Poster #68
Thursday
3-5 pm

An Innovative Gene Therapy Approach to Produce Novel Human GALC Variant with Enhanced Protein Stability and Enzyme Activity with High Levels of Mannose 6-Phosphate for Krabbe Disease

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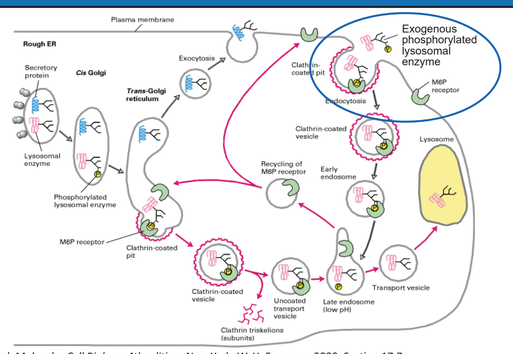
Abstract

Krabbe disease is a severe neuromuscular disease caused by deficient galactosylceramidase (GALC) activity that impairs glyco/sphingolipid metabolism resulting in accumulations of various lipids and cytotoxic psychosine. Hematopoietic stem cell transplantation is shown to be only modestly effective when initiated prior to symptoms. It is therefore critical to develop more effective therapeutic treatments. Development of effective GALC treatments for Krabbe has been hindered because GALC typically contains low levels of mannose 6-phosphate (M6P) which limits its cellular uptake and delivery to lysosomes. GlcNAc-1-phosphotransferase (PTase) catalyzes the addition of M6P onto newly synthesized lysosomal enzymes in Golgi. However, endogenous PTase has low activity and inherently inefficient for phosphorylating GALC resulting in compromised receptor binding and reduced cellular uptake. We have created a variant PTase (designated as S1S3 PTase) that has 20-fold higher specific activity for adding M6P than the wildtype enzyme. We co-expressed S1S3 PTase with mouse GALC (mGALC) to produce a highly phosphorylated version (designated as mGALC^{M6P}). mGALC^{M6P} was shown to have significantly better binding to CI-MPR and efficiently internalized into fibroblasts derived from Twitcher mice in a M6P-dependent manner. A single intracerebroventricular injection of mGALC^{M6P} in Twitcher mice at birth showed broad enzyme distribution and cellular uptake in brain resulting in increased life span and motor function as compared to WT mGALC (low M6P content) under identical experimental conditions. Human GALC (hGALC) has intrinsically low protein stability and enzyme activity which further complicates drug development. We have created a novel GALC variant with 2 strategic amino acid substitutions that improved protein stability and increased GALC enzyme activity by 8- to 10-fold as compared to wildtype hGALC. We believe that more potent gene therapy for Krabbe disease can be achieved by co-expression of stable hGALC with S1S3 PTase to produce more active enzyme with high M6P content for better cellular uptake

Introduction: M032 Gene Therapy Program for Krabbe Disease

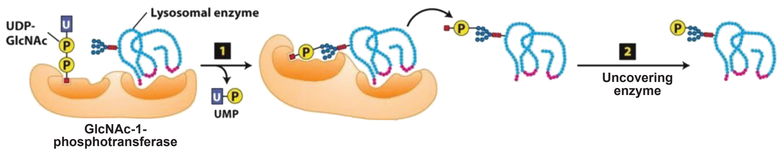
- Krabbe disease is a severe neuromuscular lysosomal storage disorder caused by mutations in the galactosylceramidase (GALC) gene.
- This leads to galactosylceramidase (GALC) enzyme deficiency and results in continuous build-up of various lipids and glycolipids including cytotoxic psychosine
- GALC deficiency causes loss of myelin sheath (protective layer around nerves) resulting in severe nerve damage
- Krabbe has many debilitating symptoms including seizures, feeding difficulties, vomiting, deafness, blindness, slurred speech and loss of motor function
- There is currently no cure or effective treatments for Krabbe
- Human GALC enzyme is difficult to express because it is highly unstable and contains only modest amounts of mannose 6-phosphate (M6P)
- Development of an effective human GALC treatment must overcome these challenges- more stable and active enzyme with higher amounts of M6P for better lysosomal targeting to increase GALC levels and address clinical symptoms

