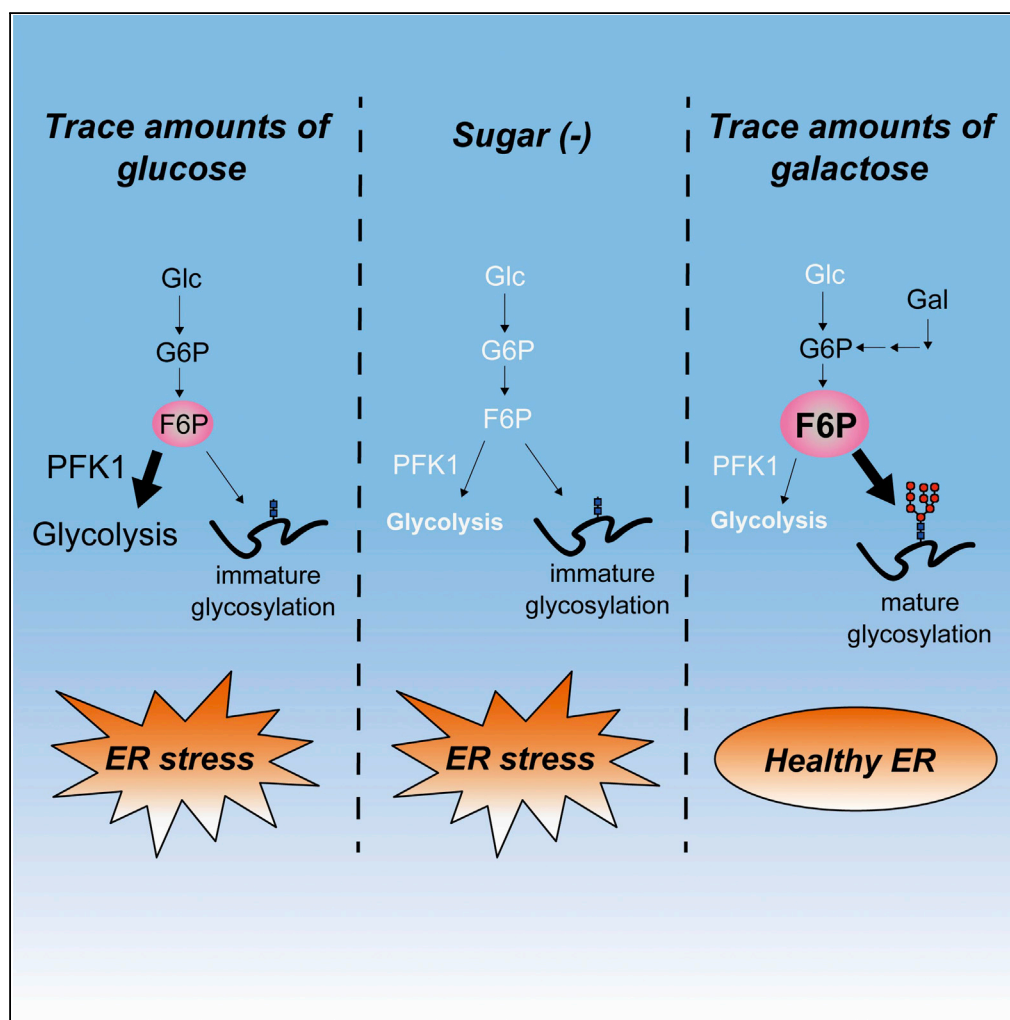


## Article

# A Trace Amount of Galactose, a Major Component of Milk Sugar, Allows Maturation of Glycoproteins during Sugar Starvation



Norio Sasaoka,  
Hiromi Imamura,  
Akira Kakizuka

kakizuka@lif.kyoto-u.ac.jp

#### HIGHLIGHTS

Trace amounts of galactose, but not glucose, allow maturation of glycoproteins

Trace amounts of galactose, but not glucose, prevent ER stress and cell death

Trace amounts of galactose raise F6P levels for the maturation of glycoproteins

## Article

# A Trace Amount of Galactose, a Major Component of Milk Sugar, Allows Maturation of Glycoproteins during Sugar Starvation

Norio Sasaoka,<sup>1</sup> Hiromi Imamura,<sup>1</sup> and Akira Kakizuka<sup>1,2,\*</sup>**SUMMARY**

Milk sugar is composed of glucose and galactose. Galactose is less suitable as an energy source than glucose. Thus, it has been a puzzle as to why mammals utilize galactose as a major component of milk sugar. Here we show that in hypoglycemic conditions, the presence of a trace amount of galactose, but not glucose, is able to maintain the production of mature glycoproteins and to abolish cell-death-inducing endoplasmic reticulum stress. In severely sugar-limited conditions, both glucose and galactose enter into the glycolytic pathway, but galactose is not able to raise the phosphofructokinase 1 activity, leading to the accumulation of fructose-6-phosphate, which in turn is utilized for the maturation of glycoproteins (e.g., growth factor receptors) and allows the activation of their intracellular signaling and prevents cell death from hypoglycemic conditions. Thus trace amounts of galactose may play unexpectedly important roles in the growth of infants and their protection during starvation.

**INTRODUCTION**

Maintenance of glucose levels is of great physiologic importance, and imbalances lead to an array of detrimental effects. Reduced glucose levels cause symptoms ranging from mild discomfort to severe confusion, seizures, and ultimately, brain damage (Agrawal et al., 2014). At the cellular level, glucose is mostly utilized as an energy source and is also required for protein glycosylation, which serves a variety of structural and functional roles in membrane-bound and secreted proteins. Glycosylation is a major form of protein modification in eukaryotes. It has been estimated that approximately half of all human proteins are glycosylated and most contain N-glycan structures (Apweiler et al., 1999). High-mannose-type N-glycans are reported to constitute approximately 40% of all N-glycans in mouse brains (Ishii et al., 2007). Furthermore, it is well known that N-glycosylation improves protein stability and regulates the function of cytokines and chemokines (Opdenakker et al., 1995). Hence, protein glycosylation plays very important roles for protein homeostasis and function.

Sugar insufficiency is one of the leading causes of endoplasmic reticulum (ER) stress. Cells cope with ER stress via three types of ER stress sensors and respond by deploying the unfolded protein response (UPR). The sensors are PKR-like ER kinase (PERK), inositol-requiring enzyme 1 $\alpha$  (IRE1 $\alpha$ ), and activating transcription factor 6 (ATF6). Following the activation of these sensors, different signaling nodes, such as c-Jun N-terminal kinase 1, and downstream transcription factors, such as X-box-binding protein 1 (XBP1), activating transcription factor 4 (ATF4), and C/EBP homologous protein (CHOP), are activated (Mori, 2009). For example, IRE1 $\alpha$  is activated by self-phosphorylation and manifests endoribonuclease activity, which cleaves a cryptic 26-bp exon from the downstream target XBP1 mRNA. The resulting unconventionally spliced mRNA encodes an alternate XBP1 protein, spliced XBP1 (XBP1s), which is a highly active transcription factor that promotes the expression of ER chaperones and molecules involved in ER-associated degradation (Yoshida et al., 2001). Notably, XBP1s-dependent activation of the hexosamine biosynthetic pathway (HBP), which converts glucose to uridine 5'-diphosphate N-acetylglucosamine (UDP-GlcNAc) for N- and O-glycosylation of proteins, has been reported to protect cells from various biological insults, such as ER stress and cardiac ischemia/reperfusion injury (Wang et al., 2014). Hence, XBP1s is particularly important for cell survival.

Most studies of mammalian sugar metabolism concentrate on glucose, owing to its central roles in not only energy generation but also protein glycosylation. Other hexoses, e.g., mannose and galactose, receive relatively little attention in metabolic and physiologic studies. Mannose is a C2 epimer of glucose and is naturally found in microbes, plants, and animals. Free mannose is found in mammalian plasma at 50–100  $\mu$ M (Alton et al., 1998). Galactose is a C4 epimer of glucose. Galactose, which is of particular

<sup>1</sup>Laboratory of Functional Biology, Graduate School of Biostudies, Kyoto University, Kyoto 606-8501, Japan

<sup>2</sup>Lead Contact

\*Correspondence:

kakizuka@lif.kyoto-u.ac.jp

<https://doi.org/10.1016/j.isci.2018.11.035>



relevance to mammals, is mostly produced by hydrolysis of the milk sugar lactose, a disaccharide with a glycosidic linkage between glucose and galactose. Free galactose concentrations in plasma are usually <10 mg/dL (0.56 mM) (Kaempf et al., 1988). The detectable plasma galactose levels in normal adult humans suggest that the dietary acquisition of modest amounts of galactose from fruits and vegetables, e.g., tomatoes, bell peppers, brussels sprouts, apples, bananas, persimmons, dates, etc., (Gross and Acosta, 1991) is beneficial. Galactose is metabolized by the enzymes of the Leloir pathway in the liver (Frey, 1996), which requires several enzymes to convert galactose to glucose-6-phosphate (G6P). In mammals, mutations in some of these enzymes can result in the genetic disease galactosemia (Leslie, 2003; Holden et al., 2004; Timson, 2006). Despite such disadvantageous features of galactose, mammals must use galactose for something important, because lactose, the milk sugar, consists of galactose and glucose as a heterodimer. Based on the evidence that lactose is an essential nutrient for mammalian babies, one of the biggest and long-lasting questions in biology has been why galactose has been evolutionally selected as a major component of lactose.

## RESULTS

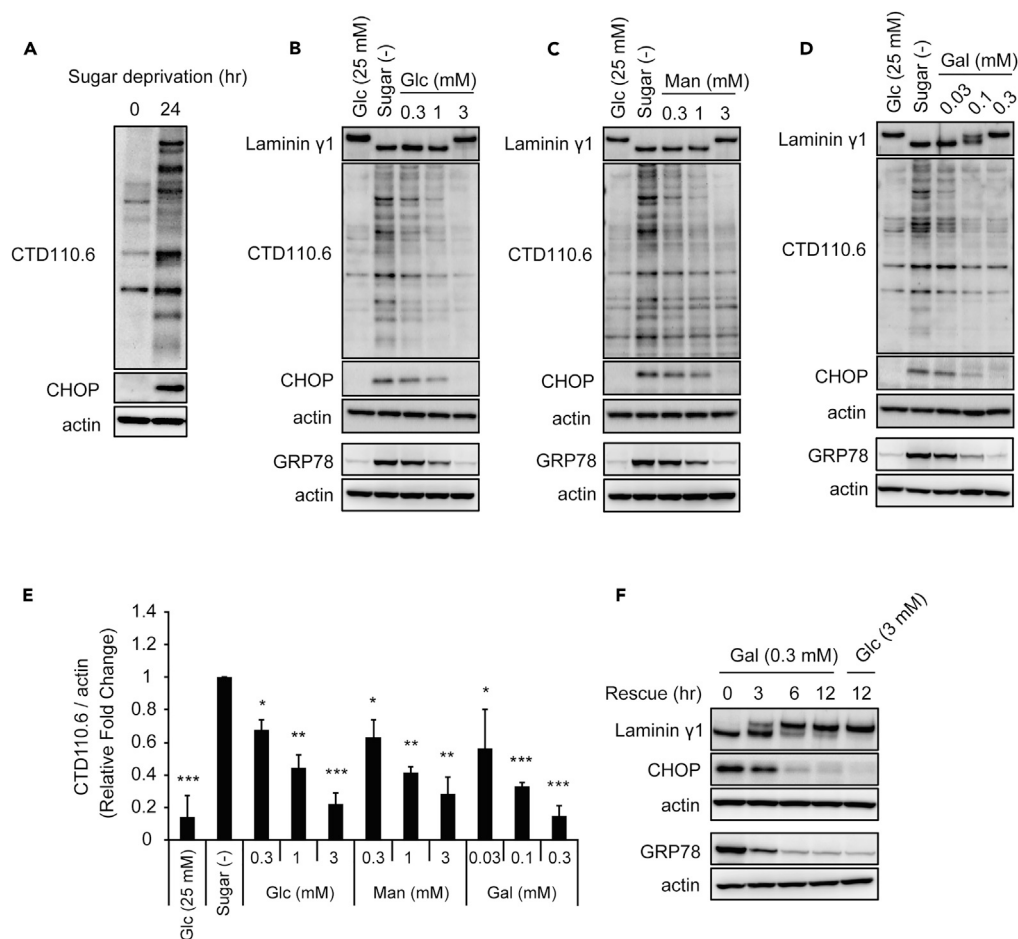
### Galactose Promotes *N*-Glycosylation Preferentially over Glucose or Mannose

Sugar deprivation induces immature *N*-GlcNAc<sub>2</sub>-modified glycoproteins, which cross-react with the O-GlcNAc-specific antibody CTD110.6 (Isono, 2011). We also independently observed that HEK293 cells produced proteins recognized by CTD110.6 antibody under sugar deprivation and that ER stress was evident under this condition (Figure 1A). We successfully identified laminin  $\gamma$ 1 and laminin  $\beta$ 2 as CTD110.6-antibody-recognized proteins, by immunoprecipitation with CTD110.6 antibody, followed by liquid chromatography-tandem mass spectrometry analyses (Figures S1A and S1B). Both of these are *N*-linked high-mannose-type glycoproteins (Fujiwara et al., 1998). Indeed, CTD110.6 antibody recognized only lower-molecular-weight or immature forms of laminin  $\gamma$ 1 and laminin  $\beta$ 2, which appeared only upon sugar starvation. We also showed that an anti-laminin  $\gamma$ 1 antibody revealed aggregate-like structures in cells under sugar starvation, most likely in the ER (Ikeda et al., 2014). Pulse-chase experiments using [<sup>35</sup>S] methionine/cysteine indicated that *N*-GlcNAc<sub>2</sub>-modified laminin  $\beta$ 2 was a newly synthesized protein rather than a cleavage product of mature *N*-glycosylated laminin  $\beta$ 2 (Figure S2). Addition of varying amounts of glucose in a dose- and time-dependent manner eliminated these immature glycoproteins and ER stress (Figures 1B and S1C). These results clearly indicated that sugar-deprivation-induced ER stress is correlated with the accumulation of immature glycoproteins in the ER.

