Mutation in the key enzyme of sialic acid biosynthesis causes severe glomerular proteinuria and is rescued by N-acetylmannosamine

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Mutations in the key enzyme of sialic acid biosynthesis, uridine diphospho-N-acetylgalactosamine 2-epimerase/N-acetylmannosaminosamine (ManNAc) kinase (GNE/MNK), result in hereditary inclusion body myopathy (HIBM), an adult-onset, progressive neuromuscular disorder. We created knockin mice harboring the M712T Gne/Mnk mutation. Homozygous mutant (GneM712T/M712T) mice did not survive beyond P3. At P2, significantly decreased Gne-epimerase activity was observed in GneM712T/M712T muscle, but no myopathic features were apparent. Rather, homozygous mutant mice had glomerular hematuria, proteinuria, and podocytopathy. Renal findings included segmental splitting of the glomerular basement membrane, effacement of podocyte foot processes, and reduced sialylation of the major podocyte sialoglycoprotein, podocalyxin. ManNAc administration yielded survival beyond P3 in 43% of the GneM712T/M712T pups. Survivors exhibited improved renal histology, increased sialylation of podocalyxin, and increased Gne/Mnk protein expression and Gne-epimerase activities. These findings establish this GneM712T/M712T knockin mouse as what we believe to be the first genetic model of podocyte injury and segmental glomerular basement membrane splitting due to hyposialylation. The results also support evaluation of ManNAc as a treatment not only for HIBM but also for renal disorders involving proteinuria and hematuria due to podocytopathy and/or segmental splitting of the glomerular basement membrane.

Introduction

The GNE gene encodes the bifunctional enzyme uridine diphospho-N-acetylgalactosamine 2-epimerase/N-acetylmannosaminosamine (ManNAc) kinase (GNE/MNK), which is ubiquitously expressed and catalyzes the first 2 committed, rate-limiting steps in the biosynthesis of 5-acetamido-2-acetate (Neu5Ac, also known as sialic acid) (Figure 1) (1–3). The epimerase enzyme is feedback inhibited by the downstream product, cytidine monophosphate Neu5Ac. Neu5Ac is the most abundant mammalian sialic acid and is found as the terminal sugar on glycoconjugates, where it functions in cellular interactions and signaling (2–4).

Mutations in the GNE gene result in the autosomal recessive neuromuscular disorder hereditary inclusion body myopathy (HIBM) (OMIM 600737). HIBM is characterized by adult-onset, slowly progressive muscle weakness and atrophy (5, 6). Serum creatine kinase levels are normal to slightly elevated and electrolytes show either a myopathic or a neuropathic pattern. Histologically, muscle fibers degenerate and develop filamentous nuclear inclusions and cytoplasmic rimmed vacuoles (5, 6). No therapy currently exists for HIBM. A GNE founder mutation (M712T) was described in Persian-Jewish HIBM families (7), and numerous other GNE mutations exist worldwide (8–10). HIBM-associated GNE mutations result in reduced activity of both GNE and MNK (11, 12), which is thought to be responsible for reduced sialic acid production.

The pathologic mechanism of muscle fiber degeneration in HIBM remains unknown (12–18). However, evidence suggests that decreased availability of sialic acid in muscle causes hyposialylation of muscle glycoproteins, whether involving glycans in general (12, 13), O-linked glycans in particular (14), polysialic acid on neural cell adhesion molecules (PSA-NCAM) (15, 16), or specific O-mannosylated glycosyl residues on α-dystroglycan (18).

One hypothesis is that provision of free sialic acid could increase glycoprotein sialylation in HIBM muscle and ameliorate the myopathic symptoms. Sialic acid might be administered in its free form, bound as glycoconjugates, or as its precursor ManNAc, which is uncharged and crosses membranes readily. ManNAc is also situated in the sialic acid biosynthesis pathway after the regulated, rate-limiting GNE step (Figure 1), so its metabolism is not subject to feedback inhibition. Residual MNK activity in HIBM patients, or ancillary kinases such as GlcNAc kinase (19), might convert ManNAc into ManNAc-6-phosphate for subsequent synthesis of sialic acid. In fact, hyposialylated, Gne-deficient mouse
Embryonic stem cells became resialylated after their growth medium was supplemented with ManNAc (16). Furthermore, incubation of cultured cells with “unnatural” ManNAc derivatives, i.e., N-levulinylmannosamine (ManLev) or N-azidoacetylmannosamine (ManNAz), resulted in incorporation of the downstream sialic acid analogs (SiaLev or SiaNAz) into cell surface glycoconjugates (20). Importantly, oral supplementation of 14C-ManNAc to healthy rats showed incorporation of the 14C label into several tissues, including kidney and muscle (21).

