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REPORT

Mutations in *PIGS*, Encoding a GPI Transamidase, Cause a Neurological Syndrome Ranging from Fetal Akinesia to Epileptic Encephalopathy

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Inherited GPI deficiencies (IGDs) are a subset of congenital disorders of glycosylation that are increasingly recognized as a result of advances in whole-exome sequencing (WES) and whole-genome sequencing (WGS). IGDs cause a series of overlapping phenotypes consisting of seizures, dysmorphic features, multiple congenital malformations, and severe intellectual disability. We present a study of six individuals from three unrelated families in which WES or WGS identified bi-allelic phosphatidylinositol glycan class S (*PIGS*) biosynthesis mutations. Phenotypes included severe global developmental delay, seizures (partly responding to pyridoxine), hypotonia, weakness, ataxia, and dysmorphic facial features. Two of them had compound-heterozygous variants c.108G>A (p.Trp36*) and c.101T>C (p.Leu34Pro), and two siblings of another family were homozygous for a deletion and insertion leading to p.Thr439_Lys451delinsArgLeuLeu. The third family had two fetuses with multiple joint contractures consistent with fetal akinesia. They were compound heterozygous for c.923A>G (p.Glu308Gly) and c.468+1G>C, a splicing mutation. Flow-cytometry analyses demonstrated that the individuals with *PIGS* mutations show a GPI-AP deficiency profile. Expression of the p.Trp36* variant in PIGS-deficient HEK293 cells revealed only partial restoration of cell-surface GPI-APs. In terms of both biochemistry and phenotype, loss of function of *PIGS* shares features with PIGT deficiency and other IGDs. This study contributes to the understanding of the GPI-AP biosynthesis pathway by describing the consequences of *PIGS* disruption in humans and extending the family of IGDs.

Recent advances in next-generation sequencing and the widespread application of whole-exome sequencing (WES) and whole-genome sequencing (WGS) have led to the discovery of the molecular basis of a growing number of congenital disorders of glycosylation (CDGs). The inherited glycosylphosphatidylinositol-anchored protein (GPI-AP) deficiencies (IGDs) are a growing group of disorders that are a subset of CDGs. To date, there are 17 IGDs that share overlapping features, including developmental delay, seizures, hypotonia, weakness, ataxia, and dysmorphic features.[1] A recent study of 4,293 parent-child triads reported that IGDs alone might account for 0.15% of all developmental disorders,[2] suggesting that IGDs could be more common than previously recognized.

In many cases, IGDs result from the failure of the GPI anchor to regulate APs, which has global consequences for development. The GPI anchor serves as a tether for APs at the external cell surface. The majority of over 150 mammalian GPI-APs act as ectoenzymes critical to many cell functions, such as the actions of hydrolytic enzymes, adhesion molecules, receptors, protease inhibitors, and complement regulatory proteins.[3] The essential role of GPI-APs in many human tissues became evident as the effects of genetic disruptions of GPI anchor biosynthesis and remodeling were identified.

Approximately 31 enzymes are integral to the post-translational modification that results in the biosynthesis of GPI-APs, and a multitude of genetic disruptions that could produce related phenotypes are possible. IGD-associated phenotypes that result from complete or partial inactivation of these GPI biosynthesis enzymes often include seizures, intellectual disability, coarse facial features, and hypotonia. Microcephaly, hearing impairment, joint contractures, skin anomalies, congenital heart defects, urinary-tract defects, and skeletal anomalies are less common features.[4–42] 17 genes in the GPI-AP biosynthesis pathway have been linked to human disease.[6–44] All of these disorders are autosomal recessive, except that *PIGA*-associated disease (MIM: 300868) is X-linked recessive.

In this report, we describe an IGD disorder resulting from recessive inheritance of variants affecting phosphatidylinositol glycan, class S (PIGS). *PIGS* (MIM: 610271) mutations were found in six individuals from three unrelated families by WES or WGS after informed consent was

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Table 1. Phenotypes Identified in Individuals with Bi-allelic *PIGS* Mutations

