

Ng Bobby George (Orcid ID: 0000-0003-0649-848X)

Predominant and novel *de novo* variants in 29 individuals with *ALG13* deficiency: Clinical description, biomarker status, biochemical analysis and treatment suggestions.

Bobby G. Ng¹, Erik A. Eklund^{1,2}, Sergey A. Shiryayev¹, Yin Y. Dong³, Mary-Alice Abbott⁴, Carla Asteggiano⁵, Michael J. Bamshad^{6,7}, Eileen Barr⁸, Jonathan A. Bernstein^{9,10}, Shabeed Chelakkadan¹¹, John Christodoulou^{12, 13}, Wendy K. Chung^{14, 15}, Michael A. Ciliberto¹⁶, Janice Cousin¹⁷, Fiona Gardiner¹⁸, Suman Ghosh¹⁹, William D. Graf²⁰, Stephanie Grunewald²¹, Katherine Hammond²², Natalie S. Hauser²³, George E. Hoganson²⁴, Kimberly M. Houck²⁵, Jennefer N. Kohler^{9, 26}, Eva Morava²⁷, Austin A. Larson²⁸, Pengfei Liu^{29, 30}, Sujana Madathil¹⁶, Colleen McCormack^{9, 26}, Naomi J.L. Meeks²⁸, Rebecca Miller²³, Kristin G. Monaghan³¹, Deborah A. Nickerson⁷, Timothy Blake Palculict³¹, Gabriela Magali Papazoglu³², Beth A. Pletcher³³, Ingrid E. Scheffer^{18, 34}, Andrea Beatriz Schenone³⁵, Rhonda E. Schnur³¹, Yue Si³¹, Leah J. Rowe²⁸, Alvaro H. Serrano Russi^{36, 37}, Rossana Sanchez Russo⁸, Farouq Thabet³⁸, Allysa Tuite³³, María Mercedes Villanueva³⁵, Raymond Y. Wang^{39, 40}, Richard I. Webster^{41, 42}, Dorcas Wilson^{43, 44}, Alice Zalan²⁴, Undiagnosed Diseases Network, University of Washington Center for Mendelian Genomics (UW-CMG), Lynne A. Wolfe⁴⁵, Jill A. Rosenfeld^{29, 30}, Lindsay Rhodes³¹, Hudson H. Freeze¹

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- (1) Human Genetics Program, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037, USA.
- (2) Lund University, Department of Clinical Sciences, Lund, Pediatrics, Lund, Sweden.
- (3) Nuffield Department of Clinical Neurosciences, University of Oxford, Oxford, OX3 9DS, UK.
- (4) Department of Pediatrics, Baystate Children's Hospital, University of Massachusetts Medical School – Baystate, Springfield, MA 01199, USA
- (5) CEMECO – CONICET, Children Hospital, School of Medicine, National University of Cordoba. Chair of Pharmacology Catholic University of Cordoba, Argentina, Cordoba, Argentina.
- (6) Department of Pediatrics, University of Washington, Seattle, WA 98195, USA.
- (7) Department of Genome Sciences, University of Washington, Seattle, WA 98195, USA.
- (8) Department of Human Genetics, Emory University, Atlanta, GA 30322 USA.
- (9) Stanford University School of Medicine, Stanford, CA 94305, USA.

(10) Stanford Center for Undiagnosed Diseases, Stanford University, Stanford, CA 94305, USA.

(11) Monash Children's Hospital, Melbourne, Australia.

(12) Brain and Mitochondrial Research Group, Murdoch Children's Research Institute, Royal Children's Hospital, Melbourne, and Department of Paediatrics, University of Melbourne, Australia.

(13) Discipline of Child & Adolescent Health, Sydney Medical School, University of Sydney, Sydney, Australia.

(14) Department of Pediatrics, Columbia University, New York, NY 10027, USA.

(15) Department of Medicine, Columbia University, New York, NY 10027, USA.

(16) Department of Pediatrics, University of Iowa Hospitals and Clinics, Iowa City, IA 52242, USA.

(17) Section of Human Biochemical Genetics, National Human Genome Research Institute, Bethesda, MD 20892, USA.

(18) University of Melbourne, Austin Health, Melbourne, Australia.

(19) University of Florida College of Medicine, Department of Pediatrics Division of Pediatric Neurology, Gainesville, FL 32610, USA.

(20) Division of Pediatric Neurology, Department of Pediatrics; Connecticut Children's; University of Connecticut; Farmington, CT 06032, USA.

(21) Metabolic Medicine Department, Great Ormond Street Hospital, Institute of Child Health University College London, NIHR Biomedical Research Center, London, UK.

(22) Division of Pediatric Neurology, Department of Pediatrics, University of Alabama at Birmingham, Birmingham, AL 35233, USA.

(23) Inova Translational Medicine Institute Division of Medical Genomics Inova Fairfax Hospital Falls Church, VA 22042, USA.

(24) Department of Pediatrics, University of Illinois at Chicago, Chicago, IL 60612, USA.

(25) Department of Pediatrics, Section of Neurology and Developmental Neuroscience, Baylor College of Medicine, Houston, TX 77030, USA.

(26) Division of Cardiovascular Medicine, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA.

(27) Department of Clinical Genomics, Mayo Clinic, Rochester, MN 55905, USA.

(28) Section of Clinical Genetics & Metabolism, Department of Pediatrics, University of Colorado, Anschutz Medical Campus, Aurora, CO 80045, USA.

(29) Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA.

- (30) Baylor Genetics Laboratories, Houston, TX 77030, USA.
- (31) GeneDx, Inc. Laboratory, Gaithersburg, MD 20877, USA.
- (32) CEMECO – CONICET, Children Hospital, School of Medicine, National University of Cordoba, Cordoba, Argentina.
- (33) Department of Pediatrics, Rutgers New Jersey Medical School, Newark, NJ 07103, USA.
- (34) University of Melbourne, Royal Children’s Hospital, Florey and Murdoch Institutes, Melbourne, Australia.
- (35) Laboratorio de Neuroquímica "Dr. N. A. Chamoles" - FESEN, Buenos Aires, Argentina.
- (36) Division of Medical Genetics Children's Hospital Los Angeles, University of Southern California, Los Angeles, CA 90027, USA.
- (37) Keck School of Medicine, University of Southern California, Los Angeles, CA 90033, USA.
- (38) MercyOne Pediatric Neurology, Des Moines, IA 50314, USA.
- (39) Division of Metabolic Disorders, Children's Hospital of Orange County, Orange, CA 92868, USA.

(40) Department of Pediatrics, University of California-Irvine, Orange, CA 92868, USA.

(41) T.Y. Nelson Department of Neurology and Neurosurgery, The Children's Hospital at Westmead, Australia.

(42) Kids Neuroscience Centre, The Children's Hospital at Westmead, Australia.

(43) Netcare Sunninghill Hospital, Sandton, South Africa.

(44) Nelson Mandela Children's Hospital, Johannesburg, South Africa.

(45) Undiagnosed Diseases Program, Common Fund, National Institutes of Health, Bethesda, MD 20892, USA.

Correspondence:

Hudson H. Freeze

Professor of Glycobiology, Director, Human Genetics Program

Sanford Children's Health Research Center

Sanford-Burnham-Prebys Medical Discovery Institute

10901 N. Torrey Pines Rd. La Jolla, CA 92037

Email: HUDSON@sbpdiscovery.org

Phone: 858-646-3142

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ABSTRACT

ALG13 encodes a non-redundant, highly conserved, X-linked UDP-N-Acetylglucosaminyltransferase required for the synthesis of lipid linked oligosaccharide precursor and proper N-linked glycosylation. *De novo* variants in *ALG13* underlie a form of early infantile epileptic encephalopathy known as EIEE36, but given its essential role in glycosylation, it is also considered a congenital disorder of glycosylation, ALG13-CDG. Twenty-four previously reported ALG13-CDG cases had *de novo* variants, but surprisingly, unlike most forms of CDG, ALG13-CDG did not show the anticipated glycosylation defects, typically detected by altered transferrin glycosylation. Structural homology modeling of two recurrent *de novo* variants, p.A81T and p.N107S, suggests both are likely to impact the function of ALG13. Using a corresponding ALG13-deficient yeast strain, we show that expressing yeast ALG13 with either of the highly conserved hotspot variants rescues the observed growth defect, but not its glycosylation abnormality. We present molecular and clinical data on 29 previously unreported individuals with *de novo* variants in *ALG13*. This more than doubles the number of known cases. A key finding is that a vast majority of the individuals presents with West syndrome, a feature shared with other CDG types. Among these, the initial epileptic spasms best responded to ACTH or prednisolone, while clobazam and felbamate showed promise for continued epilepsy treatment. A ketogenic diet seems to play an important role in the treatment of these individuals.