In order to test sialic acid replacement therapies in an animal model of HIBM, we created a gene-targeted knockin mouse homozygous for the M712T Gne mutation (GneM712T/M712T). Here, we extensively characterize this mouse, which died within 72 hours after birth, lacking a muscle phenotype. Homozygous mice had severe glomerular hematuria and podocyteopathy, including effacement of the podocyte foot processes and segmental splitting of the glomerular basement membrane (GBM), likely due to hyposialylation of specific membrane glycoproteins. Unexpectedly, these knockin mice provide a novel animal model of podocyteopathy and/or segmental splitting of the GBM, demonstrating the significance of sialic acid synthesis in kidney development and function. Administration of ManNAc to pregnant mice had a remarkably salutary effect on the survival and renal disease of homozygous pups and was associated with increased enzymatic activity of GNE, increased sialylation of kidney podocalyxin, and improved morphology of the podocyte foot processes and GBM integrity. This study supports consideration of ManNAc not only as a treatment for HIBM but also for treatment of kidney disorders characterized by segmental splitting of the GBM and/or podocyteopathy due to disturbed polyanions on podocyte membranes.

Figure 1
Intracellular sialic acid metabolism. Cytosolic glucose is converted in several steps into UDP-GlcNAc, which serves as substrate for the bifunctional, rate-limiting, committed enzyme of sialic acid biosynthesis, GNE/MNK. The GNE catalytic activity (EC 5.1.3.14) epimerizes UDP-GlcNAc to ManNAc, followed by the phosphorylation of ManNAc to ManNAc-6-phosphate (ManNAc-6-P) by the MNK kinase catalytic domain (EC 2.7.1.60). ManNAc-6-P is then further converted into Neu5Ac (sialic acid), which is activated into cytidine monophosphate–sialic acid (CMP–sialic acid) in the nucleus. CMP–sialic acid can subsequently be utilized in the Golgi complex as a substrate for the biosynthesis of sialyl-oligosaccharides by sialyltransferases. Cytosolic CMP–sialic acid displays strong feedback inhibition (dotted line) of GNE enzymatic activity by binding to its allosteric site (1), thereby contributing to the tight regulation of intracellular sialic acid biosynthesis. Even more complexity is added to this pathway by the presence of ancillary kinases, such as GlcNAc kinase (NAGK; EC 2.7.1.59) with high intrinsic MNK activity (19), which can also convert ManNAc to ManNAc-6-P. CTP, cytidine triphosphate; PEP, phosphoenolpyruvate; OGS, oligosaccharides.

Results
Generation of GneM712T/M712T knockin mice. A murine targeting vector for homologous recombination in C57BL/6j embryonic stem cells was constructed to include the M712T Gne mutation (Figure 2A). The neomycin phosphotransferase and thymidine kinase genes were introduced into the vector as positive and negative selection markers, respectively (Figure 2A). Additional LacZ (flanking exon 12 and neo) and flippase recombinase target sites (flanking neo) were inserted, allowing for potential future conditional transgenic models (22). The entire vector was sequence verified. Genotyping of the mice was performed by PCR amplification and digestion with the restriction endonuclease NlaIII (Figure 2B). Tissues of homozygous mutant GneM712T/M712T and wild-type Gne++ mice showed comparable Gne RNA transcript levels by real-time quantitative PCR. Furthermore, NlaIII digestion of amplified cDNA demonstrated homogous insertion of the M712T mutation in RNA of GneM712T/M712T mice (Figure 2C).

Early postnatal lethality. Initial matings of heterozygous mice (GneM712T/+ ) yielded 101 offspring from which only 1 GneM712T/M712T animal survived beyond P21. The remaining GneM712T/M712T offspring died at P1–P3 (Figure 2D). However, subsequent genotyping of 35 embryos at days E17–E19 showed 26% Gne++, 43% GneM712T+, and 31% GneM712T/M712T, reflecting a Mendelian distribution, statistically confirmed by goodness-of-fit testing (χ² = 0.94, P = 0.62) (Figure 2D). At E17–E19, the embryos displayed normal exteriors, normal head and body sizes, and pink skin, which indicated good circulatory and respiratory function. By P2, however, GneM712T/M712T mice were smaller than control littermates (Figure 2E), weighing 70%–100% of Gne++ mice except 1 died by P3 and had increased urinary protein. In contrast, GneM712T/+ mice appeared unaffected.

