Epigenetic Regulation of a Brain-specific Glycosyltransferase
N-Acetylglucosaminyltransferase-IX (GnT-IX) by Specific
Chromatin Modifiers*

Received for publication, January 29, 2014, and in revised form, March 2, 2014 Published, JBC Papers in Press, March 10, 2014, DOI 10.1074/jbc.M114.554311

Yasuhiro Kizuoka†, Shinobu Kitzazume‡, Kyohei Okahara§, Alejandro Villagra¶, Eduardo M. Sotomayor¶, and Naoyuki Taniguchi**

From the †Department of Systems Biology, Global Research Cluster, RIKEN, Wako 351-0198, Japan and the §Department of Immunology, H. Lee Moffitt Cancer Center and Research Institute, Tampa, Florida 33613

Background: Epigenetic mechanisms of regulating expression of glycosyltransferase genes are largely unknown.

Results: Novel epigenetic factors were identified that specifically regulate a brain-specific glycosyltransferase gene, GnT-IX (Mgat5b).

Conclusion: GnT-IX is epigenetically regulated by a combination of specific chromatin modifiers (HDAC11, OGT, and TET3) and a transcriptional factor NeuroD1.

Significance: This is the first study showing that a glycosyltransferase gene is regulated by another glycosyltransferase, OGT, in combination with histone modifications.

Expression of glycosyltransferase genes is essential for glycosylation. However, the detailed mechanisms of how glycosyltransferase gene expression is regulated in a specific tissue or during disease progression are poorly understood. In particular, epigenetic studies of glycosyltransferase genes are limited, although epigenetic mechanisms, such as histone and DNA modifications, are central to establish tissue-specific gene expression. We previously found that epigenetic histone activation is essential for brain-specific expression of N-acetylglucosaminyltransferase-IX (GnT-IX, also designated GnT-Vb), but the mechanism of brain-specific chromatin activation around GnT-IX gene (Mgat5b) has not been clarified. To reveal the mechanisms regulating the chromatin surrounding GnT-IX, we have investigated the epigenetic factors that are specifically involved with the mouse GnT-IX locus by comparing their involvement with other glycosyltransferase loci. We first found that a histone deacetylase (HDAC) inhibitor enhanced the expression of GnT-IX but not of other glycosyltransferases tested. By overexpression and knockdown of a series of HDACs, we found that HDAC11 silenced GnT-IX. We also identified the O-GlcNAc transferase (OGT) and ten-eleven translocation-3 (TET3) complex as a specific chromatin activator of GnT-IX gene. Moreover, chromatin immunoprecipitation (ChiP) analysis in combination with OGT or TET3 knockdown showed that this OGT-TET3 complex facilitates the binding of a potent transactivator, NeuroD1, to the GnT-IX promoter, suggesting that epigenetic chromatin activation by the OGT-TET3 complex is a prerequisite for the efficient binding of NeuroD1.

These results reveal a new epigenetic mechanism of brain-specific GnT-IX expression regulated by defined chromatin modifiers, providing new insights into the tissue-specific expression of glycosyltransferases.

Glycosylation is the most abundant post-translational modification in mammals and frequently takes place in a tissue-specific manner (1). Such restricted expression of glycans is essential for maintaining various physiological functions in multicellular organisms. For instance, in the nervous system, neural specific glycans such as polysialic acid and human natural killer-1 (HNK-1) are required for higher order brain functions, including learning/memory or the formation of neural networks (2, 3). Tissue-specific glycosylation depends largely on expression of glycosyltransferases, which are responsible for glycan biosynthesis. However, it is still not clear how particular glycosyltransferase genes are expressed in specific tissues or how their expression is disturbed during disease progression.

We have been investigating the functions and gene regulation of several glycosyltransferases, especially glycan-branching enzymes acting on glycoprotein, such as N-acetylglucosaminyltransferases (GnT-III, ‡V, and ‡IX(Vb)) or fucosyltransferase-8 (Fut8) (4). Among them, we and others recently found that GnT-IX (encoded by Mgat5b gene), a brain-specific glycosyltransferase, is involved in remyelination in vivo (5–7). GnT-IX forms a β1,6-branched structure on O-mannose glycans (8, 9), and the branched O-mannose glycans in activated astrocytes inhibit remyelination after myelin injury (5), indicating that the brain-specific expression of GnT-IX contributes to its function in brain. We also examined the regulatory mecha-

* This work was supported by RIKEN (Systems Glycobiology Research Project) (to N. T.) and Japan Society for the Promotion of Science (JSPS) (KAKENHI Grant 23770163).

† To whom correspondence should be addressed: Disease Glycomics Team, Systems Glycobiology Research Group, RIKEN-Max Planck Joint Research Center for Systems Chemical Biology, Global Research Cluster, RIKEN, 2-1 Hiroswa, Wako 351-0198, Saitama, Japan. Tel.: 81-48-467-8094; Fax: 81-48-467-8104; E-mail: tani52@wd5.so-net.ne.jp.

‡ The abbreviations used are: GnT, N-acetylglucosaminyltransferase; HDAC, histone deacetylase; OGT, O-GlcNAc transferase; OL, oligodendrocyte; TET, ten-eleven translocation; TSA, trichostatin A; H3Ac, acetylated histone H3; DMSO, dimethyl sulfoxide; IP, immunoprecipitation.
Regulation of GnT-IX Gene by Specific Chromatin Modifiers

nism of brain-specific GnT-IX expression and found that epigenetic histone activation but not DNA hypomethylation in the GnT-IX promoter region is pivotal for its neural specific expression (10). Two transcription factors, NeuroD1 and CTCF (CCCTC-binding factor), were also identified as transactivators for the GnT-IX promoter, but neural specific chromatin activation is likely to be hierarchically dominant because the chromatin activation state is critical for the binding of these transcription factors to the GnT-IX promoter (10). However, it remains to be clarified how chromatin at the GnT-IX gene is activated or repressed in a tissue- and gene-specific manner.