To elucidate the role of other hexoses in protein glycosylation, we then focused on mannose and galactose and examined how these sugars function in producing mature *N*-glycosylated proteins, when compared with glucose. Mannose showed an essentially identical capacity as glucose with regard to the production of mature *N*-glycosylated proteins and the reduction of ER stress (Figure 1C). Surprisingly, however, galactose was 10 times more effective at producing mature *N*-glycosylated proteins and canceling ER stress (Figure 1D). In other words, 0.3 mM galactose was just as effective as 3 mM glucose or 3 mM mannose in the production of mature *N*-glycosylated proteins and abatement of ER stress (Figures 1B–1E). Furthermore, both 0.3 mM galactose (Figure 1F) and 3 mM glucose (Figure S1C) led to mature laminin  $\gamma$ 1 production over a similar time course. We also observed that galactose had the same robust ability to produce mature *N*-glycosylated proteins and to reduce ER stress in other cell lines, including human hepatoblastoma-derived HepG2 cells and rat pheochromocytoma-derived PC12 cells (Figure S3).

### Sugar Deprivation Increases the Expression of Enzymes Involved in *N*-Glycosylation via XBP1s

Activated nucleotide sugars, such as UDP-GlcNAc and guanosine diphosphate mannose (GDP-Man), which are required for *N*-glycosylation, are derived from fructose-6-phosphate (F6P) (Dennis et al., 2009). We therefore speculated that cellular mechanisms should exist to compensate for the glycosylation failure under sugar deprivation. Based on such speculation, we performed qRT-PCR analyses on mRNAs of glycolytic pathway enzymes and observed the upregulation of *HK2*, *ALDO*, and *ENO4* mRNAs in response to sugar deprivation (Figure 2A). We also observed upregulation of mRNAs for *GFAT1* and *GMPP* (A and B), which are responsible for the production of UDP-GlcNAc and GDP-Man, respectively (Figures 2B and 2C) (Dennis et al., 2009; Szumilo et al., 1993; Ning and Elbein, 2000). mRNA for *STT3A*, but not for *STT3B*, both of which encode catalytic subunits of the *N*-oligosaccharyltransferase (OST) complex (Kelleher et al., 2003; Ruiz-Canada et al., 2009), was also upregulated (Figure 2C). Protein levels of HK2, GFAT1, GMPPA, and



**Figure 1. Galactose Promotes N-Glycosylation Preferentially over Glucose or Mannose**

(A) HEK293 cells were deprived of glucose for 24 hr. Total cell lysates were analyzed by western blot with CTD110.6 to assess N-glycosylation levels and an antibody against CHOP as an ER stress marker.

(B–D) The promoting effect of glucose (B), mannose (C), or galactose (D) for N-glycosylation. Each immunoblot is represented by laminin  $\gamma$ 1, CTD110.6, CHOP, and GRP78.

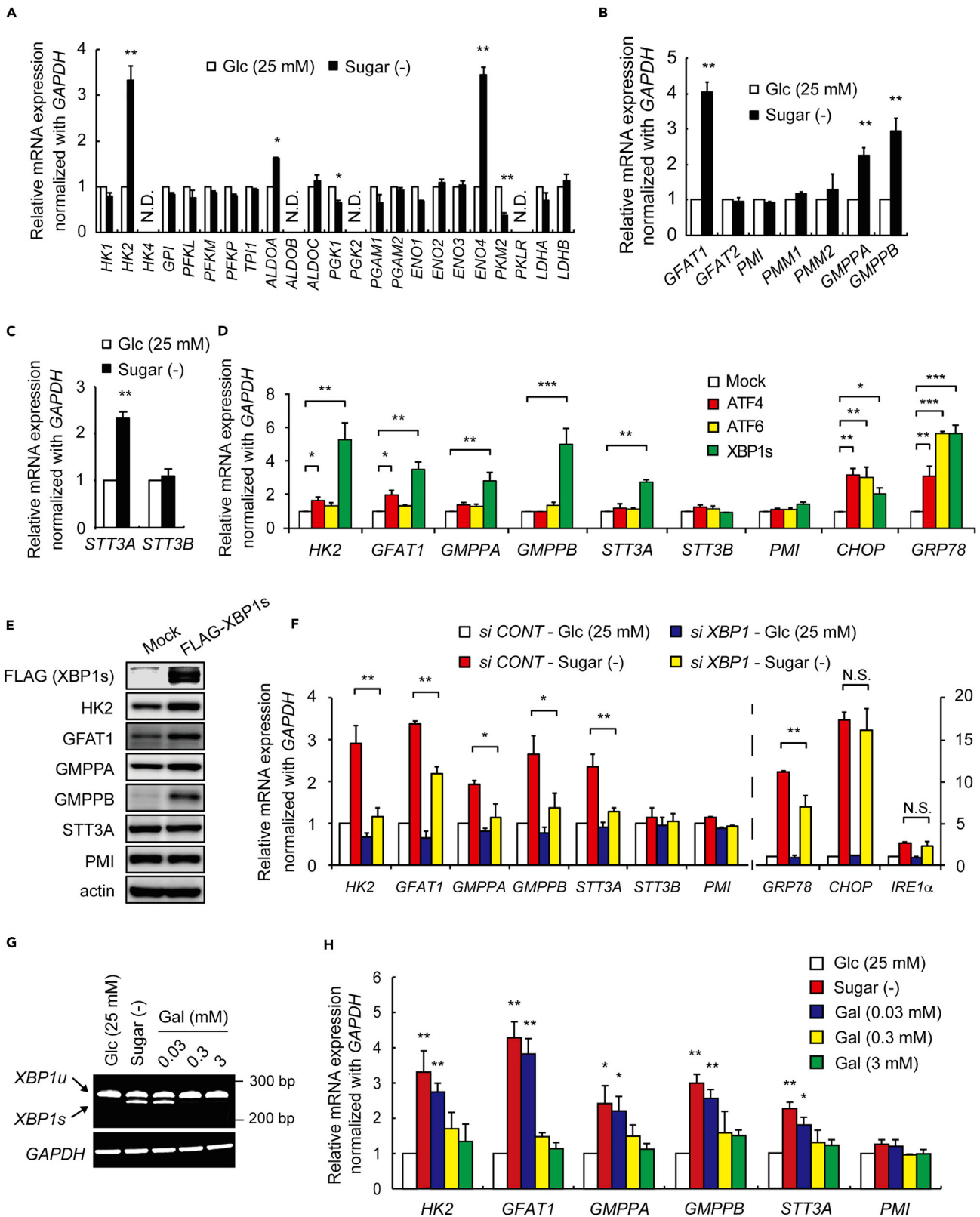
(E) Quantitative analysis of reactivity with CTD110.6 antibody in (B–D), normalized to actin intensity. The value for the sugar-free condition was set as 1. The data are the mean  $\pm$  SEM (n = 3). \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.005 by Dunnett’s test.

(F) HEK293 cells were pre-treated with sugar-free medium for 24 hr followed by re-feeding 0.3 mM galactose for indicated times. Actin was monitored as a loading control. Cells were cultured under serum-free conditions.

See also Figures S1–S3.

GMPPB were also increased by sugar deprivation (Figure S4). Subsequently, we tested whether other ER stress inducers, e.g., thapsigargin and brefeldin A, produced similar effects and found that *HK2*, *GFAT1*, *GMPPA*, *GMPPB*, and *STT3A* mRNAs and the corresponding proteins increased (Figure S5).

Under ER stress, ATF4, ATF6, and XBP1 function as transcription factors to upregulate their target genes to cope with the ER-stress-inducing conditions; the induction of these specific target mRNAs constitutes part of the UPR (Walter and Ron, 2011). We examined whether overexpression of these transcription factors would alter the expression of the same genes that were upregulated during sugar deprivation. We found that overexpression of the ER-stress-related XBP1s led to increases in transcription of all the above-mentioned genes that were upregulated during sugar deprivation (Figure 2D); overexpression of ATF4, but not ATF6, just slightly increased the abundance of *HK2* and *GFAT1* mRNAs (Figure 2D). Overexpression of ATF4 or ATF6 increased the expression of *XBP1* mRNA in the glucose(+) condition (Figures S6A and S6B) (Yoshida et al., 2001; Tsuru et al., 2016). However, overexpression of ATF4, but not ATF6, induced IRE1 $\alpha$  protein (Figure S6C), and the former, but not the latter, marginally induced the unconventional splicing



**Figure 2. Galactose Cancels the Upregulation of N-Glycosylation-Related Genes Induced by Spliced XBP1**

(A–C) Quantification of indicated gene transcripts by qRT-PCR. HEK293 cells were deprived of sugar for 24 hr, and gene transcripts in the glycolytic pathway (A), in the hexosamine biosynthetic pathway (B), and of the catalytic subunits of the *N*-oligosaccharyltransferase complex (C) were analyzed. The values are the mean  $\pm$  SEM ( $n = 3$ –6). N.D., not detected. \* $p < 0.05$ , \*\* $p < 0.01$  by Student's *t* test.

(D–F) Spliced XBP1 (XBP1s) increases glycosylation-related genes. (D) HEK293 cells were transfected with mock (white bars), ATF4 (red bars), ATF6 (yellow bars), or XBP1s (green bars) cDNA expression vectors, and cultured in 25 mM glucose condition for 24 hr. The expression levels of the indicated genes were measured by qRT-PCR, relative to the mock control, which was arbitrarily set as 1. Mannose phosphate isomerase (PMI) was included as a negative control. (E) HEK293 cells were transfected with FLAG-XBP1s and cultured in medium containing 25 mM glucose for 24 hr. Total cell lysates were analyzed by western blot. (F) Effect of XBP1 knockdown on indicated *N*-glycosylation-related gene expression in HEK293 cells treated with or without sugar deprivation for 24 hr. *GRP78* as well as *CHOP* and *IRE1 $\alpha$*  were used as positive and negative controls, respectively. The values are the mean  $\pm$  SEM ( $n = 3$ –6). N.S., not significant. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$  by Dunnett's test.

(G) XBP1 splicing assay. RNAs from HEK293 cells treated with the indicated conditions for 24 hr were examined by RT-PCR.

(H) Galactose effects on the expression of *N*-glycosylation-related genes were analyzed by qRT-PCR. HEK293 cells were treated in conditions described in (G). PMI was used as a negative control. The values are the mean  $\pm$  SEM ( $n = 3$ –6). \* $p < 0.05$ , \*\* $p < 0.01$  by Dunnett's test. Cells were cultured under serum-free conditions.

See also Figures S4–S6, Tables S1 and S2.

that generated XBP1s mRNA, albeit very weakly (Figure S6D) (Tsuru et al., 2016). The XBP1 splicing appeared to occur without inducing conspicuous IRE1 $\alpha$  phosphorylation. This observation may be explained as follows: the increased IRE1 $\alpha$  protein might weakly oligomerize by itself and induce self-phosphorylation, but the phosphorylation levels were very weak and below the detection levels of the antibody used. Overexpression of XBP1s, but not ATF4 or ATF6, clearly upregulated the protein levels of *N*-glycosylation-related genes, except for STT3A (Figure 2E). We also found that XBP1 knockdown significantly diminished the upregulation of the *N*-glycosylation-related mRNAs in response to sugar deprivation (Figure 2F). Furthermore, XBP1s production, induced by sugar deprivation, was inhibited by the addition of galactose in a dose-dependent manner (Figure 2G). Consistent with these results, the addition of a trace amount of galactose dose dependently suppressed the upregulation of the *N*-glycosylation-related mRNAs that were induced by sugar deprivation (Figure 2H).

