FIRE: THE FIRST-YEAR INNOVATION & RESEARCH EXPERIENCE

CELLEX

Impact of O-GlcNAc Posttranslational Modification on Human Ribosomal Protein S3A During Stress

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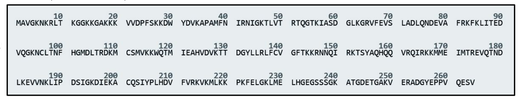
Introduction

- Human ribosomes are specialized units of translation made up of the small 40S and large 60S subunits[1].
- Human small ribosomal protein 3A (RPS3A/eS1) is part of the 40S ribosomal subunit and has unique specialized functions, including cell signaling and processing of small nuclear RNA[2,3].
- RPS3A undergoes many post-translational modifications (PTMs), including ubiquitination, acetylation, phosphorylation, and glycosylation (**Figure 1**)[4,5].
- Under cellular stress conditions, RPS3A becomes glycosylated with O-linked β-N-acetylglucosamine (O-GlcNAc) at Ser59 and Ser154[5].
- High RPS3A expression has been connected to hepatocellular carcinoma (HCC) and low tumor cell infiltration, and is being used to create predictive prognostic models[6].

Figure 1: PTMs on full-length RPS3A (~30 kDa).

Ubiquitination: multiple residues (>25 sites)
Acetylation: residues 34, 56, 249
Phosphorylation: residues 236, 237, 256, 263
O-GlcNAcylation: residues 59, 154



Research Goal:

- This study aims to determine whether the native and over-expressed RPS3A O-GlcNAcylation under MG132 treatment is associated with this protein's expression, stability, and function.

Methods

- Plated human embryonic kidney (HEK293) cells before adding a DMSO control or inducing proteotoxic stress with 10mM MG132 proteasome inhibitor.
  - Round 1: cells stressed with 2, 4, or 6μL of MG132, or 6μL DMSO as a control – incubated for 3 hours.
  - Round 2: cells stressed with 2μL of MG132, or 2μL DMSO as a control – incubated for 6 or 24 hours.
- Harvested and lysed cells after incubation with DMSO or MG132.
- Ran a Bicinchoninic assay (BCA) with lysates on a 96-well plate to measure protein levels.
- Loaded lysates with sample buffer on SDS-PAGE gel.
- Conducted Western blots with FLAG, RPS3A, Ubiquitin, O-GlcNAc, and GAPDH antibodies.
- Ran an immunoprecipitation (IP) assay to purify RPS3A.
- Quantified protein expression with densitometry analysis on ImageJ.
- Site-directed mutagenesis was conducted to create Ser59Ala (M1), Ser154Ala (M2), and Ser59/154Ala (DM) variants. Constructs were expressed in a Rabbit Reticulocyte Lysate (RRL) and subjected to Western blot analysis.

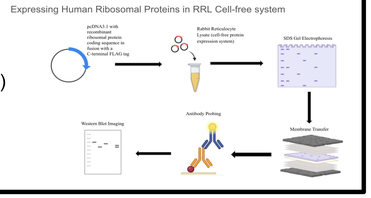


Figure 2: Protocol for expressing human ribosomal proteins in a RRL cell-free system.

Results

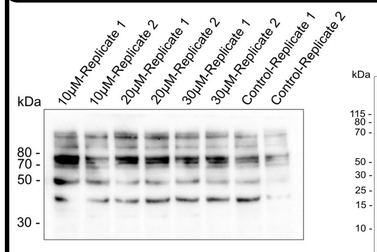


Figure 3: Round 1 O-GlcNAC

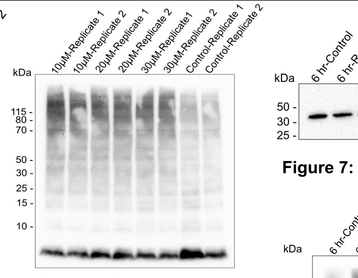


Figure 4: Round 1 Ubiquitin

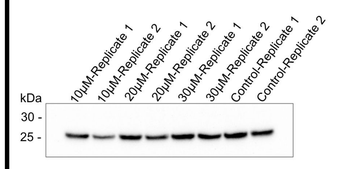


Figure 5: Round 1 RPS3A

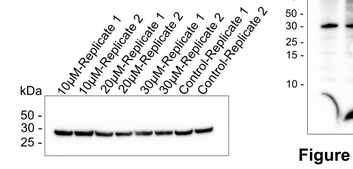


Figure 6: Round 1 GAPDH

Figures 3, 4, 5, 6: Western blots for MG132 stress experiment round 1 with a 3 hour incubation period showing O-GlcNAc, Ubiquitin, RPS3A, and GAPDH expression levels in lysates from HEK293 cells. Two replicates were loaded for each control and sample.

