Truncated, Inactive N-Acetylglucosaminyltransferase III (GlcNAc-TIII) Induces Neurological and Other Traits Absent in Mice That Lack GlcNAc-TIII*

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N-Acetylglucosaminyltransferase III (GlcNAc-TIII), the product of the Mgat3 gene, transfers the bisecting GlcNAc to the core mannoside of complex N-glycans. The addition of this residue is regulated during development and has functional consequences for receptor signaling, cell adhesion, and tumor progression. Mice homozygous for a null mutation at the Mgat3 locus (Mgat3−/−) or for a targeted mutation in the Mgat3 gene (previously called Mgat2neo††, but herein renamed Mgat3T37Δ because the allele generates inactive GlcNAc-TIII of ~37 kDa) were found to exhibit retarded progression of liver tumors. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of neutral N-glycans from kidneys revealed no significant differences, and both mutants showed the expected lack of N-glycan species with an additional GlcNAc. However, the two mutants differed in several biological traits. Mgat3T37Δ/H20648 homozygotes in a mixed or 129SvJ background were retarded in growth rate and exhibited an altered leg clasp reflex, an altered gait, and defective nursing behavior. Pups abandoned by Mgat3T37Δ/H20648 mothers were rescued by wild-type foster mothers. None of these Mgat3T37Δ/H20648 traits were exhibited by Mgat3+/− mice or by heterozygous mice carrying the Mgat3T37Δ mutation. Similarly, no dominant-negative effect was observed in Chinese hamster ovary cells expressing truncated GlcNAc-TIII in the presence of wild-type GlcNAc-TIII. However, compound heterozygotes carrying both the Mgat3T37Δ and Mgat3+/− mutations exhibited a marked leg clasp reflex, indicating that in the absence of wild-type GlcNAc-TIII, truncated GlcNAc-TIII causes this phenotype. The Mgat3 gene was expressed in brain at embryonic day 10.5 and thereafter and in neurons of adult cerebellum. The mutant Mgat3 gene was also highly expressed in Mgat3T37Δ/H20648 brain. This may be the basis of the unexpected neurological phenotype induced by truncated, inactive GlcNAc-TIII in the mouse.

The N-glycans of mammalian glycoproteins vary widely in structure, but the biological significance of this variation is largely unknown. A well studied example is the bisecting GlcNAc. This residue is transferred to the β-linked Man of the core of N-glycans by the glycosyltransferase termed N-acetylglucosaminyltransferase III (GlcNAc-TIII††, EC 2.4.1.144) (1), the product of the Mgat3 gene (2). The presence of the bisecting GlcNAc alters the lectin binding properties of a cell, a fact initially revealed by the gain-of-function Chinese hamster ovary (CHO) glycosylation mutant LEC10, which expresses GlcNAc-TIII (3). LEC10 cells are ~15-fold more resistant to ricin and ~10-fold more sensitive to the toxicity of the erythroagglutinin from Phaseolus vulgaris (E-PHA) compared with wild-type CHO cells, reflecting dramatic changes in binding of these lectins to N-linked Gal residues of cell-surface glycoproteins. Similarly, the ectopic expression of an Mgat3 cDNA reduces the expression of terminal a3-Gal residues, a key determinant in xenotransplantation (4, 5). The regulated expression of the Mgat3 gene could therefore control the binding of animal lectins like galectins to cell-surface N-glycans with a bisecting GlcNAc and thereby modulate cellular interactions.

The expression of the Mgat3 gene varies in a tissue-specific fashion in mice (6, 7) and has been correlated with the onset of hepatoma in rats (8–10) and humans (11), but not in mice (12). GlcNAc-TIII activity is elevated in human leukemia (13). Treatment of cells with forskolin increases Mgat3 gene expression (14), and overexpression of GlcNAc-TIII modulates several cellular properties, including the metastasis of B16 melanoma cells (15), the sensitivity of K562 cells to killing by natural killer cells and enhanced spleen colonization (16), and the binding of epidermal growth factor to the epidermal growth factor receptor (17). Transgenic mice overexpressing GlcNAc-TIII in liver exhibit altered secretion of certain glycoproteins (18), but no change in the development of diethylnitrosamine-induced hepatocarcinogenesis (19). In bone marrow, overexpression of GlcNAc-TIII suppresses stroma-dependent hematopoiesis (20).

To identify new functions for N-glycans with a bisecting GlcNAc, the Mgat3 gene has been inactivated by targeted mutations in the mouse. Priatel et al. (7) used a Cre-loxP strategy to delete the Mgat3 gene coding region, whereas Bhaumik et al. (12) inserted a pgkneo cassette into the Mgat3 gene to disrupt the coding region. Both mutations retard the progression of liver tumors in homozygotes treated with diethylnitrosamine and phenobarbital (12, 19). Although deletion of the Mgat3

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† The abbreviations used are: GlcNAc-T, N-acetylglucosaminyltransferase; CHO, Chinese hamster ovary; E-PHA, erythroagglutinin from P. vulgaris; PBS, phosphate-buffered saline; MALDI-TOF-MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.
gene coding region (Mgat3<sup>3</sup> mutation) causes no overt pheno-
typic traits in a mixed or C57BL/6 genetic background (7),
elimination of GlcNAc-TIII activity by disruption of the Mgat3
gene was preliminarily reported to give rise to neurological and
other consequences in mice of mixed genetic background (21).
We now describe these traits in detail and show that they are
maintained or become more severe after backcrossing 11 gen-
erations onto the 129SvJ background. By contrast, Mgat3<sup>3</sup>-<sup>3</sup>
mice do not exhibit the same traits in either mixed or inbred
genetic backgrounds. The targeted Mgat3 allele gives rise to a
truncated, enzymatically inactive GlcNAc-TIII that induces a
partial mutant phenotype in Mgat3<sup>T37</sup>/<sup>T37</sup> compound heterozy-
gotes. Humans with related nonsense mutations in the MGAT3
gene may have neurological or behavioral problems.

