Enhancing Eukaryotic Cell-Free Systems through Genetic Engineering and Optimization of Protein Production Processes

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Declaration of authorship

I, Jeffrey Lesslie Schloßhauer, hereby declare that I alone am responsible for the content of my doctoral dissertation and that I have only used the sources or references cited in the dissertation.

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Summary

Cell-free protein synthesis can circumvent the challenge of producing membrane proteins and toxic proteins in living cells. Its open environment permits the addition of specific components to enhance protein solubility and introduce novel chemical reactants, such as non-canonical amino acids, for site-specific protein modifications. Eukaryotic cell-free systems are particularly advantageous for producing complex proteins, as they support post-translational modifications and optimal protein folding conditions. However, cultivating mammalian cells, like the commonly used Chinese hamster ovary (CHO) cells for pharmaceutical protein production, is costly.

This doctoral thesis aims to develop a cost-effective cell-free system for producing difficult-to-express proteins. To achieve this, an optimal gene location for stable CHO cell line development and subsequent preparation of translationally active cell lysate was identified. The efficiency of cell-free transcription was enhanced by incorporating T7 RNA polymerase into CHO cells, while translation initiation was improved by using CHO cells expressing a mutant of the α subunit of eukaryotic initiation factor 2. Additionally, straightforward orthogonal cell-free translation was achieved by introducing orthogonal aminoacyl tRNA synthetases into CHO cells, facilitating the cell-free production of site-specifically modified membrane proteins. Furthermore, the identified locus in the CHO genome was utilized to create a tetracycline-inducible expression system, enabling both cell-free and inducible cell-based production of challenging proteins at desired time points and cell densities.

Another goal of this research was to develop a yeast-based cell-free system using *Pichia pastoris*. This system combines the benefits of low-cost production, a eukaryotic environment, and the opportunity to synthesize difficult-to-express proteins. Moreover, it could be demonstrated that statistical Design of Experiments can be performed in a short time and on a small scale to identify optimal reaction parameters, facilitating the rapid adjustment of required protein synthesis conditions.

In summary, the established methods streamlined induced cell-based expression and cell-free protein synthesis by minimizing the need for external supplements, improving protein production, and reducing costs.

Zusammenfassung

Mit der zellfreien Proteinsynthese kann die oft problematische Herstellung von Membranproteinen und toxischen Proteinen in lebenden Zellen umgangen werden. Die offene Reaktion ermöglicht die Zugabe spezifischer Komponenten, um die Löslichkeit von Proteinen zu verbessern und die Reaktion um neue chemische Reaktanten, wie nicht-kanonische Aminosäuren, für die ortsspezifische Proteinmodifikationen zu ergänzen. Eukaryotische zellfreie Systeme sind besonders vorteilhaft für die Herstellung komplexer Proteine, da sie posttranslationale Modifikationen ermöglichen und optimale Bedingungen für die Proteinfaltung unterstützen. Die Kultivierung von Säugetierzellen, wie z. B. der für die pharmazeutische Proteinproduktion häufig verwendeten CHO-Zellen, ist jedoch kostspielig.

Ziel dieser Doktorarbeit ist die Entwicklung eines kostengünstigen zellfreien Systems für die Herstellung schwer zu exprimierender Proteine. Um dies zu erreichen, wurde ein optimaler Genort für die Entwicklung stabiler CHO-Zelllinien und die anschließende Herstellung von translationsaktiven Zelllysaten ermittelt. Die Effizienz der zellfreien Transkription wurde durch den Einbau der T7 RNA Polymerase in CHO-Zellen erhöht, während die Initiierung der Translation durch die Verwendung von CHO-Zellen, die eine Mutante der α-Untereinheit des eukaryotischen Initiationsfaktors 2 exprimieren, verbessert wurde. Darüber hinaus wurde die zellfreie orthogonale Translation vereinfacht, indem orthogonale Aminoacyl-tRNA-Synthetasen in CHO-Zellen eingeführt wurden, was die zellfreie Produktion von ortsspezifisch modifizierten Membranproteinen ermöglichte. Zudem wurde der identifizierte Lokus im CHO-Genom genutzt, um ein Tetracyclin-induzierbares Expressionssystem zu schaffen, das sowohl die zellfreie als auch die induzierbare zellbasierte Produktion von anspruchsvollen Proteinen zu gewünschten Zeitpunkten und Zelldichten ermöglicht.

Ein weiteres Ziel dieser Forschung war die Entwicklung eines zellfreien Systems auf Hefebasis unter Verwendung von *Pichia pastoris*. Dieses System kombiniert die Vorteile einer kostengünstigen Produktion, einer eukaryotischen Umgebung und einer schnellen Synthese von schwer zu exprimierenden Proteinen. Darüber hinaus konnte gezeigt werden, dass die statistische Versuchsplanung in kurzer Zeit und im kleinen Maßstab durchgeführt werden kann, um optimale Reaktionsparameter zu ermitteln, was die schnelle Anpassung der erforderlichen Proteinsynthesebedingungen erleichtert.

Insgesamt zeigen die etablierten Methoden, dass sie die induzierte zellbasierte Expression und die zellfreie Proteinsynthese effizienter gestalten, indem der Bedarf an externen Zusätzen minimiert, die Proteinproduktion verbessert und die Kosten gesenkt werden.

1. Introduction

Protein production plays a pivotal role in unraveling the functions and interactions of proteins, fostering advancements in medical research, and fueling innovation across industrial biotechnological sectors. In recent decades, recombinant protein production has enabled progress in biotechnology by introducing genetic information into other organisms and thus producing proteins in large quantities. The growth of the global recombinant proteins market, which had a size of USD 3 billion in 2023, is mainly driven by recombinant proteins in modern medicine, and is expected to grow further, with an estimated market size of USD 6.47 billion in 2033[1]. Even though this growing field has tremendous potential, major challenges are the substantial production costs and time investments associated with recombinant protein production, ultimately resulting in elevated prices for the final therapeutic products.

1.1. Cell-based protein production of pharmacologically relevant proteins

The production of recombinant proteins is typically carried out in bacteria, yeasts, plants, insect cells, and mammalian cells[2,3]. The selection of the appropriate system depends on factors such as cost, lab equipment, scalability, as well as an environment for optimal folding and post-translational modifications (PTMs). Regardless of the expression organism used, the genetic information encoding the desired protein needs to be introduced into the host cells by transformation, transfection, and transduction, depending on the cell type[4,5]. *Escherichia coli* is considered the organism of choice for the production of recombinant proteins, as genetic modifications can easily be performed and high protein yields can be achieved at low cultivation costs[6–8]. Although eukaryotic PTMs are difficult to realize, much effort has been put into the generation of expression strains for the formation of disulfide bridges[9] and glycosylation[10]. However, eukaryotic proteins often aggregate due to the increased protein translation rates of *E. coli*, forming so-called inclusion bodies[11–13]. Extracting active proteins from these inclusion bodies poses significant challenges, leading to increased time and costs. Furthermore, the expression of complex proteins, which demand eukaryotic folding conditions and PTMs for optimal activity, often necessitates the use of eukaryotic protein expression systems[14]. This holds particular significance for proteins manufactured for therapeutic applications, as the glycosylation pattern not only impacts protein function but also affects protein stability, folding, transportation, and notably, the immune response[15]. Hence, the market

demand for mammalian cell factories have considerably increased. The proportion of FDA-approved biopharmaceuticals produced in mammalian cells accounted for 79% between 2015 and 2018, with FDA-approved monoclonal antibodies contributing 53% of total approvals[16]. Despite human cell lines having ideal environmental conditions for complex human proteins, 81% of approved biopharmaceuticals during the period 1987 to 2021 were produced in CHO cells[17]. The popularity of CHO cells compared to human cells is attributable to their ease of genetic manipulation, robustness, superior scalability, and lower overall costs[18]. The human-like PTMs in CHO cells meet the requirements of therapeutic proteins, such as low-immunogenic glycosylation patterns[19]. In addition, there is a low risk of contamination with human viruses, which is important for strict safety standards in the pharmaceutical industry[20]. Extensive CHO cell line development allowed several grams per liter of protein to be produced, reaching antibody titers in the range of 1.1-10 g/L[21–23].

Membrane proteins constitute another major class of pharmacologically significant proteins. Notably, G-protein-coupled receptors (GPCRs) and ion channels collectively account for 31% of human protein drug targets and a substantial 51% of small-molecule drug targets[24]. According to membrane topology prediction methods, it is assumed that 15-39% of the genome codes for membrane proteins[25]. Nevertheless, only 12,247 of 215,908 experimentally resolved membrane protein structures are currently deposited in the Protein Data Bank (PDB; accessed on February 19, 2024). The primary challenge arises from the complexity of achieving correctly folded membrane proteins, largely attributed to the hydrophobic nature of their transmembrane domains[26,27]. As a result, misfolding and aggregation during overexpression can be accompanied by the heightened risk of cellular toxicity and reduced cell growth[28–30]. This issue was addressed by lowering cultivation temperatures[31,32], employing strain engineering[33], utilizing co-expression of chaperones[34] and fusion of soluble protein tags[35] to improve the expression and solubility of membrane proteins. Moreover, studies have demonstrated that membrane proteins can undergo denaturation in substances such as sodium dodecyl sulfate, guanidine hydrochloride, and urea, followed by successful refolding in denaturant-free conditions in the presence of lipid bilayers[36–38]. Nonetheless, refolding of membrane proteins remains a major challenge[39].

1.1.1. Stable integration of genetic information into mammalian cells

The genetic information of the protein can be delivered to the expression host by transient protein expression or by long-term integration of the protein of interest through stable transfection and transduction. While transient transfection offers a rapid means to analyze expressed proteins with minimal resources and effort, the expression is inherently transient and cell-to-cell variability poses challenges[40]. To attain reproducible outcomes and isolating cells exhibiting the desired expression levels, there is often a pursuit for stable integration of recombinant deoxyribonucleic acid (DNA)[41,42]. For this purpose, the desired expression cassette can be randomly integrated into the genome through the presence of a selection marker. However, this may cause variable expression levels and genome instability due to the disruption of endogenous genes[43,44]. Alternatively, transposon systems can be used to integrate genes into the genome at specific sites in the presence of transposases and transposase recognition sites, surrounding the donor expression cassette[45]. However, transposon-based modifications can also integrate transgenes in undesired genomic locations, thereby hindering long-term stability[46]. Viruses provide another means of generating stable cell lines. While viral vectors may induce genome instability, they offer a key advantage: widely employed adenoviruses and lentiviruses infect cells and integrate their DNA with high efficacy into the host genome[47,48]. Alongside safety concerns, another disadvantage is the effort required to produce modified viruses in laboratories with an appropriate safety level[49]. In contrast to the methods mentioned above, homologous recombination can be implemented to specifically integrate desired DNA into the genome and thus circumvent the impact on endogenous genes[50]. The mechanism relies on DNA repair processes, particularly during cell division, where DNA damage, such as a double-strand break (DSB), prompts the affected DNA to utilize the sister chromatid or homologous chromosome as a template for repair[51,52]. As homologous recombination repair (HDR) occurs only in the late S and G2 phase of mitosis in somatic cells, the efficiency of the method is low[53]. Error-prone non-homologous end joining (NHEJ) is the preferred way to repair a DSB throughout the cell cycle, producing insertions and deletions at the repair junction[54]. As a result, NHEJ can be utilized to knock out genes of interest caused by mutations in essential protein domains or frame shift mutations[55].

The development of sequence-specific nucleases, such as zinc finger nucleases[56] and TALEN[57], has provided a tool to generate a DSB for subsequent HDR- or NHEJ-mediated repair. Although these sequence-specific nucleases recognize and digest genomic DNA site-

specifically, their creation is very time-consuming and costly[58]. Thus, different strategies for efficient genetic engineering became necessary.

1.1.2. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/Cas9 technology

In 2005, sections of the bacteriophage genome, called CRISPR loci, were found in the bacterial genome and linked to the defense mechanism against viruses[59]. Viruses were shown to be incapable of infecting bacteria harboring specific sequences (known as spacer) with homology to viral sequences (so-called proto-spacer) located between DNA repeats within the bacterial genome. As early as 2002, Jansen *et al*. identified CRISPR-associated (cas) genes adjacent to the CRISPR locus and hypothesized their functional relation[60]. In the following years, sequence motifs were identified which are associated with the proto-spacer sequences, termed proto-spacer adjacent motifs (PAM)[61,62]. A key to understanding the adaptive immune response was the finding that spacer sequences of bacteriophages were transcribed into small ribonucleic acids (RNAs) in *E. coli* and named CRISPR-associated RNAs (crRNAs)[63]. Finally, Deltcheva *et al*. were able to show that another small RNA, which they called trans-activating CRISPR RNA (tracrRNA), guides the Cas enzyme to its target in duplex with crRNA[64]. On this basis, Doudna and Charpentier demonstrated the fusion of crRNA and tracrRNA to form a single guide RNA (sgRNA), causing a site-specific DSB in complex with *Streptococcus pyogenes* Cas9[65]. This groundbreaking approach was soon extended, showing that the sgRNA-Cas9 complex can be tailored to precisely cleave genomic loci, facilitating the integration of foreign DNA into human cells via HDR using a repair template[66,67] (Figure 1). Since then, the functionality of the Cas9-ribonucleoprotein complex (RNP) has been adapted for many applications. A Cas9 mutant with its endonuclease activity removed, termed dead Cas9 (dCas9)[68], was developed to take advantage of the Cas9-RNP targeting function alongside an effector domain. Consequently, it has been demonstrated that gene expression can either be repressed[69] or activated[70] by fusing the repressive Krüppel-associated box domain of Kox1 or the transcriptional activator domain VP64 to dCas9. A broad spectrum of applications could be realized by fusing dCas9 with other effectors such as DNA methyltransferases[71], deaminases[72] and acetyltransferases[73]. This advancement led to the development of one of the foremost biotechnological systems in use today, which allows for gene knockout, the precise insertion of new genes, and the facilitation of epigenetic modifications.

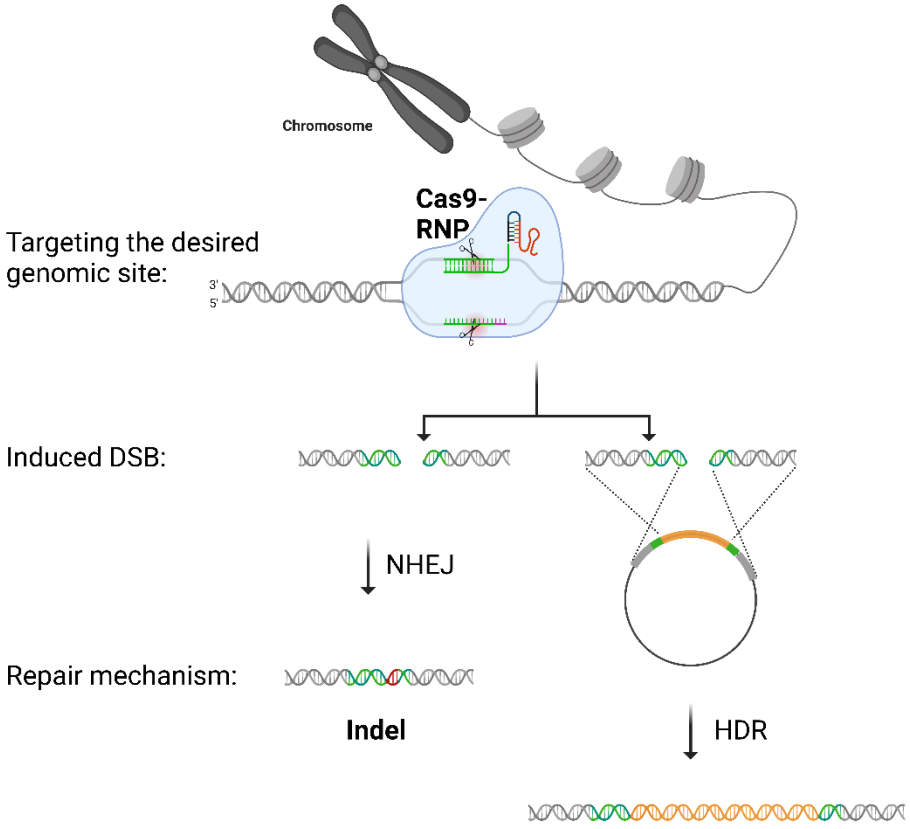

Figure 1: Scheme of CRISPR/Cas9-mediated modification of the genome. The Cas9-ribonucleoprotein complex (RNP) consists of a single guide RNA and the DNA cutting enzyme Cas9. The single guide RNA is a fusion construct composed of a target-specific CRISPR RNA sequence (green and black) and a scaffold sequence known as the trans-activating CRISPR RNA (red), connected by a linker loop (blue). A protospacer adjacent motif (pink) is located in the genomic DNA and is required for the Cas9-mediated cleavage to produce a double-strand break (DSB). Once a DSB is induced, the cell repairs the cleaved DNA by non-homologous end joining (NHEJ) resulting in insertions and deletions (Indels) at the target site (green) or by homologous recombination repair (HDR) using a transfected repair template. In this process, a gene of interest (orange) is flanked by sequences (grey and green) with homology to the genomic DNA at the target site. Created with BioRender.com.

1.1.3. Adaptable protein production using inducible expression systems

The constitutive expression of recombinant proteins, especially membrane proteins, can cause cell stress[74]. In addition, the generation of clonal cell lines for consistent protein production can substantially impact cell viability during cultivation at low cell densities after single cell deposition[75]. As an alternative approach, inducible expression systems provide the flexibility to adjust protein production levels by amplifying it at specified time points in the presence of an inducer[76]. Various inducible systems are used for the production of proteins in mammalian cells, including tetracycline (tet)-based systems[77], cumate-controllable systems[78], riboswitch-regulated approaches[79], tamoxifen[80], and rapamycin-induced[81] systems. Moreover, light-controlled systems are of particular interest as they allow spatiotemporal control over protein

expression, although the optogenetic switch leads to low induction efficiencies[82,83]. Despite the differing strengths and limitations of various inducible systems, cumate and tet-controlled systems are frequently utilized for switchable protein production in mammalian cells[84–87]. Using a tet-inducible system, Reeves *et al*. achieved the production of several milligrams of presumed opsin and its mutants[88]. They initiated GPCR expression in HEK293 suspension cells during controlled cultivation within a bioreactor. Tadauchi *et al*. employed the tet-based system to initiate the expression of both the heavy and light chains of a difficult-to-express therapeutic antibody at a desired time, aiming to enhance stable CHO cell line development and elevate final antibody titers[89].

1.2. Production of proteins outside the cell

When traditional cell-based production approaches its limits and induced protein expression through promoter leakage compromises cell viability, turning to cell-free reactions emerges as a promising alternative. Unlike cell-based approaches, cell-free protein synthesis (CFPS) eliminates the need for maintaining cell viability[90]. It operates by combining an amino acid mixture, nucleoside triphosphates (NTPs), an energy regeneration system, a template encoding a gene of interest, and a cell lysate within a suitable buffer system (Figure 2). This process enables the production of target proteins without the necessity of intact cells in a reaction timeframe typically ranging from 90 to 180 minutes[91]. The cell lysate contains crucial components such as transcription and translation factors, chaperones, ribosomes, transfer RNAs (tRNAs), and aminoacyl-tRNA synthetases (aaRS)[92,93].

A milestone in protein translation research was achieved by Matthaei and Nirenberg as they employed a cell-free system based on *E. coli* extracts to uncover the relationship between nucleotide triplets and the specific amino acids they encode[94]. Since then *E. coli*-based CFPS has been widely used for protein production because high yields can be achieved at low cost[95–98]. Similar to *E. coli* cell-based production, the capacity for PTMs in *E. coli* cell-free systems is limited, thereby complicating the production of complex eukaryotic proteins. In contrast, eukaryotic CFPS platforms such as those utilizing wheat germ extracts[99], tobacco BY-2 extracts[100], yeast extracts[101], insect cell extracts[102], rabbit reticulocyte extracts[103], CHO cell extracts[104], and human cell lysates[105] afford a range of PTMs tailored to their respective systems, albeit at a higher expense, due to lower protein yields and cell densities, as well as higher cultivation costs.

Despite variations in template features among different systems, CFPS generally categorizes into two approaches: initiating the reaction by introducing *in vitro* transcribed messenger RNA (mRNA), known as the linked reaction, or by adding DNA for the coupled transcription-translation reaction[106]. To facilitate DNA transcription into mRNA, a T7 promoter is frequently positioned upstream of the coding gene[100,107,108]. This strategy allows the usage of the exceptional processivity and specificity of the bacteriophage T7 RNA polymerase[109]. In *E. coli*-based CFPS, the supplementation of recombinant T7 RNA polymerase may be unnecessary if T7 RNA polymerase is naturally present in the cell lysate obtained from genomically recoded *E. coli* cells[110]. Conversely, in eukaryotic-based CFPS, the reaction commonly involves the addition of commercially purchased or in-house prepared T7 RNA polymerase[111–113]. Once transcription has started, cap-dependent translation initiation is a viable approach for initiating protein synthesis in cell-free systems, mirroring the typical mechanism observed in the majority of cells[114]. The cap structure is a 7-methylguanosine located at the 5' end of mRNA and is linked through a 5' to 5' triphosphate bridge to the first coding nucleotide[115]. The 7-methylguanosine cap can be recognized by the heterotrimeric cap-binding complex of eukaryotic initiation factor 4F, thereby recruiting the 43S initiation complex[116]. As part of the 43S initiation complex, the ternary complex contains the initiator methionine-charged tRNA, eukaryotic initiation factor 2 (eIF2), and GTP[117]. Upon binding to the 5' end of mRNA, the 43S initiation complex scans along the mRNA for the AUG start codon[118]. Due to the complex assembly of the translation initiation complex, protein translation initiation is the rate-limiting step of protein synthesis and thus the key to controlling the rate of protein synthesis[119,120]. Cap-independent translation based on an internal ribosome entry site (IRES) marks an efficient alternative and was first found in viruses[121]. An IRES can recruit the preinitiation complex to the start codon without the need for the 5' cap of the mRNA. Various of these highly structured regulatory elements in the 5' untranslated region exist and can be differentiated by various lengths, modes of action, structural motifs, and the requirement for additional factors[122]. The Encephalomyocarditis virus (EMCV)-IRES, often utilized for gene expression in mammalian cells, relies on the mammalian eIF4F complex and eIF2 to recruit the small ribosomal subunit[123,124]. However, phosphorylation of the alpha subunit of eIF2 (eIF2α) was reported to inhibit protein translation[125]. Previous findings indicated an elevation in eIF2α phosphorylation during cell-free reactions, which may be caused by endoplasmic reticulum (ER) stress[126]. In contrast, the cricket paralysis virus (CrPV)-IRES facilitates fast translation initiation without further initiation factors[127], hence its widespread application in eukaryotic CFPS[105,128,129].

CFPS can be further modified using signal peptides to allow translocation into the ER, thus offering PTMs. Upon the fusion of a signal peptide to the protein's N-terminus, it was shown that supplementing the open reaction with ER-derived vesicles promotes translocation into these so-called microsomes[130–132]. Demonstrating the efficacy of a melittin signal peptide (Mel), it was revealed that the requirement for microsome supplementation in the cell-free reaction could be circumvented through adjusting cell lysate preparation[102,104]. This was achieved by preserving the translocation machinery, including intact microsomes, during cell lysate preparation from *Sf*21 and CHO cells. Eukaryotic cell lysates containing microsomes could be utilized to produce ion channels[133], GPCRs[134], antibody fragments[135] and complex enzymes[136].

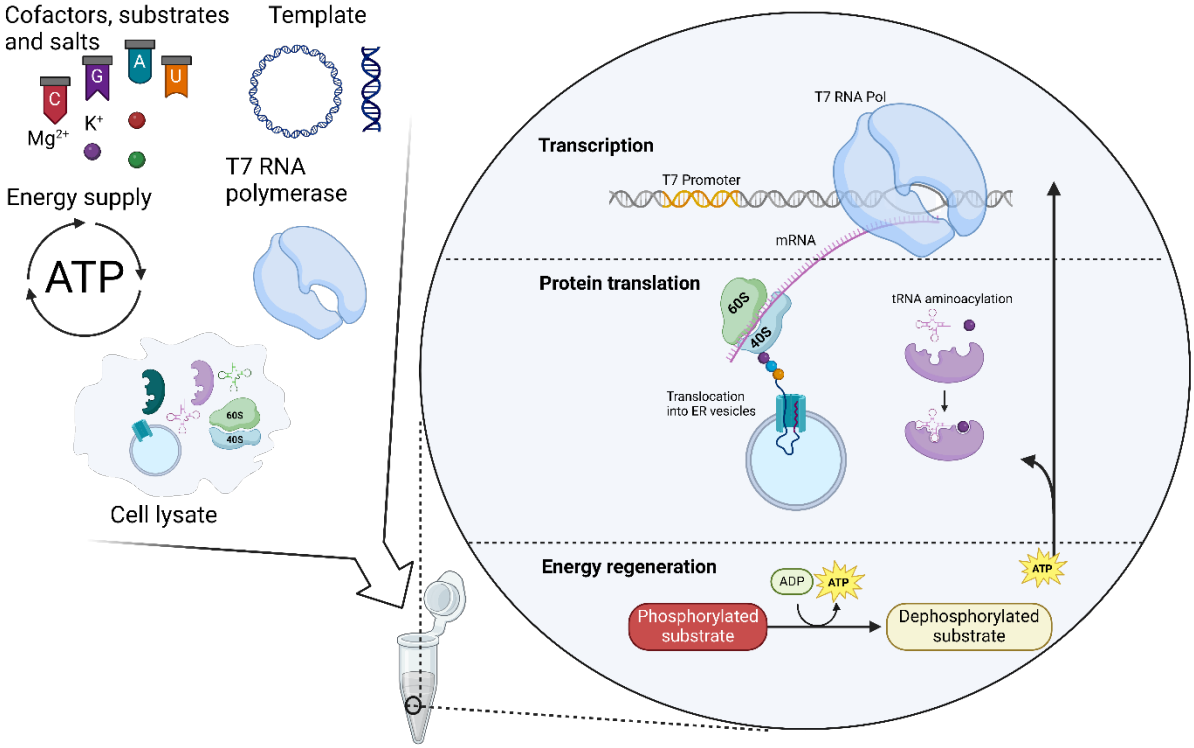

Figure 2: Scheme of cell-free protein synthesis. A cell-free protein synthesis reaction is performed in a tube based on a translationally active cell lysate and supplementary factors. After transcription of a desired template, protein translation can be performed in the presence of endoplasmic reticulum (ER) vesicles. During cell-free protein synthesis, an energy regeneration system is required to counteract the adenosine triphosphate (ATP) consumption of transcription, translation, tRNA aminoacylation, and protein folding. The figure was created with BioRender.com and is adapted from Schloßhauer *et al.*[137,138].

1.2.1. Energy regeneration as the driving force of cell-free protein synthesis

In cell-free reactions, adenosine triphosphate (ATP) is required to fuel tRNA aminoacylation, transcription, translation, and protein folding. Regeneration of ATP during CFPS can be achieved by using a secondary energy source in the form of high-energy phosphate donors including creatine phosphate, phosphoenolpyruvate, or acetate phosphate[139]. Observations revealed that in the one-pot batch format, the translation rate in the cell-free reaction containing high-energy phosphate donors decreases after several hours, varying depending on the specific cell-free system utilized[140,141]. This phenomenon is largely attributed to the consumption of high-energy phosphate substrates, leading to increasing concentrations of inorganic phosphate. Consequently, magnesium ion homeostasis is disturbed, since inorganic phosphate chelates free magnesium ions[142,143]. Several efforts have been implemented to reduce inhibitory byproducts. Removal of byproducts could be achieved using a semi-continuous cell-free reaction format[144,145]. The exchange of inhibitory components and fresh substrates via a semi-permeable membrane prolonged cell-free reactions and boosted protein yields. Kim and Swartz counteracted the reduced magnesium concentration by supplementing additional magnesium acetate during a CFPS reaction, thereby elevating chloramphenicol acetyltransferase yields[146]. Another study circumvented the accumulation of inorganic phosphate by leveraging the endogenous enzyme maltodextrin phosphorylase naturally present in the *E. coli* cell extract[147]. Through maltodextrin phosphorylation, the inorganic phosphate was recycled, generating glucose-1-phosphate for energy production. Further studies employed metabolic enzymes of the *E. coli* cell lysate to drive ATP regeneration using the phosphate-free energy source pyruvate[148] and polymeric carbohydrates[149,150]. *E. coli*-based CFPS could be enhanced by supplementing crucial cofactors like nicotinamide adenine dinucleotide (NAD) and Coenzyme A, essential for ATP production from pyruvate via acetyl-CoA and acetyl phosphate[151]. Anderson *et al.* introduced a novel eukaryotic energy regeneration system, offering an alternative to the widely employed creatine kinase/creatine phosphate (CK/CP) system[152]. In this approach, prolonged *Saccharomyces cerevisiae*-based CFPS and cost reduction were attained by exploiting glycolysis with glucose as a substrate. Despite its relatively high cost, the CK/CP system remains the predominant energy regeneration system for eukaryotic cell-free reactions to date[153–157].

1.2.2. Applications of cell-free protein synthesis platforms

Beyond the complexities of membrane protein synthesis, toxic proteins present another hurdle in cell-based methodologies as they have the potential to harm the intact cells. Given the unclear function of toxins in biological pathways and their critical use in analytical assays, inhibitor screenings, and antibody production, there is a high demand for the synthesis of toxins. Accordingly, toxins such as non-hemolytic enterotoxin[158], Shiga toxin[159], venom components[160] and enterotoxin hemolysin BL[161] could be synthesized using cell-free systems. The cell-free synthesis of unspecific peroxygenases emerges as a practical alternative for producing these fungal biocatalysts, given the inherent difficulties in achieving both homologous and heterologous expression within cells[156]. Due to the open nature of CFPS, an oxidized variant of heme can be supplemented, leading to proper folding of the heme-containing unspecific peroxygenases[136].

CFPS offers a solution to the hurdles encountered in cell-based ion channel expression and purification, achieved through the integration of microsome-based cell-free reactions with planar lipid bilayer electrophysiology[157,162]. Synthesizing ion channels on artificial scaffolds can be achieved similarly, irrespective of their localization, whether within plasma membranes or organelles, highlighting the advantage of CFPS[163]. Furthermore, the open environment of the cell-free reaction can be tailored by incorporating detergents and nanodiscs, thus enhancing the solubility of ion channels, transporters, and GPCRs[91,164]. Consequently, detergent-sensitive GPCRs could be manufactured in a properly folded state within nanodiscs with defined lipid compositions and microsome-containing cell lysates, facilitating ligand binding[165,166].

Beyond manufacturing biomolecules, CFPS allows the rapid prototyping of biosynthetic pathways, offering a means to accelerate the study of metabolic mechanisms[167,168]. Moreover, freeze-dried CFPS systems can empower *in vitro* diagnostics and biosensor-based detection of diverse molecules[169–171]. For instance, one strategy involves the detection of small molecules and pathogenic nucleic acids in portable materials, achieved through the deployment of freeze-dried cell-free reaction components[172].

With its ability to be performed in a short time, CFPS holds significant promise for screening applications. In a wheat germ-derived cell-free platform, PCR templates sourced from a cDNA library facilitated the parallel synthesis of 50 proteins, eliminating the requirement for labor-intensive cloning steps[173]. A recent study showcased the assessment of antigen-antibody interactions within a single day using high-throughput techniques[174]. Using a vast repertoire of hundreds of antibodies tailored for the spike protein of SARS-CoV-2, the investigators

identified optimal candidates by synergizing cell-free DNA assembly and CFPS methodologies, facilitated by liquid handling stations. Next-generation screening platforms have the potential to streamline the generation of virus-like particles, thereby enhancing vaccine manufacturing processes[175,176].

The versatile applications of cell-free protein synthesis and novel advancements of the system can lead to exciting new applications.

1.3. Site-specific incorporation of non-canonical amino acids into proteins at desired sites

In the context of targeted cancer therapy, antibody–drug conjugates (ADCs) offer significant potential. These compounds consist of an antibody directed against a specific cancer marker, covalently attached to a cytotoxic drug, presenting a promising avenue for treatment[177]. A common strategy is to utilize naturally present amino acids in antibodies, for instance cysteines can be coupled to drugs containing maleimides after reduction of the disulfide bonds[178,179]. The conjugation of amine groups from lysines to N-hydroxysuccinimide ester can be effectively accomplished, given the abundant presence of over 80 lysine residues within immunoglobulin scaffolds[180]. Despite efforts, ADCs exhibit heterogeneous mixtures, with drugs distributed unevenly across antibody sites[181,182]. This stereochemical heterogeneity can be bypassed through site-specific conjugation strategies, including short peptide tags, glycoengineering and insertion of non-canonical amino acids[183].

Specific conjugation via a LPXTG peptide located in the antibody can be achieved by sortase A, discovered in *Staphylococcus aureus*[184,185]. Protein trans-splicing relies on the creation of a thioester intermediate facilitated by split intein peptide tags fused to substrates that need to be conjugated[186]. A variant of this method is expressed protein ligation, where an intein tag and a synthetic peptide featuring a N-terminal cysteine are employed[187]. However, the attachment of peptide tags is restricted to either the N- or C-terminus of proteins. Instead, glycosylation sites of antibodies can be modified by azido-tagged glycans for subsequent ligation to drugs[188]. Although glycosite-specific ADCs boast high efficacy and stability, their reliance on multiple enzymes imposes substantial expenses[189,190]. A different way to introduce a reactive moiety into antibodies at desired sites is to expand the standard genetic code by non-canonical amino acids (ncaa). Across all organisms, 20 canonical amino acids are encoded through 61 codons, while the three stop codons, opal (UGA), ochre (UAA) and amber (UAG) terminate

translation[191]. Notably, a few organisms use stop codons to encode for the additional proteinogenic amino acids selenocysteine and pyrrolysine[192]. For all amino acids, the attachment to the 3' end of their cognate tRNAs is catalyzed by specific aaRS via esterification, leading to the formation of aminoacyl-tRNA along with the hydrolysis of ATP[193]. Guided by the anticodon of the aminoacyl-tRNA, amino acids are integrated into the elongating polypeptide chain when encountering the corresponding sense codon. The introduction of novel ncaa with desired properties becomes feasible, when using aaRS from evolutionary distant organisms, such as the archaeal pyrrolysyl-tRNA synthetase (PylRS)/tRNA(pyl) pair[194]. When the novel amino acid bears structural similarities to pyrrolysine, it becomes recognizable by PylRS, facilitating the attachment of ncaa to the corresponding tRNA(pyl), which contains an anticodon for the amber stop codon. As a consequence, the ncaa is integrated into the protein at the UAG codon during translation (Figure 3). Stop codons aren't exclusively recognized by tRNAs carrying the anticodon for UAG, but also by the eukaryotic release factor RF1[195]. This creates competition, where protein synthesis may terminate prematurely at the desired insertion site through RF1 binding or the protein is modified by the ncaa, resulting in full-length protein. A crucial requirement for a proficient integration of ncaa into proteins is the prevention of cross-reactivity between the endogenous amino acids, tRNAs, and aaRS, and the introduced counterparts[196]. These introduced elements are termed 'orthogonal'. Commonly used orthogonal translation systems are based on evolved archaeal PylRS[197,198], *Methanocaldococcus jannaschii* tyrosyl-tRNA synthetase (TyrRS)[199,200] and *E.coli* TyrRS[201,202] variants. The engineering of orthogonal aaRS allows for the efficient incorporation of ncaa with unique biophysical properties, such as photo-activatable crosslinkers, spin labels, and fluorescent probes, into proteins[203,204]. Furthermore, these modified proteins can be conjugated with other molecules through the introduction of reactive groups to create diverse outcomes, such as synthetic PTMs[205]. This process, known as click chemistry, is characterized by its specificity, simplicity, and broad applicability[206]. Commonly employed reactive groups are azides, alkynes, and alkenes, which can participate in reactions such as Staudinger ligation or cycloaddition to form desired conjugates[207–209]. Notably, the inverse electron-demand Diels–Alder [4 + 2] cycloaddition (IEDDA) is exceptional due to its high reaction speed, enabling sensitive applications[210,211]. In the IEDDA reaction, tetrazines are linked via relatively large and bulky reactive groups such as bicyclononyne (BCN) and strained cyclooctyne (SCO). The PylRS enzyme provides the capability to incorporate bulky BCN and SCO lysine derivatives into proteins through the modular substrate-binding pocket of certain mutants[212]. However, the N-terminal domain of PylRS presents a challenge as it can lead to enzyme aggregation[213]. To

address this issue, alternative PylRS variants lacking the N-terminal domain are currently in development[214,215].

The site-specific modification of proteins based on orthogonal systems facilitates the development of various products ranging from drug delivery by ADCs to fluorescently labeled molecules. One concern may arise from the potential toxicity of the organic solvents used to dissolve the ncaa, or from the challenge of transporting the desired ncaa across the cell membrane into the cell[216,217]. Alternatively, the cell-free reaction format enables the site-specific insertion of desired amino acids within antibodies without depending on cell growth[218].

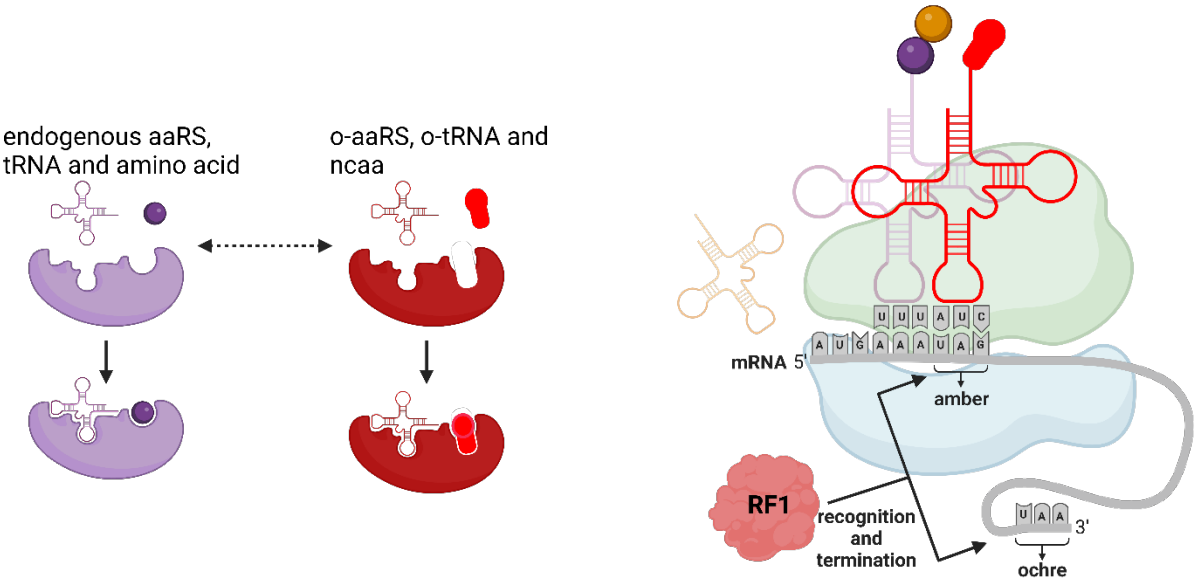

Figure 3: Scheme of orthogonal translation systems. An orthogonal aminoacyl-tRNA synthetase (o-aaRS), orthogonal tRNA (o-tRNA), and non-canonical amino acid (ncaa) must not cross-react with endogenous aaRS, tRNAs and amino acids (dashed arrow). Upon aminoacylation of o-tRNA and ncaa by the o-aaRS, the orthogonal aminoacyl-tRNA can transfer the ncaa to the growing polypeptide chain once a UAG amber stop codon is reached on the messenger RNA (mRNA). However, release factor 1 (RF1) can recognize the amber stop codon, resulting in a competition between full-length product containing the ncaa and premature termination product. Created with BioRender.com.

2. Publications

2.1. Cell Engineering and Cultivation of Chinese Hamster Ovary Cells for the Development of Orthogonal Eukaryotic Cell-free Translation Systems

Within the scope of the doctoral thesis, the work investigated whether transient and stable transfection of orthogonal mutant *E. coli* TyRS and PylRS into CHO cells could simplify subsequent synthesis of a modified GPCR in cell-free reactions. Cultivation conditions were evaluated in both shake flasks and bioreactors. *HPRT1* and *C12orf35* were examined as target sites in the CHO genome for the integration of orthogonal *E. coli* TyRS. The analysis of the resulting clone pools and cell lines provided insights into suitable hosts for cell lysate production.

Declaration of own contribution

Shared 1st authorship: I, Jeffrey Lesslie Schloßhauer, isolated clonal cell lines from the stably transfected CHO clone pools. Moreover, I characterized the CHO clone pools and clonal cell lines by qPCR, western blotting, genotyping PCR, and produced cell lysates from clonal cell lines. Furthermore, I performed the cell-free protein synthesis reactions with the subsequent analysis, wrote the manuscript, and prepared the figures. Niño Cavak and Lena Thoring designed constructs, performed transfections, and produced cell lysates. Lena Thoring, Anne Zemella, and Stefan Kubick originated the concept of the project and revised the manuscript.

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Cell Engineering and Cultivation of Chinese Hamster Ovary Cells for the Development of Orthogonal Eukaryotic Cell-free Translation Systems

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The investigation of protein structures, functions and interactions often requires modifications to adapt protein properties to the specific application. Among many possible methods to equip proteins with new chemical groups, the utilization of orthogonal aminoacyl-tRNA synthetase/tRNA pairs enables the site-specific incorporation of non-canonical amino acids at defined positions in the protein. The open nature of cell-free protein synthesis reactions provides an optimal environment, as the orthogonal components do not need to be transported across the cell membrane and the impact on cell viability is negligible. In the present work, it was shown that the expression of orthogonal aminoacyl-tRNA synthetases in CHO cells prior to cell disruption enhanced the modification of the pharmaceutically relevant adenosine A2a receptor. For this purpose, in complement to transient transfection of CHO cells, an approach based on CRISPR/Cas9 technology was selected to generate a translationally active cell lysate harboring endogenous orthogonal aminoacyl-tRNA synthetase.