Epigenetic marks, such as histone modification and DNA methylation, are central to the tissue- and cell type-specific gene program (11). Covalent histone modifications (methylation, acetylation, O-GlcNAcylation, etc.) are dynamically regulated by an interplay of adding and removing enzyme pairs, which can influence gene transcription either through direct change of chromatin structure or through binding of effector molecules. For instance, acetylation of histone tails at specific lysine residues is catalyzed by histone acetylases (HATs), whereas acetylation is removed by histone deacetylases (HDACs). These actions are highly correlated with gene activation and silencing, respectively (12). Conversely, methylation of cytosine (5mC) in a gene promoter, which correlates strongly with transcriptional silencing, had been considered to be a static modification (13). However, the recent discovery that ten-eleven translocase (TET) family enzymes (TET1-3) can convert 5mC to 5-hydroxymethyl cytosine (5hmC) led to the hypothesis that TET-mediated hydroxylation of 5mC could be part of a DNA demethylation pathway (14–16). Although TET1 and TET2 were reported to play an important role in ES cell lineage specification (17, 18), depletion of TET3 causes impairment of neural development in Xenopus (19), suggesting that TET proteins, especially TET3, might be involved in tissue-specific gene transcription. Moreover, recent studies have revealed that TET proteins bind tightly with the cytosolic/nuclear glycosyl enzyme, O-GlcNAc transferase (OGT), at transcriptional start sites to regulate gene transcription (20–22). O-GlcNAcylation by OGT on histones or histone-modifying enzymes was recently added to the growing list of epigenetic modifications (23); therefore, it has been revealed that these two emerging epigenetic factors (TET and OGT) can functionally cross-talk. Few studies have linked these epigenetic modifications to glycan expression (24); however, recent works in which an epigenetic inhibitor had a great impact on the cellular N-glycome or composition of neural glycolipid (25–27) have suggested that many glycan-related genes can be regulated by epigenetic mechanisms.

In this study, we investigated which chromatin modifiers are involved in epigenetic regulation of the mouse GnT-IX gene. To this end, we focused on enzymes involved in histone acetylation and O-GlcNAcylation. We identified one HDAC, HDAC11, and the OGT-TET3 complex as regulators of GnT-IX transcription. Meanwhile, the gene expression of other glycosyltransferases (GnT-III and -V and Fut8) was not affected by HDAC11 or the OGT-TET3 complex. Moreover, we found that recruitment of OGT-TET3 facilitates NeuroD1-dependent up-regulation of GnT-IX expression. These results suggest that a set of specific epigenetic factors (TET3-OGT and HDAC11) and a transcription factor (NeuroD1), which are highly expressed in brain, play key roles in the brain-restricted expression of GnT-IX.

EXPERIMENTAL PROCEDURES

Materials—The following antibodies were used: anti-H3K9Ac (07-352, Millipore), anti-acetyl H3 (06-599, Millipore), anti-actin (AC-40, Sigma), anti-FLAG (M2, Sigma), anti-OGT (sc-32921, Santa Cruz Biotechnology), anti-O-GlcNAC (RL2, Thermo Scientific), anti-NeuroD1 (4373, Cell Signaling Technology), anti-histone H3 (4620, Cell Signaling Technology), and normal rabbit IgG (2729, Cell Signaling Technology). siRNAs were purchased from Qiagen: control siRNA, 1027280; mouse HDAC1, S00173523; mouse HDAC2, S00173544; mouse HDAC3, S00192787; mouse HDAC11, S01063867; mouse TET1, S10962983; mouse TET2, S10439987; mouse TET3, S10968555; and mouse OGT, S100235172. Primer-probe sets for mRNA quantification were purchased from Life Technologies: control ribosomal RNA, 4308329; GnT-III, Mm00447798_s1; GnT-V, Mm00455036_m1; GnT-IX, Mm00556891_m1; Fut8, Mm00489789_m1; HDAC1, Mm02391771_g1; HDAC2, Mm00515108_m1; HDAC3, Mm00515916_m1; HDAC11, Mm0183513_m1; and TET3, Mm00805756_m1. Trichostatin A was kindly provided by Dr. Minoru Yoshida (Chemical Genetics Laboratory, RIKEN).

Cell Culture and Transfection—Neuro2A cells were cultured in DMEM supplemented with 10% fetal bovine serum. For plasmid transfection, cells plated on a 10-cm (or 6-cm) dish were transfected with 4 μg (or 1 μg) of plasmid using 10 μl (or 2.5 μl) of Lipofectamine 2000 (Life Technologies). For siRNA transfection, cells on a 10-cm (or 6-cm) dish were transfected with 200 pmol (or 80 pmol) of siRNA using 20 μl (or 8 μl) of Lipofectamine 2000. Stable HDAC transfectants were selected in the presence of antibiotics (750 μg/ml G418 for HDAC2 and 7.5 μg/ml blasticidin for the other transfectants). In some experiments, cells were treated with trichostatin A (TSA) at 0.1 μM for 24 h.