Histological analyses. Tissues of GneM712T/M712T mice and their littermates were examined between age P2 and P3. No abnormalities were identified in skeletal muscle (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI30954DS1), heart, or liver (data not shown). Moreover, immunohistochemical staining with antibodies against laminin (Supplemental Figure 1B) and dystrophin (Supplemental Figure 1C) failed to show differences between muscle sections of GneM712T/M712T mice and their wild-type littermates.
Ultrastructural analyses of the glomeruli at age P2 revealed that, compared with the slender, well-shaped glomerular foot processes of wild-type mice (Figure 4A), the podocyte foot process membranes of \textit{Gne}^{M712T/M712T} mice were flattened and largely fused, with only a few wide foot processes remaining (Figure 4B). Filtration slits were reduced in number and showed formation of tight junction–like structures (Figure 4B). In addition, the GBM showed segmental splitting of the lamina densa (Figure 4B). The size and shape of endothelial cells lining the GBM, as well as glomerular mesangial cells, appeared ultrastructurally intact.

To support these ultrastructural findings, additional analyses were performed using markers for specific glomerular compartments. The podocyte-specific markers podocin and podocalyxin (23, 24) were tested by immunoblotting kidney extracts of all genotypes. While podocin showed no difference in expression across all genotypes (at age P1) (data not shown), podocalyxin, the major sialoglycoprotein of the podocyte apical membrane (23, 24), demonstrated dramatically decreased sialylation (Figure 5E, upper gel). Expression levels of GBM markers laminin-1 (Figure 5C) and laminin B1 (Supplemental Figure 2A) were unchanged in \textit{Gne}^{M712T/M712T} kidneys, as were RNA levels of collagen type IV (Col4A3), an integral GBM component (Supplemental Figure 2B) (25–27). Immunoblotting with desmin and vascular SMA, antibodies to mesangial cell markers (28), showed similar expression levels across all genotypes (Supplemental Figure 2A). In addition, real-time quantitative PCR analysis of the endothelial cell marker CD31/Pecam-1 (29) revealed no difference in RNA expression levels across genotypes at P1 (Supplemental Figure 2B).

Serum metabolite studies on the only \textit{Gne}^{M712T/M712T} mouse that survived past weaning demonstrated elevated blood urea nitrogen levels (39 ± 10 mg/dl in \textit{Gne}^{M712T/M712T} versus 21 ± 2 mg/dl in \textit{Gne}^{+/+} mice) and increased urinary protein (>500 mg/dl protein), which indicated renal disease. All other serum metabolites tested, including creatinine and creatine kinase, were within the normal ranges (Supplemental Figure 3C). This male \textit{Gne}^{M712T/M712T} survivor was euthanized at age 8.5 months. Histologic analysis revealed no structural abnormalities in the forelimb or hindlimb. However, severe bilateral hydropnephrosis and changes consistent with glomerulopathy were found in the kidneys (Supplemental Figure 3, A and B).

\textit{Rescue by ManNAc feeding.} ManNAc, added to the drinking water at a concentration of 1 mg/ml (~0.2 g/kg/day) during matings of

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At age P2, kidneys of \textit{Gne}^{M712T/M712T} mice showed petechial hemorrhages by gross examination, but were normal in size and shape compared with kidneys of \textit{Gne}^{+/+} and \textit{Gne}^{M712T/+} littermates (Figure 3A). Histological analyses revealed cystic tubular dilatation (Figure 3B). High-magnification views of \textit{Gne}^{M712T/M712T} kidneys showed red blood cell infiltrates in the proximal and distal convoluted tubules and the collecting ducts (Figure 3C). The glomeruli of \textit{Gne}^{M712T/M712T} mice contained red blood cell infiltrates in Bowman space (Figure 3D). Of 100 glomeruli scored in each group, 64% ± 6% were affected in \textit{Gne}^{M712T/M712T} mice (n = 4) compared with 2% ± 1% in \textit{Gne}^{M712T/+} mice (n = 3) and 4% ± 4.5% in \textit{Gne}^{+/+} mice (n = 4). Immunohistochemical analysis demonstrated localization of Gne/Mnk antibodies to kidney glomeruli (Figure 3E). Examination of \textit{Gne}^{M712T/M712T} kidneys at E18 showed no histological differences compared with wild-type or heterozygous littermates (data not shown).
mice showed hemorrhages but were normal in size and shape compared with kidneys of wild-type (Gne\textsuperscript{+/+}) and heterozygous (Gne\textsuperscript{M712T/+}) littermates. Representative H&E-stained sections of renal cortex (c) and medulla (m) showing tubular dilatations in Gne\textsuperscript{M712T/M712T} mice (arrows). Scale bars: 1,000 \( \mu \text{m} \). (C) High magnification of collecting ducts, renal tubules, and urinary space, filled with rbc in Gne\textsuperscript{M712T/M712T} mice. Scale bars: 100 \( \mu \text{m} \). (D) High magnification of glomeruli (g) with rbc infiltrated into the Bowman space in Gne\textsuperscript{M712T/M712T} mice. Scale bars: 100 \( \mu \text{m} \). (E) Representative normal glomerulus (DICII filter, left panel) demonstrates abundance of Gne/Mnk protein inside the glomerular space upon immunolabeling with Gne/Mnk antibodies (FITC filter, right panel). Scale bars: 50 \( \mu \text{m} \).