System	Features	Individual 1a	Individual 1b	Individual 2a	Individual 2b	Individual 3a	Individual 3b
CNS	microcephaly	–	–	+	+	–	–
	global developmental delay	+	+	+	+	NA	NA
	hypotonia	+	+	+	+	NA	NA
	ataxia and balance problems	+	+	+	+	NA	NA
	seizures	+	+	+	+	NA	NA
	CNS atrophy	cerebellar	cerebellar	diffuse cortical	diffuse cortical	NA	NA
Ocular or visual	nystagmus	+	+	+	–	NA	NA
	cortical blindness	+	+	+	–	NA	NA
Craniofacial	coarse facial features	+	+	+	+	–	–
	arched eyebrows	+	+	+	+	–	–
	thickened helices	+	+	+	+	–	–
	broad tongue	+	+	+	+	–	–
	gingival hypertrophy	+	+	+	–	–	–
	other	preauricular tag, deep philtrum	deep philtrum	widely spaced teeth wrinkled forehead	wrinkled forehead	small chin	–
Gastrointestinal	feeding problems	oral feeding	oral feeding	gastrostomy tube for aspiration	oral feeding	NA	NA
	hepatomegaly	–	–	+	+	–	–
	constipation	–	–	–	+	NA	NA
Genitourinary	cryptorchidism	–	–	+	–	NA	NA (because the individual is female)
Musculoskeletal	hand anomalies	brachydactyly fifth-finger clinodactyly	brachydactyly, fifth-finger clinodactyly	short, stubby digits fifth-finger clinodactyly	brachydactyly, fifth-finger clinodactyly	–	–
	other	short fourth metacarpals and metatarsals	short fourth metacarpals and metatarsals	pectus carinatum, joint laxity, scoliosis	pectus carinatum, joint laxity	multiple joint contractures, consistent with fetal akinesia	multiple joint contractures, consistent with fetal akinesia
Alkaline phosphatase		145 IU/L at 1 year (reference: 25–500), 122 IU/L at 8.5 years (reference: 150–300)	116 IU/L at 8.5 years (reference: 150–300 IU/L)	98 IU/L at 7.5 years (reference: 38–126 IU/L)	130 IU/L at 9 months (reference: 38–126 IU/L)	NA	NA
Other		–	–	inguinal hernias	cardiomegaly	thickened nuchal fold	glomerular cysts, cystic hygroma

The following abbreviation is used: NA, not available.

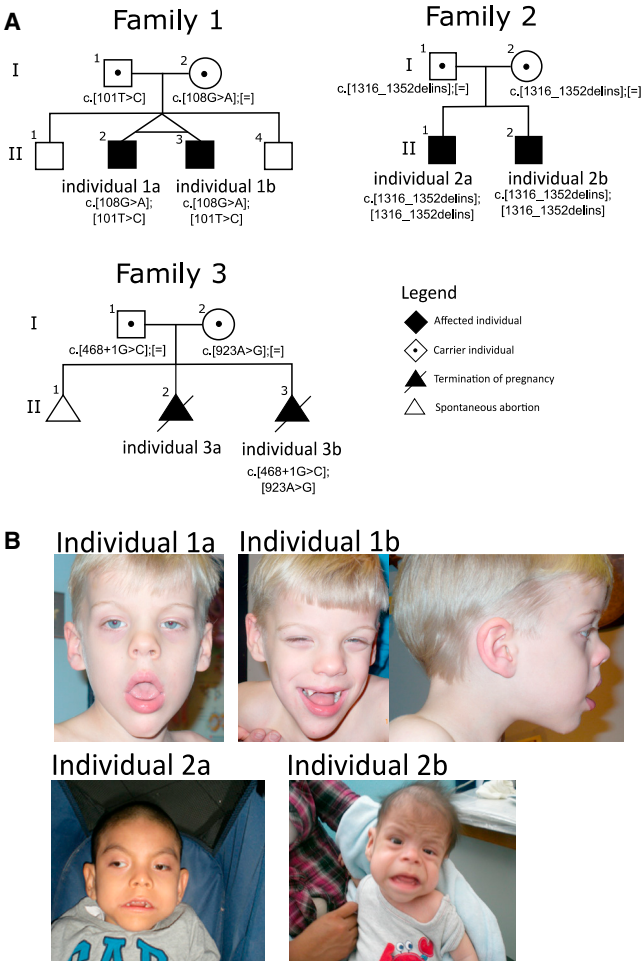

Figure 1. Pedigrees of Three Families Carrying *PIGS* Mutations in This Study and Composite Showing the Characteristics of Individuals in Families 1 and 2

(A) Pedigrees of three families.