Isolation of Primary Neurons and an Oligodendrocyte (OL)-rich Fraction—All the animal experiments were approved by the Animal Experiment Committee of RIKEN. Isolation and culture of primary neurons from embryonic C57BL/6 mouse brains were carried out as described previously (5) with slight modification. After plating neurons, Cytosine arabinofuranoside (5 μM at final concentration, Sigma) was added to the medium at 2 days in vitro to remove proliferating cells. For isolation of the OL-rich fraction, brains from C57BL/6 mice (8–10 weeks old) were collected and minced. The tissue pieces were incubated in 10 ml of PBS containing 90 units of papain, 2 mg of L-cysteine, and 0.01% DNase at 37 °C for 40 min. The supernatant was passed through a 70-μm cell strainer and mixed with 2 ml of FBS. The precipitate was resuspended with 8 ml of PBS, and the resultant supernatant was passed through a 70-μm cell strainer. The passed solution was mixed with the first passage. This procedure was repeated three more times. The collected solution was centrifuged at 500 × g for 10 min, and the pellet was resuspended with 30 ml of PBS containing 30% (v/v) Percoll. After centrifugation at 20,000 × g for 30 min, the layer between the white and red layers was collected. 2 volumes of PBS were added, and the mixture was centrifuged at 700 × g for 12 min. The pellet was resuspended with 15 ml of PBS and
Regulation of GnT-IX Gene by Specific Chromatin Modifiers

RNA Extraction, Reverse Transcription, and Real-time PCR—Total RNA from cultured cells or mouse tissues was extracted using TRI Reagent (Molecular Research Center, Inc.) according to the manufacturer’s protocol. 1 μg of total RNA was reverse-transcribed using Superscript III (Life Technologies). For real-time PCR, cDNA was mixed with TaqMan Universal PCR master mix (Life Technologies) and amplified using an ABI PRISM 7900HT. The levels of mRNA were normalized to the corresponding ribosomal RNA levels.

Chromatin Immunoprecipitation (ChIP)—ChIP assays were carried out using a SimpleChIP enzymatic chromatin IP kit (Cell Signaling Technology) according to the manufacturer’s protocol. Approximately 2 × 10^7 cells were used as starting material. 2% of the chromatin was reserved as input sample. After immunoprecipitation, real-time PCR was performed to quantify the amounts of the precipitated DNA. The data are shown as values relative to the corresponding control IgG sample (indicated as % of input in figures) or as values relative to the corresponding 2% input sample (indicated as Fold enrichment in figures).

For ChIP assays of HDAC11-FLAG, anti-FLAG M2-agarose affinity gel (Sigma) was used. After washing twice with PBS, the tissues were lysed and sonicated in buffer containing 20 mM Tris-HCl, pH 7.4, 0.3 M NaCl, 0.5% Nonidet P-40, and a protease inhibitor cocktail (Roche Applied Science). After ultracentrifugation at 100,000 × g for 15 min, the resultant supernatant was incubated with antibody for 30 min on ice. Protein G-Sepharose 4 fast flow (GE Healthcare) was then added, and the mixture was incubated for 2 h at 4 °C with gentle rotation. For IP of FLAG-tagged TET3, anti-FLAG M2-agarose affinity gel (Sigma) was used. After washing with excess volumes of TBS containing 0.1% Nonidet P-40, the precipitated material was then added (125 mM final concentration) followed by incubation for 5 min. After washing twice with PBS, the tissues were homogenized in 4 ml of buffer A (supplied in the above SimpleChIP kit) using a Potter homogenizer. Subsequent procedures were carried out as for cultured cells. Primers and probes for real-time PCR are listed in Table 1.

Plasmids—The construction of a plasmid encoding Neurod1 was described previously (10). Hdac cDNAs except Hdac2 were cloned by PCR using reverse-transcribed total RNA from mouse tissue (Hdac1, -3, -4, -5, and -6 from testis; Hdac7, -10, and -11 from skeletal muscle; Hdac8 and -9 from brain). The amplified fragments were then ligated to pCR4Blunt-TOPO (Life Technologies). All the cDNAs except Hdac2 were subcloned into pcDNA6/Myc-His A (Life Technologies). Hdac1 cDNA was ligated into the EcoRI site. The other Hdac cDNAs in pCR4Blunt-TOPO were released with NotI-Spel and then ligated to the NotI-XbaI sites of pcDNA6/Myc-His A. Tet3 cDNA was amplified by PCR using reverse-transcribed total RNA from mouse brain. The amplified fragment was digested and directly ligated to pcDNA6/Myc-His A using EcoRI-EcoRV. For HDAC11-FLAG and TET3-FLAG constructs, PCR-amplified cDNA fragments that lacked their respective stop codons were digested and ligated into p3×FLAG-CMV14 (Sigma) using EcoRI-BamHI and EcoRI-XbaI sites, respectively. pcDNA3/mouse HDAC2 was kindly provided by Dr. Akihiro Ito (Chemical Genetics Laboratory, RIKEN). Primers used are listed in Table 2.

Western Blot—Proteins were separated by 4–20% gradient SDS-PAGE using the Laemmli buffer system and then transferred to PVDF or nitrocellulose membranes. After blocking with 5% nonfat dried milk in TBS containing 0.05% Tween 20, the membranes were incubated with primary antibodies followed by HRP-conjugated secondary antibodies. Proteins were detected with SuperSignal West Dura extended duration substrate (Thermo Scientific) using an ImageQuant LAS-4000mini (GE Healthcare).

Immunoprecipitation (IP)—Cells were lysed and sonicated in buffer containing 20 mM Tris-HCl, pH 7.4, 0.3 M NaCl, 0.5% Nonidet P-40, and a protease inhibitor cocktail (Roche Applied Science). After ultracentrifugation at 100,000 × g for 15 min, the resultat supernatant was incubated with antibody for 30 min on ice. Protein G-Sepharose 4 fast flow (GE Healthcare) was then added, and the mixture was incubated for 2 h at 4 °C with gentle rotation. For IP of FLAG-tagged TET3, anti-FLAG M2-agarose affinity gel (Sigma) was used. After washing with excess volumes of TBS containing 0.1% Nonidet P-40, the

**TABLE 1**

| Primer (probe) name | Sequence |
|---------------------|----------|
| GnT-III primers     | CAAGCAGCTTTGCGCAAGCTCC and GGCGCTTCTTGGCCTGATAGTATAG |
| GnT-III probe       | 5 troop-TCGGCACGGGAAGCTCATCT-MGB-3 troop |
| GnT-V primers       | GGGAGACCACACAATCTCCGAGGCA C and TCCCAGATCGACCTACCCAA |
| GnT-V probe         | 5 troop-TCGGCAGGGTGCCGCCG-3 troop |
| GnT-IX primers      | TAGGGATGCTGAGTCCTCCGAGA C and GCCGGATCGACCTACCCAA |
| GnT-IX probe        | 5 troop-TCGGCAGGGTGCCGCCG-3 troop |
| Fus8 primers        | TCACCACGCTGAGTCCTCCCA and GAGTGGCGAAGCTGCAATCA |
| Fus8 probe          | 5 troop-TCGGCAGGGTGCCGCCG-3 troop |

