# CLN2 Disease: S1S3 Phosphotransferase Mediated Phosphorylation and N-Glycan Uncovering Significantly Increases Binding Affinity of Tripeptidyl-peptidase 1 (TPP1) to CI-MPR

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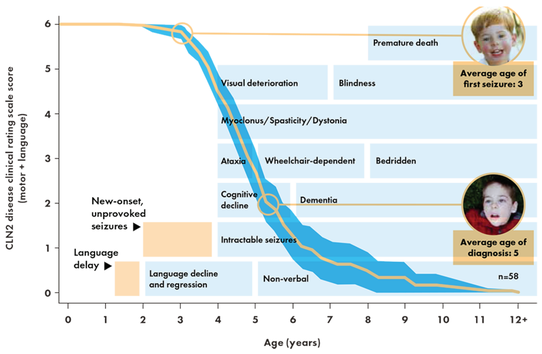
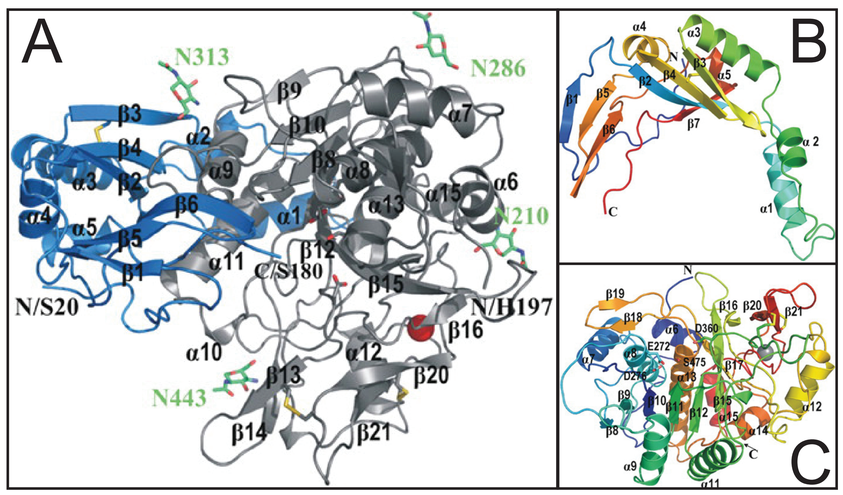
## Late Infantile Neuronal Ceroid Lipofuscinosis Type 2 (CLN2)

- CLN2 is a rare, genetically inherited disorder that affects the nervous system.
- It is caused by a deficiency of the enzyme TPP1, which leads to an accumulation of a fatty substance called lipofuscin in the brain and other tissues.
- Symptoms typically appear between the ages of 2 and 4 years, and may include seizures, progressive vision loss, motor function decline, language disturbances, and dementia.[1]
- The disease is fatal, and there is currently no known cure other than treatment for managing symptoms and improving quality of life.
- Gene therapy (GT) and enzyme replacement therapy (ERT) are being studied as potential treatment options. ERT involves direct or systematic injection of a functional copy of an enzyme to the brain.
- Brineura® (cerliponase alfa) is an ERT prescription medication used to slow loss of ability to walk or crawl in symptomatic pediatric patients 3 years of age and older with CLN2.[2]

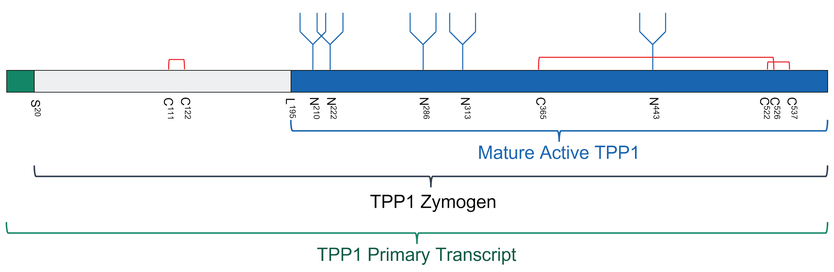



## TPP1 Structure

- Panel A: Prosegment (blue) and catalytic domain (gray),
  - N terminus ($Ser^{20}$), ends of the prosegment ($Ser^{180}$)
  - Start of catalytic domain ($His^{197}$).
  - N-linked glycan residues (green sticks).
  - Disulfide bonds (yellow lines)
  - $Ca^{2+}$-binding site (red sphere).
- Panel B: Prosegment/zymogen
  - 5 α-helices (α1-5)
  - 2 sets of antiparallel β-strands (β1-β5-β6 and β7-β2-β4-β3).
  - There is a disulfide bridge between $Cys^{111}$ (located on β3) and $Cys^{122}$ (located on β4).
- Panel C: Cartoon model of the catalytic domain; rotated by 180° around the horizontal molecule axis in respect to (A).

