



# Human UDP-galactose 4'-epimerase (GALE) is required for cell-surface glycome structure and function

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Glycan biosynthesis relies on nucleotide sugars (NSs), abundant metabolites that serve as monosaccharide donors for glycosyltransferases. *In vivo*, signal-dependent fluctuations in NS levels are required to maintain normal cell physiology and are dysregulated in disease. However, how mammalian cells regulate NS levels and pathway flux remains largely uncharacterized. To address this knowledge gap, here we examined UDP-galactose 4'-epimerase (GALE), which interconverts two pairs of essential NSs. Using immunoblotting, flow cytometry, and LC-MS-based glycolipid and glycan profiling, we found that CRISPR/Cas9-mediated GALE deletion in human cells triggers major imbalances in NSs and dramatic changes in glycolipids and glycoproteins, including a subset of integrins and the cell-surface death receptor FS-7-associated surface antigen. In particular, we observed substantial decreases in total sialic acid, galactose, and GalNAc levels in glycans. These changes also directly impacted cell signaling, as GALE<sup>-/-</sup> cells exhibited FS-7-associated surface antigen ligand-induced apoptosis. Our results reveal a role of GALE-mediated NS regulation in death receptor signaling and may have implications for the molecular etiology of illnesses characterized by NS imbalances, including galactosemia and metabolic syndrome.

Glycosylation, the enzymatic attachment of carbohydrates to proteins, lipids, and other biomolecules, is an abundant modification conserved across all clades of life (1). In mammals, glycosylation influences nearly every cell biological process, including protein quality control and secretion, adhesion and migration, and host-pathogen interactions (2–4). Consistent with this central role in mammalian physiology, aberrant glycosylation contributes to the pathology of myriad human diseases, such as developmental defects, diabetes, obesity and

metabolic syndrome, cancer, neurodegeneration, and atherosclerosis (5–13).

Virtually all glycoconjugates are assembled from nucleotide-sugars (NSs),<sup>3</sup> metabolites that donate “activated” monosaccharides to glycosyltransferases (2). In recent years, several groups observed that specific stimuli or signaling events, such as feeding or ischemic stress, trigger increased NS biosynthesis in mammalian cells, likely facilitating protein secretion and supporting the remodeling of cell-surface glycans (14, 15). Many glycosyltransferases are sensitive to NS concentrations, so changes in NS levels affect not only bulk levels of glycosylation but also specific glycosyltransferase substrate choices (16–21). These observations highlight the critical role of NS regulation in shaping downstream glycoconjugate biosynthesis and function. However, although the biochemistry of NS biosynthetic enzymes is well-understood, little is known about how cells regulate flux through NS metabolic pathways in response to signals or disease states. Furthermore, the impact of NS fluctuations on key glycosylation pathways and downstream cellular phenotypes is poorly understood, representing a major knowledge gap in cell biology.

As a first step toward understanding the mechanisms and functions of human NS regulation, we focused on UDP-galactose 4'-epimerase (GALE) as a model enzyme. Mammalian GALE interconverts two pairs of substrates: the hexose NSs UDP-Glc/UDP-Gal and the corresponding *N*-acetylhexosamine NSs UDP-GlcNAc and UDP-GalNAc (Fig. 1A) (22–24). Through these reversible epimerizations, human GALE balances the pools of four NSs essential for the biosynthesis of thousands of glycoproteins and glycolipids (2, 23, 24). Interestingly, however, GALE is not absolutely required for the biosynthesis of any of its four substrates, each of which could hypothetically be derived from independent salvage or *de novo* metabolic routes in nutrient-replete cells (2, 22). Because it acts only by interconverting existing NSs, GALE is an excellent model enzyme to study the role of dynamically balancing NS pools in cell physiology. Furthermore, GALE is significant to

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This article contains Figs. S1–S10.

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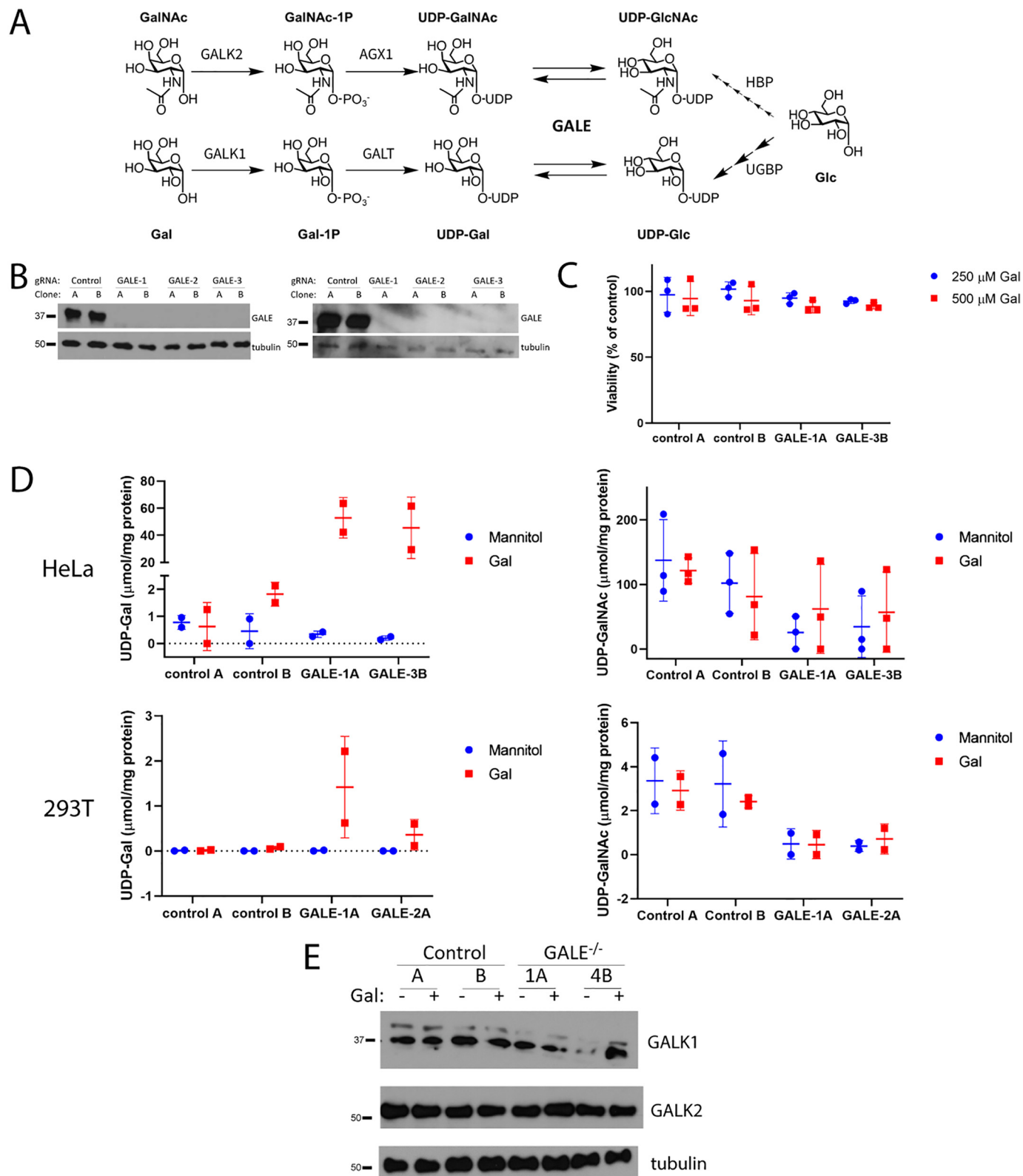
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<sup>3</sup> The abbreviations used are: NS, nucleotide sugar; GALE, UDP-galactose 4'-epimerase; FasL, Fas ligand; sgRNA, single guide RNA; HPAEC, high-performance anion exchange chromatography; PAD, pulsed amperometric detection; DMB, 1,2-diamino-4,5-methylene-dioxybenzene; MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]; GALK, galactokinase; ANOVA, analysis of variance; SNA, Sambucus nigra lectin.

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human health because it operates in the liver and hypothalamic neurons of healthy mammals to regulate Glc metabolism and satiety after feeding (14, 25, 26) and because partial loss-of-function *GALE* mutations cause a subtype of the congenital disease galactosemia and a rare form of thrombocytopenia (27–29).

To characterize the role of GALE in cell physiology, we used CRISPR/Cas9 methods to create *GALE*<sup>-/-</sup> human cell systems. Our results reveal that GALE is required to maintain NS levels and to biosynthesize a wide range of glycoproteins and glycolipids, even under nutrient-replete conditions. In particular, we show that GALE is essential for *N*-glycosylation of several cell-



surface proteins, including a subset of integrin cell adhesion proteins and the apoptotic death receptor Fas. Moreover, *GALE* deletion results in Fas hypoglycosylation and hypersensitivity to Fas ligand (FasL)-induced cell death, highlighting a previously unknown function of NS metabolism in apoptotic pathways. Our results reveal a requirement for human GALE in supporting glycoconjugate biosynthesis and cell-surface signaling and establish loss-of-function culture systems as a powerful tool for dissecting the role of NS regulation in human cell biology.