Gne\textsuperscript{M712T/+} mice, yielded no surviving homozygous Gne\textsuperscript{M712T/M712T} mice beyond age P3 from among 51 offspring (Figure 6A). However, at 5 mg ManNAC/ml (~1.0 g/kg/d), among 102 total newborns, 12 Gne\textsuperscript{M712T/M712T} pups survived beyond P3, a significantly greater number compared with the 1 survivor in the untreated group (2-tailed Fisher’s exact test, \( P = 0.01 \)) (Figure 6A). ManNAC at the administered dose (~1.0 g/kg/d) was well tolerated by the mice, and no side effects were attributed to the treatment throughout the study. Surviving Gne\textsuperscript{M712T/M712T} mice remained smaller than their wild-type littermates, weighing 70%–100%. At age P6, ManNAC treated Gne\textsuperscript{M712T/M712T} mice exhibited no abnormalities in liver, heart, or skeletal muscle tissues (data not shown). Their kidneys demonstrated significant histological improvement (Figure 6, B–D) compared with Gne\textsuperscript{M712T/M712T} mice examined at age P2 (Figure 3, B–D). Upon ManNAC treatment, there were fewer cystic tubular dilatations in the cortex and medulla (Figure 6B) and reduced red blood cell infiltrates in the tubules and the Bowman space (Figure 6, C and D). Ultrastructural analysis at age P19 showed less fusion and flattening of the podocyte foot processes including a greater number of open slit diaphragms and an improvement in the “finger shaping” of the foot processes (Figure 4, C and D). The overall integrity of the GBM was also significantly improved, although occasional segmental splitting of the lamina densa was still apparent (Figure 4, C and D).

The nursing females continued to receive ManNAC treatment until the pups were weaned (P21). Of the 12 Gne\textsuperscript{M712T/M712T} mice that survived past P3, 9 died between P6 and P12. One Gne\textsuperscript{M712T/M712T} mouse was sacrificed at age P19 for ultrastructural analysis. Two Gne\textsuperscript{M712T/M712T} mice survived past P21, when ManNAC supplementation was ceased. These 2 mice continued to grow without receiving additional ManNAC but remained smaller than their littermates (Figure 6E). At 3.5 months of age, 1 Gne\textsuperscript{M712T/M712T} survivor was sacrificed because of hydrocephalus and malocclusion. Similar events occurred in some untreated mice at different ages and were found not to be related to treatment or the disease. Skeletal muscle histology of this mouse revealed no structural or inflammatory abnormalities, but the kidneys showed mild red blood cell infiltrations in the urinary space and the tubules. The 1 surviving Gne\textsuperscript{M712T/M712T} mouse is currently 6 months old and has no obvious myopathic features.

Biochemical analyses following ManNAC feeding. Gne enzymatic activity was measured in muscle and kidney at age P2. Skeletal muscle of Gne\textsuperscript{M712T/M712T} mice showed 19.4% ± 7.5% of the Gne activity of the Gne\textsuperscript{+/+} mice (\( n = 4, P = 0.02 \)) (Figure 6F). Similar decreases in Gne activities were measured in Gne\textsuperscript{M712T/M712T} kidney extracts (10% of mean Gne\textsuperscript{+/+} kidney epimerase activities). Upon ManNAC treatment, Gne activities in Gne\textsuperscript{+/+} muscle (\( n = 3 \)) increased to 114% ± 19.7% (\( P = 0.2 \)), while Gne\textsuperscript{M712T/M712T} muscle activity (\( n = 7 \)) increased from 19.4% ± 7.5% to 31% ± 8.4% of untreated Gne\textsuperscript{+/+} mean values of muscle Gne activity (\( P = 0.05 \)) (Figure 6F).

