the invention comprises a cell lysate obtained from a *Saccharomyces cerevisiae* BY4743 strain lacking *SSA1* gene, the one or more compounds comprises cerdulatinib, and supplemented with glucose.

5 [00110] In one example, the CFPS system of the method of the invention comprises a cell lysate obtained from a *Saccharomyces cerevisiae* BY4743 strain lacking *SSA1* gene, the one or more compounds comprises dasatinib, and supplemented with glucose.

[00111] In one example, the CFPS system of the method of the invention comprises a cell lysate obtained from a *Saccharomyces cerevisiae* BY4743 strain lacking *SSA1* gene, the one or more compounds comprise cerdulatinib and nafarelin, and supplemented with glucose.

10 [00112] In some examples, the method of the invention comprises incubating the CFPS system and one or more supplemented compounds at a temperature of about 15°C to about 35°C. For example, the incubation may take place at a temperature of about 15°C, about 16°C, about 17°C, about 18°C, about 19°C, about 20°C, about 21°C, about 22°C, about 23°C, about 24°C, about 25°C, about 26°C, about 27°C, about 28°C, about 29°C, about 30°C, about 31°C, 15 about 32°C, about 33°C, about 34°C, about 35°C. In a preferred example, the method is about 22°C.

[00113] It will be appreciated by a person skilled in the art that the components of the CFPS system in the method of the invention may be assembled sequentially in any order. For example, the components of the CFPS system may be assembled sequentially in the following 20 order: first, the cell lysate; then, the reaction mix; followed by the nucleic acid template. In another example, the components of the CFPS system may be assembled sequentially in the following order: first the cell lysate; then the nucleic acid template; followed by the reaction mix. The one or more compounds which increase expression the protein may be supplemented after the first, second or third components of the CFPS system are assembled.

25 [00114] In some examples, the one or more compounds which increase protein expression may be assembled and pre-incubated with the cell lysate of the CFPS system, followed by addition of the nucleic acid template and reaction mixture of the CFPS system. It would be understood that the nucleic acid template and reaction mixture of the CFPS system may be added in any order following assembly and pre-incubation of the cell lysate with the one or 30 more compounds. For example, the cell lysate may be assembled and pre-incubated with the one or more compounds which increase protein expression, followed by addition of the nucleic acid template and lastly, followed by addition of the reaction mixture to the CFPS system. In

another example, the cell lysate may be assembled and pre-incubated with the one or more compounds which increase protein expression, followed by addition of the reaction mixture and lastly, followed by addition of the nucleic acid template to the CFPS system.

[00115] In some examples where the one or more compounds are assembled and pre-incubated with the cell lysate in the CFPS system before the addition of the reaction mix, and the nucleic acid template, the one or more compounds may be pre-incubated for up to about 20 minutes. For example, the one or more compounds may be pre-incubated in the CFPS system for about 1 minute, about 2 minutes, about 3 minutes, about 4 minutes, about 5 minutes, about 6 minutes, about 7 minutes, about 8 minutes, about 9 minutes, about 10 minutes, about 11 minutes, about 12 minutes, about 13 minutes, about 14 minutes, about 15 minutes, about 16 minutes, about 17 minutes, about 18 minutes, about 19 minutes, about 20 minutes.

[00116] In a preferred example, the one or compounds used to supplement the CFPS system are not pre-incubated with the cell lysate of the CFPS system prior to addition and assembly of the reaction mix and the nucleic acid template.

[00117] In some examples, the CFPS reaction of the invention may be set up in a suitable vessel including but not limited to a tube, a flask, a fermenter, or a bioreactor. The volume of the vessel may be about 0.5mL, about 0.75mL, about 1.0mL, about 1.5 mL, about 2.0mL, about 5.0mL, about 10.0mL, about 25mL, about 50.0mL, about 75.0mL, about 100.0mL, about 250.0mL, about 500.0 mL, about 1.0 L, about 2.5 L, about 5.0 L, about 10.0 L, about 25.0 L, about 50.0 L, about 100.0L, about 250.0 L, about 500.0L, about 1000.0L .

[00118] In another aspect, provided herein is a protein obtained by the method as described herein.

[00119] The protein yield of the protein obtained by the method as described herein may be increased by about 1.25 fold to about 44 fold compared to protein yields of the protein obtained from a CFPS system without addition of the one or more compounds which increase protein expression. For example, the protein yield may be increased by about 1.25 fold, about 1.5 fold, about 1.75 fold, about 2 fold, about 3 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, about 9 fold, about 10 fold, about 11 fold, about 12 fold, about 13 fold, about 14 fold, about 15 fold, about 16 fold, about 17 fold, about 18 fold, about 19 fold, about 20 fold, about 21 fold, about 22 fold, about 23 fold, about 24 fold, about 25 fold, about 26 fold, about 27 fold, about 28 fold, about 29 fold, about 30 fold, about 31 fold, about 32 fold, about 33 fold,

about 34 fold, about 35 fold, about 36 fold, about 37 fold, about 38 fold, about 39 fold, about 40 fold, about 41 fold, about 42 fold, about 43 fold, about 44 fold.

[00120] In another aspect, provided herein is a CFPS system supplemented with a combination of cerdulatinib and nafarelin.

5 [00121] The invention illustratively described herein may suitably be practiced in the absence of any element or elements, limitation or limitations, not specifically disclosed herein. Thus, for example, the terms "comprising", "including", "containing", etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use
10 of such terms and expressions of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modification and variation of the inventions embodied therein herein disclosed may be resorted to by those
15 skilled in the art, and that such modifications and variations are considered to be within the scope of this invention.

[00122] The invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form part of the invention. This includes the generic description of the invention with a proviso or
20 negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

[00123] Other embodiments are within the following claims and non-limiting examples. In addition, where features or aspects of the invention are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of
25 any individual member or subgroup of members of the Markush group.

EXPERIMENTAL SECTION

[00124] Non-limiting examples of the invention and comparative examples will be further described in greater detail by reference to specific Examples, which should not be construed as
30 in any way limiting the scope of the invention.

[00125] *Materials and Methods*

[00126] Generation of CFPS constructs

[00127] pJL1-sfGFP plasmid was purchased from Addgene (#102634), and modified to include 5'UTR and 3'UTR sequences for efficient cap-independent translation in yeast CFPS. Ribosome binding site downstream of T7 promoter was replaced with a tobacco mosaic virus 5'UTR fragment of Ω sequence by inverse PCR, retaining an NdeI restriction site. Similarly using inverse PCR, a stretch of 90-nt Poly(A) sequence was inserted immediately after sfGFP stop codon with a BamHI restriction site in between. sfGFP coding region was subsequently replaced by In-Fusion cloning (Takara) whereby vector was digested with restriction enzymes NdeI and BamHI (New England BioLabs Inc.) and insert was generated by standard PCR. All primers were from Integrated DNA Technologies (IDT). The primer sequences are listed in Table 1.

[00128] **Table 1.** Primer sequences used for CFPS plasmid template generation.