**Accumulation of F6P in Galactose(+) Condition**

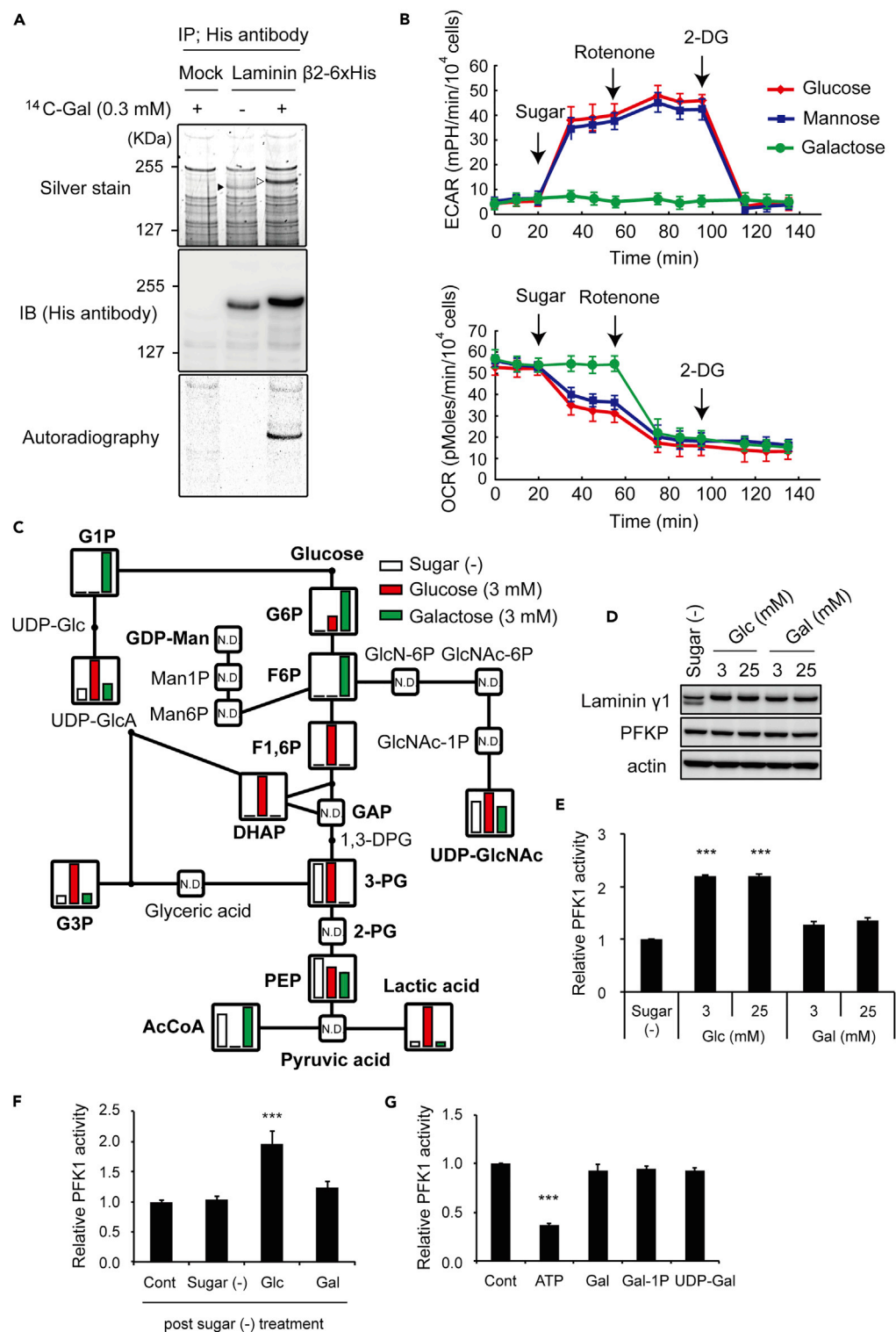
We then questioned whether added galactose is metabolized via the Leloir pathway to activated nucleotide sugars and is used in the mature *N*-glycosylated proteins, such as laminin  $\beta$ 2. We then observed that [<sup>14</sup>C]galactose was indeed incorporated in mature laminin  $\beta$ 2 (Figure 3A), demonstrating that the added galactose is incorporated as a carbon source into sugar chains of laminin  $\beta$ 2.

We next measured the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR). Glucose or mannose immediately increased ECAR, leading to a compensatory decrease of OCR. In contrast, galactose did not alter ECAR or OCR at all (Figure 3B). Rotenone, a complex I inhibitor, decreased OCR with all indicated sugar conditions. Decreased OCR induced a compensatory increase of ECAR in glucose(+) or mannose(+) medium, but not in galactose(+) medium (Figure 3B).

Given that galactose did not affect ECAR despite entering the glycolytic pathway, we expected that in the galactose(+) medium the glycolytic pathway may arrest, and then the corresponding metabolites would accumulate somewhere within the glycolytic pathway. We therefore performed a metabolome analysis with capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) to evaluate metabolite levels in HEK293 cells treated with sugar-free, 3 mM glucose, and 3 mM galactose for 6 hr (Figure 3C). In the sugar-free condition, levels of metabolites just upstream of dihydroxyacetone phosphate (DHAP) were essentially undetectable, e.g., glucose-1-phosphate (G1P), G6P, F6P, and fructose-1, 6-bisphosphate (Figure 3C). In the glucose(+) condition, the metabolites upstream of F6P appeared to be rapidly metabolized in the glycolytic pathway (Figure 3C). By contrast, in the galactose(+) condition, metabolite levels upstream of F6P dramatically increased, when compared with the sugar-free and the glucose(+) conditions. Consistent with the results of flux analyses (Figure 3B), in the galactose(+) condition, the amount of lactic acid, which reflects the ECAR, was comparable to that in the sugar-free condition, and was much lower than the amount of lactic acid in the glucose(+) condition (Figure 3C).

The level of UDP-GlcNAc even in the sugar-free condition was similar to those in the other two conditions (Figure 3C), and cells cultured in the sugar-free condition for a much longer time (e.g., 72 hours) retained the *N*-GlcNAc<sub>2</sub> modifications (Figure S7A), indicating that UDP-GlcNAc is mostly supplied by its re-usage





**Figure 3. Alteration of Metabolism in Response to Galactose**

(A) Incorporation of [ $^{14}\text{C}$ ]galactose into laminin  $\beta$ 2. HEK293 cells were transfected with mock or a C-terminal His-tagged laminin  $\beta$ 2 expression construct, and were then cultured in medium with or without 0.3 mM [ $^{14}\text{C}$ ]galactose for 24 hr. Immunoprecipitates with His antibody were analyzed by silver staining, western blot, and autoradiography.

**Figure 3. Continued**

(B) The response of ECAR (upper panel) and OCR (lower panel). HEK293 cells were stimulated with glucose (3 mM), mannose (3 mM), or galactose (3 mM), and subsequently subjected to the mitochondria inhibitor rotenone (3  $\mu$ M) and the glycolysis inhibitor 2-DG (100 mM) at the indicated times. Both datasets are from the same experiment, and each data point represents the mean  $\pm$  SEM, n = 4.

(C) Metabolomic analysis of HEK293 cells treated with sugar-free (white), glucose (3 mM, red), and galactose (3 mM, green) conditions for 6 hr. The relative quantities of the annotated metabolites are represented as bar graphs.

(D) Representative immunoblot of PFK1 in response to the indicated treatments.

(E) HEK293 cells were treated with the indicated conditions for 6 hr, and PFK1 activity was analyzed by measuring NADH consumption.

(F) HEK293 cells were sugar-deprived (SD) for 6 hr, followed by restoration with the indicated sugars (3 mM each) for 1 hr.

(G) HEK293 cells were cultured in medium containing glucose (3 mM) for 6 hr, and PFK1 activity was analyzed in a reaction mixture that included galactose (Gal) (3 mM), galactose-1-phosphate (Gal-1P) (3 mM), or UDP galactose (UDP-Gal) (3 mM). The data are the mean  $\pm$  SEM. \*\*\*p < 0.005 by Dunnett's test. Cells were cultured under serum-free conditions.

See also [Figure S7](#).

in the cells (Rome and Hill, 1986), but not by HBP. Consistently, cells cultured with azaserine or 6-diazo-5-oxo-1-norleucine, both of which are HBP inhibitors, were unaffected with regard to the maturation of *N*-glycosylated proteins or the abatement of ER stress in the presence of glucose or galactose ([Figure S7B](#)). These results further support the idea that accumulated F6P in the galactose(+) condition is preferentially utilized to produce GDP-Man for the maturation of high-mannose-type glycoproteins. It is notable that addition of bafilomycin A1 or E64d/pepstatin A, each of which is an inhibitor of autophagy, did not affect the maturation of *N*-glycosylated proteins by galactose ([Figure S7C](#)). These results appear to rule out the involvement of autophagy in the maturation of *N*-glycosylated proteins by galactose.

We next investigated whether galactose affects the expression level of phosphofructokinase 1 (PFK1), but the expression levels of PFK1 in the HEK293 cells were almost identical among all conditions ([Figure 3D](#)). We then compared PFK1 activity among the conditions. Interestingly, we found that PFK1 activity increased in parallel with the glucose concentrations of culture medium, and galactose was unable to activate PFK1 ([Figures 3E and 3F](#)). On the other hand, the PFK1 activity was not inhibited by the addition of galactose or galactose metabolites, e.g., galactose-1-phosphate and UDP galactose ([Figure 3G](#)). Taken together, these results indicate that galactose does not stimulate the PFK1 activity, leading to the accumulation of galactose-derived F6P.

**A Trace Amount of Galactose Reduces ER Stress and Cell Death in Sugar Deprivation**

Next, we investigated the effects of glucose or galactose on the prevention of cell death induced by sugar deprivation. Supplementation with 3 and 25 mM glucose increased cell proliferation in a dose-dependent manner ([Figure 4A](#)). However, 0.3 mM glucose showed no effect on cell proliferation or protection, when compared with the sugar-free condition. In contrast, 0.3 mM galactose dramatically induced cell proliferation. With 3 mM galactose, cell proliferation slightly decreased, compared with 3 mM glucose or 0.3 mM galactose. These observations may reflect a condition of patients with galactosemia, having a milieu of symptoms including cataracts, liver, and brain growth failure (Lai et al., 2009).

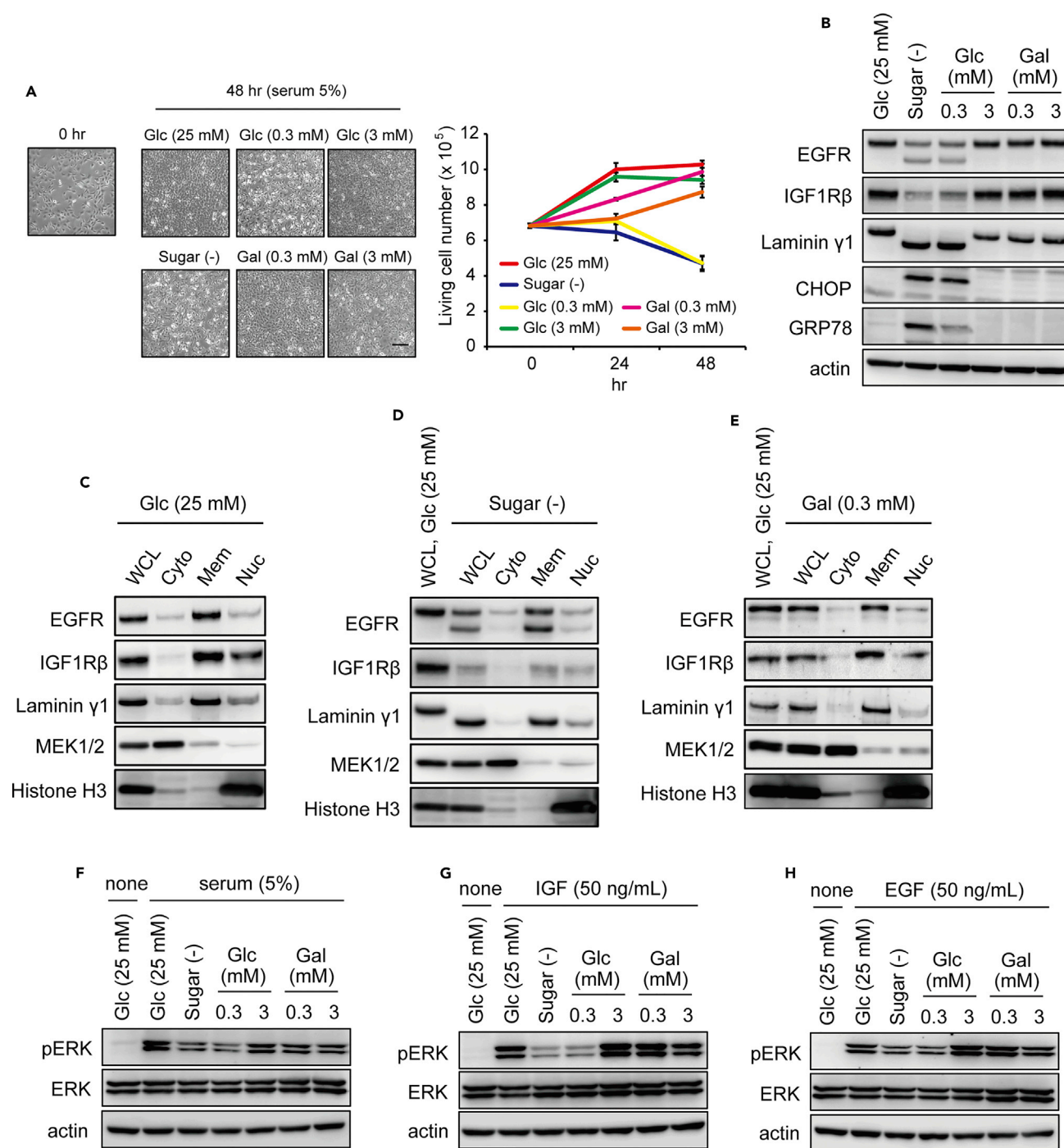
**A Trace Amount of Galactose Promotes Growth Signal Transduction via the Maturation of Protein Glycosylation**

