

# Mutations in *GMPPA* Cause a Glycosylation Disorder Characterized by Intellectual Disability and Autonomic Dysfunction

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In guanosine diphosphate (GDP)-mannose pyrophosphorylase A (*GMPPA*), we identified a homozygous nonsense mutation that segregated with achalasia and alacrima, delayed developmental milestones, and gait abnormalities in a consanguineous Pakistani pedigree. Mutations in *GMPPA* were subsequently found in ten additional individuals from eight independent families affected by the combination of achalasia, alacrima, and neurological deficits. This autosomal-recessive disorder shows many similarities with triple A syndrome, which is characterized by achalasia, alacrima, and variable neurological deficits in combination with adrenal insufficiency. *GMPPA* is a largely uncharacterized homolog of *GMPPB*. *GMPPB* catalyzes the formation of GDP-mannose, which is an essential precursor of glycan moieties of glycoproteins and glycolipids and is associated with congenital and limb-girdle muscular dystrophies with hypoglycosylation of  $\alpha$ -dystroglycan. Surprisingly, GDP-mannose pyrophosphorylase activity was unchanged and GDP-mannose levels were strongly increased in lymphoblasts of individuals with *GMPPA* mutations. This suggests that *GMPPA* might serve as a *GMPPB* regulatory subunit mediating feedback inhibition of *GMPPB* instead of displaying catalytic enzyme activity itself. Thus, a triple-A-like syndrome can be added to the growing list of congenital disorders of glycosylation, in which dysregulation rather than mere enzyme deficiency is the basal pathophysiological mechanism.

In the present study, we investigated a consanguineous Pakistani family (family A) with three siblings who suffered from alacrima and progressive feeding difficulties—including dysphagia and episodes of regurgitation, which developed within the first year of life—but showed no signs of adrenal insufficiency. All procedures were in accordance with the ethical standards of the responsible committee on human experimentation, and proper written informed consent was obtained. Further examination revealed a functional constriction of the gastric cardia (achalasia), which required surgery. All affected individuals presented with delayed developmental milestones, intellectual disability, and gait abnormalities (Figure 1A and Table 1), whereas the parents and the fourth child were

unaffected, consistent with autosomal-recessive inheritance. The considerable clinical overlap with triple A syndrome (MIM 231550), which is characterized by achalasia, congenital absence of tear flow (alacrima), adrenal insufficiency, and progressive neurologic dysfunction, prompted us to sequence *AAAS* (MIM 605378), the only known gene associated with triple A syndrome.<sup>1–3</sup>