## Results

### Human GALE is required to balance NS levels

*GALE* is the final enzyme in the Leloir pathway, a highly conserved metabolic route for the assimilation of Gal (Fig. 1A, bottom) (30). To determine the role of *GALE* in NS metabolism and glycoconjugate biosynthesis, we used CRISPR/Cas9 methods to construct multiple, single-cell-derived *GALE*<sup>-/-</sup> clones from human cell lines and confirmed successful ablation of *GALE* protein (Fig. 1B). Based on prior studies of the Leloir pathway in human patients and experimental model systems (31, 32), we hypothesized that *GALE*<sup>-/-</sup> cells might display NS imbalances under standard culture conditions and/or in the presence of supplementary Gal. In particular, Gal consumption is closely tied to adverse symptoms in galactosemic patients and laboratory models (33, 34). Therefore, we sought to determine the impact of Gal on viability and NS metabolism in our cell models. Gal supplementation did not impair cell viability in control or *GALE*<sup>-/-</sup> cells (Fig. 1C). However, both *GALE*<sup>-/-</sup> HeLa and 293T cells accumulated high levels of UDP-Gal in the presence of supplementary Gal, whereas control cells showed only a modest increase (Fig. 1D, left panels). We concluded that *GALE* is required to maintain normal NS levels in nutrient-replete human cells in the absence or presence of supplementary Gal.

Interestingly, *GALE*<sup>-/-</sup> cells also exhibited reduced basal levels of UDP-GalNAc, which was partially rescued by Gal addition in HeLa (but not 293T) cells (Fig. 1D, right panels). This effect of Gal was unexpected because there is no known human biosynthetic route to produce UDP-GalNAc from Gal. Therefore, we examined the expression of the GalNAc salvage pathway galactokinases GALK1 and GALK2 in HeLa cells to determine whether Gal supplementation or *GALE* genotype might indirectly impact GalNAc salvage. However, neither *GALE* deletion nor Gal supplementation affected GALK1 or 2 levels in HeLa cells (Fig. 1E). These results indi-

cate that Gal supplementation increases UDP-GalNAc levels through an unknown but *GALE*- and Gal-dependent mechanism in HeLa cells.

### GALE is required for glycoprotein and glycolipid biosynthesis

Given the major NS imbalances observed in *GALE*<sup>-/-</sup> cells (Fig. 1D), we hypothesized that they might display defects in the biosynthesis of glycoproteins and glycolipids containing Gal or GalNAc, such as mucin-type *O*-glycoproteins and gangliosides (35–37). Furthermore, a reduction in Gal/GalNAc moieties is predicted to decrease terminal glycan structures such as sialic acids, which are typically added to Gal or GalNAc residues of mature glycans (38). Flow cytometry assays with jacalin (a lectin that binds the Galβ1–3GalNAc Thomsen–Friedenreich antigen core of mucin-type glycoproteins (39)), wheat germ agglutinin (which binds terminal GlcNAc (40) and, with lower affinity, sialic acid (41)), and *Sambucus nigra* lectin (SNA, which binds sialic acids (42)) indicated that *GALE*<sup>-/-</sup> cells indeed have reduced levels of each species compared with control cells (Fig. 2A). Consistent with these observations, monosaccharide composition analysis revealed a substantial decrease in total sialic acid, Gal, and GalNAc levels in glycans isolated from *GALE*<sup>-/-</sup> cells compared with controls (Fig. 2B).

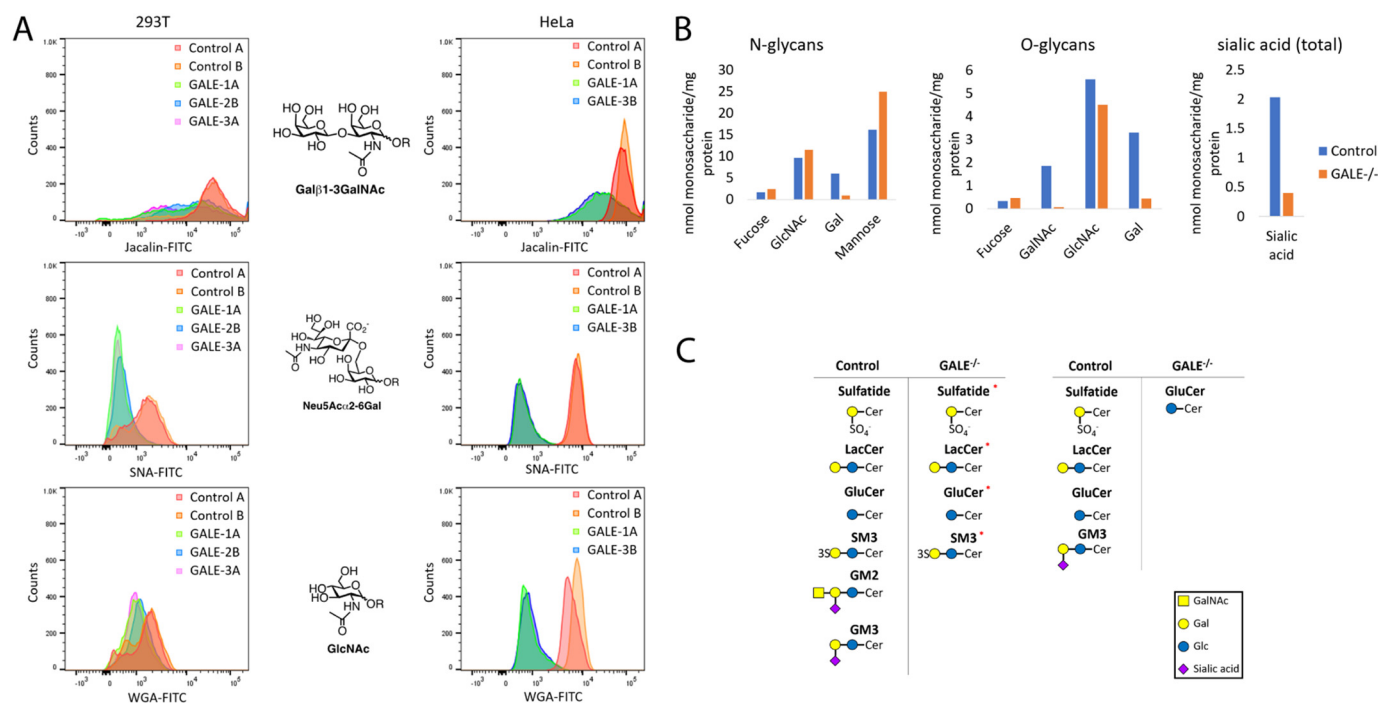
We next profiled global glycolipids and protein *N*- and *O*-linked glycans via LC-MS. *GALE*<sup>-/-</sup> and control cells bore similar levels of many lipids and glycolipid precursors, such as phosphatidylinositol, ceramide, and cardiolipin (Figs. S1–S4). However, the levels of Gal/GalNAc-containing glycolipids, such as sulfatides and several gangliosides, were greatly reduced or undetectable in *GALE*<sup>-/-</sup> cells compared with controls (Fig. 2C and Figs. S2 and S4). These results demonstrate the importance of *GALE* in maintaining the glycolipidome.

In parallel, we analyzed *N*- and *O*-linked glycans from cell-surface glycoproteins. Consistent with our lectin staining results, we observed striking deficiencies in both mucin-type *O*-glycoproteins and Gal/GalNAc-containing *N*-linked glycan structures in *GALE*<sup>-/-</sup> cells (Figs. 3 and 4 and Figs. S5–S8). Gal-responsive defects in glycoprotein synthesis have been observed in galactosemia subtypes, caused by mutations in other Leloir pathway enzymes (43), but relatively little is known about how loss of *GALE* impacts glycan synthesis in the presence of Gal. To test the importance of *GALE* in this context, we analyzed the impact of Gal supplementation on the *N*- and *O*-linked glycan profiles of *GALE*<sup>-/-</sup> cells. We found that cells treated with Gal, but not those treated with mannitol (a non-