Based on the evidence that many growth factors and their receptors are glycoproteins (Takahashi et al., 2004; Kaszuba et al., 2015), we examined the protein status of growth factor receptors, such as epidermal growth factor (EGF) receptor (EGFR) and insulin growth factor (IGF) receptor (IGFR). In the sugar-starved conditions, maturation of EGFR was clearly inhibited, and protein levels of IGF1R $\beta$  were greatly reduced ([Figure 4B](#)). Mature and immature EGFR and IGF1R $\beta$  were mainly detected in the membrane fraction ([Figures 4C–4E](#)). However, in the starved condition, responses of cells to EGF, IGF, and serum were reduced, which was evidenced by the absence of ERK phosphorylation ([Figures 4F–4H](#)). Addition of a trace amount of galactose, namely, 0.3 mM galactose, was able to correct these abnormalities. By contrast, these abnormalities were not corrected by the addition of 0.3 mM glucose ([Figures 4F–4H](#)).

**DISCUSSION**

Animals must overcome various environmental challenges. Among them, starvation might be common but most dangerous, and thus animals should be equipped to cope with such potentially fatal conditions. Starvation, especially sugar deprivation, has been known to induce ER stress, which is evidenced by the





**Figure 4. A Trace Amount of Galactose Promotes Cell Survival and Growth as well as Maturation of Growth Factor Receptors**

(A) Photographs of HEK293 cells, cultured with sugar-free, glucose, or galactose conditions for 48 hr, starting from cells at 70% confluency in each well of a 6-well plate at time 0 (left). Live cell numbers were counted by staining with trypan blue (right). The data are the mean  $\pm$  SEM ( $n = 3$ ). Scale bar, 50  $\mu$ m. (B) Expression of IGF1R $\beta$  and EGFR. HEK293 cells were treated with the indicated conditions for 24 hr, and whole-cell lysates were analyzed by western blot. (C–E) Cell fractionation to examine glycoproteins after growth in medium with (C) 25 mM glucose, (D) sugar-free, or (E) 0.3 mM galactose. Antibodies against EGFR, IGF1R $\beta$ , and laminin  $\gamma$ 1 were as in (B), and MEK1/2 and histone H3 were markers for the cytoplasmic and nuclear fractions, respectively. (F–H) ERK activation by extracellular stimulation. HEK293 cells were cultured with the indicated conditions without serum for 24 hr before stimulation with 5% serum for 5 min (F), 50 ng/mL IGF1 for 10 min (G), or 50 ng/mL EGF for 5 min (H). Western blot analysis detected phosphorylated and total ERK levels under different conditions of sugar supplementation, as indicated. Actin was used as a loading control. Cells in (B–H) were cultured under serum-free conditions.

induction of CHOP and GRP78 proteins. In this study, we observed the production of immature glycosylation forms of high-mannose-type glycoproteins, which were recognized by CTD110.6 antibody in sugar-starved cultured cells (Isono, 2011). Given that levels of GDP-Man decrease under sugar starvation, and that the molecule recognized by CTD110.6 antibody is *N*-GlcNAc<sub>2</sub> (Isono, 2011), addition of mannose to dolichol-pyrophosphate-GlcNAc<sub>2</sub> (Dol-PP-GlcNAc<sub>2</sub>) by  $\beta$ -1,4 mannosyltransferase-1 (ALG1) (Couto et al., 1984) would not occur. Thus ALG1 functions as the sensor to monitor the levels of GDP-Man in the cells; when the levels of GDP-Man become much lower than the KD value, any addition of mannose to Dol-PP-GlcNAc<sub>2</sub> would cease. In this scenario, mammalian cells utilize such naked Dol-PP-GlcNAc<sub>2</sub>, instead of mature glycans, as the substrate of OST to produce immature glycoproteins. As the presence of such immature glycoproteins appeared to be intimately coupled with the evocation of ER stress, cells appear to engage a system to induce an ER stress response via the production of immature glycoproteins under starvation. Consistently, we were able to show that ER stress leads to the expression of HK2, GFAT1, and GMPP, essential enzymes involved in the synthesis of *N*-glycosylation components (e.g., UDP-GlcNAc, GDP-Man), and that XBP1s is responsible for their expression. It is noteworthy that UDP-GlcNAc and GDP-Man are also utilized in *O*-glycosylation (Spiro, 2002). Therefore, it is also expected that maturation of not only *N*-glycosylated but also *O*-glycosylated proteins is also hindered under sugar starvation and that maturation of both *N*- and *O*-glycosylated proteins is also restored by a trace amount of galactose.

Other ER stress inducers, e.g., thapsigargin and brefeldin A, which are not related to the production of immature glycoproteins, also induce *HK2*, *GFAT1*, *GMPPA*, *GMPPB*, and *STT3A* mRNAs and the corresponding proteins, indicating that the response to immature glycoproteins is a fundamental, perhaps archetypical ER stress response.

We demonstrated that galactose is much more potent to maintain mature *N*-glycosylation of proteins than glucose and mannose. Maintenance of mature *N*-glycosylation contributes to the relief of starvation-induced ER stress and cell death and also to appropriate growth-factor-mediated signal transduction. From our metabolome data, under the galactose(+) condition accumulation of galactose-derived F6P was observed, which would be utilized in the production of nucleotide sugars required for *N*-glycosylation. However, such F6P accumulation was not observed in the glucose(+) condition. It is notable that galactose itself is utilized in other types of glycosylation. These observations indicate that galactose from milk or milk sugar would preferentially contribute to produce mature glycoproteins rather than to energy production during starvation. It is obvious that malnutrition affects the growth of infants. Furthermore, it has been shown that newborn babies experience what can be considered to be seriously starved conditions at birth (Burns et al., 2008; Adamkin and Committee on Fetus and Newborn, 2011), and that to survive these conditions, autophagy and milk play crucially important roles for the supply of nutrients as energy sources (Kuma et al., 2004). Our results indicate that milk, as a sugar source, is also necessary for the maturation of glycoproteins, which in turn contribute not only to the prevention of ER stress and cell death in many organs but also to the maintenance of growth-factor-mediated signaling for the normal post-natal growth of infants.

### Limitations of the Study

The study was conducted only in cultured mammalian cells. Therefore, for the substantiation of the beneficial functions of galactose during starvation *in vivo*, it will be necessary to perform animal experiments, examining both cellular and overall responses under similar conditions.

### METHODS

All methods can be found in the accompanying [Transparent Methods supplemental file](#).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes Transparent Methods, seven figures, and two tables and can be found with this article online at <https://doi.org/10.1016/j.isci.2018.11.035>.

### ACKNOWLEDGMENTS

We wish to thank K. Manabe, M. Ohga, and S. Sugiyama for their technical assistance and Y. Watanabe (Kyoto University) for kind support with LC-MS/MS analysis. We are grateful to Professor James A. Hejna (Kyoto University) for critical reading of the manuscript and Dr. Yamato Kikkawa (Tokyo University of Pharmacy) for the gift of the rat laminin  $\beta$ 2 antibody (C4) and plasmid. This work was supported in part by

research grants from the Mitsubishi Foundation, the Ministry of Education, Culture, Sports, Science and Technology, Japan. Also, this research was supported by the Platform Project for Supporting Drug Discovery and Life Science Research (Platform for Dynamic Approaches to Living Systems) from the Japan Agency for Medical Research and Development (AMED).

## AUTHOR CONTRIBUTIONS

N.S. performed most of the experiments. H.I. supported the experiments. N.S. and A.K. designed the project, analyzed the results, and wrote the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: May 1, 2018

Revised: November 19, 2018

Accepted: November 20, 2018

Published: December 21, 2018

## REFERENCES

- Adamkin, D.H.; Committee on Fetus and Newborn (2011). Postnatal glucose homeostasis in late-preterm and term infants. *Pediatrics* 127, 575–579.
- Agrawal, N., Jamshed, N., Aggarwal, P., and Ekka, M. (2014). Severe hypoglycemia masquerading as cerebellar stroke. *J. Family Med. Prim. Care* 3, 440–442.
- Alton, G., Hasilik, M., Niehues, R., Panneerselvam, K., Etchison, J.R., Fana, F., and Freeze, H.H. (1998). Direct utilization of mannose for mammalian glycoprotein biosynthesis. *Glycobiology* 8, 285–295.
- Apweiler, R., Hermjakob, H., and Sharon, N. (1999). On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim. Biophys. Acta* 1473, 4–8.
- Burns, C.M., Rutherford, M.A., Boardman, J.P., and Cowan, F.M. (2008). Patterns of cerebral injury and neurodevelopmental outcomes after symptomatic neonatal hypoglycemia. *Pediatrics* 122, 65–74.
- Couto, J.R., Huffaker, T.C., and Robbins, P.W. (1984). Cloning and expression in *Escherichia coli* of a yeast mannosyltransferase from the asparagine-linked glycosylation pathway. *J. Biol. Chem.* 259, 378–382.
- Dennis, J.W., Nabi, I.R., and Demetriou, M. (2009). Metabolism, cell surface organization, and disease. *Cell* 139, 1229–1241.
- Frey, P.A. (1996). The Leloir pathway: a mechanistic imperative for three enzymes to change the stereochemical configuration of a single carbon in galactose. *FASEB J.* 10, 461–470.
- Fujiwara, S., Shinkai, H., Deutzmann, R., Paulsson, M., and Timpl, R. (1998). Structure and distribution of N-linked oligosaccharide chains on various domains of mouse tumour laminin. *Biochem. J.* 252, 453–461.
- Gross, K.C., and Acosta, P.B. (1991). Fruits and vegetables are a source of galactose: implications in planning the diets of patients with galactosaemia. *J. Inher. Metab. Dis.* 14, 253–258.
- Holden, H.M., Thoden, J.B., Timson, D.J., and Reece, R.J. (2004). Galactokinase: structure, function and role in type II galactosemia. *Cell. Mol. Life Sci.* 61, 2471–2484.
- Ikeda, H.O., Sasaoka, N., Koike, M., Nakano, N., Muraoka, Y., Toda, Y., Fuchigami, T., Shudo, T., Iwata, A., Hori, S., et al. (2014). Novel VCP modulators mitigate major pathologies of rd10, a mouse model of retinitis pigmentosa. *Sci. Rep.* 4, 5970.
- Ishii, A., Ikeda, T., Hitoshi, S., Fujimoto, I., Torii, T., Sakuma, K., Nakakita, S., Hase, S., and Ikenaka, K. (2007). Developmental changes in the expression of glycogenes and the content of N-glycans in the mouse cerebral cortex. *Glycobiology* 17, 261–276.
- Isono, T. (2011). O-GlcNAc-specific antibody CTD110.6 cross-reacts with N-GlcNAc2-modified proteins induced under glucose deprivation. *PLoS One* 6, e18959.
- Kaempfer, J.W., Li, H.Q., Groothuis, J.R., Battaglia, F.C., Zerbe, G.O., and Sparks, J.W. (1988). Galactose, glucose, and lactate concentrations in the portal venous and arterial circulations of newborn lambs after nursing. *Pediatr. Res.* 23, 598–602.
- Kaszuba, K., Grzybek, M., Orłowski, A., Danne, R., Róg, T., Simons, K., Coskun, Ü., and Vattulainen, I. (2015). N-Glycosylation as determinant of epidermal growth factor receptor conformation in membranes. *Proc. Natl. Acad. Sci. U S A* 112, 4334–4339.
- Kelleher, D.J., Karaoglu, D., Mandon, E.C., and Gilmore, R. (2003). Oligosaccharyltransferase isoforms that contain different catalytic STT3 subunits have distinct enzymatic properties. *Mol. Cell* 12, 101–111.
- Kuma, A., Hatano, M., Matsui, M., Yamamoto, A., Nakaya, H., Yoshimori, T., Ohsumi, Y., Tokuhashi, T., and Mizushima, N. (2004). The role of autophagy during the early neonatal starvation period. *Nature* 432, 1032–1036.
- Lai, K., Elsas, L.J., and Wierenga, K.J. (2009). Galactose toxicity in animals. *IUBMB Life* 61, 1063–1074.
- Leslie, N.D. (2003). Insights into the pathogenesis of galactosemia. *Annu. Rev. Nutr.* 23, 59–80.
- Mori, K. (2009). Signalling pathways in the unfolded protein response: development from yeast to mammals. *J. Biochem.* 146, 743–750.
- Ning, B., and Elbein, A.D. (2000). Cloning, expression and characterization of the pig liver GDP-mannose pyrophosphorylase. Evidence that GDP-mannose and GDP-Glc pyrophosphorylases are different proteins. *Eur. J. Biochem.* 267, 6866–6874.
- Opendakker, G., Rudd, P.M., Wormald, M., Dwek, R.A., and Van Damme, J. (1995). Cells regulate the activities of cytokines by glycosylation. *FASEB J.* 9, 453–457.
- Rome, L.H., and Hill, D.F. (1986). Lysosomal degradation of glycoproteins and glycosaminoglycans. Efflux and recycling of sulphate and N-acetylhexosamines. *Biochem. J.* 235, 707–713.
- Ruiz-Canada, C., Kelleher, D.J., and Gilmore, R. (2009). Cotranslational and posttranslational N-glycosylation of polypeptides by distinct mammalian OST isoforms. *Cell* 136, 272–283.
- Spiro, R.G. (2002). Protein glycosylation: nature, distribution, enzymatic formation, and disease implications of glycopeptide bonds. *Glycobiology* 12, 43R–56R.
- Szumilo, T., Drake, R.R., York, J.L., and Elbein, A.D. (1993). GDP-mannose pyrophosphorylase. Purification to homogeneity, properties, and utilization to prepare photoaffinity analogs. *J. Biol. Chem.* 268, 17943–17950.
- Takahashi, M., Tsuda, T., Ikeda, Y., Honke, K., and Taniguchi, N. (2004). Role of N-glycans in growth factor signaling. *Glycoconj. J.* 20, 207–212.
- Timson, D.J. (2006). The structural and molecular biology of type III galactosemia. *IUBMB Life* 58, 83–89.
- Tsuru, A., Imai, Y., Saito, M., and Kohno, K. (2016). Novel mechanism of enhancing IRE1 $\alpha$ -XBP1