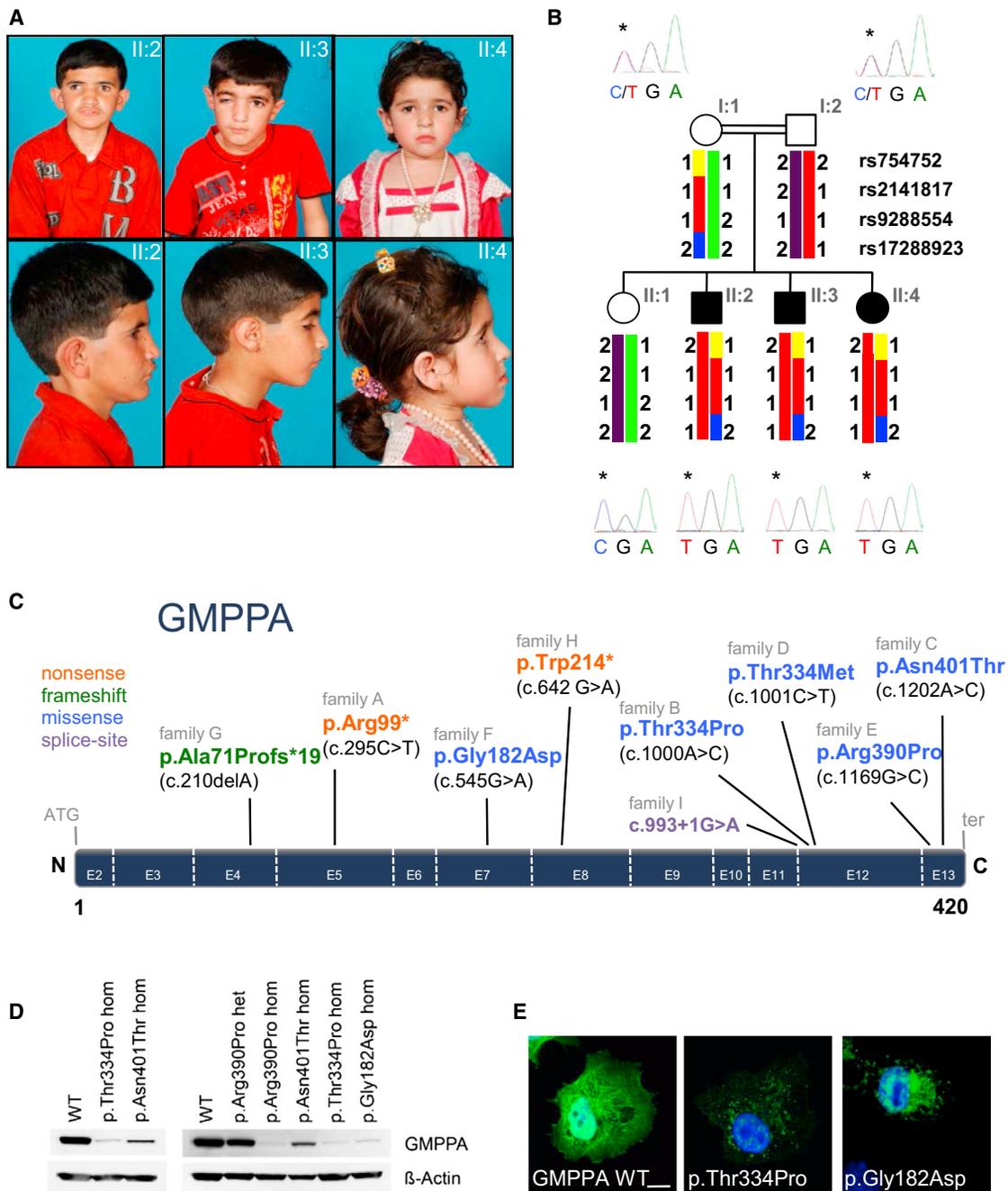
Because no mutation was identified in the coding region of *AAAS*, we performed a genome-wide linkage scan in the family by using the Affymetrix Genome-Wide Human SNP Array 6.0. We calculated LOD scores by using the program ALLEGRO<sup>4</sup> and assuming autosomal-recessive inheritance with full penetrance and a disease allele frequency of 0.0001. The analysis revealed linkage to chromosomes 2

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**Figure 1. Loss-of-Function Mutations in *GMPPA* Cause Intellectual Disability with Autonomic Dysfunction**

(A) Frontal and lateral views of the affected family members in our index family from Pakistan.

(B) Pedigree and haplotype analysis of the index family. The disease-associated region on 2q36 is shown in red. The position of *GMPPA* is between SNPs rs2141817 and rs9288554. Electropherograms show the identified mutation for individual family members.

(C) Localization of the variants identified in all families investigated.

(D) *GMPPA* abundance in protein lysates of either lymphoblastoid cell lines (left) or fibroblasts (right) obtained from affected individuals or a healthy proband. Actin served as a loading control.

(E) Transfection of COS-7 cells with wild-type Myc-tagged *GMPPA* yielded a diffuse cytoplasmic staining, which was altered upon transfection of the p.Gly182Asp and p.Thr334Pro variants. The scale bar represents 20  $\mu$ m.

and 9, thus further excluding *AAAS*, which maps to 12q13, as the underlying disease-associated gene (Figure S1, available online). The 3 Mb chromosome 2 region, with a maximum LOD score of 3.13, was flanked by markers rs754752 and rs17288923. For the 500 kb chromosome 9 region, a maximum LOD score of 3.09 was observed between rs10965932 and rs7022766.