**EXPERIMENTAL PROCEDURES**

**Materials**—UDP-[6-<sup>3</sup>H]Gal (10 Ci/μmol), UDP-[6-<sup>3</sup>H]GlcNAc (41.60
Ci/μmol), and concanavalin A-Sepharose were from Amersham
Biotechnologies. E.coli-pHAP lectins were from Vector Labs, Inc.
Bio-Gel P-2 (45–95 mesh), the DC protein assay reagent, and
AG 1-X4 resin (100–200 mesh, Cl<sup>-</sup>) were from Bio-Rad. Pronase (Streptomyces
griseus) and EDTA-free protease inhibitor tablets were from Roche
Molecular Biochemicals. Triton X-100 and bovine serum albumin
(fragment V) was from Sigma. G418, fetal bovine serum, and
α-medium were from Invitrogen. Ecolume was from ICN Biomedicals,
and N-glycanase from New England Biolabs Inc.

**Mutant Mgat3:**—The Mgat3<sup>T37</sup>/<sup>T37</sup> insertion mutation was previ-
ously generated by introducing a pgkneo gene in reverse orientation
into the Neo I site of the Mgat3 gene in WW6 embryonic stem cells
and termed Mgat3<sup>neo</sup> (12, 19). In this study, we show that a truncated
GlcNAc-TIII of ~37 kDa (predicted 371 or 374 amino acids based on
encoded stop codons) is produced from the targeted allele. Henceforth,
this mutant allele will therefore be termed Mgat3<sup>T37</sup>/<sup>T37</sup> to better reflect the
nature of its product.

**Histoglycosylation** Mgat3<sup>T37</sup>/<sup>T37</sup> progeny from chimeras generated by injec-
tion of C57BL/6 blastocysts with Mgat3<sup>T37</sup>/<sup>T37</sup> WW6 embryonic stem cells
were crossed to CD1 mice, and cousins were interbred to obtain F<sub>1</sub>, F<sub>2</sub>,
and F<sub>3</sub> generations of Mgat3<sup>T37</sup>/<sup>T37</sup> mice. The Mgat3<sup>T37</sup>/<sup>T37</sup>
mice in a CD1 mixed genetic background were generated
as described (19). To obtain compound heterozygotes, Mgat3<sup>T37</sup>/<sup>T37</sup>
mice were crossed to Mgat3<sup>T37</sup>/<sup>T37</sup> mice to generate Mgat3<sup>T37</sup>×<sup>T37</sup>
mice from the CD1 mixed genetic background. The targeted
Mgat3 gene in reverse orientation was isolated using Trizol reagent and then
amplified by PCR and Southern analysis of tail
DNA from Mgat3<sup>T37</sup>/<sup>T37</sup> mice as described (19).

**Cell Culture and Transfection**—Parental CHO cells and the gain-of-
function CHO mutant LEC10 (3) were cultured in suspension at 37°C
in complete α-medium containing 10% fetal bovine serum. CDNAs
encoding the Mgat3<sup>T37</sup> gene coding region or the Mgat3<sup>neo</sup>
were cloned into the pcdNAS.1 vector (Invitrogen). Plasmid DNA (5 μg) was linear-
ized with XhoI digestion, purified (Invitrogen), and transfected into parental CHO or LEC10 cells (~4 × 10<sup>6</sup> in 750 μl of phosphate-buffered saline (PBS) using a Bio-Rad electroporator (pulse,
0.3 kV; capacitance, 975 microfarads; and average time constant, 12
ms). Selection of stable transfecants was performed by adding G418 (1
mg/ml active weight) 24 h after plating. Colonies that survived G418
selection were isolated and characterized.

**Western and Lectin Blot Analyses**—For lectin blots, CHO cells or
kidney or brain extracts (50 μg of protein) from mice perfused with cold
PBS (pH 7.2) were electrophoresed on a 10% SDS-polyacrylamide gel
and transferred to polyvinylidene difluoride membrane. Desialylation
was performed in 25 mM sulfuric acid at 80°C for 1 h. Biotinylated
lectins (Vector Labs, Inc.) were used to detect the presence of the
complex glycan on Glyglycan II and were described in 19. To detect a
rabbit polyclonal antibody against amino acids 120–136 of mouse
GlcNAc-TIII (TRMLEKSPGRTKEKE), synthesized by the Labora-
atory for Macromolecular Analysis and Proteomics at the Albert Einstein
College of Medicine, was used as described (19).

**Matrix-assisted Laser Desorption/Ionization Time-of-Flight Mass
Spectrometry (MALDI-TOF-MS)**—To obtain kidney N-glycans for analy-
ysis, mice were perfused with PBS, and kidney pieces at 150 mg/ml
were homogenized for 5 min on ice in buffer containing 10 mM Tris-HCl
(pH 7.4), 250 mM sucrose, 0.25 mg/ml leupeptin, 0.7 mg/ml pepstatin,
0.28 mg/ml aprotinin, and 0.5 mg/ml phenylmethylsulfonyl fluoride.
Triton X-100 was added to 2% by volume; nuclei were removed by
centrifugation; glycerol was added to 20% by volume; and extracts were
stored at ~70°C. Protein concentration was measured with the Bio-
Rad protein dye reagent. N-Glycans were released from glycoproteins
immobilized on polyvinylidene difluoride membranes by treatment with
N-glycanase as described (22). Mass spectrometry was performed on a
Voyager DE Biospectrometry Workstation (PerSeptive Biosystems)
equipped with delayed extraction as described previously (22). A nitro-
gen laser irradiated samples at 357 nm, and an average of 240 scans
were taken. The instrument was operated in linear configuration (1.2-m
flight path), and an acceleration voltage of 20 kV was used after a 60-s
delay. A reflectron mode was used to produce full mass spectra to
which 0.3 μl of matrix was added and dried under vacuum (50 × 10<sup>-3</sup>
torr). Oligosaccharide standards were used to achieve a two-point ex-
ternal calibration for mass assignment of ions (23, 24). 2,5-Dihydroxy-
benzoic acid and 5-methoxyxalic acid matrix was used for neutral
oligosaccharides.