Synopsis:

Novel and recurrent *de novo* variants in ALG13 cause a neurodevelopmental disorder.

Compliance with Ethics Guidelines

Role for each contributing author: BGN, SAS, YYD performed experiments and drafted manuscript. MJB, JS, DAN, PL, JAR, KGM, TBP, RES, YS, LR performed NGS data analysis and drafted manuscript. EAE, MAA, CA, EB, JAB, SC, JC, WKC, MAC, JC, FG,SG, WDG, SG, KH, NSH, GEH, KMH, JNK, EMK, AAL, SM, CM, NJLM, RM, GMP, BAP, IES, ABS, LJR, AHSR, RSR, FT, AT, MMV, RYW, RIW, DW, AZ, LAW provided clinical evaluations and drafted manuscript. BGN and HHF supervised and drafted manuscript.

Corresponding author: Hudson H. Freeze

Conflict of interest: Bobby G. Ng, Erik A. Eklund, Sergey A. Shiryayev, Yin Y. Dong, Mary Alice Abbott, Carla Asteggiano, Michael J. Bamshad, Eileen Barr, Jonathan A. Bernstein, Shabeed Chelakkadan, John Christodoulou, Wendy K. Chung, Michael A. Ciliberto, Janice Cousin, Fiona Gardiner, Suman Ghosh, William D. Graf, Stephanie Grunewald, Katherine Hammond, Natalie S. Hauser, George E. Hoganson, Kimberly M. Houck, Jennefer N. Kohler, Eva Morava, Austin A. Larson, Sujana Madathil, Colleen McCormack, Naomi J.L. Meeks, Rebecca Miller, Deborah A. Nickerson, Gabriela Magali Papazoglu, Beth A. Pletcher, Ingrid E.

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Kristin G. Monaghan, Timothy Blake Palculict, Rhonda E. Schnur, Yue Si and Lindsay Rhodes are employees of GeneDx, Inc. Pengfei Liu and Jill A. Rosenfeld are employed by the Department of Molecular and Human Genetics at Baylor College of Medicine who receives revenue from clinical genetic testing conducted at Baylor Genetics.

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Introduction

Congenital disorders of glycosylation (CDG) are a group of nearly 140 rare metabolic disorders which can present with a broad, non-specific spectrum of clinical symptoms. The vast majority of CDG are autosomal recessive disorders, but several are caused by *de novo* variants. One such type is ALG13-CDG, which frequently presents as an early infantile epileptic encephalopathy (de Ligt et al 2012; Epi KC et al 2013; Michaud et al 2014; Deciphering Developmental Disorders 2017; Heyne et al 2019).

Asparagine-Linked Glycosylation 13 Homolog (*ALG13*) encodes a highly conserved X-linked UDP-N-Acetylglucosaminyltransferase required for the transfer of N-Acetylglucosamine

(GlcNAc) onto the extending lipid linked oligosaccharide (LLO) structure, dolichol-P-P GlcNAc (Bickel et al 2005; Gao et al 2005; Ng and Freeze 2018) (**Figure 1A**). Studies in yeast show that this early step in the LLO synthesis pathway is essential for proper N-linked glycosylation (Bickel et al 2005; Gao et al 2005). Given its critical role in N-linked glycosylation, it is not surprising that ALG13 and its UDP-GlcNAc transferase activity are conserved across all eukaryotic species.

Nearly all our knowledge about the function of ALG13 is based on biochemical and genetic analyses in *S. cerevisiae*. The majority of yALG13 protein localizes to the cytoplasm with only a portion in the endoplasmic reticulum (ER) through dimerization with yALG14, an ER transmembrane protein (Bickel et al 2005; Gao et al 2005). This ER-localized yALG13 - yALG14 heterodimer is essential for both cell viability and proper N-glycosylation, since abolishing this interaction results in profound defects in both (Bickel et al 2005; Gao et al 2005; Averbeck et al 2007).

Much less is known about the function of ALG13 in higher eukaryotes, although its strong evolutionary conservation would presumably indicate its role in the proper synthesis of LLO.

Within the LLO pathway, at least 35 genes have been identified to cause a glycosylation related disorder (Ng and Freeze 2018). To date, 24 individuals have been identified with pathogenic *de novo* variants in *ALG13* resulting in a neurodevelopmental disorder primarily characterized by

early infantile epileptic encephalopathy (Timal et al 2012; de Ligt et al 2012; Epi KC et al 2013; Michaud et al 2014; Smith-Packard et al 2015; Dimassi et al 2016; Kobayashi et al 2016; Deciphering Developmental Disorders 2017; Fung et al 2017; Hamici et al 2017; Ortega-Moreno et al 2017; Bastaki et al 2018; Galama et al 2018; Heyne et al 2019;; Demos et al 2019; Madaan et al 2019). Most of these cases were identified in sequencing studies where clinical and variant information is summarized in the supplemental material. Nearly all reported affected persons are female and harbor an apparently recurrent *de novo* variant (c.320G>A; p.N107S). Surprisingly, glycosylated serum transferrin, which is a commonly used biomarker for CDG, showed a normal glycosylation pattern in the few ALG13-CDG individuals who have been tested (Ng and Freeze 2018).

Here we present molecular data on 29 individuals with *de novo* variants in *ALG13*, including three novel variants not yet reported in the literature. Clinical information was available for 26 of these individuals, with detailed neurological findings for 24. We address the use of serum transferrin as a biomarker and show that two recurrent variants, p.A81T and p.N107S, that affect highly conserved residues, impact the function of ALG13 in a yeast complementation assay. Molecular modeling shows their potential interactions with the substrate, UDP-GlcNAc.

Methods and Materials

Clinical data

Inclusion criteria for this study required the presence of *de novo* variants in *ALG13* (Genbank NM_001099922.2, UniProt Q9NP73). Ultimately, we identified 29 individuals for which retrospective clinical data were obtained. Written consent was provided for all families in accordance with each individual's primary physician, neurologist or when required, Sanford Burnham Prebys Medical Discovery Institute approval IRB-2014-038-17.

Carbohydrate deficient transferrin analysis

As previously described (Lacey et al 2001)

Next Generation Sequencing

Next-generation sequencing (NGS) consisted of either exome sequencing (ES), genome sequencing (GS) or targeted gene panels. NGS and analysis was performed via each institutions or clinical lab services own standardized method. These methods are available upon request.

ALG13 Structural Modeling

A structural model of human ALG13 (hALG13) was generated using the Phyre2 server with the yALG13 structure (PDB code: 2JZ). MurG is the closest biological ortholog of ALG13/14 for which a high-resolution structure is currently available while bound to its substrate UDP-GlcNAc. Therefore, to get an indication of how UDP-GlcNAc might be positioned in the

hALG13 active site, we aligned the structural model of hALG13 with the structure of MurG (PDB code: 3S2U) using PyMOL.

Western blot analysis of ALG13 protein

Fibroblasts from control and affected ALG13 individuals were grown as previously described in 1g/L glucose DMEM supplemented with 10% heat inactivated FBS (Sigma). Western blot analysis was also performed as previously described using a polyclonal antibody to ALG13 [Proteintech 20810-1-AP] and a monoclonal alpha-tubulin [12G10-DSHB hybridoma bank] (Ferreira et al 2018).

