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Analysis of the Role of Aminoacyl tRNA Synthetase Genes in Global Protein Synthesis and mRNA Specific Regulation of Translation in Cancer Cells

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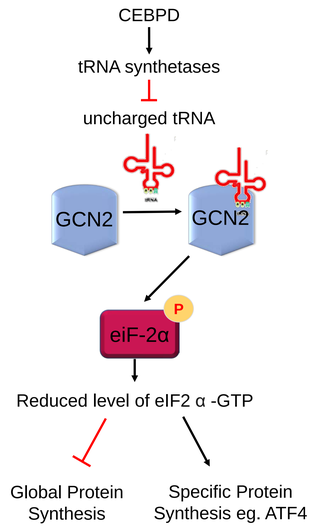
Introduction/Background

Coordinated control of transcription and translation of gene expression impels cellular fate decision under different microenvironmental stresses. Cancer cells often usurp these regulatory machineries to adapt to stress caused by the microenvironment or therapeutic interventions. The transcription factor *CEBPD* is induced by various stressors and modulates cellular adaptation and survival. RNA-seq analysis of a *CEBPD*-silenced human melanoma cell line, MB-435s, showed decreased expression of 12 aminoacyl-tRNA synthetases (aaRS), which are essential, rate limiting enzymes in the process of protein synthesis by catalyzing the addition of amino acids to their corresponding tRNA. Deletion of *CEBPD* by CRISPR/Cas9 (*CEBPD*-KO) compromised aaRS gene expression as well as global protein synthesis. However, despite this decrease in global protein production, the synthesis of certain proteins, such as ATF4, which promotes survival and/or death under stress conditions, is increased. Induction of ATF translation is expected due to activation of the GCN2 kinase by uncharged tRNAs (see model). In the current project, we sought to investigate the effect of silencing two specific aaRS genes, glutamyl-prolyl-tRNA synthetase (EPRS) and valyl-tRNA synthetase (VARS) on global protein translation and ATF4 expression, in order to address their role in *CEBPD* signaling.



Results

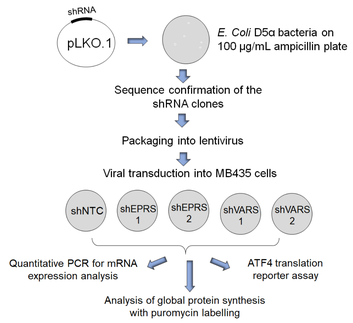


Figure 1: Schematic of experimental plan and design

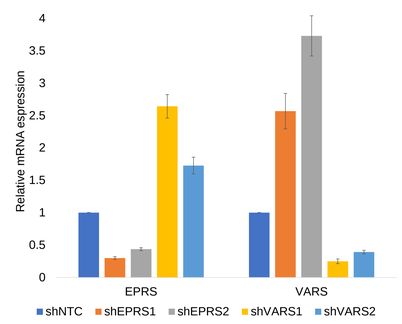


Figure 2: EPRS and VARS mRNA expression in shRNA transduced MB-435s cells (n=4)

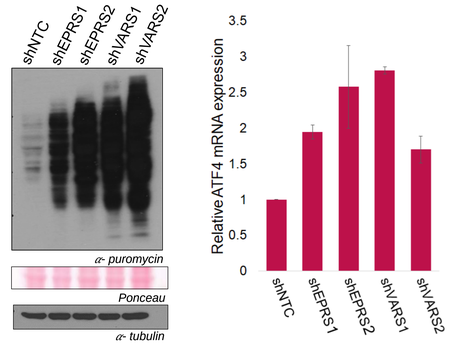


Figure 3: Protein synthesis rate measurement by puromycin pulse labelling (n=3). ATF4 mRNA expression level (n=2)

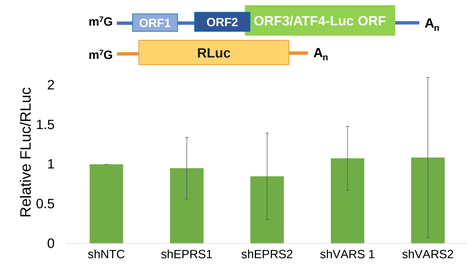


Figure 4: ATF4-5'UTR Luciferase reporter assay in *EPRS* and *VARS* silenced MB-435s cells (n=1)

