Supporting Information

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SI Materials and Methods

Preparation of Mouse Embryonic Fibroblasts. Fetuses of WT B6 mice were removed from uteri at embryonic day 14.5 d and washed once with PBS at room temperature. After the liver and head were removed, the remaining tissue was diced into fine pieces and then placed in 2 mL of PBS in a 50-mL conical tube (Falcon). The tissue was allowed to settle to the bottom of the tube, and the supernatant was then removed. A trypsin/EDTA solution (15 mL; Nacalai Tesque, Inc.) containing 1 mg/mL DNase (Wako Pure Chemical Industries) was added to the tube, which was then incubated for 1 h at 37 °C. After centrifugation of the tube at 1,000 × *g* for 5 min, the supernatant was removed and the trypsin digestion was repeated twice using the same procedure. After the final centrifugation, the obtained pellet, which contained mouse embryonic fibroblasts (MEFs), was resuspended in DMEM supplemented with 10% (vol/vol) FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The cell suspension was then added to one 15-cm tissue culture plate per fetus. After incubation at 37 °C in a 5% (vol/vol) CO_2 atmosphere for 24 h, the medium was replaced with fresh medium and the cells were further grown for 24 h.

MEFs at passage 2–3 were immortalized by transfection with pcDNA3.1-Simian Virus 40 (SV40) LT/Zeo(+) (a kind gift from Jianguo Gu, Tohoku Pharmaceutical University, Sendai, Japan) using an Amaxa MEF2 Nucleofector kit (Lonza) as directed by the manufacturer. The SV40-immortalized MEFs were selected for 3 wk in DMEM supplemented with 10% (vol/vol) FBS, 400 μg/mL zeocin (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% (vol/vol) CO_2. The selected MEFs were stored in liquid nitrogen. For experiments, MEFs were maintained in medium without zeocin and were used within 1 mo after being thawed from the frozen stock.

DTT Treatment. MEFs (2×10^6 cells) were cultured in 15-cm dishes containing 20 mL of DMEM (high-glucose environment) for 24 h at 37 °C. The cells were washed twice with 10 mL of DMEM without glucose and then further incubated for 6 h at 37 °C in 20 mL of normal-glucose DMEM with or without 2 mM DTT.

Preparation of Free Neutral Oligosaccharides. Cells were suspended in 300 μL of 20 mM Tris·HCl (pH 7.4) and 10 mM EDTA, to which 900 μL of ice-cold ethanol was then added. The cell suspension was homogenized by three 10-s vigorous agitations separated by 5-min cooling periods on ice. The resulting homogenate was centrifuged at 15,000 × *g* for 15 min, and the supernatant was recovered as the soluble oligosaccharide fraction. The obtained fraction was evaporated to dryness and was then desalted using AG50-X8 (250 μL, 200–400 mesh, H^+ form; Bio-Rad) and AG1-X2 (250 μL, 200–400 mesh, acetate form; Bio-Rad) columns, followed by passage through an InertSep GC column (150 mg/3 mL; GL Sciences).

Digitonin Permeabilization. To analyze the subcellular localization of phosphorylated oligosaccharides, MEFs (6×10^7 cells) were trypsinized, washed twice with 25 mL of ice-cold PBS, and then resuspended in 1 mL of ice-cold PBS The cells were permeabilized by the addition of 1 mL of PBS containing 0.04% (wt/vol) digitonin (CalBiochem) for 5 min at 0 °C. After centrifugation of the cell suspension at 500 × *g* for 5 min, the supernatant was recovered as the cytosol fraction. The pellet (membrane fraction) was washed twice with 2 mL of PBS, and the wash fractions were combined with the cytosol fraction. The washed membrane fraction was resuspended in 300 μL of buffer containing 20 mM Tris·HCl (pH 7.4) and 10 mM EDTA.