For whole-exome sequencing, we fragmented 1  $\mu$ g of DNA from individual II:2 by using sonification technology (Covaris). The fragments were end repaired and adaptor ligated, including the incorporation of sample index barcodes. After size selection, the library was subjected to the enrichment process with the SeqCap EZ Human Exome Library v.2.0 (Roche NimbleGen). A total of four samples

were pooled and sequenced on two lanes of an Illumina HiSeq 2000 sequencing instrument with the use of two paired-end 100 bp sequences, resulting in ~39 million reads on target with a minimum of 30× coverage for 94% of the target sequences. SNPs and short indels were called with SAMtools.<sup>5</sup> Focusing on the linkage intervals on chromosomes 2 and 9 (Figure 1), we filtered for homozygous variants not present in dbSNP (build 134) or the 1000 Genomes database (build 20110521) and for variants that were seen five times or fewer in our in-house exome database (n ~ 200). The final list contained only two variants. The first, nonsynonymous change c.19C>T (p.Pro7Ser) (RefSeq accession number NM\_057094.1) in *CRYBA2* (MIM 600836), was later identified as the known variant rs141631259, which has a reported European population frequency of 2.3% in the National Heart, Lung, and Blood Institute (NHLBI) Exome Sequencing Project Exome Variant Server (build ESP6500). The second variant, homozygous nonsense mutation c.295C>T (p.Arg99\*) (RefSeq NM\_013335.3) in guanosine diphosphate (GDP)-mannose pyrophosphorylase A (*GMPPA*), was absent from genomic variation databases. This mutation was subsequently verified by Sanger sequencing (Figure 1B).

Having identified a mutation in *GMPPA* as the likely underlying genetic defect in the Pakistani family, we screened 63 additional families affected by a combination of alacrima and achalasia but without evidence of a mutation in *AAAS*. The study was approved by the local ethics committee (Medical Faculty, Technical University Dresden; EK820897). Twenty-six individuals also suffered from adrenal insufficiency. These individuals with suspected triple A syndrome were collected at the Children's Hospital of Technical University Dresden and originate from the following countries: Germany (n = 21), Turkey (n = 12), Great Britain (n = 5), the Netherlands (n = 4), United States (n = 4), Croatia (n = 2), Australia (n = 2), France (n = 2), United Arab Emirates (n = 1), Austria (n = 1), Czech Republic (n = 1), Israel (n = 1), Italy (n = 1), Japan (n = 1), Morocco (n = 1), Poland (n = 1), Russia (n = 1), Slovakia (n = 1), and Tunisia (n = 1). Twenty-four percent of the individuals derived from consanguineous parents, and 60% derived from nonconsanguineous parents. For 16% of the individuals, it was unclear whether the parents were consanguineous or not. From eight families, we identified ten individuals with homozygous mutations in *GMPPA*. Of note, none of these individuals displayed adrenal insufficiency.

An overview on the clinical and genetic findings for these affected individuals is given in Table 1. The mutational spectrum included one frameshift mutation (c.210delA [p.Ala71Profs\*19]), one nonsense mutation (c.642G>A [p.Trp214\*]), one splice-site mutation (c.993+1G>A), and five missense mutations (c.545G>A [p.Gly182Asp], c.1000A>C [p.Thr334Pro], c.1001C>T [p.Thr334Met], c.1169G>C [p.Arg390Pro], and c.1202A>C [p.Asn401Thr]) (Figure 1C and Figure S2). None of the variants were listed in dbSNP (build 137), the 1000 Genomes Project, or the NHLBI Exome Variant Server.

Immunoblot analysis of either lymphoblastoid cell lines or fibroblasts of individuals with a homozygous missense mutation in *GMPPA* revealed that these affected individuals had lower levels of *GMPPA* than did control samples (Figure 1D), indicating that the variant transcript or protein might have had reduced stability. Whereas COS-7 cells transfected with wild-type Myc-tagged *GMPPA* showed an intense cytoplasmic labeling, Myc-tagged *GMPPA* variants p.Gly182Asp and p.Thr334Pro displayed weak signal intensity and altered localization (Figure 1E). No staining was observed for cells transfected with the p.Arg99\* and p.Ala71\* variants mimicking the p.Ala71Profs\*19 alteration (data not shown). Taken together, these findings—the mode of inheritance together with the mutational spectrum—strongly suggest that loss of function in *GMPPA* is the underlying mechanism of the disorder.

