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Supplemental Data

Mutations in GDP-Mannose Pyrophosphorylase B

Cause Congenital and Limb-Girdle Muscular Dystrophies

Associated with Hypoglycosylation of α-Dystroglycan

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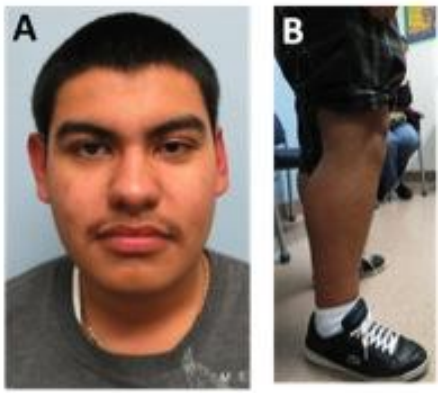

Figure S1. Ophthalmologic and Muscle Findings in P3

Asymmetric ptosis and strabismus (left-sided esotropia) (A) and pseudohypertrophy of the calf muscles (B) are seen in P3 at 16 years of age.

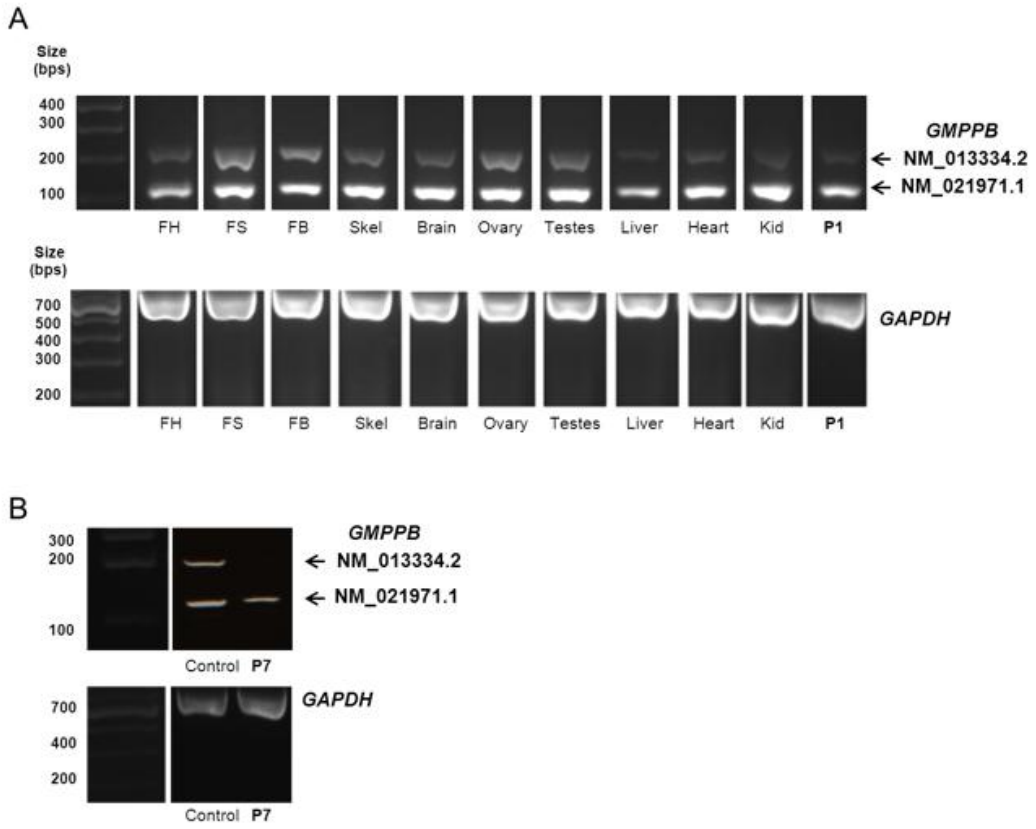

Figure S2. RT-PCR Shows Widespread Expression of *GMPPB* in Human Tissues

Human *GMPPB* is transcribed as two isoforms: NM_021971.1 and NM_013334.2. The coding DNA sequence of NM_021971.1 is 1083 bp and 10 exons long. The coding DNA sequence of NM_013334.2 is 1164 bp and 8 exons long. The first exon of NM-021971.1 encodes the 5'-UTR which is transcribed into mRNA. Exon 2-7 of NM-021971.1 and exons 1-7 of NM-013334.2 are identical. Exon 8 in NM-013334.2 contains the same sequence as exon 9 and exon 10 in NM-021971.1 but also contains an additional 81 bp. We amplified a 138 bp fragment of NM_021971.1, which shows that this isoform is strongly expressed in all tissues tested. We amplified a 222 bp fragment of NM_013334.2, which shows that it is only weakly expressed in the tested tissues. A 731 bp fragment of *GAPDH* indicates near-equivalent amounts of input cDNA. We also amplified fragments amplified from cDNA reverse transcribed from RNA extracted from P1, P7, and control fibroblasts using an RNeasy kit (Qiagen, Crawley, UK). FH = fetal heart; FS = fetal skeletal muscle; FB = fetal brain; skel = skeletal muscle; kid = kidney; P1 = cDNA from P1 fibroblasts. P7 = cDNA from P7 fibroblasts, Control = cDNA from healthy control fibroblasts.

(A) In a library of total RNA from adult and fetal tissues *GMPPB* isoform NM_021971.1 is more highly expressed than *GMPPB* isoform NM_013334.2. This is also the case for P1 fibroblast cDNA. This data also suggests that there is no developmental difference in the expression of the two isoforms.

(B) P7's expression of *GMPPB* isoform NM_021971.1 cDNA is slightly reduced compared to the control cDNA and completely absent for expression of *GMPPB* isoform NM_013334.2.