Standard 2-Aminopyridine–Labeled Oligosaccharides. The structures and abbreviations of the glycans used in this study are listed in Table S1. The 2-aminopyridine (PA)-M2A, PA-M3B, PA-M8C, and PA-M9A were purchased from Takara. A mixture of high-mannose type glycans was prepared by PNGase F (Roche) digestion of RNase B (Sigma–Aldrich) according to the manufacturer's instructions and was then labeled with PA. The oligosaccharides M5B, M6E, and M7E were prepared by mild acid hydrolysis of mannose $(Man)_{5–7}$ *N*-acetylglucosamine $(GlcNAc)_2$-pyrophosphate (PP)-dolichol that had been extracted from the microsomes of the yeast haploid, single-gene deletion mutant strains *alg3*Δ, *alg9*Δ, and *alg12*Δ, respectively (Open Biosystems), and were then labeled with PA. PA-Gn2 was prepared by labeling Gn2 (Sigma–Aldrich) with PA. PA-M1A was prepared by digestion of the PA-labeled high-mannose type glycan mixture with jack bean α-mannosidase (Seikagaku Co.) in 10 mM sodium citrate buffer (pH 4.5). PA-M4D was prepared by partial digestion of PA-M5B with *Aspergillus saitoi* α1,2-mannosidase (Seikagaku Co.) in 10 mM sodium acetate buffer (pH 5.5). PA-G(1-3)M9A was chemically synthesized as described previously (1). PA-G3M7C was prepared by digestion of PA-G3M9 with *A. saitoi* α1,2-mannosidase. PA-G3M8B was prepared by digestion of PA-G3M9A with the microsomes prepared from the yeast haploid *ams1*Δ *htm1*Δ *gls1*Δ cells (our laboratory stock) in a buffer containing 20 mM Hepes-KOH (pH 7.4) and 1% (wt/vol) digitonin. PA-G3M8C was prepared by partial digestion of PA-G3M9 with *A. saitoi* α1,2-mannosidase. After performing the enzymatic digestions, PA-Gn2, PA-M1A, PA-M4D, PA-M5B, PA-M6E, PA-M7E, PA-G3M7C, PA-G3M8B, and PA-G3M8C were purified by size-fractionation HPLC, followed by dual-gradient, reversed-phase HPLC (2). The glycan structures were confirmed by glycosidase digestions of the purified samples with jack bean α-mannosidase, *A. saitoi* α1,2-mannosidase, and microsomes obtained from *ams1*Δ *htm1*Δ *gls1*Δ cells, followed by size-fractionation HPLC.

Western Blotting. The cytosol and membrane fractions of the digitonin-permeabilized cells were mixed with Triton X-100 at a final concentration of 1% (wt/vol), and the resulting mixtures were incubated for 15 min at 0 °C. After centrifugation of the mixtures at 15,000 × *g* for 5 min, the supernatant was recovered, mixed with an equal volume of 2 × Laemmli sample buffer, and then heated for 3 min at 100 °C. The sample (1×10^5 cells) was resolved by SDS/PAGE, and the proteins were then electroblotted onto polyvinylidene fluoride membrane (Millipore) using a Trans-Blot SD apparatus (Bio-Rad). The membrane was blocked for 1 h with 5% (wt/vol) skim milk in PBS containing 0.05% (wt/vol) Tween 20 (PBST) and then incubated for 2 h with either antiprotein disulfide isomerase (anti-PDI; 1:1,000 dilution; Cell Signaling Technology) or anti-GAPDH (1:10,000 dilution; Millipore) antibodies in PBST containing 5% (wt/vol) skim milk. The membrane was washed three times with PBST and then incubated for 45 min with HRP-conjugated anti-rat IgG (for anti-PDI antibody) or anti-mouse IgG (for anti-GAPDH) in PBST containing 5% (wt/vol) skim milk. The membrane was again washed three times with PBST, and it was then incubated with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and immediately visualized using a LAS-3000 Imager (Fujifilm).

β-Hexosaminidase Activity Assay. The Triton X-100–treated cytosol and membrane fractions of the digitonin-permeabilized MEFs were used as enzyme sources for β-hexosaminidase activity assay. Each sample (5 μL) was added to 100 μL buffer containing 10 mM sodium acetate buffer (pH 5.5) and 1 mM *para*-nitrophenyl (*p*NP) β-GlcNAc, and the reaction mixtures were then incubated for 2 h at 37 °C. The reaction was stopped by the addition of 100 μL of 2 M sodium carbonate. The released *p*NP was detected at 405 nm with a Model 680 microplate reader (Bio-Rad).

Preparation and RT of Total RNA. Total RNA was prepared from MEFs cultured under normal- or low-glucose conditions using an RNeasy Mini Kit (Qiagen). cDNA was prepared from 4 μg of total RNA in 20-μL reaction mixtures using SuperScript III Reverse Transcriptase (Invitrogen) and random hexamer primers according to the manufacturer's instructions.