	Template	Description	Generated Construct	Primer Name	Sequence (5' --> 3')
1	pJL1-sfGFP	PolyA(90) insertion downstream of sfGFP	pJL1-sfGFP-PolyA90	POLYA90-F	AAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAA GTCGACCGGCTGCTAACAAAG (SEQ ID NO: 1)
2				POLYA90-R	TTTTTTTTTTTTTTTTTTTTTTTTTTT TTTCAGGATCCGTGGTGATGAT GGTGATGGGAACCCGTAATACC TGCCGCATTCACAT (SEQ ID NO: 2)
3	pJL1-sfGFP-PolyA90	TMV Ω insertion upstream of sfGFP ORF	pJL1-TMV Ω -sfGFP-PolyA90	inv-sfGFP-TMV Omega-F	ACAAACAACATTACAATTACTA TTTACAATTACATATGAGCAAA GGTGAAGA (SEQ ID NO: 3)
4				inv-pJL1-TEV Omega-R	TGTTTGTTGTTGTTGGTAATTGT TGTAATAATACTCCCTATAGTG AGTCGTATTA (SEQ ID NO: 4)
5	nLuc	nLuc insert for In-Fusion cloning	pJL1-TMV Ω -nLuc-PolyA90	inf-NdeI-6His-TMVomega-F	ATTTACAATTACATATGGTCTTC ACACTCGAAGAT (SEQ ID NO: 5)
6				inf-BamHI-nLuc-PA90-R	TTTTTTTTCAGGATCCTTATTTT CGAACTGCGG (SEQ ID NO: 6)
7	p53	p53 insert for In-Fusion cloning	pJL1-TMV Ω -p53-PolyA90	inf-NdeI-6His-TMVomega-F	ATTTACAATTACATATGCATCA CCACCACCACCACG (SEQ ID NO: 7)
8				inf-BamHI-p53-PA90-R	TTTTTTTTCAGGATCCTCAGTCT GAGTCAGGCCCTTCT (SEQ ID NO: 8)

9	MDM2	MDM2 insert for In- Fusion cloning	pJL1- TMVΩ- MDM2- PolyA90	inf- TMVomega- NdeI- MDM2-F	ATTTACAATTACATATGGCAAT ACCAACATGTCTGTACCTACTG A (SEQ ID NO: 9)
10				inf-BamHI- MDM2- PolyA90-R	TTTTTTTTCAGGATCCTTAGGGG AAATAAGTTAGCACAAATCATT GAATTGG (SEQ ID NO: 10)

[00129] Yeast extract preparation

[00130] . S288c yeast strain, BY4743 WT and BY4743 ssa1Δ knockout strains were used. S288c yeast strain, BY4743 WT and BY4743 ssa1Δ knockout strains were prepared following the same protocol. Yeast was grown in YPD broth (ForMedium™) supplemented with 50 mM Potassium Phosphate (pH 5.5) at 30°C with shaking (250 rpm) in Erlenmeyer flasks. Cells were harvested at OD 10-12 or at OD ~1.2 by centrifugation at 3,000 g for 10 min at 4°C. Cell pellets were washed 3 times in 20 mL Mannitol Buffer A (30 mM HEPES pH 7.4 w/ 5M KOH, 100 mM potassium acetate/ glutamate, 2 mM magnesium acetate/ glutamate, 2 mM DTT, 8.5 % (w/v) mannitol). Washed pellets were weighed, snap frozen in liquid nitrogen and stored at -80 °C. Frozen pellets were resuspended in Lysis Buffer containing 20 mM HEPES-KOH pH 7.4, 100 mM potassium glutamate, 2 mM magnesium glutamate, 2 mM DTT, 0.5 mM PMSF at 1.5 mL/ mg of pellet. Cells were lysed by means of high-pressure homogenization using EmulsiFlex®-C3 high pressure homogenizer (25,000 psi, single pass). Lysates were clarified by centrifugation twice at 25,000g for 5 min at 4°C. Cleared lysates were dialyzed against same lysis buffer but without mannitol using 3.5MWCO dialysis cassettes. Dialyzed lysates were centrifuged at 12,000g for 20 min at 4 °C. Aliquots of extract were flash frozen in liquid nitrogen and stored at -80°C. Final yeast extract concentration was determined using Bradford Assay (Bio-Rad).

[00131] Cell Free Protein Synthesis

[00132] CFPS reactions were set up as follows. Briefly, yeast extract was used at 50% (v/v) in a 15 μL reaction mix. Reactions were set in 1.5 mL Eppendorf tubes at 22°C for 2.5 h for creatine phosphate-driven CFPS and for 6 h for glucose-driven CFPS. T7RNAP was expressed in B121 cells and purified from 1L culture. At OD₆₀₀ of 0.5–0.6 expression of 6His-tagged T7 RNAP was induced by addition of IPTG to a final concentration of 1 mM. After an additional 4 h growth at 37 °C, cells were harvested by centrifugation, resuspended in buffer A (150 mM NaCl, 100 mM Tris:HCl pH 7.5, 2 mM MgCl₂, 1 mM β-mercaptoethanol) supplemented with 0.1 mM PMSF, 35 μg/mL lysozyme and 1 u/mL DNase I and lysed via sonication. After removal of the cell debris by centrifugation (12,000 rpm for 1 h), the clarified lysate was

loaded onto a 1 mL HisTRAP HP column (GE Healthcare) equilibrated in buffer A. The column was washed with high salt buffer B (same as buffer A except for the addition of 2 M NaCl), and the protein was eluted by a gradient of buffer C (buffer A supplemented with 0.5 M imidazole), pure fractions were pooled, concentrated and buffer-exchanged into storage
5 buffer (100 mM KCl, 20 mM Tris:HCl pH 7.5, 5 mM MgCl₂, 1 mM DTT, 50% glycerol) using 50 MWCO centricons (Amicon). The protein was stored at –20°C. Purified T7RNAP was added to the reaction mix at 0.027 mg/mL. pJ11 plasmids with target genes, purified with Maxi kit (Thermo Fisher Scientific), were used as CFPS templates. Reactions were supplemented with 0.08 mM of each of 20 amino acids, 1.5 mM of each ATP, UTP, GTP and CTP, 1.7 mM
10 DTT, 2 mM Putrescine and 0.5 mM Spermidine. Mg²⁺ and K⁺ concentrations were optimized for each extract and template concentration was optimized for creatine phosphate and glucose energy systems. Creatine phosphate-driven CFPS included 0.27 mg/mL creatine kinase (Roche), 25 mM creatine phosphate (Roche) and 1.7 mM DTT. Glucose-driven CFPS was set following established protocol and included 25 mM glucose, 0.3 mM cAMP, 10 mM potassium
15 phosphate and 4 mM DTT. Templates were used at 10 nM and 40 nM concentrations for creatine phosphate/kinase and glucose-driven CFPS respectively.

[00133] Nano-luciferase assay

[00134] Nano-luciferase activity was determined using Nano-Glo® Luciferase Assay (Promega). CFPS reactions were diluted in Nano-Glo® Buffer. Luminescence was read using
20 EnVision 2104 Multilabel Reader every 20 sec for at least 15 min, and the maximum reading was recorded. Absolute nano-luciferase yield was determined by comparing luminescence reads against standard curve generated with nano-luciferase recombinant protein purchased from Promega (Nluc-HT Protein).

[00135] Drug screen

25 [00136] A library of 1443 FDA-approved inhibitors was used for drug screen (“FDA Approved Drug Screening Library (96-well)-Z209087-200uL-L1300” library obtained from Selleck). Drugs (100 μM) were pre-incubated with yeast extract mixture containing T7RNAP and creatine kinase for 15 mins at 30°C. Reactions were set in PCR tubes in 96-well formats with no-drug and no-template controls included in the last column. No-drug controls had final
30 DMSO concentration (1%) matched to test groups. Each plate was tested once, and an average of two luciferase readings was obtained. Drug candidates were determined based on the fold change of the luminescence signal of drug-treated reactions with respect to that of untreated

controls. A cut-off value of at least 50% increase was used to select candidates for second round testing following the same method. Finalised drug candidates were further tested in a luciferase counter-screen test to eliminate false-positives whereby drugs (100 μ M) were added only after completion of CFPS reactions. Synthesized nano-luciferase was incubated with
5 drugs for 5 mins at 30°C and assayed following the method described earlier.

[00137] Western blot assay for p53 and Mdm2

[00138] Equal volumes of CFPS reactions were boiled and resolved by SDS-PAGE. CFPS products were blotted with anti-p53 (DO1, 1:1000) or anti-Mdm2 (1:1000) antibodies. Western blots from at least two independent experiments were performed and one representative image
10 was selected. Densitometry analysis was performed using Image Lab software (Bio-Rad) to determine fold increase in yields of drug-treated CFPS reactions with respect to drug-free controls.