signalling via the PERK-ATF4 pathway. *Sci. Rep.* *6*, 24217.

Walter, P., and Ron, D. (2011). The unfolded protein response: from stress pathway to homeostatic regulation. *Science* *334*, 1081–1086.

Wang, Z.V., Deng, Y., Gao, N., Pedrozo, Z., Li, D.L., Morales, C.R., Criollo, A., Luo, X., Tan, W., Jiang, N., et al. (2014). Spliced X-box binding protein 1 couples the unfolded protein response to hexosamine biosynthetic pathway. *Cell* *156*, 1179–1192.

Yoshida, H., Matsui, T., Yamamoto, A., Okada, T., and Mori, K. (2001). XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* *107*, 881–891.

**ISCI, Volume 10**

**Supplemental Information**

**A Trace Amount of Galactose, a Major  
Component of Milk Sugar, Allows Maturation of  
Glycoproteins during Sugar Starvation**

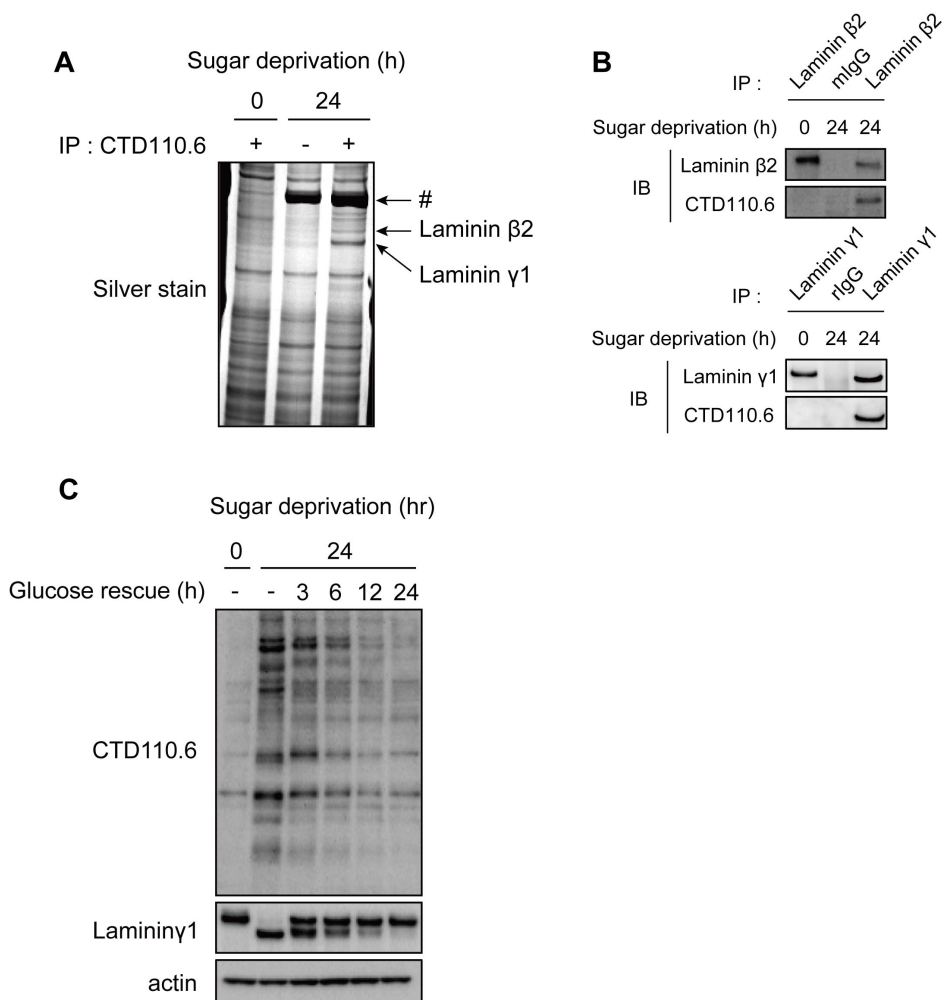
**Norio Sasaoka, Hiromi Imamura, and Akira Kakizuka**

## **Supplemental Information**

**A Trace Amount of Galactose, a Major Component of Milk Sugar, Allows  
Maturation of Glycoproteins During Sugar Starvation**

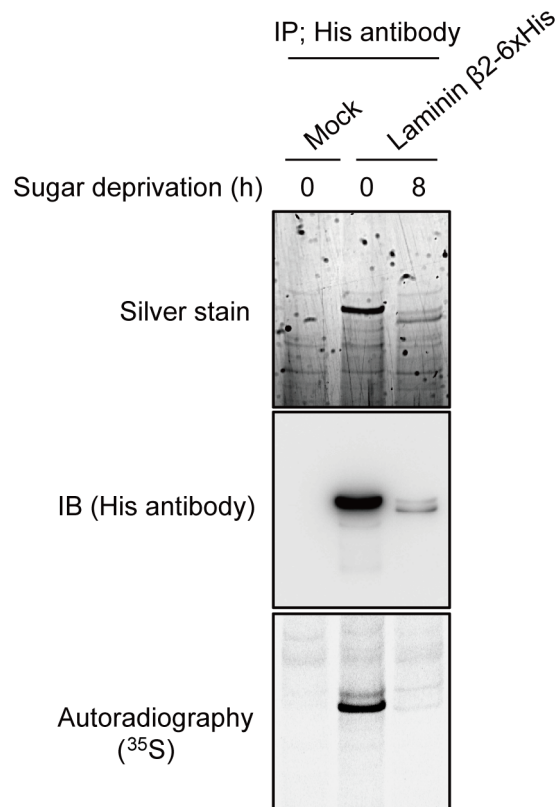
**Norio Sasaoka, Hiromi Imamura, Akira Kakizuka**





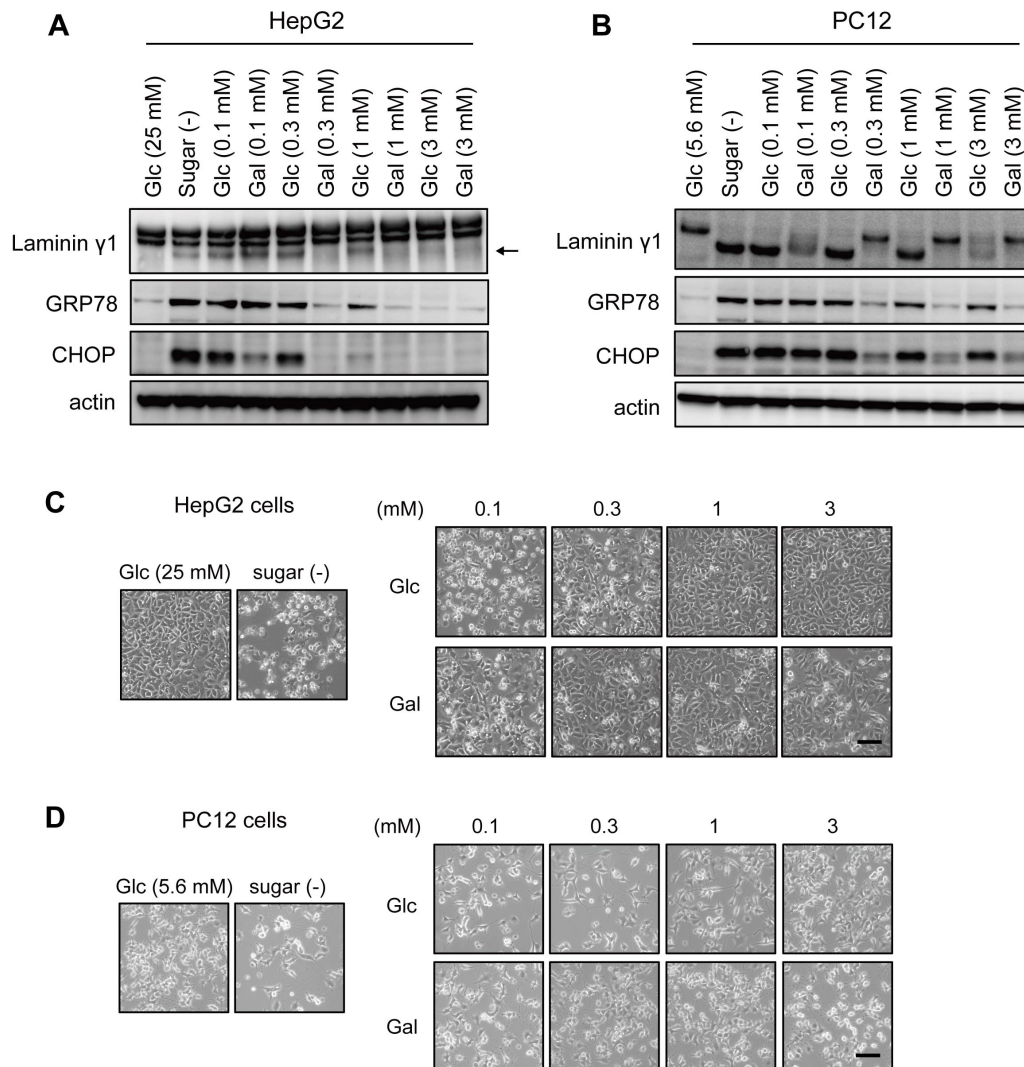
**Figure S1. Identification of *N*-GlcNAc-Modified Proteins under Sugar-Free Conditions, Related to Figure 1.**

(A) HEK293 cells were cultured in high-glucose medium (25 mM) or sugar-free medium (0 mM glucose) for 24 h. Proteins immunoprecipitated with CTD110.6 antibody were visualized on silver-stained gels. Laminin  $\beta$ 2, Laminin  $\gamma$ 1, and a non-specific signal (#) are shown. (B) HEK293 cells were treated as in **Figure 1A**, and anti-Laminin  $\beta$ 2 or anti-Laminin  $\gamma$ 1 immunoprecipitates were analyzed by western blotting with indicated antibodies. (C) HEK293 cells were grown with high-glucose medium, then deprived of glucose for 24 h. During this time, 3 mM glucose was added back to the medium for the indicated total times; thus, “24” indicates that glucose was added back to the medium immediately and remained in the medium over the full 24 h of subsequent growth, whereas “3” indicates that glucose was added back to the medium only 3 h before harvest. Western blots of cell lysates were probed with CTD110.6, anti-Laminin  $\gamma$ 1, and anti-actin antibodies. Cells were cultured under serum-free conditions.