Yeast complementation Assay

Isolation and characterization of a yALG13 mutant strain was previously described (Gao et al 2005). Survival of this strain is dependent on the expression of wild type yAlg13 protein under the control of a GAL1 promoter which is repressed by glucose and induced by galactose. Growth assays and carboxypeptidase Y (CPY) glycosylation analysis were both previously described (Gao et al 2005; Averbeck et al 2008; Gao et al 2008). The expression plasmid pRS305 containing yALG13 with a C-terminal 3x FLAG tag driven off a glucose responsive promoter was used as a template to introduce either the p.A118T (p.A81T) or p.N144S (p.N107S) yeast specific mutants. Insertion of the p.A118T (p.A81T) or p.N144S (p.N107S) mutants was carried out using a NEB Q5 site-directed mutagenesis kit.

Results

Molecular analysis

Due to the lack of a reliable biomarker for screening and identifying ALG13-CDG, all previously reported cases (N=24) were identified via NGS. In our cohort, this trend held true with all 29 individuals being identified by NGS (21 ES, 2 GS, 6 gene panel) (**Table 1**). We identified two recurrent *de novo* variants that accounted for the majority of identified individuals. The c.241G>A [p.A81T] variant was observed in 3/29 (10%) individuals, while the c.320A>G [p.N107S] variant was observed in the vast majority 23/29 (79%) (**Figure 1B, Table 1**). The c.320A>G [p.N107S] variant was also the most frequent (22/24 (92%) in previously reported cases (**Figure 1B**). We also identified several novel *de novo* variants including c.50T>A (p.I17N), c.207_209delAGA (p.E69del) and c.2915G>T (p.G972V) (**Figure 1B, Table 1**). None of the mentioned variants included in this cohort are present in gnomAD v2.1.1 or v3 (accessed 2020.5.20). *In silico* modeling each specific variant was performed using the combined annotation dependent depletion (CADD; <http://cadd.gs.washington.edu/>) scoring method and showed each variant to have a score above 20 [p.I17N (25.7), p.A81T (25.3), p.N107S (20.7), p.G972V (27)] placing all five in the top 1% of deleterious variants in the human genome.

Clinical Phenotype

ALG13 deficiency presents primarily as a neurodevelopmental disorder of varying severity. In our cohort, we were able to review clinical records for 26 individuals and found the most frequently seen symptoms were developmental delays in 26/26 (100%), seizures/epilepsy in 24/26 (92%), intellectual disability in 22/24 (92%) who could be assessed for this feature, and hypotonia in 22/26 (85%) (**Figure 2**).

Due to the severe epileptic encephalopathy previously reported in this disease, a more in-depth analysis of the epileptic manifestations was performed in those individuals (n=24) for whom detailed information was available. In the 24 individuals who presented with seizures, the mean age of onset was 6.5 (CI 4.3 - 8.7) months. The semiology was very consistent, with epileptic spasms (ES) in 20/23 (87%) individuals as the presenting semiology, and hypsarrhythmia as the initial EEG finding in 20/23 (87%) individuals with recorded initial EEG changes (**Table 2**). The spasms were treated either with adrenocorticotrophic hormone (ACTH), prednisolone (Pred) or vigabatrin (VGB). ACTH and/or Pred was described as an effective treatment for ES in eleven individuals, and ineffective in five individuals, whereas VGB was effective in five individuals and was ineffective, or sometimes even aggravated seizure activity, in four individuals. One subject showed VGB-induced changes on brain MRI scan (symmetrical diffusion restriction in the thalamus and globus pallidus), which reversed once VGB was discontinued. A plethora of anti-seizure medications was used, illuminating the pronounced pharmaco-resistance of this disorder. Benzodiazepines (clobazam (CLB), clonazepam, nitrazepam) seemed effective in a

rather high proportion of individuals (5/8-63%) where the most commonly used was CLB (used in 7/8-88%) (**Table 2**). Felbamate (FBM) was only used in two individuals but was reported to be effective in both. Topiramate was used more frequently (7 individuals), however, none of the affected individuals had a favorable outcome with this treatment. Levetiracetam, valproate and lamotrigine showed a minimal effectiveness, with a reported positive effect in 2/8, 1/7 and 1/6 individuals, respectively. Cannabidiol was used in five subjects of whom two had a positive response. Most individuals continued to show signs of an epileptic encephalopathy after the initial spasms were treated. In six individuals, a diagnosis of Lennox-Gastaut syndrome (LGS) was made following the initial diagnosis of infantile spasms (IS). Five individuals remain seizure-free on current treatment. In addition to pharmaceuticals, a ketogenic diet (KD) was widely used in our cohort (12/23-52%); eight had a sustained positive effect of the diet and one had an initial response (**Table 2**). Two individuals had placement of a vagus nerve stimulator (VNS), only one of which had a favorable response. All individuals had an MRI scan, but no consistent findings were observed. Most were either normal or had nonspecific and vaguely described findings such as cerebral atrophy and benign enlargement of the subarachnoid spaces (**Table 2**). No structural abnormalities or dysplasias have been reported as potential epileptogenic foci.

In addition to characterizing the prominent neurological phenotype, we queried other clinical symptoms occurring in these subjects.

In our cohort, ocular deficits were noted in 12/26 (46%) with cortical visual impairment present in eight of those individuals (**Figure 2**). Gastrointestinal symptoms characterized by either vomiting, GERD, reflux and the need for G-tube placement were seen in 11/26 (42%). Skeletal defects, primarily scoliosis (6/11) or osteopenia (2/11), were seen in a total of 11/26 (42%) individuals. Dysmorphic features, mainly facial were seen in 11/26 (42%) and included coarse features, high arched palate or prominent forehead to name a few (**Figure 2**). Cardiac (6/26), respiratory (5/26) and immunologic abnormalities (5/26) were all seen in < 25% of cases (**Figure 2**).

Out of the group of individuals carrying p.N107S variant, three individuals (CDG-0078, 0081 and 0135) were not included in the final clinical summary. CDG-0078 and CDG-0077 are identical twins and both carry the *de novo* p.N107S. However, in contrast to her affected sister, CDG-0078 does not have any clinical history of developmental and intellectual delays or seizure activity and is considered to be “unaffected.” One explanation for the dramatic clinical discrepancy between these two identical sisters is skewed X-inactivation of mutant *ALG13* in the “healthy” sister. However, no additional testing could be performed. For CDG-0081, we were not able to obtain a complete clinical history, although it is known that she had intractable seizures and, at 13 years of age, is the only individual in our cohort who has died (**Figure 2**). Finally, for CDG-0135, we were only provided variant information for reporting.

We did not see a consistent ALG13-CDG-specific phenotype that could be used to help differentiate this disorder from other CDG types or even other epilepsy-related disorders. This highlights the importance of finding an ALG13-CDG-specific biomarker.

Carbohydrate deficient transferrin (CDT) analysis

ALG13 is a critical component of the N-linked glycosylation pathway and it is logical to use carbohydrate deficient transferrin (CDT) analysis as a biomarker since the great majority of N-linked defects have an abnormal CDT. However, it was previously noted that several affected females who carried the recurrent p.N107S variant were tested and found to have a normal CDT result, indicating normal N-linked glycosylation, at least in hepatocytes (Smith-Packard et al 2015). In our cohort of 29 individuals, 14 had CDT testing with 14/14 (100%) found to be normal (**Figure 2**). Two individuals (CDG-0417, CDG-1017) were found to have a CDT profile suggestive of a type I CDG. CDG-0417 had two mildly abnormal CDT results detected by capillary zone electrophoresis (initial CDT at age 1yr-11mon). However, we should note that over the course of a month, this amount of this abnormal peak improved for no clear reason. A follow up analysis four years later using the more sensitive liquid chromatography mass spectrometry method showed that the abnormal peak was still detectable, but it was within the normal reference range. CDG-1017 also had a detectable abnormal peak (initial CDT at age 8yr-10mon) suggesting a type I pattern and, like CDG-0417, the amount was within the normal

reference range. While only 14/29 individuals had CDT analysis performed, it is encouraging that CDG was considered as a possible cause in nearly half the subjects. These data suggest that CDT is unlikely to be a reliable biomarker for ALG13-CDG caused by *de novo* variants, and further work will be needed to identify one.

