Optimizing the expression, solubility, and purification of human ribosomal proteins SA, S2, S7, and S12 in *E. coli* to analyze post-translational modifications *in vitro*

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FIRE: THE FIRST-YEAR INNOVATION & RESEARCH EXPERIENCE

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Introduction

Ribosomal Proteins (RPs) have been difficult to express in soluble forms.

- Recent developments have produced a new methodology for expression of some ribosomal proteins in soluble forms through a fusion protein of N-terminal 6xHis-Thioredoxin A tag to enhance solubility and enable purification respectively.[1]
- Expression of soluble RPs lowers the barrier for analysis of Post Translational Modifications (PTMs).
  - PTMs are prominent in RPs and have potential clinical implications but are poorly characterized due to purification difficulties.
- In leveraging methodology established by Correddu et al., we seek to characterize potential O-linked glycosylation of RPs through in-vitro reactions with purified, solubilized RPs.

Clinical Significance.

- Regulation of RP expression is tied to numerous cancers (colon, pancreatic, prostate, liver, and ovarian cancers), via over or under expression.[2]
- Underexpression associated with haploinsufficiency and Diamond Blackfan Anemia-like (DBA) symptoms in mice for RPS12, and DBA in general for other RPs.[3]
- RPSA associated with Isolated Congenital Asplenia.[4,5]
- RPS7 is listed as an activator of p53, and as a stabilizer of GADD54a.[4]

Methodology.

- Ligase-independent cloning of RP cDNA sequences into pNH-TrxT vector with N-terminal 6xHis-Thioredoxin A tag followed by evaluation of ideal conditions, toxicity, and solubility assessments.
- This was followed by upscaled expression and purification procedures
  - Evaluation with agarose DNA, SDS PA gel electrophoresis, western blot, BCA assays to determine success.
- To be followed by in-vitro reaction assay utilizing O-GlcNAc transferase, purified in parallel to experimental proteins.

Methods

Cloning:

- cDNA for ribosomal proteins was cloned via ligase independent cloning techniques into pNH-TrxT and evaluated through off-site sequencing
  - pNH-TrxT: contains two N-Terminal tags, a thioredoxin tag to improve solubility and an 6xHis tag for purification.[1]
  - Separated from the protein of interest by a TEV (Tobacco Etch Virus) cleavage site

Protein expression trials and large-scale expression:

- Cultures were induced by Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 18℃ with concentrations of IPTG at 0.1, 0.5, and 1 mM and at different OD_{600} at 0.2 and 0.4 to optimize expression of the ribosomal proteins.
- Further expression was conducted with 0.5 mM IPTG at 0.4 OD_{600}.
- SDS-PAGE followed by Coomassie staining, along with Western blot analysis were performed to confirm protein expression and evaluate quantity in conjunction with BCA assays.
- Larger cultures of 50 mL were grown to obtain enough material for affinity chromatography purification.

Purification and further experimentation:

- Cultures of RPSA and 12 were lysed by sonication, purified via affinity purification, then dialyzed before experimentation.
- Transformed Mutant catalytically inactive and Wild Type OGT fusion proteins with 6xHis tag into BL21 bacteria for future purification and use.
  - A similar procedure was conducted for the purification of OGT wildtype and mutant for use in future in-vitro reactions.[6]

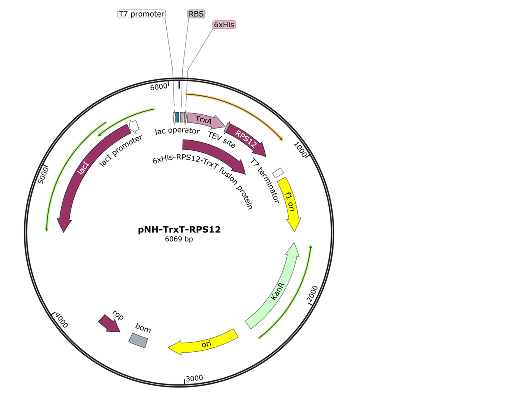

Figure 1. Plasmid map of pNH-TrxT-RPS12. The map is identical for the rest of the RPs, with the exception of the individual coding sequence for the ribosomal proteins. (Created with SnapGene).