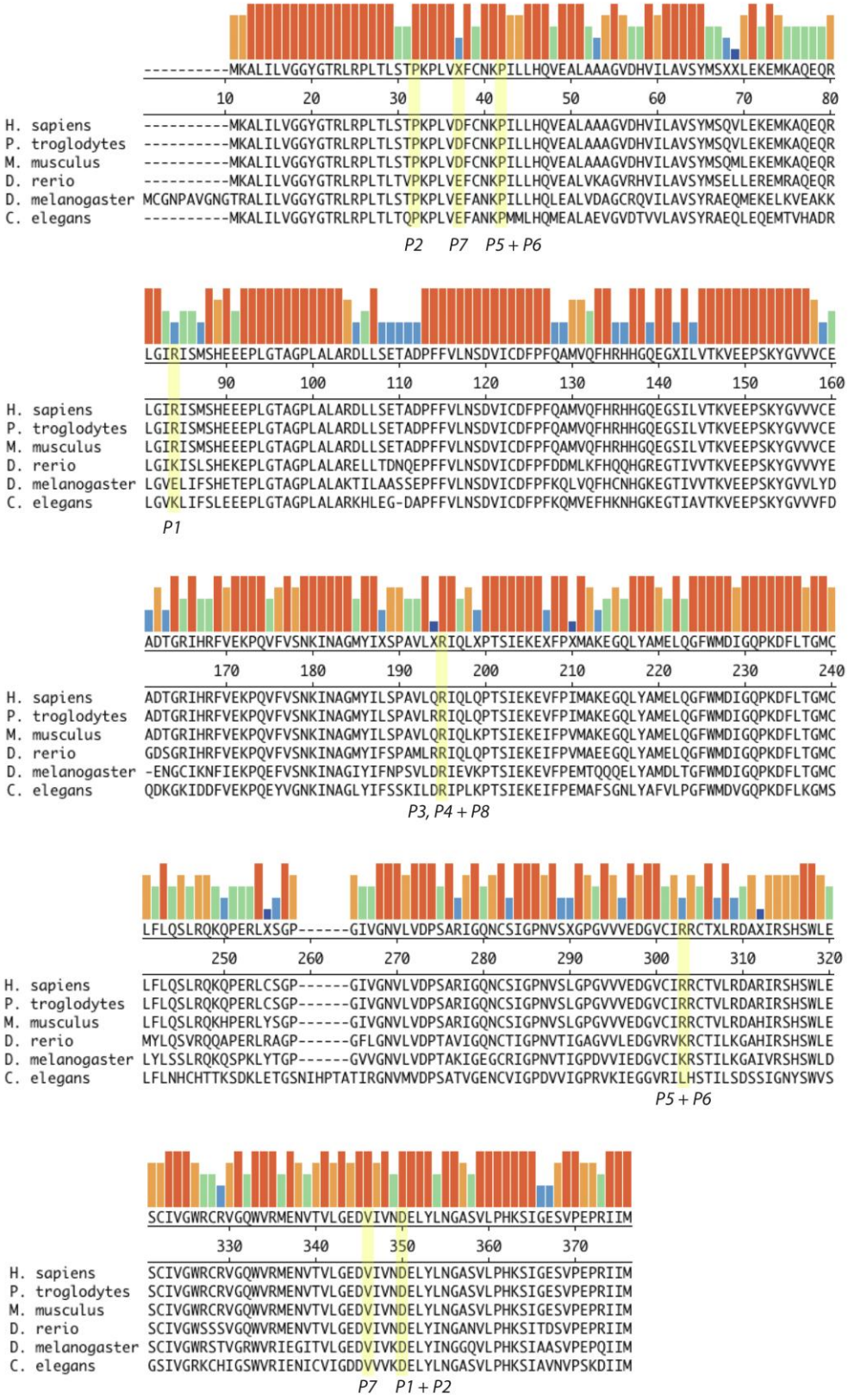

Figure S3. Protein Alignment Showing Conservation of GMPPB

Sequences are *Homo sapiens* (ENSP00000418565), *Pan troglodytes* (ENSPTRP00000025773), *Mus musculus* (ENSMUSP00000107914), *Danio rerio* (ENSDARP00000022618), *Drosophila melanogaster* (FBpp0078511), and *Caenorhabditis elegans* (C42C1.5). The height and colour of the bars indicates the degree of conservation of each amino acid