[00139] HeLa CFPS

[00140] HeLa cell lysate-based CFPS reactions from 1-Step Human Coupled IVT Kit (ThermoFisher Scientific) were set up according to manufacturer protocol with the addition of
15 a drug pre-incubation step. Bosutinib and dasatinib monohydrate at 100 μ M concentrations were added to the HeLa lysate fraction. After 30 min, accessory proteins, reaction mix and pT7-nLuc template (40 ng/ μ L) were added. Reaction was run at 30°C in 1.5 mL Eppendorf tube for 6 h.

20 [00141] CFPS time-course experiment

[00142] CFPS reactions were set as described earlier. Reactions were aliquoted into multiple individual 1.5 mL Eppendorf tubes in 15 μ L fractions. At given time points (0 - 5 hours for creatine phosphate CFPS and 0 – 8 hours for glucose CFPS) entire reaction was snap frozen in liquid nitrogen and kept at -20°C at given time point. Upon thawing, 1 μ L of reaction mix was
25 sampled out in duplicate for nLuc and ATP analysis. nLuc yield was determined as described earlier. ATP levels were measured using The CellTiter-Glo® 2.0 Assay. CFPS samples were diluted 10 times and mixed with equal volume of 10% TCA solution. Reactions were spun down at 12,000 g for 10 mins to remove precipitated proteins. 25 times diluted supernatant was mixed with equal volume of CellTiter-Glo® 2.0 reagent, incubated in dark for 10 mins and
30 luminescence signal was read. ATP levels were determined by comparing luminescence signal against ATP standard curve.

[00143] Drug dose and thermal treatment of yeast extract

[00144] 150 µg of yeast extract was diluted to 45 µl with dilution buffer of 50 mM HEPES pH7.5, 10 mM MgCl₂ with protease inhibitors. Stock solution of 180mM bosutinib in DMSO was serially diluted to 100X solution with DMSO and a subsequent dilution to 10X working solution with dilution buffer. 5 µl of 10X working solution was added to 45 µl of yeast extract, resulting in a 1% final DMSO concentration and final drug concentrations: vehicle, 0.01 nM, 0.1 nM, 1 nM, 10 nM, 100 nM, 1 µM, 10 µM, 100 µM and 1 mM. This is repeated over three technical replicates where each replicate was run on separate TMT-10 set. Lysates were incubated with drug for 3 mins at room temperature before performing heat treatment at 55°C in a PCR thermocycler for 3 mins, followed by cooling at 4°C for 3 mins. Samples were kept on ice and centrifuged at 20,000 g for 30 mins at 4°C. Supernatant containing soluble proteins after thermal treatment was transferred to a new tube with care taken to avoid disrupting pelleted proteins.

[00145] Time course treatment of yeast extract

[00146] 30 µg of yeast extract was diluted to 50 µl with dilution buffer of 50 mM HEPES pH7.5, 10 mM MgCl₂ with protease inhibitors. Lysates were incubated in a pre-heated 37°C water bath for 0, 10, 30, 60 and 120 mins. Samples taken out of water bath were placed on ice for 3 mins before centrifugation at 20,000 g for 20 mins at 4°C. Supernatant containing soluble proteins after thermal treatment was transferred to a new tube with care taken to avoid disrupting pelleted proteins.

[00147] Data processing and statistical analysis of CFPS yields

[00148] Bar graph results were obtained from at least 3 independent experiments presenting mean ± SD. Statistical analysis between two groups was performed using two-tailed Student's t-test with p < 0.05 considered significant. *p < 0.05; **p < 0.01; ***p < 0.005; NS, not significant.

[00149] Sample preparation of thermally treated proteins for mass spectrometry proteomics

[00150] Proteins were subjected to acetone precipitation by adding 4x volume of ice-cold acetone, incubated at -20°C for 12 h, then centrifuged at 14,000 g for 20 mins at 4°C. Supernatant was removed and protein pellet was air-dried. Protein pellet was reconstituted in 50 µl 8 M urea in 50 mM HEPES, pH7.5. Proteins were reduced, alkylated and digested using a slightly modified version of a previously reported protocol. In brief, TCEP was added to 10mM and incubated at 25°C for 30 mins. CAA was then added to 55 mM and incubated in the dark for 30 mins. TEAB buffer was added to dilute 8M urea to below 2M urea, and 1 µg

lysC was added with incubation for 4 h at 25°C. TEAB buffer was then added to dilute urea to below 1 M concentration and 1 µg trypsin was added with incubation for 16 h at 25°C. Digestion was quenched by addition of TFA to final 1% (v/v) concentration. Peptides were then desalted using self-packed Empore C18 stage tips. Stage tips were activated with acetonitrile, equilibrated twice with 0.1% formic acid in water. Samples were loaded on stage-tip twice and then washed twice with 0.1% formic acid in water. Peptides were eluted with 65% acetonitrile, 0.1% formic acid in water. Eluted peptides were dried by vacuum centrifugation. 5 µg of dried peptides were then resolubilized with 7 µl of 100 mM TEAB pH8.5. 3 µl of TMT-10 labelling reagent was added and incubated at room temperature for 16 h. Labelling was quenched by addition of 5% hydroxylamine and incubated for 15 mins. Samples were pooled together and diluted with 100 µl ammonium formate pH10 in water. Desalting at high pH was performed using self-packed spin columns (MoBiTec) fritted with 10 µm pore size filter and loaded with solid phase, ReproSil Pur Basic resin 10µm particle size (Dr. Maisch), in acetonitrile. Spin columns were then equilibrated with 100% acetonitrile and conditioned by passing 10 mM ammonium formate pH10 through twice. Samples were loaded and then eluted with 50% acetonitrile in 10 mM ammonium formate pH10. Fractions were dried by vacuum centrifugation and stored at -20°C before mass spectrometry analysis.

[00151] Liquid chromatography mass spectrometry proteomics

[00152] TMT-10 labelled peptides were resuspended in water with 2% acetonitrile, 0.5% acetic acid and 0.06% trifluoroacetic acid and loaded on a heated (50°C) Easy-Spray 75 µm x 50 cm column on Vanquish Neo (Thermo Scientific) liquid chromatography system coupled to an Orbitrap Eclipse Tribrid Mass Spectrometer (Thermo Scientific) with an EASY-Spray source. Peptides were resolved at a flow rate of 300 nl/min, with pre-column equilibration by 100% mobile phase A (0.1% formic acid in water) and resolved by increasing mobile phase B (80% acetonitrile in water with 0.1% formic acid) with a gradient as follows: 0-35% B for 75 mins, 35-50% B for 8 mins, 50-100% B for 3 mins, 100%B for 5 mins. Column was equilibrated with 100%A post-run. Mass spectra were collected in Data-Dependent mode with cycle time of 3 s between master scans. MS1 scans were performed in the Orbitrap with 60K resolution, AGC target of 400,000 and maximum injection time of 100 ms. MS2 scans collected by Orbitrap with 50K resolution, 42% HCD collision energy, first mass set at 110, AGC target of 75,000, and maximum injection time of 100 ms.

