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Exploring Molecular and Phenotypic Characteristics of *NAGLU* Arg234Gly and Asp312Asn Variants

Hande Kaymakcalan Celebiler[a, b] Tanyeri Barak[b] Devendra K. Rai[b] Ilyas Kaya[d] Seda Erbilgin[e] Merve Cikili Uytun[f] Didem Oztop[f] Hakan Gumus[g] Huseyin Per[g] Serdar Ceylaner[h] Icten Bozkurt[i] Maria I. Kontaridis[c, j, k, l] Kaya Bilguvar[a, m] Nilay Akhun[n] Ayse Kilincaslan[d] Ahmet Okay Caglayan[b, o] E. Zeynep Erson-Omay[b] Murat Gunel[b] A. Gulhan Ercan-Sencicek[b]

[a]Department of Translational Medicine, Acıbadem University, Istanbul, Turkey; [b]Department of Neurosurgery, Program on Neurogenetics, Yale University School of Medicine, New Haven, CT, USA; [c]Masonic Medical Research Institute, Utica, NY, USA; [d]Department of Child and Adolescent Psychiatry, Istanbul University, Istanbul Faculty of Medicine, Istanbul, Turkey; [e]Department of Child and Adolescent Psychiatry, Prof. Dr. Cemil Tascioglu City Hospital, Istanbul, Turkey; [f]Department of Child and Adolescent Psychiatry, Ankara University School of Medicine, Ankara, Turkey; [g]Division of Pediatric Neurology, Department of Pediatrics, Erciyes University, School of Medicine, Kayseri, Turkey; [h]Intergen Genetic Diagnosis and Research Center, Ankara, Turkey; [i]School of Medicine, Bahcesehir University, Istanbul, Turkey; [j]Division of Cardiology, Department of Medicine, Beth Israel Deaconess Medical Center, Boston, MA, USA; [k]Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, USA; [l]Harvard Stem Cell Institute, Harvard University, Cambridge, MA, USA; [m]Department of Genetics, Yale Center for Genome Analysis (YCGA), Yale University, School of Medicine, New Haven, CT, USA; [n]Department of Radiology, American Hospital, Istanbul, Turkey; [o]Department of Medical Genetics, Dokuz Eylul University, School of Medicine, Izmir, Turkey

Keywords
NAGLU · Mucopolysaccharidoses IIIB · Autism spectrum disorder · Lysosomal enzyme · Whole-exome sequencing

Abstract
Introduction: Mucopolysaccharidosis type IIIB is an autosomal recessive lysosomal disorder caused by variants in the α-N-acetylglucosaminidase (*NAGLU*) gene. It is a progressive neurodegenerative disorder with no treatment. Previous enzyme therapies have been unsuccessful. It is important to understand the mechanism of the disease to be able to find new treatments. ***Methods:*** We did whole-exome sequencing and standard Sanger sequencing on 7 cases of four consanguineous families diagnosed with autism spectrum disorder. ***Results:*** We identified two recurrent damaging biallelic Asp312Asn and p.Arg234Gly variants in the *NAGLU* gene. Structure modeling of these variants suggested that each variant affects the stability of the enzyme and results in a loss of activity. All affected individuals' enzymatic assay in

karger@karger.com
www.karger.com/msy

Correspondence to:
A. Gulhan Ercan-Sencicek, adife.ercan-sencicek@yale.edu

Karger

leukocytes clearly showed that α-N-acetylglucosaminidase was completely inactive. Our patients underwent magnetic resonance imaging (MRI), revealing normal findings in two of them despite progressive clinical neurodegenerative symptoms. To our knowledge, these cases represent the second and third instances of normal MRI findings documented in the literature.