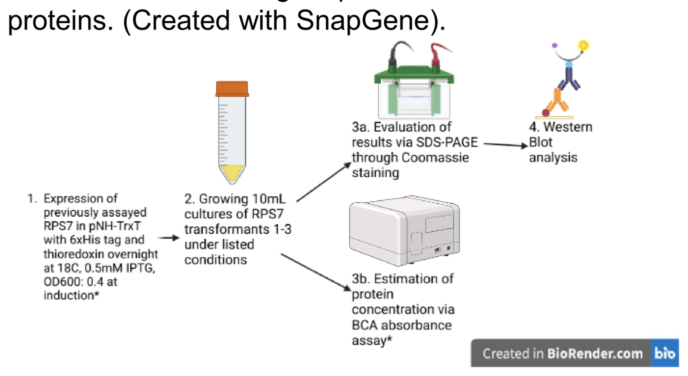

Figure 2. Bacterial Expression Trials and associated experiments (Created with BioRender.com)

Results

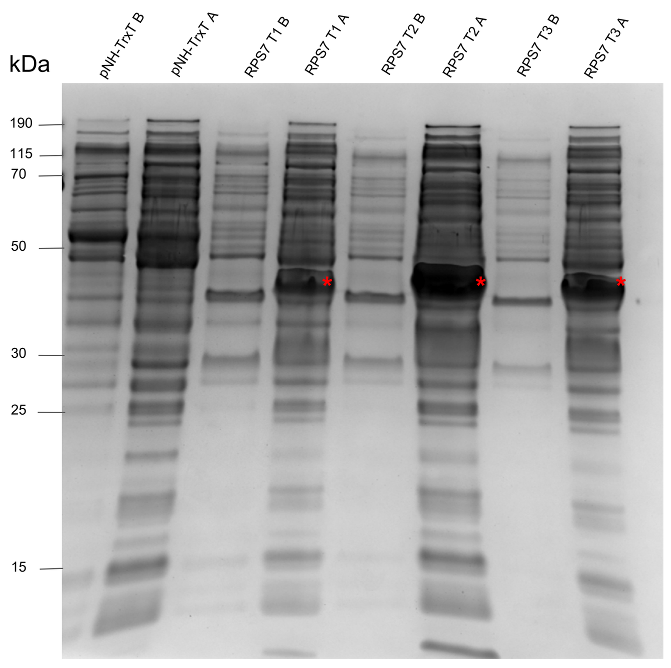

Figure 3. Whole cell lysates from *E.coli* BL21 cells, with three pNH-TrxT-RPS7 transformants and empty vector as control. Grown at 18℃ with 0.5 mM IPTG overnight, separated via SDS-PAGE and coomassie stained. The estimated size of the fusion protein 6xHis-TrxT-RPS7 is 36.1 kDa. Aliquots were taken before induction (B) and after overnight growth (A) to assess differences in banding patterns, with unmodified vector pNH-TrxT serving as a control. Of note is the dark banding pattern at ~36 kda in the overnight growth sets, indicating successful expression of the fusion protein, in addition to changes in banding patterns before and after overnight growth.

Transformant	Concentration (mg/mL)
OGT Mutant	0.3324
OGT Wild Type	0.5342
RPS12	0.4614

Table 1. BCA absorbance assay results from purified RPS12 and OGT samples. Purified samples were compared to a standard BSA curve. Results were calculated utilizing a best-fit linear equation derived from standard curve (R^2=0.997). Results suggest high protein concentration in all samples.