- [00153] Mass spectra raw files were searched with SequestHT in Proteome Discoverer 3.0 against a *Saccharomyces cerevisiae* database (retrieved Mar 2017), with following parameters: precursor mass tolerance of 10ppm, fragment mass tolerance of 0.06 Da, trypsin as enzyme with maximum 3 missed cleavages. TMT 10plex was set as quantification method, with static modification for carbamidomethyl (C) and TMT modification (Protein N-terminus, K), and dynamic modifications for acetyl (Protein N-terminus), oxidation (M) and deamidation (N,Q). A strict 1% false discovery rate was set using Percolator node, and Reporter Ions Quantifier nodes were added to workflow for TMT10 quantification. Output search files were exported as separate .txt files for each of the three replicates.
- 10 [00154] Data processing and analysis of mass spectrometry data
- [00155] The three data sets were first individually filtered for missing values and only proteins that were not missing across all samples were retained. The protein abundance at 10 nM for all three replicates and at 1 mM for one of the replicates (set 1), showed systematic shifts in the measurements and were very different from the rest of the samples that cannot be
- 15 corrected numerically. Thus, these 4 samples were left out from the downstream analysis and 0.01 nM was used as the lowest drug concentration value. Next, they were combined into a single data set using 542 common proteins across the three sets. The coefficient-of-variation (%CV) using the lowest three concentrations (0.01 nM, 0.1 nM and 1 nM) and the highest three concentrations (10 uM, 100 uM and 1 mM) for each protein in each replicate was computed.
- 20 Keeping only proteins with CV below 20% at the highest and lowest three concentrations across all three replicate sets, 457 proteins were retained for the downstream analysis. To remove the differences across the TMT sets, the data was normalized at each concentration against the lowest concentration value to derive the relative protein ratios with respect to 0.01 nM. Then for each replicate, the mean protein relative ratio at the three highest and three lowest
- 25 drug concentrations was calculated. The values were averaged across replicates and the mean fold change is computed by dividing the average at the higher concentrations by the average at the lower concentrations. Proteins were ranked and prioritized by decreasing magnitude of mean fold changes and those with greater than 1.1 fold increase were subjected to Gene Ontology (GO) enrichment analysis for biological processes, and plotted using SRplot. Protein
- 30 interactors were plotted using Cytoscape v3.10.0, using StringApp to retrieve and map STRING protein interactors. A loess curve was used to fit the changes in the protein

abundances across the nine concentrations to model the dose response curves. All analyses were carried out using R studio version 4.2.3.

[00156] *Results*

[00157] **Example 1**

5 [00158] **Screening of FDA-approved small molecule library for CFPS agonists**

[00159] A high-throughput screen was performed using a library of 1443 FDA-approved drugs on yeast CFPS to identify enhancers of heterologous protein production (Figure 1). Coupled transcription and translation reactions were set up as follows. Translation was initiated via a cap-independent mechanism by inclusion of TMV virus 5'UTR Ω sequence upstream of
 10 the nano-luciferase (nLuc) reporter gene, and transcripts were stabilised by the addition of a 90-nt Poly-A tail at the 3' end. Yeast extract fractions (50% v/v) supplemented with T7RNAP (0.027 mg/mL) and creatine kinase (0.27 mg/mL) were pre-incubated with 100 μ M drugs at 30 °C for 15 mins, allowing sufficient time for binding and onset of drug-mediated responses. Reactions were initiated by addition of nLuc plasmid DNA template (5 nM), amino acids (0.08
 15 mM each), nucleotides (1.5 mM each), creatine phosphate energy factors (25 mM), DTT (1.7 mM), putrescine (2 mM), spermidine (0.5 mM), magnesium glutamate (5 mM) and potassium glutamate (120 mM). 15 μ L CFPS reactions were set in 0.2 mL PCR tubes and incubated at 30 °C for 2.5 h. Drug candidates were identified based on the fold increase of luminescence signal observed from drug-treated reactions against drug-free controls and a 50% cut-off was applied
 20 to select hits for secondary analysis (Figure 2). Repeat experiments confirmed 8 out of 110 hits, increasing nLuc yields by 1.30 - 2.13-fold (Table 2, Figure 3). None of the positive hits were shown to directly enhance nLuc activity in a counter-screen to exclude false positives (Figure 4). Interestingly, six out of eight hits (bosutinib, cerdulatinib, afatinib/ afatinib dimaleate, neratinib and dasatinib) are tyrosine kinase inhibitors (TKIs) known to compete for
 25 ATP-binding sites of various targets including SRC, ABL, JAK, and EGFR. The remaining two are the gonadotropin-releasing hormone agonist nafarelin acetate and the antibiotic polymyxin B sulphate which disrupts gram-negative bacterial membranes.

[00160] **Table 2.** List of drug hits increasing yeast extract productivity

Table 2 List of drug hits increasing yeast extract productivity

Drug	Mode of action	Targets
Bosutinib	ATP-competitive tyrosine kinase	Tyrosine kinases BCR, ABL1, LYN, HCK, SRC

	inhibitor (BCR-ABL/SRC)	Serine/Threonine kinases	CDK2, MAP2K1, MAP2K2, MAP3K2, CAMK2G
		ATP-dependent efflux pump	ABCB1
Cerdulatinib	ATP-competitive tyrosine kinase inhibitor (SYK/JAK)	Tyrosine kinases	SYK, JAK1/2/3, TYK2
Dasatinib Monohydrate	ATP-competitive tyrosine kinase inhibitor (SRC family)	Tyrosine kinases	ABL1/2, SRC, EPHA2/5, LCK, YES1, KIT, PDGFRB, FYN, BTK, BCR, CSK, EPHB4, FGR, FRK, LYN
		Serine/Threonine kinases	ZAK, MAPK14
		Transcription factors	STAT5B, NR4A3
		Heat shock protein	HSPA8
		Phosphoribosyltransferase	PPAT
		ATP-dependent efflux pump	ABCB1
		ATP-binding cassette	ABCG2
Nafarelin Acetate	Gonadotropin-releasing hormone (GnRH) agonist	G-protein-coupled receptors	GNRHR
Afatinib	ATP-competitive tyrosine kinase inhibitor (ErbB family)	Tyrosine kinases	EGFR, ERBB2/4
		ATP-dependent efflux pump	ABCB1
		ATP-binding cassette	ABCG2
Neratinib	ATP-competitive tyrosine kinase inhibitor (HER2/EGFR)	Tyrosine kinases	EGFR, HER2
		Serum proteins	ALB, AAG
		ATP-dependent efflux pump	ABCB1
Polymyxin B sulphate	Antibiotic	LPS of gram-negative bacteria outer membrane	Displaces Ca ²⁺ and Mg ²⁺ from LPS increasing membrane permeability

[00161] Numerous drugs which reduced nLuc yields were also identified in the screen (Figure 5 and Table 3). None were direct inhibitors of nLuc (Figure 6). These comprised known protein synthesis inhibitors (hygromycin B, puromycin, gentamicin, pentamidine and paramomycin), thus validating screen efficiency. Other inhibitors likely impede the transcriptional step (doxorubicin, proflavine hemisulfate), interfere with DNA synthesis, induce DNA damage (aprotonin, ethacridine lactate and mitoxantrone) or inhibit T7RNAP

(peparin). The remaining two inhibitory compounds, calcium levofolinate (chemotherapy adjuvant), and carbenoxolone sodium (antiulcer agent) are of further potential interest.

[00162] **Table 3.** List of drug hits decreasing yeast extract productivity

Table 3 List of drug hits decreasing yeast extract productivity

Drug	Mode of action	Targets	
Hygromycin B	Antibiotic inhibits protein synthesis	Ribosomes	30S rRNA
		Ribosome-dependent ATPase	RbbA
Doxorubicin (Adriamycin)	Antibiotic induces DNA damage and apoptosis	DNA Topoisomerase	TOP1, TOP2A, TOP2B
		Nucleic acids	DNA
		Nucleolar phosphoprotein	NOLC1
Puromycin 2HCl	Antibiotic inhibits protein synthesis	Ribosomal proteins	RPL10L, RPL13A, RPL23, RPL15, RPL19, RPL23A, RSL24D1, RPL26L1, RPL8, RPL37, RPL3, RPL11,
		tRNA-protein transferase	aat
Proflavine Hemisulfate	Antiseptic intercalates DNA	Nucleic acids	DNA
Ethacridine lactate monohydrate	Antiseptic intercalates DNA	Nucleic acids	DNA
Mitoxantrone HCl	type II topoisomerase inhibitor intercalates DNA	DNA Topoisomerase	TOP2A
		Nucleic acids	DNA
Aprotinin	Serine protease inhibitor	Serine protease	PRSS1, CTRB1, PLG, KLK1
Gentamicin Sulfate	Antibiotic inhibits protein synthesis	Ribosomes	rpsL, 16S rRNA, 23S rRNA,
		Membrane	
		Synthetase	nadE
		Reductase	DHFR
Pentamidine	Antiprotozoal agent inhibits synthesis of DNA, RNA,	PRL Phosphatases	PTP1B
		tRNA-methyltransferase	TRDMT1

	phospholipids and proteins	Nucleic acids	DNA
Heparin sodium	Anticoagulant Competitive inhibitor of RNA synthesis	Serpin protease inhibitor	SERPINC1
		Coagulation factor X	F10
		Selectin	SELP
		Tyrosine kinase	FGFR1, FGFR2, FGFR4
		Fibroblast growth factor	FGF1, FGF2, FGF4, FGF19
		Cytokine	PF4
		Polymerases	T7RNAP, polA
Paromomycin Sulfate	Antibiotic inhibits protein synthesis	Ribosomes	16S rRNA, rpsJ, RPSA, RPL10L
Calcium Levofolinate	Calcium salt of folinic acid and analogue of tetrahydrofolate	Serine hydroxymethyltransferase	glyA
Carbenoxolone Sodium	Synthetic derivative of glycyrrhetic acid	Pannexon membrane channels and related innexon channels	11 β -HSD