Keywords: orthogonal translation, cell-free protein synthesis, CRISPR, amber suppression, *E. coli* tyrosyl-tRNA synthetase, *M. mazei* pyrrolysyl-tRNA synthetase, membrane protein, C12orf35

INTRODUCTION

The production of proteins represents an important building block to elucidate the fundamental biochemical signatures of cells and forms the basis for diverse industrial applications. Therefore, further development of appropriate production systems is of particular interest. Cell-based systems are commonly exploited to address questions in basic research and to meet the demand for biotechnologically relevant proteins such as drug targets. Proteins can be equipped with special properties or new reactive groups for broader applications. Although conjugation of proteins *via* cysteine residues with sulfhydryl chemical groups or coupling of lysine residues with N-hydroxysuccinimide esters is widely used, the ligation of the reacting amino acids is not homogeneously distributed and resulted in a heterogeneous protein mixture (Shadish and DeForest, 2020). In addition, there are reports of intein-based protein ligation and conjugation of proteins by cysteine transpeptidases (Debelouchina and Muir, 2017; Vogl et al., 2021). However,

protein sequence motifs located at the N- or C- terminus must be incorporated into the protein of interest to ensure appropriate protein modification. In contrast, genetic code expansion strategies have the advantage that amino acid positions in proteins can be modified site-specifically (Chung et al., 2020). This requires an orthogonal aminoacyl-tRNA synthetase (aaRS)-tRNA pair, which does not cross-react with endogenous aaRS, tRNAs and amino acids, while maintaining high specificity towards the non-canonical amino acids (ncaa). Usually, a desired sense codon in the protein will be changed to an amber stop codon, recognized by the aminoacylated orthogonal tRNA or the release factor, resulting in either amber suppressed or truncated proteins (Beyer et al., 2020). In this process, ncaa with various chemical groups such as azides, alkynes and strained alkenes can be incorporated into the protein of interest, enabling specific reactions based on azide-alkyne cycloadditions, Staudinger ligation as well as tetrazine ligation (Kenry and Liu, 2019; Mushtaq et al., 2019). Moreover, various ncaa could be incorporated into proteins, such as photocrosslinking amino acids, NMR sensitive probes and amino acids, which were equipped with post-translational modifications like phosphorylation (Chin et al., 2002; Jones et al., 2010; Rogerson et al., 2015). Albeit orthogonal systems are utilized for protein modifications *in vivo*, ncaa must be supplemented to the culture medium at high concentrations as uptake into cells is limited, thus imposing a burden on the cells (Takimoto et al., 2010; Lin et al., 2017). Furthermore, overexpression of pharmaceutical proteins including membrane proteins can lead to decreased cell viability and growth, thereby limiting these systems for further applications (Gubellini et al., 2011). On the one hand, an alternative production system based on translationally active cell lysates, offers the possibility of producing proteins such as toxins and membrane proteins that can negatively affect cell growth and viability (Khambhati et al., 2019). Due to their open character, cell-free protein synthesis (CFPS) systems in general can be supplemented by various additives, making it feasible to realize protein modifications based on orthogonal translation systems (Lu, 2017). Previous work on *Sf*21 cell-free protein synthesis systems showed that the modified EGF receptor, which is misregulated in various tumors, was translocated into microsomal structures, originating from the endoplasmic reticulum (Quast et al., 2016; Thoring et al., 2017). In the process described by Quast et al., the ncaa p-azido-L-phenylalanine (AzF) was integrated at different amino acid positions based on the orthogonal mutant *E. coli* tyrosyl-tRNA synthetase (eAzFRS)-tRNAtyr pair. Kapoor et al. demonstrated conjugation of cell-free synthesized malaria surface antigen Pfs25 to a GPI linker derivate and following immunization of mice, leading to higher anti-Pfs25 antibody titers with Pfs25-GPI compared to Pfs25 (Kapoor et al., 2018).

The cell-free system can be further improved by directly overexpressing desired proteins in host cell-lines used for the generation of cell lysate. It has been shown that expression of T7 RNA polymerase in *E. coli* eliminates the need for supplementation of the subsequent cell-free reaction (Des Soye et al., 2019). In a previous study, orthogonal pyrrolysyl-tRNA synthetase (PylRS) was expressed prior to cell disruption of *E. coli* and followed by the incorporation of two unnatural lysine derivatives into EGFP in an orthogonal CFPS reaction (Chemla et al., 2015).

In contrast to frequently used *E. coli* systems, protein expression in eukaryotic systems is often more complex but necessary to provide proteins with post-translational modifications such as glycosylation, disulfide bond formation and lipidation (Gillette et al., 2019; Tripathi and Shrivastava, 2019). Approximately 70% of clinically approved proteins are produced in Chinese hamster ovary (CHO) cells due to their robustness, growth to high cell densities, high protein yields and the ability to synthesize complex mammalian proteins (Jayapal et al., 2007). Nowadays, protein expression is often based on stable transfected cells using sequence-specific recombinases and transposon-based modifications as well as RNA-guided nuclease based integration techniques, rather than random integration methods (Matasci et al., 2011; Zhang et al., 2015; Grav et al., 2017). Of particular importance is the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology for genome editing in mammalian cells, which now makes it possible, to specifically knock out genes and insert expression cassettes at well-defined positions into the genome with high efficiency (Jinek et al., 2012; Ran et al., 2013). For protein overexpression, transcriptionally active loci in the genome that are less affected by gene silencing are preferentially targeted. To date, various loci including *HPRT*, *H11*, *COSMC*, *Rosa26* and *C12orf35* were addressed to insert desired transgenes into the CHO genome (Lee et al., 2015; Gaidukov et al., 2018; Kawabe et al., 2018; Zhao et al., 2018; Chi et al., 2019).

The objective of this study was to determine whether orthogonal aaRS can be expressed in CHO cells without diminishing the translational activity of the resulting cell lysate for CFPS. To this end, the orthogonal eAzFRS was transiently transfected into CHO cells and convenient cultivation formats were examined to attain a cell lysate optimal for modifying the G-protein-coupled adenosine A2a receptor in cell-free reactions. We also attempted to develop an orthogonal CFPS system based on stable transfection of eAzFRS into the CHO genome by targeting the *HPRT1* and *C12orf35* loci through CRISPR technology. A general workflow of the presented study is shown in **Figure 1**.

MATERIALS AND METHODS

Plasmids

The plasmids A2aRamb and A2aR for cell-free synthesis of adenosine A2a receptor (with and without an amber stop codon at amino acid position 215) were utilized as previously described (Zemella et al., 2019). A Nanoluciferase sequence was linked to the C-terminus of the receptor. The plasmid for cell-free synthesis of the modified *E.coli* tyrosyl-tRNA-synthetase (eAzFRS) in an *E.coli* based cell-free reaction was previously described (Zemella et al., 2019). The utilized PylRS-AF coding sequence contained the mutations Y306A and Y384F as

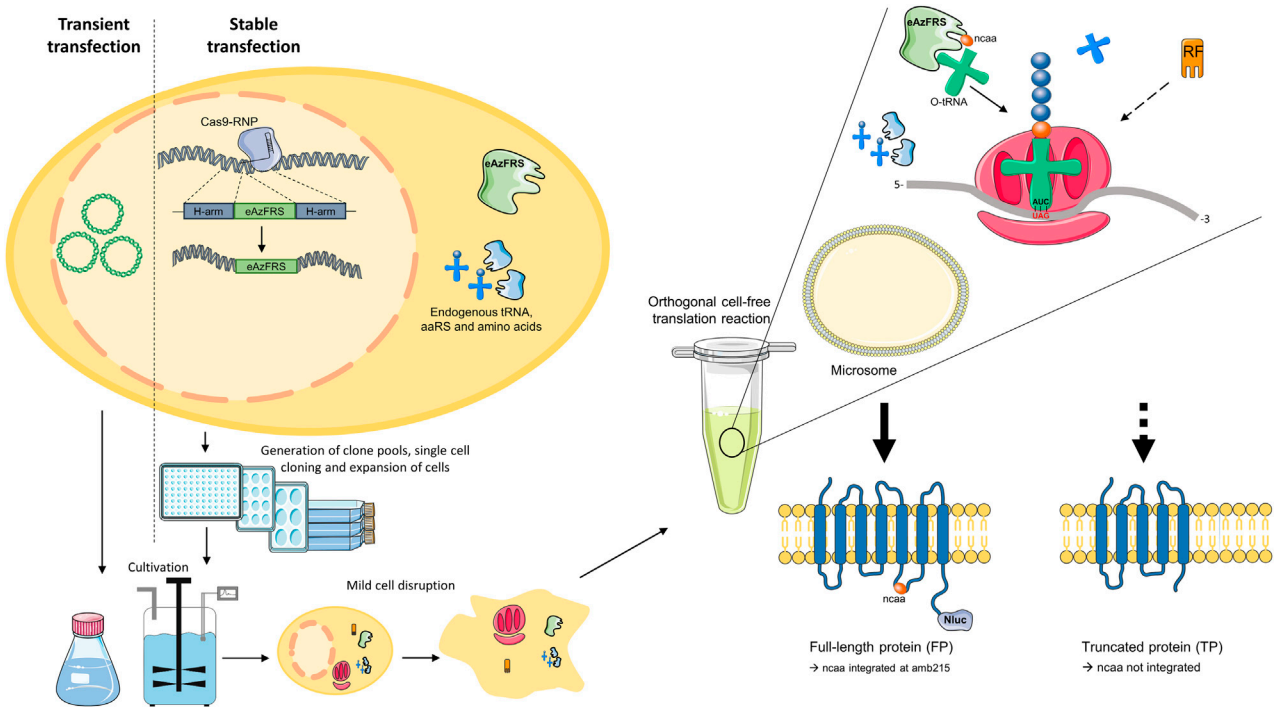

FIGURE 1 | General workflow. CHO cells were either transiently or stably transfected. While transiently transfected CHO cells were cultured in shake flasks and bioreactors for two days, stable transfected cells were subjected to selection pressure to obtain clone pools and clonal cell lines. The adenosine A2a receptor, which is C-terminally coupled to a Nanoluciferase (Nluc), was used to evaluate the developed orthogonal modification system. In the presence of orthogonal eAzFRS and orthogonal tRNA (o-tRNA), as well as the non-canonical amino acid (ncaa) p-azido-L-phenylalanine, the amber stop codon UAG at amino acid position 215 can be addressed to produce amber suppressed full-length protein (FP). Upon binding of the eukaryotic release factor (RF) termination product (TP) will be produced. Arrows indicate the direction of the workflow. The dashed arrow indicates translation termination. Parts of the figure were created by using pictures from Servier Medical Art (http://smart.servier.com/). Servier Medical Art by Servier is licensed under a Creative Commons Attribution 3.0 Unported License.

previously reported (Yanagisawa et al., 2008). Transient transfection of CHO cells was performed with pcDNA3.1(+)-eAzFRS and pcDNA3.1(+)-PylRS-AF expression vectors. A Strep-Tag was fused to the C-terminus of eAzFRS, while a 6xHis-Tag was fused to the N-terminus of the PylRS-AF. For stable transfection of CHO cells the plasmid pSpCas9(BB)-2A-GFP (Plasmid #48138 from Addgene) was used to express Cas9 enzyme linked to GFP by a 2A self-cleavage peptide. The guide RNA sequences gRNA-HPRT1-T1: 5′-GTAGAATGATCAGTC AACAG-3′ and gRNA-C12orf35-T1: 5′-GCCCCTTACAGC TGTAGATA-3′ targeting the *C12orf35* and *HPRT1* locus in the CHO genome were extracted from the literature (Zhao et al., 2018). Novel gRNA sequences gRNA-HPRT1-T2: 5′-GGG GTTGTACCGCTTGACCA-3′ and gRNA-C12orf35-T2: 5′-GCCGGGACTTAACCACTCGA-3′ were designed with the CRISPOR gRNA design tool. Gibson assembly was used to clone the gRNA sequences into the gRNA_Cloning Vector (Plasmid #41824 from Addgene) according to the protocol previously described (Mali et al., 2013). The donor vector sequence for stable transfection was designed similar to a previously described study on CHO cells (Zhao et al., 2018). Therefore, a human cytomegalovirus promoter was used to drive gene expression of the GFP or eAzFRS gene and a bovine growth hormone (BGH)-polyA sequence for termination of transcription was used. The expression cassette for the resistance gene puromycin was under the control of the phosphoglycerate kinase-1 promoter and BGH-polyA utilized to terminate transcription. The expression cassette was flanked by locus (*HPRT1* or *C12orf35*)-specific homology arms. Additionally, gRNA sequences were located at the ends of the homology arms to create a linear donor in presence of Cas9 enzyme (**Supplementary Figure S1**).

Cell Culture

CHO-K1 cells were provided from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSMZ no: ACC110) and adapted to grow in suspension. CHO-K1 cells were cultured in serum-free ProCHO5 medium (Lonza) at 37°C, 5% CO_2 and 100 rpm. Cells were seeded at densities of 1×10^6 cells/ml and grown up to 5×10^6 cells/ml in shake flasks, unless otherwise noted. Cell growth was monitored by counting cells using trypan blue staining and a Luna-FL cell counter (Logos Biosystems, Gyeonggi-do, Korea).

Transient Transfection

CHO cells were transiently transfected with eAzFRS and PylRS-AF plasmids by polyethylenimine (PEI) reagent. Therefore, cells were grown to 2×10^6 cells/ml and plasmid was added in a ratio of

1.5 μg/10^6 cells, followed by 2 μg PEI reagent/10^6 cells. Afterwards cells were incubated for four hours rotating at 37°C and 5% CO_2 and diluted to obtain a cell density of 1 × 10^6 cells/ml in 1,000 ml final volume in either 2 L shake flasks or 1 L bioreactor vessels (Biostat B-DCUII, Sartorius Stedim Biotech GmbH). CHO cells were cultivated for two days and harvested for cell lysate preparation as described previously (Thoring and Kubick, 2018).

Stable Transfection and Single-Cell Cloning

Plasmid DNA was mixed in a ratio of 2:2:1 (eAzFRS donor vector: gRNA vector: Cas9 vector) to obtain 500 ng plasmid DNA in 5 μL PBS. The DNA was further diluted by addition of 100 μL Opti-MEM serum-free medium (Gibco). Lipid based transfection was conducted by combine the diluted plamsid DNA with 2.5 μL Lipofectamine LTX (Thermo Fisher Scientific) and 0.5 μL Plus reagent. After incubation for 30 min at room temperature the transfection mixture was added to 1 × 10^6 cells/ml in 500 μL serum-free ProCHO5 medium (Lonza) in a 24-well plate. Cells were mixed gently and centrifuged for 15 min at room temperature and 400xg to increase transfection efficiency similar to a previous report (Barbu and Welsh, 2007). Following 48 h at 37°C, 5% CO_2 without shaking, cells were selected by puromycin resistance. Therefore, a concentration of 10 μg/ml puromycin in culture medium was achieved for two weeks. Positively transfected cells were pre-selected by array dilution, while maintaining the puromycin selection pressure. Clone pools were isolated and analyzed by qPCR and submitted to single-cell cloning by limiting dilution.

Fluorescent Microscopy

The GFP fluorescence of stable transfected CHO cells was visualized by a Nikon Eclipse TS2 inverted microscope combined with the NIS-Elements imaging software. A combination of a fluorescent LED and a GFP-B filter cube was used, whereby the filter cube is composed of a 470/40 nm excitation filter, a 500 nm dichroic filter and a 535 nm barrier filter. Images were recorded with a Nikon DS-Fi3 camera system.

Genotyping PCR

Genomic DNA was extracted from 10^6 CHO cells by the Quick-DNA Miniprep Plus Kit (Zymo Research) according to the manufacturer's instruction. Following genotyping primer sequences flanking the integration side of the expression cassette in the *C12orf35* locus were designed: GT-C12-T2-FW 5′-TGCATGCACCACAGAGTCAT-3′ and GT-C12-T2-RV 5′-ACAGGGCGCTTTGATGGTAA-3′. Genotyping PCR was performed by combining 0.5 μM of each primer, 5 ng/μL genomic DNA, 1x HotStar HiFidelity PCR Buffer (Qiagen), 0.05 U/μl HotStar HiFidelity DNA Polymerase (Qiagen) and distilled water in a 20 μL reaction. The following temperature profile was used: 95°C for 5 min; 40 cycles of 94°C for 15 s, 62°C for 60 s, 68°C for 5 min; 72°C for 10 min. PCR-products were run on a 1% agarose gel and product size was analyzed by comparing them to the Quick-Load 2-Log DNA Ladder (0.1–10.0 kbp, New England Biolabs).

Quantitative PCR

Quantitative real-time PCR (qPCR) was used to determine the eAzFRS mRNA levels. Therefore, mRNA was extracted with the High Pure RNA Isolation Kit (Roche) according to the manufacturer's instruction. Afterwards cDNA was prepared by using the Transcriptor First Strand cDNA Synthesis Kit (Roche). qPCR-primer sequences qRT-Gnb1-FW 5′-CCATATGTTTCT TTCCCAATGGC-3′ and qRT-Gnb1-RV 5′-AAGTCGTCGTACCCAGCAAG-3′of the housekeeping gene G protein subunit beta 1 (Gnb1) were extracted from the literature (Brown et al., 2018). The primer sequences qRT-AzFRS-FW 5′-GGATAAGAACAGCGGCAAGG-3′ and qRT-AzFRS-RV 5′-TCCATCTCCACCATAGGCAC-3′ were used for eAzFRS amplification. The qPCR reaction mix consisted of 0.5 μM qPCR primers, 2x FastStart Essential DNA Green master-mix (Roche), 10 ng cDNA and water in a 20 μL reaction. qPCR reactions were analyzed by the LightCycler 96 System (Roche). The temperature profile of the qPCR was 95°C for 5 min, followed by 38 cycles at 95°C for 20 s, 63°C for 20 s and 72°C for 20 s. A melt curve analysis was performed to confirm the specificity of amplification. Therefore, samples were heated for 5 min at 95°C and cooled down to 60°C. Afterwards the samples were heated continuously at 0.1°C/s. Relative quantification was calculated using Gnb1 as reference gene and the sample with the lowest gene expression as a control according to the Pfaffl method (Pfaffl, 2001). Measurements were conducted in triplicate and data were expressed as the mean with standard deviation.

Bioreactor Based Cultivation

Initial cell densities of 0.7 × 10^6 cells/ml, unless otherwise noted, were achieved in a 1 L bioreactor vessel connected to the control unit (Biostat B-DCUII, Sartorius Stedim Biotech GmbH). The cells were cultured while pH and oxygen supply were kept constant until a cell density of approx. 3–7 × 10^6 cells/ml was reached. During fermentation the parameters stirrer speed, pH, pO_2 and cultivation temperature were tracked over time and cell counts were measured regularly. The batch process was repeated up to three cycles, while maintaining a defined initial cell density in each cycle.

Cell-free Protein Synthesis

CHO cell lysate for CFPS was prepared to contain endogenous microsomes, taking advantage of the endoplasmic reticulum, as previously described in detail (Brödel et al., 2014; Thoring and Kubick, 2018). Eukaryotic cell-free reactions were composed of 40% CHO cell lysate, 10 μM PolyG, 30 mM HEPES-KOH (pH 7.5, Carl Roth GmbH), 100 mM sodium acetate (Merck), 3.9 mM magnesium acetate (Merck), 150 mM potassium acetate (Merck), 100 μM amino acids (Merck), 250 μM spermidin (Roche), 2.5 mM Dithiothreitol (Life technologies GmbH), 100 μg/ml creatine phosphokinase (Roche), 20 mM creatine phosphate (Roche), 1.75 mM ATP (Roche) and 0.3 mM GTP (Roche). To enable the qualitative analysis of radio-labeled proteins by electrophoresis 30 μM radioactive ^{14}C-leucine (specific radioactivity 46.15 dpm/pmol, Perkin Elmer) was added to the reaction. Furthermore, 1 U/μl T7 RNA polymerase (Agilent), 0.3 mM of UTP (Roche), 0.3 mM CTP (Roche) and 0.1 mM of

the cap analogue m7G(ppp)G (Prof. Edward Darzynkiewicz, Warsaw University, Poland) were added to the reaction to implement transcription and translation reactions simultaneously. Orthogonal cell-free reactions based on eAzFRS were further supplemented with 4 μM orthogonal $tRNA_{Tyr}$ and 2 mM p-Azido-L-phenylalanine (AzF), while PylRS-AF based reactions were supplemented with 4 μM orthogonal $tRNA_{Pyl}$ and 2 mM strained cyclooctyne (SCO). Reactions containing purified eAzFRS were supplemented with 4 μM eAzFRS unless otherwise noted. Reactions were incubated for three hours at 30°C and 600 rpm. The eAzFRS and the *in vitro* transcribed tRNAtyr were prepared as previously described in detail (Zemella et al., 2019). Briefly, the eAzFRS was synthesized in a cell-free reaction based on *E. coli* lysate using the RTS 500 E.coli HY Kit (biotechrabbit). Purification of eAzFRS was performed using Strep-Tactin Superflow high capacity columns (IBA Life Sciences) due to the presence of a C-terminal Strep tag II within the coding sequence of eAzFRS.

Autoradiography of Radiolabeled Proteins

The crude reaction mixture was precipitated after CFPS by acetone. Therefore, 3 μL of reaction mix was diluted with 47 μL distilled water followed by three times the sample volume of cold acetone. After incubation on ice for 15 min samples were centrifuged 10 min at 16,000 × g. Supernatants were discarded while precipitated proteins were dried for 30 min at 45°C to get rid of remaining acetone. Afterwards, dried protein pellets were resuspended in LDS-sample buffer (Invitrogen) containing 50 mM DTT. Samples were mixed at 1,400 rpm at room temperature. Denaturing polyacrylamide gel electrophoresis (PAGE) was carried out with NuPAGE 10% Bis-Tris Gels. Gels were stained and dried by a vacuum chamber (Unigeldryer 3545D, Uniequip), deposited on phosphor screens (GE Healthcare) for three days and screens were scanned by an imaging system (Typhoon TRIO + Imager, GE Healthcare). The presence of ^{14}C leucine in CFPS allowed the visualization of synthesized proteins based on autoradiography.

Luciferase Assay

The analysis of Nanoluciferase (Promega) activity was performed by utilizing the Nano-Glo Luciferase Assay System (Promega). Therefore, 50 μL of the assay reagent was mixed with 3 μL crude mix of a cell-free reaction and luminescence was detected by the Multimode Microplate Reader Mithras^2 LB 943 (Berthold Technologies GmbH) by using an OD2 filter. Measurements were conducted in triplicate and data were expressed as the mean with standard deviation.

Semi-Quantitative Western Blot

5×10^5 CHO cells were lysed for 30 min on ice using RIPA lysis buffer (10 mM Tris–HCl pH 8, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.5 mM EGTA, 0.1% SDS, 0.1% sodium deoxycholate), supplemented with cOmplete-ULTRA protease inhibitor cocktail (Roche). The supernatant was isolated after 10 min centrifugation (16,000 × g) at 4°C. The protein concentration was determined by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and 2.5 μg proteins were heated at 70°C for 10 min in LDS sample buffer (Invitrogen). Samples were separated by denaturing polyacrylamide gel electrophoresis with NuPAGE 10% Bis-Tris Gels. Protein transfer to a PVDF membrane was carried out by the iBlot Dry Blotting System (Invitrogen). The membrane was blocked with 5% bovine serum albumin (BSA). The C-terminally located Strep-tag II of the eAzFRS coding sequence allowed western blot analysis by using anti-Strep II (ab76949, Abcam) as primary antibody. Furthermore, anti-Actin (I-19) (sc-1616, Santa Cruz Biotechnology) was utilized as primary antibody since β-Actin served as the internal control. Primary antibodies were diluted 1: 1,000 in TBS with 0.1% Tween 20 and 5% BSA. Detection of signals was enabled by using the anti-rabbit IgG, HRP-linked antibody (#7074, Cell Signaling Technology) as secondary antibody (1:3,000 in TBS with 0.1% Tween 20 and 5% BSA). After detection of eAzFRS, removal of antibodies from the blot membrane was achieved by incubating the membrane at 50°C for 30 min in stripping buffer (0.5 M Tris HCl pH 6.8, 10% SDS, 0.8% 2-mercaptoethanol). Following detection of β-Actin was performed on the stripped PVDF membrane. Band intensities of eAzFRS signals were normalized to β-Actin signals.

Data and Statistical Analysis

The amber suppression efficiency was calculated by dividing the relative light unit (RLU) value based on cell-free reactions containing the A2aRamb construct by the RLU value based on cell-free reactions containing the A2aR construct without an amber stop codon. After multiplying the ratio by 100 the amber suppression efficiency was expressed as a percentage. The experimental data were expressed as mean values ± standard error, with "n" representing the number of biological replicates if not otherwise specified. Differences between three independent experiments were analyzed using a Welch's *t*-test. The maximum growth rate of analyzed cell lines was calculated from the slope of log-transformed data. Therefore, non-linear fitting of cell growth data was performed by using the SRichards1 function of Origin (Pro) software, Version Number 2019 (OriginLab Corporation, Northampton, MA, United States).

RESULTS

Expression of eAzFRS in CHO Cells Prior to Cell Lysate Preparation Enables Orthogonal Cell-Free Reactions

A previous study on orthogonal translation based on *E.coli* cell-free systems showed that transformation of *E.coli* with the *Methanosarcina mazei* orthogonal pyrrolysyl-tRNA synthetase prior to cell disruption circumvent time-intensive purification steps, leading to an optimized orthogonal cell-free protein synthesis system (Chemla et al., 2015). Based on this, we aimed to transiently transfect CHO cells with eAzFRS to create an eukaryotic cell-free system with the ability to site-specifically modify difficult-to-express proteins, such as the membrane protein adenosine receptor A2a (A2aR). Additionally, the goal was to identify optimal cultivation

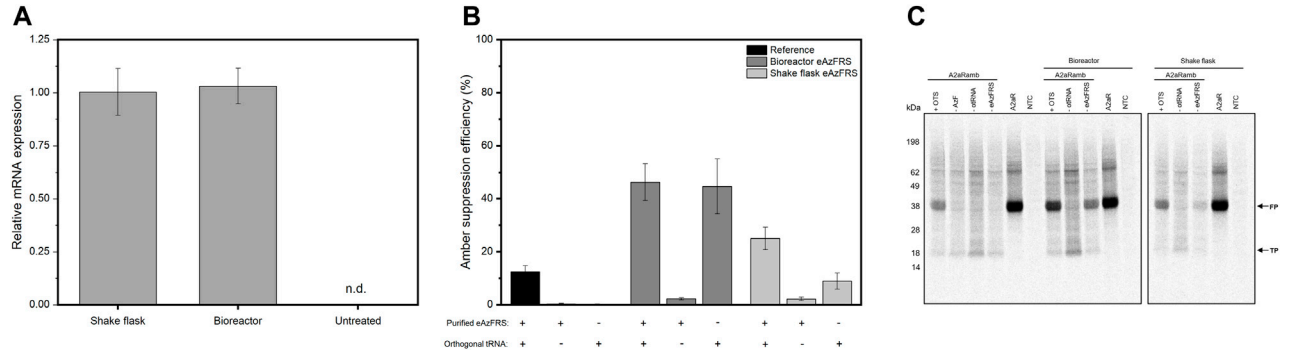

FIGURE 2 | CFPS based on transiently transfected CHO cells expressing eAzFRS. **(A)** Relative eAzFRS mRNA expression levels two days after transfection. Experiments were performed in technical triplicates and bars represent the standard deviation of the mean. **(B)** Amber suppression efficiency based on cell-free synthesized A2aR and A2aRamb constructs using reference cell lysates and cell lysates from transiently transfected CHO cells. Samples with a plus indicate the addition of either 4 μM purified eAzFRS and orthogonal tRNA, while a minus indicates the absence of the particular orthogonal component. Luminescence signals of cell-free reactions without a template (NTC) were subtracted from sample signals prior to calculation of suppression efficiency. Data are shown as mean ± SEM (n = 3). **(C)** Autoradiography of electrophoretically separated proteins based on a cell-free reaction with A2aR, A2aRamb constructs and a no-template control (NTC). n.d.: not detected, FP: Full-length protein, TP: Truncated protein, otRNA: Orthogonal tRNA, AzF: p-Azido-L-phenylalanine, OTS: Orthogonal translation system, TrCHO: Transiently transfected CHO cells.

conditions, while keeping transfection conditions constant, for producing a highly active CHO cell lysate enabling site-specific labeling of proteins in CFPS. Transiently transfected cells were either cultured in a shake flask or in a bioreactor to evaluate the influence of the culture format on cell lysate activity. Transcriptional analysis by quantitative PCR (qPCR) revealed similar eAzFRS mRNA levels for both cultivation formats (**Figure 2A**). As expected, mRNA expression could not be detected in non-treated cells due to the absence of endogenous eAzFRS. CHO cells were harvested two days post-transfection to ensure sufficient protein expression prior to cell lysate preparation. Characterization of orthogonal aaRS activity of the resulting processed cell extracts was achieved by evaluating cell-free synthesis of a reporter gene construct composed of the GPCR coding sequence containing an amber stop codon at position 215 and a C-terminal Nanoluciferase (Nluc) and is referred to as A2aRamb (Zemella et al., 2019). Once, the amber stop codon is suppressed by orthogonal tRNA-ncaa, the C-terminal Nluc activity can be correlated to the aminoacylation activity based on the orthogonal aaRS, since the luminescent output was shown to be linear (**Supplementary Figure S2**). A2aR construct without an amber stop codon was utilized to assess the efficiency of ncaa incorporation into the desired protein, referred to as amber suppression efficiency. A concentration of 4 μM purified eAzFRS was determined to be optimal in cell-free reactions based on CHO lysate (**Supplementary Figure S3**).

To exclude the possible negative effect of PEI reagent on cell lysate and thus on CFPS, a reaction set-up was included that was based on cell lysate derived from CHO cells transfected only with PEI reagent without any DNA template (**Supplementary Figure S4**). Unexpectedly, cell lysate based on CHO cells treated only with PEI resulted in higher protein expression compared to non-treated CHO cells, which may be caused by batch-to-batch variations affecting cell lysate efficiency. Considering the comparable resulting translationally active cell lysate of PEI treated CHO cells, cell lysates processed from transfected cells in a 1 L bioreactor and 2 L shake flask were analyzed for orthogonal translation in CFPS. A reference lysate based on non-treated CHO cells was utilized as a control. CFPS by using the reference lysate shows no translation after reaching the stop codon if omitting either orthogonal tRNA (otRNA) or purified eAzFRS, while combining orthogonal components led to a suppression efficiency of 12% (**Figure 2B**). In contrast, without addition of purified eAzFRS to cell-free reactions based on novel lysates, a higher amber suppression efficiency of 45% for bioreactor and 9% for shake flask based cultivations was detected. When supplementing cell-free reactions with purified eAzFRS, we came across further increase of suppression efficiencies up to 46% (bioreactor) and 25% (shake flask). Qualitative electrophoretic analysis of proteins from cell-free reactions with A2aRamb and A2aR supplemented with ^{14}C-leucine showed expression of full-length proteins (FP) and termination products (TP) as expected (**Figure 2C**). Overall, these results demonstrate the functionality of the orthogonal system by expressing the eAzFRS in CHO cells prior to cell lysate preparation.

Integration of eAzFRS Into the CHO Genome Creates a Robust System for Orthogonal Cell-Free Reactions

One should acknowledge the heterogeneous expression and the fact that eAzFRS expression only persists for a limited time if cells are transiently transfected. Here we conduct an alternative approach to generate an *in vitro* orthogonal translation system by utilizing CHO cells stably transfected with eAzFRS. Several studies have demonstrated the use of CRISPR/Cas technology to overexpress a diverse repertoire of proteins from defined genomic

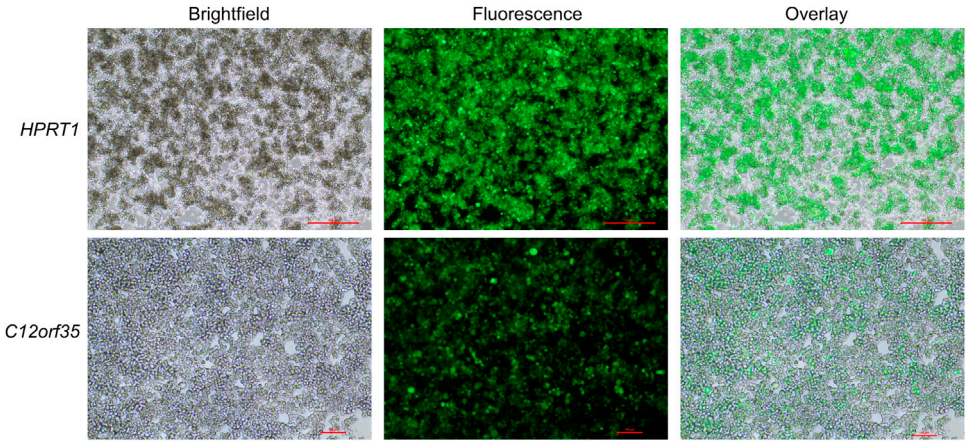

FIGURE 3 | CHO cells stable transfected with GFP. CHO cells were stable transfected by CRISPR/Cas9 to integrate GFP into the *HPRT1* or *C12orf35* locus. Cells were subjected to puromycin selection pressure to enrich positively transfected cells. Microscopic images of GFP clone pools are shown. Scale bars for *HPRT1* measure 500 µm, while scale bars for *C12orf35* measure 100 µm.

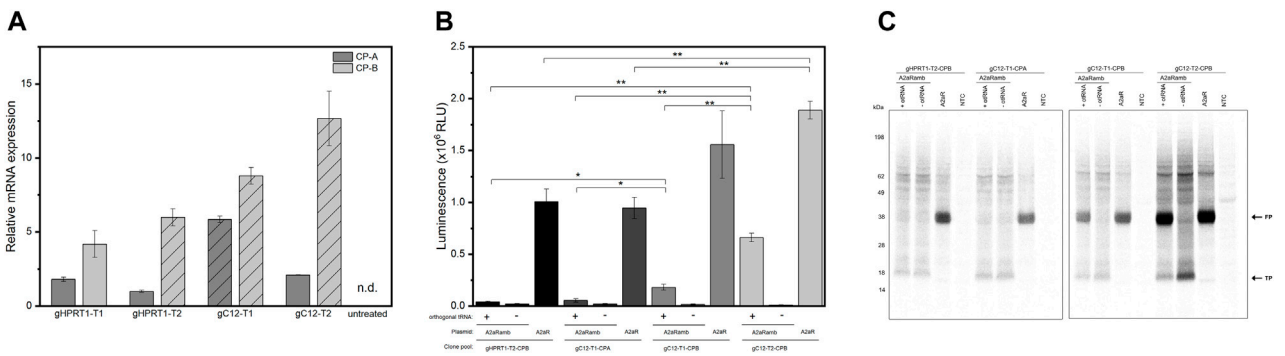

FIGURE 4 | CHO clone pools stable transfected with eAzFRS. **(A)** Relative eAzFRS mRNA expression levels of CHO clone pools. The line pattern indicates selected clone pools for cell-free synthesis. Experiments were performed in technical triplicates and bars represent the standard deviation. **(B)** Luciferase assay based on cell-free synthesized A2aR and A2aRamb constructs using cell lysates based on clone pools gHPRT1-T2-CPB, gC12-T1-CPA, gC12-T1-CPB and gC12-T2-CPB. Samples with a plus or minus indicate the addition or absence of orthogonal tRNA, respectively. Luminescence signals of cell-free reactions without a template (NTC) were subtracted from sample signals. Data are shown as mean ± SEM ($n = 3$). A Welch's *t*-test was performed to determine the statistically significant differences between samples (*p-value < 0.05; **p-value < 0.01). **(C)** Exemplary autoradiography of electrophoretically separated proteins of a CFPS based on the different clone pools. CP: Clone pool, n.d: not detected, otRNA: Orthogonal tRNA, FP: Full-length protein, TP: Truncated protein, NTC: No-template control.

TABLE 1 | Utilized guide RNA sequences

gRNA	Locus	Origin
gHPRT1-T1	*HPRT1*	gRNA from Zhao et al. (2018)
gHPRT1-T2	*HPRT1*	Novel gRNA
gC12-T1	*C12orf35*	gRNA from Zhao et al. (2018)
gC12-T2	*C12orf35*	Novel gRNA

loci in CHO cells (Lee et al., 2015; Eisenhut et al., 2018; Iwao et al., 2021). The genomic loci *HPRT1* and *C12orf35* were investigated for the generation of stable CHO cell lines using CRISPR/Cas, as reported in a recent study (Zhao et al., 2018). According to this, we have aimed to target respective loci to integrate an eAzFRS expression cassette into the CHO genome. Therefore, we analyzed four different gRNAs to modify the *HPRT1* and *C12orf35* locus of the CHO genome (**Table 1**). Either locus was targeted with one gRNA (referred to as gHPRT1-T1 and gC12-T1), which was previously identified (Zhao et al., 2018). Additionally, two gRNAs were designed that were predicted to have a higher specificity score value and less off-target effects and were referred to as gHPRT1-T2 and gC12-T2.

First, stable transfection of CHO cells was conducted by using a GFP expression cassette targeting either *HPRT1* or *C12orf35* to examine whether the locations were suitable for expression. Afterwards selection pressure (puromycin) was applied. Qualitative microscopic analysis demonstrated GFP expression of genetically modified cells (**Figure 3**). Both target sites showed a high proportion of enriched fluorescent cells, leading us to integrate an eAzFRS expression cassette into both loci. Selection of stable

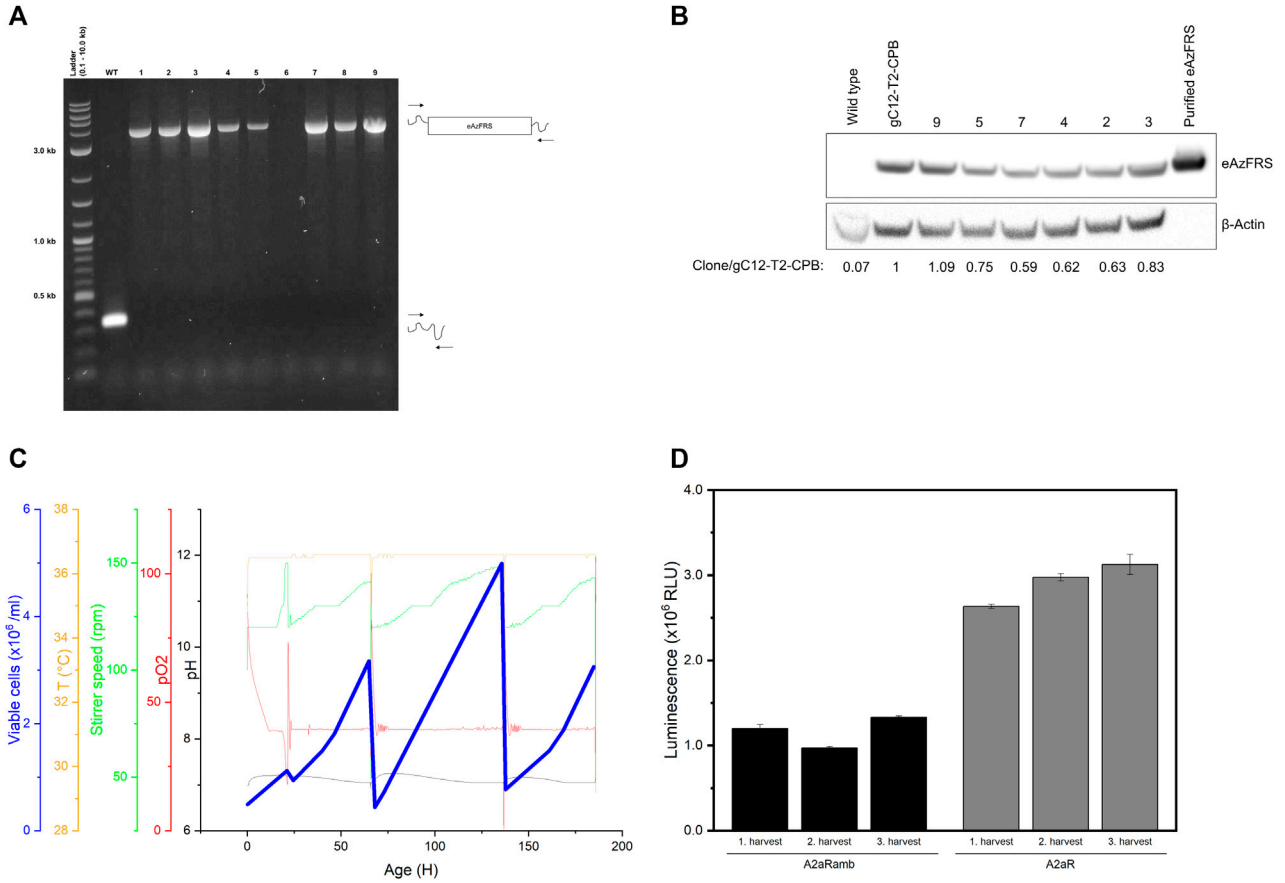

FIGURE 5 | Evaluation of clonal cell lines expressing the eAzFRS. **(A)** Genotyping PCR of clonal cell lines isolated from clone pool gC12-T2-CPB. The expected outcome is indicated by visualize primer binding (arrows) on wilde type (WT) and modified cells. A Quick-Load 2-Log DNA Ladder 0.1–10.0 kb (New England Biolabs) was used. **(B)** Semi-quantitative western blot of cell clones. eAzFRS band intensities were normalized to the internal β-Actin control. The indicated fold change is expressed as ratio of band intensity of the samples compared to clone pool gC12-T2-CPB. **(C)** Exemplary fermentation process of clone RS7. Cell harvesting was performed three times at highest peaks (blue line). **(D)** Luciferase assay of cell-free synthesized A2aR and A2aRamb constructs based on cell lysates prepared from three harvest points of a cultivation process using CHO clone RS7. Experiments were performed in technical triplicates and bars represent the standard deviation.

transfected cells was conducted by supplementation of puromycin. Two promising CHO cell clone pools for each gRNA target were evaluated by qPCR. The relative mRNA expression of eAzFRS is shown in **Figure 4A**. The change in gene expression is relative to the sample with the lowest gene expression. A 8.8-fold and 12.7-fold higher mRNA expression could be observed for the clone pools (CP) B by exploiting gC12-T1 and gC12-T2, respectively, compared to the lowest expression by using gHPRT1-T2 (CP-A). Further investigation on clone pools (marked in **Figure 4A**) by producing cell lysates highlighted their performance in orthogonal cell-free reactions. Therefore, three different cell lysates of gHPRT1-T2-CPB, gC12-T1-CPA, gC12-T1-CPB and gC12-T2-CP-B were generated and the eAzFRS activity was evaluated by the C-terminal Nluc activity. Indeed, significant higher luminescence could be observed for clone pool gC12-T2-CP-B compared to the other examined clone pools in cell-free reactions with the A2aRamb construct (**Figure 4B**). Furthermore, significant higher signals were achieved for clone pool gC12-T2-CP-B compared to gHPRT1-T1-CPB and gC12-T1-CPA using the construct A2aR without an amber position, indicating a generally higher protein production. Thus clone pool gC12-T2-CP-B was identified as the most promising candidate for subsequent isolation of CHO clones. Electrophoretic analysis supported the observed data of the luciferase assay (**Figure 4C**).