**TABLE 2**

| Primer name | Sequence |
|-------------|----------|
| HDAC1       | GAGCAACATGGCCAGACACTGAGGCA C and AGACCTGCTGAGCCAAACATGGCC |
| HDAC3       | AGCAACATGGCCAGACACTGAGGCA C and GGGAGACCACACAATCTCCGAGGCA |
| HDAC4       | AGCAACATGGCCAGACACTGAGGCA C and GGGAGACCACACAATCTCCGAGGCA |
| HDAC5       | AGCAACATGGCCAGACACTGAGGCA C and GGGAGACCACACAATCTCCGAGGCA |
| HDAC6       | AGCAACATGGCCAGACACTGAGGCA C and GGGAGACCACACAATCTCCGAGGCA |
| HDAC7       | AGCAACATGGCCAGACACTGAGGCA C and GGGAGACCACACAATCTCCGAGGCA |
| HDAC8       | GGGAGACCACACAATCTCCGAGGCA C and GGGAGACCACACAATCTCCGAGGCA |
| HDAC9       | GGGAGACCACACAATCTCCGAGGCA C and GGGAGACCACACAATCTCCGAGGCA |
| HDAC10      | GGGAGACCACACAATCTCCGAGGCA C and GGGAGACCACACAATCTCCGAGGCA |
| HDAC11      | GGGAGACCACACAATCTCCGAGGCA C and GGGAGACCACACAATCTCCGAGGCA |
| TET3        | GGGAGACCACACAATCTCCGAGGCA C and GGGAGACCACACAATCTCCGAGGCA |
| HDAC11-FLAG | GGGAGACCACACAATCTCCGAGGCA C and GGGAGACCACACAATCTCCGAGGCA |
| TET3-FLAG   | GGGAGACCACACAATCTCCGAGGCA C and GGGAGACCACACAATCTCCGAGGCA |
bound proteins were eluted by boiling with Laemmli sample buffer.

RESULTS

Histone Acetylation Selectively Up-regulates GnT-IX—We previously reported that histone acetylation is a critical factor for GnT-IX expression (10). To investigate whether glycosyltransferase genes are generally regulated by epigenetic histone acetylation, we first treated Neuro2A cells with a global HDAC inhibitor, TSA, that forcibly up-regulates the level of histone acetylation (28). We analyzed the expression of four glycosyltransferase genes that we divided into two groups, tissue-specific genes with the highest expression in brain (GnT-III and GnT-IX) and ubiquitously expressed genes (GnT-V and Fut8) (29, 30), respectively. As a result, only the expression of GnT-IX was drastically up-regulated by TSA treatment (Fig. 1A). To check the levels of histone acetylation around the transcriptional start sites of these genes, ChIP analysis was performed with anti-acetylated histone H3 (H3Ac) antibody. Consistent with the mRNA levels, only the GnT-IX gene had increased levels of H3Ac in TSA-treated cells (Fig. 1B). Although TSA is a pan-HDAC inhibitor, its effect on induction of histone acetylation and transcription is not global but is restricted to certain genes (31, 32), which is consistent with the varying effects of pan-HDAC inhibitor, TSA, on the different glycosyltransferase genes (Fig. 1B). The specific up-regulation of GnT-IX by TSA shows that GnT-IX can be selectively silenced by HDAC(s).

HDAC11 Silences the GnT-IX Gene—Next, we explored how GnT-IX gene is epigenetically silenced by HDAC(s). In mammalian cells, 11 HDACs (HDAC1-11) are expressed (33), but the precise function of each HDAC remains to be elucidated. Moreover, activity of all 11 HDACs is potentially inhibited by TSA. To examine which HDAC is involved in silencing GnT-IX, each HDAC enzyme was overexpressed in Neuro2A cells, and the mRNA levels of the glycosyltransferases were quantified. GnT-IX expression was significantly silenced by overexpression of several HDACs, such as HDAC1, -2, -3, and -11 (Fig. 2A), whereas other glycosyltransferases were moderately or slightly down-regulated, suggesting that GnT-IX gene is highly susceptible to epigenetic silencing by particular HDACs. To further examine whether these HDACs are involved in GnT-IX silencing, HDAC1, -2, -3, and -11 were knocked down by siRNA (Fig. 2, B and C). Depletion of HDAC11 resulted in the highest up-regulation of GnT-IX gene, whereas the ubiquitously expressed Fut8 gene was not up-regulated by knockdown of these HDACs, suggesting that HDAC11 might be a specific factor for epigenetic down-regulation of GnT-IX expression. ChIP analysis showed that knockdown of HDAC11 caused increased histone acetylation in the GnT-IX gene but not in the Fut8 gene (Fig. 2D), which is consistent with the up-regulation of GnT-IX mRNA by HDAC11 knockdown, suggesting that HDAC11 deacetylates histones surrounding the GnT-IX gene. In addition, ChIP analysis of exogenously expressed HDAC11-FLAG demonstrated that HDAC11 is highly recruited to the GnT-IX gene as compared with the Fut8 gene locus (Fig. 2E).

