A mouse model of a human congenital disorder of glycosylation caused by loss of PMM2

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Abstract

The most common congenital disorder of glycosylation (CDG), phosphomannomutase 2 (PMM2)-CDG, is caused by mutations in PMM2 that limit availability of mannose precursors required for protein N-glycosylation. The disorder has no therapy and there are no models to test new treatments. We generated compound heterozygous mice with the R137H and F115L mutations in Pmm2 that correspond to the most prevalent alleles found in patients with PMM2-CDG. Many Pmm2R137H/F115L mice died prenatally, while survivors had significantly stunted growth. These animals and cells derived from them showed protein glycosylation deficiencies similar to those found in patients with PMM2-CDG. Growth-related glycoproteins insulin-like growth factor (IGF) 1, IGF binding protein-3 and acid-labile subunit, along with antithrombin III, were all deficient in Pmm2R137H/F115L mice, but their levels in heterozygous mice were comparable to wild-type (WT) littermates. These imbalances, resulting from defective glycosylation, are likely the cause of the stunted growth seen both in our model and in PMM2-CDG patients. Both Pmm2R137H/F115L mouse and PMM2-CDG patient-derived fibroblasts displayed reductions in PMM activity, guanosine diphosphate mannose, lipid-linked oligosaccharide precursor and total cellular protein glycosylation, along with hypoglycosylation of a new endogenous biomarker, glycoprotein 130 (gp130). Over-expression of WT-PMM2 in patient-derived fibroblasts rescued all these defects, showing that restoration of mutant PMM2 activity is a viable therapeutic strategy. This functional mouse model of PMM2-CDG, in vitro assays and identification of the novel gp130 biomarker all shed light on the human disease, and moreover, provide the essential tools to test potential therapeutics for this untreatable disease.

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expected frequency is based on mendelian genetics. in the mannose treatment groups, females were administered 9 mg/ml mannose in drinking water for 1 week prior to mating. mannose treatment continued during pregnancy and halted after pups were born. p-values generated using chi-square test against expected mendelian distribution.

### introduction

congenital disorders of glycosylation (cdg) are rare genetic diseases in which glycosylation pathways are defective (1). phosphomannomutase 2 (pmm2)-cdg (previously known as cdg-ia, omim entry no. 212065) is the most commonly diagnosed type and is caused by loss-of-function mutations in pmm2 (2–4). pmm2 is an indispensable enzyme that catalyzes an early step of the n-glycosylation pathway by converting mannose-6-phosphate (m6p) to mannose-1-phosphate (m1p), which is the precursor of guanosine diphosphate mannose (gdp-mannose), and is necessary for the production of dolichol-linked oligosaccharides (dlos) and a myriad of n-glycans (supplementary material, fig. s1) (1,3,5). thus, deficiency in pmm2 enzymatic activity causes n-linked hypoglycosylation of oligosaccharides (dlos) and a myriad of n-glycans (supplementary material, fig. s1) (1,3,5). thus, deficiency in pmm2 enzymatic activity causes n-linked hypoglycosylation of proteins. patients with pmm2-cdg exhibit a wide range of symptoms that can include strabismus, developmental delay, failure to thrive, ataxia, susceptibility to infections, coagulopathy and an increased rate of childhood mortality (3,4,6–10). there is currently no cure or approved treatment for pmm2-cdg. moreover, no viable mouse model is available for preclinical testing of potential therapeutics.

here, we report the generation of the first viable pmm2 hypomorphic mouse model. the mutant mice carry mutations (r137h and f115l) in the highly conserved pmm2 protein that correspond to the two most prevalent mutations in patients with pmm2-cdg, r141h (nm_000303.2:c.422g>a (p.arg141his) (clinvar 7706)) and f119l (nm_000303.2:c.357c>a (p.phe119leu) (clinvar 7711)). this novel mouse model recapitulates many disease features seen in patients with pmm2-cdg. using a panel of in vitro assays, we confirmed the deficiency in the n-glycosylation pathway in mouse embryonic fibroblasts (mefVs) from pmm2<sup>r137h/f115l</sup> mutants and showed it was consistent with the deficiency in fibroblasts from patients with pmm2-cdg. we also identified a novel cellular glycosylation biomarker, glycoprotein 130 (gp130). our mouse model, novel biomarker and cell-based assays provide a platform to study the molecular pathobiology of pmm2-cdg.