Single Clone Selection Circumvents Clonal Variations and Ensures Reproducibility

On the one hand, working with a heterogeneous pool of edited cells provides a time saving method to produce cell lysates containing an endogenous orthogonal aaRS. On the other hand, reproducibility is a main issue. As a consequence, our objective was to create a stable cell line to overcome clonal variability of edited cell pools. Nine single clones were isolated from clone pool gC12-T2-CP-B. Genotyping PCR with primers flanking the site of integration revealed only a short PCR product for WT cells, as expected (**Figure 5A**). Upon successful integration of the eAzFRS expression cassette an increase of PCR product size was anticipated. The clonality of eight produced cell lines could be demonstrated due to the presence of a PCR-

product at the expected size, while clone 6 showed no PCR product. Mono-allelic knock-in of the eAzFRS expression cassette can be excluded, since only one defined PCR product correlating to the exact size of the expression cassette was observed. The eAzFRS CHO clones (RS) 2–5, 7 and 9 were subjected to semi-quantitative western blot analysis, since increased outgrowth was observed compared to RS1 and RS8. A 1.09-fold increase in band intensity was observed for clone RS9 compared to the clone pool gC12-T2-CPB, while clone RS7 showed the lowest band intensity (**Figure 5B**).

Recombinant protein expression is often associated with a metabolic burden on the cells, leading us to compare unmodified CHO cells (wild type) with CHO clones with the highest (RS9) and lowest (RS7) eAzFRS expression by evaluating their maximum growth rates. Therefore, growth curves were plotted (**Supplementary Figure S5**) and resulting growth rates were compared by a Welch's *t*-test. The maximum growth rates of WT ($0.013\ h^{-1} \pm 0.00080$), RS7 ($0.014\ h^{-1} \pm 0.00076$) and RS9 ($0.015\ h^{-1} \pm 0.0047$) were similar and there was no significant difference determined. However, cell-free reactions based on cell lysate from clone RS9 led to a lower suppression efficiency (13%) than previously observed with the cell lysate prepared from the parental pool (**Supplementary Figure S6**). In contrast, higher amber suppression could be observed by using cell lysate based on clone RS7 (**Supplementary Figure S7**). Further examination of RS7 cells by producing cell lysates highlighted their performance during the fermentation process in a 1 L bioreactor and in orthogonal cell-free reactions. In particular, it was analyzed if the absence of puromycin during the fermentation process affects the stability of eAzFRS gene expression. Consequently, we set out to cultivate clone RS7 under controlled conditions for nine days and CHO cells were harvested after three, six and nine days of cultivation as depicted in **Figure 5C**. Therefore, a so-called repeated batch mode was applied, leaving CHO cells in the bioreactor after each harvest point. Cell lysates prepared at three harvest points were analyzed by cell-free synthesis of A2aRamb and A2aR to assess the functionality of the novel orthogonal system over the period of time. Using the A2aRamb construct, it was shown that no reduced enzyme activity was observed after three harvesting times (**Figure 5D**). General protein synthesis was also not negatively affected over the cultivation period, as indicated by using A2aR construct.

Moreover, it was shown that addition of increasing concentrations of purified eAzFRS did not elevate luminescent signals in cell-free reactions with A2aRamb using the RS7 cell lysate (**Supplementary Figure S7**). On the contrary, addition of 3 μM eAzFRS seems to have a negative impact on the reaction.

Transferability of the Approach to Orthogonal PylRS

A promising orthogonal aaRS is the double mutant pyrrolysyl-tRNA synthetase (PylRS-AF), which allows large and bulky ncaa to be incorporated into proteins (Oliveira et al., 2017). A major drawback of the PylRS is the frequently observed protein instability and tendency to aggregate (Wan et al., 2014). Therefore, the intent was to check whether the PylRS can also be expressed to obtain active orthogonal aaRS in the cell lysate, thus avoiding enzyme purification and possibly accompanying enzyme inactivity. For this purpose, we transiently transfected the PylRS-AF into CHO cells and prepared cell lysate based on shake flask and bioreactor based cultivation. We could demonstrate that a suppression efficiency of 24% (bioreactor) and 42% (shake flask) was achieved by incorporating strained cyclooctyne (SCO) into the reporter construct A2aRamb (**Figure 6A**). These findings could be verified by autoradiography due to the presence of ^{14}C leucine during the reaction (**Figure 6B**).

DISCUSSION

Chemical modification of proteins is widely used to study protein function and interactions in biological environments. Applied research can also facilitate medical diagnostics and therapy by linking drugs as well as fluorescent markers to proteins as exemplified by the production of novel antibody-drug conjugates. Suitable antibodies are often coupled to the drug *via* cysteines and lysines (Shadish and DeForest, 2020). Due to many possible coupling partners, depending on the individual amino acid composition, a mixture of conjugates results, displaying different pharmacokinetic properties (Hamblett et al., 2004; Wang et al., 2005). In this context, cell-free protein synthesis can be utilized to modify therapeutically relevant proteins by means of orthogonal systems at precisely defined positions to obtain a homogeneous mixture of antibody-drug conjugates. It has been shown that site-specifically modified Her2-binding IgG antibodies based on *E.coli* cell-free systems have been coupled to the anti-cancer agent monomethyl auristatin F, delivering the antibody-drug conjugate to the antigen (Zimmerman et al., 2014).

The orthogonal aaRS/tRNA pair is usually added to the open cell-free system. On the one hand, the simple addition of a wide variety of reactants to the translation reaction is advantageous. On the other hand, the additive can negatively affect the reaction itself, as the reaction environment is highly dependent on a defined milieu. Proteins such as aaRS are typically located in a solvent that ensures protein stability but does not correspond to the optimum of cell-free reaction conditions in terms of ion concentrations. Various studies have shown that even in the case of a minor change of ion concentrations including magnesium, potassium and chloride ions, a significant effect on protein production was observed (Brigotti et al., 2003; Spice et al., 2020; Garenne et al., 2021). In particular, a reduced amber suppression was detectable with increasing eAzFRS concentrations and thus increasing buffer concentrations. Together with the time consuming purification of aaRS, an alternative approach was achieved by Chemla et al. using cell-free systems based on *E.coli* extracts, where PylRS expression was performed prior to cell disruption (Chemla et al., 2015). Besides, commonly used *E. coli* extracts for CFPS contain the T7 RNA polymerase from T7 phage to bypass the addition of purified enzyme (Köhrer et al., 1996; Des Soye et al., 2019). In contrast to *E.coli* based systems, the present study demonstrates that orthogonal aaRS originating from *E. coli* and archaea were

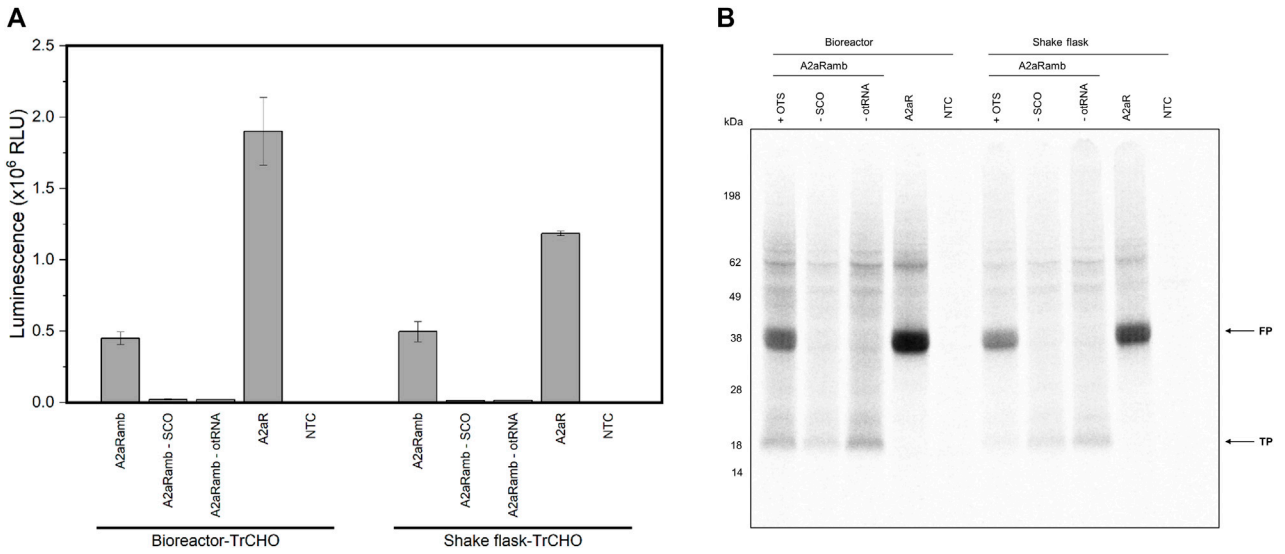

FIGURE 6 | CFPS based on transiently transfected CHO cells expressing PylRS-AF. **(A)** Luciferase assay of cell-free synthesized A2aRamb and A2aR constructs based on transiently transfected CHO cells expressing the PylRS-AF. Samples with a plus indicate the addition of the particular orthogonal component, while a minus indicates the absence of the orthogonal component. Experiments were performed in technical triplicates and bars represent the standard deviation. **(B)** Autoradiography of electrophoretically separated proteins corresponding to CFPS shown in **(A)**. FP: Full-length protein, TP: Truncated protein, NTC: No-template control, SCO: Strained cyclooctyne, otRNA: Orthogonal tRNA, OTS: Orthogonal translation system, TrCHO: Transiently transfected CHO cells.

successfully integrated into eukaryotic cells prior to cell disruption. The resulting orthogonal translation system was used to produce the complex membrane protein A2aR in cell-free systems.

It has already been shown that orthogonal aaRS were transiently transfected to modify proteins *in vivo* (Cohen and Arbely, 2016; Meineke et al., 2020). However, it has been assumed that transfection has a negative impact on the cell lysate for CFPS as cell disruption is typically performed 2–3 days after transfection and transfection reagents can negatively affect cell viability. Contrary to expectations, CFPS based on lysates of CHO cells treated with the commonly used transfection reagent PEI were shown to have similar reporter gene activity as non-treated CHO cells.

Another aspect that was examined in the present study is the cultivation format after transient transfection prior to cell disruption. Higher reporter gene activities based on A2aR were detected with cell lysates based on cultivation using bioreactors. This could be related to the controlled cultivation conditions and thus reduced cell stress in bioreactor based cultivations. Although shake flasks are easy to use, lack of oxygen control and pH control can be challenging throughout the cultivation process (Link and Weuster-Botz, 2011). Nevertheless, satisfactory reporter gene activities could be achieved based on shake flask cultivation. Thus, with the presented orthogonal translation system, cells can be efficiently enabled for orthogonal aaRS production, cost-effective cultivation in shake flasks and subsequent cell lysate preparation for CFPS can be performed in a timesaving procedure. Of particular concern is the role of the novel orthogonal CFPS based on CHO lysate for investigating membrane proteins such as the examined A2aR, since efficient translocation of membrane proteins into microsomal structures can be achieved in certain eukaryotic cell-free systems (Brödel et al., 2014; Sonnabend et al., 2017; Zemella et al., 2017). As GPCRs account for 35% of approved drug targets, the analysis of GPCRs based on interaction studies with potential ligands is of highest interest (Sriram and Insel, 2018). Fluorescence-based interaction studies offer the possibility to identify potential ligands and inhibitors by incorporating ncaa at defined positions in GPCRs subsequently conjugating them with fluorescent molecules, thus eliminating the requirement for large fluorescent fusion proteins that may affect protein interactions and functions (Lee et al., 2019). In this context, we recently demonstrated that site-specifically fluorescently labeled A2aR was excited by the C-terminal localized Nluc after substrate addition and a signal change was measured based on the conformational change of the GPCR after addition of ligand (Zemella et al., 2019). In the present study we demonstrate that transient transfection of PylRS-AF also leads to an intact orthogonal cell-free translational system for the production of site-specifically modified A2aR. Accordingly, purification of the unstable PylRS can be bypassed, which can influence the enzyme`s activity significantly.

Cell lysates based on transient transfection showed variable amber suppression efficiencies, which could be the result of the different transfection efficiencies. Alternatively, to create a cost-effective and reproducible orthogonal cell-free system for long-term use, the eAzFRS was stably transfected into the CHO cells. In particular, stable transfection by CRISPR offers the possibility to target sites in the genome that allow high expression levels and simultaneously reduce silencing (Lo et al., 2017; Li et al., 2020).

While many studies have demonstrated that *HPRT1* is suitable for high levels of antibody production in CHO cells (Wang et al., 2017; Kawabe et al., 2018), our study showed that the *C12orf35* locus is beneficial for incorporating the orthogonal eAzFRS into the CHO genome and subsequently generating active cell lysates for orthogonal translation. Disruption of the *C12orf35* gene, located in the telomeric region of chromosome 8, has been reported to affect recombinant protein production in the resulting cell lines (Ritter et al., 2016a). Using small interfering RNAs, the *C12orf35* gene was silenced and monoclonal antibody production was increased, whereas silencing of the gene and subsequent isolation of clonal cell lines resulted in fast recovery rates during the selection process. Our results are consistent with those reported by Zhao et al. who were able to generate cell lines with highest stability and anti-PD1 monoclonal antibody productivity using the *C12orf35* locus in contrast to other CHO hot spots studied such as *HPRT* and *GRIK1* (Zhao et al., 2018). However, expression of eAzFRS using the *HPRT1* locus led to lower mRNA levels and minor eAzFRS activity in cell lysates. On the one hand reduced eAzFRS transcription and production could be a result of increased epigenetic gene silencing. On the other hand *HPRT1* is a widely used target site for protein production (Wang et al., 2017; Kawabe et al., 2018). Nevertheless, the eAzFRS coding sequence is originated from *E.coli* and might have an impact on the *HPRT1* locus in CHO cells.

Addressing transcriptionally active gene loci by CRISPR ensures controlled overexpression of proteins and overcomes the unpredictable insertion of transgenes into random genomic positions. The on-target efficiency and off-target effects depend mainly on the gRNA sequence used (Wilson et al., 2018). Therefore, we designed a gRNA sequence that addresses the *HPRT1* and *C12orf35* loci and postulated that integration of eAzFRS is improved. Indeed, the novel gRNAs were shown to result in a higher eAzFRS mRNA level in comparison to the recently reported gRNA sequences (Zhao et al., 2018). This may be due to the fact that the sequence context flanking the target site and resulting gene positioning effects are of particular importance. Indeed, it has been shown that deletions in the telomeric region around *C12orf35* resulted in CHO cell lines with increased stability and high protein production rates (Ritter et al., 2016b). Epigenetic regulatory mechanisms such as DNA methylation and histone modification can strongly influence the expression level as well as gene silencing (Gibney and Nolan, 2010; Keller et al., 2019). However, it must be emphasized that off-target effects of the utilized gRNAs have not been investigated and integration of eAzFRS in other transcriptionally active gene loci cannot be excluded. In addition, mRNA expression strongly depends on the inserted gene sequence in the genomic context.

Various orthogonal aaRS would be of interest to incorporate them into CHO cells to expand the repertoire of diverse ncaa for incorporation into proteins in CFPS. In contrast, including orthogonal tRNA in the cell lysate could be challenging, since it was reported that the ratio of expression cassettes of both orthogonal tRNA and corresponding aaRS needs to be adjusted for cell-based production to achieve high suppression efficiency (Ryu and Schultz, 2006; Schmied et al., 2014). This highlights the strength of CFPS, as the tRNA can be transcribed *in vitro* followed by a titration to the cell-free system in optimal ratios.

In summary, the developed system is suitable for the incorporation of diverse orthogonal aaRS as well as other proteins of interest into the cell lysate, thus eliminating the need for the supplementation of enzymes and co-factors. As a result, a novel orthogonal eukaryotic cell-free system speeds up the production of site-specifically modified complex proteins.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

JS was involved in methodology, investigation, formal analysis and writing the original draft. NC was involved in methodology, investigation and formal analysis. AZ was involved in methodology, conceptualization, review and editing the draft. LT was involved in methodology, investigation, conceptualization, review and editing of the draft. SK was involved in methodology, conceptualization, review and editing of the draft, project administration and funding acquisition.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmolb.2022.832379/full#supplementary-material

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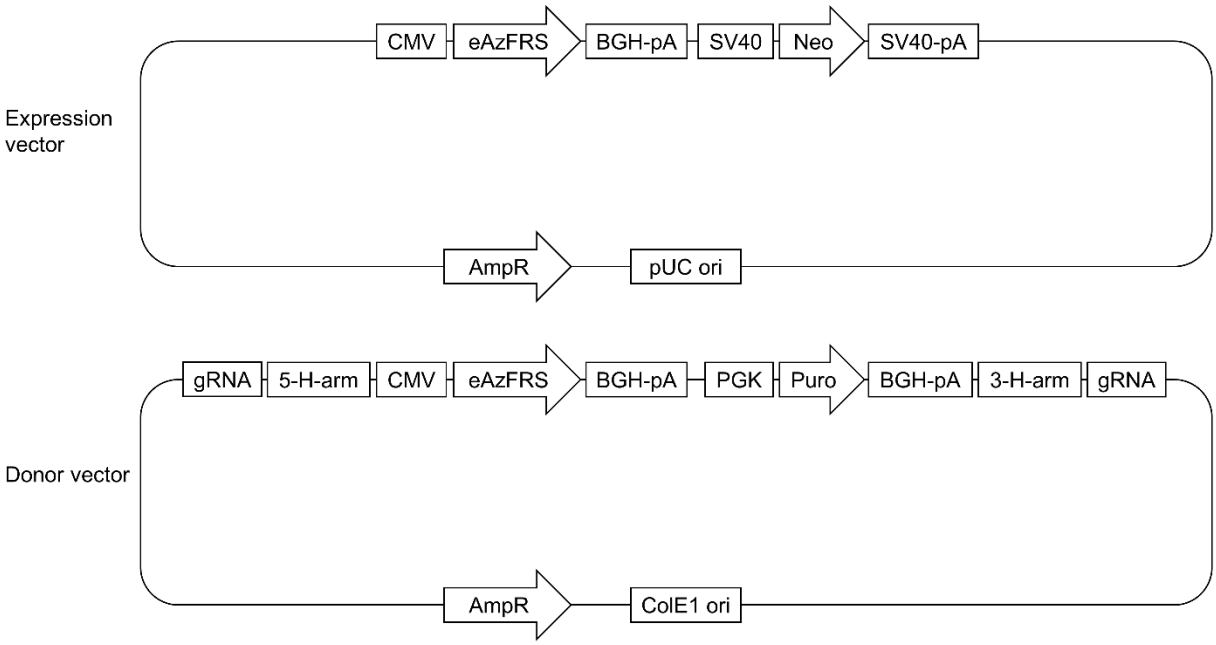
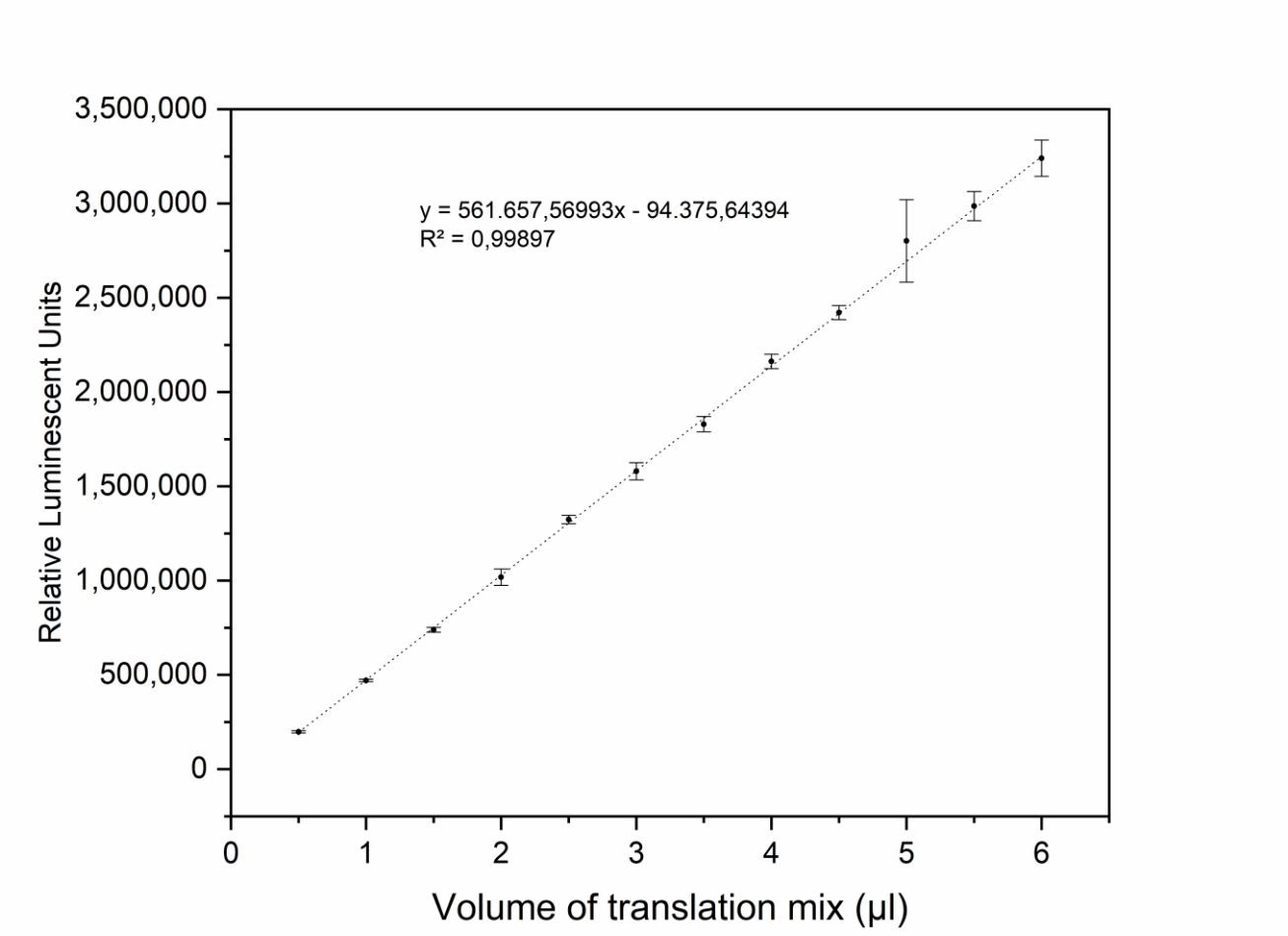
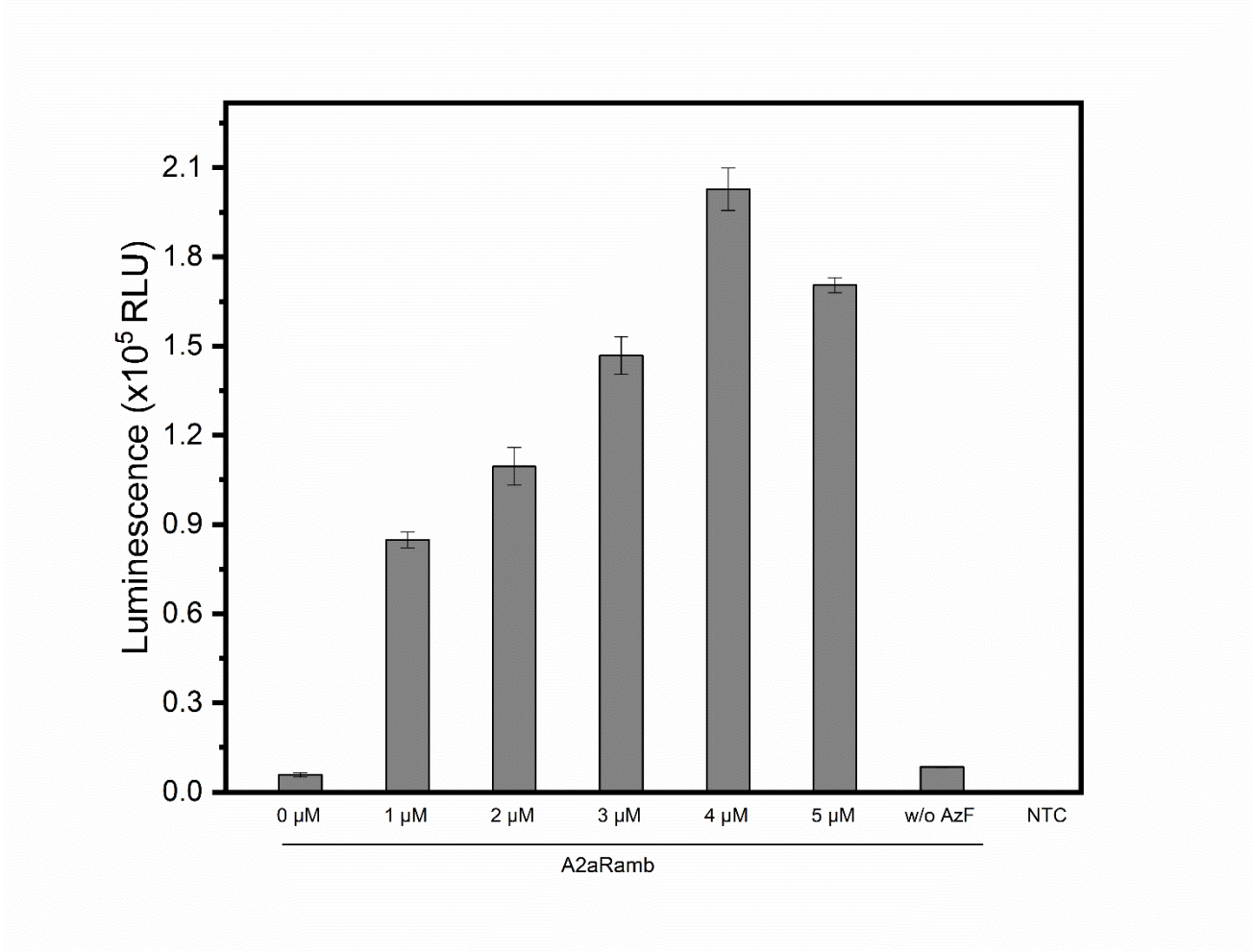
Supplementary Material



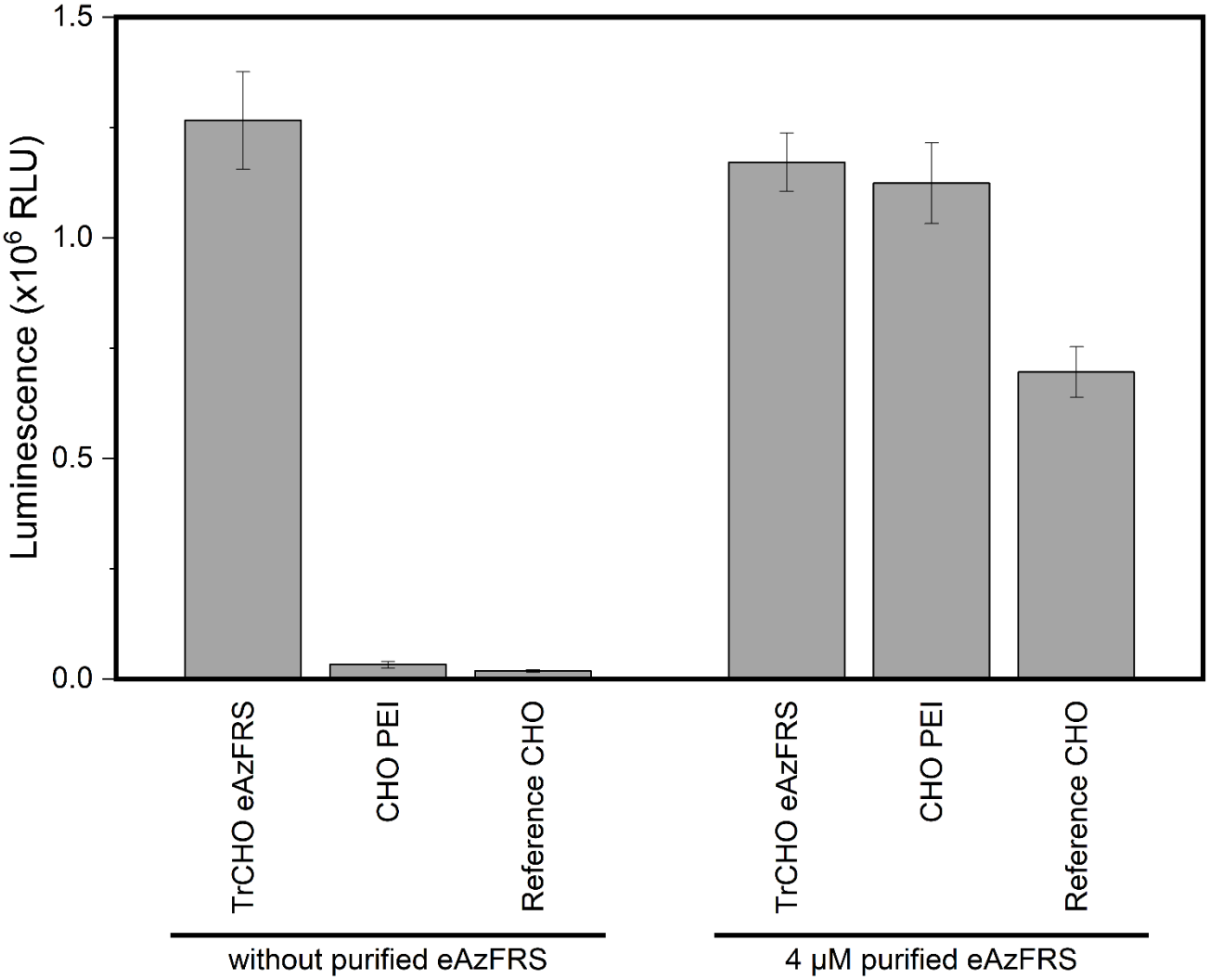
Supplementary Figure 1: Vector illustration. Expression vectors utilized for transient transfections are based on a CMV promoter for eAzFRS/PylRS-AF expression and a SV40 promoter for the expression of a neomycin resistance gene. Donor vectors utilized for homology directed repair based on CRISPR/Cas9 technology contain a CMV promoter for expression of eAzFRS and a PGK promoter for puromycin expression. Homology arms at the 5´-end and 3´-end are 700 bp pairs long. The donor cassette is flanked by gRNA recognition sequences to linearize the donor cassette after transfection with Cas9 and gRNA expression plasmids.



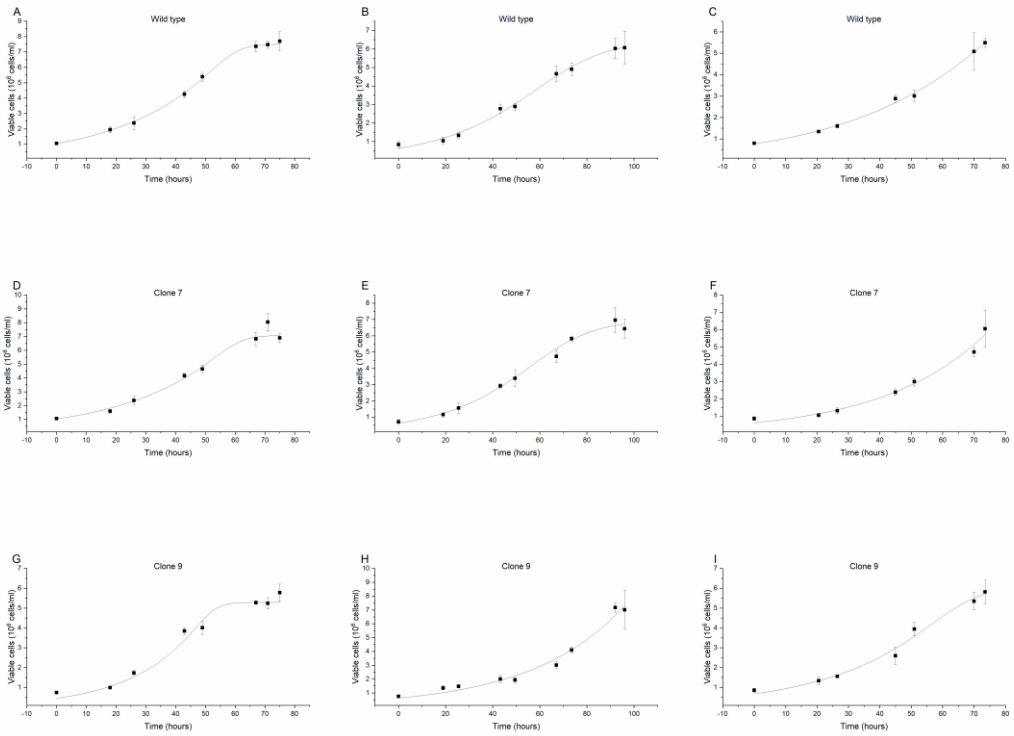
Supplementary Figure 2: Luciferase assay of cell-free reactions based on CHO cell lysate. Different volumes of the translation mix after a cell-free reaction with A2aR construct were analyzed by a luciferase assay to show linearity. Experiments were performed in technical triplicate and bars represent the mean ± standard deviation.



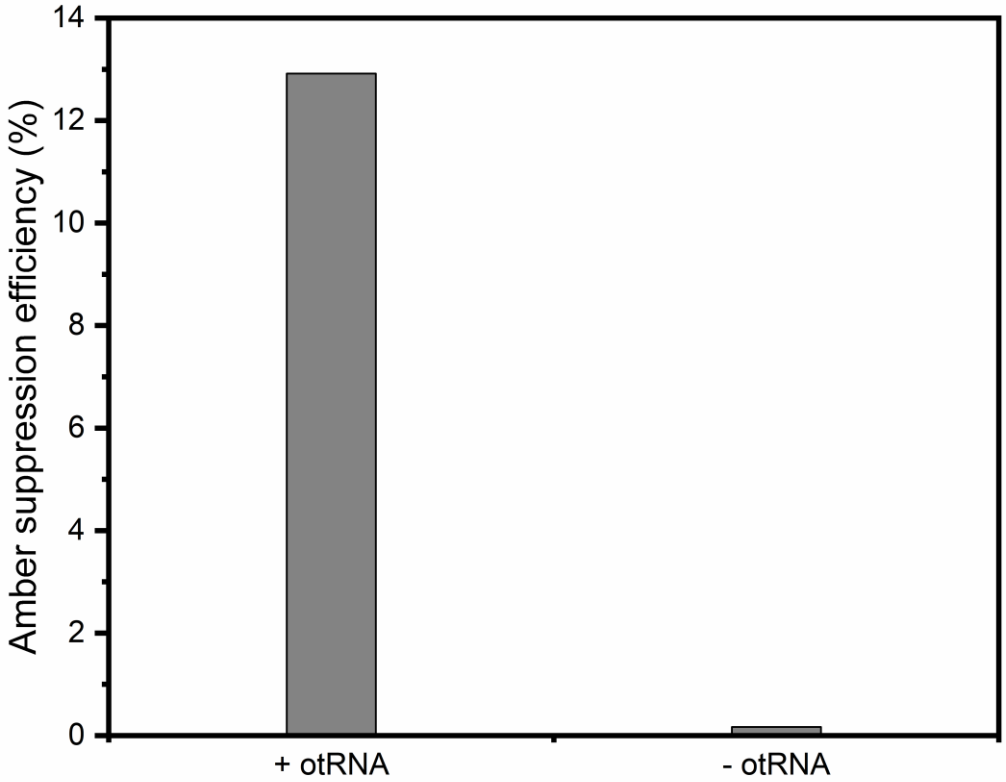
Supplementary Figure 3: Variation of the concentration of purified eAzFRS in cell-free reactions based on CHO cell lysate. The concentration of purified eAzFRS war varied from 0 to 5 µM to identify the optimal amount of enzyme for orthogonal translation in cell-free reactions based on CHO lysate without endogenous eAzFRS. The A2aRamb construct was utilized to analyze amber suppression using the luciferase assay. Experiments were performed in technical triplicate and bars represent the mean ± standard deviation.



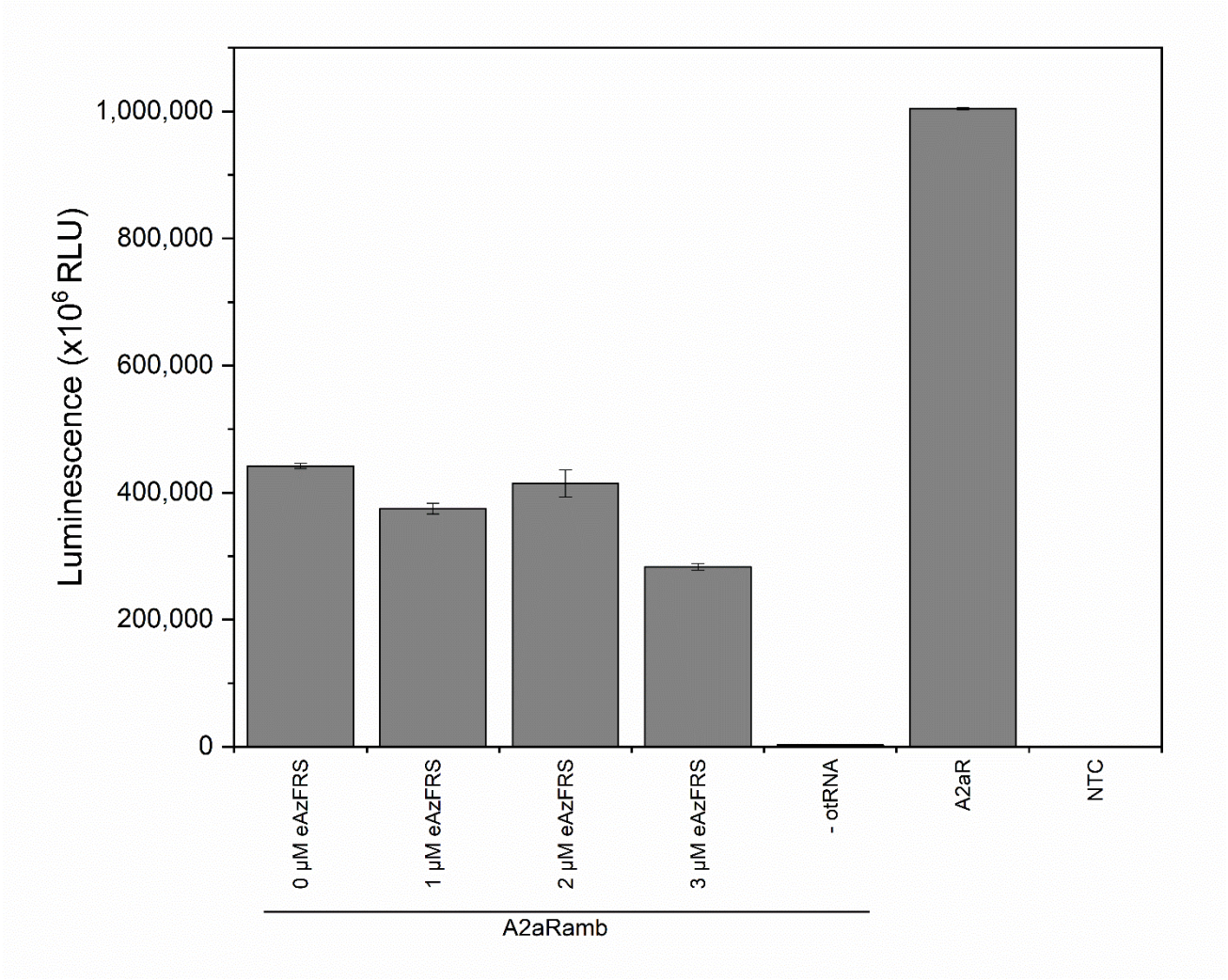
Supplementary Figure 4: Effect of PEI on CFPS based on transiently transfected CHO cells expressing eAzFRS. Luciferase assay of cell-free synthesized A2aRamb based on non-treated CHO cells (reference CHO), PEI treated CHO cells (CHO PEI) and transfected CHO cells with eAzFRS based on PEI (TrCHO eAzFRS). Cell-free reactions were performed in the presence or absence of purified eAzFRS. Experiments were performed in technical triplicate and bars represent the mean ± standard deviation.