Human *GMPPA* encodes *GMPPA*, a 420 aa protein (RefSeq NP\_037467.2) with known domains in InterPro. The predicted nucleotidyltransferase domain (amino acids 3–194 [Pfam ID PF00483]) is shared by a wide range of enzymes that transfer nucleotides onto phosphosugars.<sup>6</sup> The hexapep domain (amino acids 286–319 [Pfam ID PF00132]) is likewise found in several members of the transferase families.<sup>7</sup> The closest homolog of *GMPPA* in the human genome is *GMPPB*, which shares an identity of 32% at the protein level and a similarity of 49% (*GMPPB* isoform 1, RefSeq NP\_037466.2).

*GMPPB* catalyzes the synthesis of GDP-mannose from mannose-1-phosphate and guanosine triphosphate (GTP). GDP-mannose serves as a key component of both N-glycan and, via dolichol-P-mannose, O-glycan chains in glycoproteins.<sup>8</sup> Of note, mutations in *GMPPB* have recently been identified to cause congenital and limb-girdle muscular dystrophies with hypoglycosylation of  $\alpha$ -dystroglycan.<sup>9</sup> Most plasma membrane and secretory proteins undergo N-glycosylation while they mature in the lumen of the endoplasmic reticulum (ER) and in Golgi cisternae. In N-linked glycosylation, a branched oligosaccharide including nine mannose residues is built on a dolichol lipid anchor present in the ER membrane before being transferred to an asparagine residue of a nascent protein. Incorporation of mannose into O-glycosylated proteins likewise requires GDP-mannose for the biosynthesis of dolichol-P-mannose. GDP-mannose is also used for glycosylphosphatidylinositol (GPI)-anchored proteins that are tethered to the outer leaflet of the plasma membrane. The yeast homolog of GDP-mannose pyrophosphorylase, *VIG9* (vanadate-resistant and immature glycosylation, also termed *PSA1*, *SRB1*, and *MPG1*), was identified by a positive-selection screen with sodium orthovanadate, a substance that is less toxic in glycosylation-deficient yeast.<sup>10–12</sup> In a screen for hypoosmotic fragility, another yeast strain carrying a mutation in *VIG9* was identified, and it was speculated that defective glycosylation affected the cell-wall integrity of the yeast and led to reduced stability.<sup>13,14</sup> In contrast, the total knockout of this gene was lethal in yeast.<sup>11,15</sup>