### results

generation of a hypomorphic pmm2-cdg mouse model that mimics the human disease phenotype

we aimed to create a viable hypomorphic mouse model of pmm2-cdg. previous reports showed that pmm2 null mice die very early in embryogenesis (11) and mice of a hypomorphic line (pmm2<sup>r137h/f118l</sup>) die by mid-gestation (12). we created a hypomorphic mouse line that harbored the pmm2<sup>r137h/f115l</sup> compound heterozygous mutations (supplementary material, fig. s2), which is equivalent to the most common genotype (pmm2<sup>r141h/f118l</sup>) found in human patients with pmm2-cdg (supplementary material, fig. s3) (2,13–17). our model is distinct from a previously described pmm2 mouse model (pmm2<sup>r137h/f118l</sup>), in which the f118l mutation is equivalent to a synthetic mutation f122l in the human pmm2 protein (12). homozygous pmm2<sup>r137h/f115l</sup> embryos showed complete embryonic lethality, which is consistent with homozygous pmm2<sup>r141h/f114h</sup> mutations never being observed in human pmm2-cdg (18). significant embryonic lethality also occurred in our pmm2<sup>r137h/f115l</sup> population at > 12.5 days post coitum (dpc) (table 1). in the previous pmm2<sup>r137h/f118l</sup> model, schneider et al. (12) showed that providing mannose (9 mg/ml) in the drinking water of dams rescued embryonic lethality. the authors suggested that exogenous mannose increases the metabolic flux of mannose into the pathway and overcomes the reduced pmm2 activity caused by r137h and f118l mutations. providing our dams with the same amount of mannose did not rescue embryonic lethality in pmm2<sup>r137h/f115l</sup> pups (table 1); however, this amount of mannose significantly rescued embryonic lethality in pmm2<sup>r137h/f115l/f118l</sup> homozygous mutants (table 2). we attempted to identify prenatal gross organ defects in pmm2<sup>r137h/f115l/f118l</sup> mutant mice by histological examination of surviving embryos at 16.5 dpc; however, no significant genotype-specific histological changes in mutant versus wild-type (wt) embryos were observed at this developmental stage (data not shown).

our pmm2 hypomorphic model yields viable pmm2<sup>r137h/f115l</sup> offspring for postnatal studies, enabling comparison with the clinical and molecular features of patients with pmm2-cdg. we observed significant postnatal death in pmm2<sup>r137h/f115l</sup> mice (50.9% survival at postnatal day 65) while survival of heterozygous pups was similar to wt siblings (98.9% and 100% survival at day 65, respectively, fig. 1a). all pmm2<sup>r137h/f115l</sup> mutant mice were visibly smaller than their littermates (supplementary material, fig. s4a) and displayed lower body weight versus wt or heterozygous pmm2<sup>r137h/wt</sup> and pmm2<sup>f115l/wt</sup> littermates (fig. 1b and c). we also observed that 3 of 31 (10%) pmm2<sup>r137h/f115l</sup> mice exhibited various ocular anomalies, including appearance of secrecy and inability to open the eye (supplementary material, fig. s4b) and 2 of 31 (6%) mice displayed hind leg hypotonia (supplementary material, fig. s4c). multiple (9 of 31; 29%) pmm2<sup>r137h/f115l</sup> mice also had various degrees of kyphosis (supplementary material, fig. s4d), a disease feature documented in patients with pmm2-cdg (6,8,9). two-dimensional
Table 2  Pmm2<sup>R137H/F115L</sup> genotype caused embryonic lethality that could be reversed by mannose supplementation

<table>
<thead>
<tr>
<th>Mannose administered to dams</th>
<th>Gestational stage</th>
<th>Litters, n</th>
<th>Total events, n</th>
<th>WT/WT pups, n (%)</th>
<th>F115L/WT pups, n (%)</th>
<th>F115L/F115L pups, n (%)</th>
<th>P-value compared with expected distribution</th>
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<td>82</td>
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<td>Yes</td>
<td>Term</td>
<td>16</td>
<td>111</td>
<td>31 (27.9)</td>
<td>57 (51.4)</td>
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<tr>
<td>No</td>
<td>12.5 dpc</td>
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<td>132</td>
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<td>63 (47.7)</td>
<td>31 (24.48)</td>
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<td>14 (24.1)</td>
<td>33 (56.9)</td>
<td>11 (18.97)</td>
<td>0.4932</td>
</tr>
</tbody>
</table>

Expected frequency is based on Mendelian genetics. In the mannose treatment group, females were treated with 9 mg/ml mannose in drinking water for 1 week prior to mating. Mannose treatment continued during pregnancy and halted after pups were born. P-values generated using chi-square test against expected Mendelian distribution.