residue, for example a red bar shows that a residue is conserved across all six species. The residues altered in our muscular dystrophy cases are highlighted in yellow.

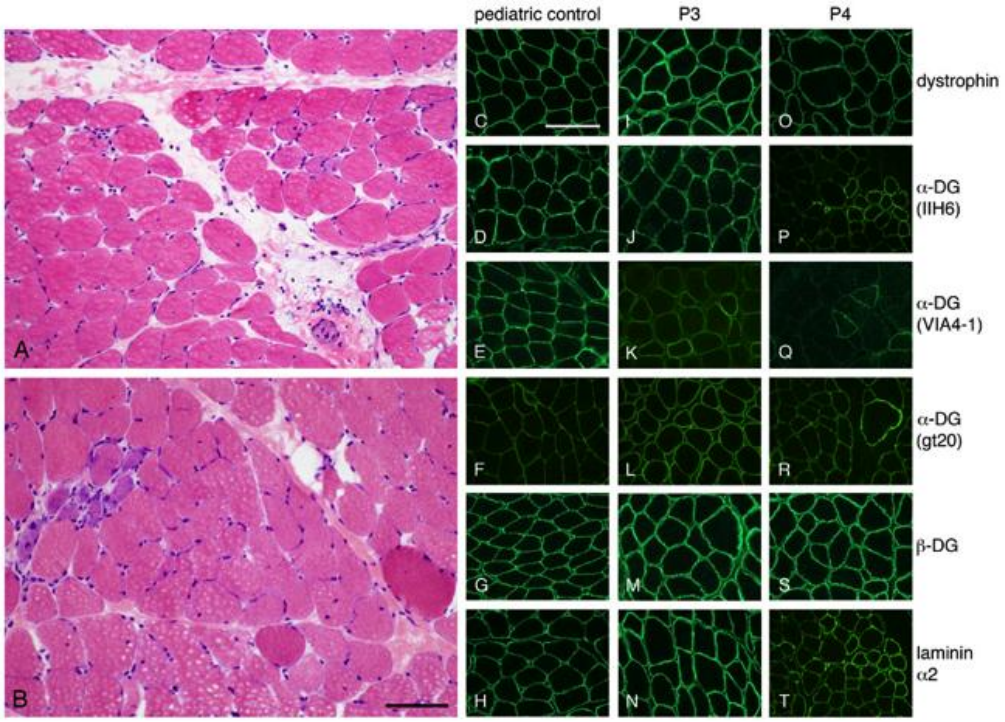

Figure S4. Histopathology and Immunostaining for P3 and P4

Mild to moderate dystrophic features were present (A = P3 and B = P4). Immunofluorescence abnormalities are shown in panels C-T. The size bars in panels B and C are 100 µm.