Immunoblots of muscle and kidney extracts labeled with anti-Gne/Mnk antibodies demonstrated 38.5% ± 27%, (\( n = 4 \)) Gne/Mnk protein in Gne\textsuperscript{M712T/M712T} muscle and 32.1% ± 7% (\( n = 3 \)) in Gne\textsuperscript{M712T/M712T} kidney tissues when compared with Gne\textsuperscript{+/+} littermates. This improved upon ManNAC treatment of Gne\textsuperscript{M712T/M712T} mice to 68.8% ± 20%, (\( n = 4 \)) in muscle and to 62.2% ± 9.7%, (\( n = 4 \)) in kidney tissues (\( P = 0.12 \) and \( P = 0.006 \) for muscle and kidney values respectively, relative to \( \beta \)-actin) (Figure 5, A and B). Immunoblots stained with antibodies against laminin-1, an integral component of the GBM (25–27), showed similar patterns across genotypes before and after treatment (Figure 5C).
We then studied the degree of sialylation of 2 heavily sialylated marker proteins, PSA-NCAM and podocalyxin. PSA-NCAM is a major sialoprotein expressed in neonatal brains (30), where its expression is regulated by the intracellular concentration of sialic acid (31). Our results showed that the expression of PSA-NCAM varied within and between genotypes, yet Gne<sup>M712T/M712T</sup> brains at P2 showed up to 80% decreased PSA-NCAM expression compared with that in Gne<sup>+/+</sup> mice (Figure 5D, upper gel). A 2%–28% increase compared with Gne<sup>M712T/M712T</sup> untreated mice following ManNAc treatment was observed (n = 14 before treatment and n = 10 after treatment, P = 0.08) (Figure 5D, lower gel). The expression of PSA-NCAM in normal muscle and kidney at P2 was low, and no change upon treatment in these tissues could be confirmed (data not shown).

In addition, the significantly decreased sialylation status of podocalyxin in untreated Gne<sup>M712T/M712T</sup> kidneys (Figure 5E, lower gel) markedly improved upon ManNAc treatment (Figure 5E, upper gel).

**Discussion**

Research into HIBM lacks an understanding of basic pathogenic mechanisms, an animal model, and an effective therapy.

We took a first step toward addressing these issues by creating a Gne gene–targeted knockin mouse mimicking the M712T mutation of Persian-Jewish HIBM patients. With the exception of I...
male, homozygous mutated (Gne<sup>M712T/M712T</sup>) mice did not survive past age P3 (Figure 2D) and showed no muscle pathology at age P2 (Supplemental Figure 1). The lack of early myopathic features recapitulates the human HIBM phenotype. In both cases, the muscle pathology may occur late or be attenuated because a modicum of sialic acid is provided through the actions of residual Gne/Mnk enzymatic activities (11, 12) (Figure 5, A and B, and Figure 6F) supplemented by ancillary enzymes such as GlcNAc kinase (NAGK) (19) and generic sugar kinases. Eventually, the Gne<sup>M712T/M712T</sup> mice may develop myopathic features, if they can be maintained well past weaning. Even very young mutant mice exhibited hyposialylation of PSA-NCAM (i.e., in brain) (Figure 5D), previously reported in skeletal muscle of HIBM patients (15) and in embryonic stem cells of Gne knockout mice (16).

Remarkably, the Gne<sup>M712T/M712T</sup> mice clearly differed from HIBM patients in their manifestation of an apparently lethal renal phenotype. The mice exhibited early glomerular disease, with red blood cell infiltrates (Figure 3, A–D), effacement of podocyte foot processes, and segmental splitting of the lamina densa of the GBM (Figure 4B), resulting in death within 72 hours after birth. In our group of human HIBM patients, we have no indication of renal abnormalities. Laboratory findings were all within normal ranges, including levels of blood urea nitrogen, creatinine, and urine 24-hour protein and glucose (32). We know of no reports of abnormal urinary laboratory values in patients with HIBM.

We showed that Gne/Mnk localized to mouse kidney glomeruli (Figure 3E), a site with high sialic acid concentrations (33, 34). Humans and mice differ in the relative importance of sialic acid to the kidney, and they certainly differ in the type of sialic acid present. Most mammalian species utilize the sialic acid Neu5Gc (N-glycolylneuraminic acid), but humans have lost the ability to synthesize Neu5Gc (35) and rely on Neu5Ac as their main sialic acid. Protein glycosylation patterns also vary; the glomerular sialoglycoproteins also diversely among species in the contingent of O- and N-linked glycosylation sites in its extracellular aminoterminal region (36). Future studies, such as employing the Cre-Lox system to create conditional Gne knockouts (22), might shed light on these issues.