[00163] **Example 2**

[00164] **Additive effects of drugs enhancing CFPS**

[00165] Varied reaction conditions were tested to see if drug activity could be further potentiated. Significant improvements in total yields were observed with lower reaction temperature (22°C versus 30°C) and removal of the drug pre-incubation step prior to initiation of transcription/translation (Figure 7A). The pre-incubation period (15 mins) reduced activity at both temperatures that could be rescued with drug treatment. Following this modified protocol, dose responsive increases (1.28 - 1.95-fold) in nLuc yield were observed, further validating hits (Figure 7B). Thereafter, drug combinations were tested for possible additive/synergistic effects. Four of the most potent drugs, bosutinib, cerdulatinib, nafarelin acetate and dasatinib, were added to CFPS reactions alone or in combination. Combining nafarelin with either cerdulatinib or bosutinib exhibited an additive effect, further enhancing nLuc production from the original 1.75~1.91-fold to 2.85~3.15-fold (Figure 7C). Interestingly, an additive effect was observed when combining bosutinib with cerdulatinib and nafarelin acetate with dasatinib, suggesting possible overlapping functions of the drugs within pairs. A similar pattern was observed with in vitro expression of p53 test protein, whereby drugs resulted in 1.98 - 2.40-fold increase in yield when added alone and as high as 3.88 and 5.47-

fold when nafarelin acetate was respectively combined with either bosutinib or cerdulatinib (Figure 7D).

[00166] **Example 3**

[00167] **Exploring functional role of bosutinib in improving yeast CFPS**

- 5 [00168] Of the four drug candidates, bosutinib alone and in combination with other hits exhibited improvement in CFPS. Given its role as an ATP-competitive kinase inhibitor, the study explored whether bosutinib increases CFPS yield by reducing background ATP consumption. More ATP could then be funneled into heterologous protein synthesis, particularly for tRNA aminoacylation. Time-course experiments revealed that the majority of
- 10 protein synthesis (>90%) occurred within the first 30 mins, reaching completion by 45 mins (Figure 8A). Bosutinib did not prolong the reaction time, but almost doubled the rate of synthesis in the period between 15-30 mins. Completion of reaction can be explained by rapid depletion of ATP to almost 0.2 mM after 45 mins (Figure 8B). No evident differences in ATP consumption were observed between bosutinib treated and untreated reactions. Moreover, the
- 15 rates of ATP consumption in reactions actively synthesizing nLuc were comparable to controls (no DNA template encoding nLuc added), indicating minimal effect of nLuc synthesis on overall ATP consumption (Figure 8B). Nevertheless, addition of bosutinib did result in altered ATP levels both in CFPS and NTC control reactions, but at a much later time. Bosutinib either suppressed or delayed regeneration of ATP that occurred after 2 hours (Figure 8B). Moreover,
- 20 the observed regeneration of ATP must have been driven by endogenous processes, as it also occurred in CFPS lacking exogenous creatine phosphate and creatine kinase added to generate ATP (Figure 8B). Yeast extracts have the capacity to generate ATP via the glycolytic pathway, with addition of glucose, cAMP and inorganic phosphate facilitating synthesis of 3.64 µg/mL of active luciferase. ATP regeneration via the glycolytic pathway occurred with a delay after
- 25 1.5 hours, with subsequent protein synthesis initiated at around 2 h. Thereafter, bosutinib's effect on ATP levels observed in the later time period was tested to determine whether it would also impact protein synthesis in glucose-driven CFPS. Interestingly, bosutinib exhibited a 3.5-fold increase in nLuc yield, attaining a similar productivity level to creatine phosphate/kinase-driven CFPS (Figure 9A).
- 30 [00169] The absolute yield of active nLuc produced using the creatine phosphate/kinase energy system was 0.77 µg/mL and increased to 1.65 µg/mL upon treatment with bosutinib. Despite using a near-identical preparation protocol, efficiency of the base yeast cell extract was

less than the reported 7 $\mu\text{g/mL}$. Drastic drops in efficiency have been reported when extracts are prepared from cells harvested at stationary phase due to metabolic shifts in response to stress and nutrient depletion. The use of standard Erlenmeyer flasks as opposed to Tunair flasks could have resulted in accumulated stress due to prolonged culture time to reach the desired
5 OD₆₀₀ of 12. For subsequent experiments, extracts were therefore prepared from yeast harvested much earlier at OD₆₀₀ of 1.2. These extracts were more efficient, and alongside optimization of template concentration, yielded 5.09 and 6.27 $\mu\text{g/mL}$ of active nLuc with the creatine phosphate/kinase and glucose energy regeneration systems respectively (Figure 9B and Figure 10A). Notably, bosutinib also shows increase in yield when the glucose energy
10 regeneration system was used (Figure 10A). The 3-fold increased nLuc yield to 19 $\mu\text{g/mL}$ is the highest thus far reported using the cost-effective glucose energy alternative. Interestingly, a time-course experiment with glucose-driven reactions supported the hypothesis of bosutinib effect on ATP consumption. As before, bosutinib increased the rate of nLuc production (Figure 10B), and reactions containing bosutinib were able to sustain higher levels of ATP in the 4 - 6
15 hour time period post initiation (Figure 10C). Moreover, the significantly later onset of nLuc production in glucose-driven reactions (3 hours compared to immediate initiation for creatine phosphate driven reactions) (Figure 8A and Figure 10B) could explain differences observed in yield between the two energy systems when DNA template concentration was increased. Similar to previous studies, saturation in yield was observed with 10 nM of template using
20 creatine phosphate as energy source. However, with glucose-based energy regeneration, a template concentration of 40 nM increased nLuc yield by 7-fold (Figure 9B). Here, increased template degradation by endogenous nucleases during the considerable 3 hour lag phase is likely mitigated by increasing input DNA levels.

[00170] **Example 4**

25 [00171] **Exploring drug effects on HeLa cell-based CFPS**

[00172] Six out of the seven positive hits identified in the screen were drugs designed to target human cells. Whether the observed outcomes could be translated to improvement of more complex eukaryotic CFPS systems was therefore investigated. HeLa cell-based CFPS reactions were tested with the compounds for improved nLuc production. Following prolonged
30 pre-incubation of the extract fraction with the drug (30 mins), yields of reactions containing bosutinib and dasatinib were 1.64 and 1.97-fold higher than corresponding controls with DMSO (Figure 11). During the prolonged incubation, increased background depletion of

endogenous ATP (up to 5mM) will occur. This reduction in total ATP levels (endogenous + regenerated) is likely responsible for the observed drug-effects, as inhibition of competing ATP consumers will not be as beneficial when ATP is not limiting. A similar drug effect was observed for yeast lysates with and without pre-incubation (Figure 7A), highlighting bosutinib and dasatinib as useful tools to suppress background ATP metabolism during preparation of eukaryotic CFPS systems and improve efficiency.