Hdac11 is known to be highly expressed in brain, kidney, heart, and muscle (34), and in brain HDAC11 was reported to regulate oligodendrocyte-specific gene expression (35). Therefore, we assumed that HDAC11 would be involved in silencing of GnT-IX in a specific brain cell type as well as in non-brain tissues. We, therefore, isolated primary neurons and an OL-rich fraction from mouse brain. Quantitative PCR results showed that GnT-IX mRNA is highly expressed in neurons as compared with OLs, whereas HDAC11 shows a higher level of expression in OLs as compared with neurons (Fig. 2F). This negative correlation suggests that HDAC11 may be implicated in cell type-specific silencing of GnT-IX in the brain. Collectively, these data suggest that among HDAC family members, HDAC11 may be a specific suppressor for GnT-IX but not for other glycosyltransferases.

The TET3-OGT Complex Is an Epigenetic Activator Specific for GnT-IX Gene—Next, we investigated the chromatin activation mechanism of GnT-IX gene. Recently, a novel chromatin activation mechanism was reported in which two epigenetic factors, OGT and TET, form a functional complex (20–22). TET molecule can recruit OGT to chromatin, and this in turn results in O-GlcNAcylation of surrounding chromatin molecules, including histones and host cell factor-1 (HCF-1, a com-
ponent of the H3K4 methyltransferase complex), leading to gene activation (20–22). Considering the fact that OGT is highly expressed in brain as compared with other tissues (36, 37) and that TET-generated 5hmC is most abundant in brain (38–40), we assumed that this OGT-TET machinery could be involved in chromatin activation of GnT-IX gene in brain. To see whether the OGT-TET complex is actually involved in activation of the glycosyltransferase genes, TET family proteins (TET1–3) were knocked down in Neuro2A cells by siRNA. As a result, we found that mRNA levels of GnT-V and -IX but not of GnT-III and Fut8 were down-regulated by TET3 knockdown (Fig. 3).

Next, we checked to see whether the OGT-TET3 complex activates GnT-IX transcription. IP experiments clearly showed that exogenously expressed TET3-FLAG can co-precipitate endogenous OGT and vice versa (Fig. 4A), which is consistent with previous studies using other cell systems (20, 21, 41). If OGT interacts with TET3 for its recruitment to chromatin and gene activation, TET3 depletion would dissociate OGT from chromatin particularly from GnT-V and GnT-IX genes. ChIP

FIGURE 2. HDAC11 silences GnT-IX gene. A, total RNAs were extracted from Neuro2A cells (control) or transfectants stably expressing HDAC1–11. The mRNA levels of GnT-III, -V, and -IX and Fut8 were quantified and normalized to those of rRNA. The mRNA/rRNA levels of HDAC-expressing samples relative to those of control samples are shown (n = 3, *, p < 0.05, Tukey-Kramer’s test). B, Neuro2A cells were treated with control siRNA (siCont), HDAC1, HDAC2, HDAC3, or HDAC11 siRNA for 24 h. Total RNAs were extracted, and the mRNA levels of GnT-IX and Fut8 were quantified and normalized to those of rRNA. The mRNA/rRNA levels of HDAC knockdown samples relative to those of control samples are shown (n = 3, **, p < 0.01, Tukey-Kramer’s test). C, Neuro2A cells were treated with control siRNA, HDAC1, HDAC2, HDAC3, or HDAC11 siRNA for 24 h. Total RNAs were extracted, and the mRNA levels of Hdac1, Hdac2, Hdac3, and Hdac11 were quantified and normalized to those of rRNA. The mRNA/rRNA levels of HDAC knockdown samples relative to those of control samples are shown (n = 3). D, Neuro2A cells were treated with control siRNA or HDAC11 siRNA for 24 h, and then the levels of H3K9Ac around the transcriptional start sites of GnT-IX and Fut8 genes were analyzed by ChIP assays (n = 3, *, p < 0.05, Student’s t test). E, HDAC11 tagged with 3×FLAG at its C terminus was overexpressed in Neuro2A cells. Recruitment of HDAC11-FLAG to the genomic region around the transcriptional start sites of GnT-IX and Fut8 genes was analyzed by ChIP assays with anti-FLAG antibody (M2)-conjugated beads. The precipitated DNA was analyzed by real-time PCR, and the amount of DNA from the transfectant (+) relative to that from mock-treated cells (−) is shown (n = 3, *, p < 0.05, Student’s t test). F, mRNA levels of GnT-IX and Hdac11 were quantified and normalized to those of rRNA in mouse primary neurons (6 days in vitro) or in the oligodendrocyte-rich fraction (OL). The mRNA/rRNA levels are shown as relative values to those in whole brain from 20-week-old male mice (n = 3, *, p < 0.05, **, p < 0.01, Student’s t test). All graphs show means ± S.E.
assays with anti-OGT antibody showed that OGT was indeed dissociated from GnT-IX gene chromatin by TET3 depletion, together with a reduced level of O-GlcNAc (Fig. 4, B and C), suggesting that OGT and TET3 molecules act as a complex on the GnT-IX gene for epigenetic activation. Surprisingly, however, such dissociation of OGT was not observed for GnT-V gene, suggesting that TET3 regulates GnT-V expression independently of OGT or in an indirect manner. Consistent with this possibility, knockdown of OGT by siRNA revealed that only GnT-IX but not other glycosyltransferases was down-regulated (Fig. 4, D and E). Together, these results indicated that the OGT-TET3 complex may be specifically required for expression of GnT-IX but not for other glycosyltransferases tested.