**Table 1. GMPPA Mutations Identified and Clinical Presentation of the 13 Affected Individuals**

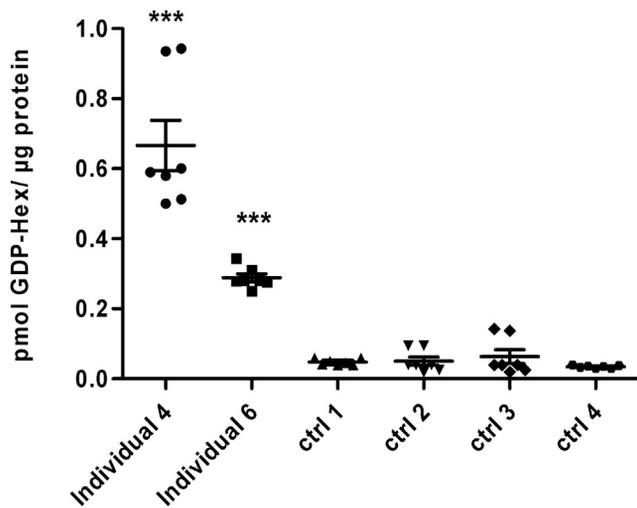
	Family A			Family B		Family C	Family D	Family E	Family F		Family G	Family H	Family I
	1	2	3	4	5	6	7	8	9	10	11	12	13
Ethnicity	Pakistani	Pakistani	Pakistani	Arabic	Arabic	Turkish	Kosovan	Pakistani	Palestinian	Palestinian	Dominican	Arabic	Moroccan
Consanguinity	yes	yes	yes	yes	yes	yes	unknown	yes	NR	NR	yes	yes	yes
Sex	male	male	female	male	male	female	male	female	female	female	female	female	female
Age at exam (years)	12	9	7	17	8	17.6	21.2	20.8	9.2	3.7	10.7	6	2.4
<b>Mutation</b>													
Location	exon 5	exon 5	exon 5	exon 12	exon 12	exon 13	exon 12	exon 13	exon 7	exon 7	exon 4	exon 8	intron 11
cDNA mutation	c.295C>T	c.295C>T	c.295C>T	c.1000A>C	c.1000A>C	c.1202A>C	c.1001C>T	c.1169G>C	c.545G>A	c.545G>A	c.210delA	c.642G>A	c.993+1G>A
Protein alteration	p.Arg99*	p.Arg99*	p.Arg99*	p.Thr334Pro	p.Thr334Pro	p.Asn401Thr	p.Thr334Met	p.Arg390Pro	p.Gly182Asp	p.Gly182Asp	p.Ala71Profs*19	p.Trp214*	-
Type	homozygous	homozygous	homozygous	homozygous	homozygous	homozygous	homozygous	homozygous	homozygous	homozygous	homozygous	homozygous	homozygous
<b>Cardinal Symptoms</b>													
Age of onset	birth	birth	birth	-	-	birth	birth	birth	birth	birth	birth	birth	birth
Achalasia	yes	yes	yes	yes	-	yes	yes	yes	yes	no	yes	yes	yes
Achalasia age of onset	3 months	1 year	1 year	-	-	7 years	2 years	birth	6 months	-	2.5 months	3 months	6 months
Alacrima	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes
Alacrima age of onset	birth	birth	birth	-	-	birth	birth	birth	birth	birth	6 years	3 months	birth
Adrenal insufficiency	no	no	no	no	no	no	no	no	no	no	no	no	no
<b>Epidermal Symptoms</b>													
Hyperkeratosis	yes	yes	yes	no	-	no	-	no	no	no	no	no	no
<b>Neurological Symptoms</b>													
Intellectual disability	yes	yes	yes	yes	-	yes	yes	yes	yes	yes	yes	yes	unknown
Milestones	delayed	delayed	delayed	delayed	delayed	delayed	delayed	delayed	delayed	delayed	delayed	delayed	delayed
Muscular hypotonia	no	no	no	yes	-	no	-	yes	yes	yes	no	-	no
Gait abnormalities	ataxia	no	no	-	-	yes	spasticity	-	no	no	yes	spasticity	no

(Continued on next page)

**Table 1. Continued**

	Family A			Family B		Family C	Family D	Family E	Family F		Family G	Family H	Family I
	1	2	3	4	5	6	7	8	9	10	11	12	13
Babinski	no	no	no	-	-	no	-	no	no	no	no	no	no
Hyperreflexia	no	no	no	no	-	no	yes	no	no	no	no	yes	no
Hyporeflexia	no	no	no	no	-	no	-	no	no	no	-	no	no
Nasal speech	no	no	no	no	-	no	-	yes	yes	yes	no speech	-	no
Sensory impairment	no	yes	no	yes	-	no	-	no	no	no	no	no	no
Hearing impairment	no	no	no	no	-	no	yes	-	yes	yes	-	no	no
Speech delay	yes	yes	yes	-	-	yes	yes	-	yes	yes	no speech	-	yes
Ptosis	no	yes	no	-	-	no	-	-	no	no	no	-	no
<b>Autonomic Nervous System Symptoms</b>													
Sweating	normal	normal	normal	normal	-	normal	-	normal	decreased	decreased	no	normal	normal
Postural hypotension	no	no	no	no	-	no	-	yes (mild)	yes	yes	no	no	no
<b>Ocular Symptoms</b>													
Visual problems	no	no	no	no	-	yes	-	no	yes	yes	yes	no	no
Nystagmus	no	no	no	no	-	no	-	yes	no	no	yes	no	no
Strabism	yes	no	no	no	-	yes	-	yes	no	no	no	no	no
Anisocoria	no	no	no	yes	-	yes	-	yes	no	no	no	no	no

The following abbreviation is used: NR, not reported.



