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# Subcellular Compartmentalization of Human Ribosomal Protein S6 Turnover

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## Introduction

- The cellular role of the assembled ribosome is known. However, the function and mechanisms of free ribosomal proteins remains understudied.[1]
- Free ribosomal proteins are present in the cytoplasm before ribosome biogenesis occurs in the nucleus and nucleolus.[2] Ribosomal protein balance is regulated at the protein level by the ubiquitin-proteasome system.[3]
- Human ribosomal protein 6 (RPS6/eS6) is a protein from the small subunit that plays a role in regulating cell growth, proliferation and metabolism downstream of the mTORC1 pathway.[4]
  - RPS6 is regulated by phosphorylation at multiple serines on its C-terminal domain.[5]
- RPS6 shuttles between the cytoplasm and nucleus via nuclear localization signals (NLS) on the C-terminal domain.[6]
  - The relationship between phosphorylation state, subcellular localization, stability, and turnover of free RPS6 is still poorly understood.
- **Objectives:**
  - Use Nuclear Export Signal (NES)-mediated cytoplasmic retention of RPS6 to study the protein's mechanism of action independent of the ribosome.
  - Determine mechanism of RPS6 stabilization and localization, including nuclear shuttling.
  - Determine post-translational modifications and extraribosomal functions of RPS6.
  - Describe functional significance of subcellular localization.
  - Understand the relationship between RPS6 post-translational modifications (PTMs) and the protein's stability, localization, and function.

## Methods

- Constructed recombinant plasmids via restriction ligation cloning and subsequent Site Directed Mutagenesis (SDM) coding for fusion proteins containing RPS6, Turquoise fluorescent protein (pmTurq), and NES.
  - pRPS6-mTurq, pRPS6-mTurq-NES (experimental)
  - pmTurq-∅, pmTurq-NES-∅ (control, addgene plasmids 60561 and 36206)
- Conducted 24 and 48 hour lipofectamine transfection into HEK293 cells.
- Transfected HEK293 cells directly on 96 well plate and measured mTurquoise fluorescence using a plate reader.
- Lysed transfected HEK293 cells for immunoblotting. Probed for mTurq using GFP antibody to detect expression of recombinant protein and for GAPDH as a loading control.
- Prepared nuclear and cytoplasmic fractions from 48 hour transfections using a proprietary extraction kit (NE-PER, Thermo Fisher).
  - Immunoblotting probed for GFP, GAPDH and Histone proteins.
- Structural figures were generated using the human 80S ribosome cryo-EM structure[7, 8] (PDB:6QZP) and the AlphaFold2 predicted RPS6 structure (UniProt P62753), both visualized in ChimeraX.[9]

## Results

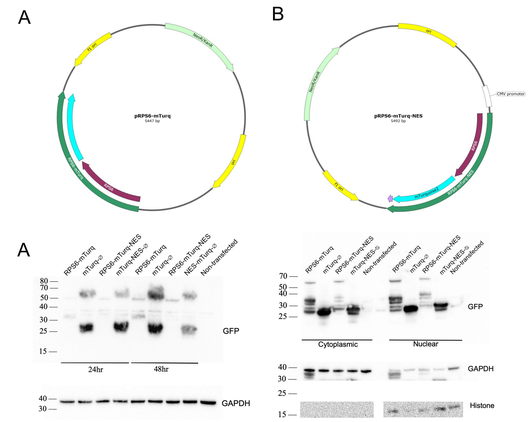


**Figure 2:** Western Blot analysis of Turquoise fluorescent fusion proteins expressed in HEK293 cells. **(A)** 24 and 48 hour lipofectamine transfections show expression of RPS6-mTurq fusion proteins compared to untransfected sample. 48 hour transfections yielded greater amounts of protein relative to 24 hour samples. RPS6 containing fusion proteins exhibit light banding at the expected MW (~57 kDa). Non-RPS6 samples show bands at ~55 kDa and ~27 kDa. Some light banding is observed in the 30-40 kDa range. Equal amount of proteins were loaded. GAPDH was used as a loading control. Samples were resolved in a 10% NuPAGE gel in MES. **(B)** Nuclear and cytoplasmic fractions of transfected HEK293 cells. Anti -GFP probing shows presence of full fusion proteins (~57 kDa) and degradation products (27-35 kDa). Empty plasmids show mTurq presence. GAPDH and Histone proteins were used as the loading controls for cytoplasmic and nuclear fractions respectively. GAPDH was observed in the nuclear fraction. Equal amount of proteins were loaded. Samples were resolved using a 4-12% NuPAGE gel in MES.

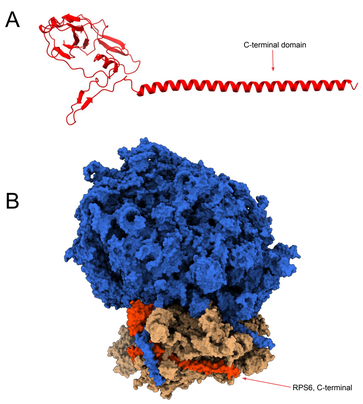


**Figure 4:** **(A)** Structure of free Ribosomal Protein S6 predicted by AlphaFold2. C-terminal domain containing phosphorylatable serine residues and NLS is indicated. **(B)** Full ribosome structure from PDB entry 6QZP including the large 60S subunit (blue) and small 40S subunit (tan) with RPS6 highlighted in red. Both structures were visualized in UCSF ChimeraX.