Quantitative Gene Expression Analysis by Real-Time PCR. Real time-PCR was performed using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The 20-μL real-time PCR mixture contained 10 μL of TaqMan Universal PCR Master Mix (Clontech), 9 μL of cDNA (0.2 μg), and 1 μL of 6-carboxyfluorescein (FAM)-labeled TaqMan probe [*immunoglobulin heavy chain-binding protein* (*BiP*), Mm00517690_g1; Applied Biosystems]. cDNA was amplified by an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 95 °C for 5 s and 55 °C for 20 s. The samples were analyzed in triplicate, and the mean number of cycles required to reach the threshold level of fluorescence detection was calculated for each sample. *GAPDH* expression was quantitated with TaqMan Rodent GAPDH Control Reagent (Applied Biosystems) to normalize the amount of cDNA in each sample.

1. Matsuo I, Totani M, Tatami A, Ito Y (2006) Comprehensive synthesis of ER related high-mannose-type sugar chains by convergent strategy. *Tetrahedron* 62(35):8262–8277.

2. Suzuki T, et al. (2008) Dual-gradient high-performance liquid chromatography for identification of cytosolic high-mannose-type free glycans. *Anal Biochem* 381(2):224–232.

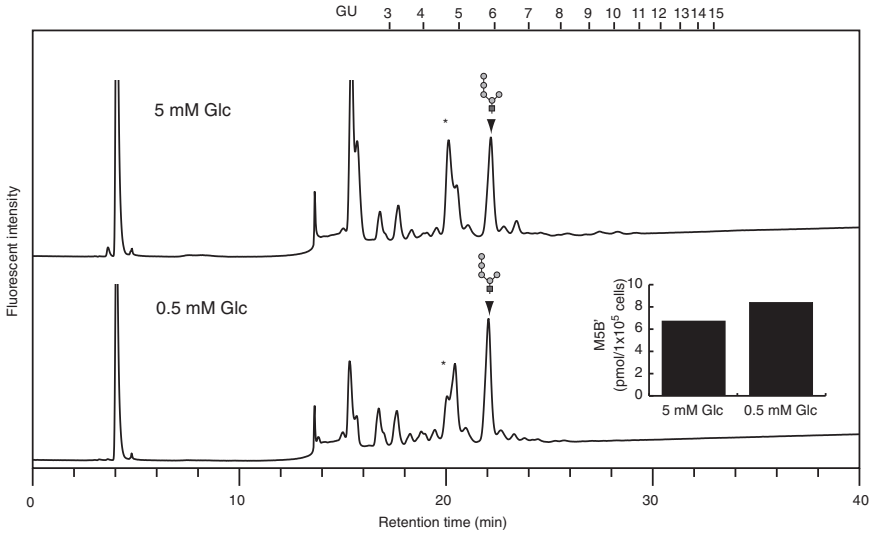


Fig. S1. Effects of glucose deprivation on the free neutral oligosaccharides. MEFs were incubated for 24 h in physiological glucose [5 mM glucose (Glc)] or glucose-deprivation (0.5 mM Glc) medium. The soluble oligosaccharide fraction was prepared, desalted, and subjected to the fluorescent labeling with PA. The labeled oligosaccharides thus obtained were analyzed by the size-fractionation HPLC. The structure of the most abundant free neutral oligosaccharide, indicated by down arrowheads, was determined as M5B′ by dual-gradient, reversed-phase HPLC. Elution positions (glucose unit, GU) of the glucose oligomer [degree of polymerization (DP) = 3–15] are indicated on top. Asterisks indicate the nonspecific peaks derived from the labeling reagents. (*Inset*) Quantitation of M5B′.