**Figure 1. Human GALE is required for NS metabolism.** A, human GALE epimerizes two pairs of NSs, UDP-Gal/UDP-Glc and UDP-GalNAc/UDP-GlcNAc. The major routes of UDP-Gal and UDP-GalNAc biosynthesis from monosaccharide salvage are outlined. UDP-Glc and UDP-GlcNAc can be biosynthesized from glucose. *AGX1*, UDP-GalNAc pyrophosphorylase; *GALK*, galactokinase; *GALT*, galactose-1-phosphate uridylyltransferase; *HBP*, hexosamine biosynthetic pathway; *UGBP*, UDP-Glc biosynthetic pathway. B, *GALE* was deleted using CRISPR/Cas9 methods and one of three *GALE*-targeting sgRNAs (denoted 1–3) in 293T (left panel) and HeLa (right panel) cells. Single-cell derived clones (denoted A or B) were lysed and analyzed by Western blotting. C, control and *GALE*<sup>-/-</sup> HeLa clones were treated with 250 or 500 μM Gal or mannitol for 72 h. Cell viability was measured by MTS assay and normalized to mannitol controls. *n* = 3 biological replicates. Error bars represent standard deviation. No statistically significant difference exists between control and *GALE*<sup>-/-</sup> cells (two-way ANOVA). D, control and *GALE*<sup>-/-</sup> HeLa cells (top panels) or 293T cells (bottom panels) were treated with 250 μM Gal or mannitol (osmolarity control) for 72 h, and UDP-Gal (left panels) and UDP-GalNAc (right panels) were quantified by high-performance anion exchange chromatography (HPAEC). *n* = 2 biological replicates for all measurements except HeLa UDP-GalNAc. Error bars represent standard deviation. Zero values indicate that the NS level falls below the detection limit of the HPAEC assays (0.15 μmol of UDP-Gal/mg protein and 3 μmol of UDP-GalNAc/mg protein at the cell lysate concentrations employed, as judged by serial dilution of standards and manual evaluation of the resulting peak). E, control or *GALE*<sup>-/-</sup> HeLa clones were treated with 250 μM Gal or mannitol for 72 h, and lysates were analyzed by Western blotting. GALK1 is the primary kinase for dietary Gal, whereas GALK2 is the primary kinase for GalNAc.



## Human GALE is required for glycome function



**Figure 2. Human GALE is required for maintenance of the glycoproteome and glycolipidome.** *A*, control and *GALE*<sup>-/-</sup> 293T cells (*left panels*) and HeLa cells (*right panels*) were stained with fluorescently (FITC) tagged jacalin, SNA, or WGA lectins, and 10,000 cells from each sample were analyzed by flow cytometry. The predominant glycan ligand of each lectin is indicated between the corresponding panels. *B*, monosaccharide composition analysis of *N*- and *O*-linked glycans was performed on control and *GALE*<sup>-/-</sup> HeLa cells. *C*, summary of glycolipid species identified in control and *GALE*<sup>-/-</sup> 293T cells (*left panel*) and HeLa cells (*right panel*) via LC-MS. Red asterisks indicate glycolipid species dramatically reduced in *GALE*<sup>-/-</sup> cells. See also Figs. S1–S4.

metabolizable osmolyte control), closely resembled the glycans of control cells (Fig. 5 and Figs. S7–S10). We concluded that human GALE is required to support the biosynthesis of a broad range of glycoproteins and glycolipids.

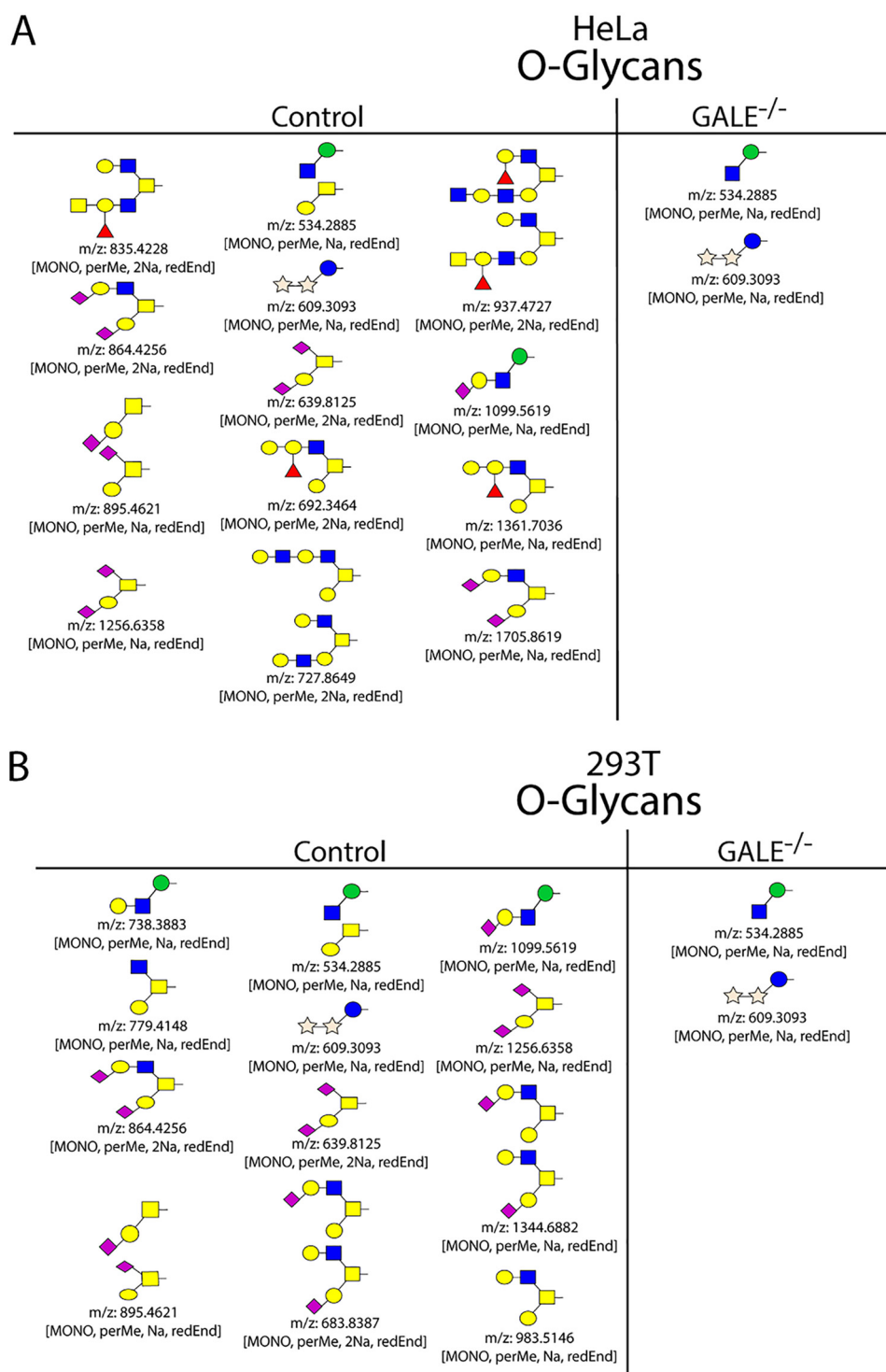
### GALE is required for cell-surface receptor glycosylation and function

We reasoned that altered glycosylation in *GALE*<sup>-/-</sup> cells might impact signaling through misglycosylated receptors. Consistent with this hypothesis, we observed substantial molecular weight shifts in several specific glycoproteins in *GALE*<sup>-/-</sup> cells compared with controls, including death receptors and integrins, major mediators of cell–matrix adhesion (Fig. 6). Gal supplementation suppressed these molecular weight changes in *GALE*<sup>-/-</sup> cells, indicating that they are caused by hypoglycosylation in the absence of GALE rather than an off-target or indirect effect of CRISPR manipulation (Fig. 6, *A* and *B*). Interestingly, although most integrins are glycoproteins (44, 45), only a subset was impacted by *GALE* deletion, suggesting specific roles of GALE activity in the biosynthesis of particular glycoconjugates (Fig. 6*B*). Our LC-MS studies indicated a relatively modest role of GALE in global *N*-glycan biosynthesis compared with *O*-glycans (Figs. 3 and 4). However, several key surface receptors, including Fas and integrin  $\beta_1$ , exhibited aberrant *N*-glycoforms, as treatment with the glycosidase PNGase F, which cleaves *N*-glycans (46), restored these proteins to their predicted molecular weights in both control and *GALE*<sup>-/-</sup> cells (Fig. 6*C*).

To determine whether GALE is required to support glycoprotein function, we focused on the death receptor Fas as a model. Fas trimerization and activation by FasL trigger the for-

mation of the death-inducing signaling complex, which recruits and activates upstream caspases, ultimately leading to downstream caspase-3 activation and apoptotic death (47, 48). No role of GALE or NS metabolism has been reported previously for death receptor signaling, but prior work has demonstrated that changes in Fas sialylation affect death-inducing signaling complex formation and the apoptotic cascade (49). Therefore, we hypothesized that Fas hypoglycosylation might affect cell death signaling in *GALE*<sup>-/-</sup> cells.