**Figure 2. GDP-Mannose Levels in Lymphoblasts of Affected Individuals and Controls**

Neutralized perchloric extracts prepared from cultures of Epstein-Barr virus (EBV)-transformed lymphoblasts were run on high-performance liquid chromatography<sup>22</sup> with a modified gradient to enhance separation of NDP-sugars. Results are expressed per  $\mu\text{g}$  of cell protein. Intracellular GDP-mannose was significantly higher ( $***p < 0.001$ ) in EBV-transformed lymphoblasts from individual 4 (p.Thr334Pro) and individual 6 (p.Asn401Thr) than in four healthy controls. The statistics were calculated with one-way ANOVA combined with Tukey's multiple-comparison test.

This background implied that a glycosylation defect might be expected in our individuals with a defect in GMPPA. Congenital disorders of glycosylation (CDGs) are grouped into two different types.<sup>16–18</sup> Whereas type I CDGs are characterized by defects in lipid-linked oligosaccharide synthesis and transfer, type II CDGs alter the processing of protein-bound sugar chains. Although there is a wide clinical spectrum, developmental delay appears to be one of the most consistent findings. Defects in O-glycosylation usually have a clinical outcome that is distinct from N-glycosylation defects and rather cause muscular dystrophy, bone or cartilage disorders, or extracellular matrix defects.

So far, two subtypes of CDGs have been linked to the enzymatic pathway that generates GDP-mannose, which is synthesized from fructose-6-phosphate via mannose-6-phosphate and mannose-1-phosphate in a three-step enzymatic reaction. Alterations in the enzymes catalyzing steps one and two of this reaction cause autosomal-recessive CDG type Ib (also known as MPI-CDG [MIM 602579]) and CDG type Ia (also known as PMM2-CDG [MIM 212065]), respectively. MPI-CDG, caused by mutations in *MPI* (MIM 154550), leads to chronic diarrhea, cyclic vomiting, and enteropathy with villus atrophy, hypoglycemia, and facultative hepatic fibrosis. However, mental and motor development is normal. In MPI-CDG, GDP-mannose is depleted and can be corrected by external mannose supplementation.<sup>19</sup> PMM2-CDG is caused by mutations in *PMM2* (MIM 601785) and is the most frequent subtype of the glycosylation disorders. Individ-

uals with the “classic” form of PMM2-CDG have psychomotor retardation, convergent strabismus, cerebellar hypoplasia, seizures, and a coagulopathy.<sup>20</sup> Common for both PMM2-CDG and MPI-CDG is the GDP-mannose deficiency that leads to detectable hypoglycosylation of numerous glycoproteins in the serum or fibroblasts of affected individuals.<sup>21</sup> In contrast, GDP-mannose levels were drastically higher in lymphoblast samples from individuals with a GMPPA defect than in those from control samples (Figure 2), whereas other nucleoside diphosphate (NDP)-sugars were unchanged (data not shown). The galactose-1-phosphate level in red blood cells of individual 6 was within normal limits as well (data not shown). Because of the increased levels of GDP-mannose, it is tempting to speculate that a mannose-depleted diet might be a therapeutic choice in individuals with a GMPPA defect. The increase in GDP-mannose levels might cause imbalances in glycosylation reactions by favoring reactions displaying a high  $K_m$  for this NDP-sugar or by inhibiting enzymes with the use of other NDP-sugars.<sup>23</sup> However, the affected individuals and control subjects showed similar N-glycosylation profiles, both for transferrin glycosylation (Table S1) and for N-glycans derived from either total serum protein or immunoglobulin G (Figure S3). Moreover, serum Apo-CIII glycosylation did not differ between controls and our individuals (data not shown). In addition, the activity of GDP-mannose pyrophosphorylase in lymphoblasts of two affected individuals did not show significant alterations (Table S2). Although our analysis of serum proteins did not display any clear alteration of N-glycosylation, the change induced by GDP-mannose overload might only be significant in restricted cell types or affect other glycosylation types (O-glycosylation, C-glycosylation, and GPI anchor synthesis) than those investigated here. Elevated GDP-mannose levels could also lead to strong perturbations in the levels of other guanine nucleotides (e.g., depletion of GTP), which could have far-reaching consequences not linked to glycosylation (e.g., dysfunction of G-proteins).