(2D) micro-computed tomography (CT) imaging revealed a more pronounced curvature of the spine in Pmm2<sup>R137H/F115L</sup> mice compared with WT (Supplementary Material, Fig. S4E). 3D micro-CT scans further detailed the abnormal skeletal features seen in Pmm2<sup>R137H/F115L</sup> mice, which included kyphosis, failed closure of the posterior fontanelle (the lower bone at the back of the skull) and defective development of the cervical spine near the base of the skull (Supplementary Material, Fig. S5).

Next, we performed histological examination of three pairs of sex-matched WT and Pmm2<sup>R137H/F115L</sup> mice at 4 weeks of age. Mild myocardial atrophy characterized by decreased thickness of both left and right ventricular walls and the interventricular septum, and decreased width of individual myofibers were observed in all three Pmm2<sup>R137H/F115L</sup> mice examined (Fig. 2A and B; Supplementary Material, Table S1). In the liver, we detected hepatocellular eosinophilic cytoplasmic hyaline bodies in Pmm2<sup>R137H/F115L</sup> mice (Fig. 2C). Mild dilation in the proximal tubule was observed consistently in the kidneys of Pmm2<sup>R137H/F115L</sup> mice, and was characterized by increased luminal space and loss of definition of the brush border of tubular epithelium (Fig. 2D). Rare degenerate cells were also present within the vascular epithelium or lumen (not shown). In contrast to patients with PMM2-CDG, we did not observe changes in the brain/cerebellum in Pmm2<sup>R137H/F115L</sup> mutant mice (Fig. 2E; Supplementary Material, Table S1). Specifically, the density of Purkinje cells in the cerebellar folia did not differ between the two groups. It should be noted that histological examination was only performed on Pmm2<sup>R137H/F115L</sup> mutant mice surviving to 4 weeks of age. Abnormal central nervous system pathology could have been present in the more severely affected mutants that died postnatally; however, we were unable to preserve these samples for histology. No histological differences were detected in lungs and spleens between WT and Pmm2<sup>R137H/F115L</sup> mice (data not shown).

Pmm2-mutant mice have dysregulated levels of circulating proteins

Patients with PMM2-CDG typically have decreased levels of circulating antithrombin III (ATIII), insulin-like growth factor (IGF)-1, IGF binding protein-3 (IGFBP-3) and acid-labile subunit (ALS) (19–21). These plasma proteins were also deficient in Pmm2<sup>R137H/F115L</sup> mice when compared with WT mice or heterozygous littersmates carrying one of the mutant alleles: ATIII was reduced by 37% (P < 0.0001), IGFBP-3 by 41% (P < 0.0001) and IGF-1 by 30% (P < 0.0001; Fig. 3A). Pmm2<sup>R137H/F115L</sup> mice had a nearly complete absence of ALS (Fig. 3B, upper), an IGF-binding protein that is normally highly N-glycosylated. In contrast to patients with PMM2-CDG (22), plasma transferrin glycosylation was normal in Pmm2<sup>R137H/F115L</sup> mice, as assayed by western blot (Fig. 3B, lower) and confirmed by additional liquid chromatography-mass spectrometry (LC-MS) protein analysis (data not shown).

To identify novel potential biomarkers in Pmm2<sup>R137H/F115L</sup> sera, we tested an array of antibodies against 83 serum proteins from WT and Pmm2<sup>R137H/F115L</sup> samples and validated our findings by enzyme-linked immunosorbent assay (ELISA). Two glycoprotein biomarkers, pentraxin 3 (PTX3) and IGFBP-1 were significantly upregulated in Pmm2<sup>R137H/F115L</sup> mice (Fig. 3C). N-glycosylated PTX3 was upregulated ~2-fold (P < 0.0001) in Pmm2<sup>R137H/F115L</sup> samples compared with the other genotypes. Strikingly, IGFBP-1, an O-glycosylated protein that binds to IGF-1 but not to ALS (23, 24), was upregulated ~13-fold in Pmm2<sup>R137H/F115L</sup> mice compared with control WT mice (Fig. 3C).