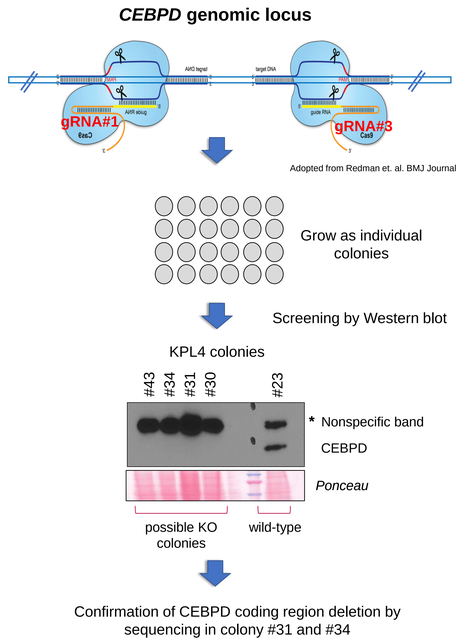


Figure 5: CRISPR/Cas9-mediated knockout of *CEBPD* in KPL4 cells

Materials and Methods

Generation of shRNA expressing constructs and viral transduction

In order to silence aaRS gene expression, short hairpin RNA (shRNA) sequences complementary to *EPRS* and *VARS* were cloned into the pLKO.1 plasmid vector. A scrambled non-targeting control (NTC) sequence was cloned into the pLKO.1 vector and used as a control shRNA. Two independent shRNA oligos were used for each of the aaRS genes. The shRNA constructs were packaged into lentivirus particles using HEK293T cells. MB-435s cells were transduced with the shRNA expressing lentiviral particles followed by selection of transduced cells with 2 µg/mL puromycin for 48 hours.

RNA isolation and quantitative PCR

The total RNA from the viral transduced MB-435s cells were isolated using GeneJET RNA purification kit (Thermo Scientific) according to the manufacturer's instructions. RNA was converted to complementary DNA (cDNA) using random hexamer primers and SuperScript III reverse transcriptase (Thermo Scientific). The level of *EPRS* and *VARS* messenger RNA (mRNA) expression was measured by real time quantitative PCR using SYBR green probe.

Puromycin pulse labelling and Western blotting

In order to measure the total protein synthesis rate, the cells were incubated for 5 minutes with 10 µg/mL puromycin containing growth media and immediately lysed with 2X Laemmli buffer. Equal amounts of cell lysates were resolved by 4-20% gradient SDS-PAGE followed by transfer to nitrocellulose membrane, probed with anti-puromycin primary antibody and HRP-conjugated secondary antibody, and visualized by Enhanced Chemiluminescence Western blotting substrate (Pierce). The membranes were stripped and subsequently probed with anti-α tubulin antibody.

Luciferase reporter assay

EPRS and *VARS* shRNA expressing MB-435s cells were co-transfected with an ATF4 5'UTR containing Firefly luciferase (Fluc) reporter plasmid and a *Renilla* luciferase (Rluc) expressing plasmid. Cells were lysed 48 hours post transfection and luciferase activity was measured using a Dual-Luciferase reporter assay system (Promega). Fluc values were normalized to Rluc values.

CRISPR/Cas9-mediated knockout of *CEBPD* in KPL4 breast cancer cells

To further elucidate this protein synthesis pathway regulated by *CEBPD* in other cell line, the CRISPR/Cas9 genome editing system was used to knock out *CEBPD* in KPL4 cells. To do this, two guide RNAs (gRNA) were designed which target the start codon and stop codon of the *CEBPD* coding region, respectively. After transfection with both gRNAs and the Cas9 protein, the cells were selected for 24h with puromycin and grown as individual colonies. Loss of *CEBPD* expression was used to screen for knock out colonies and confirmed by sequencing of the *CEBPD* genomic locus.

Result Summary

- Expression of the *EPRS* and *VARS* gene were significantly reduced by two independent shRNA in MB-435s cells. Silencing of *EPRS* caused upregulation of *VARS* and *vice versa.* This data suggests a possible feedback loop in the regulation of different aaRS genes' expression.
- Interestingly, our preliminary data suggest increased puromycin labelling after *EPRS* and *VARS* silencing in MB-435s cells. Furthermore, ATF4 mRNA expression was also upregulated in the shEPRS and shVARS cells. However, preliminary data showed unaltered ATF4-5'UTR reporter activity in the shEPRS and shVARS cells.
- CRISPR/Cas9 system with two different gRNAs against *CEBPD* successfully deleted the *CEBPD* coding region in two independent clones of the KPL4 cell line.

Future Directions

- Measurement of the total protein synthesis rate in *EPRS* and *VARS* silenced cell by metabolic pulse labelling to validate our observation with puromycin labelling.
- Repetition of the ATF4-Luc reporter assay in the aaRS silenced cells.
- Analysis of eIF2α phosphorylation and ATF4 protein expression in *EPRS* and *VARS* silenced cells.
- Analyze the effect of combinatorial silencing of *EPRS* and *VARS* on the global protein synthesis rate in MB-435s cells.
- Investigation of the effect of *CEBPD* knockout on protein synthesis pathway in KPL4 cells.

Acknowledgements

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