The elevated levels of GDP-mannose in GMPPA-deficient cells indicate that GMPPA might serve as a regulatory subunit and allow allosteric feedback inhibition of GMPPB by GDP-mannose.<sup>8</sup> GMPPA and GMPPB are indeed present in roughly equal amounts in native GDP-mannose pyrophosphorylase.<sup>24</sup> Only GMPPB displays catalytic activity,<sup>8</sup> although GMPPA is able to bind GDP-mannose.<sup>24</sup> This suggests that after a gene duplication event, *GMPPA* underwent mutations that converted its encoded protein to an allosteric subunit. Interestingly, sequence alignments indicate that compared to GMPPBs and other NDP-sugar pyrophosphorylases belonging to the same family, GMPPAs from all species are characterized by a 2 aa insertion (Pro11-Gln12 in the human enzyme) in a highly conserved motif that borders the catalytic pocket and binds the nucleotide substrate in homologous enzymes.<sup>25,26</sup> This insertion presumably inactivated the ancestral catalytic site, converting it to an allosteric site.

An analogous situation is found with the plant enzyme ADP-glucose pyrophosphorylase, which consists of two types of homologous subunits, only one of which is endowed with catalytic activity, whereas the other plays a role in allosteric regulation.<sup>26</sup>

In view of the broad expression of the mouse ortholog of *GMPPA* (Figure S4; for methods, see Rust et al.<sup>27</sup> and Hermey et al.<sup>28</sup>), the clinical symptoms of *GMPPA*-mutation-positive persons with developmental delay, alacrima, and achalasia imply that *GMPPA* is particularly relevant in neurons and autonomic nerve fibers innervating the distal esophageal sphincter or the lacrimal glands. Of note, individuals with congenital and limb-girdle muscular dystrophies with hypoglycosylation of  $\alpha$ -dystroglycan due to defects in *GMPPB* also develop neurological symptoms.<sup>9</sup> So far, none of the individuals with a mutation in *GMPPA* revealed signs of an adrenal insufficiency as described for triple A syndrome. Thus, persons suffering from alacrima and achalasia without adrenal insufficiency should primarily be sequenced for mutations in *GMPPA*.

The phenotype of individuals with *GMPPA* mutations shares aspects with hereditary sensory and autonomic neuropathies (HSANs), characterized by sensory loss or, as in the case of HSAN type III (MIM 223900), mainly autonomic dysfunction.<sup>29,30</sup> Prominent autonomic dysfunction is also found in Fabry disease (MIM 301500), Tangier disease (MIM 205400), multiple endocrine neoplasia type IIB (MIM 162300), and mtDNA depletion syndrome 6 (MIM 256810).<sup>31</sup> Further investigations are needed for addressing the role of *GMPPA* in autonomic ganglia neurons. Whether this disorder, similarly to MPI-CDG, might be a potentially treatable hereditary condition, e.g., by a mannose-depleted diet, will be an important issue for the future.

### Supplemental Data

Supplemental Data include four figures and two tables and can be found with this article online at <http://www.cell.com/AJHG>.

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### Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, <http://www.1000genomes.org/>  
Ensembl Genome Browser, <http://www.ensembl.org>  
GeneExplorer, <http://portal.ccg.uni-koeln.de/geneexplorer/>  
InterPro, <http://www.ebi.ac.uk/interpro/>  
MutationTaster, <http://neurocore.charite.de/MutationTaster/>  
NCBI, <http://www.ncbi.nlm.nih.gov/>  
NHLBI Exome Sequencing Project (ESP) Exome Variant Server, <http://evs.gs.washington.edu/EVS/>  
Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org>  
RefSeq, <http://www.ncbi.nlm.nih.gov/RefSeq>  
*Saccharomyces* Genome Database, <http://www.yeastgenome.org>

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