Pmm2-mutant fibroblasts are deficient in the PMM2 pathway

We analyzed MEFs from these mutant embryos and found that PMM2 protein levels in Pmm2<sup>R137H/WT</sup> and Pmm2<sup>R137H/WT</sup> MEFs were ~50% of levels in WT MEFs, whereas PMM2 levels in Pmm2<sup>R137H/WT</sup> MEFs were ~25–30% that of WT MEFs (Supplementary Material, Fig. S6). In vitro total PMM enzymatic activity in Pmm2<sup>R137H/WT</sup> and Pmm2<sup>R137H/WT</sup> MEFs was ~45–60% of that in WT MEFs, whereas activity in Pmm2<sup>R137H/WT</sup> MEFs was ~15–16% of WT PMM2 activity (Fig. 4A). Lower cellular PMM activity also resulted in reductions in mannose-related downstream metabolites, such as GDP-mannose (Fig. 4B) and DLO (Fig. 4C), and in global protein mannosylation (Fig. 4D). Finally, we looked at the glycosylation status of endogenous gp130, a heavily N-glycosylated protein (25). Western blot results showed that WT MEFs expressed full length, fully glycosylated gp130, whereas the Pmm2<sup>R137H/F115L</sup> MEFs expressed under-glycosylated gp130 (Fig. 4E).

PMM2 pathway activity is diminished in PMM2-CDG patient primary fibroblasts

To better understand the PMM2 pathway in CDG patients, we tested fibroblasts isolated from 10 patients with different PMM2 genotypes (Supplementary Material, Table S2). Western blots revealed that these fibroblasts expressed less PMM2 protein than PMM2 WT HFF-1 fibroblasts (Fig. 5A). As expected, total PMM activity in these patient fibroblasts was 37–89% lower than...
that of control HFF-1 cells (Fig. 5B). In addition, levels of metabolites downstream of PMM2, such as DLO (Fig. 5C) and GDP-mannose (Fig. 5D), were also reduced in patient cells. All of the CDG patient fibroblasts had a diminished ability to fully mannosylate proteins in vitro (Fig. 5E).

To demonstrate that the overall reduction in pathway activity and mannosylation was specifically due to defective PMM2 activity, we rescued the phenotype by overexpressing WT-PMM2 in patient cells. We used CDG-168 cells since they have the human mutations (PMM2\textsuperscript{R141H/F119L}) that correspond to the mutations in our mouse model. As expected, overexpressing WT-PMM2 (Fig. 6A) restored normal intracellular levels of GDP-mannose (Fig. 6B), DLO (Fig. 6C) and global mannosylation (Fig. 6D) in CDG-168 cells.

Next, we investigated whether specific glycoprotein expression was also influenced by PMM2 activity in these patient fibroblasts by western blotting. Using these skin fibroblasts, we were unable to examine the expression of the liver-specific glycoproteins (ATIII, IGF-1 and ALS) that were severely affected by PMM2 mutations in mice. However, we found that expression of glycosylated intercellular adhesion molecule-1 (ICAM-1) induced by tumor necrosis factor-\(\alpha\) (TNF\(\alpha\)) was greatly enhanced in CDG-168 fibroblasts when they overexpressed WT-PMM2 (Fig. 6E). In addition, expression of fully glycosylated gp130 and secretion of IGFBP-3 were significantly increased in CDG-168 fibroblasts that overexpressed WT-PMM2, as measured by western blotting and ELISA, respectively (Fig. 6F and G). These data are consistent with our observations that \(Pmm2\textsuperscript{R137H/F115L}\) MEFs from our mouse model expressed significantly less fully glycosylated gp130 compared with WT MEFs, and that plasma IGFBP-3 levels in \(Pmm2\textsuperscript{R137H/F115L}\) mice were significantly lower than in WT mice.

**Discussion**

Our main objective was to develop a viable mouse model that mimicked human PMM2-CDG. Previously, Schneider et al. (12) created a \(Pmm2\) hypomorphic mouse model with R137H and F118L mutations which showed complete embryonic lethality of \(Pmm2\textsuperscript{R137H/F118L}\), and was thus severely limited in its utility. In contrast, \(Pmm2\textsuperscript{F118L/F118L}\) mutants were indistinguishable from WT siblings. Our novel model is unique, since the genotype \(Pmm2\textsuperscript{R137H/F115L}\) is equivalent to the most common mutation in patients with PMM2-CDG, \(PMM2\textsuperscript{R141H/F119L}\). \(Pmm2\textsuperscript{R137H/F115L}\) mice also showed some embryonic lethality. This partial embryonic lethality could not be rescued by administering mannose to dams, in contrast to the embryonic lethality of the \(Pmm2\textsuperscript{R137H/F118L}\) genotype, which could. This difference may be due to the specific nature of the second \(Pmm2\) mutation. PMM2 with the F119L mutation (equivalent to F115L in mice) displays only 25% of the activity of WT PMM2 (27), whereas PMM2 with the F122L mutation (equivalent to F118L in mice) is expected to display a mild loss of enzymatic activity (12). It might also be due to the different genetic backgrounds of the two strains used, which was a C57Bl6-SV129 mixed background in the current study, and C57Bl6 in the Schneider et al. \(Pmm2\textsuperscript{R137H/F118L}\) mice. Differences in chow and housing could also contribute to the differences between the two studies.