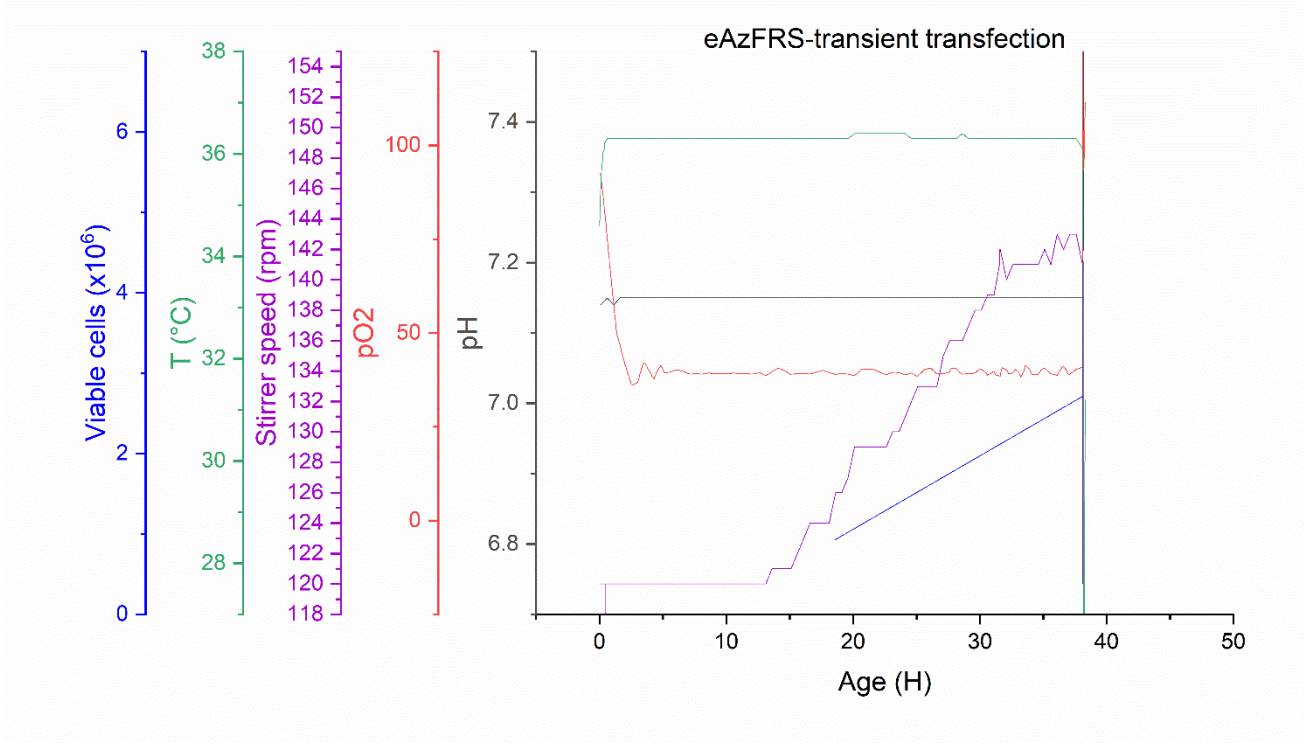
Supplementary Figure 5: Growth curves of three independent cultivations with wild type CHO (A-C), clone 7 (D-F) and clone 9 cells (G-I).



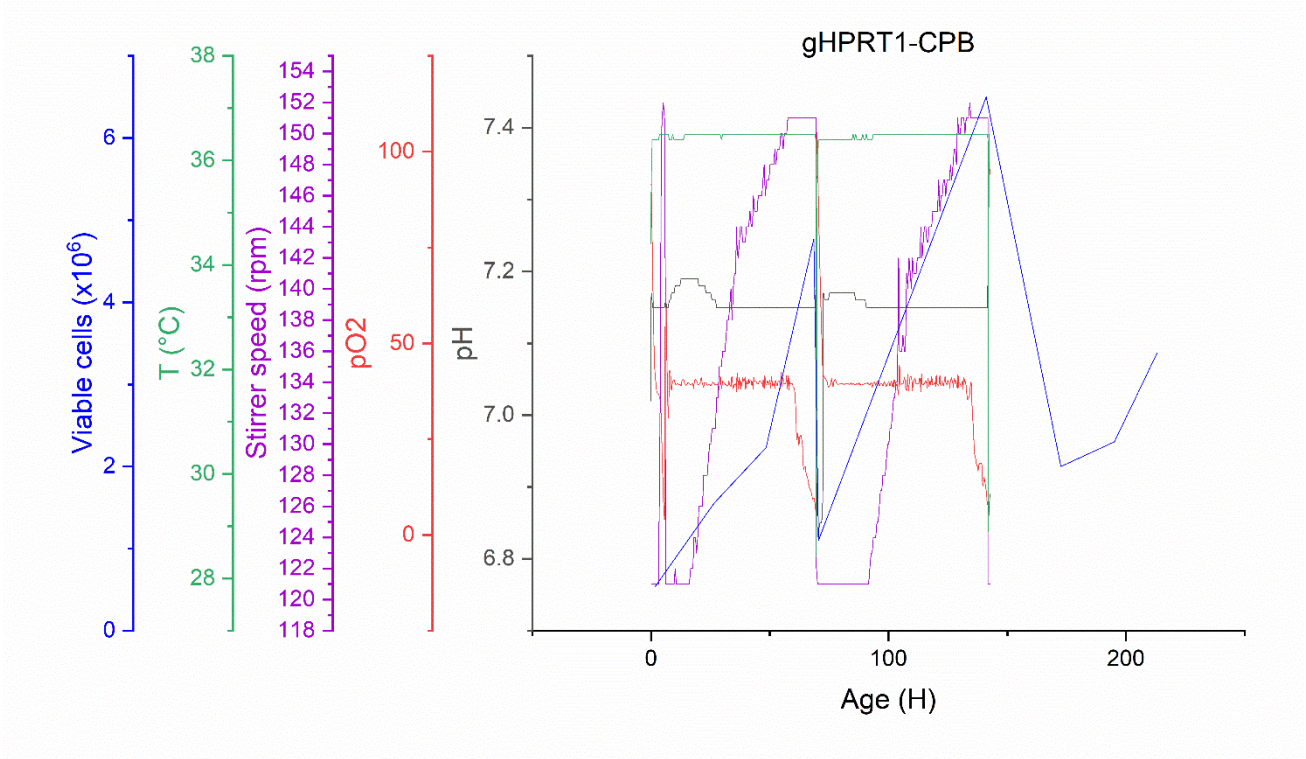
Supplementary Figure 6: Cell-free synthesis based on RS9. Suppression efficiency was calculated using the A2aRamb and A2aR construct. Luminescence signals of cell-free reactions without a template (NTC) were subtracted from sample signals prior to calculation of suppression efficiency. Samples with a plus or minus indicate the addition or absence of the orthogonal tRNA (otRNA). Experiments were performed in technical triplicate and bars represent the mean ± standard deviation.



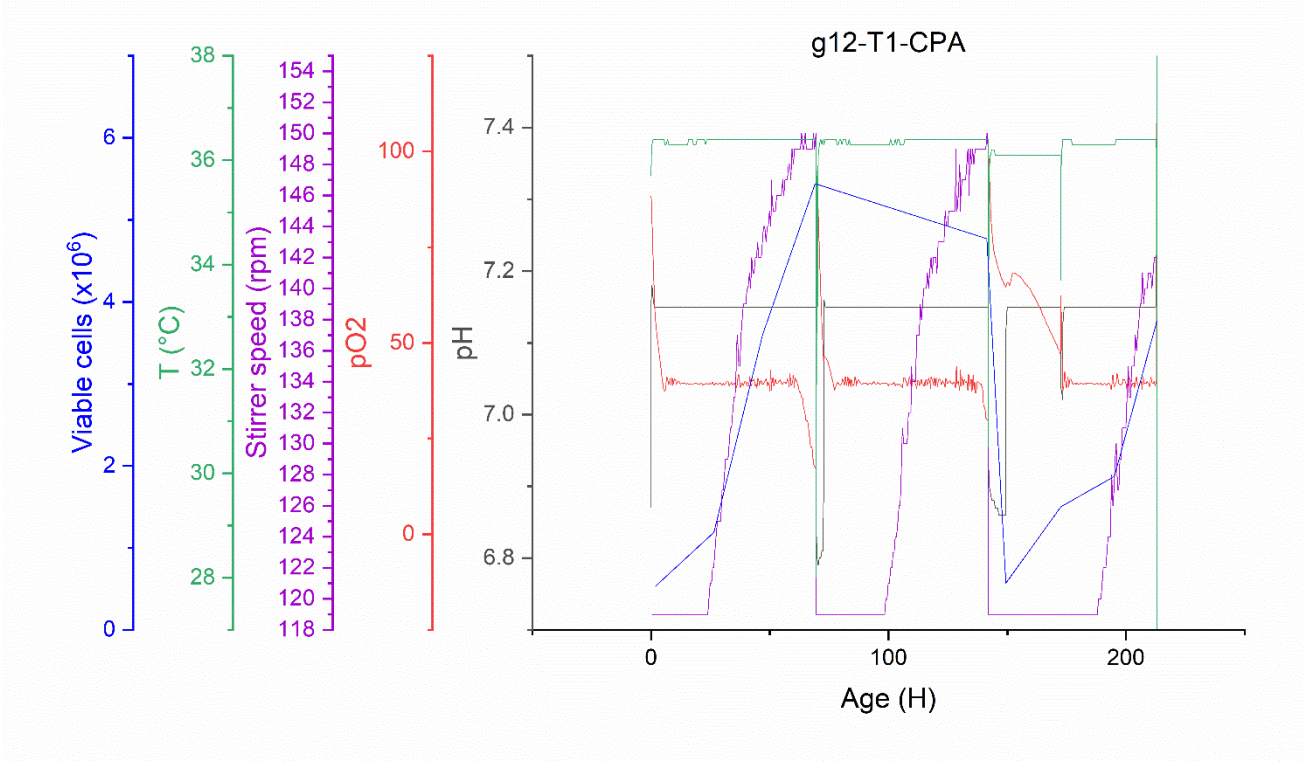
Supplementary Figure 7: Evaluation of cell-free synthesis based on RS7. Luciferase assay of cell-free synthesized A2aRamb and A2aR constructs based on CHO clone RS7. Concentrations of purified eAzFRS were varied from 0-3 µM. Samples with a minus indicate the absence of the orthogonal tRNA (otRNA). Experiments were performed in technical triplicate and bars represent the mean ± standard deviation. NTC: No-template control.



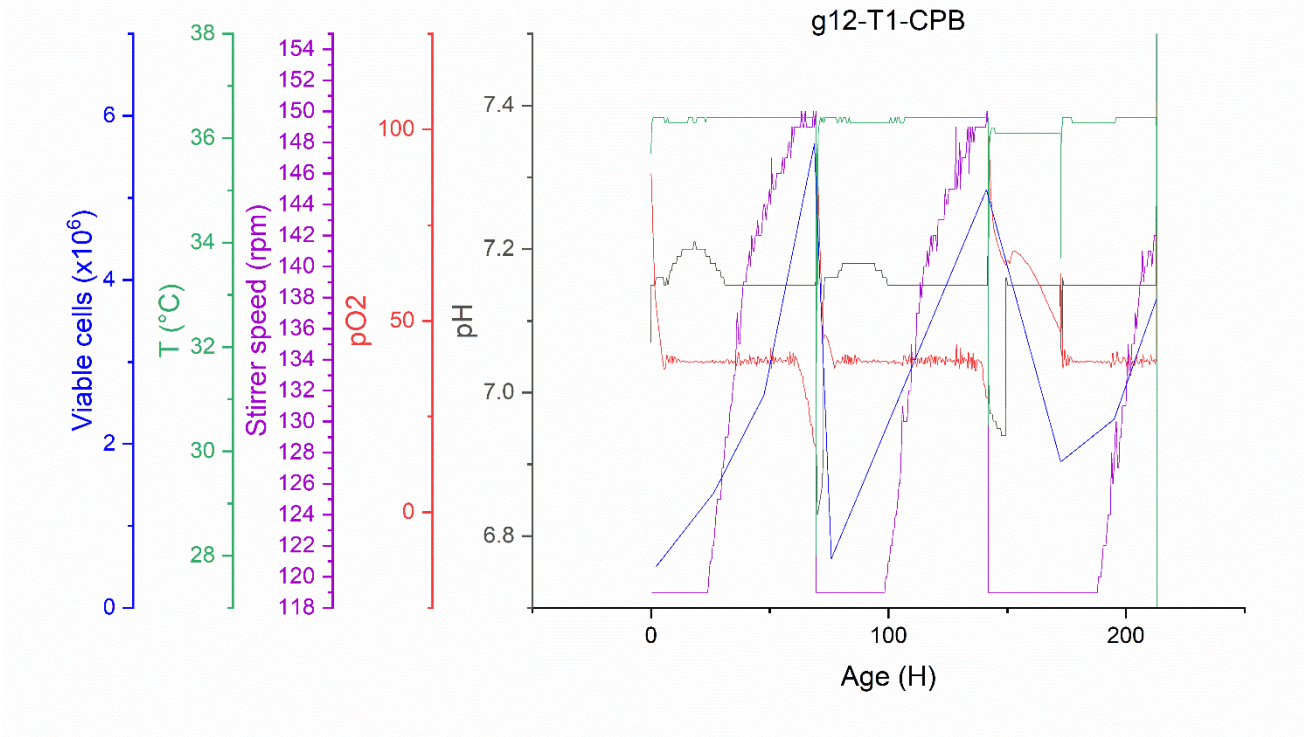
Supplementary Figure 8: Fermentation of CHO cells transiently transfected with eAzFRS.



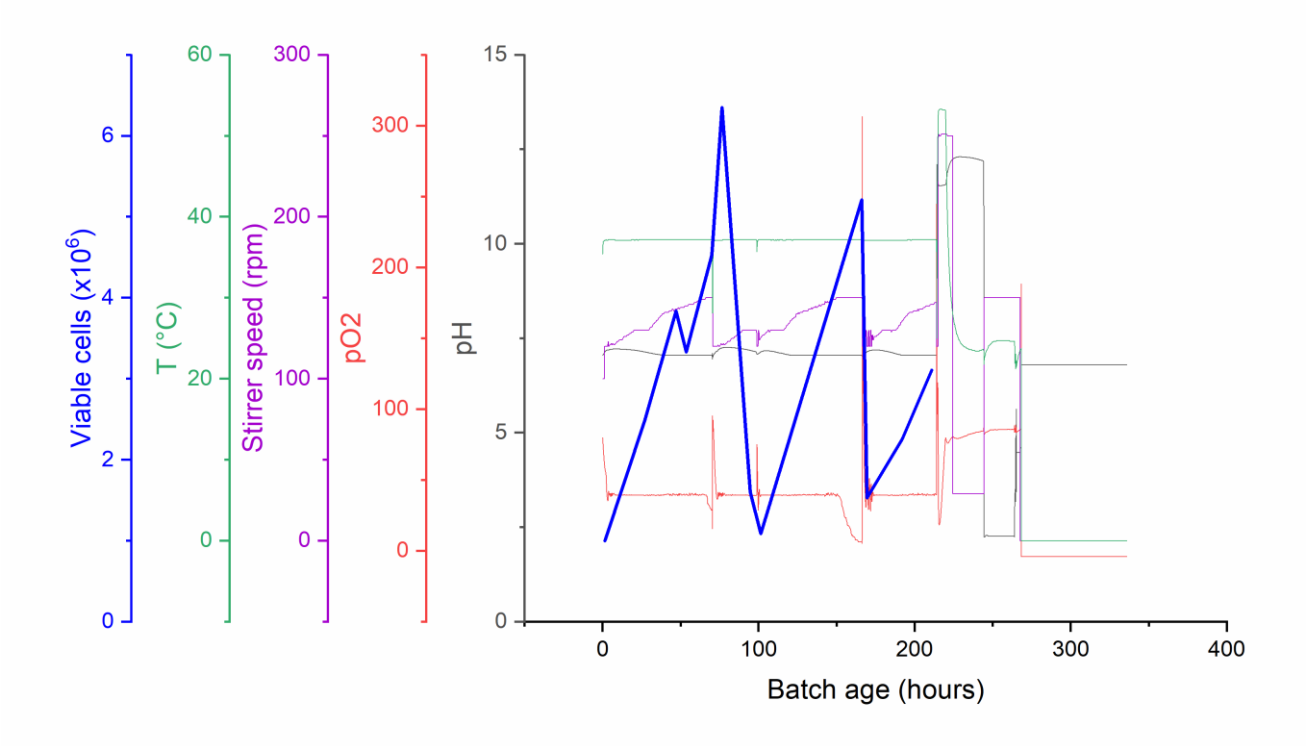
Supplementary Figure 9: Fermentation of CHO clone pool B (gHPRT1-CPB)



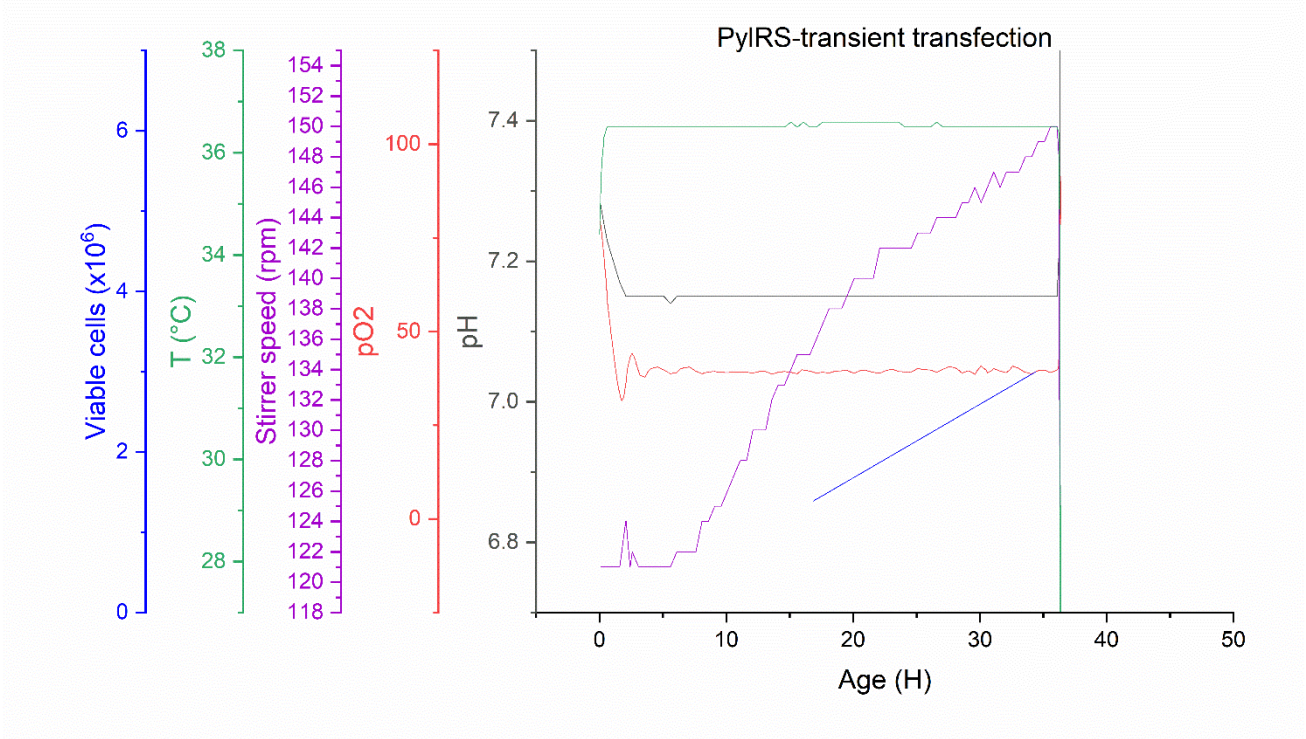
Supplementary Figure 10: Fermentation of CHO clone pool A (gC12-T1-CPA)



Supplementary Figure 11: Fermentation of CHO clone pool B (gC12-T1-CPB)



Supplementary Figure 12: Fermentation of CHO clone pool B (gC12-T2-CPB)



Supplementary Figure 13: Fermentation of CHO cells transiently transfected with PylRS.

2.2. Promoting the production of challenging proteins via induced expression in CHO cells and modified cell-free lysates harboring T7 RNA polymerase and mutant eIF2α

Building upon the initial findings of the first publication titled "Cell Engineering and Cultivation of Chinese Hamster Ovary Cells for the Development of Orthogonal Eukaryotic Cell-free Translation Systems", the objective was to identify an optimal genomic target site within CHO cells capable of harboring large expression cassettes. Hence, an inducible expression system could be developed. Additionally, T7 RNA polymerase and mutant eIF2α could be integrated into the cell lysate to further enhance CHO-based CFPS.

Declaration of own contribution

1st authorship: I, Jeffrey Lesslie Schloßhauer, originated the concept, designed the experiments, and performed the cell-based approaches, except for FACS experiments and transient transfection of T7 RNA polymerase. I prepared cell lysates for cell-free protein synthesis, except for cell lysates derived after transient transfection of T7 RNA polymerase. Moreover, I conducted cell-free protein synthesis reactions including subsequent analysis. Additionally, I wrote the manuscript and prepared the figures. FACS experiments were performed by Anja Hönow. Lena Tholen and Sofia Chatzopoulou designed the construct for transient T7 RNA polymerase expression, conducted transient transfection of T7 RNA polymerase, and carried out the corresponding cell-free protein synthesis and western blotting. Alexander Körner and I discussed the design and feasibility of the inducible expression system. Anne Zemella, Alexander Körner, and Stefan Kubick revised the manuscript.

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Original Research Article

Promoting the production of challenging proteins via induced expression in CHO cells and modified cell-free lysates harboring T7 RNA polymerase and mutant eIF2α

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ABSTRACT

Chinese hamster ovary (CHO) cells are crucial in biopharmaceutical production due to their scalability and capacity for human-like post-translational modifications. However, toxic proteins and membrane proteins are often difficult-to-express in living cells. Alternatively, cell-free protein synthesis can be employed. This study explores innovative strategies for enhancing the production of challenging proteins through the modification of CHO cells by investigating both, cell-based and cell-free approaches. A major result in our study involves the integration of a mutant eIF2 translation initiation factor and T7 RNA polymerase into CHO cell lysates for cell-free protein synthesis. This resulted in elevated yields, while eliminating the necessity for exogenous additions during cell-free production, thereby substantially enhancing efficiency. Additionally, we explore the potential of the *Rosa26* genomic site for the integration of T7 RNA polymerase and cell-based tetracycline-controlled protein expression. These findings provide promising advancements in bioproduction technologies, offering flexibility to switch between cell-free and cell-based protein production as needed.

1. Introduction

Production of biotechnologically relevant proteins can be achieved by expression of the protein of interest in cultured cells or using cell-free reaction conditions. In cell-based approaches, transient as well as stable transfection is often used for recombinant protein production [1,2]. On the other hand, protein synthesis in cell-free systems can be performed by adding DNA templates to the open reaction, which is driven by the presence of an active cell lysate containing components for transcription and protein translation including ribosomes, aminoacyl tRNA synthetases (aaRS), transcription and translation factors, viral RNA polymerase for transcription, substrates and an energy regeneration system [3,4]. The reaction environment can be further manipulated with additives, such as chaperones, mild detergents, labeled amino acids, and cofactors, to tailor protein synthesis as needed. The addition of the chaperones DnaK and GroEL significantly increased the solubility of cell-free produced proteins based on *Escherichia coli* cell lysate, while the addition of various orthogonal aaRS/tRNA pairs and disulfide isomerase to the open system allowed various non-canonical amino acids (ncaa) to be incorporated into proteins and disulfide bridges to be successfully formed [5–8]. To overcome labour-intensive preparation of supplemented purified components, enzymes were introduced into the bacterial genome and overexpressed to generate cell lysates harboring the desired enzymes for straightforward cell-free protein synthesis [9,10]. As an alternative to the commonly used cell-free protein synthesis based on *E. coli*, eukaryotic cell-free systems can be employed for the synthesis of

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complex and post-translationally modified proteins [11]. It has been shown that diverse membrane proteins can be produced in active form within 3–20 hours in eukaryotic cell-free systems [12,13]. Frequently applied cell lysates for eukaryotic cell-free protein synthesis are based on cultured human cell lines, *Spodoptera frugiperda* 21 (*Sf*21) cells, Chinese hamster ovary (CHO) cells, tobacco cells, wheat germ cells and yeast cells [14–17]. Among these, CHO cells are of critical importance for biopharmaceutical production, as they often serve as host systems for the production of therapeutic proteins [18]. The advantage of CHO cells is their robustness, scalability, and ability to allow post-translational modifications similar to those in human cells [19]. Furthermore, the in-depth analysis of the CHO cell genome offers new possibilities for advancing cell-free protein synthesis based on CHO lysates [20]. The expression of therapeutic proteins can be evaluated rapidly on a small scale in CHO cell-free systems and the identified conditions can be transferred to CHO cell-based production. To facilitate CHO based cell-free production of site-specifically modified membrane proteins, we recently demonstrated that orthogonal *E. coli* tyrosyl-tRNA synthetase can be integrated into CHO cell lysate to modify the pharmaceutically relevant G protein-coupled adenosine receptor A2a in a cell-free reaction [21].

The choice of the appropriate protein production format strongly depends on the individual protein's requirements. While cell-based production can generate large quantities of post-translationally modified proteins, cell-free synthesis can be used to produce toxic proteins and membrane proteins in a significantly reduced time on a small scale [22]. Those proteins can have a negative impact on cell viability and vitality, and may result in apoptosis when constitutively expressed. Alternatively, strict regulation using inducible promoters permits the cell-based expression of difficult-to-produce proteins to be triggered on demand [23]. Thereby, the level of putatively toxic proteins can be repressed to a level that is not harmful to the host organism. This procedure is particularly useful for large scale processes, that aim to first optimize for growth, and later for expression, when the desired cell density is achieved. Furthermore, controlled expression proves beneficial for cell line development, a process that frequently utilizes low cell densities or single cells, rendering them especially susceptible to any additional stress stimuli [24].

There are multiple inducible systems with diverse functionalities to selectively switch on or off desired genes. CHO cell systems frequently use tetracycline and cumate as inducer, as they robustly trigger pronounced protein expression [25–28]. By contrast, more recently developed light-inducible systems allow for spatiotemporal control of protein production, but currently do not provide a strong expression rate when switching on the commonly used blue light [29,30]. To ensure optimal induction of target protein expression, defined positions in the genome are essential for stable transfection of regulatory molecules such as inducible promoters, repressors, and activators, respectively. In the past, *hprt*, *C12orf35*, *Hipp11*, and *Rosa26* have been identified as target sites for stable transfection of expression cassettes because they provide reproducible results and are subject to reduced epigenetic regulation and gene inactivation [31–33].

In the present work, manipulation of cell-free transcription and translation was aimed to simplify the production of complex proteins and to increase expression levels. To this end, regulatory factors were introduced into CHO cells to produce cell lysates for enhanced cell-free protein synthesis. In order to achieve flexible production of toxic proteins and membrane proteins, a tetracycline-inducible system was developed as an alternative to cell-free protein synthesis, which allowed the controlled cell-based expression of difficult-to-express proteins from the *Rosa26* locus.

2. Material and methods

2.1. Plasmids and template generation

The plasmid pCAG-T7pol (Addgene #59926) for T7 RNA polymerase expression, pSpCas9(BB)-2A-GFP (Addgene #48138) for Cas9 expression and gRNA_cloning vector (Addgene #41824) were obtained from Addgene. The T7 RNA polymerase sequence was extracted from the UniProt database (P00573; RPOL_BPT7). The gRNA C12orf35-T2: 5′-GCC GGG ACT TAA CCA CTC GA-3′ specific for the *C12orf25* locus was designed according to our previous report [21]. The gRNA Rosa26: 5′-TCAAGCGTGAGCATAAAACT-3′ specific for the *Rosa26* locus was obtained from the literature [33]. Gibson assembly was utilized to clone the gRNA sequence into the gRNA_cloning vector (Plasmid #41824 from Addgene) according to the protocol, as previously described [34]. Plasmids based on the piX 3.0 backbone (Biotech Rabbit) are optimal for cell-free protein synthesis due to the presence of a T7 promotor and a T7 terminator as regulatory sequences for transcription. The plasmid piX4.0-NC-Luc containing a cricket paralysis virus (CrPV) internal ribosome entry site (IRES) for translation initiation was used for cell-free synthesis of firefly luciferase [35]. The piX3.0-Nluc without the CrPV IRES and with a nanoluciferase sequence (Promega) was used for the T7 RNA polymerase assay, while a CrPV IRES was cloned into piX3.0-Nluc to produce the plasmid piX3.0-CRPV-Nluc containing both, a CrPV IRES and nanoluciferase for cell-free synthesis. The pIX4.0-Luc plasmid was used to analyze cap-dependent translation initiation, as described previously [36]. Therefore, 0.1 ng/µl pIX4.0-Luc was used as template in a PCR reaction containing 10x ThermoPol Reaction Buffer (New England Biolabs), 14 mU/µl Deep Vent DNA Polymerase (New England Biolabs), 0.2 mM dNTPs, 0.5 µM of T7-Fw primer 5′-ATGATATCTCGAGCGGCCGCTAGCTAATACGACTCACTATAG-3′ and 0.5 µM of PolyA 50-Rv primer 5′-T(50)CAGATCTTGGTTAGTTAG-3′. The following temperature profile was used: 95 °C for 3 min; 30 cycles of 95 °C for 20 s, 51 °C for 20 s, 72 °C for 90 s; 72 °C for 5 min. The PCR product was purified using DNA Clean & Concentrator Kit (Zymo Research) according to the manufacturer's instructions. The pcDNA3.1-eIF2α-S52A containing a human cytomegalovirus (CMV) promoter and a HiBiT tag N-terminally was purchased from GenScript and was utilized for transient transfection of eIF2α-S52A (wildtype sequence of eIF2α extracted from UniProt P05198) in CHO cells. The donor plasmids DV-Tet-GFP and DV-T7RNAPol-Rosa26 for CRISPR/-Cas9 based modification of CHO cells were purchased from Biocat. The TRE promoter and rtTA-2a-Puro sequence was extracted from Addgene (Addgene #60495). Homology arms of the donor plasmid eAzFRS, which was described previously [21], were used to substitute homology arms of DV-T7RNAPol-Rosa26 by fusion PCR to generate the new donor sequence DV-T7RNAPol-C12orf35. The donor plasmids contain a CMV promoter for T7 RNA polymerase expression and downstream an EMCV IRES to initiate translation of Blasticidine independently of T7 RNA polymerase. The plasmids PB-Transposase-Sf21 and PB-T7RNAPol-Sf21 were purchased from Biocat. Expression of both PiggyBac Transposase and T7 RNA polymerase is driven by an OpIE-2 promoter, while the PB-T7RNAPol-Sf21 plasmid additionally contain an bleomycin resistance gene driven by an OpIE-1 promoter. PiggyBac 5- and 3-terminal inverted repeats were extracted from the literature [37] and are located at the terminal ends of the donor sequence, enabling PiggyBac Transposase to integrate the donor sequence into the *Sf*21 genome.

2.2. Cell lines and cultivation

CHO–K1 cells from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (DSMZ no: ACC110) were adapted as a suspension culture. CHO cells were grown in serum-free ProCHO5 medium (Lonza) supplemented with 4 mM Ultraglutamine (Lonza) at 37 °C, 5% CO_2 and 100 rpm in the CO_2 Multitron incubator (Infors). *Sf*21 suspension cells were cultivated at 27 °C in serum-free

Insect-XPRESS medium (Lonza).

2.3. Transient transfection and cell lysate preparation

CHO cells were transiently transfected, as described recently [21]. Briefly, 1.5 μg pcDNA3.1-eIF2α-S52A or pCAG-T7pol expression plasmid/10^6 cells and 2 μg PEI reagent/10^6 cells were incubated for 4 h at a cell density of 4×10^6 cells/ml at 37 °C, 5% CO_2 and 80 rpm in the CO_2 Multitron incubator (Infors). Afterwards, 150 ml fresh culture medium was added to achieve a 200 ml culture at a cell density of 10^6 cells/ml for two days at 37 °C, 5% CO_2 and 100 rpm. CHO cells were harvested and washed, as described previously [14]. Cell lysate was prepared by using the lysing matrix A (MP Biomedicals). The wet cell pellet was transferred to a lysing matrix A tube and cells were disrupted for 5 s and 4 m/s in the presence of dry ice in the cooling chamber of the FastPrep-24 Bead-Beating instrument (MP Biomedicals) and cell lysates were isolated as described previously [14].

2.4. Stable transfection and isolation of cells

500 ng DNA was diluted in 100 μl Opti-MEM serum-free medium (Gibco) in a ratio of 2:2:1 (linear donor vector: gRNA vector: Cas9 vector). The DNA was delivered into CHO cells by lipid based transfection using 2.5 μL Lipofectamine LTX (Thermo Fisher Scientific) and 0.5 μL Plus reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, the mixture was added to 1×10^6 cells/ml in 500 μL serum-free ProCHO5 medium (Lonza) in a 24-well plate after an incubation time of 20 min. Cells were mixed and 15 min centrifugation with $400 \times g$ at room temperature was performed according to a previous report [38]. The cells were incubated for two days at 37 °C and 5% CO_2. Selection pressure was applied by cultivating CHO cells in culture medium in the presence of 10 μg/ml Blasticidine S Hydrochloride (Sigma-Aldrich) for two weeks. Cell lysates for cell-free protein synthesis were prepared, as described above. Cells were isolated by a BD FACS Aria III flow cytometer (Becton Dickinson) and subjected to genotyping PCR, T7 RNA polymerase assays or protein expression was induced by varying tetracycline concentrations, respectively. Stable integration of T7 RNA polymerase into *Sf*21 cells was achieved using the PiggyBac Transposase system by adding pre-diluted 400 ng DNA in a ratio of 1:1 (PB-Transposase-Sf21: PB-T7RNAPol-Sf21) in 20 μl Sf-900 II medium (Gibco) to pre-diluted 2 μl Insect GeneJuice Transfection Reagent (Sigma-Aldrich) in 20 μl Sf-900 II medium (Gibco). After 15 min of incubation, 160 μl Sf-900 II medium (Gibco) was added to the transfection mixture and the final 200 μl were added to 1×10^6 cells/ml in 300 μL Sf-900 II medium (Gibco) in a 24 well plate. After 48 h incubation at 27 °C selection pressure was applied for two weeks using 250 μg/ml Zeocin (Thermo Fisher Scientific). The *Sf*21 clone pool was expanded to a 500 ml culture in shake flasks and cell lysate was prepared for cell-free protein synthesis as described above.

2.5. Cell-free protein synthesis

Cell-free protein synthesis was performed using 1.5 ml reaction tubes in 25 μl in the presence of 40% CHO cell lysates, 30 mM HEPES-KOH (pH 7.5, Carl Roth GmbH), 100 mM sodium acetate (Merck), 3.9 mM magnesium acetate (Merck), 150 mM potassium acetate (Merck), 100 μM amino acids (Merck), 250 μM spermidine (Roche), 2.5 mM Dithiothreitol (Life technologies GmbH), 100 μg/ml creatine phosphokinase (Roche), 20 mM creatine phosphate (Roche), 1.75 mM ATP (Roche), 0.3 mM of UTP (Roche), 0.3 mM CTP (Roche), 0.3 mM GTP (Roche), 0.1 mM of the cap analogue m7G (pp)G (Prof. Edward Darzynkiewicz, Warsaw University, Poland) and 10 μM PolyG. For cap-independent cell-free reactions 60 ng/μl plasmid DNA was used, while 20 ng/μl purified PCR product was added to cap-dependent reactions. Moreover, 1 U/μl T7 RNA polymerase (Agilent) was added to the cell-free reaction unless otherwise stated. The cell-free reaction was incubated for 3 h at 30 °C and 600 rpm. Cell-free protein synthesis based on *Sf*21 cell lysates was performed equivalent to CHO based cell-free reactions.

2.6. T7 RNA polymerase assay

The activity of T7 RNA polymerase in CHO cells was determined by co-transfecting gWiz-GFP (Genlantis) containing a CMV promoter and piX 3.0-Nluc containing a T7 promoter using Lipofectamine LTX (Thermo Fisher Scientific) in 96-well format according to the manufacturer's instructions. Therefore, 50 ng gWiz-GFP and 50 ng piX3.0-Nluc were combined and added to 0.5 μL Lipofectamine LTX (Thermo Fisher Scientific) and 0.1 μL Plus reagent (Thermo Fisher Scientific) in 10 μl Opti-MEM serum-free medium (Gibco). After 10 min at room temperature 10 μl transfection mixture was directly added to CHO cells and was incubated for two days at 37 °C and 5% CO_2. Technical duplicates were performed for each independent transfection. The amount of GFP positive cells was determined by the LUNA-FL Dual Fluorescence Cell Counter (Logos Biosystems) according to the manufacturer's instructions. Nanoluciferase activity was analyzed as described below. The detected relative luminescence units were divided by the amount of GFP positive cells. The fold change of samples relative to the CHO clone pool was calculated to compare independent experiments.

2.7. Luciferase assays

Firefly luciferase was analyzed by adding 50 μl Luciferase Assay Reagent (Promega) to 5 μl of translation mixture after cell-free protein synthesis. The concentration of active firefly luciferase was determined using a calibration curve. Luminescence was detected using the LB 941 luminometer (Berthold Technologies). Nanoluciferase (Promega) activity was detected using the Nano-Glo Luciferase Assay System (Promega) according to the manufacturer's instructions. Briefly, 100 μl of reagent was added to 100 μl of cells and incubated for 5 min at 300 rpm. For cell-free produced nanoluciferase 5 μl translation mixture after cell-free protein synthesis was mixed with 50 μl reagent and incubated for 3 min. Detection of HiBiT (Promega)-tagged eIF2α-S52A in CHO cells was achieved using the Nano-Glo HiBiT Lytic Detection System (Promega) according to the manufacturer's instructions. Briefly, 100 μl Nano-Glo HiBiT Lytic Reagent containing buffer, substrate and LgBiT for luciferase complex formation was added to 100 μl cell suspension and incubation was performed for 10 min at 300 rpm. The luminescence signal of the HiBiT and nanoluciferase assay was detected by the Multimode Microplate Reader Mithras 2 LB 943 (Berthold Technologies) using an OD2 filter.

2.8. Fluorescence microscopy and image analysis

GFP fluorescence of tetracycline induced CHO cells was visualized by the Olympus IX83 inverted microscope combined with the cellSens imaging software (Olympus). The FITC channel was utilized to detect GFP fluorescence. ImageJ v1.54 d software was used to analyze fluorescence images. Therefore, grayscale images were converted to green channel images and the signal intensity was adjusted equally for all images.

2.9. Western blot

The cell pellet of 5 ml CHO cell suspension was disrupted 24 h post-transfection by resuspending the pellet in 0.3 ml RIPA lysis buffer (10 mM Tris–HCl pH 8, 140 mM NaCl, 1% Triton X-100, 1 mM EDTA, 0.5 mM EGTA, 0.1% SDS, 0.1% sodium deoxycholate). After incubating cells for 30 min at 4 °C, cells were passed through a syringe tip and cell lysate was isolated by centrifugation at $16{,}000 \times g$ at 4 °C for 20 min. The protein concentration of samples was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and 11.5 μg protein was heated at 70 °C for 10 min in LDS sample buffer (Invitrogen).

Samples were separated by denaturing polyacrylamide gel electrophoresis with NuPAGE 10% Bis-Tris Gels (Invitrogen). The protein transfer to a PVDF membrane was performed using the iBlot Dry Blotting System (Invitrogen). The membrane was blocked with 2% bovine serum albumin (BSA) in TBS/T over night at 4 °C. The primary rabbit *anti*-T7 RNA polymerase antibody was diluted 1:1000 in blocking buffer and the membrane was incubated for 3 h under agitation at room temperature. After washing the membrane the 1:2000 diluted secondary anti-rabbit IgG HRP-linked antibody was incubated on the membrane for 1 h. Detection of signals was achieved using the Typhoon TRIO + imager (GE-Healthcare) after washing the membrane and incubating with ECL-detection reagent (GE-Healthcare) for 3 min.

2.10. Genotyping PCR

Genomic DNA of stable transfected CHO cells was extracted using the Quick-DNA Miniprep Plus Kit (Zymo Research) according to the manufacturer's instructions. Utilized genotyping primer sequences were designed as followed: one flanking the integration side of the expression cassette inside the *Rosa*26 locus (Rosa26: 5′-GAGGAGGAGATACCCATCTG-3′) and the second one binding in the donor sequence (Tet-GFP: 5′-GGTGCATGACCCGCAAG-3′ and T7RNAPol: 5′-TCCCGACGGATTCCCTGTTT-3′). The 20 µl PCR reaction was composed of 5x Q5 Reaction Buffer (New England Biolabs), 0.02 U/µl Q5 Hot Start High-Fidelity DNA Polymerase (New England Biolabs), 0.5 µM of each primer, 0.2 mM dNTPs and 50 ng genomic DNA. The following temperature profile was used: 98 °C for 2 min; 35 cycles of 98 °C for 10 s, 65 °C for 20 s, 72 °C for 30 s; 72 °C for 2 min. The PCR-products were run on a 1% agarose gel and product size was compared to the Quick-Load 2-Log DNA Ladder (0.1–10.0 kbp, New England Biolabs).

2.11. Data and statistical analysis

Statistical analysis was performed using Origin (Pro) software version number 2019 (OriginLab Corporation). Data were presented as mean and standard deviation (SD), when technical replicates were shown. Technical replicates were averaged for independent experiments before the mean and standard error of the mean (SEM) for the independent experiments was calculated. The difference between independent experiments was analyzed by a Student's t-test. Alternatively, the Mann-Whitney *U* test was performed to compare sample means of the T7 RNA polymerase assay, because data were not normally distributed. A p-value <0.05 was considered statistically significant.