The OGT-TET3 Complex Recruits a Brain-specific Transcription Factor, NeuroD1, to the GnT-IX Promoter—We previously identified NeuroD1, a neuronal basic helix-loop-helix transcription factor (42), as a strong transactivator for the GnT-IX promoter (10). Overexpression of NeuroD1 markedly up-regulates GnT-IX transcription in Neuro2A cells, whereas NeuroD1 fails to activate GnT-IX in a non-neural cell line (3T3-L1), in which the GnT-IX chromatin is highly
This suggests that epigenetic chromatin activation is a prerequisite for NeuroD1 binding to the GnT-IX promoter. Therefore, we expected that chromatin activation of GnT-IX by OGT-TET3 would enhance NeuroD1 recruitment. Actually, binding of NeuroD1 to the GnT-IX promoter and subsequent activation of GnT-IX transcription were both reduced by depletion of OGT or TET3 (Fig. 5, A–C). These results suggest that the OGT-TET3 complex is required for the efficient binding of NeuroD1 to the GnT-IX promoter to drive it. We also examined in vivo binding of NeuroD1 and OGT to the GnT-IX promoter. Although we failed to detect the binding of TET3 by ChIP assays with commercially available antibodies, we found that binding of endogenous NeuroD1 and OGT to the GnT-IX promoter was stronger in brain than in other tissues (Fig. 5D), which is consistent with the highest GnT-IX expression level being in the brain. This robust binding of NeuroD1 and OGT may be due to their high expression in brain (Fig. 5E). These results suggest that the OGT-TET3-dependent binding of NeuroD1 to the GnT-IX promoter may also be required for the brain-specific transcription of GnT-IX gene in vivo.

**DISCUSSION**

We have identified for the first time epigenetic factors involved in the regulation of GnT-IX gene, an epigenetic suppressor HDAC11, and an activator, the OGT-TET3 complex. The other glycosyltransferases tested were not regulated by these factors, which suggests that these chromatin modifiers selectively act on GnT-IX among the glycosyltransferase genes. In addition, our overexpression and knockdown data showed that the other HDACs (HDAC1–10) and TETs (TET1 and TET2) are not or are less involved in GnT-IX regulation, showing the specificity of HDAC11 and OGT-TET3 molecules for GnT-IX gene. Previous studies have revealed that TET proteins are essential for OGT recruitment to a CpG island in a gene promoter, leading to efficient O-GlcNAcylation of chromatin proteins (20–22, 41). Our data indicate that the OGT-TET3 complex is a prerequisite for the efficient binding of the transactivator, NeuroD1, to the GnT-IX promoter to drive it. Taking an overall view of our data, we present a stepwise model for the epigenetic regulation of GnT-IX gene (Fig. 6).

Recently, we reported that the branched O-mannose glycans produced by GnT-IX inhibit the development of OLs after

---

**FIGURE 5. Transactivation of GnT-IX by NeuroD1 is promoted by the OGT-TET3 complex.** A, Neuro2A cells were treated with control siRNA (siCont), OGT siRNA, or TET3 siRNA for 24 h and then replated on new dishes. After 24 h, the cells were transfected with the NeuroD1 expression vector followed by a further 24-h culture. Then, ChIP assays were performed with an anti-NeuroD1 antibody. A genomic region around the transcriptional start site of GnT-IX gene, including the NeuroD1 binding site, was analyzed by real-time PCR. The amounts of DNA relative to those of 2% input samples are shown (n = 3, *, p < 0.05, Tukey-Kramer’s test). B and C, Neuro2A cells were transfected with siRNA and expression plasmid as in the case of A. Total RNAs were extracted, and the mRNA levels of GnT-IX were quantified and normalized to those of rRNA. The mRNA/rRNA levels relative to those of control siRNA-treated mock sample are shown (n = 3, *, p < 0.05, Tukey-Kramer’s test) (B). Cellular proteins extracted after 24 h of NeuroD1-transfection were Western blotted with an anti-NeuroD1 or anti-actin antibody (C). D, chromatin was prepared from brain, liver, or kidney of adult mice, and ChIP assays were performed with anti-NeuroD1 or anti-OGT antibody. A genomic region around the transcriptional start site of GnT-IX gene, including the NeuroD1 binding site, was analyzed by real-time PCR. The amounts of DNA relative to those of 2% input samples are shown (n = 3, **, p < 0.01, Tukey-Kramer’s test). E, homogenates of brain, liver, or kidney from adult mice were Western blotted with an anti-NeuroD1, anti-OGT, or anti-histone H3 antibody. All graphs show means ± S.E.
Regulation of GnT-IX Gene by Specific Chromatin Modifiers

**FIGURE 6. Schematic model for the epigenetic regulation of GnT-IX.** In non-GnT-IX-expressing cells, chromatin around the GnT-IX gene is repressed by chromatin modifiers, including HDAC11. When GnT-IX chromatin is activated, TET3 recruits OGT. This OGT-TET3 complex further facilitates the recruitment of NeuroD1 to GnT-IX promoter, which in turn activates GnT-IX transcription. Pol-II, RNA polymerase II.

**REFERENCES**

1. Mooremen, K. W., Tiemeyer, M., and Nairn, A. V. (2012) Vertebrate protein glycosylation: diversity, synthesis and function. *Nat. Rev. Mol. Cell Biol.* 13, 448–462

2. Yamamoto, S., Oka, S., Inoue, M., Shimuta, M., Manabe, T., Takahashi, H., Miyamoto, M., Asano, M., Sakagami, J., Sudo, K., Iwakura, Y., Ono, K., and Kawasumi, T. (2002) Mice deficient in nervous system-specific carbohydrate epitope HNK-1 exhibit impaired synaptic plasticity and spatial learning. *J. Biol. Chem.* 277, 27227–27231

3. Weinhold, B., Seidenfaden, R., Röcke, I., Mühlenhoff, M., Schertzing, F., Conzelmann, S., Marsh, J. D., Gerardy-Schahn, R., and Hildebrandt, H. (2005) Genetic ablation of polysialic acid causes severe neurodevelopmental defects rescued by deletion of the neural cell adhesion molecule. *J. Biol. Chem.* 280, 42971–42977

4. Taniguchi, N., Miyoshi, E., Gu, J., Honke, K., and Matsumoto, A. (2006) Decoding sugar functions by identifying target glycoproteins. *Curr. Opin. Struct. Biol.* 16, 561–566