However, a significant number of \(Pmm2\textsuperscript{R137H/F115L}\) offspring survived, and showed many disease features observed clinically in patients with PMM2-CDG. The mortality rate in patients with PMM2-CDG can be as high as 20% in the early years of life (28) but the degree of pre and perinatal lethality is unknown. Our \(Pmm2\textsuperscript{R137H/F115L}\) mice also exhibited a high level of postnatal...
mortality. Patients also have significant postnatal growth failure (29), and many have additional symptoms such as kyphosis, ophthalmological problems and axial hypotonia (28). All of our surviving Pmm2R137H/F115L mutants had stunted growth, while heterozygous mice carrying a single mutation were indistinguishable from WT siblings. A small percentage of Pmm2R137H/F115L mice exhibited hind leg hypotonia, eye defects and curvature of the spine. Although seen in other CDGs, ocular anomalies are not often seen in PMM2-CDG. The hind leg hypotonia we observed is considered to be a peripheral motor neuropathy, which contrasts with the central hypotonia seen in children with PMM2-CDG. The exact causes of these differences in disease features between mutant mice and human patients are currently unknown. Although the number of individual mice affected with these additional disease features was small, our results nonetheless indicate that this new PMM2-CDG mouse model shows the broad spectrum of heterogeneity of disease presentation that is often seen in patients with PMM2-CDG.

Our Pmm2R137H/F115L mice also had molecular characteristics typical of patients with PMM2-CDG including reduced levels of IGF-1, IGFBP-3, ALS and ATIII (19–21). Although IGF-1 is not N-glycosylated, its level is 3.2-fold lower in the compound heterozygous mice, presumably due to the loss of its two stabilizing N-glycoproteins, IGFBP-3 and ALS. When IGF-1 is in a ternary complex with IGFBP-3 and ALS, its half-life is 16–20 h (23). The half-life of IGF-1 drops dramatically when it is in unbound form (1–2 min), or when bound only to IGFBP-3 (20–30 min). We speculate that the loss of N-glycosylation of IGFBP-3 and ALS led to decreased protein levels and a subsequent reduction in IGF-1 levels due to its decreased half-life. This imbalance of the IGF system is likely to be the major reason for the growth defects found in the Pmm2R137H/F115L mutant mice, and growth defects are the most consistent gross abnormality in patients with PMM2-CDG.

Next, we sought to identify novel circulating PMM2-CDG biomarkers. We found that IGFBP-1 was highly upregulated (>13-fold) in the Pmm2R137H/F115L mouse population but was nearly undetectable in the Pmm2WT/WT samples. The IGFBP-1 increase could be due to transcriptional regulation operating via an unknown mechanism to maintain IGF homeostasis. In the PMM2-deficient animals, this increase could be part of a compensatory mechanism that attempts to stabilize IGF-1 upon loss of IGFBP-3 and ALS. We also found that PTX3 was significantly upregulated in the Pmm2R137H/F115L mouse population compared with WT littermates. PTX3 binds to several members of the complement system and is an important marker for inflammation (30). Despite the fact that PTX3 has a single N-glycosylation site, Pmm2R137H/F115L mice had elevated levels of circulating PTX3. The exact mechanisms and biological significance of this upregulation are unknown at this point. As with the growth reduction phenotype, these alterations in molecular marker levels were observed in Pmm2R137H/F115L but not Pmm2WT/WT or Pmm2F115L/WT genotypes.

Transferrin glycosylation status is commonly used as a biomarker for human CDGs (31–34). However, circulating transferrin from Pmm2R137H/F115L mice did not show hypoglycosylation by western blot. This observation was supported by additional LC-MS protein analysis. Schneider et al. reported normal transferrin glycosylation in their Pmm2 hypomorphic model (12). Transferrin glycosylation was also normal in mice that have hypomorphic mutations (MpiY255C/Y255C) in phosphomannose isomerase (MPI), and show hypoglycosylation of other proteins (35). Thus, it appears that transferrin is not a reliable hypoglycosylation marker in mice, in contrast to humans.
Collectively, our biomarker analysis suggests that IGFBP-3, IGF1, ALS and ATIII could be useful biomarkers in future studies of therapeutic interventions as they are also known to be altered in patients with PMM2-CDG. In addition, IGFBP-1 and gp130 measurements may provide a larger dynamic range for tracking disease normalization by therapeutic interventions in this mouse model, although further validation will be needed to assess their relevance in patients.