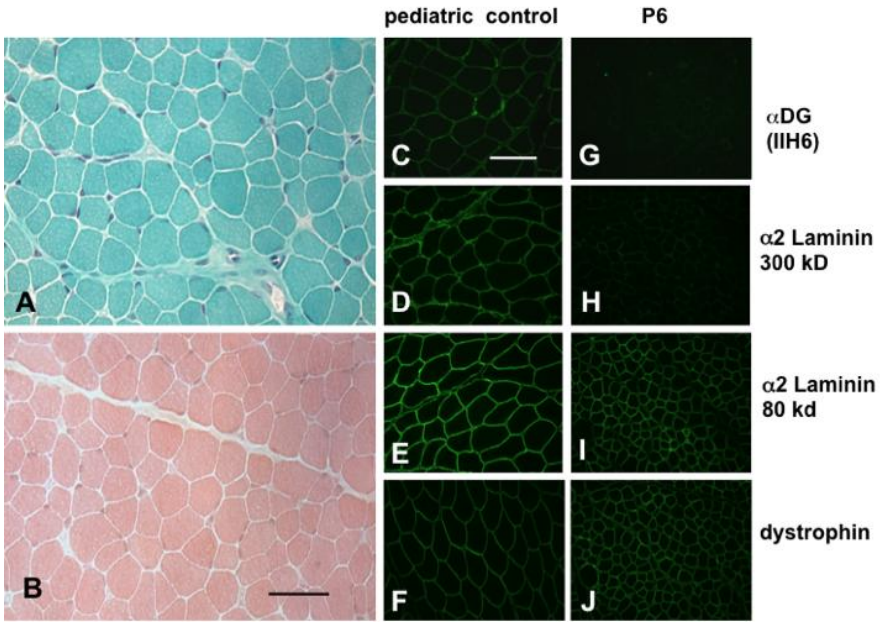

Figure S5. Skeletal Muscle Biopsy of P6 Shows Hypoglycosylation of α-DG

The left rectus femoris was biopsied at age 3. There was moderate variation in fiber size, very few necrotic and regenerating fibers (not shown) and mild interstitial fibrosis (A-Gomori's trichrome stain; B-H&E stain). Immunofluorescence showed normal expression of dystrophin and laminin-α2 (80 kD isoform), but strongly decreased staining with the glycoepitope-specific antibody IIH6 and for the laminin-α2 300kD isoform. In panels A and B the bar corresponds to 10 µm; in panels C-J the bar corresponds to 10 µm.

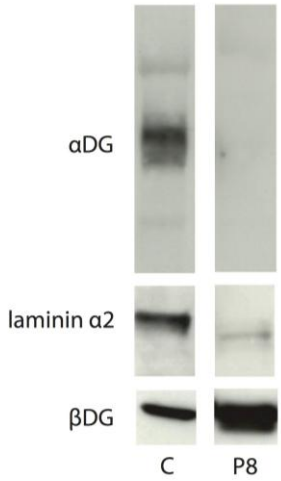

Figure S6. Immunoblot Analysis of Skeletal Muscle Protein Lysates from P8

The membrane was incubated with IIH6 and anti-β-DG and anti-laminin-α2 antibodies as previously described,[1] except that PVDF membranes were used along with Tris-Buffered Saline with Tween 20. P8 has a profound reduction in functional α-DG glycosylation. The β-DG band for P8 appears as a possible doublet, as is also seen in P1 and P4.