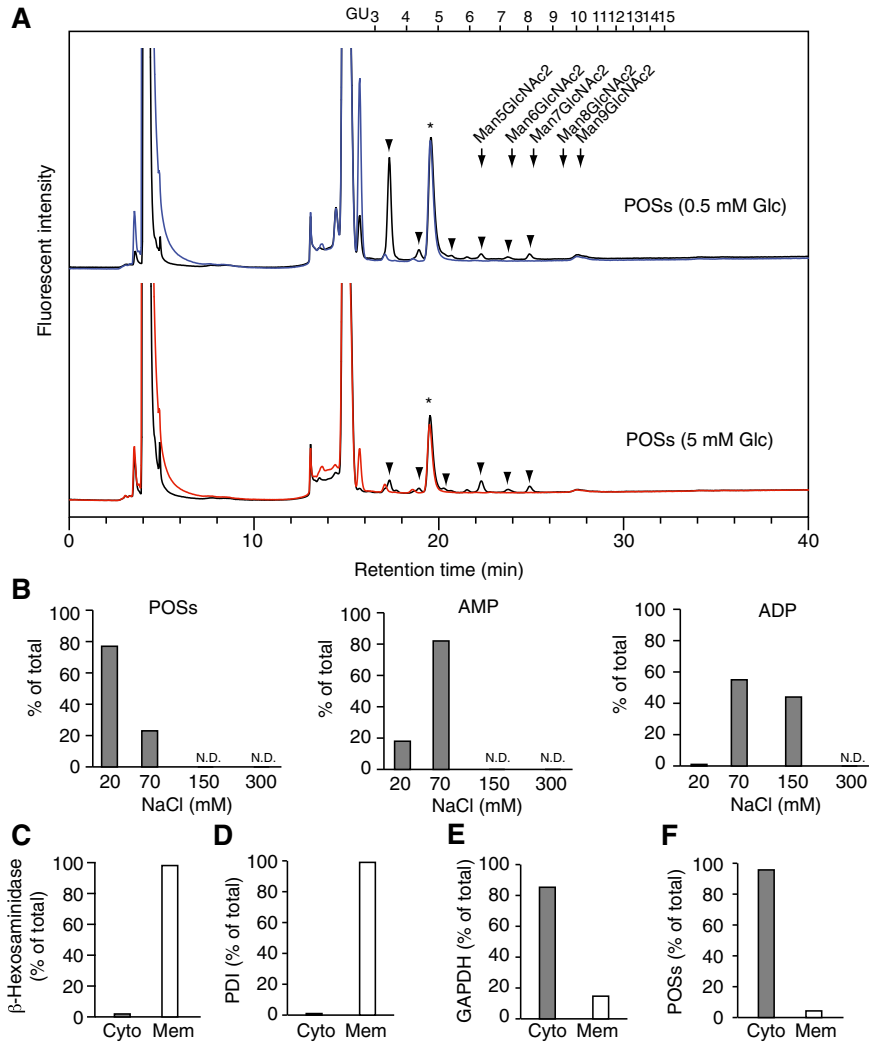


Fig. S2. Biochemical characterization of phosphorylated oligosaccarides (POSs). (*A*) MEFs were incubated under low-glucose (0.5 mM Glc) and normal-glucose (5 mM Glc) conditions. POSs were prepared from the cells and treated with (a blue line for 0.5 mM Glc and a red line for 5 mM Glc) or without (black lines) jack bean α-mannosidase. The digests were analyzed by size-fractionation HPLC. Elution positions (GU) of the glucose oligomer (DP = 3–15) are indicated on top. The elution positions of the PA-labeled glycans ($Man_{5-9}GlcNAc_2$) from RNase B are indicated by down arrows. Arrowheads indicate POSs susceptible to jack bean α-mannosidase. Asterisks indicate nonspecific peaks from the labeling reagents. (*B*) Elution profiles of POSs (*Left*), AMP (*Middle*), and ADP (*Right*) in anion exchange chromatography. Total amounts of each compound were set to 100%. N.D., not detected. (*C–F*) Subcellular fractionation of the digitonin-permeabilized MEFs that were incubated under the low-glucose condition. Cyto, cytosol; Mem, membrane fractions. (*C*) β-Hexosaminidase was detected by an enzyme assay using *p*NP β-GlcNAc. Total enzyme activity was set to 100%. PDI (*D*) and GAPDH (*E*) were detected by Western blot using anti-PDI and anti-GAPDH antibodies, respectively. The band intensity was quantitated in each fraction, and the total intensity was set to 100%. (*F*) POSs were analyzed by size-fractionation HPLC. Total amounts of POSs were set to 100%.