Using in vitro studies, we also confirmed that metabolic changes in MEFs isolated from Pmm2<sup>R137H/F115L</sup> mice were similar to changes observed in fibroblasts isolated from patients with PMM2-CDG. Levels of key metabolites such as GDP-mannose and DLO were significantly reduced in Pmm2<sup>R137H/F115L</sup> MEFs, leading to reduction in overall protein mannosylation. Similar metabolic changes were detected in vitro in 10 PMM2-CDG fibroblast cell lines with different genotypes. The reduction in, but not complete depletion of, PMM activity we observed in all the CDG patient-derived cells supports the hypothesis that disease alleles are hypomorphic, and that mutations leading to
complete loss of PMM2 are lethal [further supported by the early embryonic lethality of Pmm2 null mice (11)]. As expected, overexpression of WT human PMM2 in patient-derived fibroblasts restored PMM2 pathway metabolite levels and protein mannosylation.

Significantly, we discovered that the glycoprotein gp130 was hypoglycosylated in both Pmm2<sup>R137H/F115L</sup> MEFs and PMM2-CDG patient fibroblasts. Gp130 is a co-receptor for all members of the interleukin-6 (IL-6) receptor family and is required for signal transduction by IL-6-type cytokines (36). Its stability is enhanced by N-glycosylation at nine sites (25). We also noted that primary fibroblasts isolated from other types of CDG had reduced N-glycosylation of gp130 (unpublished results).
Patients with PMM2-CDG have not shown clinical improvement when given mannose supplements (37,38). In the present study, mannose supplementation did not improve survival of Pmm2<sup>F115L</sup>F115L homozygous pups. The mechanism is unknown, but it may involve substrate stabilization of the mutant PMM2 dimer interface or protection against thermal denaturation (39). Another possibility is that mannose supplementation increases mannose flux into the glycosylation pathway (22,40). Reducing MPI activity further enhances this flux by redirecting mannose from glycolysis toward glycosylation (35,41). Our preliminary (unpublished) results support this concept since mannose supplements (20 mg/ml mannose) rescue embryonic lethality of Pmm2<sup>F115L</sup>F115L mutant mice only when they also carry an Mpi<sup>Y255C/Y255C</sup> mutation. These results encourage further exploration of the potential therapeutic value of increasing mannose flux toward glycosylation.

In summary, our study describes the first clinically relevant mouse model of PMM2-CDG. Viable mice with the patient-relevant genotype, Pmm2<sup>F115L/F115L</sup>, were generated. This allowed, for the first time, detailed postnatal phenotypic and molecular characterization of an in vivo PMM2-CDG disease model and indeed, this model recapitulated many human disease features. We also paired our mouse model with in vitro assays analyzing fibroblasts from patients with PMM2-CDG, and showed a high degree of correlation between the two systems. This validated model can now be used to assess future glycosylation-enhancing therapies with the potential to treat this disease.

**Materials and Methods**

**Generation of Pmm2<sup>F115L</sup> and Pmm2<sup>R137H</sup> knock-in mice**

The Pmm2<sup>F115L</sup> and Pmm2<sup>R137H</sup> knock-in targeting vectors were assembled using genomic fragments of the mouse Pmm2 gene including a 3.0-kb 5' arm and a 7.6-kb 3' arm retrieved from 129S6/SvEv BAC DNA (OriGene Technologies) via an Escherichia coli-based chromosome engineering system (42), a loxP-flanked neomycin resistance cassette (Neo') and a thymidine kinase (TK) gene, which served as negative selection for genomic integration. The 3' arm contained murine Pmm2 exons 5 bearing the F115L or R137H mutations, which were created by PCR-based mutagenesis. The linearized targeting construct was electroporated into 129S6/SvEv mouse embryonic stem (ES) cells. Following homologous recombination in ES cells, an expression vector for Cre recombinase was transiently transfected to delete the Neo' cassette. Targeted ES cells were identified by PCR and sequencing analyses, and then injected into mouse blastocysts to produce chimeric mice. The chimeric mice were bred on a mixed 129/C2 background. All mutant animals were bred for germline transmission. Mice were genotyped using PCR primers spanning the position of the residual loxP site (5'-CCTCTTCATATTCTCCACAAGA3' and 5'-CCGTTAGGCCATGCACCTC-3'), generating a 168-bp PCR product in WT mice and a 270-bp PCR product in knock-in mice. Initial litters were analyzed for the presence of mutation by PCR and sequencing. All mutant animals were bred on a mixed 129 x C57BL/6J background.