3. Results

In previous studies, we demonstrated that CHO cells could be modified by transient and stable transfection such that the resulting cell lysates were ideally applicable for straightforward site-specific modification of difficult-to-produce proteins in cell-free reactions [21]. Further improvement of cell-free reaction conditions can be achieved by modifications of the transcriptional and the translational apparatus.

3.1. Harnessing CHO cell lysate containing endogenous T7 RNA polymerase for cell-free protein synthesis

One goal of the present study was to examine whether transcription in cell-free protein synthesis based on T7 RNA polymerase can be designed in a more economical and simplified way, as the enzyme represents a cost factor. Moreover, the T7 RNA polymerase is solubilized in a buffer, which contains e.g. ions and glycerol, thus can interfere with cell-free protein synthesis.

T7 RNA polymerase plays a critical role in cell-free reactions, as it is used to produce mRNA templates for protein expression by using linear and circular DNA templates. It transcribes DNA into mRNA, which then serves as a blueprint for protein production. It is widely used in various cell-free systems due to its high catalytic rate, stability and precision [39]. Consequently, the present work aimed to investigate whether viral T7 RNA polymerase can be integrated into CHO cells prior to cell lysate preparation. Subsequently we analyzed if cell lysates are suitable for cell-free protein synthesis without further addition of RNA polymerase (Fig. 1a). Therefore, CHO cells were transiently transfected with T7 RNA polymerase encoding plasmid DNA and cell lysate was generated two days post-transfection. T7 RNA polymerase expression was observed in transfected cells 24 h post-transfection by western blotting (Fig. 1b). As expected, T7 RNA polymerase could not be detected in untreated cells and cells only treated with PEI reagent.

The new cell lysate was utilized to examine T7 RNA polymerase activity in cell-free protein synthesis using a nanoluciferase (Nluc) template containing a T7 Promoter to initiate transcription and a CrPV IRES to initiate protein translation. In the absence of supplemented T7 RNA polymerase ~90% of the luminescence signal of the sample supplemented with the polymerase could be obtained, when using the novel cell lysate with endogenous enzyme (Fig. 1c). In contrast, cell-free protein synthesis based on non-transfected CHO cells only produced a luminescence signal after the addition of purified T7 RNA polymerase to the reaction.

3.2. Boosting cell-free protein synthesis by influencing cap-dependent protein translation initiation

The initiation of protein translation is the rate-determining step and has a crucial impact on the efficiency of cell-free protein synthesis [40]. Modification of the cap-dependent initiation allows precise control over protein expression levels. By varying the 5′ cap structure or the initiation factors, the expression level can be optimally controlled [41,42]. In contrast, CrPV IRES-dependent initiation, which is often used in cell-free reactions, is more difficult to control or modify because it is independent of the cap structure and initiation factors [14,43,44]. A significant regulator of translational initiation is the initiation factor eIF2α. Phosphorylation of eIF2α at Ser 52 by eIF2α kinases, often triggered by various stress responses, causes decreased eIF2 charging to GTP and thus leads to a reduction in overall protein synthesis [45,46]. Previously it was shown that mutating the phosphorylatable serine to alanine resulted in increased protein expression in CHO cells [47]. Based on this, it was intended to test whether transfection of the eIF2α-S52A mutant would lead to increased protein synthesis rates in cell-free protein synthesis (Fig. 2a). First, a N-terminal HiBiT tag, was utilized to detect successful HiBiT-eIF2α-S52A expression based on a split nanoluciferase assay, which is highly sensitive due to the strong luminescence activity of nanoluciferase. Indeed, eIF2α-S52A expression could be observed, while no luminescence signal could be obtained for untreated cells (Fig. 2b). Three independent transfections and cell lysate preparations were carried out to reproduce these findings. Subsequently, firefly luciferase was synthesized cap-dependently in eIF2α-S52A modified and unmodified cell lysates in cell-free reactions. A significant increase in protein synthesis rate was inferred based on a 3.4-fold higher luminescence signal, in lysates harboring eIF2α-S52A when compared to unmodified lysates (Fig. 2c).

3.3. Improvement of cell-free protein synthesis by integrating the T7 RNA polymerase in genomic safe harbor sites

After demonstrating enhanced cell-free protein synthesis by manipulating transcription and translation using T7 RNA polymerase or mutant eIF2α, we next intended to perform stable transfection of CHO cells to generate modified cell lysates. Stable transfection of T7 RNA polymerase should demonstrate that a suitable locus is present in the CHO genome for future gene integrations. For this purpose, the homologous *Rosa26* locus was compared to the *C12orf35* locus. In a previous study, we used *C12orf35* as a target site and showed that it is capable of expressing the orthogonal *E. coli* tyrosyl-tRNA synthetase,

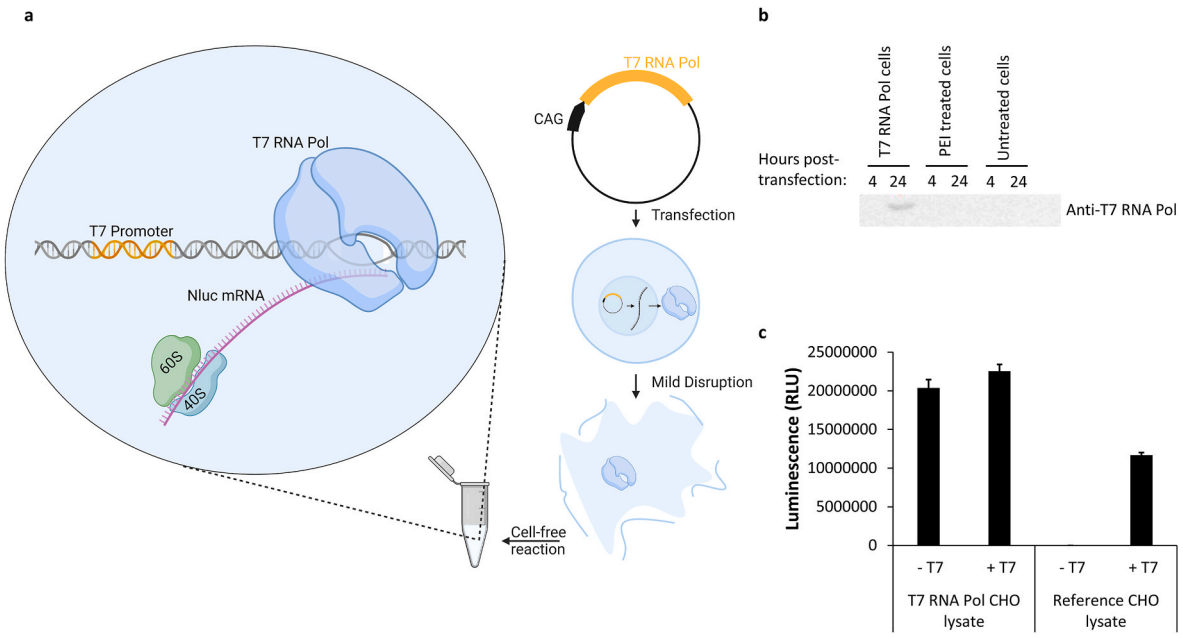

Fig. 1. Cell-free protein synthesis based on transiently transfected CHO cells expressing T7 RNA polymerase. CHO cells were transiently transfected with a T7 RNA polymerase (T7 RNA Pol) encoding expression plasmid and cell lysate preparation was achieved two days post-transfection. a) Scheme of T7 RNA polymerase expression in cells and action during cell-free reaction. b) Detection of T7 RNA polymerase expression using western blot. The uncropped image can be found in Supplementary Fig. 1 c) Cell-free synthesis of nanoluciferase was carried out with (+T7) and without (-T7) supplemented T7 RNA polymerase using a CHO cell lysate containing endogenous T7 RNA polymerase or CHO lysate without T7 RNA polymerase (reference lysate). Measurements were performed in technical triplicate. Data are shown as mean ± SD.

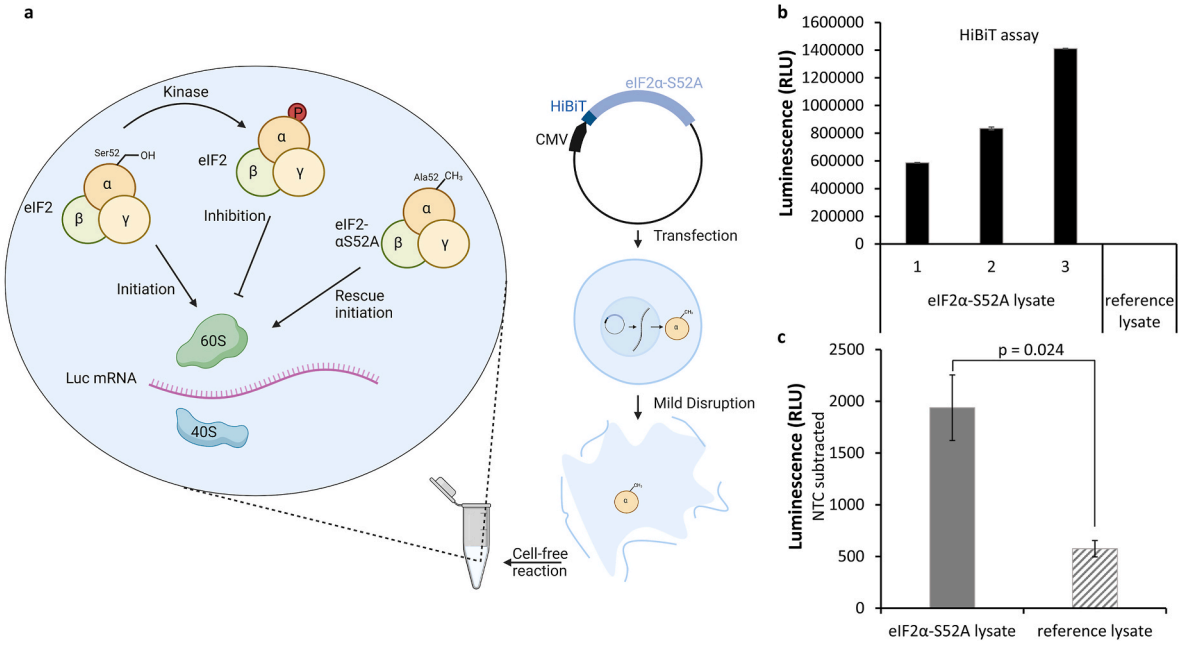

Fig. 2. Cell-free protein synthesis based on transiently transfected CHO cells expressing eIF2α-S52A. CHO cells were transiently transfected with an eIF2α-S52A expression plasmid, containing a HiBiT tag at the N-terminus, and cell lysate preparation was achieved two days post-transfection. a) Scheme of eIF2α-S52A expression in cells and action of eIF2α-S52A during cell-free protein synthesis. b) Detection of eIF2α-S52A expression using the HiBiT assay. Three independent transfections and cell lysate preparations (sample 1–3) were performed and data is expressed as technical duplicate. c) Cell-free synthesis of firefly luciferase utilizing CHO cell lysate containing endogenous eIF2α-S52A. A cell-free reaction without luciferase template (No-template control, NTC) was subtracted from sample values for background subtraction. Three independent transfections were performed to produce eIF2α-S52A CHO lysates (grey), while six independent cell lysate preparations were performed from non-transfected CHO cells referred as to reference lysate (diagonal lines). Data are shown as mean ± SEM. Statistical significance was observed by a *t*-test.

resulting in translationally active cell lysates that can be used for orthogonal cell-free protein synthesis [21]. Gaidukov et al. demonstrated that the *Rosa26* locus, which is commonly used for transgene site-specific integration in human and mouse cell lines, is also present in homologous form in the CHO genome [33]. Loci were analyzed using a T7 RNA polymerase assay based on a dual reporter system (Fig. 3a). A T7

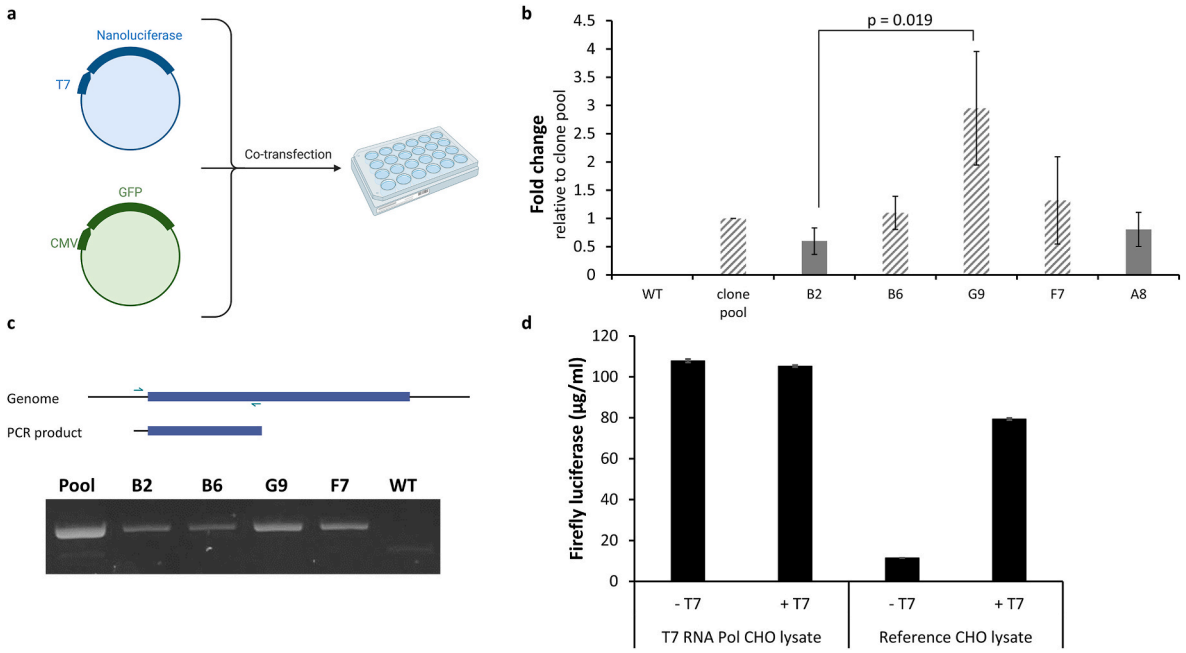

Fig. 3. Generation of stably transfected CHO cells expressing T7 RNA polymerase. CHO cells were stably transfected by CRISPR/Cas9 to integrate a T7 RNA polymerase expression cassette into *Rosa26* in the CHO genome. a) Scheme of the T7 RNA polymerase assay used for the analysis of T7 RNA polymerase expressing CHO cells. A nanoluciferase plasmid driven by a T7 promoter and an expression plasmid containing a CMV promoter upstream of a GFP gene was utilized to evaluate T7 RNA polymerase expressing CHO cells. b) T7 RNA polymerase assay. The fold change is relative to the clone pool from which the cell lines were selected from. Independent transfections (grey: n = 4; diagonal lines: n = 6) were carried out. Data are shown as mean ± SEM. A Mann-Whitney *U* test was used to detect statistical significance. c) Genotyping PCR was performed using a primer pair binding inside and outside of the donor template. The resulting PCR products were analyzed by agarose gel electrophoresis. The uncropped image can be found in Supplementary Fig. 3) d)Cell-free synthesis of firefly luciferase was carried out with (+T7) and without (-T7) supplemented T7 RNA polymerase using a CHO cell lysate containing endogenous T7 RNA polymerase or CHO lysate without T7 RNA polymerase (reference lysate). Measurements were performed in technical duplicate. Data are shown as mean ± SD.

promoter upstream of the Nluc gene was utilized to evaluate transcriptional efficiency corresponding to the luminescent signal. At the same time, another plasmid was co-transfected, which contains a CMV promoter upstream of a GFP gene for constitutive expression. The luminescence signal was normalized to the GFP positive cells to compensate for differences such as cell number and transfection efficiency. The clone pools enriched by selection pressure were transfected and analyzed in two independent experiments. A 88 ± 29 fold higher luminescence signal was obtained with the *Rosa26* clone pool compared to the *C12orf35* clone pool. After isolation of clonal cell lines from the promising *Rosa26* pool, again the T7 RNA polymerase assay was performed to evaluate the outgrown T7 RNA polymerase expressing cell lines. The CHO clone G9 achieved the highest T7 RNA polymerase activity among the tested clones and significantly higher activity than CHO clone B2 (Fig. 3b). After further expansion of cell lines, genotyping PCR was performed by using a primer which binds inside the T7 RNA polymerase expression cassette and a primer which binds outside of the expression cassette to ensure gene integration at the *Rosa26* genomic site (Fig. 3c). CHO clone A8 showed decreased cell viability and was not examined further. The T7 RNA polymerase expression cassette seems to be integrated at the desired *Rosa26* locus, indicated by the PCR product. As a result, CHO G9 clone was the ideal candidate to produce cell lysate for cell-free protein production. Consequently, it was examined whether the cell lysate containing the T7 RNA polymerase can be used for cell-free protein synthesis. The firefly luciferase plasmid containing a T7 promoter upstream of the coding sequence, was added to the cell-free reaction to evaluate the new CHO lysate. Cell-free reactions with and without the addition of commercial T7 RNA polymerase were performed by using the novel T7 RNA polymerase harboring lysate and a reference lysate, which was derived from non-modified CHO cells. As expected, no signal was detected without the addition of T7 RNA polymerase to the cell-free reaction based on unmodified CHO cells (Fig. 3d). In contrast, with the newly developed cell lysate, a comparable luminescence signal was obtained without the addition of T7 RNA polymerase compared to the cell-free reaction with further addition of commercial T7 RNA polymerase.

We transferred the approach to *Sf*21 cell-free reactions by a PiggyBac transposase system to stably transfect T7 RNA polymerase into *Sf*21 cells. Although, T7 RNA polymerase activity was also detected during cell-free protein synthesis, further enzyme addition to the cell-free reaction is of distinct advantage (Supplementary Fig. 2).

3.4. Tetracycline-driven gene expression at the Rosa26 locus

Once established that the *Rosa26* locus is well adapted for integration of genes for cell-free protein synthesis without affecting the sensitive translational active lysate, we further investigated whether this locus can be utilized to control gene expression in cells by addition of an induction agent. Consequently, we decided to develop a tetracycline inducible system at the *Rosa26* genomic site to flexibly switch between cell-free and cell-based production depending on the application.

For this purpose, we designed an all-in-one donor vector containing the tetracycline-inducible tetracycline-response element (TRE) coupled to a CMV promoter upstream of a GFP gene (Fig. 4a). Downstream of the inducible expression site, the reverse tetracycline-controlled transactivator (rtTA) was under the control of a constitutive CMV promoter. Upon addition of tetracycline, the rtTA can bind to the TRE element and start transcription. The rtTA was fused to a puromycin resistance gene via a self-cleavage peptide site to allow enrichment and isolation of clonal cells. The large construct of 4554 bp (without plasmid backbone) was stably transfected into the *Rosa26* locus using CRISPR/Cas9. Correct integration was confirmed by genotyping PCR (Supplementary Fig. 3). Without the addition of tetracycline to the culture medium only weak fluorescence could be detected by microscopy (Fig. 4b). As anticipated,

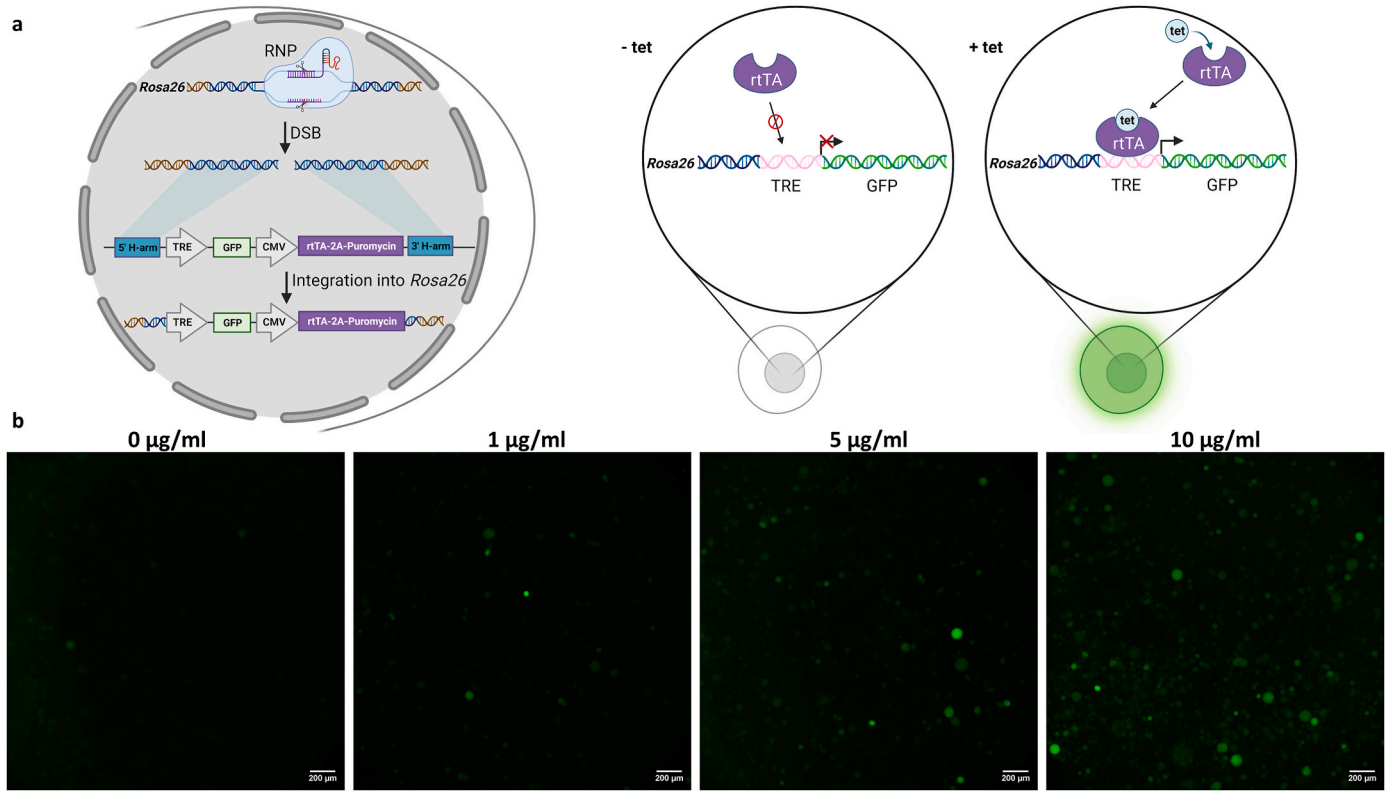

Fig. 4. Tetracycline-based induction of GFP expression at *Rosa26* in CHO cells. CHO cells were stably transfected by CRISPR/Cas9 with an all-in-one donor template to integrate a GFP expression cassette controlled by a tetracycline-responsive element (TRE) and an expression cassette containing the reverse tetracycline-controlled transactivator (rtTA) driven by a CMV promoter into *Rosa26* in the CHO genome. a) Scheme of CRISPR/Cas9 based modification of CHO cells to create cells capable of tetracycline (tet) -based induction of GFP expression in the presence (+tet) or absence (-tet) of tetracycline. b) A clonal cell line was isolated and incubated in the absence (0 µg/ml) and presence of increasing tet concentrations (1–10 µg/ml). CHO cells were analyzed by fluorescence microscopy two days after tet addition and images represent the fluorescence detection by using the FITC channel.

elevating tetracycline levels in the medium resulted in increased fluorescence signals. Thus, it was demonstrated for the first time that an inducible system could be successfully used at the *Rosa26* locus in the CHO genome.

4. Discussion

Eukaryotic cell-free protein synthesis is an ideal reaction format to rapidly produce complex proteins that are difficult to manufacture. However, protein synthesis under cell-free conditions is costly, often limiting protein production in larger quantities. In the present study, we demonstrated that cell-free protein synthesis based on CHO cell lysates can be more efficient and cost-effective by modifying the host cells. The potential of enhancing cell-free cap-dependent translation initiation was recently demonstrated by overexpression of GADD34 and K3L in human cells [48]. It was shown that increased levels of the truncated protein phosphatase GADD34 and the vaccinia virus protein K3L prior to cell disruption resulted in decreased eIF2α phosphorylation and increased cell-free protein production based on human cell lysate. We have previously shown that eIF2α phosphorylation is significantly increased in the absence of the specific kinase PERK inhibitor C38 in the cell-free reaction [36], thus the presence of eIF2α-S52A in the cell lysate can compensate for translation initiation in cell-free reactions. Underhill et al. transiently transfected CHO cells with the mutant eIF2α to prevent phosphorylation and could achieve a 3-fold increase of reporter gene activity compared to control cells, which is in accordance with our findings, based on the mutant eIF2α cell lysate [47].

Frequently, the CrPV IRES is utilized for translation initiation in eukaryotic cell-free systems because it is independent of the cap structure and translation initiation factors [49,50]. Although the Encephalomyocarditis virus (EMCV) IRES is commonly used in mammalian cell lines, its effectiveness in cell-free systems was poor [14,51,52]. The EMCV IRES operates independently of the cap structure, but unlike CrPV IRES, initiation factors such as eIF2 are required [53]. Therefore, using the novel mutant eIF2α cell lysate, translational initiation rates, based on diverse IRES, could potentially be increased. T7 RNA polymerase is a significant cost factor of cell-free protein synthesis, hence *E.coli* based cell-free protein synthesis systems often utilize extracts based on host cells, which have the T7 RNA polymerase integrated into the genome [54]. Here, we were able to establish an eukaryotic cell-free production system that does not require the exogenous addition of T7 RNA polymerase. While lysates based on the expression of orthogonal *E. coli* tyrosyl-tRNA synthetase at the *C12orf35* locus could be successfully used, expression of T7 RNA polymerase at the same target site was not effective. This could be due to the larger coding sequence of the T7 RNA polymerase (~2.65 kbp) to be inserted in contrast to the orthogonal aaRS (~1.27 kbp). Histone deacetylation or DNA methylation of the gene are known to decrease recombinant protein production during ongoing cultivation [55], thus epigenetic silencing could result in low T7 RNA polymerase expression at *C12orf35*.

While the *Rosa26* locus has become standard as target site in human and mouse cell lines for many years, it has been shown that the homologous *Rosa26* in the CHO genome can be used to exchange expression cassettes for constitutive expression [33,56,57]. In the present work, *Rosa26* was found to be a suitable target site that allows for the incorporation of large expression cassettes, such as the complete regulatory units required for induced expression in CHO cells. Thus, the *Rosa26* target site provides a safe, predictable location in the CHO genome where foreign DNA can be introduced for inducible protein expression without disrupting normal gene function. Thus, expression of

the desired proteins can be regulated under specific conditions without undesirable effects on other genetic processes.

It is often observed that the tetracycline-based system may have low basal activity in the absence of tetracycline or the derivative doxycycline [58–60]. We found minimal basal activity of the established system in the absence of tetracycline, but at the same time a significant increase in expression with increasing tetracycline concentration. Alternatively, other inducible systems that exhibit lower basal activity, such as light-inducible systems or riboswitches, could be used in the future, depending on the desired level of expression [29,61]. Furthermore, it would be interesting to use the established system for the induction of toxic proteins to compare them with cell-free protein synthesis or to produce initially cell-free characterized toxins and membrane proteins in a cell-based manner. Moreover, targeted S52A modification of endogenous eIF2α by CRISPR/Cas prior to cell disruption could shift the balance between phosphorylated and non-phosphorylated eIF2α to enhance translation initiation without stressing cells by permanently increasing eIF2α activity. Combined recombinant expression of orthogonal aaRS, T7 RNA polymerase and eIF2α-S52A can be subject of gene silencing and a burden for living cells, when inserted in undefined loci. In the future, combined stable transfection of desired proteins could be conceivable using the *Rosa26* locus to improve cell-free protein synthesis.

5. Conclusion

In this study, we increased the protein synthesis rate by modification of translation initiation using a eIF2α-S52A modified CHO cell lysate. Additionally, the integration of T7 RNA polymerase into the CHO cell lysate by transient and stable transfection decreased costs of cell-free protein synthesis. The utilization of the *Rosa26* locus enabled us to develop an effective tetracycline-controlled expression system. Anticipating the future, we are confident that the combination of the cost-effective cell-free and the cell-based *Rosa26* inducible system, provide flexible production of difficult-to-express proteins for diverse applications in biotechnology.

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CRediT authorship contribution statement

Jeffrey L. Schloßhauer: Conceptualization, Methodology, Formal analysis, Visualization, Investigation, Writing – original draft. **Lena Tholen:** Methodology, Supervision. **Alexander Körner:** Methodology, Writing – review & editing. **Stefan Kubick:** Supervision, Writing – review & editing. **Sofia Chatzopoulou:** Investigation. **Anja Hönow:** Resources. **Anne Zemella:** Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.03.011.

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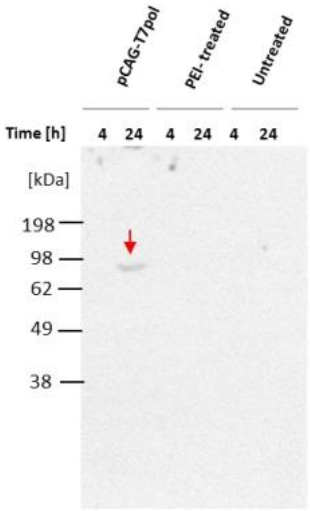
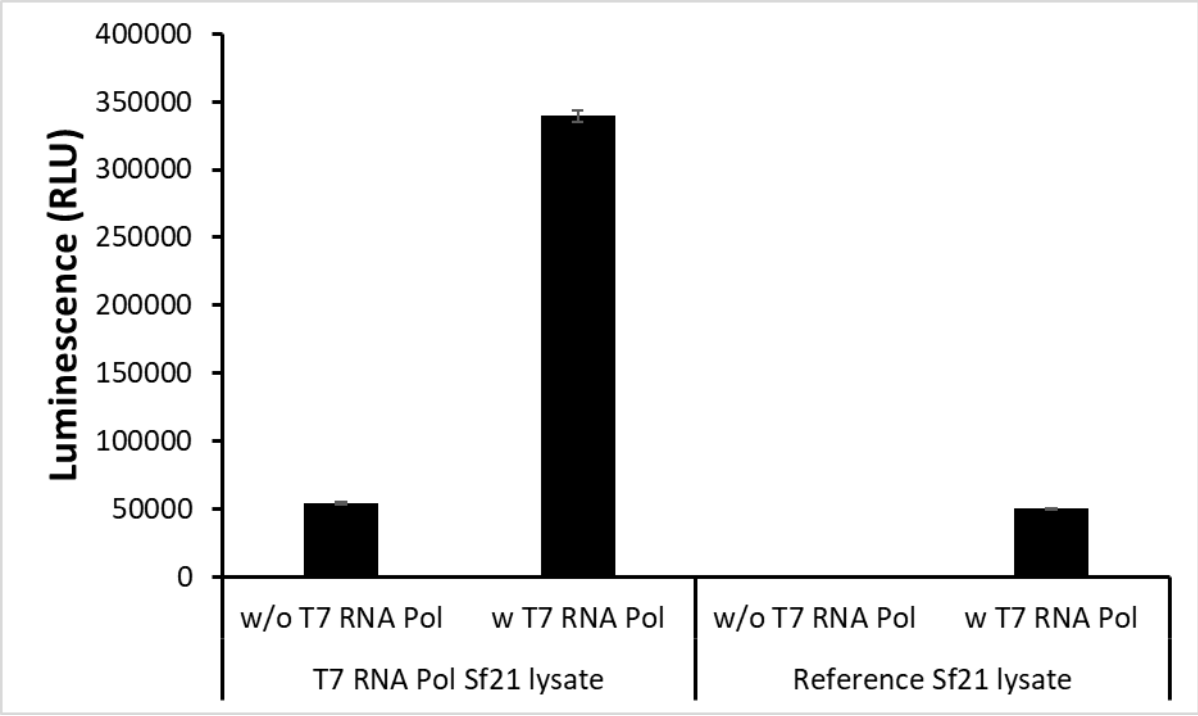
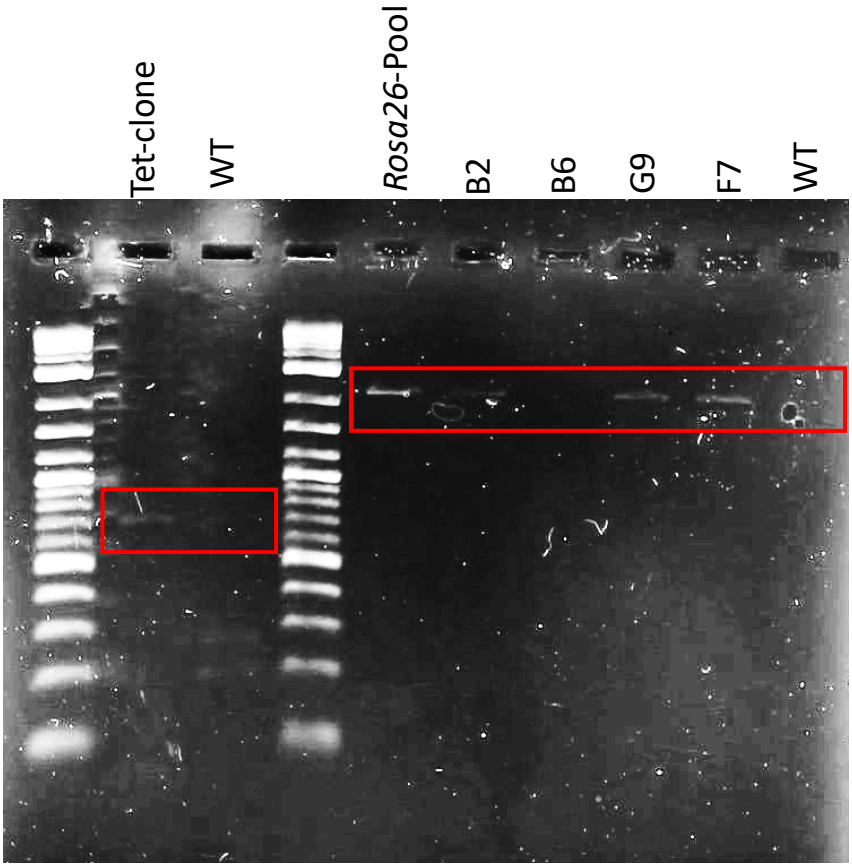
Supplementary Information



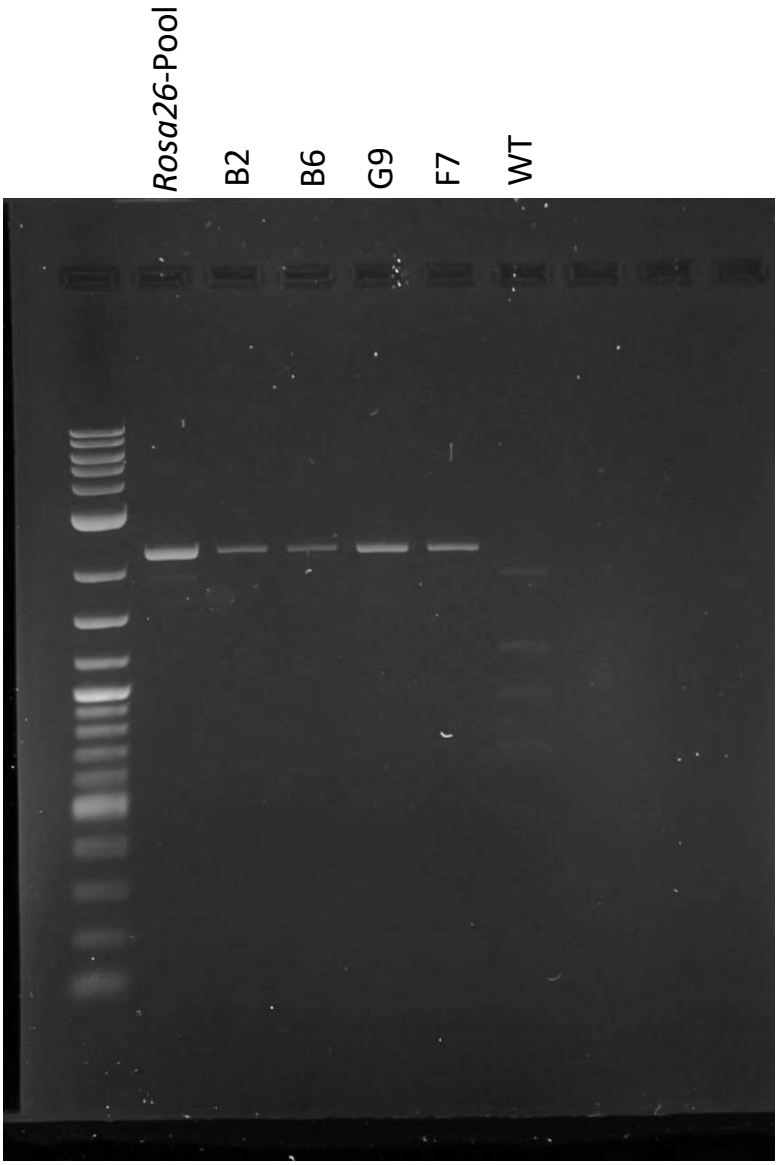
Supplementary Figure 1: Western blot. Detection of T7 RNA polymerase expression using western blot. Displayed is the uncropped image of Figure 1b. A SeeBlue Plus2 Pre-Stained Standard (Life technologies) was used. The red arrows indicates the protein band of T7 RNA polymerase.



Supplementary Figure 2: Cell-free synthesis of luciferase based on *Sf*21 cell lysates containing endogenous T7 RNA polymerase. *Sf*21 cells were stable transfected using PiggyBac Transposase. Positively stable transfected cells were enriched in the presence of the selection marker Zeocin. Cell-free synthesis of the Luciferase was carried out with (w) and without (w/o) supplemented T7 RNA polymerase using a *Sf*21 cell lysate containing endogenous T7 RNA polymerase or *Sf*21 lysate without T7 RNA polymerase (reference lysate). Measurements were performed in technical duplicate. Data are shown as mean ± SD.



Supplementary Figure 3: Genotyping PCR. Genotyping of the tetracycline-inducible CHO cell line and the T7 RNA polymerase expressing CHO cell lines was performed by PCR using a primer pair binding inside and outside of the donor template. The resulting PCR products were visualized by agarose gel electrophoresis. Red boxes indicate the expected PCR products. Lane 1 & 4: Quick-Load 2-Log DNA Ladder 0.1–10.0 kb (New England Biolabs). Lane 2: tetracycline-inducible CHO cell line, Lane 3 & 10: Wildtype CHO cells (WT), Lane 5: *Rosa26* clone pool expressing T7 RNA polymerase, Lane 6-9: CHO clones expressing T7 RNA polymerase. Genotyping of T7 RNA polymerase expressing cells was repeated and is presented in Supplementary Figure 4.



Supplementary Figure 4: Repeated genotyping PCR of T7 RNA polymerase expressing cells. Genotyping was repeated due to the absence of PCR-products for clone B6. Lane 1: Quick-Load 2-Log DNA Ladder 0.1–10.0 kb (New England Biolabs), Lane 2: *Rosa26* clone pool expressing T7 RNA polymerase, Lane 3-6: CHO clones expressing T7 RNA polymerase, Lane 7: Wildtype CHO cells (WT).

2.3. A Cost-Effective *Pichia pastoris* Cell-Free System Driven by Glycolytic Intermediates Enables the Production of Complex Eukaryotic Proteins

While the other publications addressed CHO-based approaches for producing complex proteins, the aim of this work in the doctoral thesis was to further simplify CFPS and drastically reduce the costs of eukaryotic CFPS by utilizing *P. pastoris* cell lysate. Therefore, an alternative energy regeneration system based on glycolysis was developed, and a statistical Design of Experiments was applied. It was demonstrated that the novel yeast-based cell-free system is capable of producing site-specifically modified proteins and can produce membrane proteins in their active form.

Declaration of own contribution

1st authorship. I, Jeffrey Lesslie Schloßhauer, originated the concept, designed the experiments, and performed them, including optimizing the cultivation and cell disruption of *P. pastoris* and *S. cerevisiae*, followed by cell lysate preparation for cell-free protein synthesis. Moreover, I examined protein translocation in ER vesicles in cell-free protein synthesis using different signal peptides, developed a new energy regeneration system, and carried out the statistical Design of Experiments and orthogonal protein translation. Additionally, I synthesized and prepared ion channels for electrophysiological measurements, although I did not perform or analyzed the electrophysiological measurements myself. Moreover, I wrote the manuscript and prepared the figures, except for the electrophysiological approaches. Srujan Kumar Dondapati conducted electrophysiological measurements, corresponding analysis, and prepared electrophysiological figures. Anne Zemella and I discussed the project design. Anne Zemella and Stefan Kubick revised the manuscript.

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A Cost-Effective *Pichia pastoris* Cell-Free System Driven by Glycolytic Intermediates Enables the Production of Complex Eukaryotic Proteins

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Abstract: Cell-free systems are particularly attractive for screening applications and the production of difficult-to-express proteins. However, the production of cell lysates is difficult to implement on a larger scale due to large time requirements, cultivation costs, and the supplementation of cell-free reactions with energy regeneration systems. Consequently, the methylotrophic yeast *Pichia pastoris*, which is widely used in recombinant protein production, was utilized in the present study to realize cell-free synthesis in a cost-effective manner. Sensitive disruption conditions were evaluated, and appropriate signal sequences for translocation into ER vesicles were identified. An alternative energy regeneration system based on fructose-1,6-bisphosphate was developed and a ~2-fold increase in protein production was observed. Using a statistical experiment design, the optimal composition of the cell-free reaction milieu was determined. Moreover, functional ion channels could be produced, and a G-protein-coupled receptor was site-specifically modified using the novel cell-free system. Finally, the established *P. pastoris* cell-free protein production system can economically produce complex proteins for biotechnological applications in a short time.