Natural M6P Receptor Pathway Enables Phosphorylated Exogenous Lysosomal Enzymes Cellular Uptake for Treatment of Lysosomal Storage Diseases



Lodish H, Berk A, Zipursky SL, et al. Molecular Cell Biology. 4th edition. New York: W. H. Freeman; 2000. Section 17.7

Phosphorylation of Lysosomal Enzymes is Mediated by GlcNAc-1-Phosphotransferase That is Inherently Inefficient in Cells



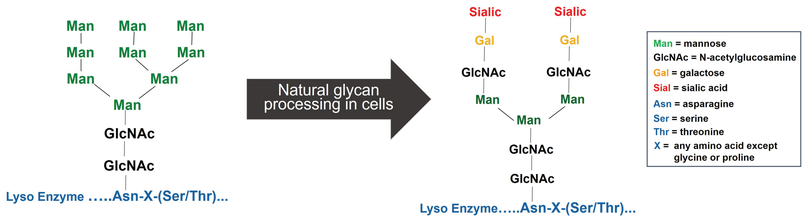
GlcNAc-1-phosphotransferase catalyzes the transfer of GlcNAc-1-phosphate from sugar donor UDP-GlcNAc onto certain terminal mannose residues of the N-linked oligosaccharides in cis-Golgi

NAGPA (N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase), also known as uncovering enzyme, removes covering GlcNAc group to reveal mannose 6-phosphate (M6P)

Section 17.7 Protein Glycosylation in the ER and Golgi Complex, Molecular Cell Biology, 8th edition, 2016

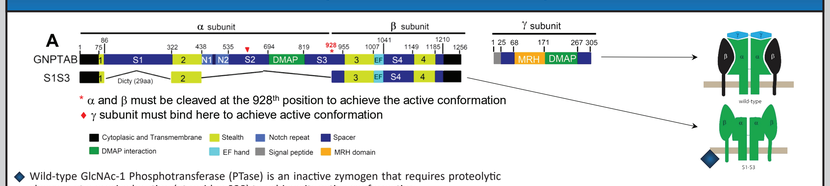
- GlcNAc-1-Phosphotransferase is a large and complex protein that is responsible for adding phosphate groups to mannose residues on N-linked glycans of newly synthesized lysosomal enzymes in Golgi to create mannose 6-phosphate (M6P)
- Phosphorylation of lysosomal enzymes is an inherently inefficient process in nature, it is further compromised when overexpressing lysosomal enzyme
- Increasing GlcNAc-1-Phosphotransferase activity within cells for producing therapeutic lysosomal enzymes with higher M6P content has not been achievable thus far with traditional ERT and gene therapy approaches

The Problem: Most N-glycans on Lysosomal Enzymes are Not Phosphorylated and Processed to Complex-Type Structures During Over-Expression



- Identical Man9 N-glycan structure is added to glycoproteins in endoplasmic reticulum during synthesis; N-glycan processing primarily occurs in Golgi
- Phosphorylation of recombinant lysosomal enzymes is highly inefficient in cells leading to mostly complex-type structures
- Complex-type N-glycans are typical of plasma proteins and do not enable cellular uptake or delivery of exogenous lysosomal enzymes to lysosomes
- No current technology can increase phosphorylation within cells

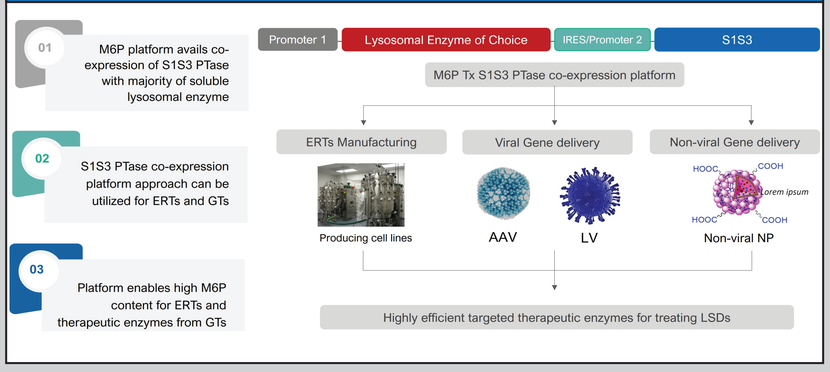
S1S3 Variant Has Key Attributes That Enable Its Use for Development of Best-In-Class Recombinant Enzymes and Gene Therapies