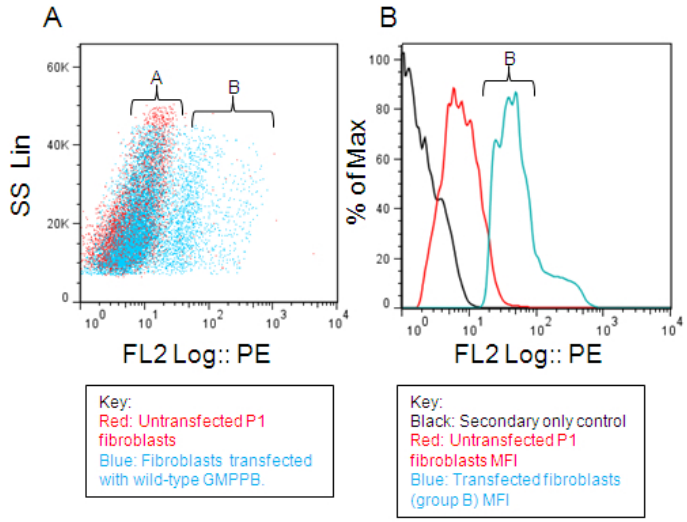

Figure S7. α-DG Glycosylation of P1 Fibroblasts Is Increased by Introduction of Wild-Type *GMPPB*

We transfected affected subjects' fibroblasts with wildtype *GMPPB* using Gene Juice (Merck Millipore, Nottingham, UK) in serum free media. These cells were grown in DMEM (Gibco for Life Technologies, Paisley, UK) supplemented with 20% fetal bovine serum (FBS, PAA, UK) and plated on 60 mm plates so that they were 90% confluent on the day of transfection. Approximately 10,000 fibroblasts were analyzed per sample per experiment. 12-16 hours after transfection, we quantified α-DG glycosylation of the cells by flow cytometry. P1 fibroblasts harbor the compound heterozygous *GMPPB* mutations c.1000G>A and c.220C>T. Transfection with the wildtype cDNA increased the amount of glycosylated α-DG (assessed by flow cytometry and the IIH6 antibody). Approximately 10,000 fibroblasts were analyzed per sample per experiment. Untransfected P1 fibroblasts had a MFI of 24.7 while the transfected P1 fibroblasts had a MFI of 55.6 ($p = 0.0015$). 23.5% of the transfected fibroblasts (bracketed population B) had an MFI of 76.2. Untransfected P1 fibroblasts were compared to P1 fibroblasts transfected with an empty vector and no significant change was observed in the MFI between the two populations (data not shown). SS Lin = side scatter linear scale.