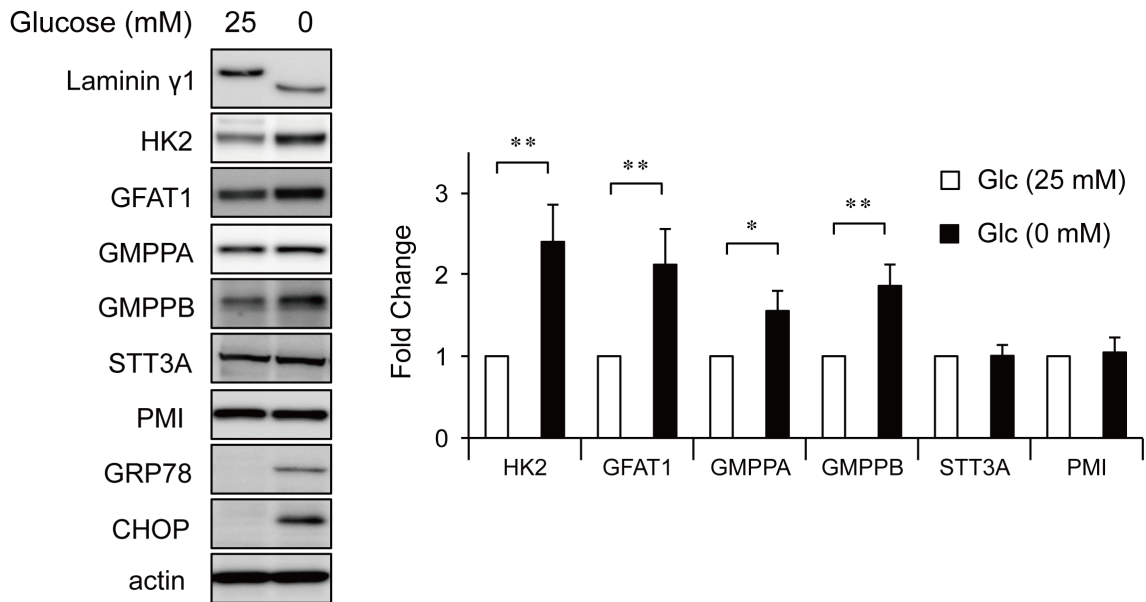
**Figure S2. *N*-GlcNAc-Modified Laminin  $\beta$ 2 is a Newly Synthesized Product, Related to Figure 1.**

HEK293 cells were transfected with mock or C-terminal 6xHis-tagged Laminin  $\beta$ 2. At 24 h post-transfection, cells were cultured in serum-free medium containing 50  $\mu\text{Ci}$  of [ $^{35}\text{S}$ ]methionine/cysteine (PerkinElmer) for 4 h. Following the pulse-labeling, cells were subjected to sugar deprivation for 8 h. Immunoprecipitates pulled down with an anti-His-tag antibody were analyzed by silver staining, western blotting, and autoradiography.



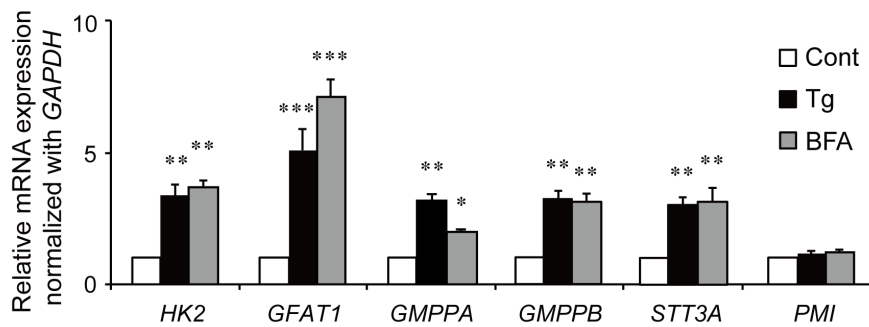
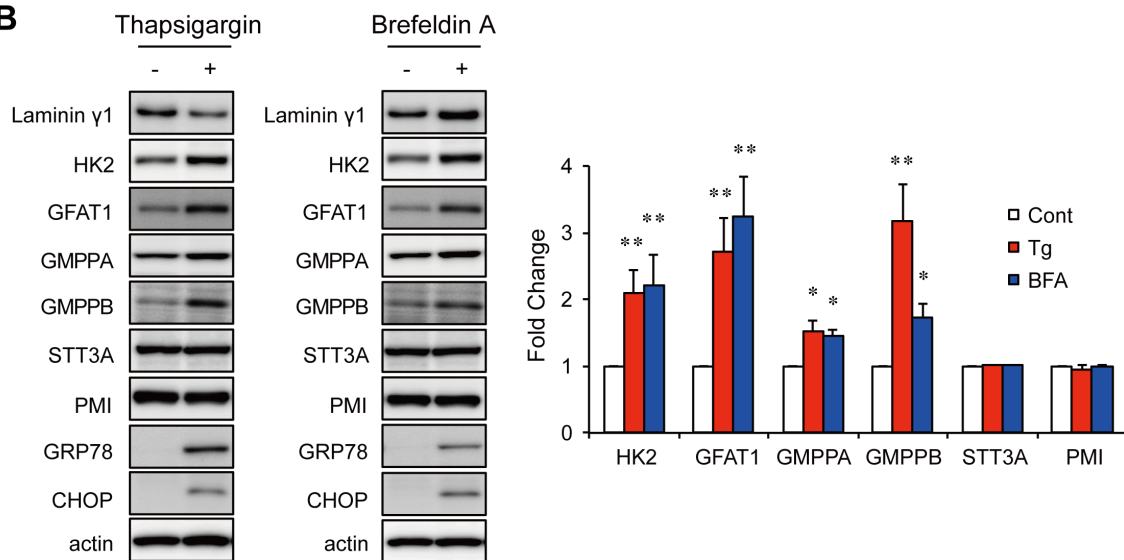
**Figure S3. Galactose Preferentially Promotes Protein *N*-Glycosylation over Glucose in Low-Sugar Conditions in HepG2 and PC12 cells, Related to Figure 1.**

The effect of limiting amounts of galactose or glucose on *N*-glycosylation of Laminin  $\gamma$ 1, and the ER stress marker CHOP, in HepG2 (A) and PC12 cells (B). In A and B, each immunoblot shows the Laminin  $\gamma$ 1 cellular *N*-glycosylation level and CHOP as ER stress marker. The arrow indicates an immature lower molecular weight Laminin  $\gamma$ 1 in HepG2 cells. Data are representative of three independent experiments. Photographs of HepG2 (C) and PC12 cells (D), cultured with the indicated concentrations of glucose or galactose for 8 h and 30 h, respectively. In PC12 cells, the culture medium was replaced with 50 ng/mL neurite growth factor in low-glucose DMEM with 1% FBS and 1% HS for 24 h prior to treatment. Scale bar, 50  $\mu$ m. Cells were cultured under serum-free conditions.



**Figure S4. Expression Levels of *N*-Glycosylation-Related Proteins in HEK293 Cells, Comparing 25 mM Glucose to Sugar-Free Medium, Related to Figure 2.**

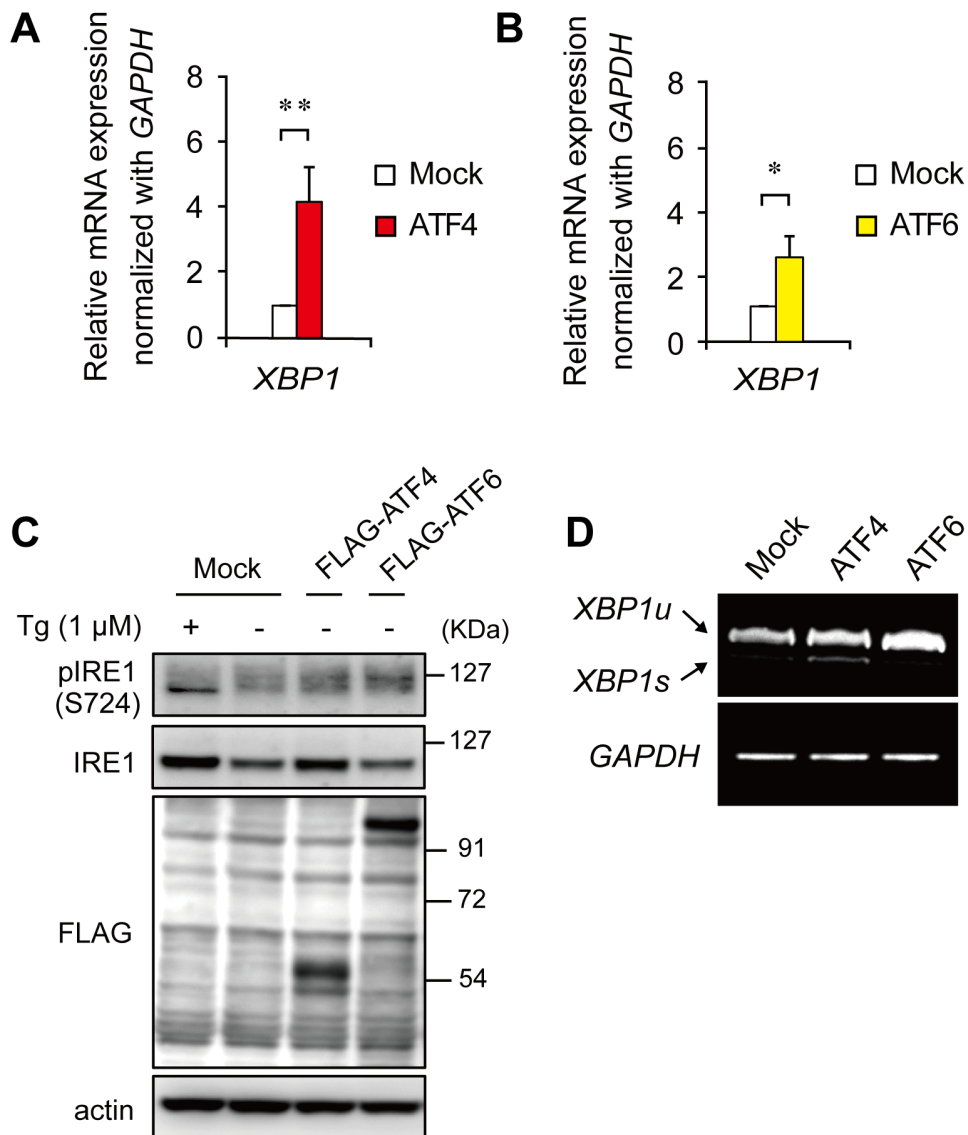
Expression levels of proteins related to sugar-free upregulated mRNA levels (see **Figures 2A-2C**) were analyzed by western blotting using the indicated antibodies (left panels). Cells were cultured under serum-free conditions. Quantitative analysis of the protein levels normalized to actin is shown (right panels). The data are the mean  $\pm$  SEM ( $n = 3$ ). \*  $P < 0.05$ , \*\*  $P < 0.01$  by Student's  $t$  test.

**A****B**

**Figure S5. The ER Stress Inducers Thapsigargin and Brefeldin A also Upregulate *N*-Glycosylation-Related Genes, Related to Figure 2.**

(A) RT-qPCR analysis of *N*-glycosylation-related genes in HEK293 cells after treatment with 0.1% DMSO (Cont, white color), 1  $\mu$ M thapsigargin (Tg, black color) or 3  $\mu$ g/mL brefeldin A (BFA, grey color) for 24 h. *PMI* was used as a negative control.