(B) Photos of individuals from families 1 and 2: individuals 1a and 1b at 5.5 years of age, individual 2a at 7.5 years, and individual 2b at 9 months.

approved by the ethics committees of the institutions where the families were seen. We hypothesize that *PIGS* mutations cause a deficiency of GPI-AP biosynthesis in these individuals and that this results in severe global developmental delay, hypotonia, seizures, weakness, balance problems, ataxia, and dysmorphic facial features. The biochemistry and phenotype of this putative IGD are discussed in the context of the other known GPI-deficiency disorders.[1]

An overview of the affected individuals' symptoms is presented in Table 1. Additional detailed clinical descriptions are provided in the Supplemental Data. In family 1, individuals 1a and 1b are monozygotic twin brothers born to unrelated parents of European descent (Figure 1A). In family 2, individuals 2a and 2b are the only children born to Mexican parents with an otherwise unremarkable family history (Figure 1A). These individuals share a phenotype of hypotonia, severe global developmental delay, seizures, visual impairment, CNS atrophy on radiological studies, and hand anomalies (including brachydactyly and clinodactyly). Characteristic facial features, as seen in Figure 1B, include coarse facies with arched eyebrows, thickened helices, gingival hypertrophy, broad tongue, and nystagmus. Two fetuses, 3a and 3b, from family 3 had multiple joint contractures, consistent with fetal akinesia. In addition, individual 3a had a thickened nuchal fold, and individual 3b had glomerular cysts and a cystic hygroma. The pregnancies were terminated after 19 weeks for fetus 3a and after 13 weeks for fetus 3b.

By WES, we found heterozygous *PIGS* mutations, including c.108G>A (p.Trp36*) and c.101T>C (p.Leu34Pro) (GenBank: NM_033198.3), in individuals 1a and 1b in family 1. WGS was performed for individuals 2a and 2b in family 2, and they were found to have the homozygous mutation c.1316_1352delCCACCA CCCTTACCTCCCTGGCGCAGCTTCTGGGCAAinsGGTT GCT (p.Thr439_Lys451delinsArgLeuLeu) within a region of homozygosity (ROH). Although the parents were not known to be consanguineous, both were from the same small rural community, which most likely explains the homozygosity. This in-frame deletion-and-insertion event was not observed in the ExAC Browser or NHBLI Exome Sequencing Project Exome Variant Server. It results in the insertion of 7 bases and the deletion of 37 bases within a highly conserved region of *PIGS*. *In silico* analyses predicted this variant to be damaging. Finally, two individuals (3a and 3b) were compound heterozygous for the missense mutation c.923A>G (p.Glu308Gly) and the splicing mutation c.468+1G>C (Figure 2).

To study the expression of *PIGS* in individuals 1a and 1b, we used B lymphoblastoid cell lines (LCLs) established by Epstein-Barr virus immortalization of peripheral-blood mononuclear cells (PMBCs) of these individuals, as well as healthy control individuals, for real-time PCR and western blotting. The results indicated a decrease in *PIGS* expression of up to 50% in qPCR and a significant decrease in protein levels in western blotting (Figure 3). This indicates that the stop codon introduced by c.108G>A results in the low PIGS mRNA and protein levels in these individuals.

We next assessed whether the GPI-anchoring process was deficient in individual cells. To determine whether individual cells had reduced cell-surface expression of GPI-APs, we stained whole-blood samples from four affected individuals and control individuals with fluorescent antibodies for GPI-APs (CD16, CD55, and CD59), as well as with fluorescein-labeled proaerolysin (FLAER), which binds to the GPI anchor itself, and performed fluorescence-activated cell sorting (FACS) analysis to assess relative fluorescence.[28] Analysis on granulocytes indicated that individual cells had less signal of CD16 (all individuals) and CD55 and CD59 (individuals 1a and 1b) than age-matched control cells (Figure 4).

