Validating CRISPR'd PMM deficient neurons for modeling PMM2-CDG

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Background

Glycosylation
- The enzyme-mediated process by which carbohydrates (glycans) are added to nascent proteins.

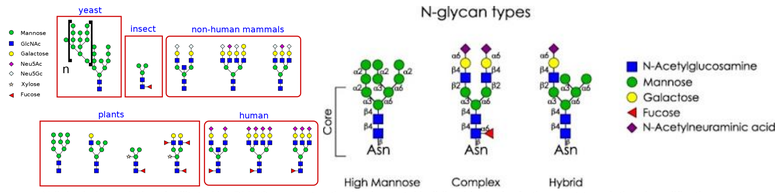
N-linked Glycosylation
- The subtype of glycosylation in which glycans are added to asparagine (N) residues of proteins.

Phosphomannomutase-2 (PMM2)
- An enzyme that transforms the sugar mannose into a usable form for developing a core structure for further glycosylation.

PMM2-CDG
- A congenital disorder of glycosylation in which PMM2 activity is deficient, leading to reduced glycosylation and various phenotypic defects.

CRISPR-Cas9
- A gene editing technology that uses a guide RNA to excise DNA fragments at specified locations, after which natural repair mechanisms can be used to insert an edited gene.

N2a Cells
- An immortalized line of mouse neuroblasts, rapidly dividing neuron precursor cells.



N-linked glycosylation, facilitated by PMM2, generates the fundamental building blocks for further post-translational modification.

Objective

To utilize CRISPR gene editing to render PMM deficient neurons that can be used for the characterization of PMM2-CDG in a cellular model. This requires both genetic modification and proteomic validation of N2a cells.

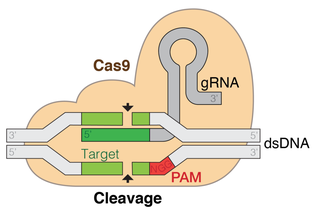
Genetic Modification of Neurons

Using CRISPR to modify neurons for reduced PMM activity

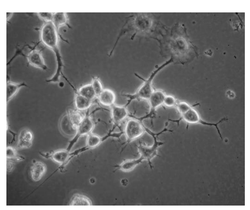
CRISPR gene editing was used to inactivate both PMM1 and PMM2, the two forms of phosphomannomutase, in mouse neuroblasts. Specialized guide RNAs were developed that could target the 2nd exon in PMM1 and PMM2. By excising these genes, their PMM activity would be removed.

Assessing the proteomic activity of CRISPR'd cells

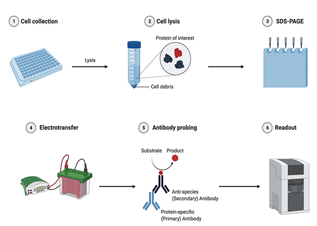
To assess the efficacy of the N2a gene editing, the cells were screened using Western blot and densitometry for quantifying general protein expression, a PMM enzymatic assay for comparing PMM activity, and N-linked glycan analysis for broad changes in glycosylation.



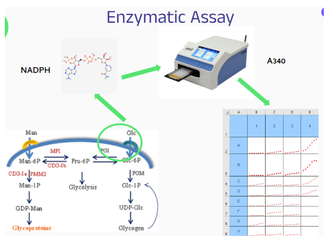
CRISPR uses a guide RNA to localize the Cas-9 excision machinery to a gene of interest for precise editing.



N2a cells are an immortalized mouse neuroblast line that can be rapidly divided and used in nervous system study.



Western blot allows for protein quantification through the electric separation of different protein bands according to size. Target bands can then be visualized and compared using an antibody probe that can be imaged.



The metabolism of mannose can be biased to occur in a reverse reaction, producing NADPH. Using photo spectroscopy on NADPH in different protein samples can act as a reference for PMM enzymatic activity.

Methods for Screening Proteomic Activity

Western Blot Comparison

Western blot allows for the comparison of general protein expression amongst different cell samples. This is visualized through gel electrophoresis bands, and quantified through digital densitometry. This can be used to determine if CRISPR'd cells have lower levels of PMM expression.

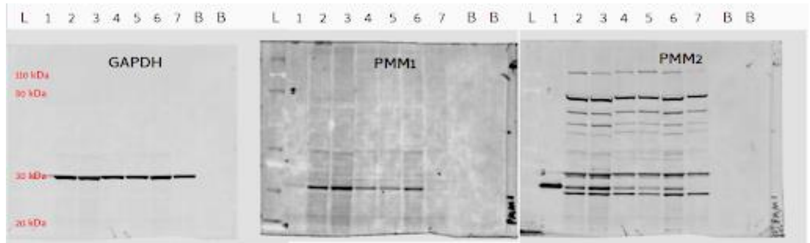
Enzymatic Assay

The PMM enzymatic assay reverses the mannose metabolism of cells in order to assess relative levels of PMM activity using photo spectrometry methods. This can be used to determine if CRISPR'd cells that have lower levels of PMM expression also exhibit properties of reduced PMM enzymatic activity.