ALG13 Structural Modeling

The protein structure for *S. cerevisiae* yALG13 and the *Pseudomonas aeruginosa* ALG13 ortholog, MurG, are both known (Hu et al 2003; Wang et al 2008). MurG is a single polypeptide whose N-terminal domain has a high degree of homology to ALG14 and a C-terminal domain to ALG13 (Gao et al 2008). Furthermore, MurG is required to carry out a similar enzymatic reaction as the human ALG13:ALG14 heterodimer. Due to the structural conservation of yALG13 and MurG to human ALG13, we aimed to model and then determine the potential impact the p.A81T and p.N107S variants may have on human ALG13 protein.

From this alignment, human p.A81 and p.N107 are both situated close to the predicted position of the GlcNAc moiety of UDP-GlcNAc, the donor substrate (**Figure 3A, Figure 3B**). The p.N107 in human ALG13 is predicted to be positioned close to the homologous p.Q288 in *P. aeruginosa* MurG (Brown et al 2013) (**Figure 3B**), which hydrogen bonds with the C3 hydroxyl group, and is part of the DDHQ motif that is homologous to the NNHQ motif in human ALG13. The p.A81 in human ALG13 is homologous to p.A260 in *P. aeruginosa* MurG, which is part of a

hydrogen bond network with p.N291 and p.A125, that could be important in the correct positioning of p.N124, which binds to the C4 and C6 hydroxyls of the GlcNAc moiety. Further enzymatic and biophysical analyses are needed to determine the roles that p.N107 and p.A81 play in human ALG13 function, as well as how the disease-causing mutations affect this. Hence, significant changes to the side chain chemistry of either of these residues is likely to affect the structure of the ALG13 active site. We speculate these variants could affect their affinity and /or specificity for the activated monosaccharide carried by the UDP. What is clear is that neither the p.A81T nor the p.N107S variants affect the stability of mutant ALG13 protein (**Figure 4**).

Yeast complementation Assay

Deletion of *yALG13* causes a severe growth defect and ultimately lethality

(<https://www.yeastgenome.org/locus/S000003015>). A conditional null mutant *yALG13* strain is available, but its survival depends on the presence of wild type *yALG13* driven off a GAL1-responsive promoter (i.e. when galactose is provided in the absence of glucose, the strain will grow). We took advantage of a previously described method using this mutant strain and the ability to express *yALG13* under the control of a glucose responsive promoter (Gao et al 2005). When we expressed highly conserved equivalent *yALG13* mutants and shifted to selection under glucose (i.e. the rescued *yALG13* under galactose is repressed), the p.A118T and p.N144S mutants both rescued the growth defect in a similar fashion to wild type (**Figure 5**). However,

unlike wild type yALG13, neither the p.A118T nor p.N144S mutants were capable of restoring glycosylation of a commonly used biomarker (carboxypeptidase Y, CPY) for yeast glycosylation mutants (**Figure 5**) (Avaro et al 2002). Importantly, Western blot analysis determined neither expressed mutation affected the stability of yALG13 when compared to wild type (**Figure 5**). These data suggest in yeast the p.A118T and p.N144S variants likely affect the function of ALG13.

Discussion

Here we present data on 29 newly reported individuals who were found to have *de novo* variants in *ALG13*, which more than doubles the number of known cases. Previously *de novo* variants in *ALG13* were shown to cause a neurodevelopmental disorder characterized by varying degrees of developmental and intellectual disabilities and epilepsy. Together with our novel cases, the total number of individuals affected with ALG13-CDG who have been identified is now 53.

The epileptic phenotype in the ALG13-CDG subjects described here is strikingly homogenous and is consistent with previous publications (Fung et al 2017; Ortega-Moreno et al 2017; Madaan et al 2019). It emerges in the usual time for ES, with a mean age of 6.5 months at the debut of the seizures, where the peak age at the debut of spasms in the whole group is 6-8 months (Riikonen 2001). Most individuals show the electrophysiological pattern of hypsarrhythmia in their first EEG, and all show developmental arrest, thus fulfilling the criteria for West syndrome. This age-dependent epileptic encephalopathy syndrome often develops later on into another age-

dependent syndrome, LGS, and many of the individuals in our cohort displayed a fully developed LGS or partial symptoms thereof (Lombroso 1983). Only five individuals remained seizure-free on treatment. Traditionally, three main treatment approaches to ES exist, corticosteroids (usually Pred or ACTH), and the GABA aminotransferase inhibitor VGB (γ -vinyl-GABA). In a recent review of all clinical studies involving these agents, ACTH seems to be the most effective single treatment, whereas a combination of VGB and ACTH also shows promise (Riikonen 2020). In our cohort ACTH and Pred seemed superior to VGB in both effectiveness and side-effects, which is also supported by previous case descriptions (Epi KC et al 2013; Kobayashi et al 2016; Madaan et al 2019). We therefore suggest that ACTH or Pred to be used as the first line treatment of ALG13-related ES, possibly with an extended period of weaning as several individuals had seizure recurrence during tapering. In the continued care for these individuals, a large number of different AEDs have been used in our cohort, highlighting widespread pharmaco-resistance.

Amongst the different drugs, two stood out as potentially favorable alternatives, benzodiazepines (most commonly CLB) and FBM). FBM is a drug initially approved for LGS that was previously restricted due to unusual cases of fatal aplastic anemia and hepatic failure (Shah et al 2016), but now is seeing a revival as a rescue agent in unresponsive IS (Dozieres-Puyravel et al 2020); its use in ALG13-related epileptic encephalopathy should certainly be further studied. A KD is a powerful treatment option in some epileptic encephalopathies such as Dravet syndrome (Dressler

et al 2015), and other metabolic conditions such as GLUT1-deficiency (Kass et al 2016). In ALG13-CDG with epileptic encephalopathy, we only found one report of successful treatment using a KD (Smith-Packard et al 2015), and it is unclear from the other reports whether it has been tried on this cohort of individuals. In our cohort, however, as many as 12 individuals were being treated with, or were previously treated with a KD; eight showed a sustainable response, whereas one showed an initial response to the diet. This is very encouraging and suggest a KD may be an important potential alternative/complement to pharmaceuticals in this disease.

One important issue we were not able to fully address was the role X-chromosome inactivation (XCI) plays in ALG13 deficiency. In our cohort, only three individuals were reported to have XCI analysis and all were found to have random XCI from whole blood samples. Because of the strong neurological phenotype, XCI in whole blood may not fully represent what is happening in the brain.

Despite the clear role of ALG13 in glycosylation, the common CDG biomarker transferrin was not as reliable for ALG13-CDG as it is for other CDG types. This is reminiscent of SLC35A2-CDG, which is also an X-linked disorder caused by *de novo* variants and like ALG13-CDG can give unreliable or unexpected CDT results. However, unlike SLC35A2-CDG, which is due to loss of function variants, we hypothesize that the recurrent p.N107S and other *de novo* variants are likely gain of function variants. While it is unclear what that gain of function is, this could potentially explain why CDT is not abnormal.

As is the case with all LLO synthesis proteins, ALG13 is an essential component of the glycosylation machinery. In both yeast and humans, it is the only known enzyme capable of carrying out the transfer of a GlcNAc onto Dol-P-P-GlcNAc to generate Dol-P-P-GlcNAc-GlcNAc, which serves as a substrate for ALG1, the next enzyme in LLO biosynthesis (**Figure 1A**). Human deficiencies in ALG13 should cause a profound glycosylation defect like that seen in the preceding (DPAGT1-CDG) and subsequent (ALG1-CDG) LLO steps (Ng et al 2019; Ng et al 2016). However, despite the proven role of ALG13 in glycosylation, individuals with *de novo* variants do not demonstrate a clear glycosylation abnormality, at least not in serum glycoproteins or skin fibroblasts (data not shown). Interestingly, this is also true for the ALG13 specific binding partner, ALG14, where biallelic variants cause both congenital myasthenic syndrome-15 (ALG14-CMS) and also a disorder characterized as an early lethal neurodegeneration with myasthenic and myopathic features (Cossins et al 2013; Schorling et al 2017).