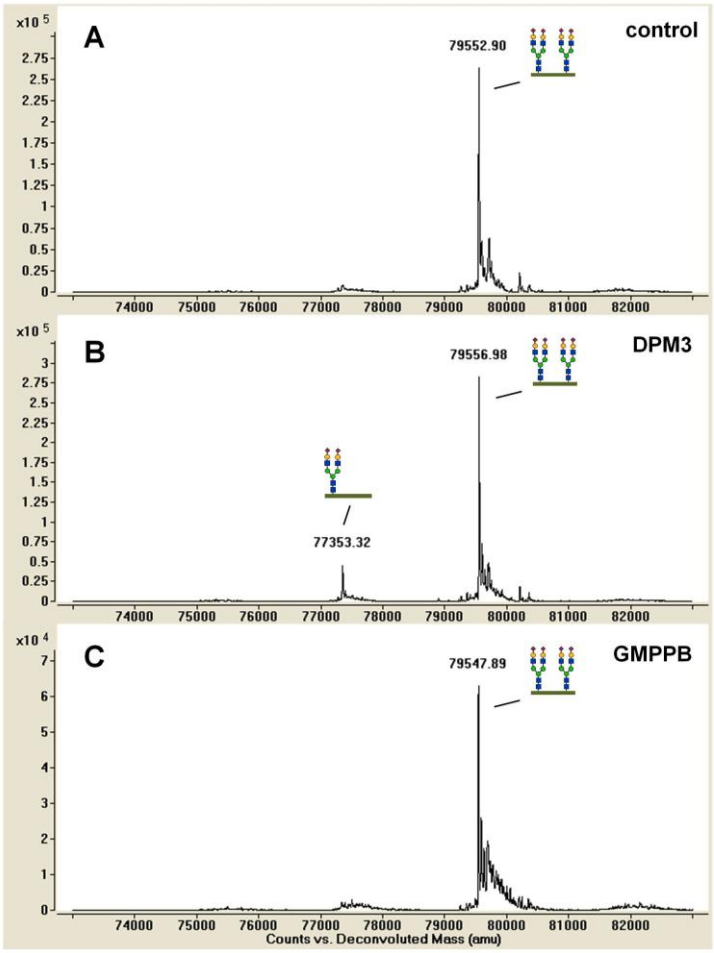

Figure S8. High-Resolution Mass Spectrometry of Intact Transferrin Isolated from Serum for Analysis of N-glycosylation

A 5 µl serum sample was incubated with anti-transferrin beads as described.[2] The eluate was analyzed on a microfluidic 6540 LC-chip-QTOF instrument (Agilent Technologies) using a C8 protein chip. Data analysis was performed using Agilent Mass Hunter Qualitative Analysis Software B.04.00. The Agilent BioConfirm Software was used to deconvolute the charge distribution of raw data to reconstructed mass data.

(A) Serum transferrin of a control, showing transferrin with two complete N-glycans as the dominant peak.

(B) Serum transferrin of an individual with DPM3-CDG,[3] showing a partial loss of complete N-glycans (mass 77353), which is characteristic for CDG type I defects.

(C) Serum transferrin of GMPPB in P6 showing a normal N-glycosylation of transferrin, without signs of a loss of complete N-glycans.

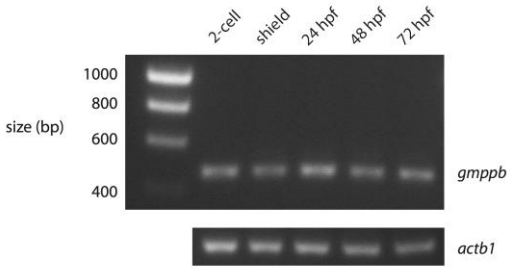

Figure S9. RT-PCR Shows Expression of *gmppb* throughout Early Zebrafish Development

We performed RT-PCR on RNA extracted from wildtype zebrafish embryos at the five developmental stages indicated. Expression of *gmppb* was detected at each stage, using PCR amplification of a 500 bp fragment of *gmppb* cDNA. An *actb1* fragment indicates near-equivalent amounts of input cDNA.

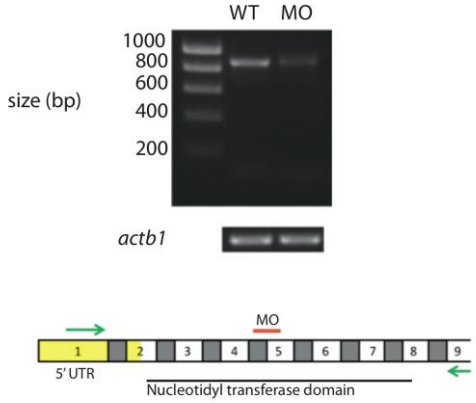

Figure S10. The *gmppb* Splice Blocking Morpholino Disrupts RNA Splicing

We extracted RNA from 48 hpf wildtype zebrafish embryos (WT) alongside embryos injected with the *gmppb* splice blocking morpholino (MO) (2.5 ng, 5 ng and 7.5 ng pooled). We performed RT-PCR and PCR amplified a ~900 bp fragment of *gmppb* cDNA using primers (indicated by green arrows on schematic diagram) that bind either side of the morpholino binding site (indicated by red line). A clear reduction in band intensity is seen in the MO embryos, indicating that the morpholino disrupts correct mRNA splicing. An *actb1* fragment indicates near-equivalent amounts of input cDNA.

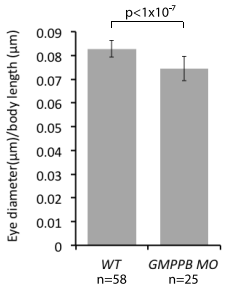

Figure S11. *gmppb* MO Embryos Have Significantly Smaller Eyes than Wild-Type

We measured the eye diameter of *gmppb* morphants and wildtype embryos and normalized each eye diameter measurement with the embryo's body length. Statistical significance was assessed using an unpaired two-tailed t-test.