Neither *GALE* deletion nor Gal treatment altered the cell-surface expression of Fas (Fig. 7*A*). Interestingly, however, *GALE*<sup>-/-</sup> cells were significantly sensitized to FasL-induced killing (Fig. 7*B*). This result reflects a specific effect on FasL-induced death rather than general susceptibility to apoptosis because no analogous effect was observed when cells were treated with the broad-spectrum serine/threonine kinase inhibitor staurosporine (Fig. 7*C*). Next, to confirm the mechanism of sensitization to FasL in *GALE*<sup>-/-</sup> cells, we examined the apoptotic executioner caspase-3 (47). Compared with controls, *GALE*<sup>-/-</sup> cells exhibited increased caspase-3 cleavage (indicative of its activation) at early time points after FasL exposure (Fig. 7*D*). Finally, we examined the GALE-dependent glycan determinants that influence Fas signaling. Prior work by Bellis and colleagues demonstrated that up-regulation of the ST6Gal-I sialyltransferase in tumor cells potentiates Fas sialylation, reducing its responsiveness to ligand activation (49). Based on these reports and our monosaccharide composition and glycoprotein profiling data (Figs. 2, *A* and *B*, and 3–5), we hypothesized that the hypersensitivity of *GALE*<sup>-/-</sup> cells to FasL was caused by Fas hyposialylation. Consistent



**Figure 3. Human GALE is required for global O-linked glycoprotein biosynthesis.** *A*, summary of O-glycan species identified in control and GALE<sup>-/-</sup> HeLa cells via LC-MS. *m/z* values are given for each species. See also Fig. S5. *B*, summary of O-glycan species identified in control and GALE<sup>-/-</sup> 293T cells via LC-MS. *m/z* values are given for each species. *MONO*, monoisotopic mass; *perMe*, permethylated; *redEnd*, reducing end; *xLi* or *xNa*, lithium or sodium adduct with *x* ions. See also Fig. S6.

with this idea, Gal supplementation reversed the sensitivity to FasL in GALE<sup>-/-</sup> cells (Fig. 7E), indicating a GALE-dependent causal link between receptor glycosylation (Figs. 3, 4, and 6, A and C) and activity (Fig. 7). Taken together, these results demonstrate that GALE is required to support the biosynthesis of sialylated *N*-glycans on Fas and other surface

proteins and that loss of GALE function dysregulates glycoprotein receptor signaling.

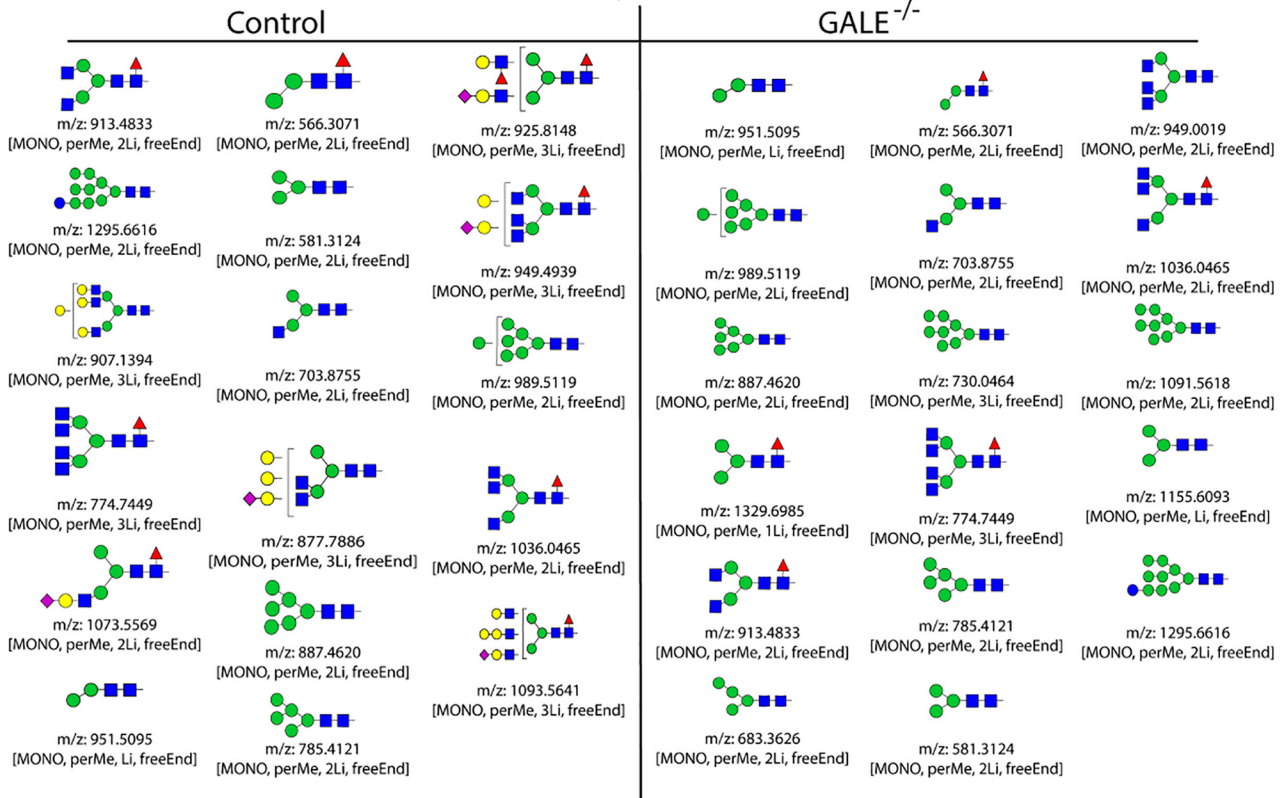
## Discussion

Regulation of human NS levels has profound implications for normal physiology and disease, but it remains poorly under-