The morphologic abnormalities of podocytes and GBM in the Gne<sup>M712T/M712T</sup> mouse represent surprising and important findings. Podocytes are glomerular epithelial cells that provide the architecture of the glomerular filtration apparatus, including interdigitating foot processes, slit diaphragms, and the intercellular urinary spaces (23, 37, 38). The negatively charged sialic acid residues on glycoproteins such as α-dystroglycan, α3β1-integrin, and podocalyxin are essential for the function of podocyte foot processes (24, 33, 34) and they act as antiadhesion molecules, assisting in maintaining an open urinary space, filtration slits, and Bowman space. Disruption of podocalyxin or neutralization of its negative charge resulted in dissociation of podocalyxin from the actin cytoskeleton and led to foot process effacement (39). Indeed, we demonstrated the presence of hyposialylated podocalyxin in Gne<sup>M712T/M712T</sup> mouse kidney extracts (Figure 5E) along with effacement of foot processes and malformed filtration slits (Figure 4B). Some forms of glomerular disease (such as minimal change nephrosis; ref. 40) exhibit hyposialylation of foot process glycoproteins, with subsequent deformation of podocyte membranes and proteinuria (33, 34, 37, 38, 41–43).
Effacement of podocyte foot processes has also been reported for mice lacking podocalyxin (44), nephrin (45), or the giant protocadherin mFAT1 (46), all leading to proteinuria and death within 72 hours of birth. Other laboratory models have been created to study the phenomenon of effacement of foot processes due to general hyposialylation; these include sialidase inoculation in mice, resulting in hyposialylation of podocalyxin (41), supplementation studies with purine aminonucleoside (47), and perfusion of a polycation protamine sulfate (48). To our knowledge, our Gne$^{M712T}$ knockin mouse is the first genetic model demonstrating podocyte flattening and fusion events due to hyposialylation, resulting in proteinuria.

Interestingly, our mutant mice also displayed hematuria, a feature that can be attributed to structural GBM defects (Figure 4B), which allow red blood cells to gain access to Bowman space, as occurs in thin GBM disease (49). The segmental GBM splitting in our knockin mice might have resulted from defects in expression levels or charge of specific proteins. In contrast to the findings on podocalyxin (Figure 5E), testing of other compartment-specific markers, some of which are affected in renal GBM disorders, did not reveal any defects in expression levels in Gne$^{M712T/M712T}$ kidneys. These included GBM markers laminin-1, laminin B1, and Col4A3 (dysregulated in Alport disease) (25–27, 50) (Figure SC and Supplemental Figure 2) and the podocyte marker podocin (data not shown).
shown) (38). Advanced investigations are required to further elucidate the glomerular phenotype in our GneM712T/M712T mice. Nevertheless, the severe renal findings of podocytopathy and GBM splitting are likely responsible for the proteinuria, hematuria, and early death in our mice.

Although the exact cause of neonatal lethality in the GneM712T/M712T mice remains elusive, their rescue by ManNAC suggests that sialic acid deficiency was involved. Outcome parameters included survival (Figure 6A), improved renal histology (Figure 6, B–D), including a markedly improved integrity of the GBM (Figure 4, C and D), less flattened and fused podocyte foot processes (Figure 4, C and D), increased sialylation of renal podocalyxin (Figure 5E), and increased sialylation of brain PSA-NCAM (Figure 5D), all likely related to augmented production of sialic acid. Since ManNAC supplementation increased skeletal muscle Gne epimerase activity (Figure 6F) and Gne/Mnk protein expression (Figure 5, A and B), we suggest that ManNAC improved catalytic activity by stabilizing the normal and the mutant enzymes. Similar stabilization effects on other proteins have been demonstrated using natural or artificial ligands or chaperones (51, 52). The effects of ManNAC on Gne mutations other than M712T deserve further investigation.

GneM712T/M712T mice can be used to determine the optimal sialic acid repletion therapy for HIBM. Our knockin mice may eventually develop myopathic features similar to the adult-onset myopathy of HIBM patients; treatment could then be geared toward preventing the specific muscle disease. We recently observed a transient but significant improvement in the muscle strength of HIBM patients unexpectedly provide a unique opportunity to study basic mechanisms and targeted therapies of podocyte injury and/or GBM splitting, for which appropriate model systems are sparse (23, 38, 56, 57). This is especially true for disorders involving proteinuria and/or hematuria for which the etiology is unknown and may be related to changes in charge (sialylation) of GBM components. Increased survival of the M712T Gne knockin mice could serve as an absolute outcome parameter for potential therapeutic interventions, and resolution of renal disease could provide a graded measure of response.