[00173] **Example 5**

[00174] **Thermal proteomics approach for target identification**

[00175] Thermal stability-based proteomics methods such as the cellular thermal shift assay (CETSA) or thermal proteome profiling (TPP) coupled to mass spectrometry (MS) can identify proteins whose thermal stability is affected by small molecule action. In particular, isothermal dose response (ITDR) analysis allows the identification of dose-dependent effects on protein thermal stability at elevated temperatures, enabling CETSA application to a variety of cellular systems for identification of possible targets of small molecules. Yeast extracts were treated with bosutinib at 9 different concentrations (0.01 nM to 1 mM) along with a vehicle control prior to heat treatment at 55°C. Proteins stabilized by bosutinib binding at this elevated temperature were then quantified by tandem mass tag (TMT) labelling, and dose response curves and pathway analysis performed (Figure 12A). A total of 545 proteins were fully quantified from the treated yeast lysates across the three replicates. Proteins with high coefficient-of-variation (%CV) greater than 20% at the lowest and highest three drug concentrations were filtered away, leaving 457 proteins which were quantified. Relative protein ratios were computed by normalizing against the lowest concentration (0.01 nM), and mean fold-changes were obtained by dividing the mean protein abundance in the highest three concentrations against the mean of the lowest three concentrations across the three replicates (Tables 4 - 9).

[00176] **Table 4.** Table summarizing the list of top 20 hits from 457 proteins quantified after filtering, reporting the protein relative ratios with respect to 0.01nM, coefficient of variation (%CoV) across the three TMT replicates and arranged in decreasing order of the magnitude of mean fold change.

Accession	Description	ProtDescription	GeneName

P40150	Ribosome-associated molecular chaperone SSB2 OS= <i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) GN=SSB2 PE=1 SV=2	Ribosome-associated molecular chaperone SSB2	SSB2
P10591	Heat shock protein SSA1 OS= <i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) GN=SSA1 PE=1 SV=4	Heat shock protein SSA1	SSA1
P40040	Protein THO1 OS= <i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) GN=THO1 PE=1 SV=1	Protein THO1	THO1
P35691	Translationally-controlled tumor protein homolog OS= <i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) GN=TMA19 PE=1 SV=1	Translationally-controlled tumor protein homolog	TMA19
P16547	Mitochondrial outer membrane protein OM45 OS= <i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) GN=OM45 PE=1 SV=2	Mitochondrial outer membrane protein OM45	OM45
P06208	2-isopropylmalate synthase OS= <i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) GN=LEU4 PE=1 SV=1	2-isopropylmalate synthase	LEU4
P52286	Suppressor of kinetochore protein 1 OS= <i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) GN=SKP1 PE=1 SV=2	Suppressor of kinetochore protein 1	SKP1
P53184	Nicotinamidase OS= <i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) GN=PNC1 PE=1 SV=1	Nicotinamidase	PNC1
P32861	UTP--glucose-1-phosphate uridylyltransferase OS= <i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) GN=UGP1 PE=1 SV=1	UTP--glucose-1-phosphate uridylyltransferase	UGP1
P41940	Mannose-1-phosphate guanylyltransferase OS= <i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) GN=PSA1 PE=1 SV=2	Mannose-1-phosphate guanylyltransferase	PSA1
Q12177	Uncharacterized protein YLL056C OS= <i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) GN=YLL056C PE=2 SV=1	Uncharacterized protein YLL056C	YLL056C
P04786	DNA topoisomerase 1 OS= <i>Saccharomyces cerevisiae</i> (strain ATCC 204508 / S288c) GN=TOP1 PE=1 SV=2	DNA topoisomerase 1	TOP1
P42940	Probable electron transfer flavoprotein subunit beta OS= <i>Saccharomyces</i>	Probable electron transfer	CIR1

	cerevisiae (strain ATCC 204508 / S288c) GN=CIR1 PE=1 SV=1	flavoprotein subunit beta	
P47018	Maintenance of telomere capping protein 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=MTC1 PE=1 SV=1	Maintenance of telomere capping protein 1	MTC1
P15705	Heat shock protein STI1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=STI1 PE=1 SV=1	Heat shock protein STI1	STI1
P00937	Multifunctional tryptophan biosynthesis protein OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TRP3 PE=1 SV=2	Multifunctional tryptophan biosynthesis protein	TRP3
P32773	Transcription initiation factor IIA large subunit OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=TOA1 PE=1 SV=1	Transcription initiation factor IIA large subunit	TOA1
P37292	Serine hydroxymethyltransferase, mitochondrial OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=SHM1 PE=1 SV=2	Serine hydroxymethyltransferase, mitochondrial	SHM1
P46680	Actin-interacting protein 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=AIP1 PE=1 SV=1	Actin-interacting protein 1	AIP1
P00950	Phosphoglycerate mutase 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c) GN=GPM1 PE=1 SV=3	Phosphoglycerate mutase 1	GPM1

[00177] **Table 5.** Table summarizing the protein relative ratios with respect to 0.01nM the list of top 20 hits from 457 proteins quantified, coefficient of variation (%CoV) across the three TMT replicates and arranged in decreasing order of the magnitude of mean fold change.

GeneName	Drug_0.01nM_R1	Drug_0.1nM_R1	Drug_1nM_R1	Drug_100nM_R1	Drug_1uM_R1	Drug_10uM_R1	Drug_100uM_R1	Drug_1mM_R1
SSB2	0.000	0.281	0.259	0.337	0.805	0.639	0.651	0.686
SSA1	0.000	-0.006	0.117	0.035	0.406	0.343	0.254	0.228
THO1	0.000	0.336	0.427	0.627	0.593	0.656	0.624	0.644
TMA19	0.000	0.011	0.038	0.282	0.326	0.239	0.168	-0.329
OM45	0.000	0.024	-0.009	0.128	0.342	0.313	0.445	1.048
LEU4	0.000	0.179	0.143	0.359	0.707	-0.004	0.366	-0.541
SKP1	0.000	-0.123	0.065	0.034	0.163	0.082	-0.002	-0.957
PNC1	0.000	-0.003	-0.188	-0.348	0.164	0.103	0.272	0.457
UGP1	0.000	-0.073	0.163	-0.019	0.362	0.191	0.207	-0.342

PSA1	0.000	0.215	0.305	0.114	0.270	0.359	0.265	0.906
YLL056C	0.000	-0.013	0.091	0.234	0.266	0.238	0.505	1.090
TOP1	0.000	0.173	0.207	0.844	0.464	0.441	0.535	-0.135
CIR1	0.000	0.040	0.240	1.018	0.083	0.369	0.443	-0.042
MTC1	0.000	-0.163	-0.052	0.001	0.214	0.157	0.242	-0.745
STI1	0.000	0.019	-0.029	0.117	0.186	0.004	0.103	-1.430
TRP3	0.000	0.065	0.025	0.248	0.208	0.184	0.241	-0.261
TOA1	0.000	0.186	0.191	0.059	0.472	0.362	0.424	0.112
SHM1	0.000	-0.014	-0.045	0.162	0.215	0.124	0.196	0.311
AIP1	0.000	0.126	0.173	0.395	0.338	0.221	0.350	-0.631
GPM1	0.000	-0.338	-0.009	-0.248	0.004	0.032	-0.084	0.446

[00178] **Table 6.** Table summarizing the protein relative ratios with respect to 0.01nM the list of top 20 hits from 457 proteins quantified, coefficient of variation (%CoV) across the three TMT replicates and arranged in decreasing order of the magnitude of mean fold change.