While ALG13 and ALG14 are both ubiquitously expressed proteins, it is possible that deficiencies in either could cause a tissue-specific disorder. This has for example been seen with a few CDG types that primarily affect the liver (e.g. MPI-CDG, TMEM199-CDG, CDCC115-CDG, ATP6AP1-CDG) (Marques-Da-Silva et al 2017).

While ALG13's role in N-glycosylation has been well documented in yeast, its role in humans is less clear and more complicated because, at some point within its evolution from *S. cerevisiae* to *H. sapiens*, there was a dramatic change in the ALG13 protein. *S. cerevisiae* ALG13 (Uniprot P53178) is a small 202 amino acid (aa) protein consisting only of a glycosyltransferase 28 (GT28) domain. However, in humans, multiple transcripts of *ALG13* (Uniprot Q9NP73) occur with the canonical transcripts encoding a 1137aa protein, two intermediate forms of 1059aa and 954aa, and the smallest form of 165aa. This smaller 165aa form most closely resembles the single, essential yeast protein. Interestingly, the canonical human isoform contains not only the glycosyltransferase 28 domain, but also several other domains including an OTU deubiquitinase domain (OTUD), a TUDOR domain and a Proline rich domain (**Figure 1B**).

Transcript 3 lacks 78 amino acid of the N-terminal GT28 domain just before the p.A81T hotspot, while transcript 4 lacks 105 amino acid of the N-terminal GT28 domain prior to the p.N107S hotspot. (**Figure 1B**). Both of these proteins likely lack GT28 activity due to the loss of many critical amino acids required for substrate binding and catalytic activity. We are able to detect three of four isoforms (**Figure 4**); however, it is very possible isoform 4 is not detectable due to the loss of the antibody epitope.

It is unclear what roles the different transcripts play in the pathology of ALG13 deficiency. We hypothesize that only the long and short forms could potentially harbor a functional GT28

domain, but to date no functional studies have proven the long form has catalytic activity. Thus, it is unclear if the long and short forms compensate for one another. Studies have shown it is possible to completely delete the long isoform form, but not the short form, suggesting the short form is the essential glycosyltransferase required for glycosylation (Gao et al 2019).

The functional significance of these additional domains within ALG13 is unknown. These other, non-glycosyltransferase domains are found together as a separate gene in zebrafish (<https://zfin.org>). In zebrafish, *zgc:92907* is most similar to the small ALG13 isoform seen across all organisms, while the gene annotated as *ALG13* does not contain the GT28 domain required for GT28 activity but does contain the OTUD family domain. It is unclear when during evolution these quite different genes fused into the single gene seen in humans and other vertebrates.

It is tantalizing to speculate what functions these additional domains within the long isoform of ALG13 are performing. For example, the ovarian tumor deubiquitinase (OTUD) family domain of ALG13 contains the conserved catalytic triad of amino acids (Asp239, Cys242, His345) required for deubiquitinase DUB activity (Mevisen et al 2013). However, when expressed in bacteria, the purified ALG13 OTUD domain lacked DUB activity towards a Ub propargylamide (Ub-PA) substrate but did have activity toward an artificial haloalkyl substrate (Mevisen et al 2013). Is it possible that ALG13-dependent DUB activity is restricted to a very small select set of protein targets, like those in the LLO pathway? Interestingly, DPAGT1, which catalyzes the

proceeding step to ALG13, has been found to be ubiquitinated at Lys48 within a critical cytoplasmic loop required for UDP-GlcNAc binding (Udeshi et al 2013). Could the long isoform of ALG13 DUB activity regulate DPAGT1 activity via deubiquitination? These, and many other questions remain unanswered about the function of ALG13.

In conclusion, we present data on 29 individuals found to specifically harbor *de novo* variants in *ALG13*, allowing us to expand both the clinical phenotype and molecular understanding of this disorder. Clinical and pharmacological data suggest certain anti-seizure medications could potentially be prioritized as a first line therapies, while others could be avoided. Furthermore, non-pharmaceutical alternatives such as a KD could have beneficial effects on suppression of seizures and should be considered. We identified several novel *de novo* variants, additional cases of the recurrent p.N107S and p.A81T. Structural modeling predicts both the p.A81T and p.N107S variants may affect the ALG13 active site and UDP-GlcNAc interface. Finally, we show expression of either recurrent variant in an *ALG13* mutant yeast model restores the observed growth defect but does not correct the N-glycosylation abnormality, suggesting that both residues are important for normal glycosylation in yeast.

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Figure Legend

Figure 1 – Lipid linked oligosaccharide pathway highlighting the role of ALG13 and a schematic showing the location of *de novo* variants identified in *ALG13* (A) Schematic showing the role of ALG13 in LLO synthesis. (B) Schematic showing the four primary *ALG13* transcripts with the positions of each *de novo* variant within the ALG13 protein. Variants identified in this study have been placed on the top portion, while previously reported variants are on the lower portion. The number of individuals identified is also listed as (n=). The solid triangles denote the catalytic triad required for the deubiquitinase domain active site D239, C242, H345.

Figure 2 – Clinical summary for 26 individuals with *de novo* variants in ALG13. General clinical summary for 26 individuals found to have *de novo* variants in ALG13.

Figure 3 – Structural homology model of ALG13, showing the predicted positions of recurrent *de novo* mutations relative to UDP-GlcNAc. (A) N107, A81 and UDP-GlcNAc shown as sticks, colored according to the element, with carbon represented in yellow in UDP-

GlcNAc and mauve in ALG13. Structural elements of ALG13 labelled according to (Wang et al 2008). (B) shows an overlay of ALG13 in mauve, and MurG (PDB: 3S2U, PMID: 22973843) in grey, with H-bonds observed in the structure represented by dashed black lines.

Figure 4 – Western blot analysis of ALG13 from fibroblasts. Available fibroblasts were used for whole cell extracts to detect endogenous ALG13 protein levels. Alpha tubulin was used as a loading control to assure equal protein levels.

Figure 5 – Yeast complementation assay using an ALG13 deficient yeast strain.

(A) An ALG13 deficient yeast strain was grown under selection conditions allowing for expression of wild type yALG13 when grown in the presence of galactose. Transfection and expression of various yALG13 mutants were performed as previously described (Gao et al 2005). (B) Western blot analysis of glycosylated carboxypeptidase Y under various complementation and growth conditions was performed in triplicates with representative data shown. (C) Western blot analysis showing the transfected levels of yALG13-Flag tagged protein.

Table 1 – Genotypes identified in 29 individuals found to have *de novo* variants in ALG13.

Genotypes from 29 individuals along with their sex, inheritance status, *ALG13* variant and the method of detection are listed. Nucleotide numbering for cDNA uses +1 as the A of the ATG translation initiation codon in the reference sequence (Genbank: NM_001099922.2, UniProt:

Q9NP73), with the initiation codon as codon 1.

Table 2 – Neurological summary for 42 individuals with *de novo* variants in *ALG13*

Detailed neurological summary for 25 individuals in our cohort found to have *de novo* variants in *ALG13*. Additionally, available information for 17 previously reported individuals is also provided. Bolded drugs indicate a favorable response effect, non-bolded ones indicate uncertain response and grey indicates no response or unacceptable side effects.

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Table 1 – Genotypes identified in 29 individuals with *de novo* variants in *ALG13*.