**Characterization of Mouse Phenotypes**—Mice were maintained in a
tail barrier facility with food and water provided ad libitum and weighed on
the same day at the same time each week. To measure gait, each hind
paw was dipped in Indian ink, and the mouse was allowed to walk
undisturbed on a 4.5 × 3.5-foot piece of Whatman filter paper. The
distance between hind footsteps was measured by the manual method
as described (25). To record the altered leg clasp reflex, each mouse was
suspected by the tail for a maximum of 2 min and photographed using a
video camera. The time of holding of the rear leg clasp was recorded.

**In Situ Hybridization**—Brain sections prepared by cryostat from
mice perfused with PBS were thawed at room temperature for 30 min, fixed in 4%
paraformaldehyde and PBS at room temperature for 15 min, and
washed three times with PBS. The sections were acetylated twice in 0.1
m triethanolamine and acetic anhydride at room temperature for 10 min
and washed twice with PBS, followed by dehydration in 30, 60, 70, 90,
and 100% ethanol. After drying for 5 min at room temperature and in 0.2× SSC for 1–2 min, prehybridization
was carried out in a moist chamber with 50% formamide in 5× SSC
at 45°C for 3 h. For hybridization, 35S-UTP-labeled antisense and sense
RNAs encoding the Mgat3<sup>T37</sup> gene coding region were prepared from a
mouse Mgat3 cDNA (6) using T7 or SP6 RNA polymerase (Promega);
probes were hydrolyzed; and hybridization, washing, and autoradiog-
raphy were performed as described previously (26).

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RESULTS

In Vivo Products of the Mgat3<sup>T37</sup> Gene—Previous studies showed that the insertion of a pgk<sub>neo</sub> gene in reverse orientation into the NeoI site of the Mgat3 gene abrogates GlcNAc-TIII activity from mouse tissues (12). However, the stop codons that occur in the pgk<sub>neo</sub> reverse sequence (Fig. 1A) did not cause nonsense-mediated decay, as was obtained previously with a similarly disrupted Mgat1 gene coding exon (27, 28). Northern analysis (Fig. 1B) and reverse transcription-PCR (data not shown) revealed read-through transcripts from Mgat3 gene promoter(s) that were present at similar or greater levels than transcripts from the endogenous Mgat3 gene in brain and kidney. The size of the novel transcripts was ~6.5 kb and included the ~4.7-kb Mgat3 gene coding and untranslated regions with the ~1.8-kb antisense transcript from the pgk<sub>neo</sub> cassette. Transcription was from Mgat3 gene promoter(s) because reverse transcription-PCR of kidney and brain transcripts from homozygous mutant mice using an oligo(dT) primer for reverse transcription and primers 63 and 99 (Fig. 1A) for PCR gave the predicted product (data not shown), and Western analysis (see below) revealed a protein product. Sense transcripts of ~1.8 kb that would encode an active neomycin phosphotransferase protein were not detected by Northern analysis in kidney (Fig. 1B) or brain (data not shown) RNA. The signal present in all lanes at ~1.8 kb in Fig. 1B presumably comes from cross-hybridization of the probe with 18 S RNA because it was present in wild-type mice lacking the pgk<sub>neo</sub> gene.

A truncated GlcNAc-TIII predicted to comprise 371 or 374 amino acids was produced from the ~6.5-kb transcripts. It was detected by Western analysis of mouse tissues, faintly in LEC10 CHO cells, and readily in CHO transfectants using a peptide affinity-purified anti-GlcNAc-TIII antibody made against N-terminal amino acids 120–136 (Fig. 1C). The disrupted Mgat3 gene that gives rise to the truncated product will henceforth be referred to as the mutant Mgat3<sup>T37</sup> allele. Truncated GlcNAc-TIII was also present in brain extracts from Mgat3<sup>T37/T37</sup> mice (data not shown). Wild-type GlcNAc-TIII migrated at a molecular mass of ~70 kDa, just above a nonspecific band detected in kidney and brain. GlcNAc-TIII assays of stable CHO transfectants overexpressing wild-type Mgat3 cDNA gave an activity of 17.9 nmol/mg/h compared with <0.01 nmol/mg/h for cells expressing truncated GlcNAc-TIII in the same vector. Both types of transfectant expressed equivalent activities of unrelated glycosyltransferases such as β4galactosyltransferase and GlcNAc-TI (data not shown). Previous studies detected no GlcNAc-TIII activity in kidney or brain extracts of Mgat3<sup>T37/T37</sup> mice (12).