Plain Language Summary

Mucopolysaccharidosis type IIIB (MPS IIIB) is an autosomal recessive lysosomal disorder caused by mutations in the α-N-acetylglucosaminidase (*NAGLU*) gene. Whole-exome sequencing and Sanger sequencing identified recurrent damaging biallelic variants (p.D312N and p.R234G) in the *NAGLU* gene in affected individuals and confirmed by enzymatic essays. Four patients underwent magnetic resonance imaging (MRI). Interestingly, 2 patients showed normal MRI findings despite having progressive clinical neurodegenerative symptoms. These cases represent the second and third instances of normal MRI findings in MPS IIIB reported in the literature. Structure modeling indicated that the identified mutations (p.D312N and p.R234G) affect the stability of the enzyme, leading to a loss of activity.

Introduction

Mucopolysaccharidoses (MPS) refer to a group of hereditary disorders that are caused by excessive buildup of mucopolysaccharides due to deficiencies in specific enzymes (lysosomal hydrolases) responsible for breaking down these molecules, also known as glycosaminoglycans (GAGs). There are seven distinct types and 13 subgroups of MPS by their associated enzyme deficiency, each with unique clinical and radiologic features [1, 2]. MPS is rare, with an estimated overall incidence of 1 in 25,000 [3].

MPS III, also known as Sanfilippo syndrome, is a progressive central nervous system and other systems, beginning in childhood, with progressive neurocognitive deterioration and loss of functional abilities, and premature death. The incidence of MPS III is approximately 1 in 70,000 live births [3, 4]. There are four autosomal recessive subtypes (types A, B, C, and D) of Sanfilippo syndrome. Each subtype is caused by a deficiency of a different enzyme that degrades the GAG which leads to substrate accumulation and cellular dysfunction [5]. At present, there is no disease-modifying treatment for individuals with Sanfilippo syndrome. However, disease-specific therapies are being investigated for this condition. These include enzyme replacement therapy, substrate reduction therapy, hematopoietic stem cell transplantation, and gene therapy, some of which have advanced to the mid-to-late stages of clinical development [6].

Among the MPS III disorders, mucopolysaccharidosis type IIIB (MPS IIIB), also known as Sanfilippo B syndrome, is an autosomal recessive lysosomal disorder caused by variants in the *NAGLU* gene which encodes lysosomal enzyme N-acetyl-glucosaminidase [7–9]. A recent study has demonstrated accumulations of GAGs, secondary lipids, and enlarged lysosomes in iPSC-derived neural stem cells and neurons in MPS IIIB patients with *NAGLU* variants [10]. The deficiency or absence of this key enzyme results in the accumulation of heparin sulfate which belongs to the GAG family [7–9, 11]. In addition, autosomal dominant mutation in *NAGLU* has been reported in patients with a milder, late-onset, sensory-motor Charcot-Marie-Tooth disease type 2V neuropathy (MIM#616491) [12]. Patients with MPS IIIB usually present with normal or near-normal development at birth followed by progressive behavioral problems that emerge with continued intellectual decline; finally, there is an onset of dementia and loss of motor function, resulting in death usually in the 2nd to 3rd decade of life [8, 9, 13]. Many of the symptoms of MPS IIIB correspond to autism spectrum disorder (ASD), such as language delay, impaired social communication, epilepsy, sleep problems, and hyperactivity [8, 14, 15]. Most of the time, ASD is diagnosed without any suspicion of MPS IIIB, resulting in delayed supportive therapy and genetic counseling. As shown in our cases and a case by Rezayi et al. [16], magnetic resonance imaging (MRI) findings can be normal, and this finding should not be used alone to rule out MPS IIIB. Despite clinical trials of enzyme therapy for patients with MSP IIIB, currently, there is no cure available [17–19]. Supportive interventions aim to preserve function, enhance abilities, and improve the overall quality of life for individuals affected by MPS IIIB and their families [6].

Here, we presented structure modeling of two recurrent variants in the *NAGLU* gene in seven MPS IIIB cases followed by 7 years along with their associated clinical information. Structure modeling of these variants suggests that each mutation adversely affects the stability of the enzyme and causes a loss of activity.