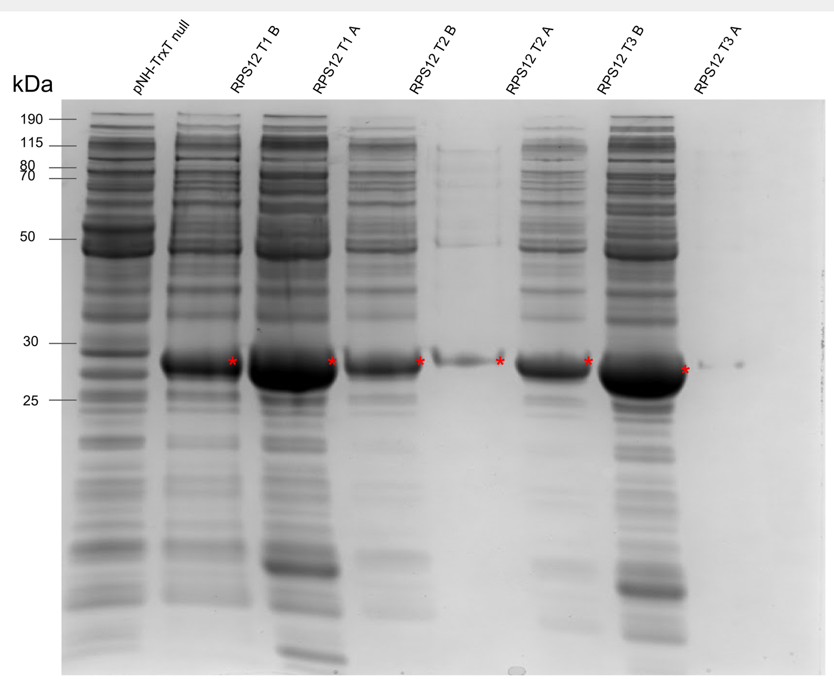

Figure 4. Expression trials with three pNH-TrxT-RPS12 transformants and empty vector as control. Grown at 18℃ with 0.5 mM IPTG overnight, separated via SDS-PAGE and coomassie stained. The estimated size of the fusion protein 6xHis-TrxT-RPS12 is 28.5 kDa, Aliquots were taken before induction (B) and after overnight growth (A) to assess differences in banding patterns. Of note is the leaky expression demonstrated by the presence of the target band in both before and after aliquots, with increased expression in after aliquots.

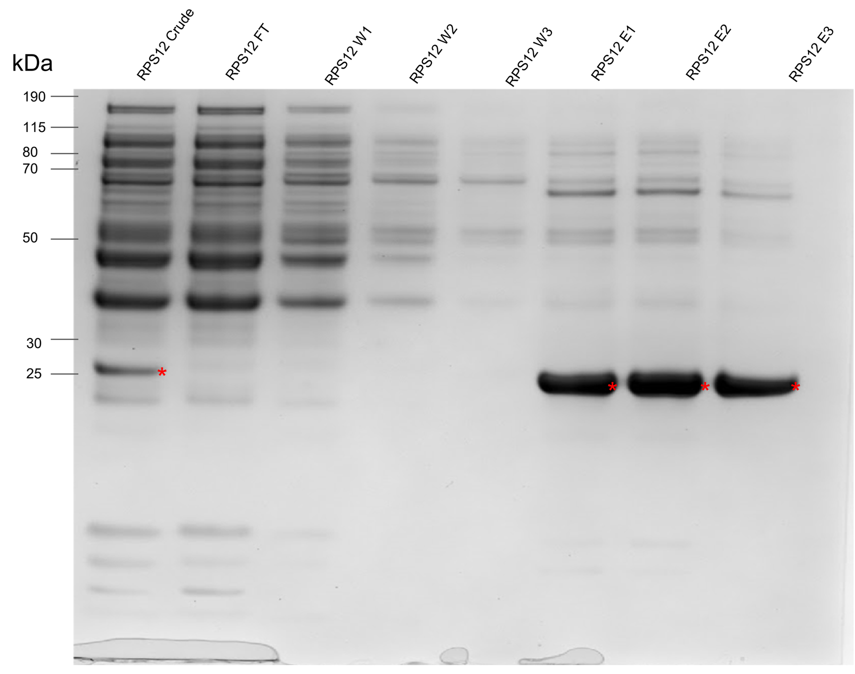

Figure 5. Purification of pNH-TrxT-RPS12 transformant one (see Figure 4). Grown at 18℃ with 0.5 mM IPTG overnight, separated via SDS-PAGE and coomassie stained. The estimated size of the fusion protein 6xHis-TrxT-RPS12 is 28.5 kDa, Aliquots were taken of the crude lysate, the flowthrough after binding to the beads, the three wash stages, and the three elution stages. Of note is the presence of the target band in the initial crude lysate, and the strong presence of the target band in each of the three eluent aliquots, indicating a successful purification.