5. Kanekiyo, K., Inamori, K., Kitazume, S., Sato, K., Maeda, J., Higuchi, M., Kizuka, Y., Korekane, H., Matsuo, I., Honke, K., and Taniguchi, N. (2013) Loss of branched O-mannosyl glycans in astrocytes accelerates remyelination. *J. Neurosci.* 33, 10037–10047

6. Inamori, K., Endo, T., Ide, Y., Fuji, S., Gu, J., Honke, K., and Taniguchi, N. (2003) Molecular cloning and characterization of human GnT-IX, a novel β1,6-N-acetylgalactosaminyltransferase that is specifically expressed in the brain. *J. Biol. Chem.* 278, 43102–43109

7. Kaneko, M., Álvarez-Manilla, G., Kamar, M., Lee, I., Lee, J. K., Troupe, K., Zhang, W. j., Osawa, M., and Pierce, M. (2003) A novel β1,6-N-acetylgalactosaminyltransferase V (Gnt-Vb). *FEBS Lett.* 554, 515–519

8. Inamori, K., Endo, T., Gu, J., Matsuo, I., Ito, Y., Fujii, S., Iwasaki, H., Nari-matsu, H., Miyoshi, E., Honke, K., and Taniguchi, N. (2004) N-Acetylgalactosaminyltransferase IX acts on the GlcNAc β1,2-Man-α1-Ser/Thr moiety, forming a 2,6-branched structure in brain O-mannosyl glycan. *J. Biol. Chem.* 279, 2337–2340

9. Lee, J. K., Matthews, R. T., Lim, J. M., Swanier, K., Wells, L., and Pierce, J. M. (2012) Developmental expression of the neuron-specific N-acetylglucosaminyltransferase Vb (Gnt-Vb/X) and identification of its in vivo O-GlcNAcylation of NeuroD1 for GnT-IX activation because NeuroD1 was also reported to be modified by OGT (47). O-GlcNAc modification exerts a wide variety of functions depending on the OGT substrate (48). In our case, OGT, a sugar transferase, positively regulates the transcription of another sugar transferase, GnT-IX, which is connected by epigenetics. Meanwhile, OGT is considered as a master sensor for nutrients as it uses UDP-GlcNAc as the high energy donor substrate, which is the end point of the hexosamine biosynthetic pathway (48). The biosynthesis of UDP-GlcNAc integrates the metabolic pathways of glucose, an amino acid (glutamine), a fatty acid (acetyl-CoA), and a nucleotide (UTP). Therefore, nutrient flux to the cells would affect the levels of both cellular UDP-GlcNAc and cellular O-GlcNAc modification. Notably, other glycosyltransferases, including GnT-IX, also use UDP-GlcNAc as the donor substrate. Therefore, nutrient flux and the subsequent metabolic pathway could regulate GnT-IX activity by both donor-substrate use and OGT-mediated gene transcription. We recently proposed that cellular glycosylation forms a functional cycle called the “glycan cycle” comprising nutrient flux, biosynthesis of sugar nucleotide, sugar transfer, biological action, degradation, and recycling (49). We still do not fully understand this cycle at the molecular level, but we believe that the present study provides new insights into how epigenetic regulation of glycogenes is integrated into the glycan cycle.
glycan products in comparison with those of its paralog, Gnt-V. J. Biol. Chem. 287, 28526–28536.

10. Kizuka, Y., Kizatsume, S., Yoshida, M., and Taniguchi, N. (2011) Brain-specific expression of N-acetylglucosaminyltransferase IX (Gnt-IX) is regulated by epigenetic histone modifications. J. Biol. Chem. 286, 31875–31884.

11. Bonasio, R., Tu, S., and Reinberg, D. (2010) Molecular signals of epigenetic states. Science 330, 612–616.

12. Bannister, A. J., and Kouzharides, T. (2011) Regulation of chromatin by histone modifications. Cell Res. 21, 381–395.

13. Suzuki, M. M., and Bird, A. (2008) DNA methylation landscapes: provocative insights from epigenomics. Nat. Rev. Genet. 9, 465–476.

14. Wu, H., and Zhang, Y. (2011) Mechanisms and functions of Tet protein-mediated 5-methylcytosine oxidation. Genes Dev. 25, 2436–2452.

15. Williams, K., Christensen, J., and Helin, K. (2012) DNA methylation: TET proteins-guardians of CpG islands? EMBO Rep. 13, 28–35.

16. Tahiliani, M., Koh, K. P., Shen, Y., Pastor, W. A., Bandukwala, H., Brudno, Y., Agarwal, S., Iyer, L. M., Liu, D. R., Aravind, L., and Rao, A. (2009) Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science 324, 930–935.

17. Ito, S., Shen, L., Dai, Q., Wu, S. C., Collins, L. B., Swenberg, J. A., He, C., and Zhang, Y. (2011) Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxycytosine. Science 333, 1300–1303.

18. Koh, K. P., Yabuuchi, A., Rao, S., Huang, Y., Cunniiff, K., Nardone, J., Laibo, A., Tahiliani, M., Sommer, C. A., Mostoslavsky, G., Lahesmaa, R., Orkin, S. H., Rodrig, S. J., Daley, G. Q., and Rao, A. (2011) Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. Cell Stem Cell 8, 200–213.

19. Xu, Y., Xu, C., Kato, A., Tempel, W., Abreu, J. G., Bian, C., Hu, Y., Hu, D., Zhao, B., Cerovina, T., Diao, J., Wu, F., He, H. H., Cui, Q., Clark, E., Ma, C., Barbara, A., Veenstra, G. J., Xu, G., Kaiser, U. B., Liu, S. X., Sjugre, S. P., He, X., Min, J., Kato, Y., and Shi, Y. G. (2012) Tet3 CXXC domain and dioxygenase activity cooperatively regulate key genes for Xenopus neural and neuronal development. Cell 151, 1200–1213.

20. Chen, Q., Chen, Y., Bian, C., Fujiki, R., and Yu, X. (2013) TET2 promotes histone H3K4 acetylation during gene transcription. Nature 493, 561–564.