Mice were bred and maintained in a barrier facility under pathogen-free conditions at WuXiAppTec (Shanghai, China). For mannose treatment studies, pregnant dams and pups were continuously supplied with 5 mM mannose in their drinking water. Mice were housed on 12 h/12 h light/dark cycle and given ad libitum access to food and water.
Cell culture including MEF isolation and growth conditions

HFF-1 cells were from ATCC. PMM2-CDG patient-derived fibroblasts were isolated as described (43–46). HFF-1s and PMM2-CDG fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 30% fetal bovine serum (FBS) (Invitrogen). MEFs were harvested from embryos at 12.5 dpc. MEFs were cultured in DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 2 mM GlutaMAX (Life Technologies), 100 units/ml penicillin and 100 µg/ml streptomycin, at 37°C with 5% CO₂.

Mouse embryo fixation, staining and histology

For histological analysis, mouse embryos were harvested at different days of development. Whole embryos were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned (3-µm thick) and stained with hematoxylin and eosin (H&E) according to standard laboratory protocols.

Micro-CT imaging

Mice were anesthetized by 1% pentobarbital sodium (50 mg/kg, i.p.) for micro-CT scans with a GE eXplore Locus (GE Healthcare). Mice were anesthetized by 1% pentobarbital sodium (50 mg/kg, i.p.) for micro-CT scans with a GE eXplore Locus (GE Healthcare). All scans were performed in the same mode: 80 kV X-ray voltage, 450 µA current, 0.045 mm effective pixel size, 360° tomographic rotation and a rotation step of 0.9° with an exposure time of 400 ms for each view. All scans were reconstructed as 3D images by eXplore MicroView 2.0 analysis software with the same parameters.

Western blotting of mouse plasma and cell culture samples

Mouse plasma samples were diluted 1:1000 in phosphate-buffered saline (PBS). Control BL/6 mouse plasma was either untreated (intact glycosylation) or deglycosylated with N-glycanase (PROzyme) per the manufacturer’s protocol. Cell lines were grown overnight. The next day, cell pellets were frozen at -80°C until LC-MS analysis was performed.

GDP-mannose quantification via LC-MS

Cells were plated at 2.5 × 10⁶ cells per well in 6-well plates and grown overnight. The next day, cells were allowed to equilibrate for 1 h in basal labeling media [RPMI (Life Technologies) containing 3% dialyzed FBS and supplemented with non-essential amino acids and -glutamine with no glucose or mannose]. Cells were then incubated in basal labeling media with or without 5 µM glucose for 4 h. Next, cells were promptly washed in ice-cold ammonium carbonate buffer (7.2 g/l, pH 7.4) and placed on dry ice. Metabolites were extracted with 80/20 methanol/water at −20°C containing an internal standard, U-13C₅ U-15N₁ ²H₁₅ glutamic acid. Samples were dried, sealed and stored at −80°C until LC-MS analysis was performed.

GDP-mannose was quantified by coupling an ultra-performance LC (UPLC) column (Waters) with a Xevo TQ-S mass spectrometer. Separation was accomplished using reverse phase ion pairing chromatography, applying an Acuity UPLC HSS T3, 1.8 µm, 2.1 × 50 mm column (Waters). Mobile phase A consisted of 95/5 water/methanol plus 15 mM acetic acid, 10 mM tributylamine, pH 4.95, and phase B was methanol. A linear gradient was run at 0.4 ml/min unless otherwise noted: 0 min, 2% B; 1 min, 2% B; 5 min 60% B; 5.3 min 90% B, 0.3 ml/min; 5.5 min, 2% B, 0.375 ml/min; 6 min, 2% B; 7 min 2% B. The source parameters applied were: capillary voltage 3 kV, desolvation temperature 300°C, desolvation gas 800 l/h. GDP-mannose was monitored in negative ionization mode, with the 604 → 442 transition, 24 V collision energy and cone voltage of 62. Quantitation was performed against an external calibration curve.