Mgat3<sup>T37/T37</sup> and Mgat3<sup>3/3</sup> Mice Express a Similar Spectrum of Non-biased Complex N-Glycans—Mgat3<sup>T37/T37</sup> mice from predominantly C57BL/6 or 129<sup>N</sup>−<sup>−</sup> genetic backgrounds exhibit no overt phenotype (7), whereas Mgat3<sup>T37/T37</sup> mice on a mixed genetic background were observed, in a preliminary report, to possess several distinctive traits different from those of wild-type littermates (21). To investigate the phenotype associated with the Mgat3<sup>T37</sup> mutation and to determine whether the discrepancy with the Mgat3<sup>3/3</sup> mutation was due to genetic background, Mgat3<sup>3/3</sup><sup>−</sup> and Mgat3<sup>T37/T37</sup> mice were generated on a similar mixed background (predominantly 129<sup>N</sup>−<sup>−</sup> and CD1) (19) and bred through cousin matings to obtain wild-type, heterozygous, and homozygous progeny for phenotypic comparisons. The lack of complex N-glycans with a bisecting GlcNAc was confirmed in homozygotes by analyzing the N-glycans released by N-glycanase from kidney glycoproteins of Mgat3<sup>T37/T37</sup>, Mgat3<sup>3/3</sup>, and wild-type littermates by MALDI-TOF-MS (Fig. 2). Spectra from two wild-type and two homozygous mutants of each strain were distinguished only by the presence (in wild-type) and absence (in mutants) of glycans with masses predicted for bi-, tri-, or tetraantennary N-glycans with an unsubstituted HexNAc residue (Table I). Based on previous lectin blot analyses (7, 12, 19) and reports on similar analyses of kidney N-glycans from other mice (29, 30), this “extra” HexNAc residue present only in wild-type N-glycans represents the bisecting GlcNAc. The spectra show a remarkable reproducibility in the profile of N-glycans synthesized by the kidneys of unrelated mice from the two strains (Table I). It is important to note that the presence of C-terminally truncated GlcNAc-TIII in Mgat3<sup>T37/T37</sup> kidney did not significantly change the spectrum of complex N-glycans expressed by Mgat3<sup>T37/T37</sup> compared with Mgat3<sup>3/3</sup> mice.

Mgat3<sup>T37/T37</sup> Mice Exhibit Retarded Growth—At birth, Mgat3<sup>T37/T37</sup> homozygotes in the mixed genetic background appeared normal, but grew more slowly than littermates, especially after weaning (<p><sup>2</sup></p><p><sup>0</sup></p> ~ 0.0017 for males and <p><sup>2</sup></p><p><sup>0</sup></p> ~ 0.0001 for females by repeated measurement data analysis using the random effect model) (Fig. 3). Similar results were obtained with Mgat3<sup>T37/T37</sup> mice obtained after backcrossing the Mgat3<sup>T37</sup> mutation onto the 129<sup>N</sup>−<sup>−</sup> background for 11 generations (data not shown). Although smaller than littermates, Mgat3<sup>T37/T37</sup> mice are healthy and have lived up to 2 years. Histological examination of all major tissues from the progeny of mixed background or eleven generation 129<sup>N</sup>−<sup>−</sup> crosses did not reveal any obvious abnormalities in Mgat3<sup>T37/T37</sup> mice.3 Mice heterozygous for the Mgat3<sup>T37</sup> mutation in either genetic background did not exhibit retarded growth. Homozygous Mgat3<sup>3/3</sup> mice lacking GlcNAc-TIII also did not have a growth-retarded phenotype in the genetic backgrounds examined (Fig. 3) (7, 19).

Mgat3<sup>T37/T37</sup> Mice Have an Altered Leg Clasp Reflex—Several mouse mutants with an inactivating mutation in a neurally expressed molecule exhibit a characteristic leg clasp reflex when held suspended by the tail (for examples, see Refs. 31–33). Northern analysis has previously shown that the Mgat3 gene is highly expressed in adult brain (6, 7). In Fig. 4A, it is shown that the Mgat3 gene became transcriptionally active in brain at embryonic day 10.5 and that expression was maintained at a similar level through embryogenesis and birth. In situ hybridization of brain sections showed that in adult brain, the Mgat3 gene was highly expressed in neurons of the hippocampus (Fig. 4B). The Mgat3 gene was not detected by Northern analysis of RNA from WW6 embryonic stem cells or by lectin histochemistry, but E-PHA binding was induced in neurite extensions by in vitro differentiation of embryoid bodies.4

When Mgat3<sup>T37/T37</sup> mice in either a mixed or 129<sup>N</sup>−<sup>−</sup> background were suspended upside down by the tail, they retracted their hind and fore limbs toward the trunk, and in some cases, they rolled upward into a ball (Fig. 5). This reflex became obvious at 2 weeks and was most pronounced up to 6 months, and older Mgat3<sup>T37/T37</sup> mice took up the position more slowly. By contrast, wild-type and heterozygous littermates of both strains extended their fore and hind legs when held in the same inverted position and did not clasp their hind legs no matter how long they were suspended, and neither did mice homozygous for the Mgat3<sup>3/3</sup> mutation. Therefore, Mgat3<sup>3/3</sup> mice lacking GlcNAc-TIII altogether and heterozygous mice expressing wild-type and truncated GlcNAc-TIII did not exhibit the al-

3 J. D. Marth, personal communication.

4 M. Bhaumik and P. Stanley, unpublished data.
Terminated leg clasp reflex. However, all mice expressing only the truncated form of GlcNAc-TIII had a marked leg clasp reflex. Unfortunately, no cell type-specific alteration has been correlated with this response in any mutant model, and examination of overall brain morphology and brain sections from 
Mgat3<sup>T37/T37</sup> mice by light and electron microscopy also revealed no significant abnormalities.<sup>3</sup>

Mgat3<sup>T37/T37</sup> Mice Have an Altered Gait—Mgat3<sup>T37/T37</sup> mice and their littersmates were examined for gait by footprint analysis after inking their hind footpads. Whereas wild-type and heterozygous mice walked in a straight line, the tracks of both male and female Mgat3<sup>T37/T37</sup> mice formed a distinct S shape (Fig. 6). Only the Mgat3<sup>T37/T37</sup> progeny of heterozygous matings exhibited this trait, and it was maintained throughout 11 generations of backcrossing to 129<sup>sv</sup>. The distance between hind footpad prints was increased and more variable for Mgat3<sup>T37/T37</sup> mice (Fig. 6). Neither Mgat3<sup>T37/+</sup> heterozygotes nor Mgat3<sup>T37/-</sup> mice exhibited this phenotype in male or female mice from 6 weeks to 4.3 months of age. Thus, only mice expressing two copies of the Mgat3<sup>T37</sup> allele had an altered gait.