Methods

GneM712T/M712T mice. GneM712T/M712T knockin mice were generated by targeting the M712T (ATG to ACG) mutation of exon 12 of the murine Gne gene (Gne, Unl, GenBank NM_015828) (Figure 2A). The mutant mice were maintained in the C57BL/6J background. Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care International–accredited specific pathogen–free facility in accordance with the Guide for the care and use of laboratory animals (NIH publication no. 85-23. Revised 1985). Cages were ventilated in a temperature- and light-controlled environment (22°C, 30%-70% humidity, 12-hour light/12-hour dark cycle). The mice were fed irradiated chow (Prolab SPF Isopro 3000; PMI Nutrition International) and sterile water ad libitum. All euthanasia was performed by CO2 inhalation followed by cervical dislocation. For Mendelian distribution studies, 4 pregnant mice at E17–E19 were euthanized, and embryos were retrieved by cesarean section and euthanized by decapitation. All mouse procedures were performed in accordance with protocol GO4-3 and were approved by the Institutional Animal Care and Use Committee of the National Human Genome Research Institute.

Molecular analysis. Mouse genotyping was performed on tail genomic DNA or cDNA isolated from kidney or skeletal muscle using standard protocols. Total RNA was isolated from murine tissues using the TRIzol reagent (Invitrogen), and cDNA was prepared using the SuperScript III system (Invitrogen). PCR amplifications were performed across the M712T mutation with genomic DNA as template, using the primer set 5′-AGCACTTCCGGAGTTTGATG-3′ and 5′-ATTGCGCTTCGCAAAACATGTGA-3′ (Figure 2B) or with cDNA as template (Figure 2C), using the primer set 5′-GCCAAGACCATTCAGAAC-3′ and 5′-GGGTCCCTCCGGAGCTTGG-3′ and PuReTaq Ready-To-Go PCR beads (GE HealthCare), using standard PCR conditions. PCR fragments were digested with NlaIII and/or HpaII at 37°C to verify the mutation status (Figure 2, B and C). Quantitative real-time PCR was performed on RNA isolated from kidney and skeletal muscle, utilizing Assays-On-Demand (Applied Biosystems) for Gne (Assay ID mm00607939_s1), Pecam-1 (Assay ID mm00476702_m1), Col4A3 (Assay ID mm01269206_g1), and β-actin (Assay ID mm0450174_s1) on an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems).

Clinical chemistry screen. Retrosorbital blood samples (100–150 μl) from age-matched, weaned male mice weighing at least 15 grams were obtained bimonthly after pretreatment with a topical anesthetic (0.5% tetracaine HCl; Bausch & Lomb Pharmaceuticals). Samples were allowed to clot (30 minutes at room temperature) in MicroPrep centrifuge tubes (IRIS International) and sterile water ad libitum. All euthanasia was performed by CO2 inhalation followed by cervical dislocation. The samples were centrifuged at 1,500 g for 10 minutes and stored at −80°C until analysis. Clinical chemistry screens were performed at the Department of Laboratory Medicine at the NIH and included monitoring of creatinine, blood urea nitrogen, albumin, total protein, uric acid, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, amylase, creatine kinase, and lactate dehydrogenase. In addition, reagent strips for protein urinalysis were used to assess proteinuria (Chemstrip 2GP; Roche Diagnostics).

Antibodies. A rabbit polyclonal antibody was custom prepared against a Gne/Mnk peptide comprising amino acids 588–607 (EAYASGMAQLREKLVHDDE), coupled to keyhole limpet hemocyanine, and affinity purified against the corresponding antigenic peptide (Convance). The following additional primary antibodies were commercially obtained: dystrophin (catalog no. ab15277; Abcam); laminin-1 (catalog no. L9393; Sigma-Aldrich); podocalyxin (catalog no. PODX15-A; Alpha Diagnostic International); podocin (catalog no. P0372; Sigma-Aldrich); laminin β1 (catalog no. MAB1928; Millipore); desmin (catalog no. 1466-1; Epitomics); VSMA (α-SMA) (SPM332; GeneTex Inc.); PSA-NCAM (catalog no. MAB5324; Millipore); and β-actin (catalog no. AAN01; Cytoskeleton).