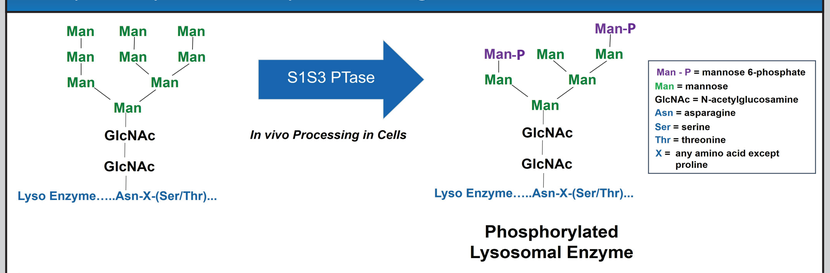
- Wild-type GlcNAc-1 Phosphotransferase (PTase) is an inactive zymogen that requires proteolytic cleavage at a precise location (at residue 928) to achieve its active conformation
- No known way to increase proteolytic cleavage and enzyme activation within cells for increasing enzyme activity for improving phosphorylation
- S1S3 PTase is a truncated, pre-activated variant with significant advantages:
  - Has proper conformation without need for γ-subunit
  - Has 20-fold higher specific activity
  - Enables efficient phosphorylation of soluble lysosomal enzymes
  - Small gene size enables its incorporation into expression constructs for S1S3 PTase co-expression with therapeutic enzymes within cells for ERT and gene therapy applications

Lin Liu et al. Engineering of GlcNAc-1-Phosphotransferase for Production of Highly Phosphorylated Lysosomal Enzymes for Enzyme Replacement Therapy, Mol Ther Methods Clin Dev. 2017 Jun 16; 5: 59–65.

M6P Therapeutics' Unique S1S3 PTase Co-Expression Platform for Producing Therapeutic Lysosomal Enzymes with High M6P Levels M6P for ERTs and from GTs

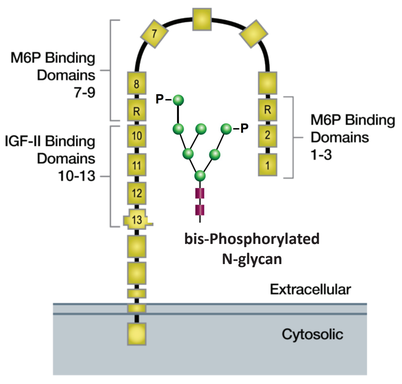


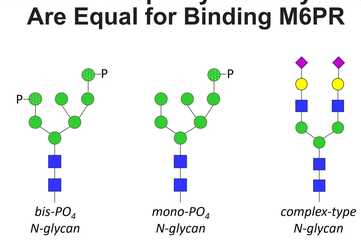
The Solution: S1S3 PTase Co-Expression Platform Ensures Production of Therapeutic Lysosomal Enzymes with High Levels of M6P



- Co-expression of S1S3 PTase with therapeutic protein produces lysosomal enzyme with high levels of M6P to enable much improved drug targeting
- Cation-independent M6P receptor (CI-MPR) is present on nearly all cells for cellular uptake of exogenous M6P-bearing lysosomal enzymes

Structure of IGF-II/Cation-Independent M6P Receptor (IGF-II/CI-MPR) and Measured Binding Affinities of Carbohydrate Ligands





Ligand	Binding affinity (Apparent K_D; nM)*
bis-phosphorylated N-glycan	2
mono-phosphorylated N-glycan	>6000
complex type N-glycan	No binding

Adapted from Tong et al., 1989

M6PT's S1S3 Co-Expression Platform has Significant Advantages over Conventional ERT and GTs Approaches