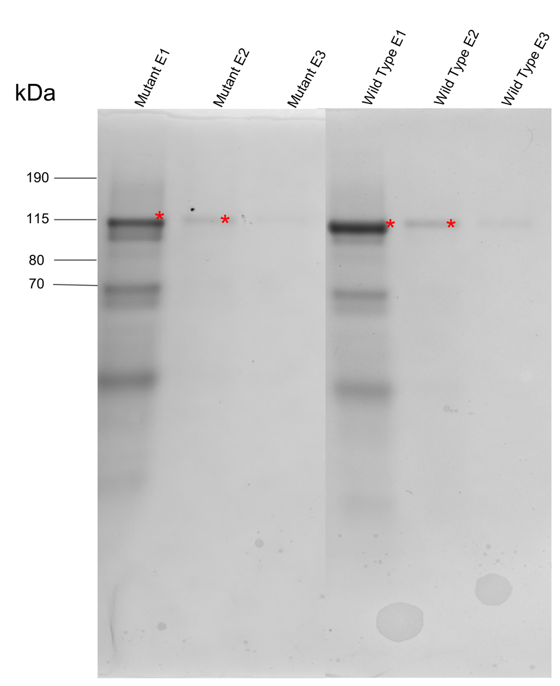

Figure 6. Purification of OGT catalytically inactive mutant (left) and wild-type (right) proteins. Only the elution is shown. The estimated molecular weight of 8xHis-OGT is ~118 kDa. Aliquots were taken of the three elution stages to evaluate the effectiveness of the purification of the two enzymes. Of note is the presence of a strong target band in each of the first eluent aliquots with declining presence in the following two aliquots.

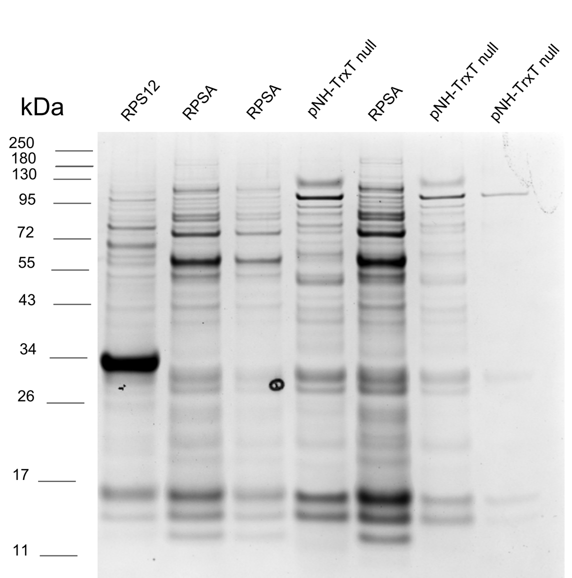

Figure 8. Dialysis of three purified pNH-TrxT-RPSA eluents (see Figure 7) and purified empty vector (null) as control. Dialyzed in salt buffer overnight, then twice for two hours. The estimated size of the fusion protein 6xHis-TrxT-RPSA is 46.9 kDa. Aliquots taken from each dialyzed sample of pNH-TrxT-RPSA and null (three from each).

