A Subcellular Fractionation Method to Enrich Endoplasmic Reticulum from *Saccharomyces cerevisiae* and Human Dermal Fibroblasts with ALG9 Mutation

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Background

Glycosylation is the cellular process of enzymatic addition of sugars to protein, forming complex glycoproteins that are vital for cellular function. In the endoplasmic reticulum (ER), during the N-glycosylation process (see Figure 1), ALG9 also known as α1,2-mannosyltransferase facilitates the addition of mannose residues to a growing oligosaccharide chain, which is subsequently added to nascent polypeptides[1,2]. Faulty glycosylation secondary to mutations in the *ALG9* gene result in a rare subtype of congenital disorder of glycosylation (CDG), termed ALG9-CDG or CDG-1L, characterized by seizures, psychomotor retardation and brain atrophy[5]. Establishing a CDG-1L disease model is critical to identify potential therapeutic options. This research describes the utility of *Saccharomyces cerevisiae* as a CDG-1L disease model to examine human mutations in *ALG9*.

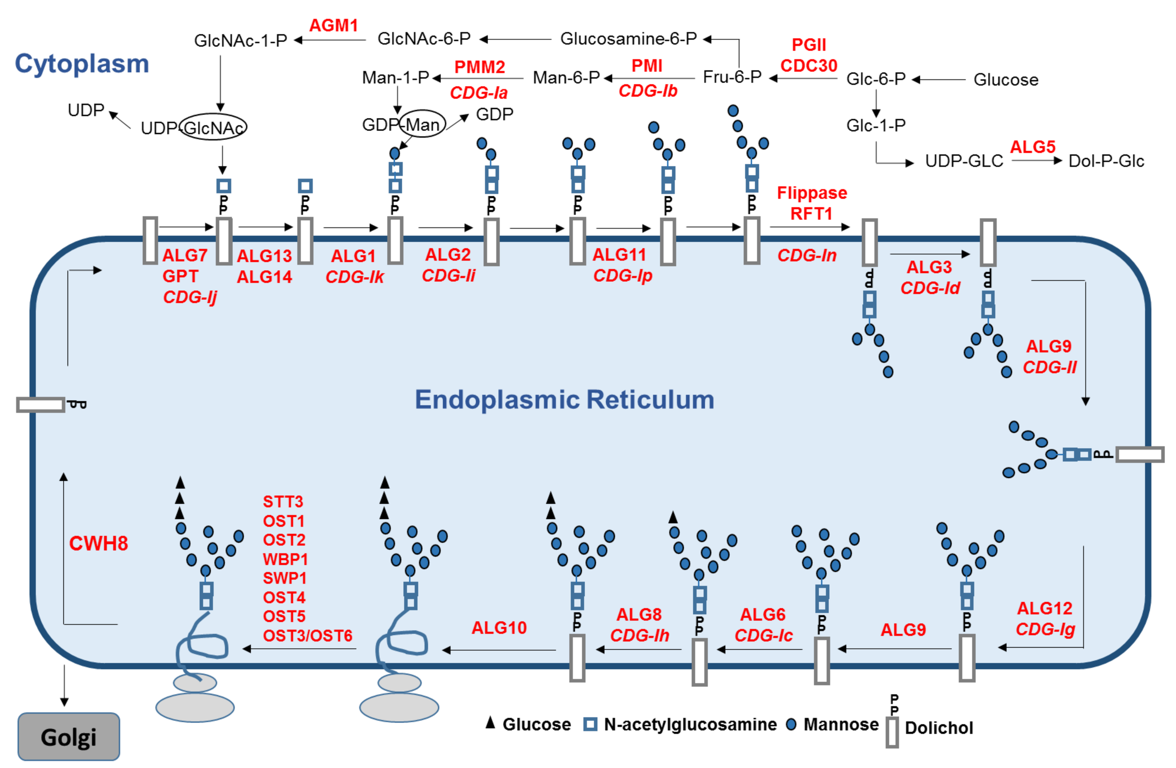


Figure 1. **Pathway of lipid linked oligosaccharide synthesis of nucleotide-activated sugar and associated defects in Congenital Disorders of Glycosylation[2].**

Aim

The goal of this study was to develop a subcellular fractionation protocol to isolate the ER that may be utilized for further functional *in vitro* analysis of human ALG9.