Inbreeding of Mgat3<sup>T37/T37</sup> Mice Reveals a Nurturing Defect—Inbreeding between male and female Mgat3<sup>T37/T37</sup> littermates of the F<sub>1</sub> generation in a mixed genetic background was performed to determine whether Mgat3<sup>T37/T37</sup> phenotypic traits were affected by modifying genes. The reduced growth rate (Fig. 3), altered leg clasp reflex (Fig. 5), and altered gait (Fig. 6) traits were inherited by all progeny. Interestingly, however, crosses between first generation Mgat3<sup>T37/T37</sup> mice gave a higher proportion of pups that died soon after birth compared with progeny from inbreeding of Mgat3<sup>T37/+</sup> or Mgat3<sup>T37/+</sup> littermates (Table II). This effect was even more dramatic when second generation F<sub>2</sub> progeny were intercrossed. Almost all the progeny of F<sub>2</sub> Mgat3<sup>T37/T37</sup> × Mgat3<sup>T37/T37</sup> crosses died within 48 h (Table II). There was only one litter of five in which four pups survived to maturity. When these pups were subsequently mated to each other, all 52 progeny from five litters of two crosses died within 48 h, showing that the phenotype bred true.

Third generation Mgat3<sup>T37/T37</sup> pups had no visible milk patch in the first 24 h after birth and were usually scattered around the cage. They died within 24–48 h of birth. The mother was often agitated and paced around the cage. Following backcrossing of the Mgat3<sup>T37</sup> mutation onto the 129<sup>sv</sup> background and inbreeding of eleventh generation Mgat3<sup>T37/T37</sup> mice, the nurturing defect was apparent even in the first generation. All F<sub>1</sub> 129<sup>sv</sup>T37/T37 × 129<sup>sv</sup>T37/T37 progeny died within 24–48 h without being suckled. The combined data suggest that segregation of one or more genes that ameliorate the nurturing defect in early generation, mixed background Mgat3<sup>T37/T37</sup> mice occurred upon inbreeding. In contrast, inbreeding of Mgat3<sup>T37/+</sup> or Mgat3<sup>T37/-</sup> mice produced mainly healthy pups that were suckled by their mother (Table II). Thus, no nurturing defect was observed following inbreeding of wild-type littersmates as expected or of mice completely lacking GlcNAc-TIII (Table II).

To further investigate the basis of the Mgat3<sup>T37/T37</sup> nurtur-
ing defect, reciprocal fostering experiments were performed (Table III). They showed that about half of the unfed
Mgat3T37⁄T37 pups transferred within 24 h of birth to a foster
mother were capable of nursing and had no suckling defect. Fostering is rarely 100% efficient, and in addition, mutant pups
that were not rescued by transfer to a foster female may have
been neglected by the birth mother for too long. Ten of the
rescued male and female Mgat3T37⁄T37 pups that grew to ma-
turity were mated, and all their progeny died within 24–48 h,
as would be predicted from Table I. When the reciprocal ex-
change was performed, Mgat3T37⁄T37 mothers that were ne-
eglecting their own pups were perfectly capable of nursing
Mgat3T37⁄T37 pups, showing that they would feed pups that had
already been suckled soon after birth (Table III). In a mixed
transfer experiment, an Mgat3T37⁄T37 mother did not feed her
five Mgat3T37⁄T37 pups, even when stimulated by the addition of
two wild-type pups. She fed and nurtured the latter exclu-
sively, allowing her own pups to die. The combined results
suggest a problem with recognition by Mgat3T37⁄T37 mothers of
Mgat3T37⁄T37 pups that is caused by the expression of trun-

**Fig. 2. MALDI-TOF-MS.** Kidney gly-
coproteins were extracted from two mice
of each strain lacking GlcNAc-TIII (Mgat3T37⁄T37 and Mgat3T37⁄T37) and their
wild-type Mgat3T37⁄T37 littermates. N-Gly-
cans released by N-glycanase treatment
were subjected to MALDI-TOF-MS in the
negative ion mode. Spectra from one
mouse in each group are shown. The pre-
dicted N-glycan structures represented by
these masses are given in Table I.
cated, inactive GlcNAc-TIII, but is not induced by a lack of the GlcNAc-TIII enzyme.