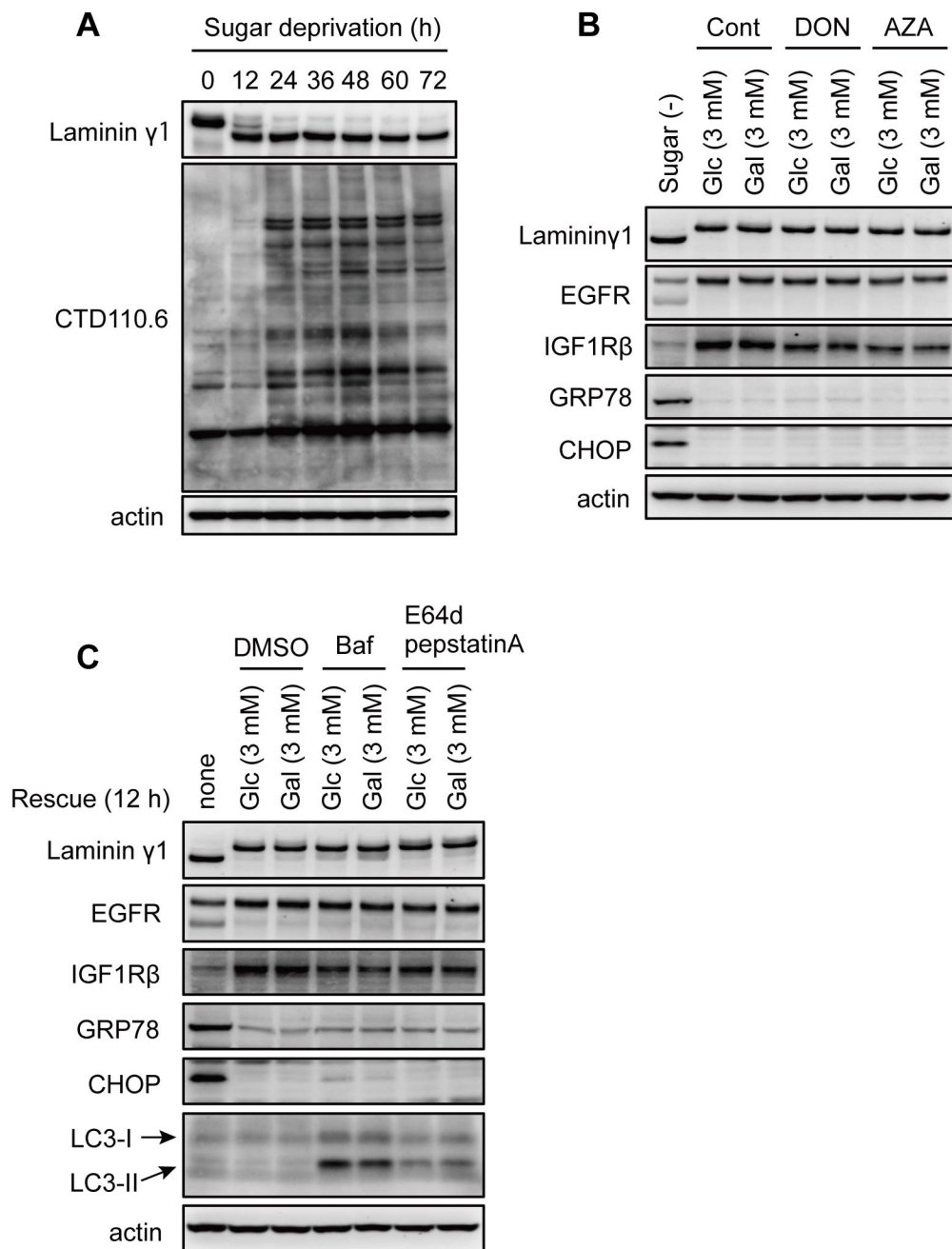
(B) Western blotting showing the levels of *N*-glycosylation-related proteins under the treatment conditions described in (A). Cells were cultured under serum-free conditions. Quantitative analysis of protein levels normalized to actin is shown (right panels). The data are the mean  $\pm$  SEM (n = 3). \*  $P < 0.05$ , \*\*  $P < 0.01$  by Dunnett's test (vs. Cont.).



**Figure S6. Expression of XBP1 was Induced by the Forced Expression of ATF4 or ATF6, Related to Figure 2.**

(**A and B**) HEK293 cells were transfected with Mock and FLAG-ATF4 (**A**) or FLAG-ATF6 (**B**) for 24 h. The amount of *XBP1* was determined by RT-qPCR. The data are the mean  $\pm$  SEM (n = 3). \*  $P < 0.05$ , \*\*  $P < 0.01$  by Student's *t* test. (**C**) HEK293 cells were transfected with empty (Mock), ATF4-, or an ATF6-expression vector and cultured for 48 h, followed by a 12h treatment with 0.1% DMSO or thapsigargin (Tg). Cell lysates were then subjected to western blotting analysis with the indicated antibodies. (**D**) Confirmation of overexpression efficacy of ATF4 or ATF6 in HEK293 cells by XBP1 splicing assay. Cells were cultured under serum-free conditions.





**Figure S7. Maturation of Glycoproteins by Galactose is Independent of Autophagy, Related to Figure 3.**

(A) Western blotting analysis of HEK293 cells cultured in the absence of sugar for the indicated times. Each immunoblot was probed with CTD110.6, anti-Laminin  $\gamma$ 1, or anti-actin antibodies. (B) HEK293 cells cultured in medium containing 3 mM glucose or 3 mM galactose with or without HBP inhibitors (1 mM DON or 1 mM AZA) for 24 h were harvested, and then cell lysates were subjected to western blotting analysis with

the indicated antibodies. (C) HEK293 cells were cultured in glucose-free medium for 24 h, and then treated with medium containing 3 mM glucose or 3 mM galactose with or without autophagy inhibitors for 12 h. Inhibitors were bafilomycin A1 (1  $\mu$ M, a lysosomal acidification inhibitor) and E64d/pepstatin A (10  $\mu$ M each, both of which are lysosomal proteases inhibitors). Cell lysates were then analyzed by western blotting with the indicated antibodies. The conversion of LC3-I to LC3-II indicates autophagic activity. Cells were cultured under serum-free conditions.

<b>Name of Primer</b>	<b>sequence (5' - 3')</b>
EcoRI-mATF4-F	cggaattccATGACCGAGATGAGCTTCCTGAACAGC
mATF4-XbaI-R	gctctagaTTACGGA ACTCTTCTTCCCCCTTGCC
ClaI-mATF6-F	ccatcgatcATGGTGGTGGTGGCAGCGGCGCCGAGC
mATF6-Asp718I-R	gctctagaTTAGACACTAATCAGCTGGGGGAAAAG
ClaI-mXBP1s-F	ccatcgatcATGGTGGTGGTGGCAGCGGCGCCGAGC
mXBP1s-XbaI-R	gctctagaTTAGACACTAATCAGCTGGGGGAAAAG

**Table S1. List of Primers Used for Constructions, Related to Figure 2, S6, and Methods.**

Gene	Forward sequence (5' - 3')	Reverse sequence (5' - 3')
<i>Hexokinase 1 (HK1)</i>	AGGTGAAGAAGAGGATGCGG	CGAAGGAGGGCAGCATCTTAAC
<i>Hexokinase 2 (HK2)</i>	GAGGATGAAGGTAGAAATGGAGCGA	GTAGCACACACGTAGGTGGG
<i>Glucokinase (HK4)</i>	CTACTACGAAGACCATCAGTGCGA	CATTCTGCATCTCTCCATGTAGC
<i>Glucose-6-phosphate isomerase (GPI)</i>	GATGGACCAGCACTTCCGCA	TCTCACACCCAAAGCAGTTGAT
<i>Phosphofructokinase, liver (PFKL)</i>	TGACAAGAGGTTTGACGAGGCCA	TGGGCGAGGAGCTTGAAAT
<i>Phosphofructokinase, muscle (PFKM)</i>	CCTGTGTAGTGAGCCTCTCTGGTAA	TCAGCTTCAGGGCTTCGTCAA
<i>Phosphofructokinase, platelet (PFKP)</i>	CGTGCAGATGACTCAGGATGT	TTGATGGCAAGTCGCTTGATGG
<i>Aldolase, fructose-bisphosphate A (ALDOA)</i>	CGCAGGAGGAGTATGTCAAGC	ATAGGCGTGGTTAGAGACGAAGA
<i>Aldolase, fructose-bisphosphate B (ALDOB)</i>	GCTGCAAACAAGGAGGCAAC	CTGTGAAGAGCGACTGGGTG
<i>Aldolase, fructose-bisphosphate C (ALDOC)</i>	CCTACCTTCTCTATGGGCGTG	CGCTTGATGAACTCCTCAGTGG
<i>Triosephosphate isomerase 1 (TPI1)</i>	AGCAGACAAAGGTCATCGCAGATAA	GGGTGTTGCAGTCTTGCCAGTA
<i>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</i>	CCTGCACCACCAACTGCTTA	CAGTGATGGCATGGACTGTG
<i>Phosphoglycerate kinase 1 (PGK1)</i>	TCCGAGCTTCACTTTCCAAGCTA	TTCTGTGGCAGATTGACTCCTACC
<i>Phosphoglycerate kinase 2 (PGK2)</i>	CCGGATTCTTGATGAAGAAGGAAC	CTGTCTGCCACTTTGGCTC
<i>Phosphoglycerate mutase 1 (PGAM1)</i>	GGAGGCGCTCTATGATGTC	TCTTCTGTGAGGTCTGCATACCTG
<i>Phosphoglycerate mutase 2 (PGAM2)</i>	ACAGGCCTCAACAAGGCAGAAA	CCTTGCTAATGGAGTTGTAGTAGGG
<i>Enolase 1 (ENO1)</i>	AGAGGTTTACCACAACCTGAAGAAT	TCTCCAGGATGTTGGGAGCA
<i>Enolase 2 (ENO2)</i>	CAGTGGGAGCTGAGAGCTTT	GTGGCATCTTGCCGTATTTG
<i>Enolase 3 (ENO3)</i>	GAGAACAATGAGGCCCTGGAG	AAGATCGTACTTCCATTGCGATAG
<i>Enolase 4 (ENO4)</i>	CGAGATGCTTATGGAAATGCAGAA	AGTGATCTGCTGTTGACCTCTT
<i>Lactate dehydrogenase A (LDHA)</i>	AATTTGGTCCAGCGTAACGTGA	ATCTTCCAAGCCACGTAGGT
<i>Lactate dehydrogenase B (LDHB)</i>	CTAAGTGGATTACCCAAACACCGC	CTGCTGGATGAATGCCAAG
<i>Pyruvate kinase-Muscle (PKM2)</i>	GGGTCGAGCAGGATGTTGATATG	ATGTTCTTCCCTTCTCTCCAG
<i>Pyruvate kinase-Liver and RBC (PKLR)</i>	GAAATCCTGGAGGTGAGCGAC	GCCCAATCATCATCTTCTGAGCC
<i>Mannose phosphate isomerase (PMI)</i>	TTCCGGCCAGTTGAGGAGATTGTAA	TCATGGCTCATGGTCTGCTT
<i>Phosphomannomutase 1 (PMM1)</i>	TTATGTGTTTCCGAGAACGGG	CCAGGTGGTCTGGATGGTC
<i>Phosphomannomutase 2 (PMM2)</i>	AGAAGAACGCATTGAGTCTACGA	GCAAACCTTTCCGTAGATCTGCT
<i>Glutamine--fructose-6-phosphate transaminase 1 (GFAT1)</i>	ATATTTGAGCAGCCAGAGTCTGTC	CCTTTATGTGATCCTCAAACCACC
<i>Glutamine--fructose-6-phosphate transaminase 2 (GFAT2)</i>	ATACTATGAGAGGTCGGGTGAATTT	AACCATCACAGGAAGCTCAGTC
<i>GDP-mannose pyrophosphorylase A (GMPPA)</i>	AAACCCAGCACATTATCAGTGAC	TGCCATCTGCTGATTACG
<i>GDP-mannose pyrophosphorylase B (GMPPB)</i>	GAGGAACCCCTCAAGTACGG	GCCTGCGTTGATCTTATTGGAC
<i>Stt3a, subunit of the oligosaccharyltransferase complex (STT3A)</i>	AGCTCCTCGTCTTCATGTTTCC	AGACTCCAATGCCAGAGAGAAT
<i>Stt3b, subunit of the oligosaccharyltransferase complex (STT3B)</i>	TGCAGGTGCTGTGTTCTTAG	CCCAAGTCGTAGGTTGATGCTC
<i>DNA damage inducible transcript 3 (CHOP)</i>	CCAGCAGAGGTCACAAGCAC	ACTCTGTTCCGTTTCTGGTT
<i>Heat shock protein family A (Hsp70) member 5 (GRP78)</i>	CAACATGGATCTGTCCGGTC	TTGGAATTCGAGTCGAGCCAC
<i>X-box binding protein 1 (XBP1)</i>	TTACGAGAGAAAACCTCATGGCC	GGGTCCAAGTTGCCAGAATGC
<i>eukaryotic translation initiation factor 2 alpha kinase 3 (PERK)</i>	CATCCTCATCTCACAGGCAAAG	TCATTCCAGCTACTGTCATTGGC
<i>endoplasmic reticulum to nucleus signaling 1 (IRE1)</i>	GGTGGCCTTCATCATCACCTAT	CTGCAGGAGCTGGATCTTCT
<i>activating transcription factor 6 (ATF6)</i>	CACCCACTAAAGGCCAGACG	CGTGATTAGGGAGCTGTGTGAC

**Table S2. List of Primers Used for RT-qPCR, Related to Figure 2, S5, S6, and Methods.**

## **TRANSPARENT METHODS**

### **Cell Cultures and Plasmid Transfection**

HEK293 and HepG2 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM, Nacalai Tesque), and supplemented with 10% fetal bovine serum (FBS) at 37°C in 5% CO<sub>2</sub>. PC12 cells were maintained in low-glucose DMEM (5.6 mM glucose) with 10% FBS and 5% horse serum. Cells were initially seeded in each medium, washed once with PBS and cultured in DMEM without glucose (Nacalai Tesque, referred as "sugar-free") supplemented with various concentrations of monosaccharides (glucose, mannose, and galactose) without FBS. When needed, HEK293 cells were transfected with expression plasmids using Lipofectamine LTX with Plus reagent (Invitrogen), according to the manufacturer's protocol.