Keywords: cell-free protein synthesis; yeast; *Pichia pastoris*; protein production; energy regeneration; design of experiments; signal sequence; orthogonal system

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1. Introduction

Pichia pastoris has been established as a highly common organism for recombinant protein production. This yeast offers an ideal balance between genetic manipulability, streamlined protein production, and the ability to post-translationally modify proteins [1,2]. Straightforward manipulation of the yeast genome, compared to mammalian cells, allows for the introduction of foreign genes and metabolic engineering with reduced effort [3]. Another advantage is the availability of regulatable promoters, such as the methanol-inducible AOX1 promoter, as a highly controllable genetic switch that enables precise control of target protein expression [4]. *P. pastoris* is easily scalable and also known for its ability to produce high levels of recombinant proteins, efficiently secrete proteins, and realize complex post-translational modifications, including glycosylation [5–7]. The presence of yeast-like glycans can lead to adverse reactions when glycosylated proteins were applied for human applications [8]. In contrast to the model organism *Saccharomyces cerevisiae*, which produces proteins with high mannose glycosylation, *P. pastoris*-based protein production results in fewer immunogenic glycosylation patterns and can grow to

higher cell densities [9]. The simple handling during cultivation and transformation, as well as a doubling time of 1–3 h, lead to time-saving protein production in *P. pastoris* [10].

The properties of *P. pastoris* in recombinant protein production make it useful for cell-free synthesis of proteins, especially toxic proteins and membrane proteins [11,12]. Most importantly, it allows for the rapid production of proteins without depending on the growth and maintenance of living cell cultures. Proteins can be produced up to 60 µg/mL within 90–180 min in yeast-based cell-free systems, substantially accelerating the production of biotechnologically demanding proteins [13,14]. Furthermore, cell-free protein synthesis allows for precise control over production conditions, which can lead to higher yields and quality of the proteins produced [15]. In this approach, translationally active cell lysate, a reaction mix containing an energy regeneration system and substrates for transcription and translation, and T7 RNA polymerase are added to the open reaction environment to start cell-free protein synthesis based on a gene of interest downstream of a T7 promoter located on linear or circular DNA templates (Figure 1a). Other supplements can be added to the open system, such as detergents for solubilization of membrane proteins, the addition of components for site-specific modification of proteins, and cofactors to enhance protein activity [16].

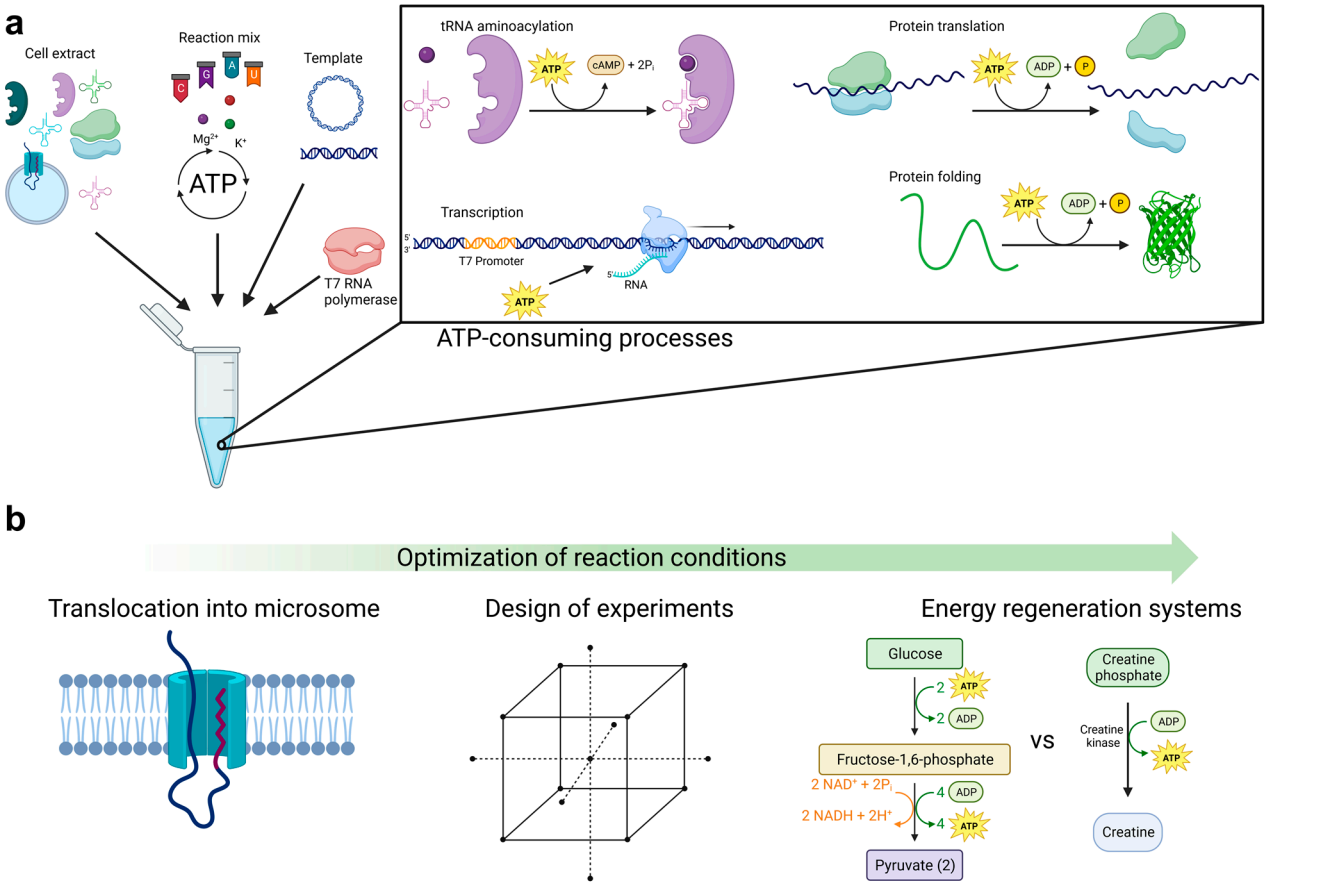

Figure 1. Cell-free protein synthesis with adjusted reaction conditions. (**a**) Eukaryotic cell-free protein synthesis is based on the utilization of a translationally active cell extract containing endogenous components, including aminoacyl tRNA synthetases, ribosomes, tRNAs, and microsomes. Moreover, a linear or circular DNA template, a T7 RNA polymerase, and a reaction mix containing defined concentrations of nucleotides, amino acids, and ions are needed. Additionally, an energy regeneration system is part of the reaction mix due to the ATP-consuming processes in cell-free protein synthesis. (**b**) Optimization strategies to obtain an effective *P. pastoris* cell-free protein synthesis system.

Processes during cell-free protein production, including transcription, aminoacylation of tRNAs, ribosome dissociation, and protein folding, require ATP. Therefore, high-energy phosphor donors are often used to keep energy requirements in balance [17]. However, the reagents and energy sources required for cell-free protein synthesis are expensive, especially for large-scale protein production [18]. *Escherichia coli* represents a more economical way to produce proteins in cell-free reactions, also due to other energy regeneration systems. Although *E.coli* cell-free protein synthesis is widely used, the prokaryotic cell lysate offers limited post-translational modifications and thus fewer issues in the production of complex proteins [19]. On the other hand, post-translational modifications and optimal folding conditions are possible in eukaryotic cell-free protein production systems based on mammalian, insect, and tobacco cells [20–22]. Moreover, it was shown that membrane proteins can be translocated into vesicles derived from the endoplasmic reticulum (ER), termed microsomes, to facilitate the production of active ion channels in CHO and insect cell-free systems [23]. However, the cultivation and preparation of translationally active cell lysates is associated with increased time and cost, compared to *E. coli*-based cell-free protein synthesis. In fact, yeast-based cell-free protein synthesis can be used to produce complex proteins at a relatively low investment of both time and expense. In recent years, various protocols have been developed to establish cost-effective *S. cerevisiae*-based cell-free protein synthesis based on different cultivation, disruption, and lysate processing methods, as well as additives to the cell-free reaction [24–26]. Various protocols could be transferred to *P. pastoris*, and new methods could be established to use *P. pastoris* lysates in a cell-free format to synthesize, for instance, virus-like particles that are difficult to produce [27–29].

In the present work, *P. pastoris* cells were treated under different conditions to generate translationally active cell lysates. Evaluation of different signal sequences demonstrates the possibility of simultaneous production enhancement and translocation into microsomes (Figure 1b). A statistical experimental design was created to show that reaction conditions could be modified with a low time requirement and high informative value. An alternative source based on glycolysis intermediates for energy regeneration aimed to make the established cell-free yeast expression system more productive and less expensive to produce complex proteins, including the ion channels KvAP and KcSA.

2. Materials and Methods

2.1. Plasmids

The plasmids Adora2a-amb-Nluc and Adora2a-Nluc (with and without an amber stop codon at amino acid position 215) contain a melittin signal peptide and were used as previously described [30]. The plasmid DNA coding for the voltage-gated potassium channel KvAP and the potassium channel KcSA were used as previously described [31,32]. The ppα-GFP plasmid contains an α-mating factor prepro peptide fused to GFP and was purchased from Biocat. The firefly luciferase plasmid was utilized as previously described [33]. All plasmids for cell-free protein synthesis contain a cricket paralysis virus (CrPV) internal ribosome entry site (IRES) for translation initiation. The Mel-GFP plasmid containing a melittin signal peptide was utilized as previously described [34].

2.2. Yeast Cells and Cultivation

P. pastoris cells were obtained from the Leibniz Institute DSMZ—German Collection of Microorganisms and Cell Cultures GmbH (DSMZ no: 70382), while the *S. cerevisiae* strain S288C was obtained from the American Type Culture Collection (ATCC no: 204508). *P. pastoris* and *S. cerevisiae* cells were cultured at 29 °C and 300 rpm in YPD (1% yeast extract, 2% peptone, 2% dextrose) medium using baffled shake flasks, unless otherwise noted. Shake flasks were filled up to 20% culture volume capacity.

2.3. Cell Lysate Preparation

P. pastoris cells were harvested at a backscatter signal of ~5500 a.u. measured by the Cell Growth Quantifier (CGQ) online biomass monitoring system (Scientific Bioprocessing,

Baesweiler, Germany). Cells were immediately cooled down to 4 °C and centrifuged for 10 min at 500× *g*. The cell pellet was washed three times with washing buffer (30 mM HEPES-KOH (pH 7.4), 100 mM KOAc, 2 mM DTT) using centrifugation steps of 5 min at 4 °C and 500× *g*. After the final wash step, for each 1 g wet cell pellet, 1 mL cell disruption buffer (30 mM HEPES-KOH (pH 7.4), 100 mM KOAc, 2 mM DTT), and 1 × cOmplete ULTRA Protease Inhibitor Cocktail (Roche, Basel, Switzerland) were used for resuspension. Bead-based homogenization was performed by adding 1 mL resuspended cells to a 2 mL lysing tube containing a lysing matrix (MP Biomedical, Irvine, CA, USA). The yeast cells were disrupted by applying four cycles of 35 s, unless otherwise noted, at 6 m/s using the FastPrep-24 Bead-Beating instrument (MP Biomedicals, Irvine, CA, USA) in the presence of dry ice in the cooling chamber. High-pressure homogenization was performed at 35 KPSI using the CF1 Cell Disruptor (Constant Systems, Daventry, United Kingdom) by applying one or two cycles at 4 °C. Disrupted cells were initially centrifuged at 1500× *g* at 4 °C for 5 min, followed by centrifugation of the supernatant for 10 min at 8000× *g* and 4 °C. The supernatant was isolated, and the cell lysates were supplemented with Baker's yeast tRNA (final concentration of 5 µg/mL) and RNasin (Promega, Madison, WI, USA) Ribonuclease Inhibitor (final concentration of 150 U/mL). Cell lysates were stored at −80 °C. *S. cerevisiae* was disrupted by bead-based homogenization equivalent to *P. pastoris*.

2.4. Cell-Free Protein Synthesis

Initially performed cell-free reactions were composed of 35% *P. pastoris* cell lysate, 10 µM PolyG, 100 ng/µL plasmid, 30 mM HEPES-KOH (pH 7.4, Carl Roth, Karlsruhe, Germany), 6 mM magnesium acetate (Merck, Darmstadt, Germany), 150 mM potassium acetate (Merck, Darmstadt, Germany), 100 µM amino acids (Merck, Darmstadt, Germany), 250 µM spermidine (Roche, Basel, Switzerland), 2.5 mM Dithiothreitol (Life technologies, Darmstadt, Germany), 100 µg/mL creatine phosphokinase (Roche, Basel, Switzerland), 20 mM creatine phosphate (Roche, Basel, Switzerland), 0.5 mM ATP (Roche, Basel, Switzerland), 0.5 mM GTP (Roche, Basel, Switzerland), 0.5 mM of UTP (Roche, Basel, Switzerland), 0.5 mM CTP (Roche, Basel, Switzerland), and 1 U/µL T7 RNA polymerase (Agilent, Santa Clara, CA, USA). A total of 30 µM radioactive ^{14}C-leucine (Perkin Elmer, Rodgau, Germany) was added to the reaction to enable the qualitative analysis of radio-labeled proteins by gel electrophoresis. Additionally, ^{14}C isoleucine and ^{14}C valine were added when indicated. Cell-free reactions were incubated for three hours at 28 °C and 600 rpm, unless otherwise noted. Cell-free reactions without the addition of plasmid to analyze translational activity of cell lysates using endogenous mRNA were modified by omitting the plasmid and T7 RNA polymerase. Orthogonal cell-free reactions were further supplemented with 2 µM mutant *E. coli* tyrosyl-tRNA synthetase [35,36] (eAzFRS), 3 µM orthogonal tRNAtyr, and 2 mM p-azido-L-phenylalanine (AzF). The purified eAzFRS and the orthogonal tRNAtyr were prepared as previously described in detail [30]. Creatine phosphokinase (Roche, Basel, Switzerland) and creatine phosphate (Roche, Basel, Switzerland) were substituted by either 20 mM glucose (Sigma-Aldrich, St. Louis, MI, USA), glucose-6-phosphate (Sigma-Aldrich, St. Louis, MI, USA), fructose-6-phosphate (Sigma-Aldrich, St. Louis, MI, USA), fructose-1,6-bisphosphate (Sigma-Aldrich, St. Louis, MI, USA), 3-phosphoglyceric acid (Sigma-Aldrich, St. Louis, MI, USA), phosphoenolpyruvate (Sigma-Aldrich, St. Louis, MI, USA), glycerol (Sigma-Aldrich, St. Louis, MI, USA), or glycerol-3-phosphate (Sigma-Aldrich, St. Louis, MI, USA) in cell-free reactions based on alternative energy regeneration systems. A total of 330 µM nicotinamide adenine dinucleotide (Sigma-Aldrich, St. Louis, MI, USA), 150 µM cyclic AMP (Sigma-Aldrich, St. Louis, MI, USA), and 20 mM potassium phosphate (Merck, Darmstadt, Germany) were added to cell-free reactions based on glycolytic intermediates, unless otherwise noted. Cell-free reactions with ppα-GFP were performed in 96-well plates, and fluorescence was measured using the LightCycler 96 instrument (Roche, Basel, Switzerland). Modifications of the cell-free reaction composition were mentioned in the text.

2.5. Design of Experiments and Data Analysis

The Minitab software version 21.4.1 was utilized to design and analyze the conducted experiments. The fluorescence signal produced from ppα-GFP was the analyzed response. A full factorial experimental design with three factors on two levels (presence or absence of the tested factor) was performed with three replicates. A total of 24 totals runs, including 8 base runs, were carried out in one block. A significance level of 0.05 was used to interpret the results. The central composite design was performed as a two-level factorial (half fraction) plan using 90 randomized total runs in three blocks and a rotatable design (distance of each axial point from the center (α = 2.82843)). The design included 64 cube points, 8 center points in the cube, 14 axial points, and 4 center points in the axis. Analysis of variance and backward elimination of terms was performed by using an α-to-remove value of 0.1 to generate a robust model.

2.6. Autoradiography of Radiolabeled Proteins

After cell-free protein synthesis, a 5 µL translation mixture was mixed with 45 µL of water, and proteins were precipitated by the addition of 150 µL acetone. Protein precipitation was performed on ice for 15 min, and the precipitated pellet was isolated by centrifugation for 10 min at 4 °C and 16,000× *g*. A total of 20 µL LDS sample buffer (Invitrogen, Carlsbad, CA, USA) containing 50 mM DTT was used to resuspended pellets after drying for 45 min at 45 °C to remove acetone. Samples were mixed at 1400 rpm for 30 min at room temperature. Denaturing polyacrylamide (14%) gel electrophoresis (PAGE) was based on the SureCast Gel Handcast System (Invitrogen, Carlsbad CA, USA). Subsequently gels were stained with SimplyBlue SafeStain (Thermo Scientific, Waltham, MA, USA) and dried by a Unigeldryer 3545D vacuum chamber (UniEquip, Martinsried, Germany) and deposited on phosphor screens (GE Healthcare, Chicago, IL, USA) for three days, and incubated screens were scanned by the Amersham Typhoon RGB (GE Healthcare, Chicago, IL, USA). The presence of ^{14}C-labeled amino acids in cell-free reactions allowed for the visualization of the produced proteins based on autoradiography.

2.7. Fluorescence Microscopy

The translation mixture was centrifuged at 16,000× *g*, 4 °C, for 15 min to enrich the microsomes in the pellet. The supernatant was discarded, and the pellet was resuspended in an equal amount of PBS. The samples were transferred to an 18-well flat µ-Ibidi-Slide (Ibidi, Gräfelfing, Germany). The GFP fluorescence was visualized via excitation by a 488 nm laser, and emitted light was captured at 505 nm using an LSM 510 meta laser-scanning microscope (Zeiss, Oberkochen, Germany) equipped with a Plan-Aprochromat 63×/1.4 oil objective.

2.8. Luciferase Assay

Firefly luciferase activity was detected using Luciferase Assay Reagent (Promega, Madison, WI, USA). Therefore, 50 µL of reagent was added to 5 µL of translation mixture after cell-free protein synthesis, and luminescence was detected by the LB 941 luminometer (Berthold Technologies, Bad Wildbad, Germany). The concentration of active luciferase was determined using a calibration curve.

2.9. Electrophysiological Measurements

The translation mixture of cell-free-produced KcSA and KvAP was centrifuged for 10 min at 16,000× *g* at 4 °C and the pellet was resuspended in an equal-volume PBS. The resuspended pellet contained the microsomal vesicles and was utilized for electrophysiological measurements. Planar bilayer experiments were performed, as explained previously [37]. Lipid bilayers were formed from 1, 2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) (Avanti Polar Lipids, Albaster, AL, USA). Lipids were dissolved in octane (Sigma Aldrich, Munich, Germany) at a concentration of 10 mg/mL. A total of 10 mM HEPES, pH 7.45, and 150 mM KCl (Sigma Aldrich (Fluka), Munich, Germany) were used as an

electrolyte buffer. For KcSA recordings, a buffer with pH 4.0 was used. A total of 5 µL of the vesicles resuspended in PBS was added to the chamber containing the buffer, and we waited until there was a visible response. For current measurements, different voltages were applied to analyze the functional properties. The cavity contains the non-polarizable working electrode containing an Ag/AgCl layer deposited on the underlying Cr/Au layer. Briefly, 180 µL of electrolyte solution was added to the measurement chamber of an Orbit 16 system (Nanion Technologies, Munich, Germany). A single-channel amplifier (EPC-10, HEKA Electronic Dr. Schulze GmbH, Lambrecht, Germany) was connected to the multiplexer electronics port of the Orbit16 system. Recordings were performed at a sampling rate of 50 kHz with a 10 kHz Bessel filter. Data were analyzed with Clampfit (Molecular Devices, Sunnyvale, CA, USA).

3. Results

3.1. Enhancement of Protein Production with Cell-Free Pichia pastoris Systems by Specific Adjustment of Cell Disruption Conditions

Primarily, the growth behavior of the *P. pastoris* cells was examined. Therefore, online biomass monitoring was utilized to analyze cell growth over time in flasks with and without baffles (Figure 2a). As expected, growth in baffled flask resulted in an extended log phase due to increased aeriation. Although the highest growth rate was achieved after 16 h (Supplementary Figure S1), the cultivation was continued to 24 h and yeast cells were harvested at a backscatter signal of ~5500 a.u. to reach higher cell densities. In the next step, the aim was to identify a disruption method that could be used for processing a large number of yeast cells. While it has already been shown that high-pressure homogenization is effective in breaking the stable cell wall of *P. pastoris* while maintaining a translationally active lysate [38], bead-based homogenization with different bead compositions was also applied to gently disrupt the yeast cells. Bead-based homogenization was initially performed in a 2 mL lysing tube, but it can be scaled up to 50 mL tubes. The evaluation was performed via direct detection of the translational activity of the resulting cell lysates. For this purpose, the production of radioactively labeled proteins in the presence of the endogenous mRNA of the crude lysates by supplementation of ^{14}C-labeled amino acids in cell-free protein synthesis was analyzed. After two hours of cell-free protein synthesis, the reaction was precipitated by acetone and compared against a reaction at 0 h based on an autoradiographic analysis of the running pattern in SDS-PAGE. In fact, the high-pressure homogenization showed only a low translational activity in contrast to the bead-based homogenization (Figure 2b). Therefore, further lysing matrix tubes with different materials were utilized to disrupt the yeast cells. The success of disruption was observed by the release of RNA, which resulted in increased absorption at 260 nm. Recognizable by the highest absorption signal, the disruption with 0.5 mm-diameter yttria-stabilized zirconium oxide beads (lysing matrix Y) was the most successful when applying identical amount of cycles (4×) and a disruption time of 35 s (Figure 2c). Subsequently, the disruption time during a cycle was varied, and only two cycles were applied to reduce the burden on the active components in the lysate, but we still obtained a high proportion of disrupted cells. Although a higher release of RNA was observed with increasing disruption time, the translational activity was almost equal, as indicated by the intensity of ^{14}C leucine-labeled endogenous proteins in the autoradiograph (Figure 2d). Consequently, two cycles and a time of 15 s were used for the efficient disruption of *P. pastoris* cells in the following experiments.

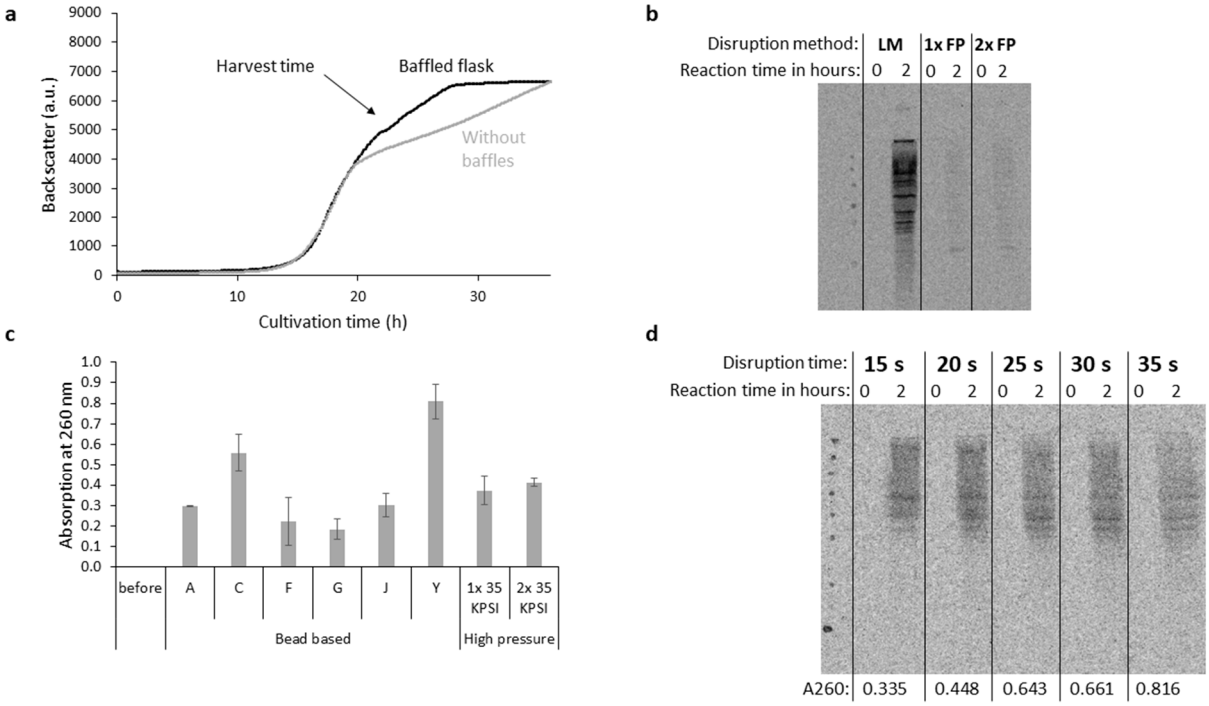

Figure 2. Identification of mild cell disruption parameters for cell lysate preparation. (**a**) Cell growth of *P. pastoris* cells was monitored over time by using an online biomass sensor. Cultivation in shake flasks with and without baffles was compared. The arrow indicates the harvest time when the yeast cells were disrupted for cell lysate preparation. (**b**) *P. pastoris* was disrupted by bead-based homogenization (lysing matrix C, LM) and high-pressure homogenization, referred to as French press (FP). Cell lysate was used for cell-free reactions containing initially ^{14}C isoleucine, ^{14}C leucine, and ^{14}C valine to monitor protein translation of endogenous mRNA. After completion of the cell-free reaction the samples were precipitated by acetone, separated by SDS-PAGE, and visualized by autoradiography. (**c**) *P. pastoris* cells were disrupted with the indicated bead-based lysing matrix and French press. Cell disruption by using bead-based homogenization was carried out in four cycles of 35 s, while French press-based cell disruption was carried out at 35 KPSI in either one or two cycles. The absorption of the pre-diluted resulting cell lysate was measured at 260 nm. Measurements were performed in two independent cell lysate preparations. Data are shown as mean ± SD. (**d**) Bead-based homogenization was carried out in two cycles by using lysing matrix Y by varying the time for each of the cycles. Afterwards, the absorption was measured at 260 nm, and cell-free reactions containing ^{14}C leucine were utilized to monitor protein translation of endogenous mRNA. Afterwards, samples were precipitated by acetone, separated by SDS-PAGE, and visualized by autoradiography.

3.2. Effect of Signal Sequences on Cell-Free P. pastoris Synthesis

Signal sequences play an essential role in cell-free protein synthesis by allowing for the precise control of protein expression as well as the proper folding of proteins produced in the optimal environment. These signal peptides, also often referred to as translocation sequences, can direct the efficient translocation of the co- or post-translationally produced protein into the ER. The presence of microsomes in the processed *P. pastoris* lysate was detected by the presence of the ER marker NADPH cytochrome c reductase (Supplementary Figure S2). Honeybee melittin signal peptide (Mel) can be used to enhance the co-translational translocation of complex proteins in cell-free systems [39]. In contrast, in recombinant protein production using *P. pastoris* cells, the *S. cerevisiae* α-mating factor prepro peptide (ppα) is frequently adopted as a post-translational signal sequence [40–42]. In this study, the translocation of GFP with either of the two signal sequences was examined microscopically. A much higher fluorescence intensity of microsomal structures was detected by the N-terminal fusion of the ppα signal sequence in contrast to the weaker fluo-

rescence signal for Mel-GFP (Figure 3). Nevertheless, an increase in the overall translational efficiency could be achieved with both signal sequences (Supplementary Figure S3).

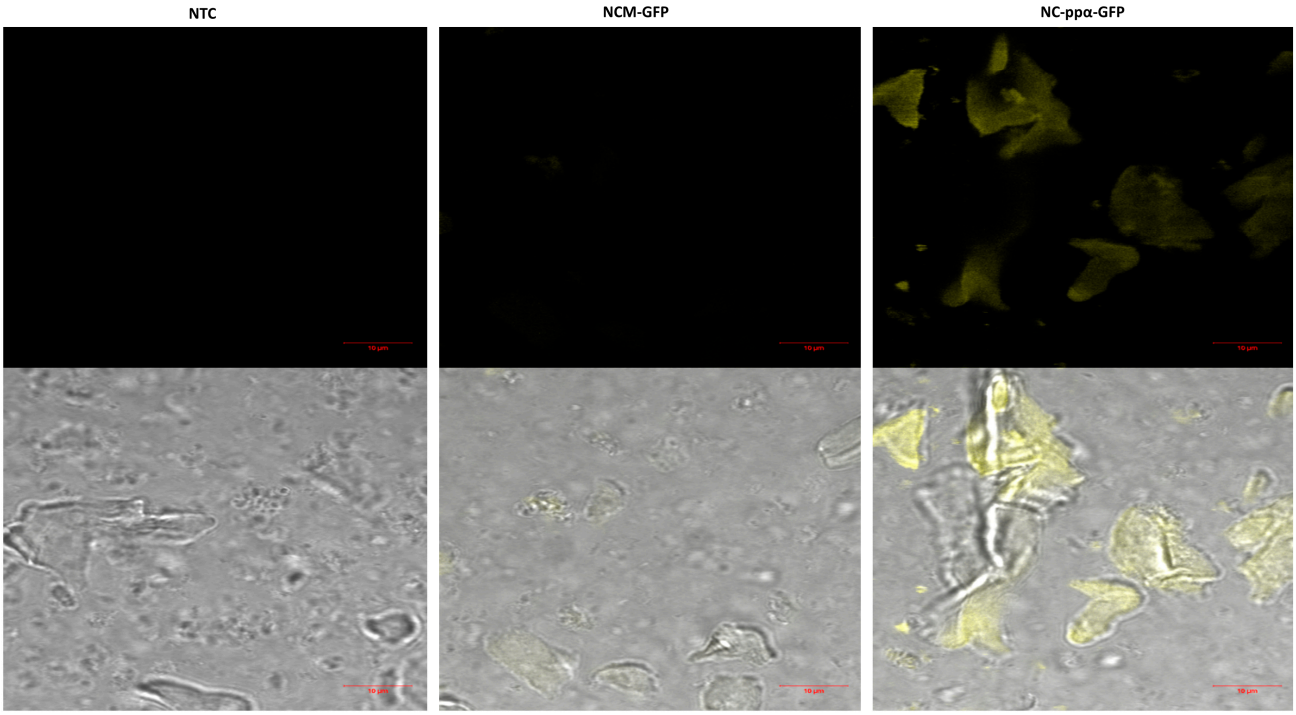

Figure 3. Visualization of protein translocation into ER derived microsomes. Cell-free reactions based on *P. pastoris* lysate were carried out in the presence of either no template (no template control, NTC), melittin signal peptide fused to GFP (Mel-GFP), or α-mating factor prepro peptide fused to GFP (ppα-GFP). Microsomes were centrifuged after the cell-free reaction and solubilized in PBS. Fluorescence was visualized via confocal microscopy after excitation at 488 nm. The excitation with the 488 nm laser is shown at the top, while an overlay of the bright field and the fluorescence image is shown at the bottom.

3.3. Boosting the Performance of Yeast-Based Cell-Free Protein Synthesis through Alternative Energy Regeneration Systems

Maintaining an adequate energy level is essential for cell-free protein synthesis, as protein production requires energy-intensive processes. To meet this need, various approaches of energy regeneration have been used. A common method in eukaryotic cell-free protein synthesis is the use of substrates such as phosphoenolpyruvate (PEP), creatine phosphate (CP), and acetyl phosphate, which are enzymatically converted by the presence of the enzymes in the lysate or by enzyme addition [17]. These substrates provide an important source of high-energy compounds needed for protein synthesis. However, these substrates can pose a problem as high-energy phosphate bond donors due to their high cost and inhibitory effects on cell-free protein synthesis, which is mainly caused by the rapid production of phosphate [43,44]. Although it has been shown that *E. coli*-based cell-free synthesis can proceed efficiently with alternative energy sources, energy regeneration based on creatine kinase (CK) and CP is widely used in eukaryotic systems [45]. Alternative attempts to switch to inexpensive glycolysis intermediates have already been demonstrated with *S. cerevisiae* cell-free protein synthesis [46]. Anderson et al. were able to fuel their glucose energy system in the presence of supplemented cyclic AMP (cAMP) and exogenous phosphate. Additionally, nicotinamide adenine dinucleotide (NAD) is an important cofactor of glycolysis and is frequently supplemented in PANOx-based *E.coli* cell-free protein synthesis [47,48]. Therefore, NAD, cAMP, and potassium phosphate (KPO_4) were added to cell-free reactions containing glycolytic intermediates. To examine whether energy from glycolysis can be harnessed for cell-free protein synthesis, the following glycolytic inter-

mediates were tested: glucose, glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), fructose-1,6-bisphosphate (F-1,6-P), 3-phosphoglyceric acid (3-PGA), and PEP. As glycerol is a carbon source commonly used in recombinant protein production for *P. pastoris*, the potential to enable cell-free protein synthesis via energy production from glycerol and glycerol-3-phosphate (Glycerol-3-P) was investigated (Figure 4a). Initial experiments with glycolysis intermediates in the *P. pastoris* cell-free reaction have shown that the use of the CP/CK system still works most efficiently (Supplementary Figure S4). Hence, the processing of the crude lysate was subsequently modified. Similar to *E. coli*-based cell-free production as reported previously [49,50], the dialysis and size exclusion chromatography were omitted in order to retain a large set of cell lysate components that may have a significant effect on glycolysis present in the cell-free reaction. The cell-free reaction was monitored in real time by using ppα-GFP as a template. Strikingly, GFP production was increased ~2-fold in the presence of F-1,6-P in contrast to the previously used CP/CK system (Figure 4b). Although it was shown that increased F-6-P and 3-PGA resulted in higher fluorescence signals, the supplemental addition of F-6-P and 3-PGA did not notably increase protein yields when 20 mM F-1,6-P was present in the cell-free reaction (Supplementary Figure S5). To demonstrate the broad application of F-1,6-P to other cell-free systems, identical parameters were transferred to a cell-free synthesis of GFP based on *S. cerevisiae* lysate. Indeed, GFP could be successfully produced in the presence of F-1,6-P (Figure 4c). Thus, transferability to other fungal-based cell-free systems is feasible. In the conventional energy regeneration system, CK and CP have to be supplemented, whereas in the novel system, the addition of F-1,6-P is sufficient through the presence of glycolytically active enzymes in the cell lysate.

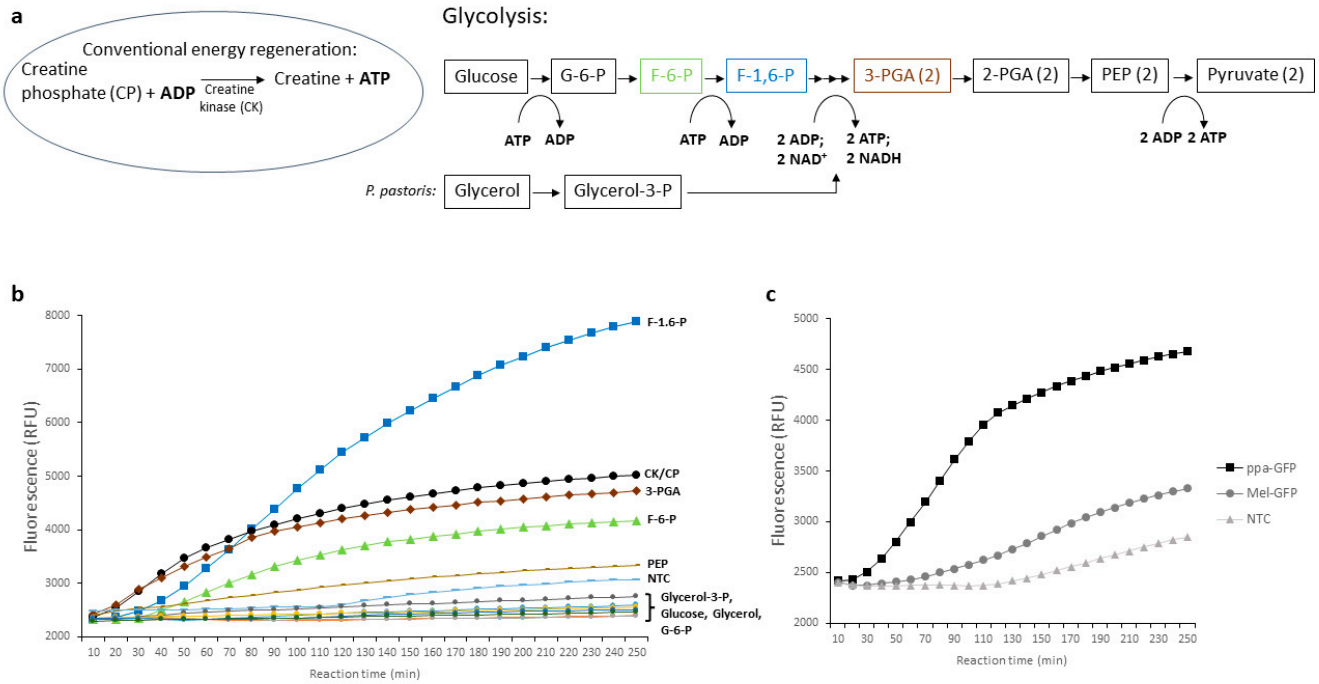

Figure 4. Alternative energy system for cell-free protein synthesis. (**a**) Scheme of the conventional energy regeneration in eukaryotic cell-free protein synthesis and an overview of glycolytic substrates for an alternative energy regeneration in *P. pastoris* cell-free systems. The colors of F-6-P (green), F-1,6-P (blue), and 3-PGA (brown) correspond to the graphs in (**b**). (**b**) Real-time fluorescence measurement of *P. pastoris* cell-free reactions containing ppα-GFP as template. The utilized energy source is indicated at each graph and is visualized in different colors to increase the readability. (**c**) Real-time fluorescence measurement of cell-free reactions based on *S. cerevisiae* lysate using F-1,6-P as energy source. Utilized templates were indicated as squares (ppα-GFP), circles (Mel-GFP), and triangles (no template control, NTC).

3.4. Increasing the Efficiency of Cell-Free P. pastoris Protein Synthesis Using Statistical Experimental Designs

Changing single parameters, such as glycolysis intermediates, buffer, and ions, to adjust the cell-free environment by the one-factor-at-a-time method is often time-consuming and costly but can also miss interactions between various parameters of cell-free protein synthesis. Consequently, there are numerous strategies to adjust the optimal composition for cell-free reactions based on statistical experimental designs. In addition, the use of crude lysate for cell-free synthesis raises the question of the optimal level of essential components. As a first step, we investigated whether the energy-regenerating system based on F-1,6-P can be influenced by the cofactors NAD, cAMP, and KPO_4, similar to those reported previously [46]. A full factorial experimental design with two levels (presence or absence of the tested factor) was performed with three replicates. Significant two-way and three-way interactions could not be detected (Figure 5a and Supplementary Table S1). A significant negative effect on the overall synthesis performance was observed at higher NAD concentrations, while KPO_4 and cAMP had no significant effect on cell-free protein synthesis at all (Figure 5b). Consequently, the three factors were not supplemented to the cell-free reaction for further experiments.

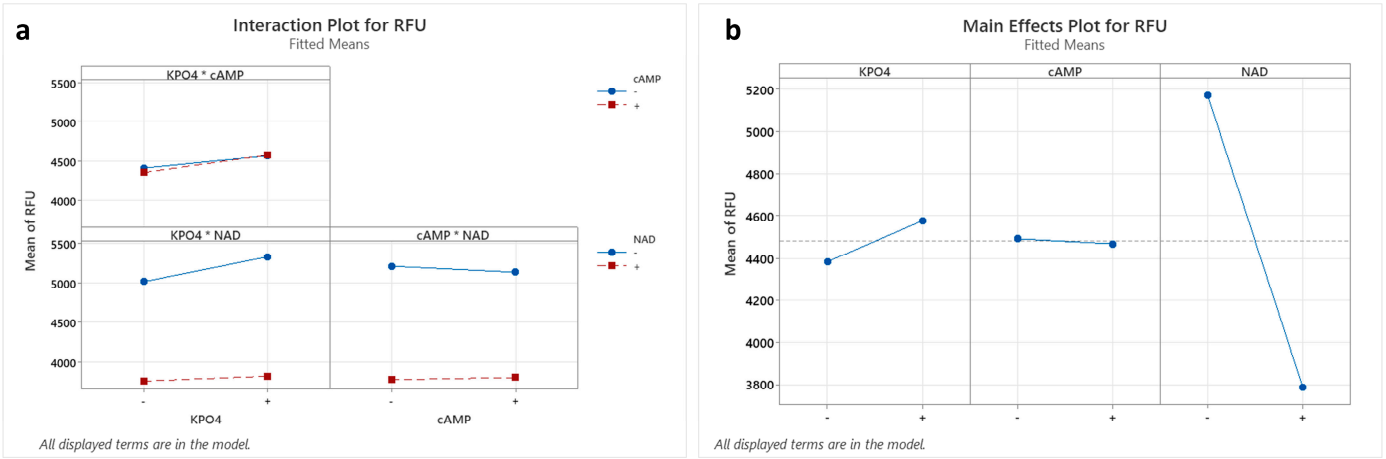

Figure 5. Full-factorial design evaluating the influence of NAD, cAMP, and KPO_4 on cell-free protein synthesis. Cell-free reactions were carried out to evaluate the influence of NAD, cAMP, and KPO_4 on general protein synthesis expressed through GFP fluorescence measurement. Therefore, a full-factorial design with two levels—absence (−) or presence (+) of the factor—was performed with three independent replicates. (**a**) Interaction plot. (**b**) Main effect plot.