**Figure 1:** **(A)** pRPS6-mTurq and **(B)** pRPS6-mTurq-NES recombinant plasmid maps, with expected molecular weights for expressed fusion proteins of 56.2kDa and 58kDa respectively. Plasmid constructs were made via restriction cloning and subsequent SDM. Constructs were sent to Plasmidsaurus for sequence confirmation.

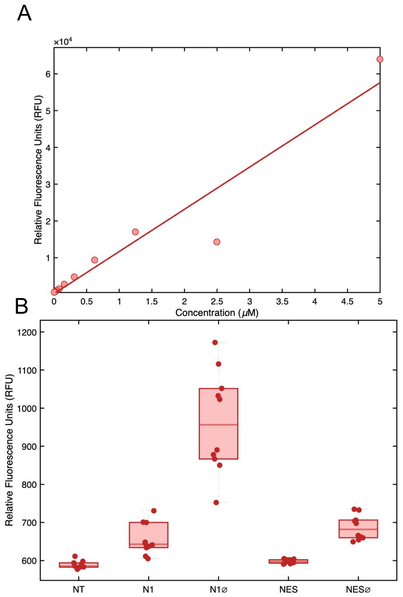


**Figure 3:** 96 well plate fluorescence assay on HEK-293 transfections. **(A)** Fluorescein standard curve was used to convert RFU to concentration of mTurq. Y = 11500 RFU/μM, $R^2$ = 0.9231. **(B)** Fluorescence assay results including non-transfected cells, RPS6-mTurq (N1), empty mTurq (N1∅), RPS6-NES (NES), and mTurq-NES (NES∅). Data shown as median and interquartile range. 10 biological replicates were tested for each control. Results show a small amount of RPS6-mTurq and mTurq-NES presence, a larger relative mTurq-∅ presence and near baseline RPS6-mTurq-NES signal.

## Discussion

- GFP immunoblotting confirmed expression of mTurquoise fusion proteins in HEK293 system (Fig 2A).
  - 48 hour transfections may yield clearer results in future experiments relative to 24 hour.
  - RPS6 samples showed signal from full fusion proteins around 57kDa and non-specific GFP binding around 33kDa. Same signal may be due to RPS6-mTurq degradation products.
  - Non-RPS6 samples exhibit banding around 55 kDa and 27 kDa which may indicate presence of an mTurq dimer.
    - The expected molecular weights of mTurquoise is 27kDa.
- Nuclear-Cytoplasmic preparations showed RPS6 and RPS6-NES accumulation in both fractions (Fig 2B).
  - This may be due to increased expression of RPS6-NES overloading nuclear export system.
- Nuclear-Cytoplasmic preps yielded greater resolution of degradation products relative to crude lysate.
  - Different-sized degradation products indicate differential breakdown of RPS6.
- Both fractions showed lower relative intensity of RPS6-NES vs RPS6 bands indicating that stabilization may occur in the nucleus.
  - Presence of RPS6-NES and RPS6 pmTurq fusion protein in the cytoplasm show that the construct is stable and detectable. This result was consistent between the crude lysate and Nuclear-Cytoplasmic prep.
- Nuclear samples showed presence of GAPDH. Studies have shown that GAPDH is involved in DNA repair.[10]
  - Nuclear GAPDH presence does not indicate a poor separation of fractions. Future experiments will use a purely cytoplasmic loading control to confirm nuclear fraction purity.
- Lack of histone signal in cytoplasmic fraction indicate nuclear proteins are not present in the cytoplasm.
  - Inconsistent histone banding may be due to issues with the histone antibody.
- The fluorescence assay showed a small signal for RPS6-mTurq, however immunoblotting showed dark banding. Degradation of RPS6 in the cytosol may cause improper folding of mTurq.
- Low RPS6-NES fluorescence was observed, suggesting that free RPS6 is partially being degraded in the cytosol.

## Future Directions

- Conduct titration experiments in HEK293 cells to determine the amount of transfected construct required for optimal expression of the mTurq and RPS6-mTurq fusion proteins to prevent overload of the nuclear import-export pathways.
- Optimize nuclear-cytoplasmic extraction using adequate protease inhibitors, and cytoplasmic and nuclear markers.
- Conduct HEK293 transfection experiments using proteasome inhibitor (MG132) to investigate the role of the ubiquitin-proteasome system on cytoplasmic RPS6 stability and degradation.
- Immunopurify RPS6-mTurq-NES to analyze post-translational modifications and their role in RPS6 stability and degradation.
- Validate protein expression from NES-containing constructs by fluorescence microscopy in fixed HEK293 cells.
- Conduct plate reader fluorescence measurements in transfected HEK293 cells normalizing against a construct containing Red Fluorescent Protein.
- Validate observations using an enucleated rabbit reticulocyte cell-free expression system.
- Visualize RPS6 fusion proteins using AlphaFold.

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