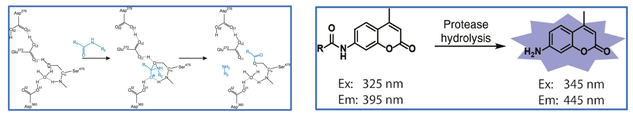



- TPP1 is a 563-residue preproprotein with a cleavable N-terminal 19-residue signal peptide.
- The proenzyme (zymogen) (residues 20-563) is a monomer that undergoes proteolytic cleavage in the lysosome.
- This endoprotease cleavage occurs at residue 195 zymogen to form an active, mature TPP1 (residues 196-563) enzyme.

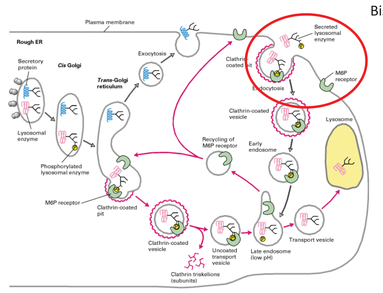



## TPP1 Must Be Proteolytic Processed for Activity

- TPP1 is a serine protease with a Ser, Asp, and Glu catalytic triad adapted for low pH catalysis.
- The autoactivation of TPP1 involves two intramolecular proteolytic cleavage at pH 3.0 of the 66-kDa inactive proenzyme to the 46-kDa mature enzyme.
- The exopeptidase activity of TPP1 catalyzes the cleavage of variety of tripeptide sequences (pH optimum of 4.8).
- Activity Assay was developed using Ala-Ala-Phe-7-$NH_2$-4-Methylcoumarin with a fluorescence Ex (345 nm)/ Em (445 nm)

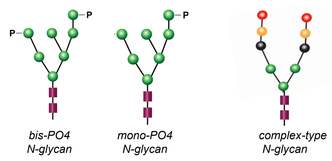



## CI-MPR is the Main Receptor Responsible for Targeting Lysosomal Proteins to Lysosomes




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

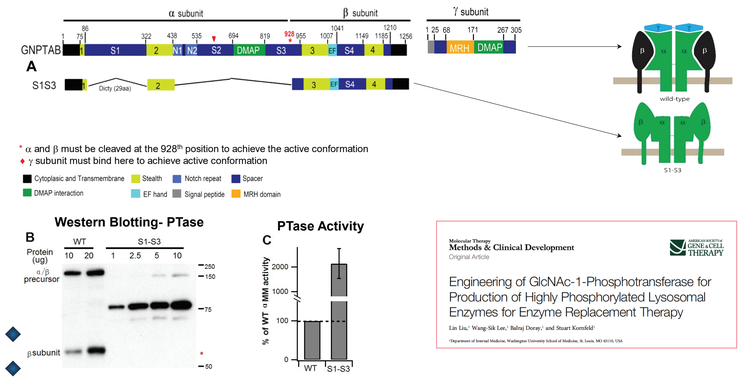
Not all phosphorylated glycans are equal. Bis-phosphorylated glycans have ~3,000x greater affinity for the CI-MPR than Mono-phosphorylated glycans




| N-Glycan | Binding affinity (Apparent $K_D$; nM)* |
|---|---|
| Bis-phosphorylated | 2 |
| Mono-phosphorylated | >6000 |
| Complex | No binding |

Do et al. Ann Transl Med 2019: 7(13):291

## S1S3 Truncated PTase has ~20X Greater Activity than Wild-Type PTase that Enables Better Phosphorylation of Lysosomal Enzymes




Molecular Therapy Methods & Clinical Development
Original Article

Engineering of GlcNAc-1-Phosphotransferase for Production of Highly Phosphorylated Lysosomal Enzymes for Enzyme Replacement Therapy

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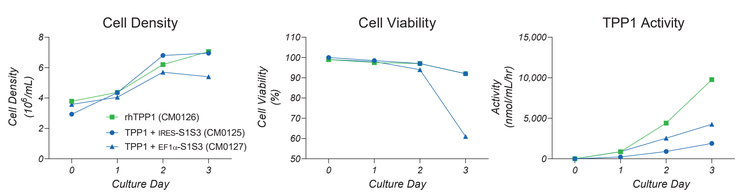
## Project Goals

- *Evaluate the expression and phosphorylation of TPP1*
- *Evaluate the expression and phosphorylation of TPP1 co-expressed with S1S3 PTase*
- *Perform biochemical characterization of both versions of TPP1*

## Expression of TPP1 in the presence/absence of S1S3 phosphotransferase

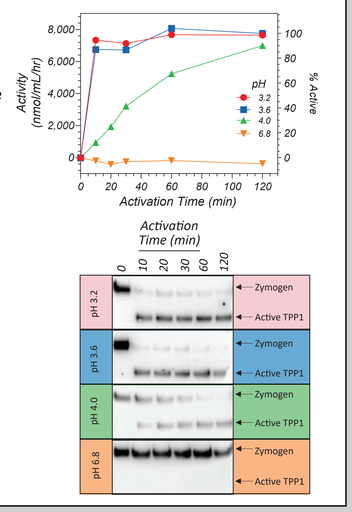
- Gibco Expi293 Transient Expression System, Cell viability and density are determined: Trypan Blue 0.4% and Invitrogen Countess II FL




- The TPP1 co-expressed with the S1S3 phosphotransferase (CM0125) resulted in a lower yield compared to the one without (CM0126).
- The S1S3 expression system was changed from IRES promoter to an EF1-α promoter in the second batch of TPP1 with S1S3 co-expression (CM0127).
- The cell viability of CM0127 has dropped significantly on the 3rd day however, yield of TPP1 enzyme was doubled compared to CM0125.
- The increase in yield is 2-fold in all Day1, Day2 and Day3 and does not correspond to loss of cell viability.