Truncated GlcNAc-TIII Does Not Act in a Dominant-negative Fashion—
The product of the Mgat3T37 gene is a 371- or 374-amino acid GlcNAc-TIII that is missing 165 or 163 C-terminal amino acids (Fig. 1C). The fact that heterozygous Mgat3T37+/H11001 mice have 50% GlcNAc-TIII activity (12) shows that truncated GlcNAc-TIII does not act in a dominant-negative fashion to inhibit wild-type GlcNAc-TIII, as was recently reported for a GlcNAc-TIII point mutant (34). A lack of a dominant-negative effect was confirmed by overexpressing a cDNA encoding the Mgat3T37 gene in LEC10 CHO cells, which express GlcNAc-TIII activity (3), due to a gain-of-function mutation that activates transcription of the endogenous Mgat3 gene.5 Overexpressed full-length and truncated GlcNAc-TIII were readily detected by Western analysis, although endogenous GlcNAc-TIII was not detected under the same conditions in whole cell

### Table 1

| Predicted neutral, complex N-glycan structure | Predicted mass ([M + Na]+) | Area under peak |
|---------------------------------------------|----------------------------|----------------|
| Biantennary                                 |                           |                |
| G_Gn_M_Gn_F                                | 1810.6                    | 2.9            |
| G_Gn_F/M_Gn_F                              | 1956.1                    | 3.8            |
| *G_Gn_M_Gn_F                               | 2012.9                    | 7.4            |
| *G_Gn_F/M_Gn_F                             | 2013.8                    | 7.2            |
| *G_Gn_F/Gn_M_Gn_F                          | 1997.8                    | 3.3            |
| *G_Gn_F/Gn_M_Gn_F                          | 2160                      | 7.1            |
| *G_Gn_F/Gn_M_Gn_F                          | 2306.1                    | 25.6           |
| *G_Gn_F/Gn_M_Gn_F                          |                         | 20.8           |
| Triantennary                                |                           |                |
| G_Gn_F(F)_M_Gn_F                           | 2468.2                    | 3.6            |
| G_Gn_F(F)_M_Gn_F                           | 2614.4                    | 8.4            |
| *G_Gn_F(F)_Gn_M_Gn_F                       | 2671.4                    | 3.2            |
| *G_Gn_F(F)_Gn_M_Gn_F                       | 2817.5                    | 8.3            |
| Tetraantennary                              |                           |                |
| G_Gn_F(F)_M_Gn_F                           | 2833.6                    | 2.9            |
| G_Gn_F(F)_M_Gn_F                           | 2979.7                    | 3.7            |
| G_Gn_F(F)_M_Gn_F                           | 3125.8                    | 5.7            |
| *G_Gn_F(F)_Gn_M_Gn_F                       | 3096.8                    | 1.8            |
| *G_Gn_F(F)_Gn_M_Gn_F                       | 3182.9                    | 2.1            |
| *G_Gn_F(F)_Gn_M_Gn_F                       | 3329.1                    | 2.8            |

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FIG. 3. Growth retardation of Mgat3T37+/T37 and Mgat3+/+ mice. Mice were weighed each week beginning at day 10 or later after birth. The progeny of between 5 and 11 litters of each type were followed. Error bars represent S.E.

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FIG. 4. Expression of the Mgat3 gene in brain. A, embryos were dissected from C57BL/6 mice at different days postcoitus. Total RNAs prepared from heads at each embryonic (E) stage, at birth, and from an adult were electrophoresed on a denaturing gel and probed with the Mgat3 gene coding region probe V (see Fig. 1). After stripping, the blot was reprobed to detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts. B, a section of adult mouse brain was probed with an antisense 35S-UTP-labeled Mgat3 gene probe to detect transcripts by in situ hybridization.

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TABLE I

Neutral N-glycans from kidneys of Mgat3T37+/T37 and Mgat3+/+ Mice

Shown is a summary of MALDI-TOF spectra in Fig. 2. N-Glycan structures are written with the nonreducing end at the left. G, Gal; Gn, GlcNAc; M, Man; F, Fuc; Gn, bisecting GlcNAc; *, structures with a bisecting GlcNAc. Predicted masses are given; observed masses can be seen in the spectra in Fig. 2. The area under the peak for each mass is given relative to all major species observed and is an average of data obtained from two mice of each respective genotype. Mice were 2–3 months of age.

| Predicted mass ([M + Na]+) | Area under peak |
|----------------------------|----------------|
| Mgat3T37+/T37              |                |
| Mgat3+/+                   |                |

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5 X. Yang and P. Stanley, unpublished data.
heterozygous interbreeding were used to obtain Mgat3<sup>T37/Δ</sup> compound heterozygous mice expressing a single dose of truncated GlcNAc-TIII and no wild-type GlcNAc-TIII. These mice exhibited a marked leg clasp reflex similar to Mgat3<sup>T37/T37</sup> mutants (Fig. 5). In a series of male and female Mgat3<sup>T37/Δ</sup> mice from 2 weeks to 6 months of age, the response occurred in all, but was generally somewhat slower in being achieved and was generally held for a shorter period than observed with Mgat3<sup>T37/T37</sup> mice. The other distinctive traits of Mgat3<sup>T37/T37</sup> mice, including altered gait, reduced weight, and altered nurturing, were not readily observed in Mgat3<sup>T37/Δ</sup> heterozygotes. It seems that a single copy of the Mgat3<sup>T37</sup> gene is not sufficient to cause a significant reduction in weight or the altered gait or nurturing defect typical of Mgat3<sup>T37/T37</sup> homozygotes. The combined data support the conclusion that truncated, inactive GlcNAc-TIII causes the Mgat3<sup>T37/T37</sup> phenotype, although effects due to the pgkneo gene in the Mgat3 locus cannot be excluded.