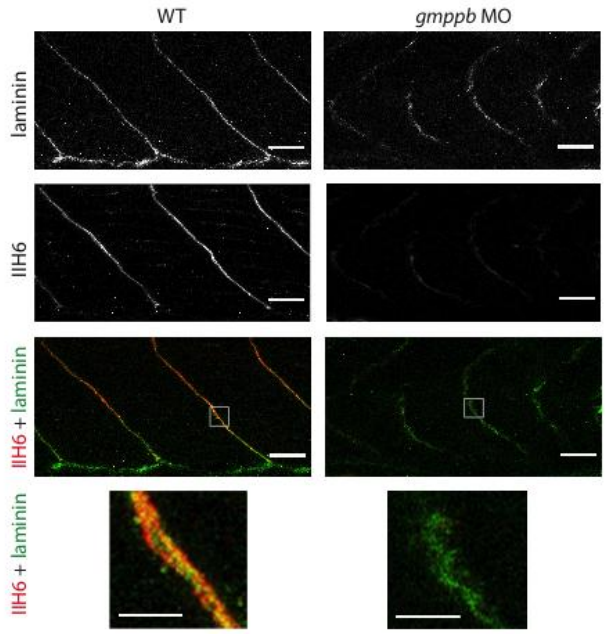

Figure S12. Staining Using IIH6 and Anti-laminins Antibodies Shows that α-DG of *gmppb* Morphants Is Hypoglycosylated Relative to Wild-Type

Antibodies used are against laminins, (polyclonal antibody L9393, Sigma-Aldrich, Dorset, UK), and glycosylated α-DG (monoclonal antibody IIH6, from Professor Kevin Campbell). Staining of laminins revealed abnormal myosepta structure, while the fluorescent intensity of IIH6 epitope was significantly reduced in *gmppb* morphants. White square indicates magnified area. Scale bar = 25 µm except for magnified area where scale bar = 6 µm.

Table S1. Primers Used to Analyze *GMPPB* Expression in Human Tissues

Gene	Fragment size (bp)	Direction	Primer Sequence (5'–3')
GMPPB	138 (NM_021971.1) or 222 (NM_013334.2)	Forward	gtggtcgaagatggtgtgtg
		Reverse	cactgtcacgttctccatgc
GAPDH	731	Forward	cccatcaccatcttccagga
		Reverse	ttgtcaccaggaatgagc

Table S2. Primers Used in *GMPPB* pcDNA 3.1/V5-HIS TOPO Construction and Mutagenesis

Function	Direction	Primer Sequence (5'–3')
Amplify *GMPPB* CDS from IMAGE clone	Forward	atgaaggcactgatcttagtgg
	Reverse	catgatgatacgaggctctggc
Introduce p.Asp334Asn (P1 and P2)	Forward	gggtgaggacgtcatagttaat**a**atgagctctacctc
	Reverse	gaggtagagctcat**t**attaactatgacgtcctcacc c
Introduce p.Pro22Ser (P2)	Forward	gacgctgagcacc**t**cgaagccactggt
	Reverse	accagtggcttcg**a**ggtgctcagcgtc
Introduce p.Arg185Cys (P3, P4 and P8)	Forward	gcagtgctgcgg**t**gcatccagctgc
	Reverse	gcagctggatgc**a**ccgcagcactgc
Introduce p.Pro32Leu (P5 and P6)	Forward	ggacttctgcaataagc**t**catcttgctgcaccaag
	Reverse	cttggtgcagcaagatg**a**gcttattgcagaagtcc
Introduce p.Arg287Gln (P5 and P6)	Forward	ggtgtgtgtatcc**a**gcggtgcacggtg
	Reverse	caccgtgcaccgc**t**ggatacacacacc
Introduce p.Asp27His (P7)	Forward	ccgaagccactggtg**c**acttctgcaataagc
	Reverse	gcttattgcagaagt**g**caccagtggcttcgg
Introduce p.Val330Ile (P7)	Forward	cagtgctgggtgaggac**a**tcatagttaatgatgag
	Reverse	ctcatcattaactatga**t**gtcctcacccagcactg

The bold nucleotide indicates the mutation.