	CDG - ID	Status	Sex	Inheritance	cDNA Position	Protein Position	Method of detection
1	CDG - 0075	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	Panel
2	CDG - 0077*	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	Panel
3	CDG - 0078*	Unaffected	F	<i>de novo</i>	c.320A>G	p.N107S	Panel
4	CDG - 0079	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
5	CDG - 0080	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
6	CDG - 0081	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
7	CDG - 0082	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	GS
8	CDG - 0083	Affected	M	<i>de novo</i>	c.320A>G	p.N107S	ES
9	CDG - 0085	Affected	F	<i>de novo</i>	c.241G>A	p.A81T	Panel
10	CDG - 0086	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	Panel
11	CDG - 0088	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
12	CDG - 0089	Affected	F	<i>de novo</i>	c.241G>A	p.A81T	ES
13	CDG - 0092	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	Panel
14	CDG - 0101**	Affected	M	<i>de novo</i>	c.2915 G>T	p.G972V	ES
15	CDG - 0125	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
16	CDG - 0133	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
17	CDG - 0134	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
18	CDG - 0135	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
19	CDG - 0136	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
20	CDG - 0139	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
21	CDG - 0140**	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
22	CDG - 0141	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
23	CDG - 0417	Affected	F	<i>de novo</i>	c.241G>A	p.A81T	ES
24	CDG - 0431	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	GS
25	CDG - 0453	Affected	F	<i>de novo</i>	c.50T>A	p.I17N	ES
26	CDG - 0456	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
27	CDG - 0457	Affected	F	<i>de novo</i>	c.207_209del AGA	p.E69del	ES
28	CDG - 0458	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES

29	CDG - 1017	Affected	F	<i>de novo</i>	c.320A>G	p.N107S	ES
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*CDG - 0077 and CDG - 0078 are identical twins

**CDG - 0101 and CDG - 0140 were found to be mosaic.

Table 2 – Neurological summary for 42 individuals with *de novo* variants in *ALG13*

<u>Case</u>	<u>Sex</u>	<u>Mutation</u>	<u>Age at sz start</u>	<u>Initial semiology</u>	<u>EEG at diagnosis</u>	<u>MRI findings</u>	<u>Current and previous AED drugs</u>	<u>Other interventions</u>	<u>Epilepsy outcome</u>	<u>DD/ID</u>
CDG-0075	F	p.N107S	6 months	GTCS	Mu/G	normal	VPA	no	N/A	yes
CDG-0077	F	p.N107S	10 months	IS	H	BESS	ACTH, VGB, LEV, TPM	no	N/A	yes
CDG-0079	F	p.N107S	6 months	IS	H	normal	ACTH, LEV, ESX, TPM, GBP, LTG	Keto	Myoclonic sz, moderate control	yes
CDG-0080	F	p.N107S	3 months	IS	H	normal	ACTH, Pred, VGB, B6	no	Poor	yes
CDG-0082	F	p.N107S	6 months	IS	H	lack of WM	Pred, VGB, ACTH, LEV, VPA, ZON, LCM, CBD, NZM	Keto	N/A	yes
CDG-0083	M	p.N107S	7 months	IS	H	cerebral atrophy	ACTH, VGB, VPA, ESX, CLB, LTG	Keto	Frontal lobe sz/Nocturnal sz.	yes
CDG-0085	F	p.A81T	9 months	IS	H	normal	ACTH	no	sz free w/o AED	yes
CDG-0086	F	p.N107S	2 months	IS?	H?	BESS/normal	ZON, LTG, RUF, LEV, CLB, CBD	no	Myoclonic sz, head drops, LGS; mild	yes
CDG-0088	F	p.N107S	6 months	IS	H	VGB changes	VGB, TPM, ACTH, FBM, Pred	no	N/A	yes
CDG-0089	F	p.A81T	1 month	IS	H	BESS, thinning CC	N/A	Keto	Good control on keto	yes

CDG-0092	F	p.N107S	1 month	IS	H	normal	ACTH, Pred, B6, VGB , PHB , TPM	Keto	IS and GTCS, controlled on AED/keto	yes
CDG-0125	F	p.N107S	4-5 months	IS	H	mild cerebral atrophy	CBD, CLB, CZP, LEV, Pred , LCM	no	N/A	yes
CDG-0133	F	p.N107S	no sz	no	normal	normal	N/A	N/A	N/A	yes
CDG-0134	F	p.N107S	4 months	IS	H	normal	Pred, VGB	no	sz free w/o AED	yes
CDG-0136	F	p.N107S	3 months	IS	H	PVL	VPA, TPM, CZP, CLB, LEV, NZM, LTG, ESX, PHB	VNS, Keto	LGS	yes
CDG-0139	F	p.N107S	5 months	IS	H	normal	Pred, CZP, B6, VGB	Keto	sz free on Keto	yes
CDG-0140	F	p.N107S	18 months	ES	H	normal	ZON, LEV	no	GC	yes
CDG-0141	F	p.N107S	6 months	N/A	N/A	cortical atrophy	LTG, VPA, PER	no	Myoclonic sz, LGS, refractory	yes
CDG-0417	F	p.A81T	1 month	IS	H	progressive atrophy	Pred , VGB, LEV, CLB, TPM, VPA, OXC, LTG	Keto, initial effect	Focal tonic sz	yes
CDG-0431	F	p.N107S	5 months	IS	H	mild cerebral atrophy	ACTH , CLB, LEV, TPM, LTG, OXC	Keto	N/A	yes
CDG-0453	F	p.I17N	10 months	Eye deviation	Mu	normal	LEV, CLB, CBD	VNS	LGS, sz free on treatment	yes
CDG-0456	F	p.N107S	6 months	absence w eye flutter	Fo	BESS	PHB, TPM, ACTH	Keto	M/GT, sz free on keto	yes
CDG-0457	F	p.E69del	24 months	IS	evolving H	normal	VGB, VPA, FBM	no	LGS	yes
CDG-0458	F	p.N107S	6 months	IS	H	cerebral atrophy	Pred , VGB, ACTH , ZON, CBD	Keto	LGS, refractory	yes

CDG-1017	F	p.N107S	7 months	IS	H	normal	ACTH, LEV, LTG	Keto	Complex partial sz	yes
Madaan et al., 2019	F	p.N107S	5 months	IS	H	normal	ACTH	no	infrequent sz	yes
Fung et al., 2017	F	p.N107S	4 months	IS	N/A	N/A	3 (non-specified)	N/A	50 % reduction on treatment	severe
Ortega-Moreno et al., 2017	F	p.N107S	5 months	IS	N/A	N/A	N/A	N/A	LGS	N/A
Bastaki et al., 2018	F	p.N107S	N/A	IS	H	N/A	N/A	N/A	N/A	yes
Galama et al., 2018	M	p.N107S	4.5 months	IS	H	hypoplasia of CC/DM	VGB, Pred , VPA, NZM, LTG, LEV	no	Pred stopped ES; LEV stable GTCS	yes
DDD Study, 2017	F	p.N107S	N/A	N/A	N/A	N/A	N/A	N/A	Sz	yes
DDD Study, 2017	F	p.N107S	N/A	N/A	N/A	N/A	N/A	N/A	Sz	yes
Hamici et al., 2017	F	p.N107S	N/A	IS	H	N/A	N/A	N/A	N/A	yes
Epi4K Consortium, 2016	F	p.N107S	1-2 months	Tonic/IS	H	VGB changes	VGB, Pred , TPM, LEV, CZP, ZON	no	Ongoing sz myoclonic	profound
Kobayashi et al., 2016	F	p.N107S	6 months	IS	H	cerebral atrophy	ACTH	N/A	Reduced sz on ACTH	yes
Dimassi et al., 2016	F	p.N107S	2 months	IS	H	mild global atrophy	N/A	N/A	Spasms continue	yes
Smith-Packard et al., 2015	F	p.N107S	8 months	IS	H	N/A	ACTH	Keto	Complex partial sz at 5 yrs	severe

Michaud et al., 2014	F	p.N107S	4 months	IS	H	cerebral atrophy	N/A	N/A	Focal sz, multifocal EEG	severe
Epi4K Consortium, 2013	F	p.N107S	1 month	IS	H	BESS	ACTH	N/A	Spasms returned on taper	yes
Epi4K Consortium, 2013	F	p.N107S	4 months	IS	H	normal	N/A	N/A	LGS	severe
de Ligt et al., 2012	F	p.N107S	N/A	N/A	N/A	atrophy, delayed myelin	N/A	N/A	Seizure unspecified	profound
Timal et al., 2012	M	p.K94E	N/A	N/A	N/A	N/A	N/A	N/A	Refractory sz	N/A

Abbreviations: ACTH, adrenocorticotrophic hormone; B6, pyridoxine; BESS, benign enlargement of the subarachnoidal spaces; CBD, cannabidiol; CC, corpus callosum; CLB, clobazam; CZP, clonazepam; DM, delayed myelinization; ESX, ethosuximide; FBM, felbamate; Fo, focal; GTCS, generalized tonic-clonic seizure; GBP, gabapentin; H, hypsarrhythmia; IS, infantile spasms; LCM, lacosamide; LGS, Lennox-Gastaut syndrome; LEV, levetiracetam; LTG, lamotrigine; Keto, ketogenic diet; Mu, multifocal; NZM, nitrazepam OXC, oxcarbazepine; PHB, phenobarbital; Pred, prednisolone; PVL, Periventricular leukomalacia; RUF, rufinamide; SZ, seizure; TPM, topiramate; VGB, vigabatrin, VNS, vagus nerve stimulator; VPA, valproate; WM, white matter; ZON, zonisamide. N/A, Not available.