**DISCUSSION**

The targeting of genes in the mouse genome can generate mutant alleles that are unexpectedly informative and provide insight into mutations that may impact human health. Our strategy to target the Mgat3 gene was designed to generate a null allele due to nonsense-mediated decay of mutant transcripts, as occurred previously with the Mgat1 gene similarly disrupted in its single coding exon by the pgkneo gene cassette in reverse orientation (27, 28). However, the two nonsense codons introduced into the Mgat3 gene by disrupting the coding region with the pgkneo cassette did not lead to degradation of transcripts, and we show here that a truncated, stable GlcNAc-TIII protein is produced from the Mgat3<sup>T37</sup> allele. Many lines of evidence have shown that this truncated protein completely lacks GlcNAc-TIII activity and cannot make N-glycans with the bisecting GlcNAc (Fig. 2 and Table I) (12, 19). In addition, only Mgat3<sup>T37/T37</sup> mice gave a serum antibody response to N-glycans containing a bisecting GlcNAc, as might be expected if Mgat3<sup>T37</sup> mice are tolerant to this “self-antigen.”

We have shown in this work that mice homozygous for the Mgat3<sup>T37</sup> mutation that express truncated GlcNAc-TIII from two mutant alleles suffer neurological and other consequences. However, their phenotype does not arise from the lack of GlcNAc-TIII activity because mice in which the Mgat3 gene coding region is deleted share none of the same traits. The complete absence of GlcNAc-TIII was shown previously (7) and in this study to have no overt phenotypic consequences. However, a more subtle biological consequence was uncovered when Mgat3<sup>Δ/Δ</sup> mice were challenged with the carcinogen diethylnitosamine. Mgat3<sup>Δ/Δ</sup> mutants exhibit retarded tumor progression in liver compared with wild-type controls (19). This same phenotype occurs in Mgat3<sup>T37/T37</sup> mice (12) and therefore must result from a lack of GlcNAc-TIII activity. This is the only phenotype so far known to require active GlcNAc-TIII. However, GlcNAc-TIII is expressed in B cells, and it may well be that Mgat3<sup>Δ/Δ</sup> mice have immunological phenotypes yet to be discovered.

The data presented herein and previously (12) support the conclusion that the Mgat3<sup>T37</sup> mutation is the cause of the Mgat3<sup>T37/T37</sup> phenotype. First, backcrossing of the mutation to strain 129<sup>sv</sup> for 11 generations showed that the expected small size, altered leg clasp reflex, altered gait, and nurturing defect were present only in Mgat3<sup>T37/T37</sup> homozygotes as observed in the mixed genetic background. Moreover, the nurturing phenotype was manifest immediately in 129<sup>sv</sup> Mgat3<sup>T37/T37</sup> females, whereas homozygous mutants in the

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<sup>6</sup> J.-H. Lee, M. Scharff, and P. Stanley, unpublished data.
mixed CD1/129SvJ background required one intercrossing before this phenotype became obvious (Table II). This suggests the presence of modifying genes that modulate the phenotype, as was observed in GlcNAc-TII/−/− mice from different genetic backgrounds (30). Second, the lack of an overt phenotype in mice with the Mgat3+/−/null mutation also breeds true in C57BL/6 and 129SvJ (7) and mixed genetic (this study) backgrounds. Finally, compound heterozygotes expressing one copy of the Mgat3+/− allele in a null background exhibited the leg clasp reflex, although not as robustly as Mgat3+/−/− homozygotes (Fig. 5). This result supports the conclusion that the leg clasp phenotype is a direct consequence of expressing truncated, inactive GlcNAc-TIII and that twice as much gene product leads to a stronger leg clasp reflex as well as to the other traits of Mgat3+/−/− mice. In that case, the compound heterozygote should behave like the wild-type mice, as do both Mgat3+/+ and Mgat3+/− heterozygotes. The genes neighboring the human MGAT3 gene in a region of chromosome 22 that is well conserved in mouse chromosome 15 encode synaptogyrin-1, TAK1-binding protein-1, and cAMP-responsive element-binding protein-2, respectively (40). Mice homozygous for a null mutation in synaptogyrin-1 have no overt phenotype (41), whereas mice lacking cAMP-responsive element-binding protein-2 have microphthalmia (42), a phenotype that was not present in Mgat3+/−/− homozygotes. TAK1-binding protein-1 null mutant mice have not been described, but would be expected to be defective in transforming growth factor-β signaling and probably to be affected in heart function (43). A gain-of-function phenotype could arise if the phosphotransferase activity encoded by the neo gene were expressed. For example, expression of the neomycin phosphotransferase from the Men1 locus is lethal in heterozygotes (44), whereas sense transcripts from the neo gene were not apparent in RNA of brain or kidney tissue from Mgat3+/−/− mice (Fig. 1B), making this possibility unlikely. In addition, extensive Southern analyses using three probes to regions 5′ and 3′ of the targeting vector gave no evidence for genomic DNA rearrangements adjacent to the targeted locus (12). Although it is theoretically possible that the pgkneo cassette in the Mgat3 locus activates expression of a silent neighboring gene, it seems most likely that truncated, inactive Glc-
Mgat3 gene and generation from either the mixed CD1/129 or eleventh generation-backcrossed 129 backgrounds were crossed, and their progeny were monitored as soon as possible after birth for milk in the stomach and for care and feeding by the mother. The majority that died within 24–48 h were the progeny of Mgat3T37T37 × Mgat3T37T37 crosses (boldface). About half of the pups survived compared with <4% in Table II. Similarly, when pups from wild-type or heterozygous crosses were fostered by Mgat3T37T37 mothers, the majority survived, showing that mothers expressing only truncated GlcNAc-TIII can feed pups that are not Mgat3T37T37 homozygotes.