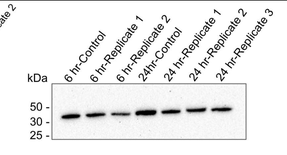


Figure 7: Round 2 RPS3A

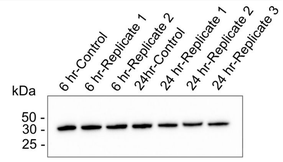


Figure 8: Round 2 GAPDH

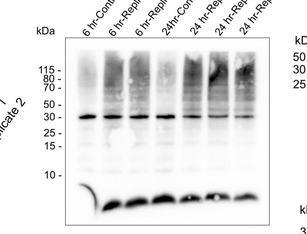


Figure 9: Round 2 Ubiquitin

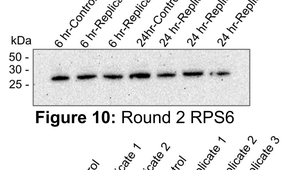


Figure 10: Round 2 RPS6

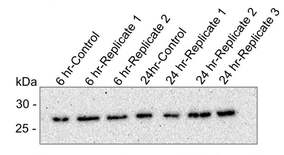
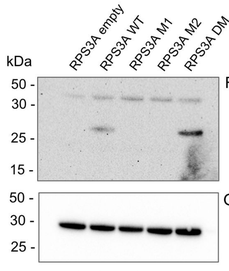


Figure 11: Round 2 RPS3A IP



Figures 7, 8, 9, 10, 11, 12, 13: Western blots for MG132 stress experiment round 2 with a 6 hour or 24 hour incubation time showing RPS3A, GAPDH, Ubiquitin, and RPS6 expression levels in lysates, and RPS3A, Ubiquitin, and RPS6 levels in RPS3A IPs from HEK293 cells. One control (DMSO) was added for the 6 hour and 24 hour rounds. Two replicates of 2μM MG132 were loaded for the 6 hour round and 3 replicates were loaded for the 24 hour round.

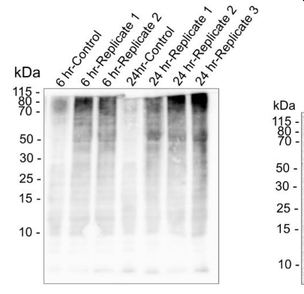


Figure 12: Ubiquitin RPS3A IP

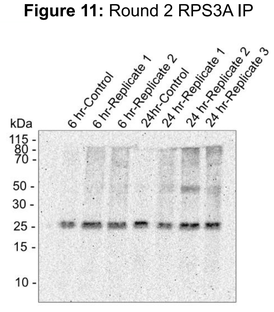


Figure 13: RPS6 in RPS3A IP

Figure 14: Coupled transcription/translation of pcDNA3.1+/C-FLAG RPS3A wild type (WT), M1, M2, and DM in RRL. Reactions were separated by denaturing gel electrophoresis. The gel was probed with anti-FLAG and anti-GAPDH antibodies for Western blot.

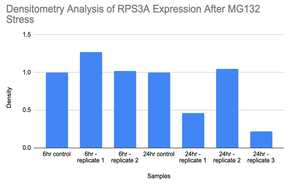


Figure 15: Native RPS3A normalized densitometry analysis after MG132 stress.

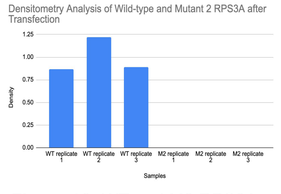


Figure 16: WT and M2 RPS3A normalized densitometry analysis.

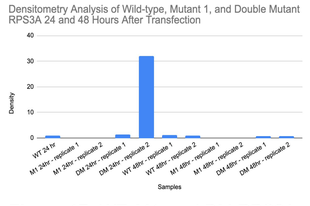


Figure 17: WT, M1, and DM RPS3A normalized densitometry analysis.

Discussion

- After transfection, protein expression in RPS3A M1 and M2 dropped compared to the WT, while RPS3A DM increased.
- In MG132 stress experiments, O-GlcNAc levels increase slightly, and RPS3A show variations in expression levels that are still inconclusive.
- Results suggest that RPS3A levels may decrease upon MG132 stress in a time-dependent manner, but more samples are needed for proper quantification.
- Ubiquitin signals increased across the replicates for round 1 and round 2 in a dose and time dependent manner, indicating that MG132-induced stress increases accumulation of ubiquitinated proteins as expected.
- The RRL system found the WT was expressed at a basal level, the DM level was higher, and M1 and M2 did not show expression. These results are consistent with the HEK293 cells transfected with the same mutant constructs, indicating that the effects on expression are not transfection dependent.
- The IP shows RPS3A was detected, along with ubiquitinated proteins associated with it, as well as RPS6, suggesting that other proteins from the small subunit may have been pulled down in the purification process.

Future Directions

- Inducing different stressors on RPS3A, such as DNA damage, UV radiation or nutrient deprivation, to determine the impact on protein levels, PTMs, and functionality.
- Conduct HEK293 transfections with and without the induced stress to determine if different concentrations of, or combining, M1 and M2 can force protein expression.
- Measure mRNA levels of M1 and M2 to determine if impaired expression is at the level of protein stability.
- Test the effect of other substitutions on RPS3A expression to determine the role of O-GlcNAc.

References

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