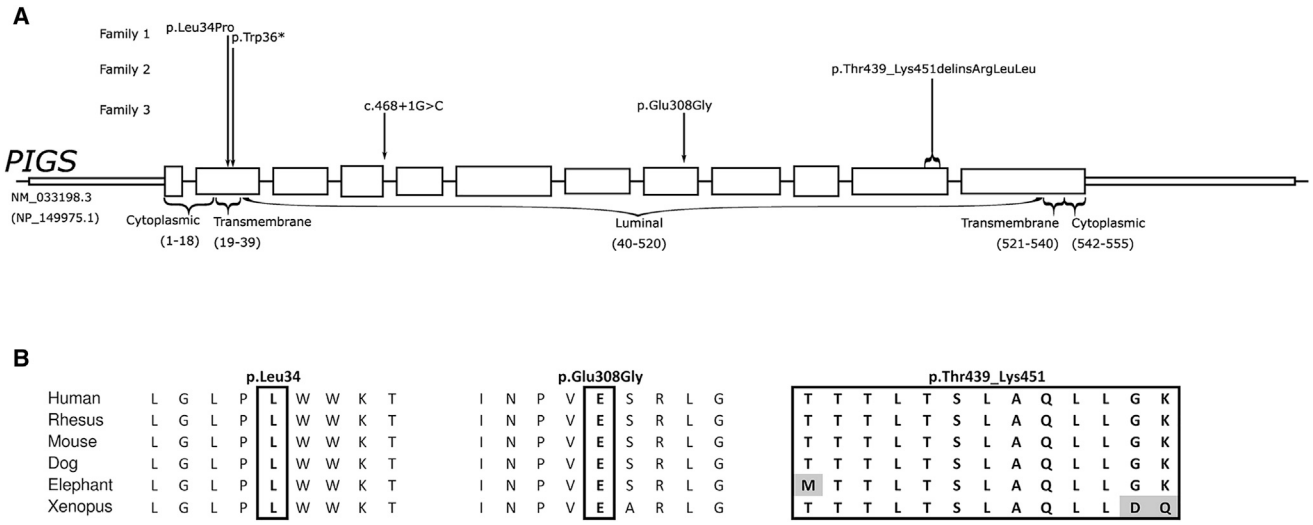

Figure 2. Mutations in *PIGS*
(A) Location of the mutations in *PIGS* and the corresponding protein. Introns are not drawn to scale.
(B) Conservation in vertebrates of the amino acids affected by missense mutations and indels.

In all of these individuals, the level of FLAER in lymphocytes was also significantly downregulated (Figure 5). Thus, the compound-heterozygous mutations c.108G>A (p.Trp36*) and c.101T>C (p.Leu34Pro) in family 1 and the homozygous mutation c.1316_1352del CCACCACCCTTACCTCCCTGGCGCAGCTTCTGGGCAA insGGTTGCT (p.Thr439_Lys451delinsArgLeuLeu) in family 2 in *PIGS* result in low amounts of GPI-AP in peripheral white blood cells. We conclude that insufficient amounts of PIGS and/or defective PIGS function in individual cells leads to reduced amounts of GPI-AP at the cell surface.

To study the role of each mutation found in individuals 1a and 1b, we established PIGS-knockout HEK293 cells with the CRISPR-Cas9 system and transfected them with wild-type or mutant pME-FLAG hPIGS (a strong SRαpromoter), pTK-FLAG hPIGS (a weaker, thymidine kinase promoter), or pTA-FLAG hPIGS (a weak TATA-box-only promoter). FACS analysis was performed 3 days after transfection with FLAER, CD55 (DAF), and CD59.

The results showed that, compared with the wild-type, the Trp36* mutant only partially restored the surface expression of GPI-APs on HEK293 cells even when driven by a strong promoter, whereas Leu34Pro mutants showed slight but significant reduction in restoration even when driven by a weak promoter (Figures 6 and 7). It is interesting to note that the p.Leu34Pro variant is in a helical transmembrane domain and affects a residue with a low missense tolerance ratio (MTR).[43] Western blots from cell lysates of N-terminal FLAG-tagged Trp36* mutants with a FLAG-tagged antibody showed almost no FLAG-PIGS expression, whereas similar expression was observed between the wild-type and the Leu34Pro mutant (Figure 8). Western blots from cell lysates of C-terminal HA-tagged Trp36* mutants with an HA-tagged antibody showed two truncated isoforms of PIGS-HA (Figure 8). This indicates that the PIGS proteins that start from the downstream methionines have some residual activities.

We next analyzed the amount of GPI-AP in amniocytes from individual 3b by using FLAER, as well as GPI markers

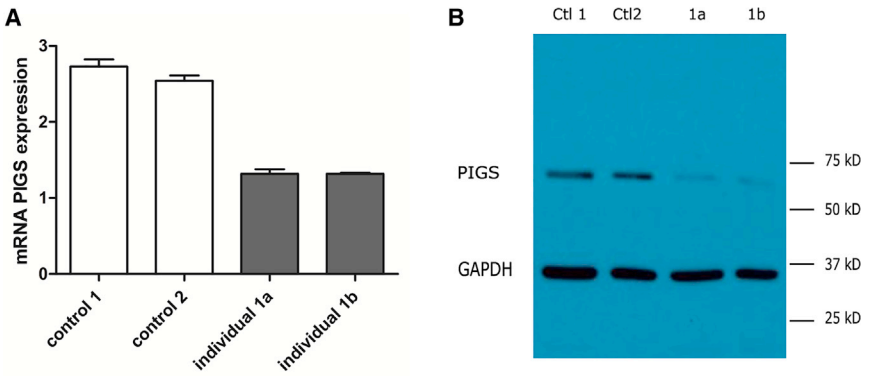

Figure 3. *PIGS* Expression in Individuals with *PIGS* Mutations
(A) Real-time PCR on subject LCL extracts shows that the affected males have reduced transcript levels of PIGS. RNA extractions from LCLs of individuals 1a and 1b were subjected to qRT-PCR according to the ΔCt method. The results were normalized to TBP expression from quadruplicate experiments. Error bars represent standard errors (n = 3).
(B) Western blot using a specific antibody against human PIGS and anti-GAPDH as a reference protein on LCLs from individuals 1a and 1b.