Gene Name	Drug_0.01nM_R2	Drug_0.1nM_R2	Drug_1nM_R2	Drug_100nM_R2	Drug_1uM_R2	Drug_10uM_R2	Drug_100uM_R2	Drug_1mM_R2
SSB2	0.000	0.480	0.141	0.340	0.214	0.885	0.593	0.791
SSA1	0.000	0.478	0.164	0.564	0.321	0.822	0.659	0.878
THO1	0.000	0.288	0.276	0.349	0.453	0.658	0.417	0.568
TMA19	0.000	0.455	0.007	0.508	0.293	0.756	0.621	0.758
OM45	0.000	0.017	0.056	0.161	0.203	0.363	0.243	0.513
LEU4	0.000	0.357	0.332	0.420	0.546	0.806	0.717	0.851
SKP1	0.000	0.362	0.253	0.434	0.419	0.757	0.643	0.747
PNC1	0.000	0.010	-0.112	-0.345	0.117	0.283	0.210	0.374
UGP1	0.000	0.055	0.192	0.081	0.095	0.620	0.355	0.488
PSA1	0.000	0.474	0.209	0.439	0.131	0.630	0.473	0.849
YLL056C	0.000	0.084	0.124	0.046	0.148	0.390	0.180	0.537
TOP1	0.000	0.044	-0.044	-0.120	0.147	0.567	0.290	0.170
CIR1	0.000	0.185	-0.094	0.064	-0.033	0.330	0.085	0.228
MTC1	0.000	-0.004	0.091	0.113	0.203	0.330	0.327	0.465
STI1	0.000	0.043	-0.004	0.041	0.135	0.181	0.644	0.246
TRP3	0.000	0.007	0.193	0.103	0.338	0.552	0.561	0.348
TOA1	0.000	-0.034	-0.424	-0.274	0.032	-0.059	0.100	0.337
SHM1	0.000	-0.072	0.152	-0.062	0.184	0.442	0.115	0.375
AIP1	0.000	0.211	0.244	0.258	0.358	0.539	0.422	0.405
GPM1	0.000	0.075	0.351	0.244	0.214	0.806	0.401	0.444

[00179] **Table 7.** Table summarizing the protein relative ratios with respect to 0.01nM the list of top 20 hits from 457 proteins quantified, coefficient of variation (%CoV) across the three TMT replicates and arranged in decreasing order of the magnitude of mean fold change.

Gene Name	Drug_0.01nM_R3	Drug_0.1nM_R3	Drug_1nM_R3	Drug_100nM_R3	Drug_1uM_R3	Drug_10uM_R3	Drug_100uM_R3	Drug_1mM_R3
SSB2	0.000	0.157	0.037	0.152	0.494	0.332	0.483	0.439
SSA1	0.000	0.061	0.002	0.338	0.466	0.214	0.430	0.535
THO1	0.000	0.342	0.250	0.595	0.522	0.599	0.391	0.519
TMA19	0.000	-0.095	-0.034	0.106	0.128	0.207	0.291	0.128
OM45	0.000	-0.042	-0.079	0.134	0.141	0.167	0.136	0.451
LEU4	0.000	0.455	0.282	0.358	0.843	0.460	0.679	0.494
SKP1	0.000	0.080	0.027	0.255	0.350	0.395	0.275	0.428
PNC1	0.000	-0.021	-0.025	0.041	0.263	0.222	0.270	0.415
UGP1	0.000	0.024	0.033	0.334	0.338	0.343	0.356	0.265
PSA1	0.000	0.164	-0.153	0.387	0.456	0.222	0.249	0.495
YLL056C	0.000	0.059	-0.074	0.313	0.136	0.259	0.226	0.167
TOP1	0.000	-0.070	-0.296	0.282	-0.033	0.068	-0.141	0.135
CIR1	0.000	0.435	0.375	0.325	0.548	0.669	0.805	0.355
MTC1	0.000	-0.028	-0.098	0.015	0.213	0.121	0.182	0.210
STI1	0.000	0.009	-0.080	0.228	0.178	0.209	0.617	0.236
TRP3	0.000	-0.102	0.006	0.099	0.074	0.087	-0.075	0.443
TOA1	0.000	0.020	-0.064	0.393	0.195	0.205	0.237	0.338
SHM1	0.000	0.090	-0.043	0.347	0.242	0.289	0.235	0.508
AIP1	0.000	0.054	0.061	0.466	0.330	0.381	0.332	0.350
GPM1	0.000	0.010	0.053	0.479	0.252	0.239	0.235	0.365

[00180] **Table 8.** Table summarizing the protein relative ratios with respect to 0.01nM the list of top 20 hits from 457 proteins quantified, coefficient of variation (%CoV) across the three TMT replicates and arranged in decreasing order of the magnitude of mean fold change.

GeneName	cov1_first3	cov2_first3	cov3_first3	cov1_last3	cov2_last3	cov3_last3
SSB2	10.5%	17.6%	5.7%	0.6%	10.1%	5.3%
SSA1	4.9%	17.2%	2.4%	4.4%	7.7%	11.1%
THO1	15.0%	10.9%	11.9%	1.6%	8.3%	7.2%
TMA19	1.3%	18.9%	3.3%	3.5%	5.3%	5.7%
OM45	1.2%	2.0%	2.7%	6.5%	9.4%	12.4%

LEU4	6.4%	13.2%	15.6%	18.0%	4.7%	8.3%
SKP1	6.6%	12.5%	2.8%	4.1%	4.3%	5.5%
PNC1	7.3%	4.6%	0.9%	8.3%	5.7%	7.0%
UGP1	8.5%	6.9%	1.2%	0.8%	9.2%	3.4%
PSA1	10.6%	16.6%	11.0%	4.6%	13.2%	10.7%
YLL056C	4.0%	4.3%	4.6%	13.0%	12.3%	3.2%
TOP1	7.5%	3.1%	10.4%	4.6%	14.4%	9.8%
CIR1	9.1%	10.0%	15.6%	3.6%	8.5%	15.5%
MTC1	5.7%	3.8%	3.5%	4.2%	5.6%	3.1%
STI1	1.7%	1.8%	3.4%	4.8%	18.1%	16.5%
TRP3	2.3%	7.8%	4.2%	2.8%	8.2%	18.8%
TOA1	7.4%	15.5%	3.0%	3.0%	13.9%	4.9%
SHM1	1.6%	8.0%	4.8%	3.5%	11.6%	10.2%
AIP1	6.1%	8.9%	2.3%	6.3%	5.1%	1.7%
GPM1	12.8%	13.2%	2.0%	5.7%	16.0%	5.2%

[00181] **Table 9.** Table summarizing the protein relative ratios with respect to 0.01nM the list of top 20 hits from 457 proteins quantified, coefficient of variation (%CoV) across the three TMT replicates and arranged in decreasing order of the magnitude of mean fold change.

Gene Name	mean_Ratio1_first3	mean_Ratio2_first3	mean_Ratio3_first3	mean_Ratio1_last3	mean_Ratio2_last3	mean_Ratio3_last3	Grand mu_firrst3	Grand mu_last3	mean FoldChange
SSB2	1.14	1.17	1.05	1.56	1.70	1.34	1.12	1.53	1.37
SSA1	1.03	1.17	1.01	1.23	1.73	1.32	1.07	1.43	1.33
THO1	1.20	1.14	1.15	1.56	1.46	1.42	1.17	1.48	1.27
TMA19	1.01	1.13	0.97	1.15	1.64	1.16	1.04	1.32	1.27
OM45	1.00	1.02	0.97	1.30	1.30	1.20	1.00	1.27	1.27
LEU4	1.08	1.18	1.20	1.14	1.73	1.46	1.15	1.45	1.26
SKP1	0.99	1.16	1.03	1.03	1.64	1.29	1.06	1.32	1.25
PNC1	0.96	0.98	0.99	1.14	1.22	1.24	0.98	1.20	1.23
UGP1	1.02	1.06	1.01	1.15	1.41	1.25	1.03	1.27	1.23
PSA1	1.13	1.18	1.01	1.24	1.58	1.26	1.11	1.36	1.23
YLL056C	1.02	1.05	1.00	1.30	1.30	1.16	1.02	1.25	1.23
TOP1	1.09	1.00	0.92	1.40	1.28	1.02	1.01	1.23	1.23
CIR1	1.07	1.02	1.22	1.33	1.16	1.54	1.10	1.34	1.22
MTC1	0.95	1.02	0.97	1.15	1.30	1.13	0.98	1.19	1.21
STI1	1.00	1.01	0.98	1.04	1.29	1.29	1.00	1.21	1.21
TRP3	1.02	1.05	0.98	1.16	1.40	1.12	1.02	1.23	1.21
TOA1	1.09	0.91	0.99	1.31	1.10	1.20	1.00	1.20	1.21
SHM1	0.99	1.02	1.01	1.12	1.25	1.27	1.01	1.21	1.20
AIP1	1.07	1.11	1.03	1.22	1.37	1.28	1.07	1.29	1.20