A

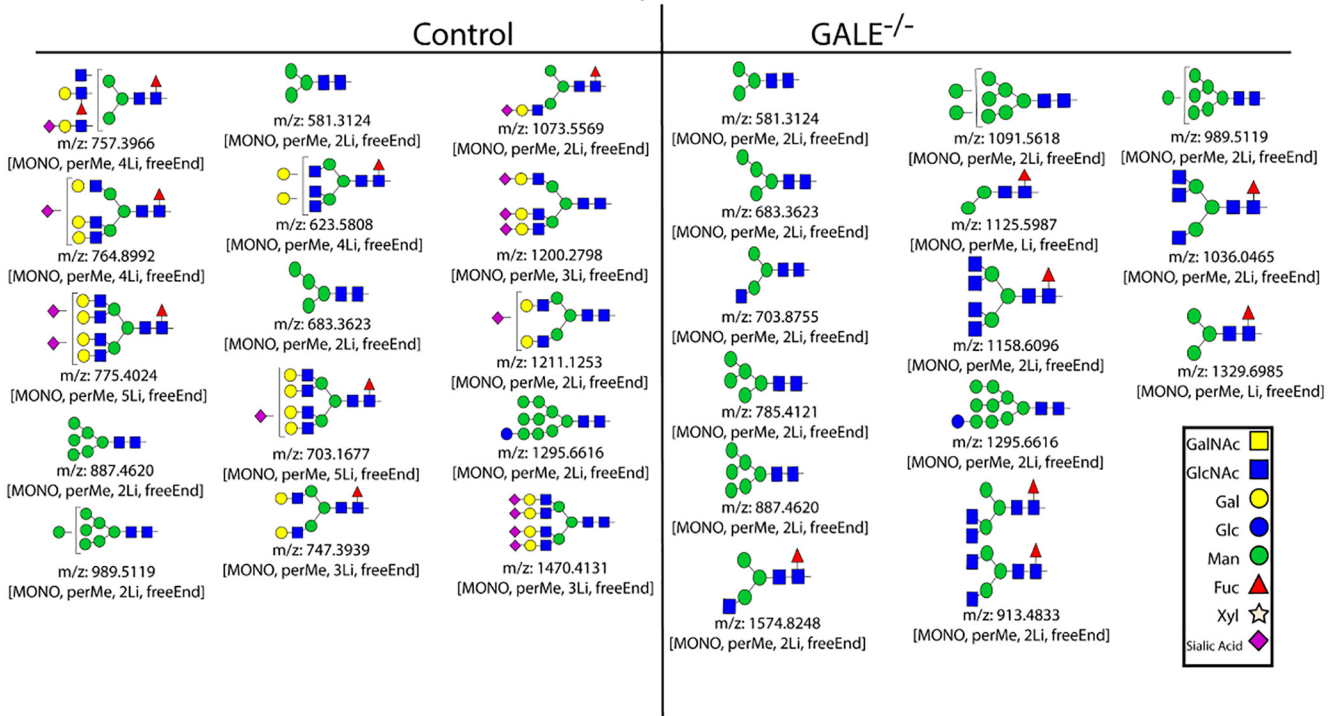
293T

N-Glycans

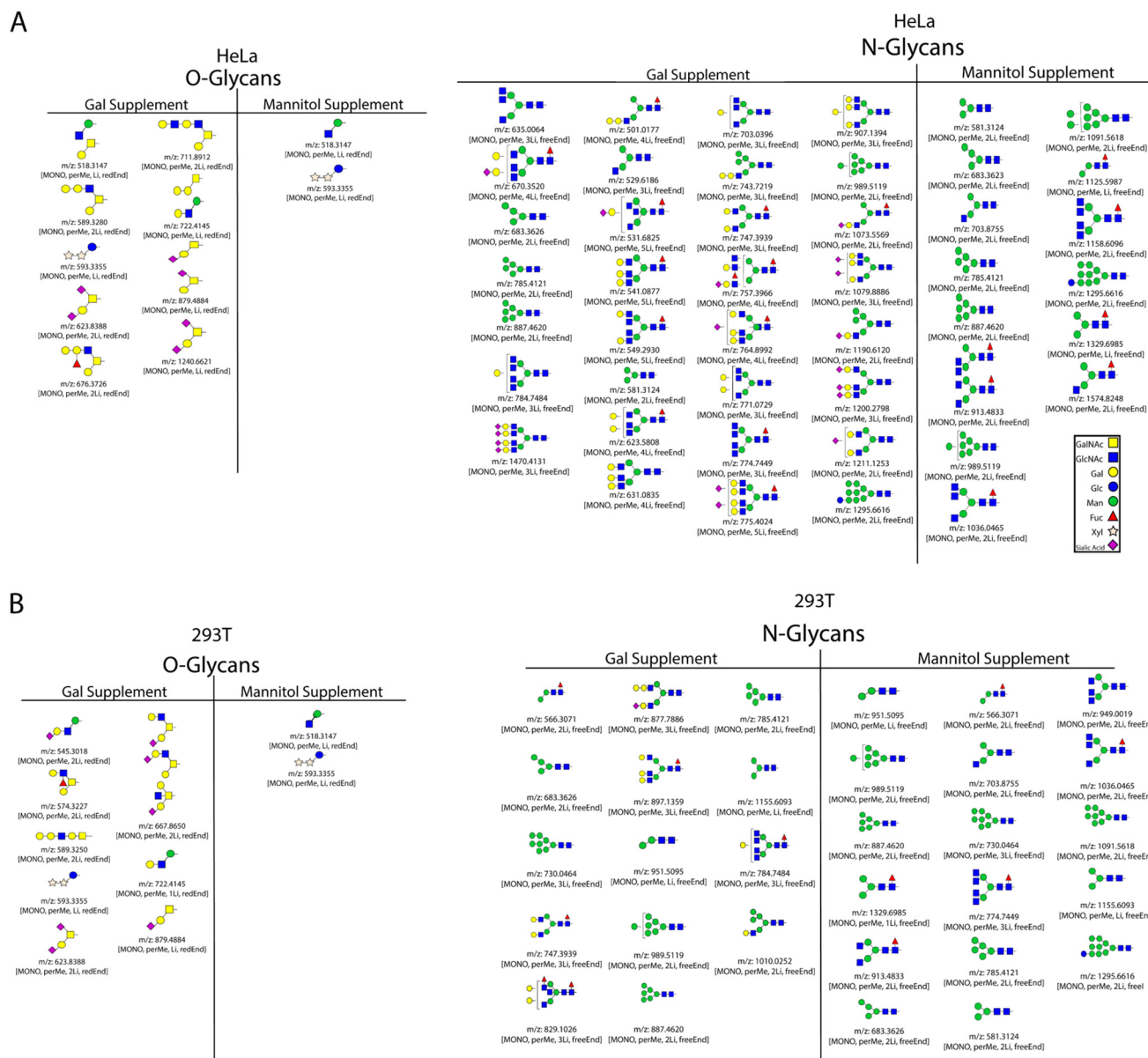


B

HeLa  
N-Glycans







**Figure 5. Gal supplementation restores protein glycosylation in *GALE*<sup>-/-</sup> cells.** *A*, summary of O-glycan (left panel) and N-glycan (right panel) species identified by LC-MS in *GALE*<sup>-/-</sup> HeLa cells treated with 250  $\mu$ M Gal or mannitol (osmolarity control) for 72 h. *m/z* values are given for each species. See also Figs. S8 and S9. *B*, summary of O-glycan (left panel) and N-glycan (right panel) species identified by LC-MS in *GALE*<sup>-/-</sup> 293T cells treated with 250  $\mu$ M Gal or mannitol (osmolarity control) for 72 h. *m/z* values are given for each species. *freeEnd*, nonreducing end; *Fuc*, fucose; *Man*, mannose; *MONO*, monoisotopic mass; *perMe*, permethylated; *redEnd*, reducing end; *xLi* or *xNa*, indicates lithium or sodium adduct with x ions; *Xyl*, xylose. See also Figs. S7 and S10.

stood. Our results shed new light on the role of *GALE*, a model NS metabolic enzyme, in the biosynthesis and function of a wide range of glycoconjugates.

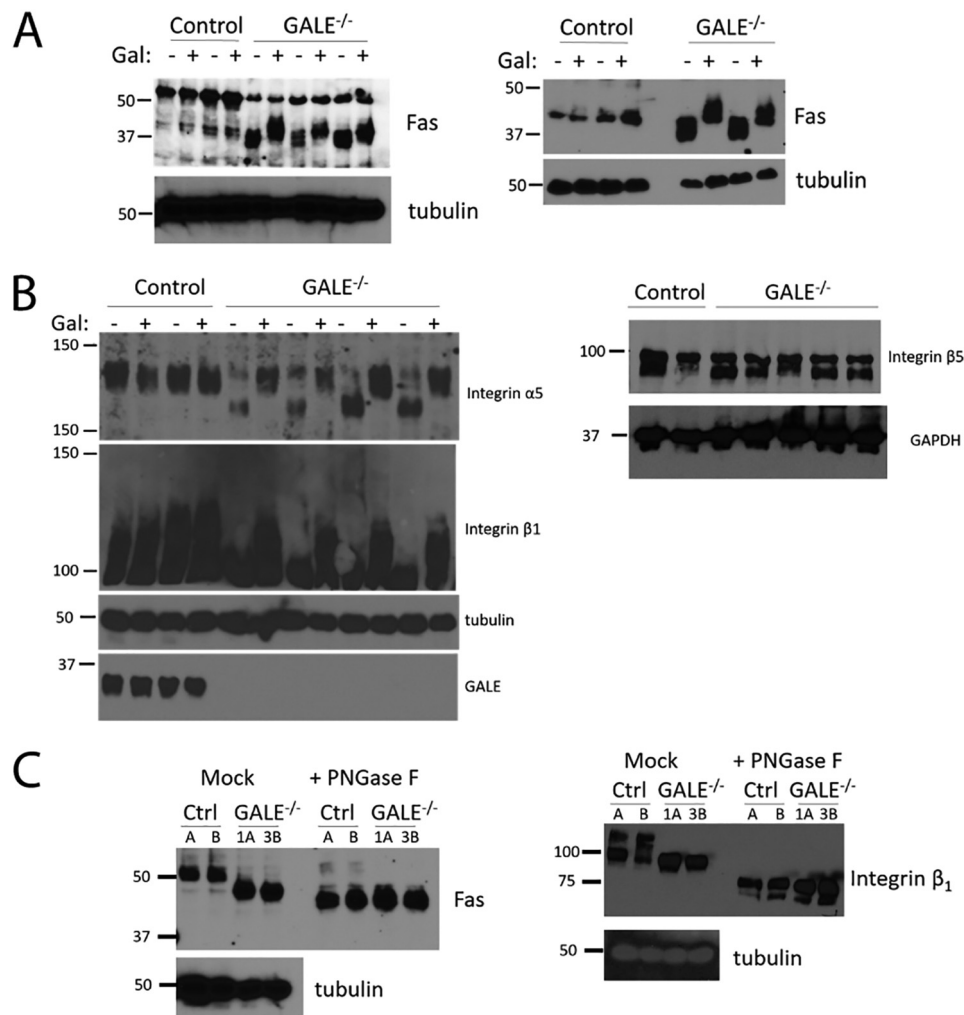
### *GALE* governs human NS levels

*GALE*<sup>-/-</sup> cells have significantly reduced levels of UDP-GalNAc and UDP-Gal compared with controls (Fig. 1D). Based on these observations, we propose that *GALE* is required for biosynthesis and balancing of these key NSs despite the presence of free Gal and Gal/GalNAc-containing glycoproteins in serum,

which could hypothetically be salvaged in a *GALE*-independent manner (50, 51). Our results support some conclusions of prior studies performed in other systems. For example, consistent with the idea that *GALE* is required to balance NSs, and in agreement with data from galactosemic patients and earlier experimental models (32, 34, 52), we observed a large increase in UDP-Gal in *GALE*<sup>-/-</sup> cells in the presence of supplementary Gal (Fig. 1D). On the other hand, our results challenge some conclusions of prior work. For instance, in *GALE*-deficient human patients and some model systems, Gal is acutely toxic,

**Figure 4. Human *GALE* is required for global N-linked glycoprotein biosynthesis.** *A*, summary of N-glycan species identified in control and *GALE*<sup>-/-</sup> 293T cells via LC-MS. *m/z* values are given for each species. See also Fig. S7. *B*, summary of N-glycan species identified in control and *GALE*<sup>-/-</sup> HeLa cells via LC-MS. *m/z* values are given for each species. *freeEnd*, nonreducing end; *Fuc*, fucose; *Man*, mannose; *MONO*, monoisotopic mass; *perMe*, permethylated; *redEnd*, reducing end; *xLi* or *xNa*, lithium or sodium adduct with x ions; *Xyl*, xylose. See also Fig. S8.