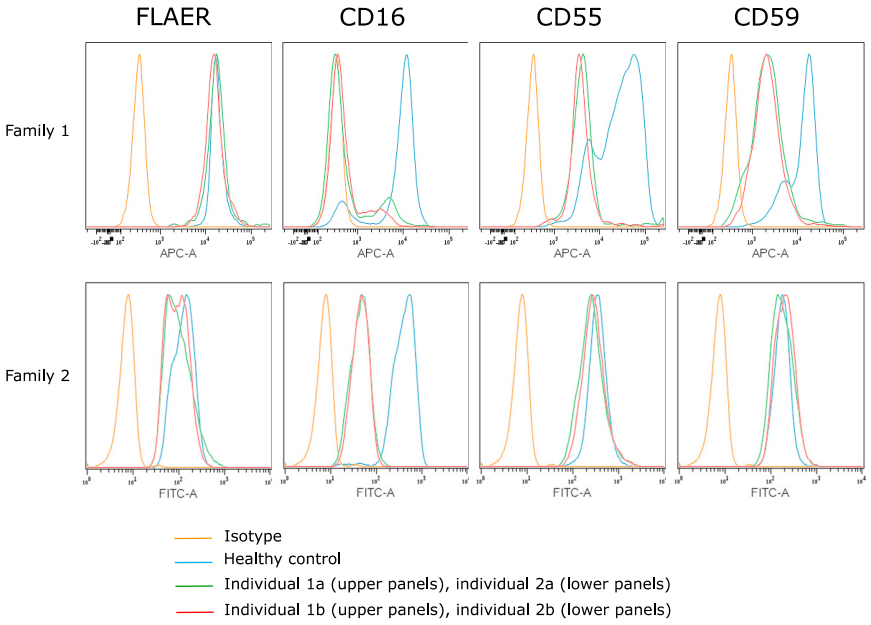

Figure 4. Impact of the *PIGS* Mutations on Individual Granulocyte Cell-Surface GPI-APs

Red blood cells were lysed in BD FACS lysing solution (BD Biosciences) from fresh blood of the individuals in families 1 and 2 and control cells, and then the samples were stained with GPI-AP markers (FLAER, CD16-FITC, CD55-FITC, and CD59-FITC) for 20 min on ice. The nonspecific binding was washed before analysis by the BD FACSCanto II system. Shown is a representative analysis of the amount of cell-surface GPI-AP on granulocytes from triplicate experiments.

CD24, CD55, CD59, and CD73 (Figure 9). CD3, a non-GPI-AP marker, was used as a negative control. FACS analyses indicated decreases in all used markers in the individual cells, and the highest reduction was found for CD24, which was 85% lower than in similar gestation-age control cells.

Here, we describe a recessive genetic GPI biosynthesis disorder caused by bi-allelic *PIGS* mutations that result in loss of function and decreased GPI-AP expression on flow cytometry. The index subjects share a core phenotype of coarse facial features, seizures, hypotonia, and developmental delay, which overlap the phenotype of other IGDs that result from failure to synthesize functional GPI-APs. Contractures as seen in family 3 are also seen with *PIGA*, *PIGY* (MIM: 616809), and *PIGG* (MIM: 616917) mutations.[9,29,44]

This disorder results from the failure of the GPI transamidase complex, which includes PIGS,[45] to transfer the GPI anchor to the precursor protein bearing a GPI-attachment signal sequence. PIGS and PIGT are members of the GPI transamidase complex and have been demonstrated to be essential for the formation of carbonyl intermediates during the transfer of the GPI group to the protein.[45,46] The *PIGS* pattern of reduced GPI-AP resembles the *PIGT* (MIM: 610272)-associated reduction in CD16 and FLAER on granulocytes.[32]

The biochemical analysis of the consequences of the *PIGS* variants supports this observation. We have shown that individuals 1a and 1b in family 1 have low amounts of PIGS mRNA and protein. PIGS is