Methods

We performed whole-exome sequencing (WES) and Sanger sequencing on seven individuals from consanguineous families who were diagnosed with ASD between

the ages of 4–11. Their diagnoses were confirmed according to the Diagnostic and Statistical Manual of Mental Disorders, Fifth edition (DSM-5) criteria [20] or the Childhood Autism Rating Scale (CARS) [21]. Intelligence testing was made by using the Wechsler Intelligence Scale for Children-Revised (WISC-R) [22, 23] or the Stanford-Binet Intelligence Scales [24, 25].

All procedures in this study comply with Yale University's Human Investigation Committee and Human Research Protection Program. The Institutional Review Board of Istanbul University, Istanbul Medical School, Turkey, approved this study, and informed written consent forms were obtained from all involved subjects by their referring physicians.

Whole-Exome Sequencing

Peripheral blood samples were collected from all available family members, and genomic DNA was used to produce the exome-captured sequencing library by SeqCap EZ MedExome Target Enrichment Kit (Roche Sequencing) and the NimbleGen 2.1M human exome array (Roche NimbleGen, Inc.), according to the manufacturer's protocol by the Yale Center for Genome Analysis (YCGA). The enriched libraries were sequenced by the Illumina Genome Analyzer IIx and HiSeq2500 with paired-end chemistries.

DNA Sequencing and Variant Identification

The exome analysis pipeline follows the GATK 3.2 best practice workflow for alignment and variant calling [26]. Reads were first mapped to the human reference hs38DH using BWA MEM v0.7.10 [27]. After removing PCR duplicates using Picard's MarkDuplicates, v1.118, the GATK 3.2-2 software was used to perform indel realignment, base quality score recalibration, and the generation of GVCF files using HaplotypeCaller. The target regions used for the exome were a combination of the exome kit target regions, padded by 40 bp on either side, plus all RefGene coding regions, padded by 15 bp. Once GVCF files were generated, joint variant calling was performed, and variants were filtered using either hard filtering or variant quality score recalibration. Variant annotations were made with ANNOVAR [28] and in-house programs. In all sequenced affected samples, 85% or more of targeted bases had 8 or more independent reads (online suppl. Table 1; for all online suppl. material, see https://doi.org/10.1159/000542367).

We excluded the variants with minor allele frequency >0.001 in 1000 Genomes, dbSNP, as assessed by the Genome Aggregation Database (gnomAD) and our in-house exome sequencing data from unrelated Turkish sample cohort. Scores from Sorting Intolerant From Tolerant (SIFT) [29], PolyPhen [30], LRT prediction [31], MutationTaster prediction [32], MetaLR prediction [33], meta-analytic support vector machine [33], Genomic Evolutionary Rate Profiling (GERP++) [34], and Combined Annotation-Dependent Depletion (CADD) [35] (online suppl. Table 2) were used to determine possible changes in the protein structure. We included only protein-altering variants, including frameshift, start loss, stop gain or loss, missense variants, canonical splice-site variants, and inframe indels affecting protein-coding regions.

Sanger Sequencing

Sanger sequencing confirmed segregation of the *NAGLU* variants with the phenotype. Briefly, genomic DNA was amplified using the Kapa Taq ReadyMix (Kapa Biosystems), 10 μM each of the forward and reverse primers, 5 ng DNA, and H_2O to 25 μL. Primers were designed by Primer3 online tool and their specificity to the regions that were determined by Ensembl BLAST. Primer sequences are available upon request. PCR products were sequenced at W.M.Keck Center, and sequence chromatograms were analyzed using Sequencher 4.9 (Gene Codes).

Protein Structure Modeling

The crystal structure of *NAGLU* (the Protein Data Bank [PDB]: 4XWH) was used to generate structural models. Mutation of residues 234 and 312 was performed by importing the protein in UCSF Chimera molecular visualization system and selecting backbone-dependent rotamers (Dunbrack rotamer library [36]) of residues glycine and asparagine, respectively [37]. After performing the mutation, the protein structure was minimized with 100 steps of steepest descent minimization of 0.02 Å step size and 10-step intervals of updates. For energy minimization, the entire protein except for residues within 5 Å range of the mutated residue was fixed. Additional analysis of the effect of mutations on the stability of the protein was performed by uploading the protein (PDB: 4XWH) on DynaMut web server [38] and analyzing one mutation at a time. Protein's structure models were rendered using PyMOL v2.4 (The PyMOL Molecular Graphics System, Version 2.4, Schrödinger, LLC).