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**Figure 6. GALE is required for glycosylation of specific cell-surface receptors.** *A*, control and GALE<sup>-/-</sup> 293T cells (*left panel*) and HeLa cells (*right panel*) were treated with 250 μM Gal or mannitol for 72 h, and lysates were analyzed by Western blotting. Each pair of lanes (± Gal) represents a distinct clone. *B*, control or GALE<sup>-/-</sup> HeLa clones were treated with 250 μM Gal or mannitol for 72 h, and lysates were analyzed by Western blotting. Integrins α<sub>5</sub> and β<sub>1</sub> (*left panel*) but not β<sub>5</sub> (*right panel*) are hypoglycosylated in GALE<sup>-/-</sup> cells. *C*, lysates from control and GALE<sup>-/-</sup> HeLa cells were treated with PNGase F and analyzed by Western blotting, indicating GALE-dependent *N*-glycosylation of Fas (*left panel*) and integrin β<sub>1</sub> (*right panel*). Within treatment groups (± PNGase F), each lane represents a distinct clone. *Ctrl*, control.

leading to severe symptoms or impairing cell health and proliferation (34, 53, 54). It has been proposed that the accumulation of intermediate metabolites or toxic byproducts might account for these harmful effects (55). However, we found no evidence of Gal toxicity in our GALE<sup>-/-</sup> cell systems (Fig. 1C). It may be that the accumulation of intermediate metabolites affects only specific cell or tissue types, perhaps because of other differences in Gal metabolism. Testing this hypothesis will be an important focus of future studies.

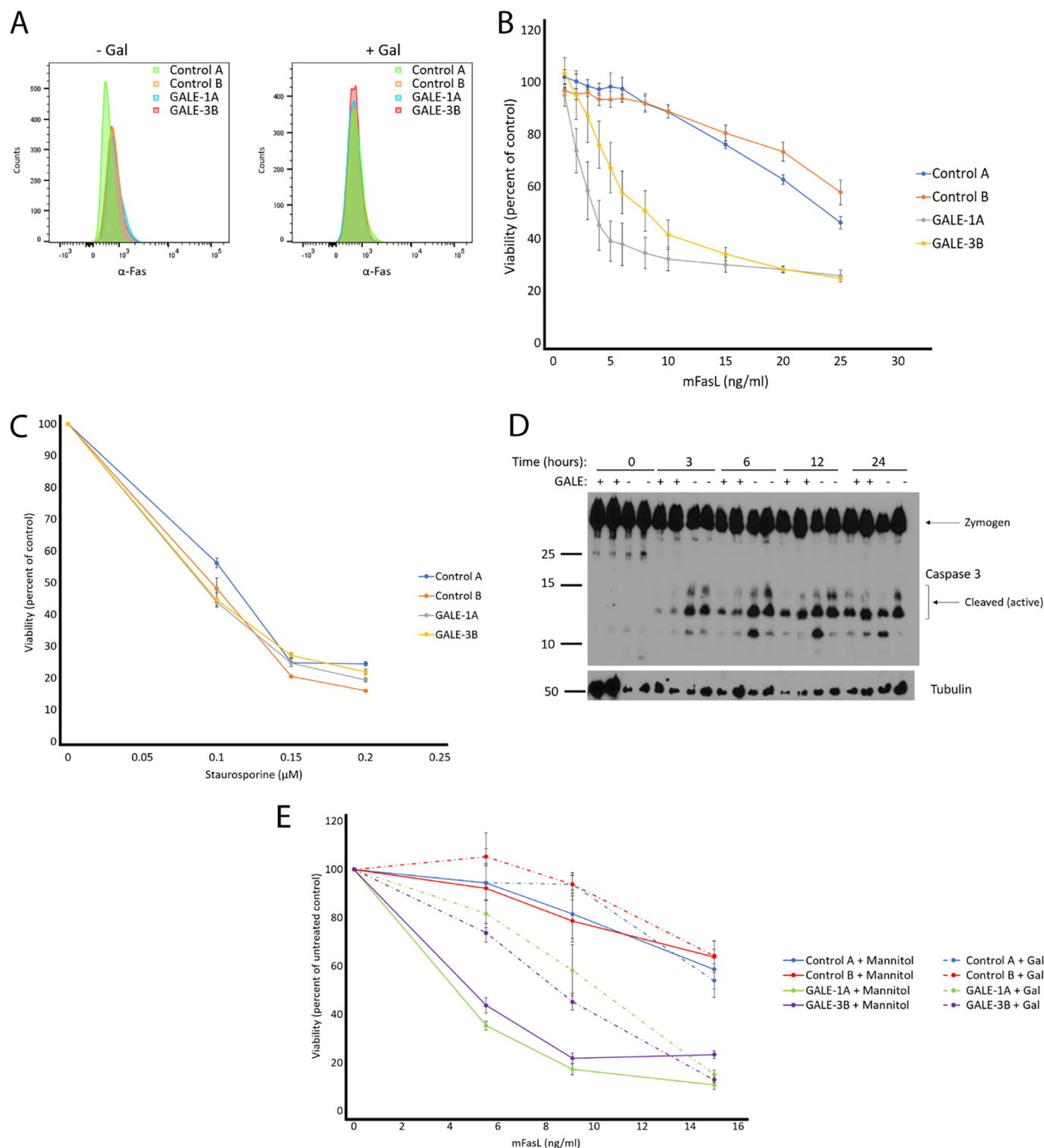
### GALE is required to support glycoconjugate biosynthesis

Although aberrant glycosylation has been reported in other subtypes of galactosemia (43, 56–63), little is known about how loss of GALE affects glycan synthesis in human cells in the presence or absence of supplementary Gal. Here we show that GALE deletion dramatically affects glycoconjugate biosynthesis with greatly reduced Gal, GalNAc, and sialic acid content in cell-surface glycans of GALE<sup>-/-</sup> cells (Fig. 2, *A* and *B*), impacting both *N*- and *O*-linked glycoproteins (Fig. 3). Moreover, our

glycolipid profiling suggests that GALE is essential for biosynthesis of such species as myelin gangliosides, including lactosylceramide and sulfatides (Fig. 2C). GM3 ganglioside is a regulator of leptin signaling and has been linked to development of insulin resistance (64, 65), sulfatides are major components of myelin and are believed to play crucial roles in neuronal differentiation (66, 67), and deficiencies in lactosylceramide biosynthesis cause locomotor deficits and abnormal brain development in mice (68). Therefore, GALE-dependent glycolipid biosynthesis may be required for normal nutrient responses and neuronal physiology *in vivo*. Our data may also provide a functional explanation for the previously reported postprandial transcriptional up-regulation of GALE in multiple tissue types (14, 25), suggesting that GALE could be critical for balancing NS pools and supporting glycoprotein and glycolipid biosynthesis after feeding.

Our results may also have unexpected implications for our understanding of human cell metabolism. For example, we observed that Gal supplementation largely restored biosynthe-





**Figure 7. GALE is required for Fas N-glycosylation and function.** *A*, control or  $GALE^{-/-}$  HeLa cell clones were treated with 250 μM Gal or mannitol for 72 h, stained with an anti-Fas antibody, and analyzed by flow cytometry. *B*, control and  $GALE^{-/-}$  HeLa cell clones were treated with the indicated concentrations of FasL for 24 h, and cell viability was measured by MTS assay.  $n = 3$  biological replicates. Error bars represent mean ± S.E.  $p < 0.05$  for all FasL concentrations of 6 ng/ml or more, comparing control with  $GALE^{-/-}$  (two-way ANOVA, post hoc one-way ANOVA). *C*, control or  $GALE^{-/-}$  HeLa cell clones were treated with the indicated concentrations of staurosporine for 24 h, and cell viability was measured by MTS assay.  $n = 3$  biological replicates. Error bars represent S.E. *D*, control and  $GALE^{-/-}$  HeLa cell clones were treated with 2.5 ng/ml FasL for the indicated times, and lysates were analyzed by Western blotting. *E*, control or  $GALE^{-/-}$  HeLa clones were treated with 250 μM mannitol or Gal for 72 h and then with the indicated concentrations of FasL for an additional 24 h. Cell viability was measured by MTS assay.  $n = 3$  biological replicates. Error bars represent S.E.  $p < 0.05$  comparing mannitol-treated versus Gal-treated  $GALE^{-/-}$  cells at 5.5 ng/ml and 9.1 ng/ml FasL (three-way ANOVA, post hoc Tukey's honest significant difference test).