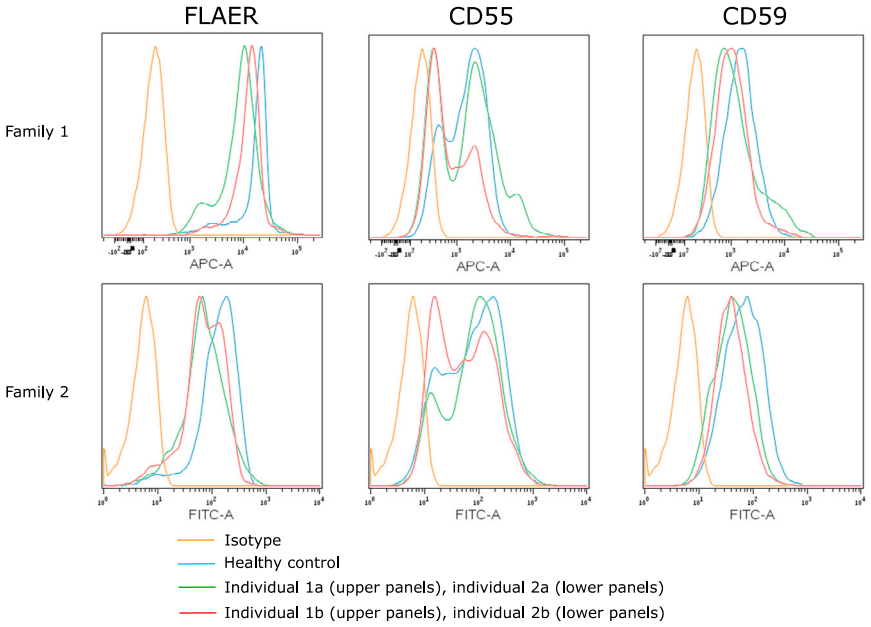

Figure 5. Impact of the *PIGS* Mutations on Individual Lymphocyte Cell-Surface GPI-Aps

Flow-cytometry analysis of lymphocytes from the same experiments described in Figure 4. Shown is a representative analysis of the amount of cell-surface GPI-AP on lymphocytes from triplicate experiments.

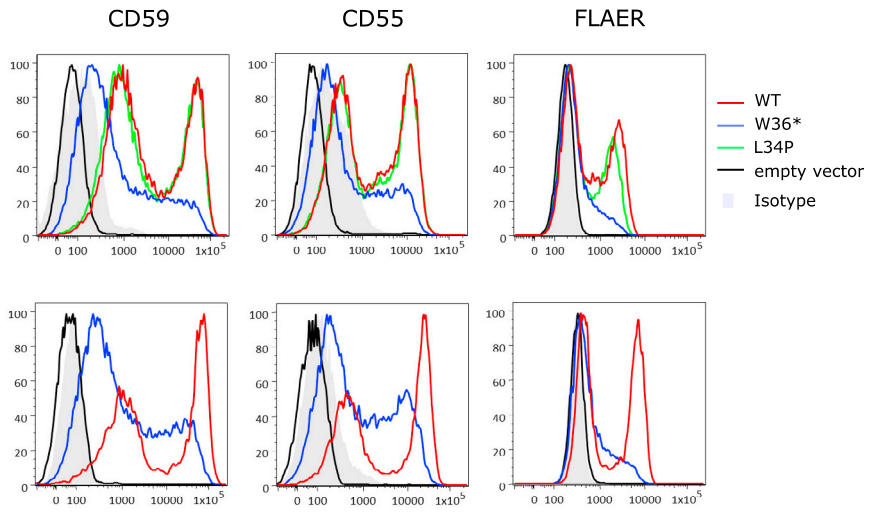

Figure 6. PIGS Functional Assay for Mutations Found in Individuals 1a and 1b
PIGS-deficient HEK293 cells were transfected with wild-type or mutant pME-FLAG hPIGS (top) or pME-HA hPIGS (bottom). FACS analysis was performed 3 days later.

downregulated or expressed as a truncated isoform in PIGS-deficient HEK293 cells transfected with mutant *PIGS* (c.108G>A [Trp36*]), which leads to a decrease in PIGS function, as demonstrated by rescue assays of GPI-AP expression at the cell surface. In addition, all available blood samples from affected individuals showed reduced cell-surface expression of GPI-APs, including CD16 in granulocytes and FLAER in lymphocytes. It is worth noting, however, that whereas individuals 1a and 1b showed reduced CD55 and CD59, we found these to be normal for individuals 2a and 2b. The low signals seen for all GPI-AP markers in individual 3b amniocytes correlates with the severe phenotypes caused by the mutations found in individuals 3a and 3b.