Global protein mannosylation quantification

Cells were seeded at 3 × 10⁶ cells per well in 96-well plates and grown overnight. The next day, cells were incubated with serum-free DMEM (Life Technologies) containing 0.5 g/l glucose, 20 µCi ²H-mannose and 4 µCi ³⁵S-methionine for 8 h at 37°C. Cells were harvested in 400 µl Tris-EDTA, 650 µl DMEM was added and the cells were spun at 3500 rpm for 10 min. Pellets were frozen at −20°C overnight. The next day, cell pellets were resuspended in 100 µl 10 mM Tris–HCl pH 7.4 with 1% NP40. Next, 20 µl of each lysate was mixed with 5 µl BSA (10 mg/ml), 175 µl water and 200 µl TCA 20%, then placed on ice for 2 h. Samples were then centrifuged at 13 000 rpm for 20 min and the supernatant was discarded. The precipitate was resuspended in 50 µl of 0.2 N NaOH by pipetting, vortexing and then adding to 5 ml of scintillation fluid. Radioactivity was counted with the Beckman scintillation counter dual labeling program (channel 1 = 3H, channel 2 = 35S, channel 3 none) for 2 min. Final data were calculated as follows: ³⁵S = W2/0.632 and ²H = W3-35S, where W = window.
DL0 determination

Cells were seeded at 6 × 10^4 cells per well in 48-well plates and grown overnight. The next day, the cells were washed with PBS, and 200 µl DMEM without glucose containing 4 µCi/ml [2-3H]-mannose was added and incubated at 37 °C for 1 h. The labeling medium was then removed and cells were washed with PBS. Cells were then trypsinized, transferred to the 96-well polypropylene cell culture plate and pelleted. Next, cells were lysed in 200 µl chloroform:methanol (C.M, 2:1) and vortexed for 1 min. The mixture was then centrifuged at 13 000 rpm for 10 min and the C.M layer was removed. The extraction was repeated twice more with 200 µl C.M (2:1) each. The final pellet was allowed to air dry, and 200 µl water was then added. The samples were vortexed for 1 min, centrifuged as above and the water was removed. This step was repeated twice more. Samples were then extracted with 100 µl chloroform:methanol:water (10:10:3) three times, incubated for 10 min and centrifuged for each extraction to ensure maximum recovery. Samples were next dried and 150 µl of scintillation cocktail was added. Incorporation of [3H]-mannose into DLO was measured by radioactivity and counted with a MicroBeta reader (Perkin Elmer). CellTiter-Glo (Promega) cell viability values were used to normalize each sample for the number of cells. Samples were split after trypsinizing and half tested with CTG, half extracted for DLO levels.

ELISA assays

The following ELISA kits were used: mouse IGFBP-3 (R&D Systems, MGB300), mouse IGFBP-1 (R&D Systems, MG100), mouse antithrombin III (Abcam, ab10880), mouse PTX3 (R&D Systems, MPTX30), mouse IGFBP-1 (R&D Systems, DYI588-05) and human IGFBP-3 (R&D Systems, SGB300). All assays were run according to the manufacturer’s protocol. In some cases, the standard curve was adjusted to include more low concentrations to increase confidence in dilute samples. For most kits, plasma samples were diluted 1:1000 in PBS and then final dilutions were prepared in the appropriate assay buffer. For PTX3, the samples were diluted 1:100 and then to 1:300 in appropriate buffer. For IGFBP-1, the WT/WT, F115L/WT and R137H/WT samples were diluted 1:100 and then to 1:200 in appropriate buffer.

Lentiviral over-expression of WT-PMM2 in PMM2-CDG patient fibroblast cells

Lentiviruses directing over-expression of the cDNA for WT-PMM2 and empty lentiviral vectors were generated by transfecting 293T cells with PLVX-RES-ELF1α-NEO-based over-expression plasmids with packaging (psPAX2) and envelope (pCMV-VSVG) plasmids in low antibiotic growth media, alongside no vector cells to provide the selection resistance control. Twenty-four hours post-transfection, cells were exchanged into growth media. Viral supernatants were collected and pooled for infection of target cells.

The desired cell lines were generated by seeding CDG-168 cells at a level giving 20–40% confluence 24 h later. Viral supernatants were then added to the cells and polybrene added to 8 µg/ml, plates spun at 1200g for 60 min at 37 °C, and incubated overnight. The next day, the supernatant was replaced with growth media and cells selected with 200 µg/ml Geneticin (Invitrogen).

Statistics

P-values were generated using one-way ANOVA followed by Tukey post-hoc tests for multiple comparisons or chi-squared test, using Prism v.6 (GraphPad).

Study approval

Mice were bred and maintained at WuXi AppTec. All animal work was performed in accordance with protocols approved by the WuXi AppTec Institutional Animal Care and Use Committee.

Supplementary Material

Supplementary Material is available at HMG online.

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Conflict of Interest statement. B.C., M.C., G.A.S., G.H., J.P., Y.Ch., C.S., M.D., L.S., S.-S.M.S., and S.J. are employees of and have ownership interest in Agios Pharmaceuticals. All other authors have declared no conflicts of interest.

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