Due to the large number of parameters affecting the cell-free reaction itself, we focused on factors which were shown to be essential in cell-free protein synthesis. Hence, HEPES (pH 7.4), potassium acetate (KOAc), magnesium acetate ($Mg(OAc)_2$), adenosine triphosphate (ATP), nucleoside triphosphates without ATP (NTPs w/o ATP), F-1,6-P, and an amino acid mix were included in a central composite design to obtain meaningful information on the optimal composition of the cell-free reaction, while other components of the cell-free reaction were kept constant. A total of 90 cell-free reactions were carried out in a 96-well plate in the presence of a GFP template to evaluate the fluorescence signal as a response for the statistical analysis. A backward elimination with an α of 0.1 for removing terms from the model was utilized to create a robust model. The model explains 89.68% of the variation in the response and has a high predictive ability (R^2-pred: 83.24%) for new observations (Supplementary Table S2). The model fits the experimental data, evidenced by the *p*-value 0.296 for the lack-of-fit test. The analysis of variance can be found in Table 1. Significant main effects (white background) are shown for all tested factors except for ATP, while the main effect of ATP was not significant (grey background) and was thus not included in the model (Figure 6). Moreover, a non-linear behavior, indicated by the curvature in the

main effect plots, could be detected for KOAc, $Mg(OAc)_2$, and NTPs w/o ATP. Significant interactions between KOAc and NTPs w/o ATP, as well between the energy regeneration system F-1,6-P and the factors $Mg(OAc)_2$, HEPES, and NTPs w/o ATP, could be observed (Table 1 and Supplementary Figure S9). Further information can be found in Supplementary Figures S8–S10.

Table 1. Model summary and analysis of variance of the central composite design.

Source	*p*-Value
Model	0.000
Linear	0.000
KOAc	0.000
$Mg(OAc)_2$	0.000
HEPES	0.000
Amino acids	0.094
NTPs w/o ATP	0.000
F-1,6-P	0.000
Square	0.000
KOAc*KOAc	0.000
$Mg(OAc)_2$*$Mg(OAc)_2$	0.000
NTPs w/o ATP*NTPs w/o ATP	0.078
Two-Way Interaction	0.000
KOAc*NTPs w/o ATP	0.001
$Mg(OAc)_2$*F-1,6-P	0.000
HEPES*F-1,6-P	0.003
NTPs w/o ATP*F-1,6-P	0.000
Error	
Lack-of-Fit	0.296
Pure Error	
Total	

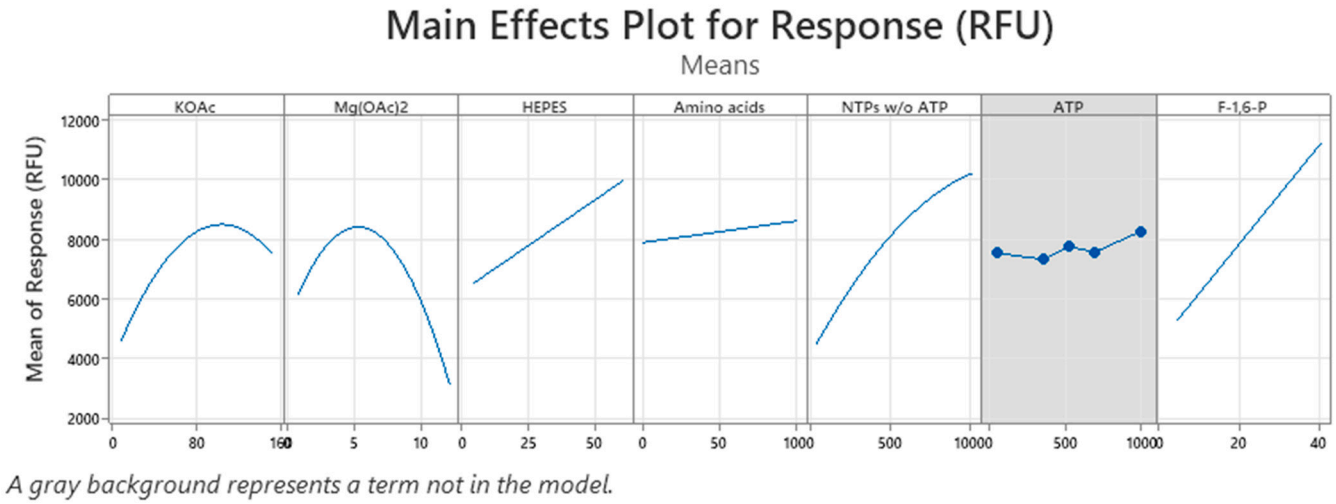

Figure 6. Main effect plots of the central composite design. Cell-free reactions were carried out in a 96-well plate, and fluorescence was measured as response to evaluate the central composite design based on seven factors. Main effect plots were generated for each factor. A white background represents a term which is in the generated model, while a grey background indicates a term which is not in the model. The x-axis indicates the tested concentration range of each factor.

Based on the prediction of the generated model, we produced firefly luciferase in cell-free reactions to estimate protein yields using the established cell-free system. Therefore, *P. pastoris* cells were independently cultivated and disrupted. As a result, five different *P. pastoris* cell lysates were utilized to produce luciferase based on predicted optimal

concentrations in *P. pastoris* cell-free reactions (Table 2). The mean protein concentration 24.5 ± 0.3 µg/mL of active luciferase could be determined. It is important to emphasize that no signal sequence was used, so the N-terminal fusion of Mel or ppα peptides could lead to higher protein yields.

Table 2. Predicted optimal concentrations for *P. pastoris* cell-free protein synthesis. Tested parameters are indicated in grey.

Components	Predicted Final Concentration
KOAc	141.5 mM
HEPES pH 7.4	60 mM
$Mg(OAc)_2$	7.6 mM
F-1,6-P	40 mM
NTPs w/o ATP	1 mM
Amino acids	100 µM
ATP	0.5 mM
T7 RNA Polymerase	1 U/µL
P. pastoris lysate	35%
Spermidine	0.25 mM
PolyG	5 µM
DTT	1 mM
Nanoluciferase plasmid	100 ng/µL

3.5. Site-Specific Modification in P. pastoris Cell-Free Reactions by Using an Orthogonal Translation System

To demonstrate the broader scope of the novel system, the possibility of modifying proteins at defined amino acid positions was investigated. Site-specific modification of proteins in cell-free protein synthesis is of high importance as it allows for the directed adaptation of proteins to specific functions. By introducing non-canonical amino acids at precisely defined sites in the protein, the activity, stability, or interaction with other molecules can be tailored [51,52]. This opens up possibilities for the development of customized biotechnological applications, such as therapeutic proteins with improved properties [53]. The orthogonal aminoacyl tRNA synthetase (aaRS), orthogonal tRNA, and the non-canonical amino acid (ncaa) necessary for site-specific modification can be conveniently added to the cell-free reaction without consideration of cell viability, as in cell-based applications [54,55]. In this process, the endogenous aaRS, tRNAs, and amino acids must not cross-react with the orthogonal components (Figure 7a). A competition for the recognition of the amber stop codon exists between aminoacylated orthogonal tRNA and the eukaryotic release factor (eRF1) to either suppress the amber stop codon or terminate protein translation. Since the present novel cell-free system based on *P. pastoris* is intended to be a cost-effective alternative for the production of complex proteins, we further aimed to demonstrate that the system is also suitable to modify complex proteins in a target-specific manner. For this purpose, the mutant orthogonal *E. coli* tyrosyl-tRNA synthetase/tRNAtyr pair was used, which is frequently employed in eukaryotic-cell-based and cell-free systems to modify proteins site-specifically with diverse ncaa such as p-azido-L-phenylalanine (AzF) and p-propargyloxyphenylalanine [30,35,36]. The pharmacologically relevant G-protein-coupled adenosine receptor A2a (Adora2a) was site-specifically modified in a cell-free *P. pastoris* synthesis by the insertion of an amber stop codon at amino acid position 215. C-terminal fusion of a nanoluciferase (Adora2a-amb-Nluc) was used to determine successful amber stop codon suppression by detecting luminescence. The suppression efficiency could be obtained by comparing the synthesis of Adora2a-amb-Nluc with a cell-free reaction of Adora2a with a C-terminal nanoluciferase without an amber stop codon in between (Adora2a-Nluc). While a suppression efficiency of 36% was achieved, no amber

stop codon readthrough was observed in the absence of orthogonal tRNAtyr, indicating the high specificity of the orthogonal system (Figure 7b).

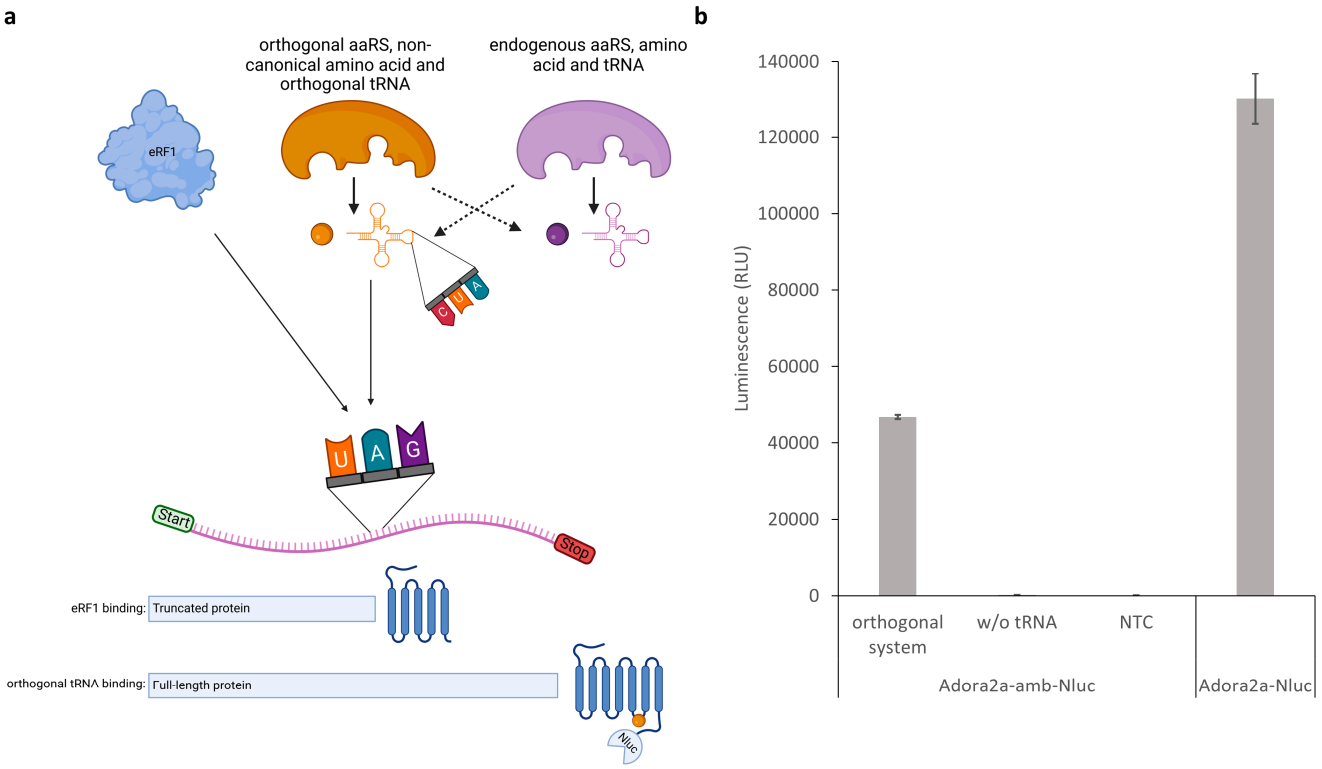

Figure 7. Site-specific protein modification in *P. pastoris* cell-free systems by using an orthogonal system. (**a**) Scheme of site-specific incorporation of a non-canonical amino acid into the GPCR Adora2a-amb-Nluc. Dashed arrows indicate no interaction. (**b**) The mutant orthogonal *E. coli* tyrosyl-tRNA synthetase, orthogonal tRNAtyr, and p-azido-L-phenylalanine (AzF) were added to a cell-free reaction based on *P. pastoris* lysate containing the Adora2a-amb-Nluc template. A no-template control (NTC) and a control reaction without orthogonal tRNAtyr were utilized. Adora2a-Nluc without an amber stop codon was used to examine the amber suppression efficiency. Luminescence was detected after completed cell-free reactions. Measurements were performed in technical triplicate. Data are shown as mean ± SD.

3.6. Functional Characterization of the Potassium Channels KcSA and KvAP Reconstituted into Planar Lipid Bilayers

Due to the presence of microsomes, we aimed to produce functionally active ion channels using the *P. pastoris* cell-free system. Planar lipid bilayer-based reconstitution is widely used for measuring the functionality of potassium channels [56,57]. Planar lipid bilayer electrophysiology involves creating a lipid bilayer across a cavity, dividing it into two compartments with buffer solutions on both sides. After forming the bilayer, the sample, including proteins like KcSA and KvAP, is added. This results in the reconstitution of the ion channels in the planar lipid bilayer. Subsequently, a voltage clamp is applied to initiate the gating of ion channels between open and closed states, facilitating the transport of ions. The movement of ions generates a current, which is recorded during measurements.

Following the addition of the KcSA-incorporated microsomal fraction to the DPhPC lipid bilayer, we observed transient, discrete conductance changes in the form of single-channel conductance with amplitudes of 5–7 pA at −100 mV (Figure 8a). Electrophysiological recordings showed a clear single channel activity with several sub-conductance levels at different voltages, similar to McGuire and Blunck [57]. In between, there are several sub-conductance levels (2–3 pA) rapidly flickering between the closed and half levels of the full-conductance state. All-time histograms were plotted for all recordings, and we

noticed sub-conductance to full-conductance peaks corresponding to the KcSA channel activity. Similar types of currents recorded at +60 mV are also shown in Supplementary Figure S11, with a histogram showing two peaks corresponding to the opening and closing of the channel. We were able to observe a range of conductance behaviors ranging from a large peak corresponding to closed levels and several smaller peaks corresponding to sub-conductance, single-channel, and cooperative multi-conductance gating levels (Figure 8b). Multi-conductance levels might correspond to the insertion of more than a single channel. Moreover, several macro currents were observed, which were not shown in this draft as they are often irrelevant and most often end up in the rupturing of the underlying lipid bilayers. These macro currents might either be due to the rapid insertion of several ion channels or to the osmotic stress of the microsomal fraction. Rarely, these macro currents were also seen in negative controls, where only a sample without the expressed protein was added.

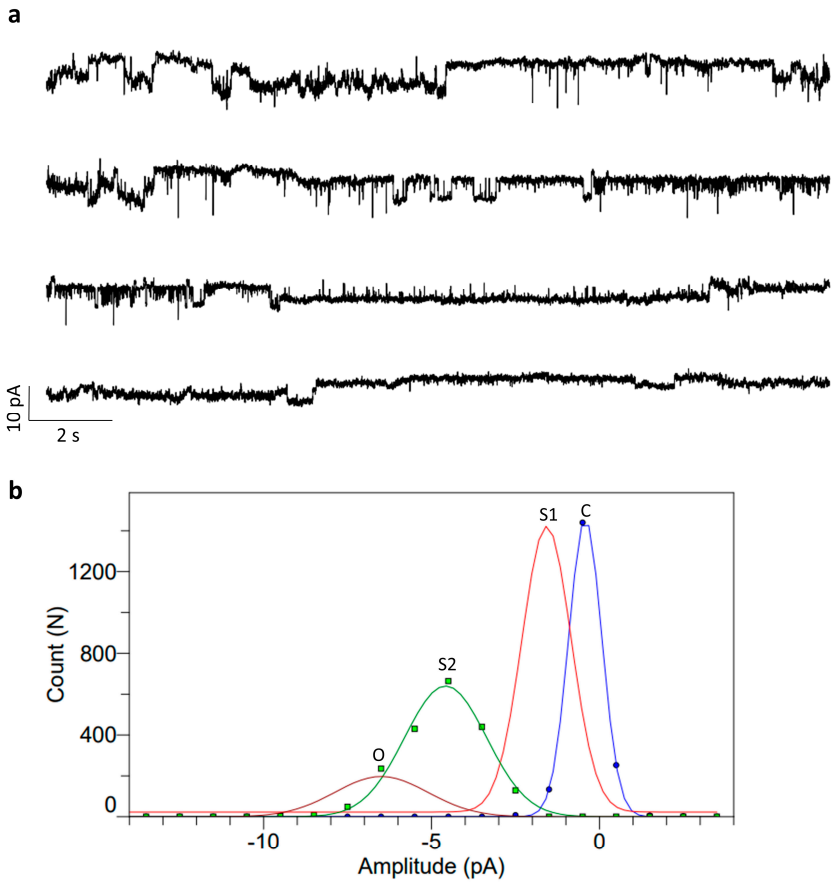

Figure 8. Planar lipid bilayer electrophysiology measurements from the DPhPC bilayers after the addition of KcSA containing vesicles. (**a**) Representative current traces of single-channel activity of KcsA in pure DPhPC bilayers at pH 4.0 (N > 5) at −100 mV. (**b**) All time amplitude histograms of traces of the above single-channel trace showing multi-conductance peaks (C: closed; S1: sub-conductance 1; S2: sub-conductance 2; and O: full open conductance). Buffer: 10 mM HEPES, 150 mM KCl, pH 4.0.

KvAP activity was measured after adding the microsomal fraction harboring the active protein onto the planar lipid bilayer. After several minutes, there were different types of response observed. We focused on measuring the single-channel recordings from the reconstituted lipid bilayers. Single-channel conductance was measured from the KvAP-reconstituted DPhPC lipid bilayers, as shown in Figure 9a. The measured currents correspond to a single channel conductance of around 120 pS, with several sub-conductance levels in between, transitioning from full-length conductance to closed conductance in

accordance with Devaraneni et al. [58]. All time histograms showed multi-conductance peaks corresponding to the partially opened channel (5 pA) and full-length conductance (12 pA) (Figure 9b). Similar activity was also observed in a different experiment at +60 mV (Supplementary Figure S12a,b). In the controls, there were some leaky large currents that increased over time, resulting in the rupturing of the bilayer. These events were also observed with the same frequency in the case of KvAP-reconstituted lipid bilayers; hence, they were considered unrelated to the presence of KvAP. However, in the case of KvAP samples, macroscopic currents resulting from the stochastic opening of several channels were observed, showing rectification of the currents in one polarity. These macroscopic currents varied from bilayer to bilayer and were not considered for analysis.

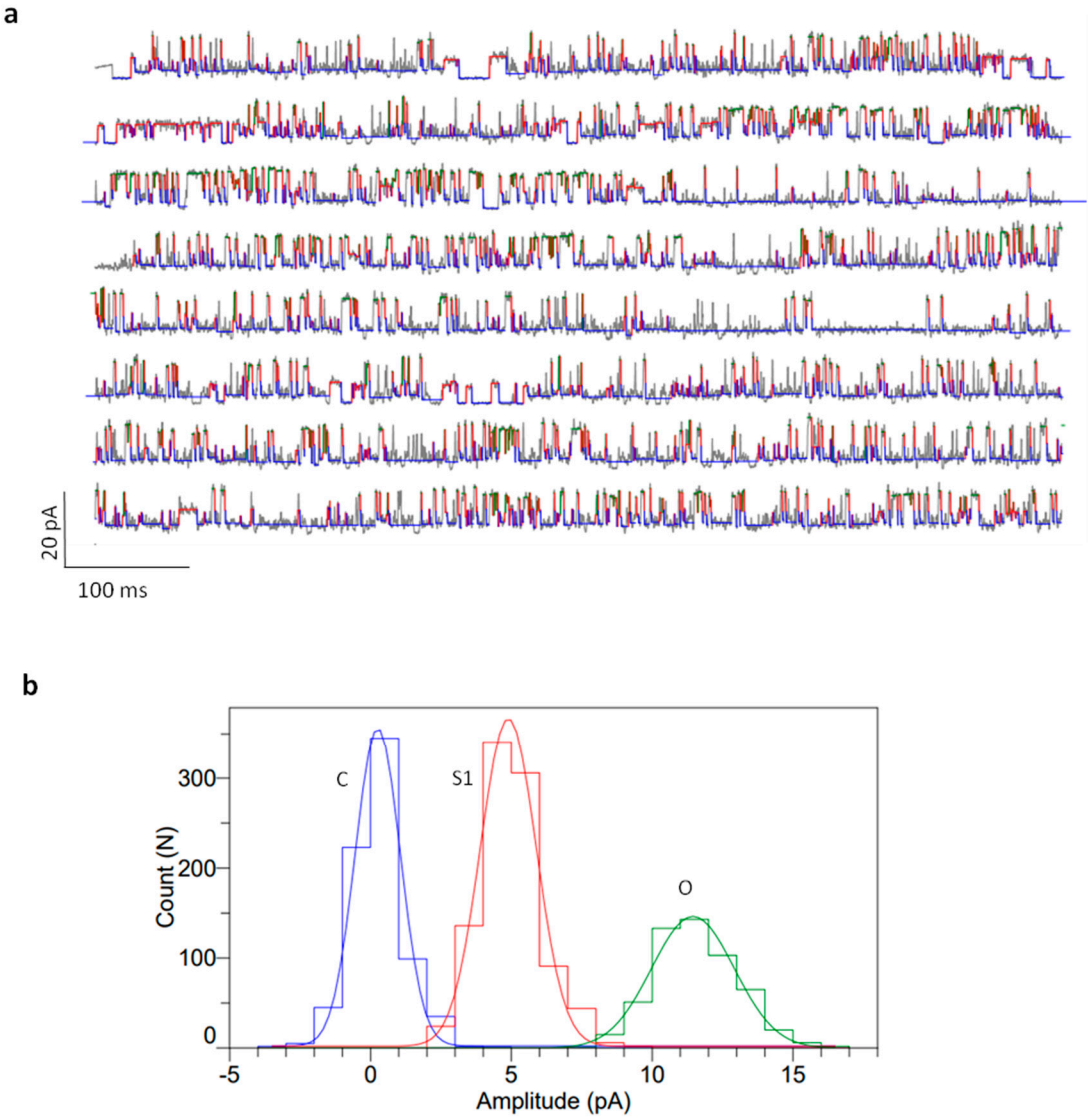

Figure 9. Planar lipid bilayer electrophysiology measurements from the DPhPC bilayers after the addition of KvAP-containing vesicles. (**a**) Representative current traces of single-channel activity of KvAP in pure DPhPC bilayers at pH 7.45 (N > 5) at +100 mV. The color corresponds to the conductance peaks in (**b**). (**b**) All time amplitude histograms of traces of the above single-channel trace showing multi-conductance peaks (C: closed in blue; S1: sub-conductance 1 in red; and O: full open conductance in green). Buffer: 10 mM HEPES, 150 mM KCl, pH 7.45.

4. Discussion

P. pastoris plays a prominent role in recombinant protein production. Although the scalability of eukaryotic cell-free systems for the production of difficult-to-produce proteins is limited due to high costs, a more economical low-cost system would be of outstanding interest in the manufacture of proteins on a larger scale. In the present work, the developed cell-free protein production system based on the yeast *P. pastoris* could provide a low-cost alternative to already established eukaryotic cell-free systems.

In the present study, we demonstrated that the N-terminal fusion of Mel and ppα signal sequences can achieve increased protein production and translocation into microsomes, allowing diverse complex proteins to be optimally folded and post-translationally modified. In this process, the pre sequence is expected to be removed by signal peptidases in the ER, while the pro sequence is cleaved in the Golgi. However, it is reported that ppα-signaling peptides can lead to accumulation outside of the ER [59]. On the other hand, different mutant ppα-signaling peptides were established [60] and could conceivably be used in the future for cell-free synthesis, providing efficient translocation during recombinant protein production.

With the use of the novel energy regeneration system, which utilizes F-1,6-P as a substrate, cell-free protein synthesis can be realized even more economically. On the one hand, F-1,6-P is also a high-energy phosphate bond donor, but in contrast to the widely used substrate CP, there is no need for additional CK supplementation since a large proportion of enzymes of glycolysis seem to be present in the cell lysate in an active form. The fact that the cell lysate can be directly used for cell-free protein synthesis without further chromatographic purification means that a comparably low time requirement as for *E. coli* cell lysate generation is necessary. Interestingly, the use of F-1,6-P could be transferred to *S. cerevisiae* cell-free protein synthesis without further optimization, empowering cell-free protein synthesis based on this model organism. Hence, it is conceivable to substitute the CK/CP system by F-1,6-P for other cell-free systems.

In fact, it was shown in this study that supplementation of cAMP and KPO_4, as well as NAD as an important cofactor of glycolysis, had no effect or a negative effect, respectively, on the production of GFP, although a positive effect on *S. cerevisiae*-based cell-free synthesis has already been shown [46]. Therefore, it can be assumed that the components are present in sufficient and balanced form in the cell lysate, which are otherwise unavailable for the cell-free reaction due to dialysis and chromatography. These results were in accordance with a previous report on *E. coli*-based cell-free reactions using F-1,6-P as an energy source without NAD and coenzyme A addition [61].

The capability of running cell-free syntheses in a 96-well format has shown that 90 reactions can be performed in parallel to enable statistical analyses of the optimal environment of the reaction components. This means that the cell-free system can be adapted to new conditions with little effort and at low cost. The model constructed here represents a high predictive power, with an R^2-pred of 83.24%, and the predicted optimal concentrations of HEPES, $Mg(OAc)_2$, ATP, NTPs w/o ATP, and amino acids are in agreement with the recent results of a statistical experiment with cell-free protein synthesis based on *P. pastoris* [62]. It is noticeable that increasing HEPES and NTPs without ATP and F-1,6-P levels resulted in elevated protein synthesis. The preference for higher HEPES concentration may be caused by the omitted rebuffering of the lysate, whereas the optimum at high NTP concentrations could be explained by a high lysate concentration, as described by Takahashi et al. [63]. In addition, the high F-1,6-P concentration seems to function optimally as an energy source for ATP. Among other things, magnesium ions are important cofactors in complexes with ATP for glycolytic enzymes such as aldolase, phosphoglycerate kinase, and pyruvate kinase, explaining the significant interactions of F-1-,6-P with $Mg(OAc)_2$ [64]. Surprisingly, no significant interaction of ATP with magnesium ions was detected, although it is known that high ATP levels can affect magnesium-dependent processes, such as protein synthesis, considerably owing to their high affinity for magnesium ions [65,66]. This could indicate that higher ATP concentrations than assayed might have a negative effect on protein synthesis.

Although cell-free *P. pastoris* protein synthesis functions through the presence of endogenous amino acids, the addition of exogenous amino acids was shown to have a positive effect on GFP production. Therefore, substitution with a more economical amino acid source would be desirable. Recently, it has been shown that cell-free syntheses based on *E. coli*, *Streptomyces venezuelae*, and *P. pastoris* by common nutrient-rich media such as peptone can be effectively used as an amino acid source for cell-free protein production [67].

Site-specific modification allows for precise control of chemical changes at defined sites, facilitating the production of tailored proteins with improved properties and functions, for instance, to produce antibody-drug conjugates [68]. Previously it was shown that AzF was incorporated into trastuzumab IgG to establish a yeast-based platform for the production of antibody-drug conjugates [69]. While it has already been shown that cell-free orthogonal translation systems have the potential to modify difficult-to-produce proteins [30,70], the present results demonstrate that the cell-free site-specific modification of difficult-to-produce proteins in *P. pastoris* lysates is associated with significantly lower costs.

Given that *P. pastoris* cells are susceptible to genetic modification, cell-free synthesis can be improved by inserting diverse beneficial protein-coding sequences into the genome. It was shown that T7 RNA polymerase was inserted into the *P. pastoris* genome under the control of a constitutively expressing GAP promoter to drive transcription via a T7 promoter in yeast cells [71]. Additionally we recently showed that inserting the orthogonal aaRS into the CHO genome can reduce costs and time, while achieving sufficient amber suppression to site-specifically modify membrane proteins [72]. Hence, incorporating these enzymes would strengthen the novel yeast cell-free protein synthesis.

The potential to use a cell-free *P. pastoris* system for recombinant protein production has already been demonstrated by Aw and Polizzi [38]. By overexpressing the global regulator of ribosome biogenesis FHL1, a 3-fold increase in protein yields could be achieved. Furthermore, using their developed cell-free *P. pastoris* system, Wang et al. recently demonstrated that CrPV IRES can initiate protein translation most efficiently among the 14 IRESs tested [29].

In summary, a cost-effective *P. pastoris* cell-free system was successfully established to produce functionally active complex proteins. Furthermore, it was also demonstrated that this system can be used for targeted protein modification, which opens up promising possibilities for future biotechnological applications.

5. Conclusions

In this study, we identified efficient cell disruption conditions to produce translationally active cell lysate for cell-free protein synthesis based on *P. pastoris*. The cell-free environment was optimized via the statistical experimental design, and the translocation of proteins into ER vesicles was shown. Furthermore, we utilized the mutant orthogonal *E. coli* tyrosyl-tRNA synthetase/tRNAtyr pair to introduce AzF into the GPCR Adora2a-amb-Nluc. In addition, we increased the protein yield by ~2-fold using fructose-1,6-bisphosphate as an energy regeneration system. Finally, the novel system was shown to produce active ion channels.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/bioengineering11010092/s1. Supplementary Figure S1: Growth rate of P. pastoris. P. pastoris was cultivated in shake flasks with (black dots) and without baffles (grey dots). Cell growth was monitored over time and growth rates were calculated using an online biomass sensor. Supplementary Figure S2: Cytochrome c Reductase (NADPH) assay. P. pastoris cell lysate was prepared and the Cytochrome c Reductase (NADPH) activity was determined using the Cytochrome c Reductase (NADPH) assay (Sigma-Aldrich) according to the manufacturer's instruction. A positive control containing Cytochrome c Reductase (NADPH) from rabbit liver was additionally analyzed. Measurements were performed in technical duplicate. Data are shown as mean ± SD. Supplementary Figure S3: Cell-free protein synthesis with and without melittin signal peptide. EYFP and the ETB receptor were synthesized in P. pastoris cell-free reactions in the presence of 14C leucine. A melittin signal peptide (Mel) was inserted (+) or was omitted (−) in

utilized templates. Proteins in the translation mixture were precipitated by acetone after cell-free protein synthesis. The autoradiography of radiolabelled proteins is visualized. Supplementary Figure S4: Cell-free protein synthesis based on different energy sources. Nanoluciferase (Promega) was synthesized in cell-free reactions based on P. pastoris cell lysate, which was processed by size exclusion chromatography. Luminescence was analyzed after cell-free protein synthesis by the Nano-Glo Luciferase Assay System (Promega). Measurements were performed in technical duplicate. Data are shown as mean ± SD. Supplementary Figure S5: Cell-free protein synthesis containing F-1,6-P. Cell-free synthesis of ppα-GFP based on P. pastoris cell lysate was performed in the presence of 20 mM F-1,6-P. Different concentrations of F-6-P and 3-PGA were applied during cell-free reactions. Fluorescence was detected after cell-free protein synthesis. Results are displayed as endpoint measurement. Supplementary Figure S6: Model equation of the full-factorial design. The model equation corresponds to Supplementary Table S1 and Figure S5 and is shown in uncoded units. Supplementary Figure S7: Residual plots of the full-factorial design. The influence of NAD, cAMP and KPO4 were analyzed based on a full-factorial design. Corresponding model information, analysis of variance and plots can be found in Supplementary Table S1 and Figure S5, respectively. Residual plots are shown and were prepared using Minitab software. Supplementary Figure S8: Model equation of the central composite design. The model equation corresponds to Table 1 and is shown in uncoded units. Supplementary Figure S9: Interaction plots of the central composite design. Interaction plots of the central composite design corresponding to Table 1 are shown and were prepared using Minitab software. Supplementary Figure S10: Residual plots of the central composite design. Residual plots corresponding to Table 1 are shown and were prepared using Minitab software. Supplementary Figure S11. Planar lipid bilayer electrophysiology measurements from the DPhPC bilayers after the addition of KcSA containing vesicles. (A) Representative current traces of single channel activity of KcsA in pure DPhPC bilayers at pH 4.0 (N > 5) at −100 mV. (B) All time amplitude histograms of traces of the above single channel trace showing multi-conductance peaks (C: closed, and C: full open conductance). Buffer: 10 mM HEPES, 150 mM KCl, pH 4.0. Supplementary Figure S12. Planar lipid bilayer electrophysiology measurements from the DPhPC bilayers after the addition of KvAP containing vesicles. (A) Representative current traces of single channel activity of KvAP in pure DPhPC bilayers at pH 7.45 (N > 5) at +60 mV. (B) All time amplitude histograms of traces of the above single channel trace showing multi-conductance peaks (C: closed, S1: sub-conductance 1 and C: full open conductance). Buffer: 10 mM HEPES, 150 mM KCl, pH 7.45. Supplementary Table S1: Model summary and analysis of variance of the full-factorial design. Supplementary Table S2: Model summary and analysis of variance of the central composite design corresponding to Table 1.

Author Contributions: J.L.S. was involved in methodology, investigation, conceptualization, formal analysis, and writing the original draft. S.K.D. was involved in methodology, writing the original draft, and formal analysis. A.Z. was involved in conceptualization, project administration, review, and editing the draft. S.K. was involved in conceptualization, project administration, and review and editing of the draft. All authors have read and agreed to the published version of the manuscript.

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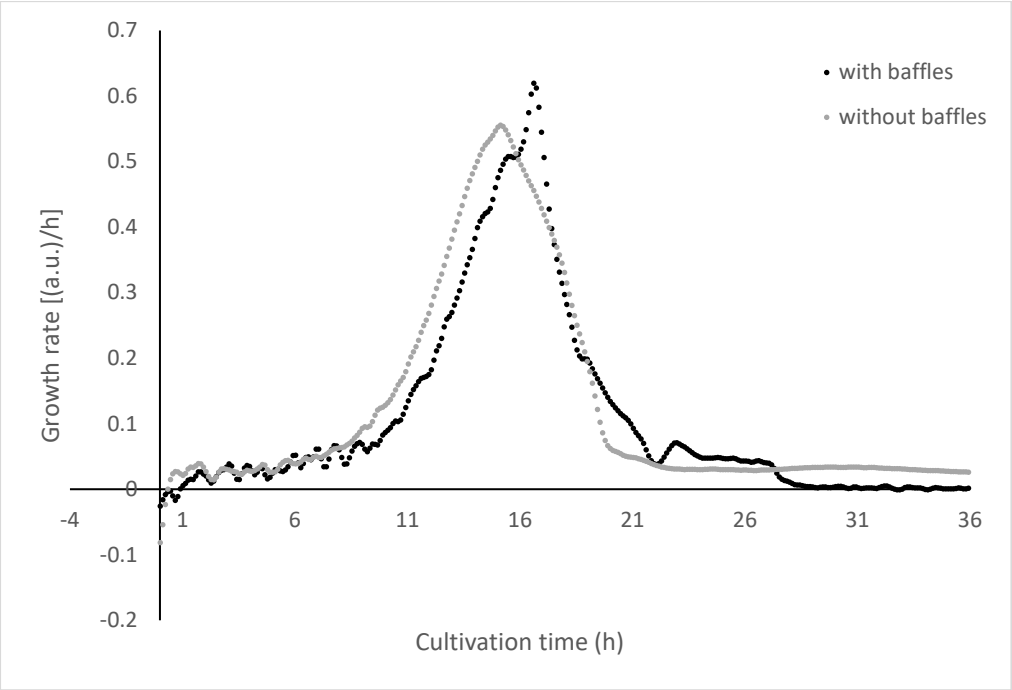
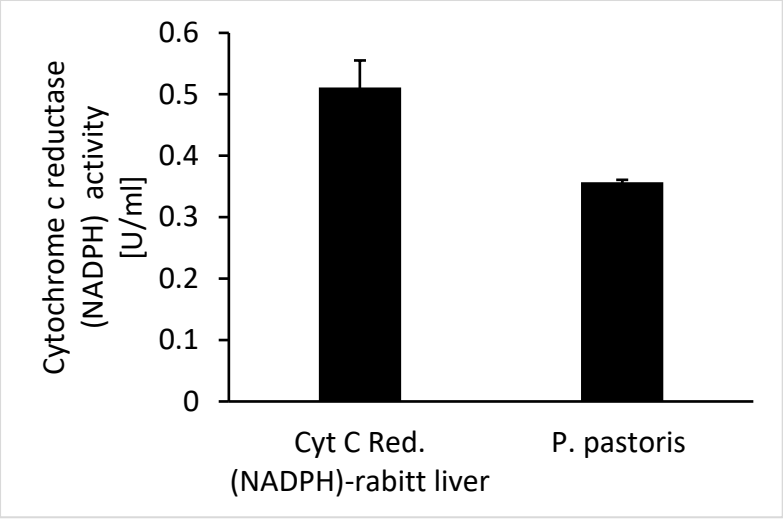
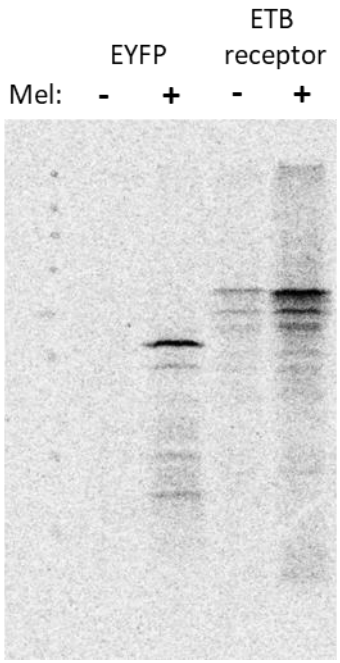
Supplementary Materials



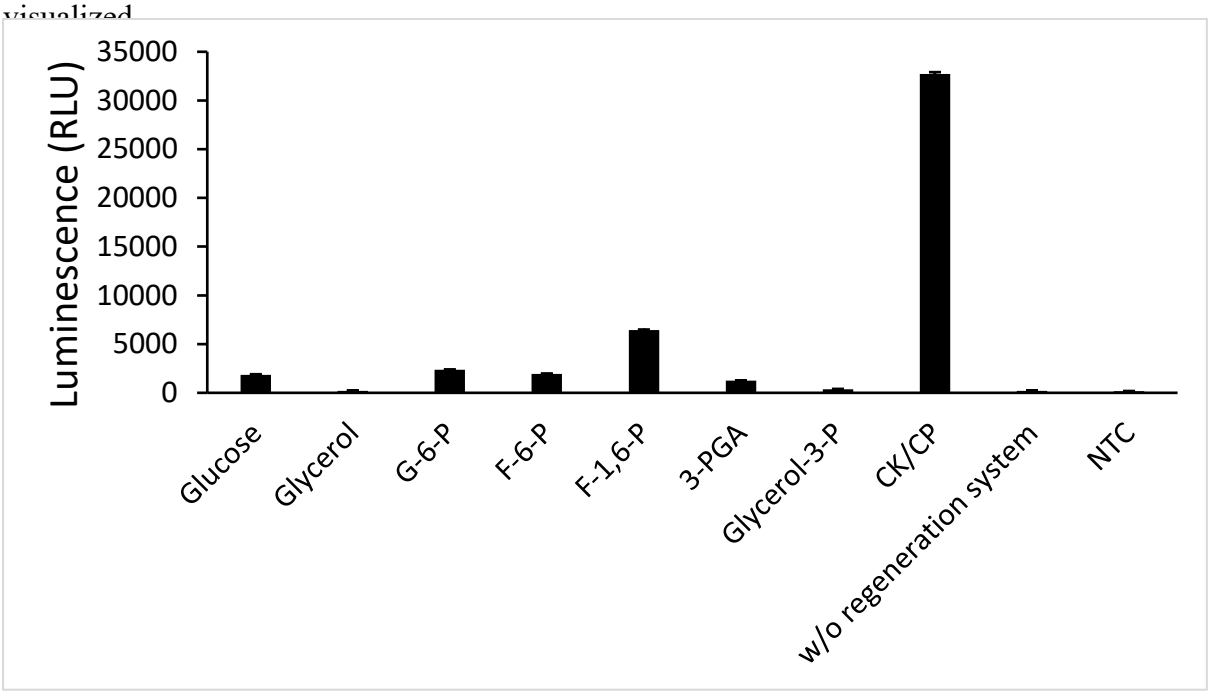
Supplementary Figure S1: Growth rate of P. pastoris. *P. pastoris* was cultivated in shake flasks with (black dots) and without baffles (grey dots). Cell growth was monitored over time and growth rates were calculated using an online biomass sensor.



Supplementary Figure S2: Cytochrome c Reductase (NADPH) assay. *P. pastoris* cell lysate was prepared and the Cytochrome c Reductase (NADPH) activity was determined using the Cytochrome c Reductase (NADPH) assay (Sigma-Aldrich) according to the manufacturer's instruction. A positive control containing Cytochrome c Reductase (NADPH) from rabbit liver was additionally analyzed. Measurements were performed in technical duplicate. Data are shown as mean ± SD.