Members of the Undiagnosed Diseases Network

Maria T. Acosta
Margaret Adam
David R. Adams
Pankaj B. Agrawal
Mercedes E. Alejandro
Justin Alvey
Laura Amendola
Ashley Andrews
Euan A. Ashley
Mahshid S. Azamian
Carlos A. Bacino
Guney Bademci
Eva Baker
Ashok Balasubramanyam
Dustin Baldrige
Jim Bale
Michael Bamshad
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Pinar Bayrak-Toydemir
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Gill Bejerano
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Sirisak Chanprasert
Hsiao-Tuan Chao
Gary D. Clark

Terra R. Coakley
Laurel A. Cobban
Joy D. Cogan
Matthew Coggins
F. Sessions Cole
Heather A. Colley
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Andrew B. Crouse
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Surendra Dasari
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Neil A. Hanchard
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Kevin S. Smith
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Lilianna Solnica-Krezel
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Joan M. Stoler
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Kathleen Sullivan
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Mustafa Tekin
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CONFLICT OF INTEREST

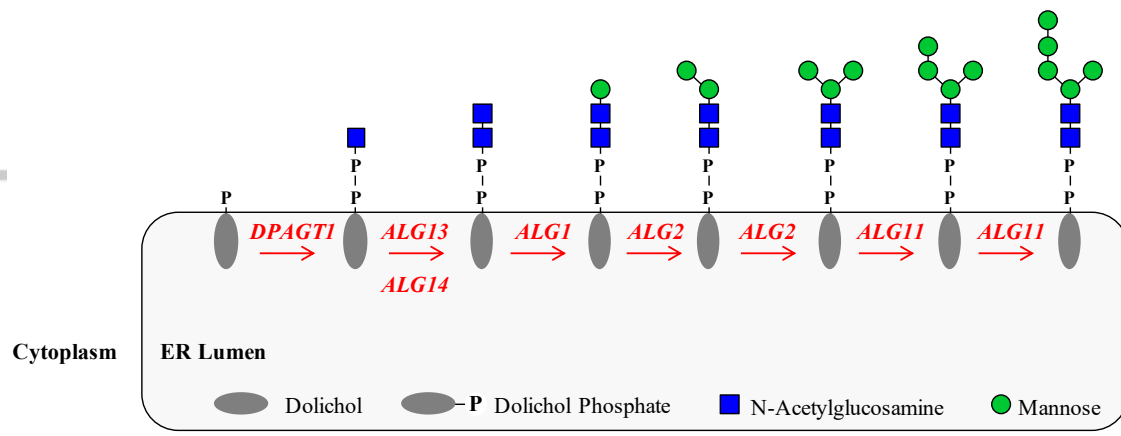
Conflict of interest: Bobby G. Ng, Erik A. Eklund, Sergey A. Shiryayev, Yin Y. Dong, Mary Alice Abbott, Carla Asteggiano, Michael J. Bamshad, Eileen Barr, Jonathan A. Bernstein, Shabeed Chelakkadan, John Christodoulou, Wendy K. Chung, Michael A. Ciliberto, Janice Cousin, Fiona Gardiner, Suman Ghosh, William D. Graf, Stephanie Grunewald, Katherine Hammond, Natalie S. Hauser, George E. Hoganson, Kimberly M. Houck, Jennefer N. Kohler, Eva Morava, Austin A. Larson, Sujana Madathil, Colleen McCormack, Naomi J.L. Meeks, Rebecca Miller, Deborah A. Nickerson, Gabriela Magali Papazoglu, Beth A. Pletcher, Ingrid E. Scheffer, Andrea Beatriz Schenone, Leah J. Rowe, Alvaro H. Serrano Russi, Rossana Sanchez Russo, Farouq Thabet, Allysa Tuite, María Mercedes Villanueva, Raymond Y. Wang, Richard I. Webster, Dorcas Wilson, Alice Zalan, Lynne A. Wolfe, and Hudson H. Freeze declare that they have no conflict of interest.

Kristin G. Monaghan, Timothy Blake Palculict, Rhonda E. Schnur, Yue Si and Lindsay Rhodes are employees of GeneDx, Inc. Pengfei Liu and Jill A. Rosenfeld are employed by the Department of Molecular and Human Genetics at Baylor College of Medicine who receives revenue from clinical genetic testing conducted at Baylor Genetics.

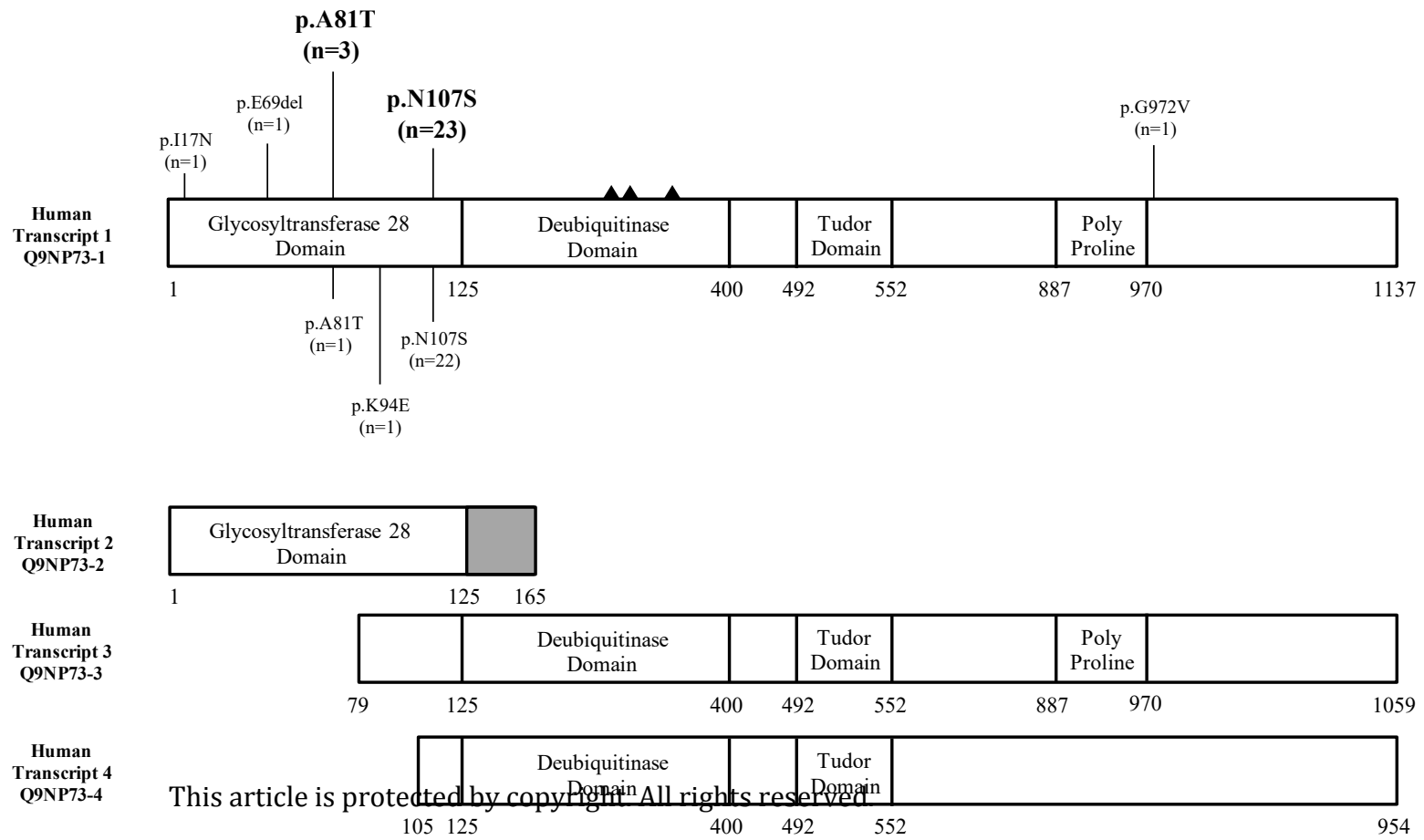
SIGNED FOR ALL AUTHORS:

A handwritten signature in black ink, appearing to read "Hudson H. Freeze". The signature is written in a cursive style with a long horizontal line extending to the right.

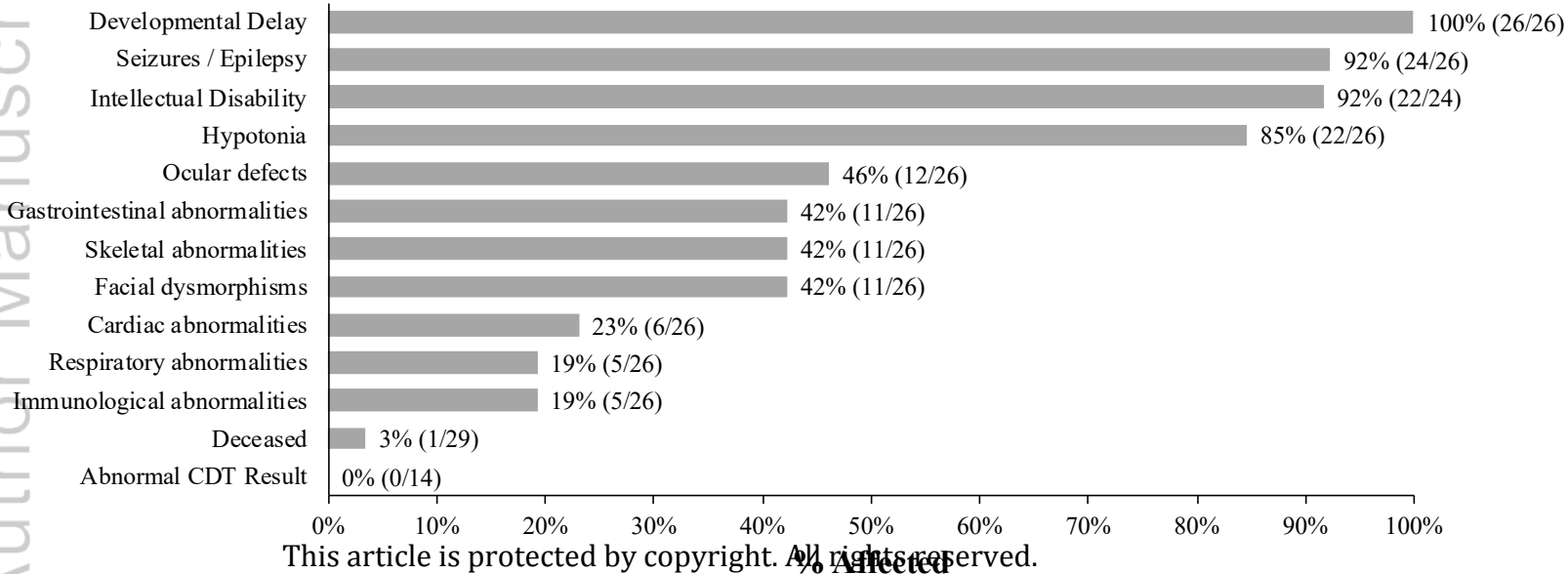
(A)



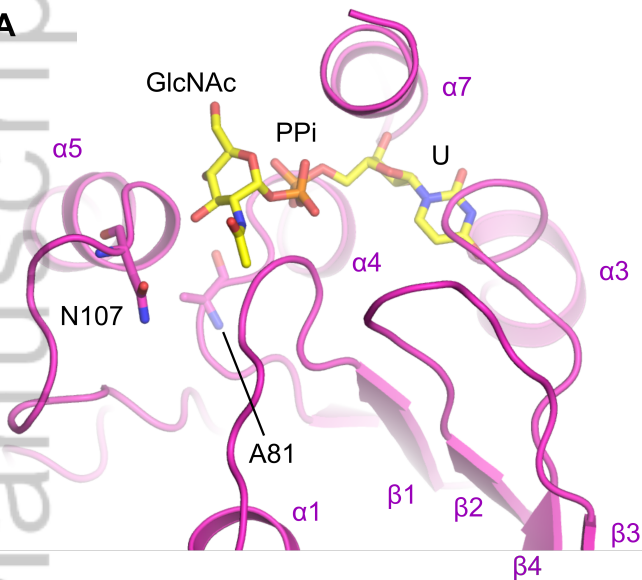
(B)



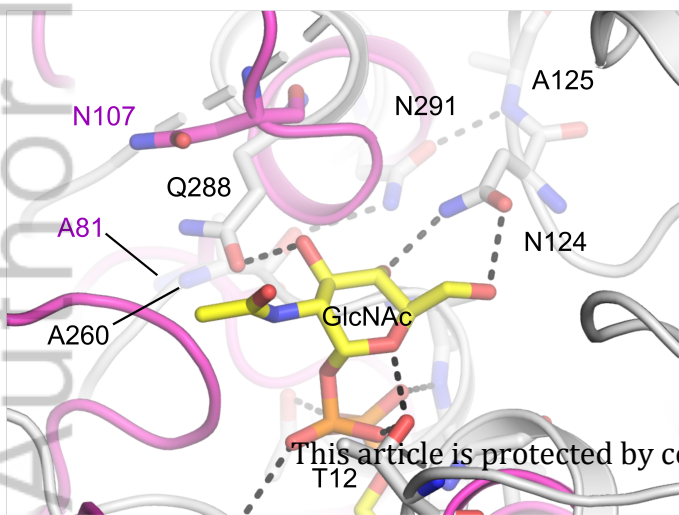
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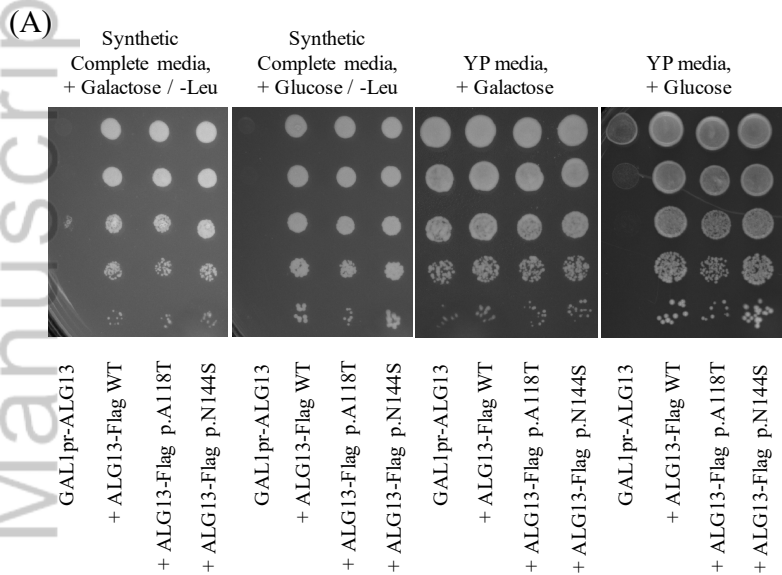


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