For ERTs

ERTs are produced with high M6P content to enable better targeting to lysosomes

- Potential for increased efficacy across more tissue types
- Potential for best-in-class treatments
- Enable alternative dosing strategies and schedules
- Potential for new treatments for LSDs with no current treatment

For Gene Therapies

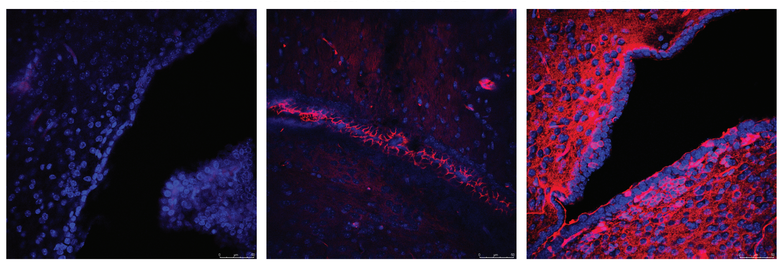
Only gene therapy approach that ensures production of lysosomal enzymes with high M6P content

- Other GT approaches may solve in vivo enzyme expression problem but cannot produce lysosomal enzymes with sufficient M6P for cellular uptake (i.e., cross-correction) of non-transduced cells
- M6PT has the only technology able to achieve adequate in vivo phosphorylation thereby ensuring cross-correction of non-transduced cells
- More potent gene product enables use of lower AAV dosages → reduces safety and tolerability risks

Fulfilling the original promise of ERTs and maximizing the potential of cross-correction for GTs

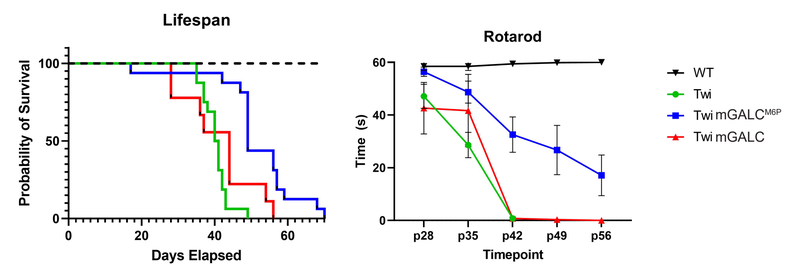
Highly Phosphorylated Mouse GALC (mGALC^{M6P}) Enzyme Exhibits Much Broader Distribution in Brain of Krabbe Mice Post-ICV Injection

GALC Biodistribution 24 hrs Post-dosing by Immunohistochemistry with Anti-mGALC 1^{o} Antibodies



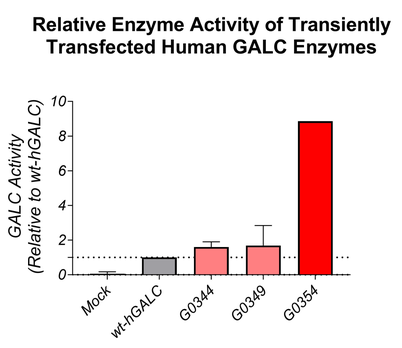
Native mGALC = recombinant mouse GALC expressed without S1S3 PTase
mGALC^{M6P} = recombinant mouse GALC expressed with S1S3 PTase

Single ICV Administration of Highly Phosphorylated mGALC^{M6P} Enzyme Improves Survival and Rotarod Performance in Twitcher Mice



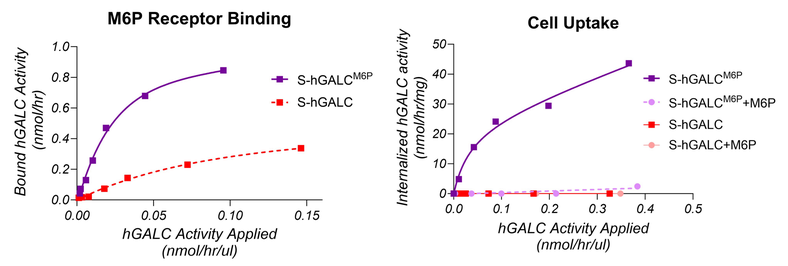
mGALC = recombinant mouse GALC expressed without S1S3 PTase
mGALC^{M6P} = recombinant mouse GALC expressed with S1S3 PTase