Culturing Cells

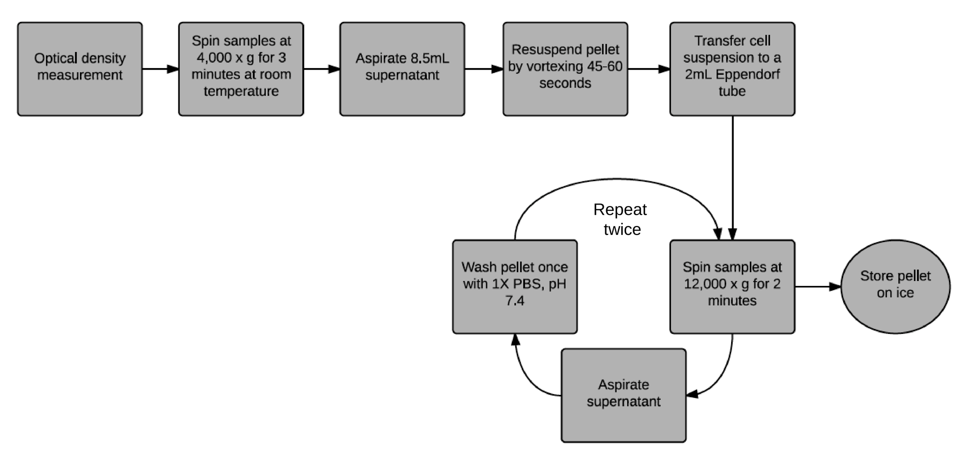
Strain/Cell	Description
SS328 (SS)	*S. cerevisiae*; temperature-sensitive, uracil-auxotroph SS328 strain with fully functional ALG9
YG414 (YG)	*S. cerevisiae*; derived from SS328 (SS328 with Dalg9:: KanMX) with deletion of ALG9 gene
YG414 / HSALG9 Y286C (4)	*S. cerevisiae*; YG414 cells with inserted plasmid bearing *ALG9* p.Y286C mutation
ALG9Δ (ALG)	*S. cerevisiae* cells with full deletion of *ALG9* gene obtained from Dalhousie University
Wild type (WT)	Clinical strain of *S. cerevisiae* obtained from the Saint John Regional Hospital
Dermal fibroblasts (MW)	Cells were obtained from a human CDG-IL patient and are homozygous for *ALG9* p.Y286C mutation

Table 1. **Yeast strains and mammalian cells utilized for ER isolation protocol.**

Five strains of *S. cerevisiae* obtained from DMNB glycerol stocks (Table 1) were cultured for use in this experiment. For *S. cerevisiae* cell culture, 15mL inoculating tubes containing 10mL YPD agar broth media were inoculated with 50µl glycerol stock yeast cells. For each strain, input required is 6 inoculated tubes. Cells were incubated for 72 hours at 30˚ C, with continuous shaking at 250 rpm. Following incubation, cultures were placed on ice. Optical density (OD600) were used to measure cell growth. Fibroblast cells were grown and maintained in 100mm plates using DMEM/F-12 (1:1) with 10% FBS until 80% confluency.

Harvesting Cells

Figure 2. **Steps involved in the harvest of *S. cerevisiae* cells.**



S. cerevisiae cells were separated from the culture medium by centrifugation and re-suspended in 1X PBS, pH 7.4 to wash the cell pellet for subsequent ER isolation (Figure 2).

Human dermal fibroblast cells were harvested in 1X PBS and then cell pellet was subjected to ER isolation (Figure 3). See fibroblast image on the right.