N-glycan Analysis

N-glycan analysis involves the use of mass spectrometry to assess changes in widespread glycosylation, by showcasing relative distribution of both simple and complex glycan structures. This can be used to determine if a lack of PMM activity can translate to broad-spectrum changes in the glycoproteome.

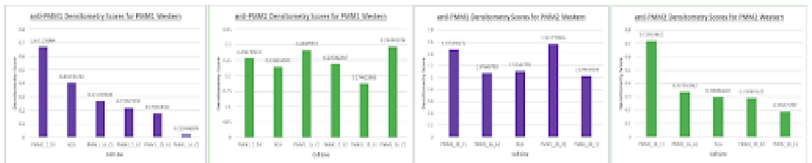
Results of Proteomic Screening



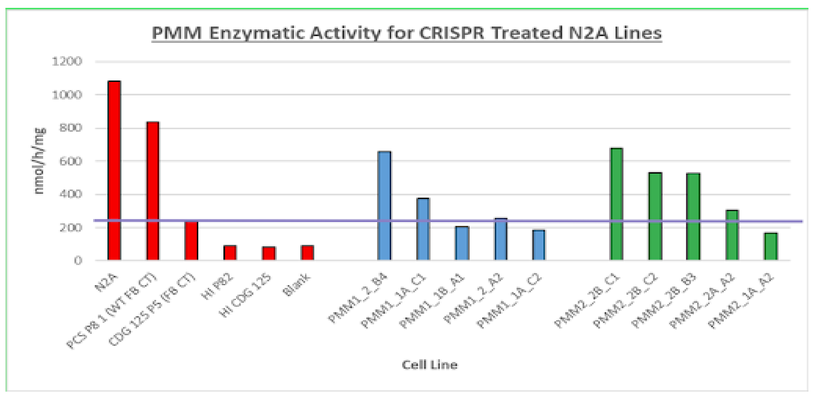
Protein bands for a loading control (GAPDH), PMM1, and PMM2 were separated using gel electrophoresis. Band intensity is proportional to protein expression.

Cell Line Legend

L) Ladder **1)** HPMM2 **2)** N2a **3)** PMM1_2_B4 **4)** PMM1_2_A2 **5)** PMM1_1B_A1 **6)** PMM1_1A_C1 **7)** PMM1_1A_C2 **B)** - Blank

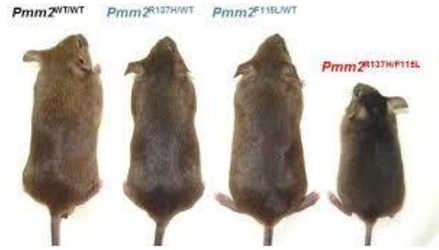


Densitometry is the use of imaging software to compare the intensity of various bands, allowing for direct quantification and comparison of different sample intensities.

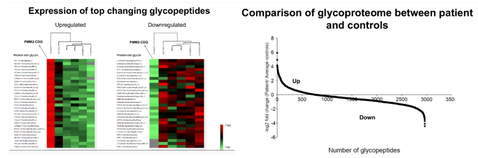


The PMM enzymatic activity of the modified neuroblast lines were compared to a patient fibroblast line as a control, allowing for the identification of cell lines with reduced PMM activity.

Existing Models for PMM2-CDG



Current models for PMM2-CDG include a mouse model, which showcases the phenotypes of the two mutations in phosphomannomutase-2 that render the disorder, R137H and F115L. However, the model has failed to generate mice with deadly R137H/R137H genotypes, as these are embryonic lethal.



Data from human patient post-mortem samples have also been used to showcase changes in the glycoproteome for PMM2-CDG patients, however statistical analysis is impossible due to reduced sample size.

A cellular model, rendered possible by the proliferation of these cell lines, is vital to accurately characterizing PMM2-CDG.

Developing Treatments for PMM2-CDG

PMM2-CDG patients often possess striking facial structure deviations.

Common symptoms of PMM2-CDG include
- Hypotonia (reduced muscle mass)
- Strabismus (eye misalignment)
- Developmental Delay (failure to reach movement milestones)
- Failure to Thrive (weight and height decrease)
- Intellectual Disability

Severe symptoms of PMM2 CDG include
- Hydrops Fetalis (fluid buildup around infant)
- Liver Dysfunction
- Blood clotting disorders

More than 800 patients have been identified worldwide as having PMM2-CDG. The disease is also reported to have a **20% infant mortality rate** due to organ failure resulting from complications in glycosylation.

The development of a cellular model for the disease will help to provide a platform for further research into new therapeutics and treatments for PMM2-CDG.