### Table II

**Nurturing defect of Mgat3T37T37 mice**

| Cross                  | No. litters (no. of pairs) | No. progeny | No. that died in 24–48 h |
|------------------------|-----------------------------|-------------|--------------------------|
| Wild-type Mgat3T37T37  | 7 (2)                       | 61          | 0 (<1%)                  |
| F1 CDI/129 (+/+ × +/+)+ | 10 (2)                      | 92          | 4 (4%)                   |
| Heterozygous Mgat3T37T37 | 11 (2)                     | 112         | 0 (<1%)                  |
| F1 CDI/129 (T37/T37 × T37+/+) | 7 (3)              | 83          | 10 (12%)                 |
| Homozygous Mgat3T37T37 | 8 (2)                       | 68          | 11 (16%)                 |
| F2 CDI/129 (T37/T37 × T37/T37) | 15 (6)           | 93          | 89 (96%)                 |
| F3 CDI/129 (T37/T37 × T37/T37) | 5 (2)              | 52          | 52 (100%)                |
| Homozygous Mgat3T37T37 | 10 (6)                      | 62          | 3 (5%)                   |
| F1 CDI/129 (ΔΔ × ΔΔ)   | 12 (8)                      | 99          | 14 (14%)                 |
| F2 CDI/129 (ΔΔ × ΔΔ)   | 11 (8)                      | 111         | 16 (14%)                 |
| Heterozygous Mgat3T37T37 | 15 (3)                    | 74          | 16 (21%)                 |
| F1, 129T37+/− (T37/T37) | 4 (3)                      | 10          | 10 (100%)                |

### Table III

**Rescue of Mgat3T37T37 pups by fostering**

Mice of the Mgat3 genotype indicated were crossed, and pups from Mgat3T37T37 females that would die within 24–48 h were transferred to foster females that had given birth in the last 3 days and were either of wild-type or heterozygous Mgat3T37T37 genotype. About half of the pups survived compared with <4% in Table II. Similarly, when pups from wild-type or heterozygous crosses were fostered by Mgat3T37T37 mothers, the majority survived, showing that mothers expressing only truncated GlcNAc-TIII can feed pups that are not Mgat3T37T37 homozygotes.

| Mgat3 cross | No. pairs | No. pups transferred | Fostered Mgat3 genotype | No. that survived >48 h |
|-------------|-----------|----------------------|--------------------------|-------------------------|
| Homozygous mutant (T37/T37 × T37/T37, F1 CDI/129) | 6 | 57 | Wild-type or heterozygous (+/+, T37+/+) | 25 (44%) |
| Wild-type or heterozygous (+/ × +/+, T37+/ × T37+/+) | 4 | 22 | Homozygous mutant (T37/T37, F1 CDI/129) | 16 (72%) |

**Fig. 7.** The Mgat3T37 gene product does not behave in a dominant-negative fashion. CHO cells, which lack GlcNAc-TIII, or LEC10 cells, which express GlcNAc-TIII, were transfected with a cDNA encoding the Mgat3 gene coding region or the Mgat3 targeting vector. Glycoproteins from stable transfectants were extracted in detergent and electrophoresed, and those expressing the bisecting GlcNAc were detected by blotting with biotinylated E-PHA.

NAc-TIII causes the phenotype arising from the Mgat3T37T37 mutation.

The overall phenotype of Mgat3T37T37 mice appears to be unique. The altered gait in mice lacking another glycosyltransferase, GlcNAc-TII, appears to be due to muscular problems, and the nurturing defect of homozygous mutant females was toward heterozygous pups (30). The specific inability of Mgat3T37T37 mothers to recognize mutant progeny, although they suckled wild-type or heterozygous pups, also differs from the nurturing defective phenotypes of cyclin D1−/− (32), fosB−/− (46), and fyn−/− (47) mice. The fostering experiments showed that the milk of Mgat3T37T37 mothers is not toxic and that they can nurse Mgat3T37T37 progeny effectively (Table III) and so have no problem with milk production or milk ejection like mice lacking oxytocin (48). Mgat3T37T37 pups that had been neglected for many hours could be completely rescued (albeit not at 100% efficiency) and therefore had no difficulty in feeding or digesting milk. Therefore, Mgat3T37T37 pups differ from those with a sucking defect (49–51). Most similar to Mgat3T37T37 are fosB−/− mothers. However, fosB−/− mothers do not recognize any pups regardless of genotype, whereas Mgat3T37T37 mothers specifically did not recognize only their own pups.

The biochemical basis of the complex phenotype in mice carrying the Mgat3T37 mutation is not due to a lack of GlcNAc-TIII activity, as clearly shown by the absence of a similar phenotype in Mgat3T37/− mice. Because the spectrum of complex N-glycans appears to be essentially identical in kidney glycoproteins from both strains of mice (Fig. 2), there appears to be no significant effect of truncated GlcNAc-TIII on the activities of other glycosyltransferases that synthesize complex N-glycans in kidney. However, overexpression of truncated GlcNAc-TIII in CHO transfectants led to reduced ricin binding, suggesting that altered Golgi functioning could play a part in causing the Mgat3T37T37 phenotype. Although the mechanism...
of action of truncated GlcNAc-TIII will be difficult to determine, the phenotype of Mgtat-/- mice suggests that humans with mutations in the MGA3 gene that lead to truncated, inactive GlcNAc-TIII might present with neurological or behavioral problems similar to, but milder than, those observed in patients with certain congenital disorders of glycosylation (52). To date, most congenital disorders of glycosylation mutations are missense and lead to relatively severe consequences in compound heterozygous carriers. However, more subtle pathologies may arise from mutations that have a milder effect on enzyme activity or from mutations in glycosyltransferases that are not required for development, such as GlcNAc-TIII.

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Truncated GlcNAc-TIII Induces Neurological Traits in Mice

26309
Truncated, Inactive N-Acetylglucosaminyltransferase III (GlcNAc-TIII) Induces Neurological and Other Traits Absent in Mice That Lack GlcNAc-TIII

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