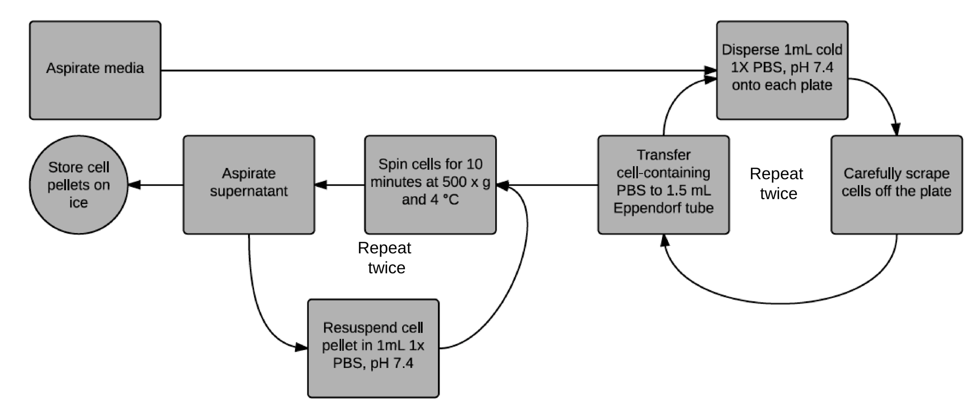


Figure 3. **Steps involved in the harvest of dermal fibroblast cells.**

ER Fractionation Method

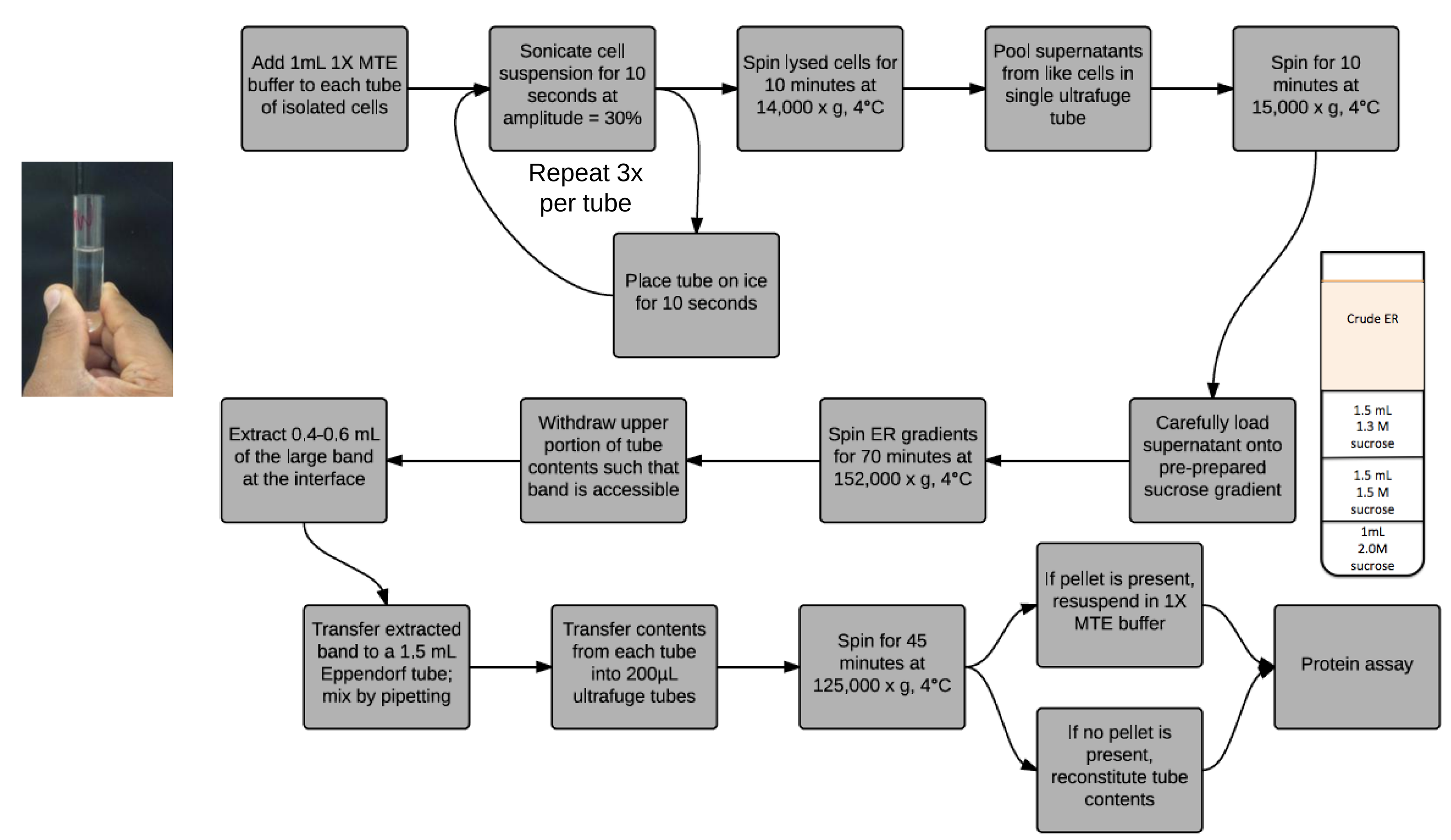


Figure 4. **ER isolation from *S. cerevisiae* and human dermal fibroblast cells using differential centrifugation.**

Using discontinuous sucrose step-gradients ER membranes are captured at the interface of the 1.3 M and 1.5 M sucrose layers[6]. ER fraction is further enriched by an additional centrifugation step ensuring high ER purity. As shown in the figure distinct ER band contained within the interfaces of each sucrose concentration is visible. A cautionary note that tubes containing sucrose gradients must be handled with extreme care to prevent perturbation of this separation. This modified method was adapted from Petros Bozidis et al. Current Protocols in Cell Biology, Volume 1.3.27.1.