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sis of UDP-GalNAc- and GalNAc-bearing glycans in *GALE*<sup>-/-</sup> cells (Figs. 1D and 5). These results were surprising because Gal is thought not to enter the well-characterized pathway for UDP-GalNAc biosynthesis (69, 70). We suggest two possible explanations for these observations. First, Gal supplementation may up-regulate the activity of salvage enzymes such as GALK1 and 2, allowing cells to more efficiently recycle GalNAc monosaccharides from serum glycoproteins. Our results indicate that expression of GALK1 and GALK2 is unaffected by Gal treatment or *GALE* deletion (Fig. 1E), but future studies of GALK enzymatic activity will be needed to further test this hypothesis. Second, very high levels of Gal may result in its noncanonical entry into the hexosamine biosynthetic pathway, which biosynthesizes UDP-GlcNAc from Glc. In this scenario, sufficient UDP-GalNAc may be produced to restore glycoprotein biosynthesis. This hypothesis remains to be tested, but significant substrate promiscuity has been documented previously in mammalian hexosamine metabolism (71–73). Experiments are ongoing to investigate these two mutually compatible possibilities.

### *GALE* loss triggers receptor hypoglycosylation and dysfunction

Our results demonstrate that *GALE* is required to support cell-surface receptor signaling even in nutrient-replete human cells, with significant effects on death receptor function (Fig. 7). Aberrant glycosylation has long been known to affect apoptotic receptor signaling. For example, oncogene activation up-regulates sialyltransferase expression and glycoprotein sialylation in colon adenocarcinomas, promoting cell migration and resistance to galectin-mediated apoptosis (74–76). Similarly, hypersialylation of Fas by ST6Gal-I protects tumor cells from Fas-mediated apoptosis (49). Given the dramatic loss of global sialylation in *GALE*<sup>-/-</sup> cells (Figs. 2, A and B, 3, and 4), we propose that Fas hyposialylation accounts for their hypersensitivity to FasL-induced apoptosis (Fig. 7B). Consistent with this notion, we observed *GALE*-dependent differences in FasL-mediated caspase-3 activation (Fig. 7D). Importantly, however, these results are not attributable to a general predisposition to apoptosis because no such hypersensitivity was observed in response to staurosporine (Fig. 7C). The effect of Gal supplementation on FasL response in *GALE*<sup>-/-</sup> cells is less pronounced at high FasL doses (Fig. 7E). This observation may be due to subtle variations in Fas *N*-glycosylation between Gal-treated control and *GALE*<sup>-/-</sup> cells and/or glycan-independent activation of Fas above a certain threshold concentration of FasL.

Beyond Fas, we also observed aberrant glycosylation of a subset of integrins in *GALE*<sup>-/-</sup> cells (Fig. 6, B and C). Glycosylation is well-known to impact the function of several integrins, particularly  $\beta_1$  (75, 77–84), and a very recent study suggested that *GALE* in particular may be necessary for integrin  $\beta_1$  function in platelet homeostasis (29). Based on this work and our own results, we speculate that *GALE* activity may be required in some contexts for normal integrin-mediated attachment to the extracellular matrix, cell adhesion and migration, or tissue homeostasis. Experiments are underway to test this hypothesis.

Finally, our results may have implications for understanding global protein *N*-glycosylation in both model systems and

galactosemic patients. Past studies of mammalian *GALE* have largely focused on its role in *O*-glycan biosynthesis (35, 85–87). Therefore, it is especially noteworthy that we discovered alterations in the *N*-glycans of several cell-surface receptors (Fig. 6C). Altered *N*-glycans have been implicated in other subtypes of galactosemia based on observations in both human patients (43) and animal models (56). However, the role of mammalian *GALE* in supporting *N*-glycan biosynthesis has received little attention in any clinical or experimental context. We propose that altered *N*-glycans may contribute to the pathology of *GALE*-deficient galactosemia and that *GALE* may play a critical role in healthy tissue under normal conditions that trigger glycosylation changes, such as feeding or stress (14, 15).

### Conclusions

NS metabolic enzymes have been extensively characterized at the biochemical level. However, the mechanistic basis and functional effects of dynamic changes in NS pools in cells and organisms remain poorly understood. We deleted *GALE*, a key node in NS metabolism, in human cell systems as a model for NS dysregulation. Our data demonstrate that human *GALE* is required for normal glycoconjugate biosynthesis and receptor signaling even in nutrient-replete cells. Moreover, we anticipate that the dramatic absence of cell-surface Gal, GalNAc, and sialic acid in *GALE*<sup>-/-</sup> cells, combined with the ability to restore normal glycosylation with simple Gal supplementation, will make *GALE*<sup>-/-</sup> cells an attractive model system for studying glycan function in normal cell physiology, metabolic syndrome, thrombocytopenia, and galactosemia.

### Experimental procedures

#### Cell culture

293T and HeLa cells were cultured in DMEM containing 10% FBS, 100 g/ml streptomycin, and 100 units/ml penicillin in 5% CO<sub>2</sub> at 37 °C. For monosaccharide supplementation experiments, cells were preconditioned with 250  $\mu$ M mannitol or Gal (Sigma-Aldrich) for 72 h, with replenishment of monosaccharide every 24 h.

#### Generation of *GALE*<sup>-/-</sup> cell lines

*GALE*<sup>-/-</sup> HeLa and 293T cell lines were constructed essentially as described previously (88). Briefly, cells at ~50% confluency were stably transduced with a LentiCas9 virus obtained from the Duke Functional Genomics Facility in the presence of 4  $\mu$ g/ml Polybrene. After overnight incubation, the medium was replaced, and cells were allowed to recover for 48 h before selection with 3  $\mu$ g/ml (HeLa) or 5  $\mu$ g/ml (293T) blasticidin. Cells were passaged until an uninfected control plate had no live cells remaining. Following selection, cells were infected with lentiviruses bearing one of three single guide RNA (sgRNA) sequences targeting the *GALE* coding sequence or a “safe harbor” AAVS1-targeting control sgRNA (89). After sgRNA infection, cells were selected for stable sgRNA expression with 1.5  $\mu$ g/ml (HeLa) or 0.5  $\mu$ g/ml (293T) puromycin with continued presence of blasticidin. Following drug selection, clonal lines were generated via limiting dilution and assayed for *GALE* deletion via Western blotting. The *GALE*

sgRNA sequences used were as follows: GALE-1, GAGAAGG-TGCTGGTAACAGG; GALE-2, GGAGGCTGGCTACTTG-CCTG; GALE-3, GCCAGGTGCCATGGCAGAGA.

### Western blotting

Protein samples were quantified by BCA assay according to the manufacturer's protocol (Thermo Fisher). Samples with equal protein amounts were separated by SDS-PAGE using standard methods (90) and electroblotted onto a PVDF membrane (Thermo Fisher, 88518). After blocking in Tris-buffered saline with 0.1% Tween (TBST) and 2.5% dry milk, the blots were incubated overnight at 4 °C in primary antibody with 5% BSA in TBST. The following day, the blots were washed three times in TBST, incubated with an appropriate horseradish peroxidase-conjugated secondary antibody (Southern Biotech, 1:5000) for 1 h at room temperature, washed three times in TBST, and developed via ECL according to the manufacturer's instructions (WesternBright ECL, Advansta). The following primary antibodies were used: GALE (Abcam, ab 118033, 1:1000), tubulin (Sigma-Aldrich, T6074, 1:10,000), Fas (Cell Signaling, C18C12, 1:1000), caspase-3 (Cell Signaling, 8G10, 1:1000), FAS-associated protein with death domain (Abclonal, A5819, 1:5000), integrin  $\alpha_5$  (Cell Signaling, 4705, 1:1000), integrin  $\beta_1$  (Santa Cruz, sc-13590, 1:1000), integrin  $\beta_5$  (Cell Signaling, D24A5, 1:1000), and GAPDH (Cell Signaling,  $^{14}$ C10, 1:5000).

### Flow cytometry

For both lectin- and antibody-labeled flow cytometry, cells were harvested in 135 mM KCl/15 mM sodium citrate with 10 min of gentle rocking and resuspended in PBS with 2% BSA at 1 million cells/ml. For lectin labeling, cells were rotated for 30 min at 4 °C with fluorescent lectin in PBS/BSA, washed three times with PBS/BSA, and fixed with 2% paraformaldehyde for 20 min before analysis on a FACSCanto II cytometer (BD Biosciences). The lectins used were fluorescein-SNA (Vector Laboratories, FL-1301-2, 5  $\mu$ g/ $10^6$  cells), fluorescein-wheat germ agglutinin (Vector Laboratories, FL-1201, 50  $\mu$ g/ $10^6$  cells), and fluorescein-jacalin (Vector Laboratories, FL-1121, 50  $\mu$ g/ $10^6$  cells).