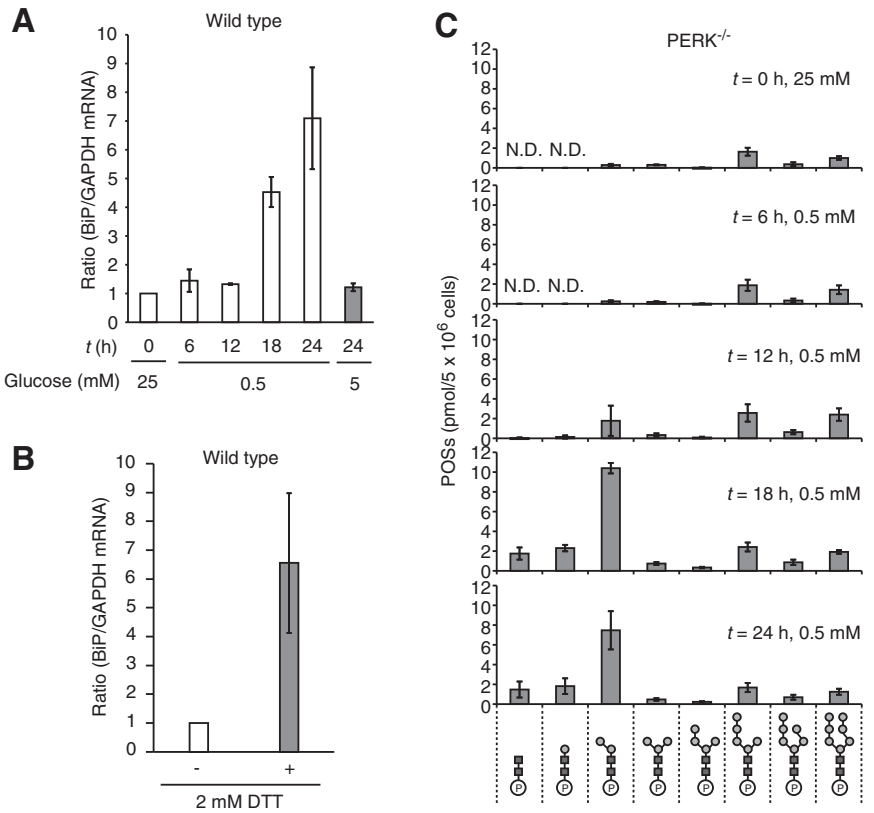


Fig. S3. Effects of unfolded protein response (UPR) on the release of POSs. (*A*) MEFs were incubated under normal-glucose (5 mM) or low-glucose (0.5 mM) conditions for the indicated time. The mRNA expression levels of *BiP* were quantitated by the real-time PCR and normalized by those of *GAPDH*. (*B*) MEFs were incubated for 6 h in the normal-glucose medium in the presence (+, gray bar) or absence (–, white bar) of 2 mM DTT. The mRNA expression levels of *BiP* were quantitated by the real-time PCR and normalized by those of *GAPDH*. Error bars indicate SDs from three independent experiments. (*C*) PERK$^{-/-}$ MEFs pre-cultured under high-glucose conditions (t = 0 h, 25 mM Glc) were incubated for the indicated time under low-glucose conditions (t = 6–24 h, 0.5 mM Glc). POSs were dephosphorylated, labeled with PA, and then analyzed by size-fractionation HPLC.