Mouse histology. Mouse tissues were collected, formalin fixed (10%) and paraffin embedded. Tissue sections (5 μm) were stained with H&E following standard procedures (American Histolabs) or subjected to immunohistochemistry with a variety of primary antibodies (Gne/Mnk, dystrophin, and laminin-1). Formalin-fixed tissues were deparaffinized in HistoClear II (National Diagnostics) and dehydrated in a series of ethanol solutions. Antigen retrieval was performed for sections that were to be stained with antibodies against Gne/Mnk (by boiling 5 minutes in citric acid–based solution; Vector Laboratories) and against dystrophin (by boiling in 1 mM EDTA according to the manufacturer’s protocol; AbCam). The
sections were blocked (2% BSA, 10% donkey serum, and 0.1% Triton X-100 in PBS) and incubated with primary antibodies (Gne/Mnk 1:50; laminin 1:25; dystrophin 1:50) overnight at 4°C, followed by incubation with the secondary antibody, Alexa Fluor 488–conjugated donkey anti-rabbit (1:500 in blocking solution) (Invitrogen). The sections were mounted in VECTASHIELD Mounting Medium (Vector Laboratories) and viewed and digitally imaged with a Zeiss Axiosvert 200M microscope (Zeiss).

**Western blotting.** Mouse tissues (age P2) were extracted, homogenized in Celllytic buffer consisting of a mild detergent, bicine buffer, and 150 mM NaCl (Sigma-Aldrich) supplemented with protease inhibitors (Complete Mini; Roche Applied Science). The lysates were sonicated and cleared by centrifugation (1,000 g for 10 minutes), and the resulting supernatants were assayed for protein (BCA Protein Assay; Pierce Biotechnology). For the purposes of protein quantification, the lysates were sonicated and cleared by centrifugation (1,000 g for 10 minutes), and the resulting supernatants were visualized with ECL (ECL Western Blotting Detection Reagents; GE Healthcare). Densitometry was performed on the digital images obtained with a Kodak CR500 system (Kodak). The protein levels were normalized to those of β-actin to correct for differences in protein loading and/or transfer.

**Electron microscopy.** Kidney samples were fixed overnight at 4°C in 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and washed with cacodylate buffer. After fixation with 1% Oso4, for 2 hours and a second wash with 0.1 M cacodylate buffer, the tissues were serially dehydrated in ethanol and embedded in Eponate 12 resin (Ted Pella). Thin sections (~80 nm) were obtained using a Leica Ultracut UCT Ultramicrotome (Leica Microsystems), placed onto 400 mesh copper grids, and stained with saturated uranyl acetate in 50% methanol followed by lead citrate. The grids were viewed with a Philips 410 transmission electron microscope (FEI Company) at 80 kV, and images were recorded on Electronic Image Film SO-163 (Kodak).

**ManNac administration.** Breeding pairs of 6-week-old GneM712T/+ mice were divided into 3 groups. Group I consisted of 9 GneM712T/+ breeding pairs, which were administered untreated sterilized tap water. Group II consisted of 1 breeding pair of Gne−/− mice (wild-type control) and 6 GneM712T/+ breeding pairs, who were administered water containing 1 mg/g (−0.2 g/kg/day) ManNac (Sigma-Aldrich). That dose was selected based on previous evidence of the safety of ManNac (administered at a single dose of 0.142 g/kg/day) in a study performed in humans (21). Group III consisted of 1 Gne−/− breeding pair and 7 GneM712T/+ breeding pairs, who were administered water supplemented with 5 mg/ml (~1.0 g/kg/day) ManNac. Water was changed twice weekly. Nursing females continued to be supplied with ManNac. All mice were weaned from ManNac at 21 days. Selected whole litters were euthanized at age P2, P6, and P19 for histological, genetic, biochemical, or ultrastructural analysis.

**Gne enzymatic assays.** Mouse kidney and skeletal muscle (quadriceps) tissues were homogenized and subjected to the Gne-epimerase enzymatic assay as previously described (11, 58). This assay was based on incubation with radiolabeled substrate UDP-[3H]GlcNac (catalog no. ART 1136; American Radiolabeled Chemicals Inc.) and detection of radiolabeled product [3H]ManNac upon separation of oligosaccharides by high-pH anion-exchange chromatography with pulsed amperometric detection (BioLC carbohydrate analyzer and Carbopac PA-100 Column; Dionex) (32).

**Statistics.** Differences between data groups were evaluated for significance using the 2-tailed Student’s t test of unpaired data. For Mendelian distribution analysis, a goodness-of-fit (χ2) test was performed, while for comparisons of survival between treated and untreated mice of all genotypes (Gne−/−, GneM712T/+ , and GneM712T/M712T), a 2-tailed Fisher’s exact test using a 2 × 3 table was employed. All data are presented as the mean ± SD. A P value less than 0.05 was considered statistically significant.

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