Table S3. Primers Used to Analyze *gmppb* Expression in Early Zebrafish Development, and Splicing Disruption in *gmppb* Knockdowns

Function	Direction	Primer Sequence (5'–3')
Expression analysis and MO-flanking	Forward	tacagcagcaggtcaatcgt
Expression analysis	Reverse	acaacaatggtgccctctct
MO-flanking	Reverse	gttctgcccaatcactgctg

The sequences of the *actb1* primers have been previously described.[4]

Table S4. Variant Filtering of Exome Data for P1

Filter	Number of Variants
Total number of high quality PASS variants	47146
Removed variants in dbSNP132, 1000G, UK10K, controls	1991
Functional variants	482
Loss-of-function variants (heterozygous)	22
Genes with 2 functional variants (possible compound heterozygous)	2 (*MAN1B1* and *GMPPB*)

Table S5. Population Frequency of *GMPPB* Mutations Identified in Affected Individuals

Case	Genomic Position (Build 37)	Mutation (cDNA Change)	Amino Acid Residue Change (Protein)	Sequencing Method	rsID	MAF (Source)
P1	3:49760726	c.220C>T	p.Arg74*	Exome	None	None
	3:49759268	c.1000G>A	p.Asp334Asn		None	None
P2	3:49761096	c.64C>T	p.Pro22Ser	Sanger	None	None
	3:49759268	c.1000G>A	p.Asp334Asn		None	None
P3	3:49759946	Hom c.553C>T	p.Arg185Cys	Exome	None	None
P4	3:49759946	Hom c.553C>T	p.Arg185Cys	Sanger	None	None
P5	3:49760905	c.95C>T	p.Pro32Leu	Exome	None	None[b]
	3:49759490	c.860G>A	p.Arg287Gln		rs142908436	0.00041[a]
P6	3:49760905	c.95C>T	p.Pro32Leu	Exome	None	None[b]
	3:49759490	c.860G>A	p.Arg287Gln		rs142908436	0.00041[a]
P7	3:49761081	c.79G>C	p.Asp27His	Sanger	rs142336618	0.001[a]
	3:49759280	c.988G>A	p.Val330Ile		rs199922550	0.001[c]
P8	3:49759946	Hom c.553C>T	p.Arg185Cys	Exome	None	None

MAF = minor allele frequency (out of 1). [a]MAF data from UK10K twins cohort. [b] Although this change has not been reported, another variant involving this nucleotide has (3:49760905C>A; MAF = 0.000077).[5] [c]MAF data from ClinSeq study.[6]

Table S6. Percentage Identity of GMPPB Ortholog of Five Diverse Eukaryotic Species with Human GMPPB

Species	GMPPB Ortholog Name	Ensembl Identifier	% ID of GMPPB Ortholog with Human GMPPB
Pan troglodytes	GMPPB	ENSPTRP00000025773	99.7
Mus musculus	GMPPB	ENSMUSP00000107914	98.1
Danio rerio	GMPPB	ENSDARP00000022618	81.4
Drosophila melanogaster	CG1129	FBpp0078511	70.2
Caenorhabditis elegans	TAG-335	C42C1.5	63.8

Orthologs identified by BLAST alignment of the *H. sapiens* GMPPB sequence (ENSP00000418565) against the genomes of the species shown.

Table S7. Transfection with Wild-Type *GMPPB* Increases α-DG Glycosylation for P1 Fibroblasts

	P1	Control
Untransfected cell MFI	24.7	82
Transfected cell MFI (all cells)	55.60	80.9
Transfected cell w/ higher MFI than untransfected cells (subpopulation)	76.2	n/a
n	3	3
p	0.0015	NS

MFI= mean fluorescence intensity of IIH6 positive cells. N= number of repeat experiments. P values are for a paired two-tailed *t*-test comparing untransfected cell MFI to transfected cell MFI (total). P<0.05=significant. NS= not significant.

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