For antibody labeling, aliquots of cells were incubated with primary antibody for 30 min at 4 °C and then washed three times with PBS/BSA. Then the cells were washed twice with 2% BSA/PBS and incubated with a secondary antibody for 30 min. After the final incubation, the cells were fixed with 2% paraformaldehyde for 20 min before analysis on a FACSCanto II cytometer (BD Biosciences). The antibodies used were Fas (Biolegend, 3056020, 0.5  $\mu$ g/ $10^6$  cells) and goat anti-mouse Alexa Fluor 488 (Thermo, A11001, 1  $\mu$ g/ $10^6$  cells).

### Glycolipid profiling

Lipid extraction was performed using a modified Bligh-Dyer method (91). Briefly, cell pellets were resuspended in 1.6 ml of PBS and transferred into a 17-ml glass tube with a Teflon-lined cap. Then 4 ml of methanol and 2 ml of chloroform were added to create a single-phase Bligh-Dyer solution (chloroform:methanol:PBS, 1:2:0.8). The mixture was vigorously vortexed for 2 min and then sonicated in a water bath at room temperature for 20 min. After centrifugation at 3000  $\times$  g for 10 min, superna-

tants were transferred to fresh glass tubes and acidified by adding 100  $\mu$ l of 37% HCl. After mixing, acidified solutions were converted into two-phase Bligh-Dyer systems by adding 2 ml of PBS and 2 ml of chloroform. After centrifugation at 3000  $\times$  g for 10 min, the lower phase was collected and dried under nitrogen. Lipid extracts were stored at -20 °C until LC-MS analysis. For lipidomic analysis, samples were dissolved in 200  $\mu$ l of solution of chloroform:methanol (2:1), and 20  $\mu$ l was injected for normal-phase LC-MS analysis.

Normal-phase LC was performed on an Agilent 1200 Quaternary LC system equipped with an Ascentis silica HPLC column (5  $\mu$ m, 25 cm  $\times$  2.1 mm, Sigma-Aldrich). Mobile phase A was chloroform:methanol:aqueous  $\text{NH}_4\text{OH}$  (800:195:5 by volume), mobile phase B was chloroform:methanol:water:aqueous  $\text{NH}_4\text{OH}$  (600:340:50:5 by volume), and mobile phase C was chloroform:methanol:water:aqueous  $\text{NH}_4\text{OH}$  (450:450:95:5 by volume). The elution was performed as follows. 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The gradient was then changed to 100% mobile phase C over 3 min, held at 100% C for 3 min, and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The LC eluent (total flow rate of 300  $\mu$ l/min) was introduced into the electrospray ionization source of a high-resolution TripleTOF 5600 mass spectrometer (Sciex). The instrument settings for negative-ion electrospray ionization and MS/MS analysis of lipid species were as follows: ion spray voltage = -4500 V, curtain gas = 20 psi, ion source gas 1 = 20 psi, declustering potential = -55 V, and focusing potential = -150 V. MS/MS analysis used nitrogen as the collision gas. Data analysis was performed -55 V; FP, using Analyst TF1.5 software (Sciex).

### Glycan profiling

Frozen cell pellets were resuspended in methanol:water (4:1.5) and sheared using a 20-gauge needle-equipped syringe. Delipidation was achieved by adding chloroform to a final ratio of 2:4:1.5 chloroform:methanol:water. Samples were incubated for 2 h, centrifuged, decanted, and incubated in the same chloroform/methanol/water mixture overnight. The next day, samples were centrifuged, resuspended in 4:1 acetone:water, and incubated on ice for 15 min. The samples were centrifuged, and the supernatant was decanted and incubated with acetone:water. Delipidated protein was dried under nitrogen and stored at -20 °C until analysis.

For *N*- and *O*-glycan analysis, 2–4 mg of dried protein powder was used per sample. *N*- and *O*-glycans were released enzymatically or chemically, respectively, and isolated as described previously (92). Following cleanup, glycans were permethylated as described previously (93), dried under nitrogen, and stored at -20 °C until analysis.

Permethylated glycans were resuspended and mixed with an internal standard before analysis via reverse-phase LC-MS/MS on a Thermo Scientific Velos Pro mass spectrometer as described previously (92). Glycan structures were manually interpreted based on in-house fragmentation rules, augmented by GlycoWorkbench and GRITS Toolbox semiautomated soft-



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ware solutions (94, 95). All glycan structures are depicted using standard symbol nomenclature for glycobiology (96).

### Glycan monosaccharide analysis

Complete hydrolysis of isolated *N*- and *O*-glycans to monosaccharides was performed using 2 M TFA prior to analysis by HPAEC with pulsed amperometric detection (PAD). 100  $\mu\text{g}$  of dry glycan sample was dissolved in 50  $\mu\text{l}$  of ultrapure water and 50  $\mu\text{l}$  of 4 M TFA and mixed thoroughly. The samples were hydrolyzed at 100 °C for 4 h. The hydrolyzed samples were cooled to room temperature and then centrifuged at 2000 rpm for 2 min. Acid was removed by complete evaporation under dry nitrogen flush, followed by two rounds of coevaporation with 100  $\mu\text{l}$  of 50% isopropyl alcohol each time. Dry samples were resuspended in 142  $\mu\text{l}$  of ultrapure water, and 70  $\mu\text{g}$  of sample was injected for HPAEC-PAD analysis.

For neutral and amino sugar analysis, HPAEC-PAD profiling was performed on a Dionex ICS-3000 system equipped with a CarboPac PA1 column (4  $\times$  250 mm), guard column (4  $\times$  50 mm), and PAD using standard Quad waveform supplied by the manufacturer. An isocratic mixture of 19 mM NaOH with 0.95 mM NaOAc was used at a flow rate of 1 ml/min for 20 min. Neutral and amino sugars were identified and quantified by comparison with an authentic standard mixture of L-fucose, D-galactosamine, D-glucosamine, D-galactose, D-glucose, and D-mannose using Thermo Scientific Chromeleon software (version 6.8).

For sialic acid analysis, 50  $\mu\text{g}$  of sample was hydrolyzed at 80 °C using 2 M acetic acid for 3 h. Released sialic acids were isolated by spin filtration using a 3000-kDa molecular mass cut-off centrifugal device (Nanosep 3K Omega, PALL Life Sciences, OD003C34). The flow-through containing released sialic acid was dried and derivatized using 1,2-diamino-4,5-methylene-dioxybenzene (DMB) (Sigma-Aldrich, D4784-10MG) as described previously (97). Fluorescent DMB-derivatized sialic acids were analyzed by reverse-phase HPLC using a Thermo-Dionex UltiMate 3000 system equipped with a fluorescence detector. Samples were isocratically eluted with 9% acetonitrile (9%) and 7% methanol in ultrapure water over 30 min using an Acclaim 120-C18 column (4.6  $\times$  250 mm, Dionex) at a flow rate of 0.9 ml/min. The excitation and emission wavelengths were set at 373 nm and 448 nm, respectively. DMB-derivatized sialic acids were identified and quantified by comparing the elution times and peak areas with known authentic standards of *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid using Chromeleon software (version 6.8).

### Cell viability assays

Cells were plated into 96-well plates at 5000 cells/well in phenol red-free DMEM and allowed to recover for 24 h. FasL (Adipogen, AG-40B-0130-C010) or staurosporine (LC Labs, S-9300, 0.1–0.2  $\mu\text{M}$ ) was added and incubated for the indicated times. Then cell viability was assessed by adding soluble formazan (MTS assay, Promega) at a 1:1 dilution according to the manufacturer's protocol and incubating for 1 h at 37 °C, 5% CO<sub>2</sub> before measuring absorbance at 490 nm.

### NS analysis

Cells were cultured to confluency in 10-cm culture dishes, detached using 0.25% trypsin at 37 °C, and washed three times with PBS at 4 °C. Cells were lysed in methanol on dry ice, vigorously vortexed, and centrifuged to pellet proteinaceous solids. Pellets were resuspended in 8 M urea, and total protein was quantified by BCA assay. Methanol supernatants (containing NSs) were SpeedVac-dried and resuspended in 80 mM Tris-HCl (pH 7.4). Samples were separated on a Dionex CarboPac PA1 column (Thermo) using a Dionex ICS-5000+ SP HPAEC instrument and photodiode array. 10  $\mu\text{l}$  of each sample was injected onto the column after pre-equilibration with 1 mM NaOH (solvent A). Samples were eluted at a flow rate of 1 ml/min using solvent A and solvent B (1 mM NaOH and 1 M NaOAc) as follows: 0–100% B for 0–28 min and 100% B for 2 min. Samples were quantified using absorbance at 260 nm and compared with a series of authentic UDP-Gal or UDP-GalNAc standards at known concentrations (Sigma-Aldrich). Peak areas were integrated using Chromeleon software, and cellular NS concentrations were calculated using standard curves and total protein amounts.

### Quantification and statistical analysis

No randomization was performed for these studies. Quantitative experiments (e.g. MTS viability assays) were performed with a minimum of three biological replicates originating from independent cultures; for HPAEC metabolite analysis, a minimum of two biological replicates from independent cultures was employed. The number of biological replicates and statistical tests used are given in the figure legends. All Western blots are representative of at least two experiments from biologically independent samples.

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