Biochemical Test in Blood/Urine

Enzyme activity was measured on peripheral blood leukocytes by Willink Biochemical Genetics Unit (Manchester, UK), Duzen Laboratory (Ankara, Turkey), and Gazi University Medical School (Ankara, Turkey).

Table 1. The clinical characteristics of the cases

	TURCK2-1	TURCK2-2	NG1893-1	NG1893-4	ITF1-1	ITF1-5	NG3307
Current age, years	20	24	23	27	19	18	18
Mutation	p.Asp312Asn	p.Asp312Asn	p.Asp312Asn	p.Asp312Asn	p.Arg234Gly	p.Arg234Gly	p.Arg234Gly
Gender	Female	Male	Female	Female	Male	Male	Male
Psychiatric diagnoses	ASD, ID	ASD, ID, ADHD	ID, ASD	ASD, ID	ASD, ADHD	ASD, ID, ADHD	ASD, ADHD
Age of first psychiatric evaluation	8 yr	4 yr	8 months	4 yr	5 yr	6 yr	3 yr
Age of ASD diagnosis	8 yr	4 yr	~2 yr	4 yr	11 yr	10 yr	10 yr
Age of MPS IIIB diagnosis	10 yr	14 yr	16 yr	20 yr	12 yr	11 yr	11 yr
Epilepsy (+/–)	+	+	+	+	–	–	–
Coarse face	+	+	–	+	–	–	+
Sleep disorder	–	–	–	+	+	–	–
Past or current medications	Quetiapine, VPA	Risperidone, biperiden, VPA	Risperidone	VPA, clonazepam	MPH	MPH, atomoxetine, aripiprazole	Aripiprazole, MPH, atomoxetine, risperidone

ASD, autism spectrum disorder; ID, intellectual disability; ADHD, attention-deficit/hyperactivity disorder; VPA, valproic acid; MPH, methylphenidate.

Results

In this study, we report clinical and genetic findings of 7 patients followed by 7 years, from four consanguineous pedigrees. All affected patients came to clinical attention initially due to autistic features, epilepsy, aggressive behaviors, or hyperactivity (Table 1). Our WES data analysis and PCR-based Sanger sequencing identified two pathogenic recurrent *NAGLU* mutations in four families (Fig. 1a). Both variants are classified as pathogenic under the following criteria: the missense variant has a minor allele frequency of less than 0.001 in the gnomAD. Additionally, scores from predictive tools such as SIFT, PolyPhen, LRT, MutationTaster, MetaLR, and meta-analytic support vector machine indicate that the variants are predicted as deleterious or potentially damaging, with a CADD score of ≥20. Furthermore, a GERP++ score of ≥3 suggests high conservation across other species (Fig. 1b; online suppl. Table 2). Following the genetic tests, MPS IIIB in families NG-3307, ITF1, and TURCK2 was also biochemically confirmed (online suppl. Table 3) suggesting that these two recurrent homozygous variants act through a loss-of-function mechanism.

ITF1 Family

ITF1-1 is a 19-year-old male who was diagnosed with ASD (CARS: 36.5) at the age of 11 and diagnosed with MPS IIIB at 12 years old. He had uncontrollable aggressive behavior and attention-deficit/hyperactivity disorder (ADHD). He was born via cesarean section without complications. He started walking by 18 months, using words by 3 years, and making sentences by 5 years. His intellectual and language ability showed progressive deterioration, and his anxiety level started to increase around the age of 8. His current speech is dysprosodic and echolalic and includes only two- or three-word sentences. He has difficulty with social interactions with his peers. He has sensitivity to sounds and repetitive behavior such as stirring and spilling food, filling bags with stuff, and hiding things. He has severe uncontrollable aggressive behavior and sleep problems. He is not toilet trained. He can feed himself. His ophthalmologic