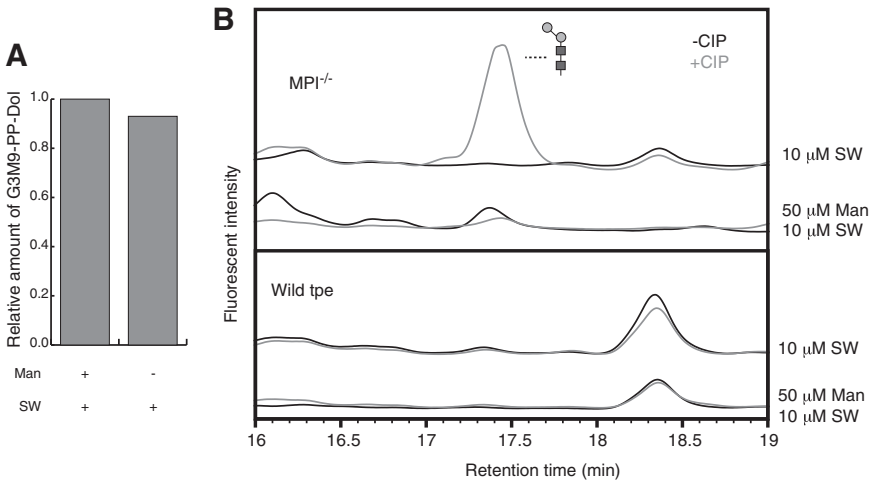
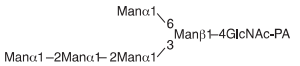
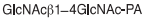
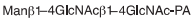
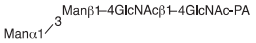
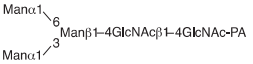
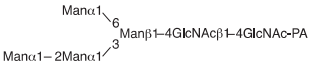
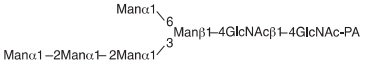
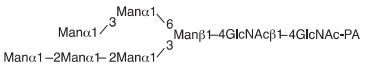
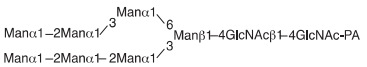
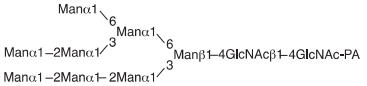
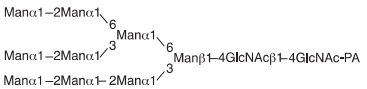
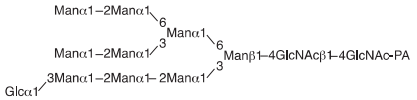
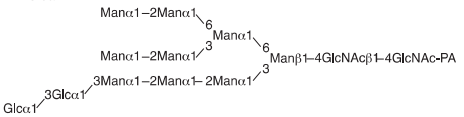
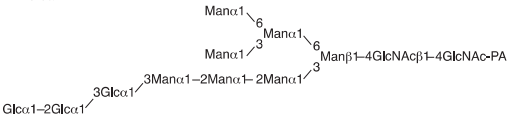
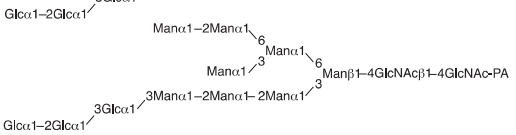
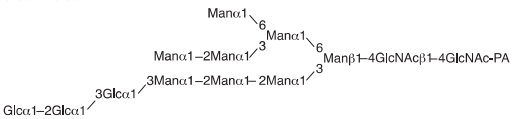


Fig. S4. Effects of the mannose deprivation on dolichol-linked oligosaccharide (DLOs) and POSs in the WT MEFs. (*A*) WT MEFs were incubated for 24 h in the culture medium containing 5 mM Glc and 10% (vol/vol) dialyzed FBS, which is supplemented with 10 μM swainsonine (SW) alone or with 10 μM SW and 50 μM Man. DLOs were prepared from the cells, and their oligosaccharide moiety was released by mild acid hydrolysis. After the fluorescent labeling of the released oligosaccharides with PA, the PA-oligosaccharides were analyzed by size-fractionation HPLC. The amounts of $Glc_3Man_9GlcNAc_2$-pyrophosphate-dolichol (G3M9-PP-Dol) from the WT MEFs treated with SW and Man were set to 1.0. (*B*) MPI$^{-/-}$ (*Upper*) and WT (*Lower*) MEFs were incubated as in *A*. POSs were prepared from the cells and treated with (+) or without (–) calf intestine alkaline phosphatase (CIP). After the fluorescent labeling of the released oligosaccharides with PA, the PA-labeled oligosaccharides were analyzed by size-fractionation HPLC. The glycan structure of $Man_2GlcNAc_2$-P shown in the HPLC chart was determined by dual-gradient, reversed-phase HPLC. The SW treatment of MPI$^{-/-}$ MEFs induced the generation of $Man_2GlcNAc_2$-P, whereas no $Man_2GlcNAc_2$-P was detected in the WT MEFs treated under the same conditions.

Table S1. Structures and the abbreviations of the standard PA-labeled glycans used in this study

Abbreviations	Glycan structures
M5B′	
Gn2	
M1A	
M2A	
M3B	
M4D	
M5B	
M6E	
M7E	
M8C	
M9A	
G1M9A	
G2M9A	
G3M7C	
G3M8B	
G3M8C	
G3M9A