21. Deplus, R., Delatte, B., Schwinn, M. K., Defrance, M., Méndez, J., Murphy, N., Dawson, M. A., Volkmar, M., Putmans, P., Calonne, E., Arighi, L. C., Levine, R. L., Bernard, O., Mercher, T., Solary, E., Urh, M., Daniels, D. L., and Fuks, F. (2013) TET2 and TET3 regulate H3K4 methylation through OG/T and SET1/COMPASS. EMBO J. 32, 645–655.

22. Vella, P., Scelfo, A., Jammula, S., Chiacchiera, F., Williams, K., Cueto, M. A., Asselbergs, F., and Atadja, P. (2002) Cloning and functional characterization of a unique O-GlcNAc transferase with multiple tetratricopeptide repeats. J. Biol. Chem. 277, 25748–25755.

23. Gao, L., Cueto, M. A., and Hart, G. W. (1997) Dynamic glycosylation of nuclear and cytosolic proteins: cloning and characterization of a unique O-GlcNAc transferase in multiple tetratricopeptide repeats. J. Biol. Chem. 272, 9308–9315.

24. Suzuki, Y., Yanagisawa, M., Ariga, T., and Yu, R. K. (2011) Histone acetylation is the quantum mechanics of biology. J. Cell Biol. 194, 557–560.

25. Vella, P., Scelfo, A., Jammula, S., Chiacchiera, F., Williams, K., Cueto, M. A., Asselbergs, F., and Atadja, P. (2002) Cloning and functional characterization of a unique O-GlcNAc transferase with multiple tetratricopeptide repeats. J. Biol. Chem. 277, 25748–25755.

26. Luo, C., and Bujalowski, K. (2011) Histone acetylation and gene expression profiling of neural HDAC inhibition. Nucleic Acids Res. 41, 8072–8084.

27. Haberland, M., Montgomery, R. L., and Olson, E. N. (2009) The many roles of histone deacetylases in development and physiology: implications for disease and therapy. Nat. Rev. Genet. 10, 32–42.

28. Lopez-Atalaya, J. P., Ito, S., Valor, L. M., Benito, E., and Barco, A. (2013) Genomic targets, and histone acetylation and gene expression profiling of neural HDAC inhibition. Nucleic Acids Res. 41, 8072–8084.

29. Nishimoto, K., Mita, S., Gu, J., Mizuno-Horikawa, Y., Miyoshi, E., Dunnis, J. W., and Taniguchi, N. (2006) Demonstration of the expression and the enzymatic activity of N-acetylglucosaminyltransferase IX in the mouse brain. Biochem. Biophys. Acta 1760, 678–684.

30. Haller, G., Schmidt, W. M., Ziegler, B., Holzer, S., Müllauer, L., Bilban, M., Zieblinski, C. C., Drach, I., and Zöchbauer-Müller, S. (2008) Genome-wide transcriptional response to 5-aza-2'-deoxycytidine and trichostatin a in multiple myeloma cells. Cancer Res. 68, 44–54.

31. Roeder, R. G., Brown, M., and Kato, S. (2011) GlcNAcylation of histone H2B facilitates its monoubiquitination. Nat. Rev. Genet. 12, 557–560.

32. Andrali, S. S., Qian, Q., and Ozcan, S. (2007) Glucose mediates the transcriptional response to 5-aza-2'-deoxycytidine and trichostatin a in multiple myeloma cells. Cancer Res. 68, 44–54.

33. Hart, G. W., Slawson, C., Ramirez-Correag, G., and Lagerlof, O. (2011) Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease. Annu. Rev. Biochem. 80, 825–858.

34. Fujiki, R., Hashiba, W., Sekine, H., Yokoyama, A., Ito, S., Imai, Y., Kim, J., He, H. H., Igarashi, K., Kanno, J., Ohtake, F., Kitagawa, H., Imai, Y., Matsui, M., Hada, M., Okumura, T., Sugawara, A., and Yokoyama, A. (2014) TET3-OGT interaction increases the stability and the presence of OG/T in chromatin. Genes Cells 19, 52–65.

35. Miyata, T., Maeda, T., and Lee, J. I. (1999) NeuroD is required for differentiation of the granule cells in the cerebellum and hippocampus. Genes Dev. 13, 1647–1652.

36. Shen, S., Li, J., and Casaccia-Bonnefille, P. (2005) Histone modifications affect timing of oligodendrocyte progenitor differentiation in the developing rat brain. J. Cell Biol. 169, 577–589.

37. Fujiki, R., Hashiba, W., Sekine, H., Yokoyama, A., Chikanishi, T., Ito, S., Imai, Y., Kim, J., He, H. H., Igarashi, K., Kanno, J., Ohtake, F., Kitagawa, H., Roeder, R. G., Brown, M., and Kato, S. (2011) GlcNAcylation of histone H2B facilitates its monoubiquitination. Nature 480, 557–560.

38. Capotosto, F., Guernier, S., Lammers, F., Waridel, P., Cai, Y., Jin, J., Conaway, J. W., Conaway, R. C., and Herr, W. (2011) O-GlcNAc transferase catalyzes site-specific proteolysis of HCF-1. Cell 144, 376–388.

39. Aprigliano, S. S., Qian, Q., and Ozcan, S. (2007) Glucose mediates the transcription of NeuroD1 by O-linked glycosylation. J. Biol. Chem. 282, 15589–15596.

40. Hart, G. W., Slawson, C., Ramirez-Correag, G., and Lagerlof, O. (2011) Cross talk between O-GlcNAcylation and phosphorylation: roles in signaling, transcription, and chronic disease. Annu. Rev. Biochem. 80, 825–858.

41. Taniguchi, N. (2009) From the /H9253 N-acetylglucosaminyltransferase IX (GnT-IX) is regulated by epigenetic histone modifications. J. Biol. Chem. 284, 34469–34478.