### **Construction of Expression Vectors**

Mouse full-length ATF4, ATF6, and spliced XBP1 (XBP1s) were amplified from cDNA from B16F10 cells using the indicated primers (see Table S1), and subcloned into EcoRI-XbaI sites, ClaI-Asp718I sites, and ClaI-XbaI sites of the pFLAG-CMV2 vector, respectively. C-terminal 6×His-tagged rat Laminin β2 was obtained by subcloning the cDNA insert into the pcDNA3-6×His vector.

### **Reagents**

D-glucose, D-mannose, D-galactose, L-glutamine, sodium pyruvate, and 2-deoxy-D-glucose (2-DG) were purchased from Nacalai Tesque. Thapsigargin (Tg) was obtained from WAKO. Rotenone, oligomycin, bafilomycin A1, E64d, pepstatin A, and UDP-Galactose (UDP-Gal) were purchased from Sigma-Aldrich. Brefeldin A (BFA) and galactose-1-phosphate (Gal-1P) were obtained from Millipore. Azaserine (AZA) and 6-diazo-5-oxo-1-norleucine (DON) were purchased from Santa Cruz Biotechnology.

### **Western Blotting**

Cells were lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 25 mM Tris-HCl, pH 7.6) containing a protease inhibitor cocktail (Roche), sonicated, and centrifuged at 15,000 ×g for 10 min. The protein content of supernatants was quantified using a BCA assay kit (Nacalai Tesque). Proteins

were separated on pre-cast 5–20% gradient polyacrylamide gels (WAKO) and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were then incubated overnight with primary antibodies, described below. ECL anti-rabbit or anti-mouse IgG HRP-linked whole antibody (GE Healthcare, 1:2,500) was used as the secondary antibody, and signals were detected using ECL Western blotting detection reagent (GE Healthcare) and ImageQuant LAS4000 (GE Healthcare). Protein bands were quantified by densitometry analysis using ImageJ software. Actin was used as a loading control.

### **Antibodies**

Antibodies against Laminin  $\gamma$ 1 (sc-5584) and CHOP (sc-793) were purchased from Santa Cruz Biotechnology. Laminin  $\beta$ 2 antibody (C4) was kindly provided by Dr. Kikkawa (Tokyo University of Pharmacy and Life Sciences, Japan). Antibodies against HK2 (C64G5), GRP78 (C50B12), MEK1/2 (47E6), Histone H3 (D1H2), ERK (9102), phospho-ERK (9101), IRE1 $\alpha$  (14C10), EGFR (D38B1), and IGF1R $\beta$  (D23H3) were purchased from Cell Signaling. Antibodies against GFAT1 (EPR4854), PMI (EPR10234), phospho-IRE1 $\alpha$  (EPR5253), and PFKP (1D6) were obtained from Abcam. The antibody against actin (clone C4) was from Chemicon. Anti-*O*-GlcNAc (CTD110.6) antibody was obtained from Covance. Anti-His (OGHis) and anti-LC3 (PM036) antibodies were purchased from MBL. Anti-GMPPA (15517-1-AP), anti-GMPPB (15094-1-AP), and anti-STT3A (12034-1-AP) antibodies were obtained from Proteintech. Anti-FLAG (M2) antibody was purchased from Sigma-Aldrich.

### **Immunoprecipitation**

HEK293 cells were lysed in lysis buffer (150 mM NaCl, 1% Nonidet P-40, 5% glycerol, 1 mM EDTA, 25 mM Tris-HCl, pH 7.4) containing protease inhibitor cocktail and incubated for 2 h at 4°C with CTD110.6 antibody (1:1,000), Laminin  $\gamma$ 1 antibody (1:1,000), or Laminin  $\beta$ 2 antibody (1:1,000). The immunocomplexes were bound to Protein A/G PLUS-agarose (Santa Cruz) overnight at 4°C and washed five times with TBS. For LC-MS/MS analysis, the immunocomplexes were eluted with 0.1 M glycine-HCl (pH 3.0) and immediately neutralized with 2 M Trizma base, and for other experiments the immunocomplexes were eluted with 1 $\times$  SDS sample buffer and heat-denatured at 90°C for 5 min.



### **LC-MS/MS Analysis and Identification of *N*-GlcNAc Modified Proteins**

To identify the CTD110.6-interacting bands, gels were silver-stained using Sil-Best Stain One (Nacalai Tesque) and immunoblotted with CTD110.6 antibody. Each identified band was digested in the gel by modified trypsin (Promega). The extracted samples were analyzed by LC-MS/MS using an LTQ mass spectrometer (Thermo Fisher Scientific) combined with Paradigm MS4 HPLC (Michrom BioResources). Both MS and MS/MS spectra were searched against the Mascot database (Matrix Science) to identify the proteins.

### **RT-qPCR**

Total RNA was isolated from cells using the SV total RNA isolation kit (Promega). For cDNA synthesis, total RNA (1 µg) was reverse transcribed using ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO), according to the manufacturer's protocol. qPCR was performed on a LightCycler PCR System (Roche Diagnostics) and carried out at 98°C for an initial 2 min followed by 45 cycles of denaturation at 98°C for 10 sec, annealing at 60°C for 10 sec, and extension at 68°C for 30 sec using KOD SYBR qPCR Mix (TOYOBO). Primer sets are listed in Table S2. Primers were designed and selected with the use of LightCycler Probe Design software 2.0 (Roche Applied Bioscience). The *GAPDH* was used as an internal control for normalization of target gene expression.

### ***XBPI* splicing assay**

To assess the variant *XBPI* by PCR, sequence of the primers were used as same as qPCR, corresponding to nucleotides 428-449 and 689-710 of the human *XBPI* cDNA generating two PCR products of 283 (unspliced *XBPI*, *XBPI<sub>u</sub>*) and 257 (spliced *XBPI*, *XBPI<sub>s</sub>*) base pairs. Cycling condition was carried out at 95°C for an initial 30 sec followed by 28 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 10 sec and extension at 72°C for 5 sec using PrimeSTAR Max DNA polymerase (TaKaRa). The PCR products were separated on a polyacrylamide gel. The *GAPDH* was used as an internal control.

### **RNA Interference**

siRNAs for *PERK* (s18102), *IRE1* (s200430), *ATF6* (s22688), and *XBPI* (s14913) were obtained from Ambion (LifeTechnologies) using the Silencer Select siRNA series.

Target siRNA or control siRNA (Silencer Select Negative Control No.1 siRNA; Ambion) was transfected into HEK293 cells using Oligofectamine (Invitrogen) in each well of 6-well plates following the manufacturer's instructions. The cells were cultured for 72 h prior to use in the knockdown experiments. .

### **ECAR and OCR Measurements**

ECAR and OCR were measured using the Seahorse XF96 Flux Analyzer in accordance with the manufacturer's instructions (Seahorse Bioscience, USA). The XF96 microplate was coated with type I collagen (Nitta Gelatin) and left for at least 1 h at room temperature. Briefly, cells were seeded in a microplate in the corresponding cell growth medium and then incubated at 37°C in 5% CO<sub>2</sub> for 24 h. Before changing the assay medium, cells were exposed to sugar-free conditions for 3 h. Assays were initiated by replacing the medium with 175 µL assay medium supplemented with 4 mM glutamine and without sugars. The microplates were incubated at 37°C for 2 h to equilibrate the temperature and pH of the media before measurement. After incubation, the plate was loaded on the analyzer. The concentrations of compounds sequentially added to the medium (175 µL) of the cell plate during the course of the experiment were 24 mM sugar, 27 µM rotenone, and 1 M 2-DG in volumes of 25 µL each, resulting in final concentrations of 3 mM sugar, 3 µM rotenone, and 100 mM 2-DG, respectively. The final injection was 2-DG, a glucose analog, which inhibits glycolysis through competitive binding to glucose hexokinase, the first enzyme in the glycolytic pathway. All values of ECAR and OCR were normalized to cell number. Data shown are the representative mean ± SEM from three independent experiments.

### **Carbon-14 Labeling Study**

D-[1-<sup>14</sup>C]galactose (PerkinElmer) was used for labeling sugar chains on 6×His-tagged Laminin β2. Cells transfected with mock or a 6×His-tagged Laminin β2 expression construct were plated in 6-well plates, and maintained for 24 h. When cells were 100% confluent, the medium was changed to sugar-free medium or 0.3 mM [1-<sup>14</sup>C]galactose medium for 24 h. After cells were harvested and lysed, 6×His-tagged Laminin β2 was

pulled down using a His-tagged Protein PURIFICATION KIT (MBL), followed by SDS-PAGE and autoradiography. Detection of the labeled Laminin  $\beta$ 2 by autoradiography was performed using an FLA7000 image analyzer (Fujifilm).

### **Metabolomic Analysis**

HEK293 cells were seeded in three 10 cm dishes and incubated in growth media until they reached 90% confluence, then treated with medium that was sugar-free, or supplemented with 3 mM glucose or 3 mM galactose, for 6 h. The respective media were removed from the plates, and cells were washed twice with 5% mannitol solution (10 mL first and then 2 mL), treated with 800  $\mu$ L of methanol (WAKO), and let stand for 30 sec. The cell extract was then treated with 550  $\mu$ L of Milli-Q water containing internal standards (Human Metabolome Technologies (HMT)) and let stand for another 30 sec. The extract was obtained and centrifuged at 2,300  $\times g$  and 4°C for 5 min, and then 800  $\mu$ L of the upper aqueous layer was centrifugally filtered through a Millipore 5-kDa cutoff filter (UltrafreeMC-PLHCC, HMT) to remove macromolecules (9,100  $\times g$ , 4°C, 120 min). The filtrate was centrifugally concentrated and resuspended in 50  $\mu$ L of Milli-Q water for metabolome analysis at HMT and analyzed by a CE-TOFMS system (Agilent Technologies). Signal peaks were annotated according to the HMT metabolite database based on their m/z values with the migration times. Detected metabolites were plotted on metabolic pathway maps using VANTED software.

### **Spectrophotometric PFK1 Activity Assay**

PFK1 activity was measured with slight modifications to the described procedure (Deng et al., 2008). Cells were washed with 1  $\times$  PBS, pH 7.4, and incubated in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5 mM DTT, protease inhibitor cocktail) for 10 min on ice. Briefly, the assay was performed by adding 5  $\mu$ g cell lysate to 100  $\mu$ L of reaction buffer containing 50 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM ATP, 0.2 mM NADH (Nacalai Tesque), 5 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.1 mM AMP, 1 mM NH<sub>4</sub>Cl, 5 mM fructose-6-phosphate, 5 units/mL of triose phosphate isomerase (Sigma-Aldrich), 1 unit/mL of aldolase (Sigma-Aldrich), and 1 unit/mL of  $\alpha$ -glycerophosphate dehydrogenase (Sigma-Aldrich). The mixture was incubated at room temperature, and absorbance at 340 nm, which reflects the NADH

concentration, was determined each minute for up to 20 min using a DU640 spectrophotometer (Beckman).

### **Statistical Analysis**

Each experiment was conducted at least three times with consistent results. The gel or blot representative of each experiment is presented. The statistical significance was analyzed using Student's *t* test or Dunnett's test.

### **SUPPLEMENTAL REFERENCE**

Deng, H., Yu, F., Chen, J., Zhao, Y., Xiang, J., and Lin, A. (2008). Phosphorylation of Bad at Thr-201 by JNK1 promotes glycolysis through activation of phosphofructokinase-1. *J. Biol. Chem.* 283, 20754-20760.