In addition to having biochemical similarities, disruptions of *PIGS* and *PIGT* result in phenotypic similarities. This is consistent with the fact that both PIGS and PIGT are components of the GPI transamidase complex and are essential for the formation of the carbonyl intermediates necessary when the transamidase complex transfers the GPI group to the protein.[45,46]

The phenotypic features resulting from disruption of *PIGS*, including coarse facies, developmental delay, hypotonia, and seizures, overlap not just those of other IGDs but also those of other types of CDGs (MIM: PS212065) and numerous other Mendelian disorders. Many of these disorders can be distinguished on the basis of physical exam findings or biochemical findings (e.g., peroxisomal disorders and mucopolysaccharidoses). The findings of brachytelephalangy in conjunction with seizures and intellectual disability could be considered for DOORS syndrome (MIM: 220500),[47] which is caused by mutations in *TBC1D24* (MIM: 613577). Laboratory findings that can help distinguish *PIGS*-associated disorders from other Mendelian disorders include flow-cytometry analysis with reduced CD16 expression (with or without reduced CD55 expression), a lack of elevated serum tissue-nonspecific alkaline phosphatase (observed to be elevated in a number of other IGDs but normal in others), a lack of 2-oxoglutaric aciduria (occasionally seen with *TBC1D24* mutations), and normal serum transferrin isoelectric focusing (abnormal with several

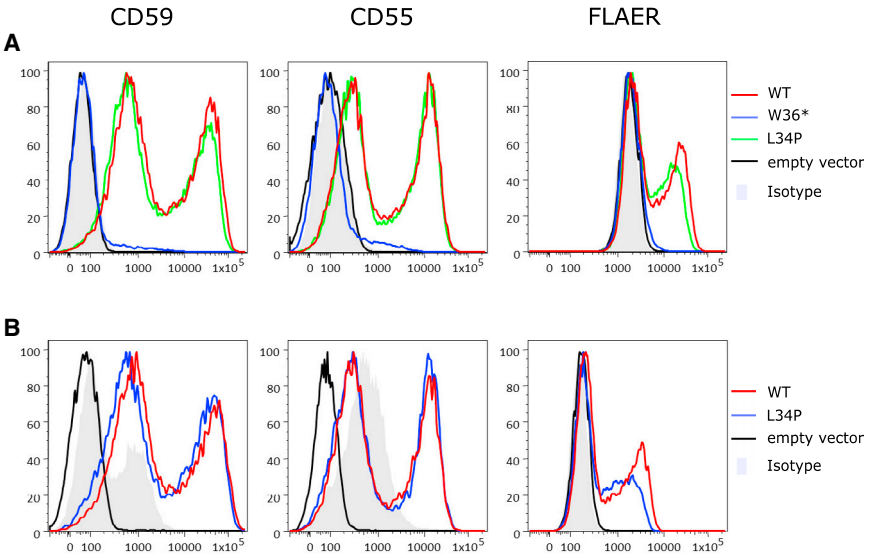

Figure 7. PIGS Functional Assay with pTK and pTATA Promoters for Mutations Found in Individuals 1a and 1b
(A) PIGS-knockout HEK293 cells were transfected with wild-type or mutant pTK-FLAG hPIGS (PIGS driven by a weaker, thymidine kinase promoter). FACS analysis was performed 3 days later. The geometric means decreased by 97% and 24% for CD59 by 65% and 19% for FLAER with the c.108G>A (p.Trp36*) and c.101T>C (p.Leu34Pro) mutations, respectively.
(B) PIGS driven by a weak TATA-box-only promoter. The geometric means decreased by 18% for CD59 and by 25% for FLAER with the c.101T>C (p.Leu34Pro) mutation.

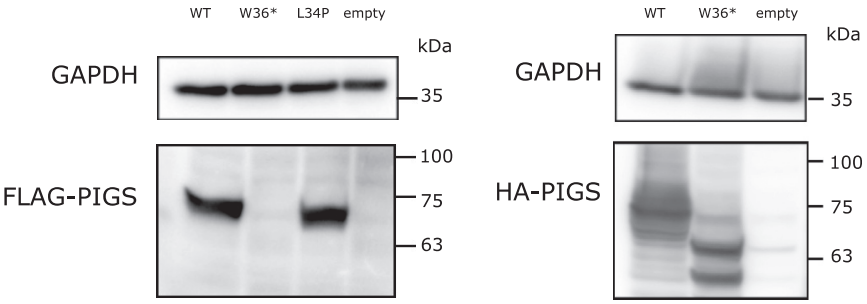

Figure 8. Expression of FLAG- and HA-Tagged PIGS in PIGS-Deficient HEK293 Cells Transfected with *PIGS* Mutants
Cell lysates from PIGS-deficient HEK293 cells transfected with N-terminal FLAG-tagged and C-terminal HA-tagged wild-type or mutants were used for western blot analysis using FLAG tag and HA tag antibodies, respectively.