Novel Human GALC Variant with Two Strategic Amino Acid Substitutions Has Significantly Increased Protein Stability and Enzyme Activity



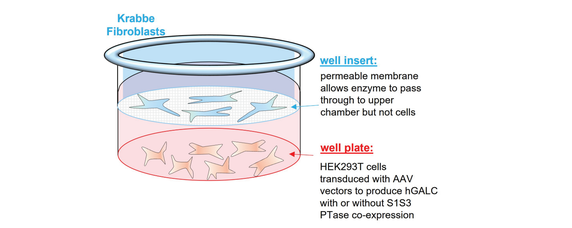
Novel S-hGALC is significantly more stable and has 8-10X higher enzyme activity than wildtype hGALC

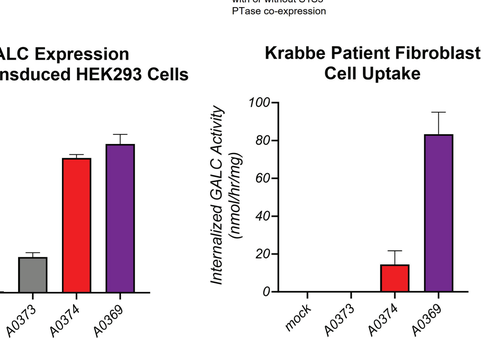
Combination of Novel hGALC Construct with S1S3 PTase Co-Expression Yields Much More Stable Enzyme with Improved M6P Receptor Binding and Cellular Uptake

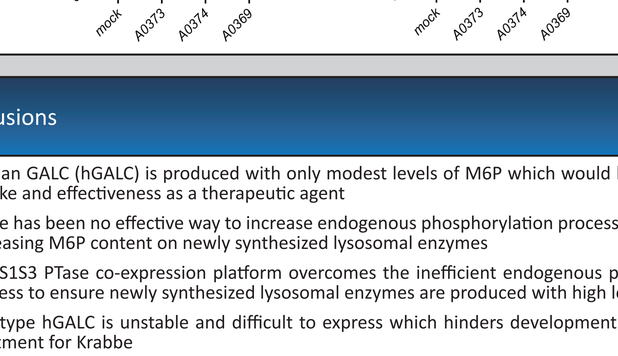


S-hGALC = recombinant stable hGALC expressed without S1S3 PTase
S-hGALC^{M6P} = recombinant stable hGALC expressed with S1S3 PTase

AAV GTx Produces Novel Stable hGALC with High M6P Levels when Co-expressed with S1S3 for Efficient Cross-Correction of Krabbe Patient Fibroblast Cells







Conclusions

- Human GALC (hGALC) is produced with only modest levels of M6P which would limit its cellular uptake and effectiveness as a therapeutic agent
- There has been no effective way to increase endogenous phosphorylation process within cells for increasing M6P content on newly synthesized lysosomal enzymes
- Our S1S3 PTase co-expression platform overcomes the inefficient endogenous phosphorylation process to ensure newly synthesized lysosomal enzymes are produced with high levels of M6P
- Wildtype hGALC is unstable and difficult to express which hinders development of an effective treatment for Krabbe
- We have created a novel hGALC enzyme with 2 strategic amino acid substitutions that yields significantly higher protein stability and enzyme activity
- We have created an innovative gene therapy approach for producing more stable hGALC with high M6P content via S1S3 PTase co-expression
- Novel highly phosphorylated, stable hGALC produced from AAV vector is shown to be efficiently internalized in patient-derived fibroblasts and suggests this could be a more effective gene therapy for Krabbe

Future Studies

- Evaluate GTx for cross-correction and substrate reduction, particularly psychosine, in cellular models of Krabbe
- Determine whether GTx can improve various cellular dysfunction in Krabbe cellular models
- Evaluate GTx in animal models of Krabbe disease for hGALC expression and biodistribution, including the CNS
- Determine whether GTx increases survival in animal models of Krabbe disease
- Determine whether GTx can reduce/prevent psychosine accumulation in Krabbe mouse models