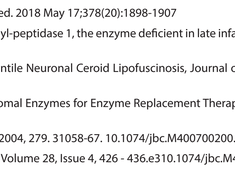
## TPP1 Zymogen Is Efficiently Activated at pH <3.6 after 1 hr at 37°C

- TPP1 is produced in the cell as an inactive zymogen.
- Once the TPP1 zymogen reaches the lysosomal it can be activated either by a lysosomal serine protease or/and extremely acidic lysosomal conditions (<pH 4.5) the zymogen undergoes endo-protease cleavage at $L^{195}$ to activate the enzyme[3-6].
- To study TPP1 activation, the zymogen was incubated in different pHs at 37°C and samples collected over 2 hrs. Once a sample was collected the activation was stopped by the dilution 10-fold with HEPES, pH 6.8.
- Activation was measured by measuring activity or by Western blot to monitor relative mobility.
- TPP1 activity was determined at pH 4.8 for 1 hr at 37°C using the Ala-Ala-Phe-7-$NH_2$-4-Methylcoumarin substrate mentioned earlier.
- The actual hydrolysis of the zymogen was monitoring the increase in relative mobility by Western blot analysis where a TPP1 monoclonal antibody was used to develop the blot.
  - TPP1 zymogen has a relative mobility of ~67 kDa
  - Active TPP1 has relative mobility of ~44 kDa




## Activated TPP1 Has Optimal Activity at pH 4.8

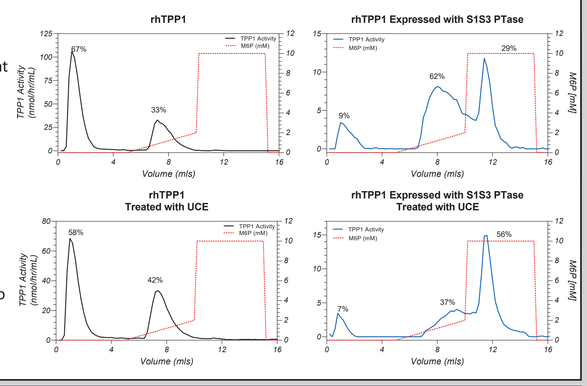
- TPP1 zymogen was activated with pH 3.6 buffer and aliquots made to evaluate the effect of pH on activity
- A pH range form 2.2 to 6.0 was tested, and pH 4.8 had the highest activity with pH 4.5 and pH 5.0 slightly lower. There was no detectable activity at pH 6.8 (data not shown)




## Cross-Correction via CIMPR Uptake Should be More Efficient with Well Phosphorylated TPP1 Co-expressed with S1S3 PTase

- To measure binding to the CIMPR, bovine CI-MPR was purified from FBS and coupled to to Bio-Rad NHS activated Affi-Gel 15 at 1 mg/ml of ligand to resin.
- An 1ml column was packed and a two buffer system was use. Buffer A (NaPhosphate, pH 6.8) was used to equilibated the column. Buffer B (NaOAc, pH 4.8) contained 10 mM M6P and was used to elute the bound TPP1.
- 1 mg of TPP1 was loaded onto the column and the chromatograph developed as indicated by the dotted line on the graphs below. Fractions were collected and TPP1 activity measured.

- TPP1 co-expressed with S1S3 PTase increased the amount of material that can interact with CIMPR by >50%
- 29% of the TPP1 co-expressed with S1S3 PTase required 10 mM M6P to compete it off. Indicating very good binding to CIMPR
- Treatment with uncovering enzyme (UCE) reveals that >25% of total glycan on TPP1 co-expressed with S1S3 PTase still have their terminal GlcNAc attached to M6P compared to 9% of TPP1 expressed alone.




## Conclusions

- Optimal pH for the activation of TPP1 zymogen is 3.4-3.6.
- TPP1 co-expressed with S1S3 PTase increased CIMPR receptor binding by >50%.
- Treatment of TPP1 with UCE suggests that >25% of the glycans on TPP1+S1S3 PTase is covered. Thereby protecting TPP1 from potential serum or interstial phosphatases.

## Acknowledgments

Dr. Lin Liu and Dr. Andrew Hedman for their valuable contribution in preparation of the plasmids carrying S1S3 phosphotransferase gene.

## References

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