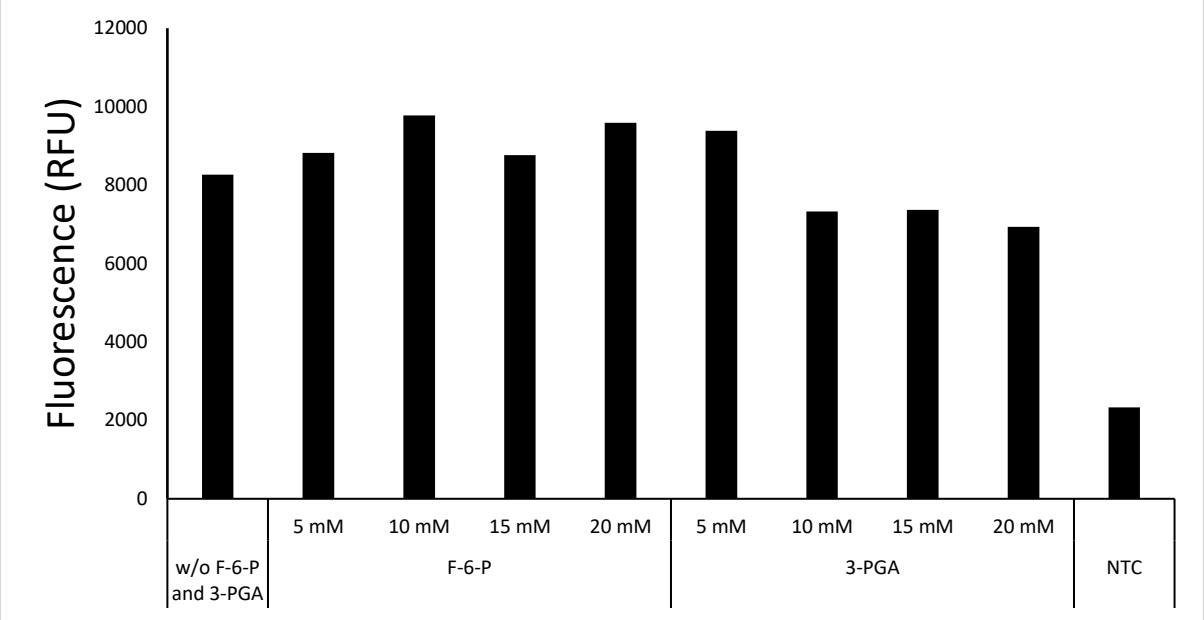


Supplementary Figure S3: Cell-free protein synthesis with and without melittin signal peptide. EYFP and the ETB receptor were synthesized in *P. pastoris* cell-free reactions in the presence of ^{14}C leucine. A melittin signal peptide (Mel) was inserted (+) or was omitted (-) in utilized templates. Proteins in the translation mixture were precipitated by acetone after cell-free protein synthesis. The autoradiography of radiolabelled proteins is visualized



Supplementary Figure S4: Cell-free protein synthesis based on different energy sources. Nanoluciferase (Promega) was synthesized in cell-free reactions based on *P. pastoris* cell lysate, which was processed by size exclusion chromatography. Luminescence was analyzed

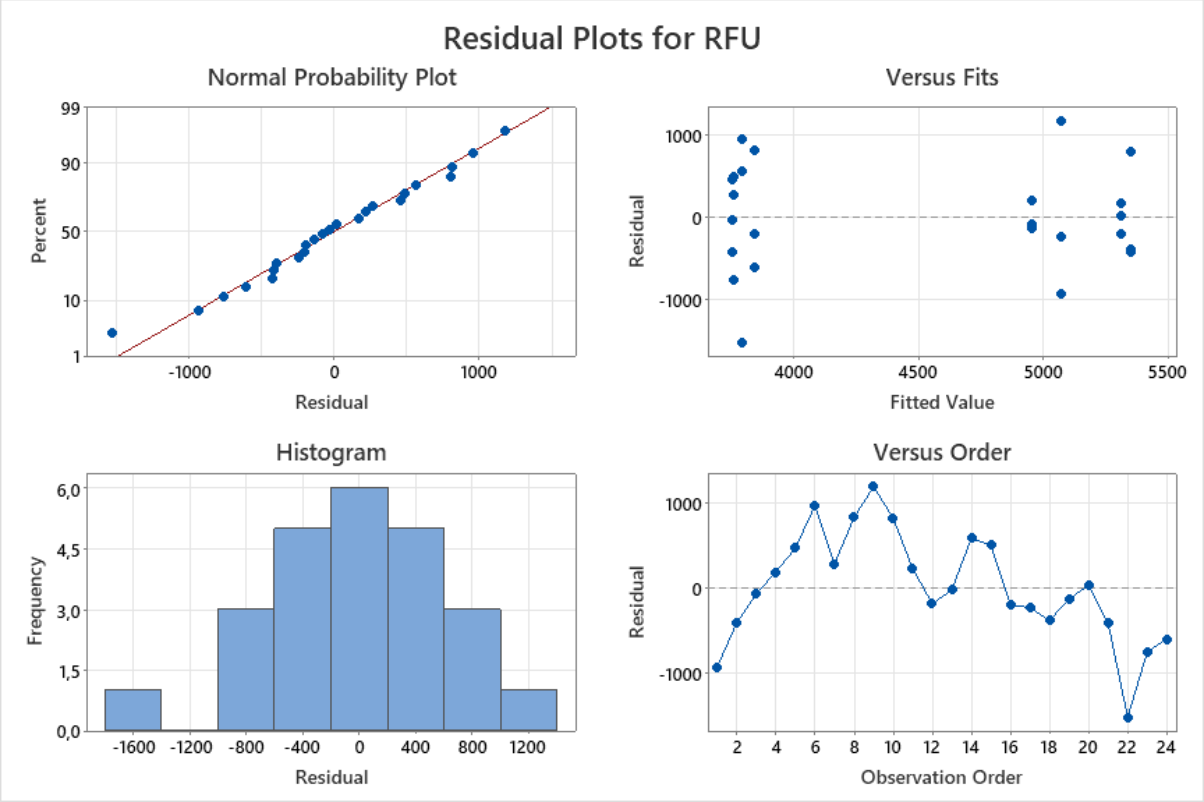
after cell-free protein synthesis by the Nano-Glo Luciferase Assay System (Promega). Measurements were performed in technical duplicate. Data are shown as mean ± SD.



Supplementary Figure S5: Cell-free protein synthesis containing F-1,6-P. Cell-free synthesis of ppα-GFP based on *P. pastoris* cell lysate was performed in the presence of 20 mM F-1,6-P. Different concentrations of F-6-P and 3-PGA were applied during cell-free reactions. Fluorescence was detected after cell-free protein synthesis. Results are displayed as endpoint measurement.

Response (RFU) = 4480 + 95 KPO4 – 12 cAMP – 693 NAD + 17 KPO4*cAMP – 64 KPO4*NAD + 27 cAMP*NAD – 4 KPO4*cAMP*NAD

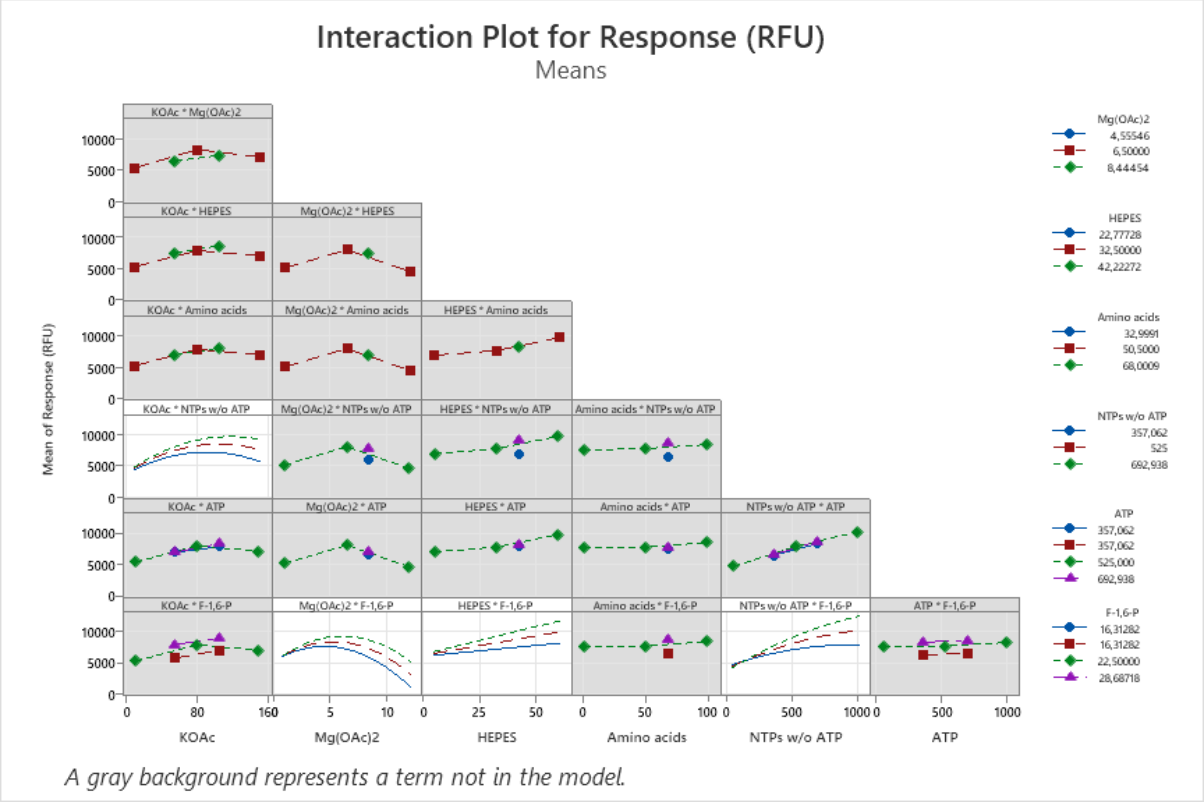
Supplementary Figure S6: Model equation of the full-factorial design. The model equation corresponds to Supplementary Table 1 and Figure 5 and is shown in uncoded units.



Supplementary Figure S7: Residual plots of the full-factorial design. The influence of NAD, cAMP and KPO_4 were analyzed based on a full-factorial design. Corresponding model information, analysis of variance and plots can be found in Supplementary Table 1 and Figure 5, respectively. Residual plots are shown and were prepared using Minitab software.

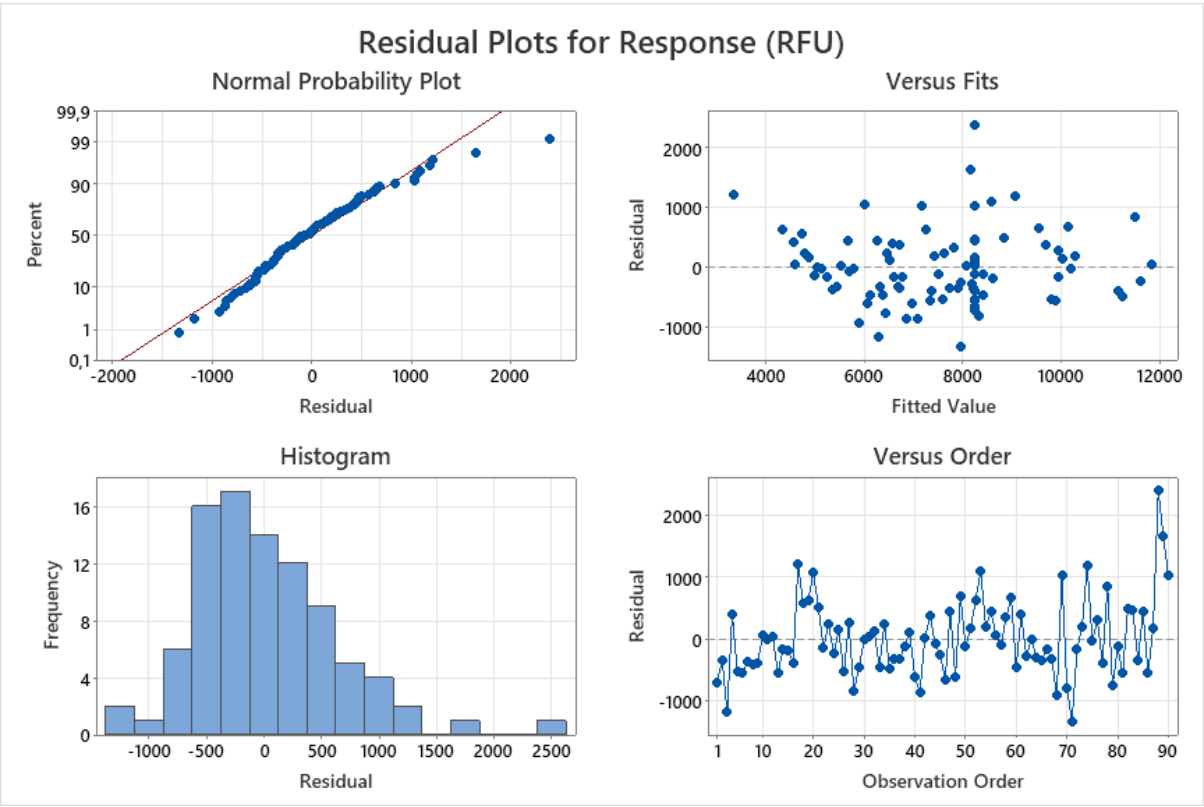
Response (RFU) = 5783 + 53.6 KOAc + 569 Mg(OAc)2 – 36.2 HEPES + 7.23 Amino acids – 5.01 NTPs w/o ATP – 386.2 F-1,6-P – 0.4263 KOAc*KOAc – 114.1 Mg(OAc)2*Mg(OAc)2 – 0.00386 NTPs w/o ATP*NTPs w/o ATP + 0.0667 KOAc*NTPs w/o ATP + 28.68 Mg(OAc)2*F-1,6-P + 4.34 HEPES*F-1,6-P + 0.4285 NTPs w/o ATP*F-1,6-P

Supplementary Figure S8: Model equation of the central composite design. The model equation corresponds to Table 1 and is shown in uncoded units.

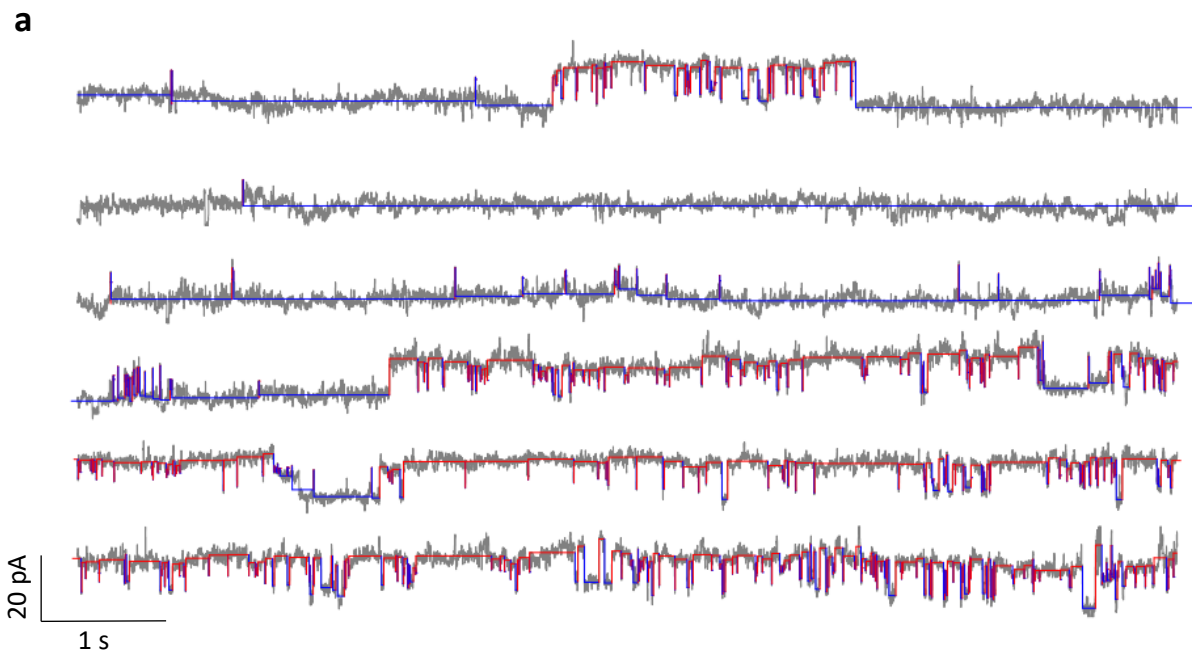


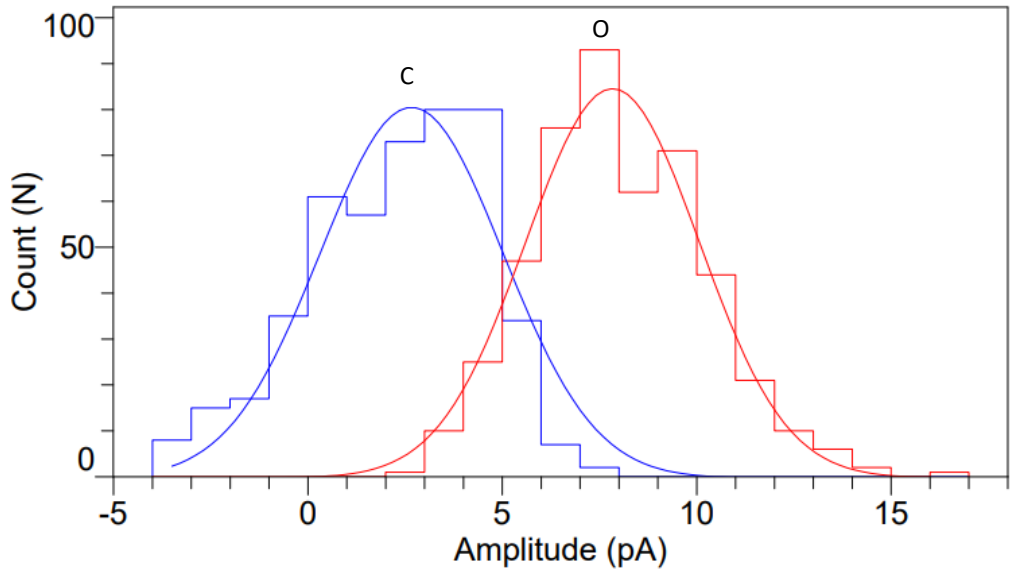
A gray background represents a term not in the model.

Supplementary Figure S9: Interaction plots of the central composite design. Interaction plots of the central composite design corresponding to Table 1 are shown and were prepared using Minitab software.



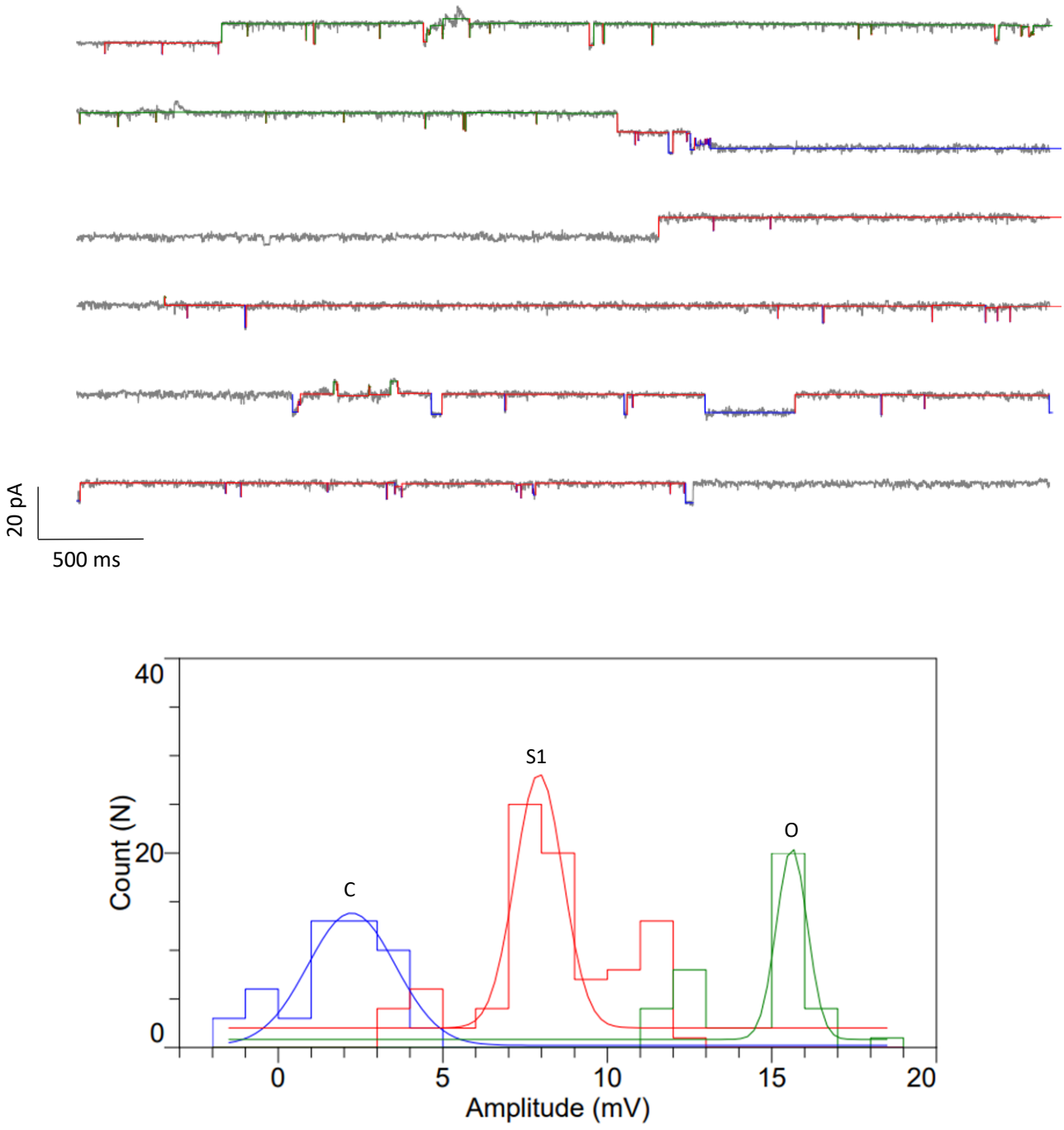
Supplementary Figure S10: Residual plots of the central composite design. Residual plots corresponding to Table 1 are shown and were prepared using Minitab software.





Supplementary Figure S11. Planar lipid bilayer electrophysiology measurements from the DPhPC bilayers after the addition of KcSA containing vesicles. A) Representative current traces of single channel activity of KcsA in pure DPhPC bilayers at pH 4.0 (N > 5) at -100 mV. B) All time amplitude histograms of traces of the above single channel trace showing multi-

conductance peaks (C: closed, and C : full open conductance). Buffer: 10mM HEPES, 150mM KCl, pH 4.0.



Supplementary Figure S12. Planar lipid bilayer electrophysiology measurements from the DPhPC bilayers after the addition of KvAP containing vesicles. A) Representative current traces of single channel activity of KvAP in pure DPhPC bilayers at pH 7.45 (N > 5) at +60 mV. B) All time amplitude histograms of traces of the above single channel trace showing multi-conductance peaks (C: closed, S1: sub-conductance 1 and C : full open conductance). Buffer: 10mM HEPES, 150mM KCl, pH 7.45.

Supplementary Table S1: Model summary and analysis of variance of the full-factorial design.

S	R^2	R^2-adj	R^2-pred
764.548	55.90%	36.61%	0.77%

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	7	11854617	1693517	2,90	0,037
Linear	3	11733296	3911099	6,69	0,004
KPO4	1	217313	217313	0,37	0,551
cAMP	1	3433	3433	0,01	0,940
NAD	1	11512550	11512550	19,70	0,000
2-Way Interactions	3	120912	40304	0,07	0,976
KPO4*cAMP	1	6575	6575	0,01	0,917
KPO4*NAD	1	97080	97080	0,17	0,689
cAMP*NAD	1	17258	17258	0,03	0,866
3-Way Interactions	1	409	409	0,00	0,979
KPO4*cAMP*NAD	1	409	409	0,00	0,979
Error	16	9352537	584534		
Total	23	21207154			

Supplementary Table S2: Model summary and analysis of variance of the central composite design corresponding to table 1.

S	R^2	R^2-adj	PRESS	R^2-pred	AICc	BIC
667.62	89.68 %	87.92%	55025830	83.24%	1447.35	1478.36

Source	DF	Seq SS	Contribution	Adj SS	Adj MS	F-Value	P-Value
Model	13	294419365	89.68%	294419365	22647643	50.81	0.000
Linear	6	235352852	71.69%	235352852	39225475	88.00	0.000
KOAc	1	20355056	6.20%	20355056	20355056	45.67	0.000
$Mg(OAc)_2$	1	21780696	6.63%	21780696	21780696	48.87	0.000
HEPES	1	28466325	8.67%	28466325	28466325	63.87	0.000
Amino acids	1	1279414	0.39%	1279414	1279414	2.87	0.094
NTPs w/o ATP	1	78940714	24.05%	78940714	78940714	177.11	0.000
F-1,6-P	1	84530647	25.75%	84530647	84530647	189.65	0.000
Square	3	29484887	8.98%	29484887	9828296	22.05	0.000
KOAc*KOAc	1	6359531	1.94%	8182454	8182454	18.36	0.000
$Mg(OAc)_2$*$Mg(OAc)_2$	1	21706273	6.61%	22319426	22319426	50.07	0.000
NTPs w/o ATP*NTPs w/o ATP	1	1419083	0.43%	1419083	1419083	3.18	0.078
2-Way Interaction	4	29581625	9.01%	29581625	7395406	16.59	0.000
KOAc*NTPs w/o ATP	1	4914213	1.50%	4914213	4914213	11.03	0.001
$Mg(OAc)_2$*F-1,6-P	1	7620995	2.32%	7620995	7620995	17.10	0.000
HEPES*F-1,6-P	1	4356863	1.33%	4356863	4356863	9.77	0.003
NTPs w/o ATP*F-1,6-P	1	12689554	3.87%	12689554	12689554	28.47	0.000
Error	76	33874843	10.32%	33874843	445722		
Lack-of-Fit	67	30952666	9.43%	30952666	461980	1.42	0.296
Pure Error	9	2922177	0.89%	2922177	324686		
Total	89	328294207	100.00%				

3. Overarching discussion

The production of proteins often forms the basis for elucidating interactions in biological systems, generating therapeutics and diagnostics, as well as powering advancements across various biotechnological domains. Currently, there are numerous options for producing recombinant proteins, for instance in bacteria, plant cells, yeast cells, insect cells, and mammalian cells. Each expression host offers distinct advantages and disadvantages, but they all share the common characteristic of expressing the desired proteins within living cells. This approach often encounters complications when producing proteins that negatively affect living cells. CFPS provides an alternative route to streamline the production of these complex proteins, albeit with high costs when using eukaryotic systems. In this study, CFPS was optimized to both reduce costs and simplify the process through the modification of cell lines. Two main strategies were employed for this purpose. Primarily, the adaptation of CHO cell lines to the conditions of CFPS was undertaken. This enabled, for the first time, the enhancement of mammalian-based CFPS through the incorporation of desired proteins into a translationally active cell lysate via transfection. Additionally, a novel CFPS system based on *P. pastoris* was developed, with parameters optimized through a statistical Design of Experiments. For the first time, F-1,6-P was shown to completely substitute the commonly used CP/CK energy regeneration system, leading to improved eukaryotic cell-free protein production. The flow chart in Figure 4 illustrates the relationships between the publications.

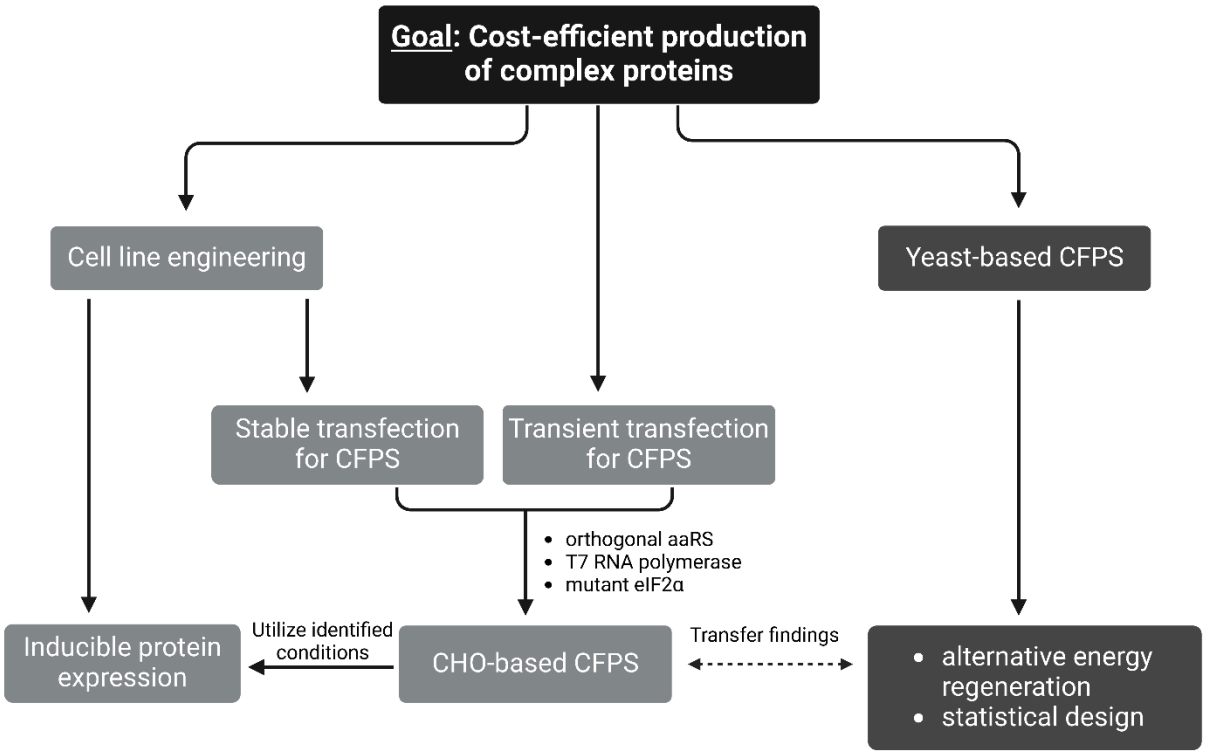


Figure 4: Flow chart of the doctoral thesis. Arrows indicate the flow to the specific approaches, which were realized during the doctoral work and their connections between the published findings. The dashed arrow indicates the possibility to transfer the findings from yeast-based to CHO-based cell-free reactions. Grey: CHO-based achievements. Dark grey: Yeast-based achievements. Created with BioRender.com.

3.1. Modification of the CHO genome

First, an engineered CHO-based cell-free system was developed to strengthen the synthesis of site-specifically modified membrane proteins. For this purpose, CHO cell lines were genetically modified, and the cell lysate was adapted so that orthogonal aaRS are present in the translationally active cell lysate. The analyzed loci *HPRT1* and *C12orf35* were characterized in detail for their roles in the recombinant expression of *E. coli* TyrRS and subsequent CFPS, with *C12orf35* identified as the optimal target site. Based on this, further investigation of target sites in the CHO genome was conducted to identify an optimal site for the generation of stable CHO cell lines harboring large transgenes. Therefore, the homologous *Rosa26* locus was compared against *C12orf35*. Due to the specificity and ease of use of the CRISPR/Cas9 system, the *Rosa26* locus was found to be a suitable site in the CHO genome to generate CHO cell lines with desired properties. Although the integration of large expression cassettes was successfully demonstrated in this work, it generally represents a challenge in genome editing. A donor template with large homologous arms is often used via HDR-directed repair to correct CRISPR/Cas9-induced DSBs. In contrast, Suzuki *et al*. demonstrated that homology-

independent targeted integration (HITI)-mediated repair is active throughout the entire cell cycle, achieving significantly higher editing efficiencies compared to HDR-mediated repair[219]. Multiple studies have confirmed the efficiency of HITI-mediated repair, which could streamline the insertion of desired DNA sequences at specific genomic sites in the future[220–222]. The combination of site-specific recombinases and transposases with CRISPR/Cas9 can further enhance and simplify stable transfection, facilitating the rapid generation of cell lines for cell-free protein production. In this regard, Pallarès-Masmitjà *et al*. utilized Cas9-PiggyBac transposase fusions to take advantage of the Cas9-RNP recognition and the PiggyBac transposase transfer efficiency of DNA payloads in human and mouse cells[223].

In the present work, the complete regulatory unit of a tet-inducible expression system, as a large donor sequence, was integrated into *Rosa26*. This integration enables the transfer of findings from the cell-free system to direct induced cell-based expression, potentially enabling scalable production of larger quantities of membrane proteins.
While the tet-controlled system demonstrated a substantial enhancement in GFP production at *Rosa26* upon tet addition, minimal promoter leakage was detected in its absence. Leakage from various inducible systems can pose challenges[224–226], particularly when expressing highly cytotoxic proteins[227]. In a recent study, cytotoxic effects during the production of difficult-to-express GPCRs M4 and GPR120 were minimized through the utilization of a cumate-switchable system[228]. The study elucidated that in the absence of cumate, stable CHO cell pools exhibited reduced metabolic burden and ER stress compared to cells overexpressing GPCRs. Integrating a cumate-controlled system into *Rosa26* would also bring the advantage of being regulated independently of antibiotics like tet. This would alleviate regulatory constraints on the production of therapeutic proteins.

3.2. Integration of desired proteins into the cell lysate promotes CFPS

Beyond stable transfection, it was shown that aaRS could be integrated into CHO cells using transient transfection for orthogonal cell-free translation. This method provides rapid evaluation of promising proteins for stable integration into CHO cells to improve CFPS. Building on this, transient transfection of T7 RNA polymerase and mutant eIF2α demonstrated further enhancement of CFPS. The combination of the examined proteins in one cell lysate could be advantageous for streamlined cell-free reactions. However, it needs to be determined

whether combined expression might adversely affect cell growth and the translation activity of the resulting cell lysate for CFPS.

Regarding the influence of eIF2α on CFPS, recent studies have demonstrated that the cap-dependent translation initiation process can be enhanced by modulating proteins that regulate the phosphorylation status of eIF2α in human cell-free systems[229,230]. Existing research has demonstrated that decreased phosphorylation of mutant eIF2α in embryonic fibroblast cells resulted in a remarkable 30-fold increase in translation within cell-free reactions utilizing the corresponding embryonic fibroblast cell lysate[231]. Thus, the approach developed here, incorporating mutant eIF2α into the cell lysate, could potentially augment translation in human cell lysates.

Engineering of endogenous eIF2α in cells may be promising. Conversely, targeted mutations in the eIF2α of the host cell line or stable transfection of this essential endogenous protein may significantly affect endogenous protein translation.

3.3. Site-specific modification of proteins

CFPS can significantly complement cell-based expression of GPCRs and other difficult-to-express proteins. It allows for rapid screening of GPCRs in small-scale experiments. In this regard, Köck *et al*. screened different nanodisc compositions to identify an optimal lipid composition for GPCR folding in 96-well plates using *E. coli* cell lysates[166]. Using a reconstituted cell-free protein synthesis, it was shown that that a gene library of endothelin receptor type-B was utilized for directed evolution *in vitro*[232].

This doctoral thesis further demonstrated the potential for cell-free site-specific modification of GPCRs to improve the evaluation of these membrane proteins. By coupling fluorophores to ncaa, optimal amino acid positions can be evaluated in cell-free reactions. Once suitable positions are identified, these findings can provide insights into cellular localization and trafficking via fluorescence microscopy. However, the analysis of ligand binding by GPCRs often necessitates evaluating conformational changes[233], which cannot be sufficiently resolved by the insertion of a single fluorophore. Since ligand binding of GPCRs is transmitted to the C-terminal domain, the integration of fluorescent fusion proteins in the third intracellular loop and at the C-terminus of the α2A-adrenergic receptor allowed monitoring of conformational rearrangements by fluorescence resonance energy transfer (FRET)[234].

As with many proteins that naturally occur as oligomers, GPCRs also form homodimers and heterodimers[235,236]. Asher *et al*. examined GPCR oligomerization in living cells using self-labeling SNAP tags to detect structural dynamics upon ligand binding[237]. Although the incorporation of fluorescent proteins (~ 27kDa) and self-labeling tags including SNAP-, Halo- and CLIP tags (~ 20-33 kDa)[238] is frequently used[239–241], the size and localization restricted to the N- or C-terminus can strongly influence the function and interactions of proteins. Furthermore, the size of the tags can contribute to creating additional space between the interaction partners being examined[242]. This problem could be circumvented by inserting a tetracysteine CCXXCC motif into the β2 adrenoceptor and subsequent reaction with the fluorogenic dye FlAsH[243]. However, the specific tetracysteine motif can disrupt the structure and function of the protein, and it was also observed that the fluorogenic dye binds other cysteine-containing proteins non-specifically[244]. Alternatively, a proximity ligation assay revealed the assembly of $GABA_B$ receptors into a GPCR receptor complex after *Sf*21-based cell-free reactions[245]. Nonetheless, primary antibodies against the GPCRs are required.

In this study, exclusively amber stop codons were utilized for incorporating ncaa. To integrate two distinct reactive fluorophores, it would be advantageous to target two separate codons. Previous studies demonstrated dual labeling by incorporating ncaa for subsequent click reactions using site-specific modification strategies based on introduced amber and ochre stop codon positions, indicating the potential for FRET applications[246,247]. Wang *et al*. were able to evolve tRNA(Pyl) corresponding to the PylRS to decode for quadruplet codons using orthogonal ribosomes[248]. In this process, fluorophores could be introduced into calmodulin through a combination of quadruplet codons and amber stop codons, followed by analysis using click chemistry and FRET. However, the PylRS, which is frequently utilized due to its acceptance of bulky ncaa, has an unstable N-terminal domain that can result in aggregation or loss of activity[213]. While the present work showed the circumvention of enzyme purification through PylRS integration into the cell lysate, enzyme instability can also be mitigated by co-expressing the target protein and PylRS[249,250]. This approach avoids the need for enzyme purification and storage, enabling the direct use of PylRS in the same cell-free reaction for site-specific modification in CHO, *Sf*21, and *E. coli* lysates. Moreover, it was demonstrated that a PylRS from *Methanomethylophilus alvus* can overcome the instability issues of the utilized *M. mazei* PylRS due to the absence of the N-terminal domain[214]. Additionally, the *M. alvus* and *M. mazei* PylRS are mutually orthogonal, allowing dual site-specific modifications. To enable the incorporation of large, bulky ncaa with *M. alvus* PylRS, it was proven that a mutant library

could be used to transfer structurally diverse ncaa, accepted by *M. mazei* and *M. barkeri* PylRS, to *M. alvus*[251].

3.4. Cost-effective cell-free protein production based on *P. pastoris*

Despite the reduction in CFPS costs from integrating desired proteins into CHO cell lysates, the high cost of media and labor-intensive processes keep cultivation expenses elevated. To address this, a new cell-free system based on the yeast *P. pastoris* has been created. In addition to the well-understood and simple integration into the *P. pastoris* genome, along with both constitutive and induced expression, the cultivation costs and time are significantly reduced compared to other eukaryotic systems[252]. Hence, the yeast-based cell-free system presents a promising approach for the production of difficult-to-express proteins. This doctoral thesis aimed to optimize energy regeneration, a significant cost factor in cell-free systems. Applying the alternative glycolysis-based energy regeneration system to CHO-based CFPS is feasible, given the successful implementation in *S. cerevisiae* cell-free reactions.

To enable large-scale application of the new cell-free systems, it is essential to address cost factors such as amino acid and nucleotide sources. Instead of supplementing cell-free reactions with single amino acids, common nutrient-rich media were shown to be substrates for various cell-free systems[253]. Furthermore, leveraging the endogenous NTP metabolism could potentially replace the costly addition of NTPs in cell-free reactions. However, the synthesis of purine and pyrimidine nucleotides is an energy-intensive process, which requires multiple enzymes[254]. Nonetheless, in *E. coli*-based cell-free reactions, the inclusion of PEP as an energy source facilitated nucleotide regeneration without requiring the addition of exogenous NTPs[96]. Calhoun and Swartz achieved a significant cost-reduction by exchanging NTPs for nucleoside monophosphate in an *E.coli*-based CFPS using glucose as energy source[255]. Wang *et al*. demonstrated that supplementation of polyphosphate kinase in *E. coli* CFPS can be utilized to phosphorylate nucleosides by adding inorganic polyphosphate[256].

After cost reduction, scalability of both cell lysate production and cell-free reactions becomes crucial for commercial application. Zawada *et al*. achieved linear scalability up to 100 L cell-free *E.coli* reactions to produce human granulocyte-macrophage colony-stimulating factor[257]. They first optimized the production in a 96-well format by Design of Experiments and utilized the optimization parameters to scale up the cell-free reaction, yielding 700 mg/L protein after 10 hours. Moreover, eukaryotic cell-free reactions were scaled up from 100 µL to 1 L reactions

using tobacco BY-2 cell lysates[258]. In the present work, the feasibility of optimizing process parameters through statistical experimental design was demonstrated, requiring minimal effort yet yielding significant insights. Having established the scalability of the novel system, the next pivotal step lies in exploring lyophilization of the cell lysate, ensuring sustained activity and paving the path for its commercial practicability. For instance, studies revealed the potential to apply CFPS for decentralized bioproduction by improved lyophilized *E.coli* cell lysates[96,259]. Statistical experimental design with *P. pastoris* lysates allowed for the rapid identification of optimal parameters for cell-free reactions. It also demonstrated the feasibility of parallel cell-free synthesis in a 96-well format. Miniaturizing cell-free reactions to nanoliter volumes and utilizing liquid handling stations enables semi-automatic screening of entire libraries in a short period[260,261].

In conclusion, this study successfully optimized CFPS to produce challenging proteins at lower costs, focusing on CHO cell lines and a novel *P. pastoris*-based CFPS system. Key advancements included the integration of orthogonal aaRS, T7 RNA polymerase, and mutant eIF2α into CHO cells, and the development of cost-effective energy regeneration methods in yeast. These innovations, along with the induced expression approach, have the potential to enhance the efficiency of protein production in various biotechnological applications, with findings that can be transferred between yeast and CHO CFPS systems.

List of publications

Ramm, F., Jack, L., Kaser, D., **Schloßhauer, J. L.**, Zemella, A., & Kubick, S. (2022). Cell-Free Systems Enable the Production of AB5 Toxins for Diagnostic Applications. Toxins, 14(4), 233. https://doi.org/10.3390/toxins14040233

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