Results

Sonication proved the most effective in disrupting the yeast cell wall with minimal organelle damage. To qualitatively measure ER enrichment, immunoblot analysis of ER fractions and total lysates were performed (Figure. 5, 6 & 7). Blots were probed with yeast and human specific antibodies, detecting ER and cytosol resident proteins to qualitatively estimate the level of enrichment and level of purity of each fraction. To confirm that glycosylation is impaired in cells with *ALG9* mutation, blots were probed for carboxypeptidase Y (CPY), an N-glycosylated yeast vacuolar protein synthesized in the ER and transported to the cytosol. Hypo-glycosylation of CPY was observed in total lysates and ER fractions from mutant *ALG9* mutant yeast strains (*ALG9*Δ & YG414) when compared to the functional strain (SS328) as noted by the alpha, beta and gamma forms of CPY (triplet bands) in these samples (Figure 5 & 6). Furthermore Dol-P Mannose Synthase (Dol-P Man), an ER resident protein, was highly enriched in ER fractions in both human dermal fibroblasts and *S. cerevisiae* cells (Figure 6 & 7). Note: Human (3 subunits) and *S. cerevisiae* (1 subunit) Dol-P Man differ in molecular weight[7] (Figure 6).

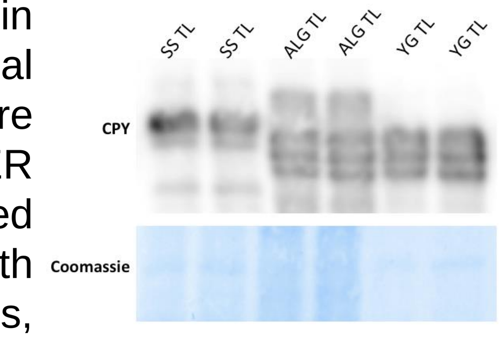


Figure 5. Total lysate yeast strains; SS328, *ALG9*Δ, and YG414. Blot probed with CPY

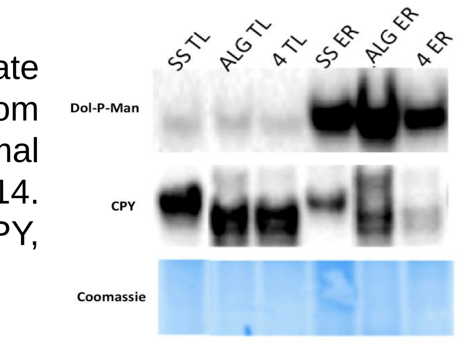


Figure 6. Total lysate and ER fractions from SS328, *ALG9*Δ, dermal fibroblast, and YG414. Blot probed with CPY, and Dol-P-Man.

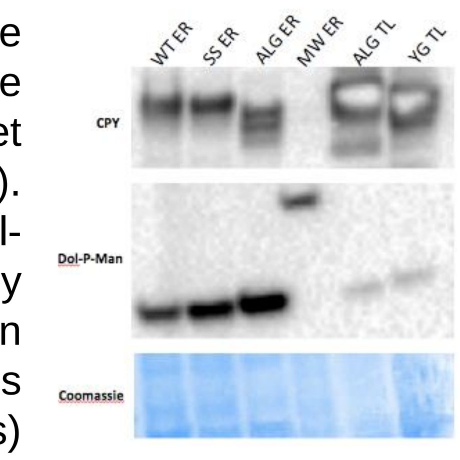


Figure 7. Total lysate and ER fractions from SS328, *ALG9*Δ, and YG414/HS*ALG9* yeast strains. Blots probed with CPY and Dol-P Man.

Discussion

Optimization of the endoplasmic reticulum fractionation method allows for biological characterization of ALG9 in cellular development and function. Enriched ER fractions could be employed in fluorophore-assisted carbohydrate electrophoresis (FACE) analysis to study carbohydrate fingerprinting, which allows for the comparison of lipid-linked oligosaccharides between mutant and non-mutant strains. Carbohydrate fingerprinting will allow for a better understanding of the effects of the *ALG9* mutation at a molecular level and may assist in the development of rescue experiments for this rare disorder.

References

1. Aebi M. N-linked glycosylation in the ER. *Biochimica et Biophysica Acta*. 2013:1833:2430-2437
2. Burda P, Aebi M. The dolichol pathway of N-linked glycosylation. *Biochimica et Biophysica Acta.* 1999:1426:239-257
3. Weinstein M *et al*. Clinical Report: CDG-IL: An Infant with a Novel Mutation in the ALG9 Gene and Additional Phenotypic Features
4. Petros Bonzidis *et al.* Isolation of endoplasmic reticulum, mitochondria, and mitochondria-associated membrane fractions from transfected cells and from human cytomegalovirus-infected primary fibroblasts. *Current Protocols in Cell Biology.* Volume 1. 3.27.1
5. Colussi P, Taron, C, Mack, J, Orlean, P. Human and Saccharomyces cerevisiae dolichol phosphate mannose synthases represent two classes of the enzyme, but both function in Schizosaccharomyces pombe. 1997; 94(15): 7873-7878.

Acknowledgements

Tess Robart and Alyson Zwicker were funded by Foundation Glycosylation summer studentship support. Thomas Pulinilkunnil is funded by NSERC and NBHRF.