CDGs). These can help support the diagnosis for those whose access to sequencing is limited or in the interpretation of variants of unknown significance identified on genetic testing.

It is noteworthy that pyridoxine hydrochloride improved the seizures in one of the affected probands. This success has been observed in other IGDs.[28,48] Given that many of the IGDs can manifest with early-onset seizures, a trial of pyroxidine hydrochloride would be warranted as part of the epilepsy treatment. Similarly, the diagnosis of an IGD should be considered among the causes of pyridoxine-responsive epilepsies. The ketogenic diet could be another effective treatment for epilepsy given that Joshi et al. reported two *PIGA*-deficiency-affected siblings who became seizure-free on a ketogenic diet.[49]

Collectively, these data provide evidence of the existence of a *PIGS*-disease relationship that shares some of the characteristics of IGDs. Studies using an animal model could represent a good strategy for investigating the pathophysiology of *PIGS* mutations. Because GPI-APs are widely expressed during mammalian development and their absence frequently results in lethal global deficits in knockout mouse models, a preferred strategy might involve using CRISPR-Cas9 to knock human *PIGS* mutations into mice. As more IGDs are identified, it might become possible to discover genotype-phenotype correlations that could be useful in predicting the severity of a condition on the basis of the disrupted step in the pathway.[1]

Accession Numbers

The mutations reported in this paper have been deposited in LOVD at https://databases.lovd.nl/shared/genes/PIGS.

Supplemental Data

Supplemental Data include more clinical details on the affected individuals and can be found with this article online at https://doi.org/10.1016/j.ajhg.2018.08.014.

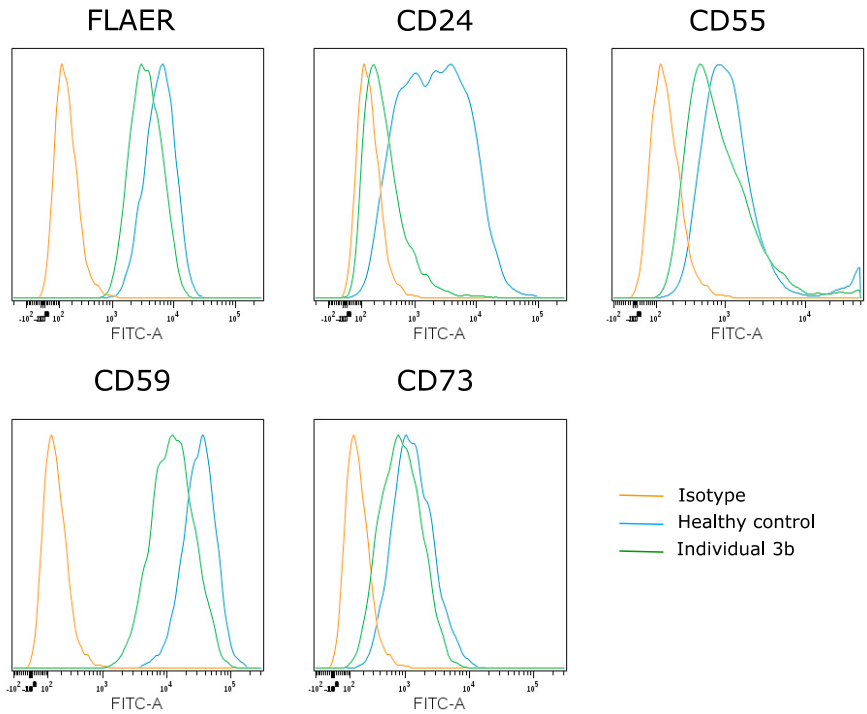

Figure 9. Impact of the *PIGS* Mutations on Individual 3b Amniocyte Cell-Surface GPI-APs
15-week-old amniocytes derived from individual 3b and similarly aged amniocytes from healthy control individuals were stained with FLAER, CD24, CD55, CD59, and CD73 for 1 hr on ice, nonspecific bindings were washed, and cells were then fixed in paraformaldehyde before analysis by the BD FACSCanto II system. Shown is a representative analysis of the amount of cell-surface GPI-AP on amniocytes of individual 3b and a control individual from triplicate experiments.

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Declaration of interests

D.L.P. and R.J.T. are employees of Illumina Inc., which sells technologies that can be used to diagnose this condition.

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Web Resources

ExAC Browser, http://exac.broadinstitute.org/
GenBank, http://www.ncbi.nlm.nih.gov/genbank/
Leiden Open Variation Database (LOVD), https://databases.lovd.nl/shared/genes/PIGS
OMIM, http://www.omim.org/
UniProt, http://www.uniprot.org/uniprot/

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