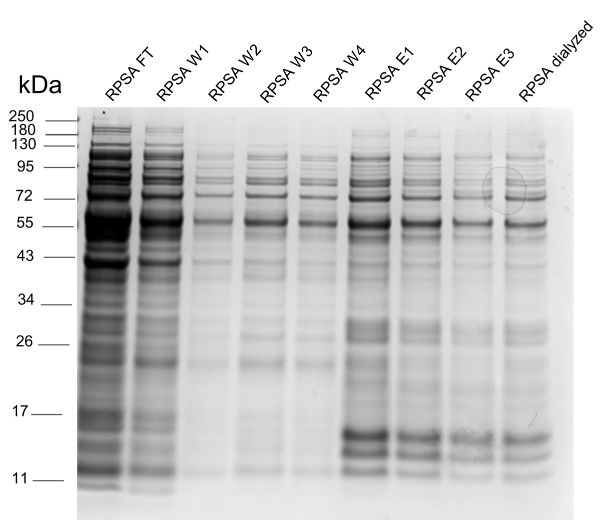

Figure 7. Purification of pNH-TrxT-RPSA transformant. Grown at 18℃ with 0.5 mM IPTG overnight, separated via SDS-PAGE and coomassie stained. The estimated size of the fusion protein 6xHis-TrxT-RPSA is 46.9 kDa. Aliquots were taken of the crude lysate, the flowthrough after binding to the beads, four wash stages, and three elution stages. Of note is the presence of the target band in the initial crude lysate (not shown here), and the strong presence of the target band in each of the three eluent aliquots, indicating a successful purification. Smaller weight bands may indicate degradation products of the His tag.

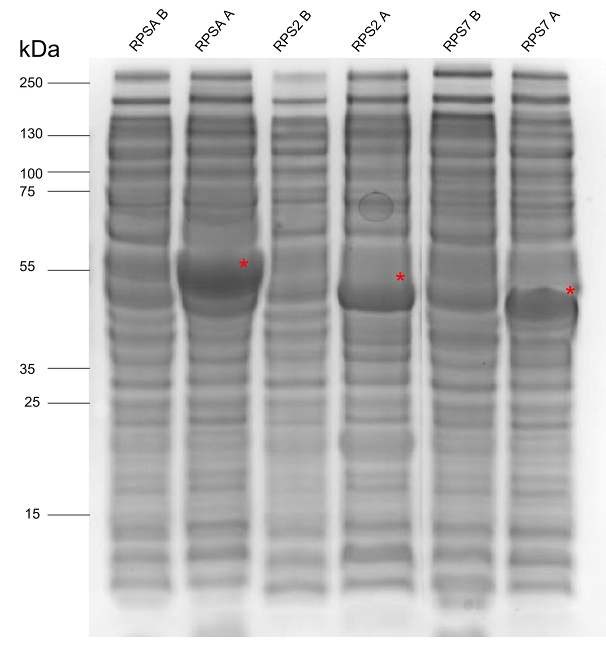

Figure 9. Expression trials with pNH-TrxT-RPSA, pNH-TrxT-RPS2, and pNH-TrxT-RPS7. Grown at 18℃ with 0.5 mM IPTG overnight, separated via SDS-PAGE and coomassie stained. The estimated size of the fusion protein 6xHis-TrxT-RPSA is 46.9 kDa, for RPS2 is 45.3 kDa, RPS7 is 36.1 kDa. Aliquots were taken before induction (B) and after overnight growth (A) to assess whether the expression of the ribosomal proteins was induced by IPTG. Of note is the dark banding pattern at ~45 kDA for RPSA and RPS2, and ~36 kDa for RPS7, indicating successful expression of the fusion protein.

Discussion

Overview

- Despite utilization of Corredu protocol, some proteins (RPS2, RPS7) remained insoluble.
- Successful solubilization and purification of RPS12, RPSA demonstrate basic viability of the protocol for some RPs, but we can conclude that the use of Thioredoxin as a stabilizing element is not universally successful.
- Earlier attempts to express other RPs demonstrated toxicity in BL21 cells, forcing the development of alternative expression methods.

Future Directions

- In-vitro reactions with OGT remains the objective.
- After confirmation of glycosylation or lack thereof, we will pursue exploration of modified residues via Mass Spectrometry followed by site-directed mutagenesis to confirm sites.
- Present challenges in going forward include limited culture size, limited solubility of some RPs, and toxicity of others.
- Future endeavors will focus on expanding expression to enable greater material collection and improving solubility of target proteins.

References

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