GPM1	0.93	1.11	1.01	0.98	1.48	1.21	1.02	1.22	1.20
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[00182] Two hundred and fifty one proteins were identified with at least 10% increase in protein abundance in the presence of bosutinib. Of these, the cytosolic HSP70 chaperone proteins SSA1 and SSB2 were the top 2 proteins stabilised, with up to ~1.6-fold dose-responsive increases in abundance measured (Figure 12B and Figure 12C). Furthermore, known interactors of SSA1 and SSB2 (SKP1 and STI1) are present within the top 20 drug-stabilised proteins, strongly implicating the ATP-consuming HSP70 chaperones as bosutinib targets (Figure 12D). Gene set enrichment analysis of stabilised proteins identified 77 significantly enriched biological pathways. The most enriched pathways included proteasomal ubiquitin-independent proteins (PRE1, PRE3-9, PUP1-3) and other metabolic pathways, such as dicarboxylic acid, glutamine, homoserine, hexose and gluconeogenesis metabolism (Figure 12E). Several components of these pathways utilize ATP (e.g. proteasome complex, PCS60, THR1, PCK1 and PYC2), and non-specific inhibition by bosutinib may contribute to reducing background lysate ATP metabolism. a proteomics time-course experiment was performed at 37°C to monitor protein stability during a CFPS reaction (Figure 13). SSA1 and SSB2 protein levels remained consistent, indicating that the dose-dependent stabilising effects of bosutinib were not confounded by intrinsic thermal instability.

[00183] **Example 6**

[00184] **Chemoproteomic-guided strain engineering to improve CFPS**

[00185] The BY4743-*Ssa1Δ* strain from the homozygous knockout collection was selected to investigate if single gene knockout could reproduce drug-mediated enhancement of yeast CFPS. Extracts from BY4743 WT and *Ssa1Δ* strains were prepared as previously described. As reported earlier, deletion of *SSA1* gene displayed no effect on cell growth (Figure 14). Glucose-driven CFPS reactions produced 30% more nLuc (9.1 µg/mL) when *Ssa1Δ* extract was used (Figure 15A). Moreover, addition of bosutinib resulted in significantly greater improvement of WT extract (2.6-fold) than the *Ssa1Δ* extract (2.0-fold). A similar pattern was observed with two other TKI drug hits (cerdulatinib and dasatinib), but not with nafarelin acetate GnRH agonist (Figure 15B). The lysates were further treated with drug combinations previously shown to improve yield (Figure 7C). Up to 49.7 and 28.1 µg/mL of nLuc was made using combined cerdulatinib and nafarelin treatment in parental and *Ssa1Δ* strains respectively. A very similar pattern was observed when comparing expression yields of another test protein, the E3 ligase Mdm2, in parental and *Ssa1Δ* extracts. In absence of drugs, yields were higher in

*Ssa1*Δ extract. Drug addition also resulted in less improvement of yield when added to the *Ssa1*Δ extract. Notably, expression yield of Mdm2 could be improved up to 44 times using the nafarelin and cerdulatinib combination. Figures 15C and 15D confirm that same drug effects are observed upon synthesis of a different and more complex protein, Mdm2. The effects appear to be greater but follow a similar pattern. Lysate from SSA1 deletion strain is expressing more Mdm2 compared to that from the WT strain (up to 7 times more) (Figure 15C). Drugs alone and in combination (Cer+Naf) increase Mdm2 production in both the WT and SSA1Δ extracts (Figure 15C). Lastly, drug effects are greater on WT lysates compared to the SSA1 lysate (Figure 15D).

10 [00186] **Equivalents**

[00187] The foregoing examples are presented for the purpose of illustrating the invention and should not be construed as imposing any limitation on the scope of the invention. It will readily be apparent that numerous modifications and alterations may be made to the specific embodiments of the invention described above and illustrated in the examples without departing from the principles underlying the invention. All such modifications and alterations are intended to be embraced by this application.

Claims

1. A method of increasing expression of a protein in a cell-free protein synthesis (CFPS)
5 system, wherein the CFPS system comprises a cell lysate, a reaction mixture, and a nucleic acid template that encodes the protein, the method comprising:
 - i) supplementing the CFPS system with one or more compounds which increase expression of the protein; and
 - 10 ii) incubating the CFPS system and the one or more compounds which increase expression of the protein under conditions that allow expression of the protein,wherein the expression of the protein is increased compared to a CFPS system expressing the same protein that is not supplemented with the one or more compounds.
- 15 2. The method according claim 1, wherein the one or more compounds which increase expression of the protein is selected from the group consisting of a kinase inhibitor, a gonadotropin-releasing hormone receptor agonist, and an antibiotic.
3. The method according to claim 2, wherein the gonadotropin-releasing hormone receptor agonist is nafarelin.
- 20 4. The method according to claim 2, wherein the kinase inhibitor is an ATP-competitive kinase inhibitor.
5. The method according to claim 4, wherein the ATP-competitive kinase inhibitor is selected from the group consisting of bosutinib, cerdulatinib, dasatinib, neratinib, and afatinib.
- 25 6. The method according to any one of claims 2 to 5, wherein the kinase inhibitor is bosutinib and the gonadotropin-releasing hormone receptor agonist is nafarelin.
7. The method according to any one of claims 2 to 5, wherein the kinase inhibitor is cerdulatinib and the gonadotropin-releasing hormone receptor agonist is nafarelin.
8. The method according to any one of claims 2 to 7, wherein the kinase inhibitor and the
30 gonadotropin-releasing hormone receptor agonist decrease ATP consumption by metabolic processes not necessary for expression of the protein.

9. The method according to claim 2, wherein the antibiotic targets gram-negative bacteria, and wherein the antibiotic targeting gram-negative bacteria is polymyxin B.
10. The method according to any one of the claims 2 to 9, further comprising supplementing the CFPS system with an energy source.
- 5 11. The method according to claim 10, wherein the energy source is a carbohydrate.
12. The method according to claim 11, wherein the carbohydrate is a monosaccharide.
13. The method according to claim 12, wherein the monosaccharide is glucose.
14. The method according to claim 10, wherein the energy source is creatine phosphate.
15. The method according to any one of claims 1 to 14, wherein the cell lysate is obtained
10 from a eukaryotic cell.
16. The method according to claim 15, wherein the eukaryotic cell is a yeast cell or a human cell.
17. The method according to claim 16, wherein the yeast cell is *Saccharomyces cerevisiae* or *Pichia pastoris*.
- 15 18. The method according to claim 17, wherein the *Saccharomyces cerevisiae* is selected from the group consisting of S288C strain and BY4743 strain.
19. The method according to claim 16, wherein the human cell is a Henrietta Lacks (HeLa) cell.
20. The method according to any one of claims 16 to 19, wherein one or more genes in the
20 eukaryotic cell is genetically modified.
21. The method according to claim 20, wherein the one or more genes in the eukaryotic cell is genetically modified by deletion of the one or more genes, and wherein the one or more genes encode a protein target of the one or more compounds which increase expression of the protein.
- 25 22. The method according to claim 21, wherein the gene is *SSA1*, and wherein the *SSA1* gene encodes a SSA1 protein.
23. The method according to any one of claims 1 to 22, wherein the protein is a heterologous protein.
24. The method according to claim 23, wherein the heterologous protein is a eukaryotic
30 protein.
25. The method according to claim 24, wherein the eukaryotic protein is post-translationally modified.

26. A protein obtained by the method according to any one of claims 1 to 25.

27. A cell-free protein synthesis (CFPS) system supplemented with a combination of cerdulatinib and nafarelin.