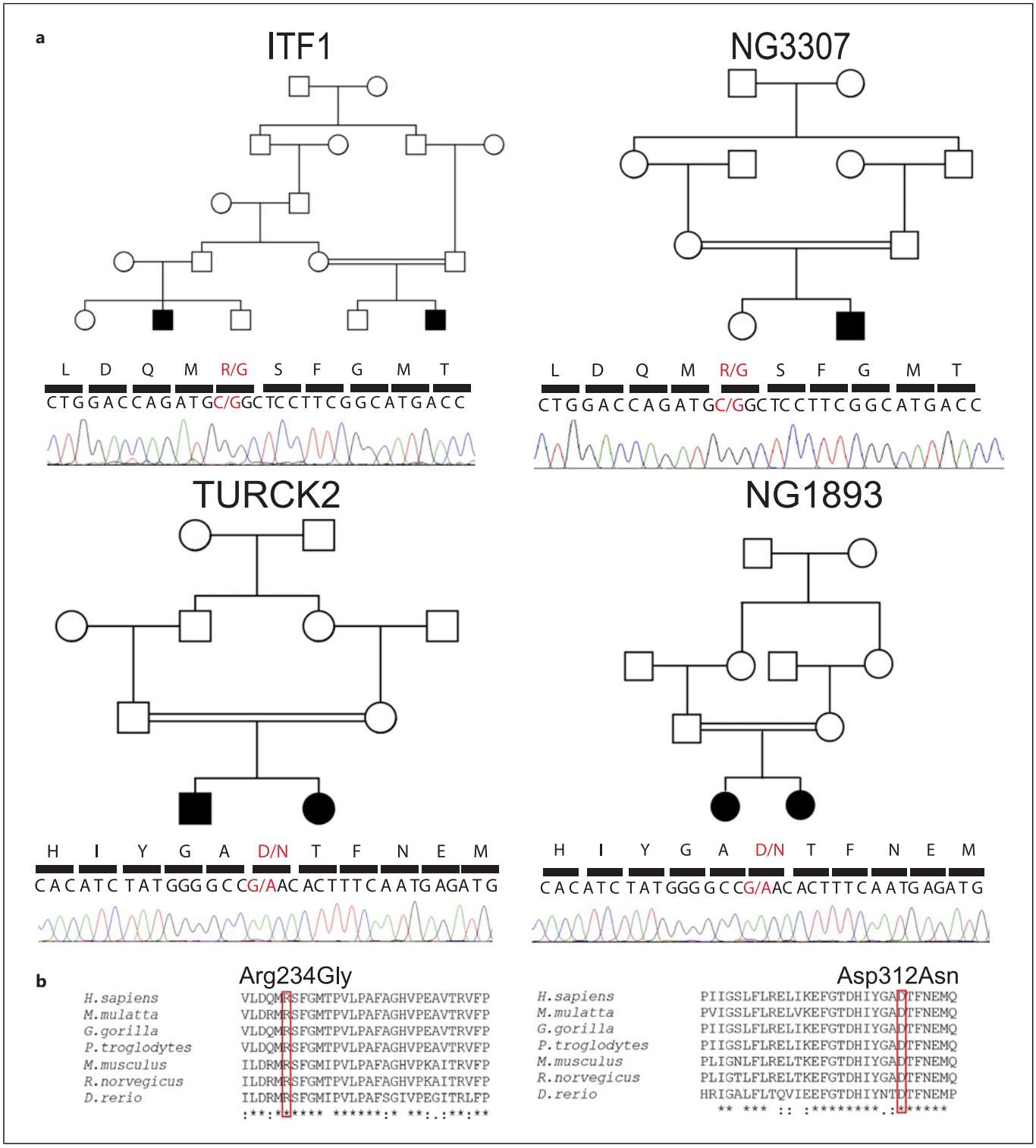

Fig. 1. a Pedigree structures of families with MPS IIIB and ASD. Affected members are shown by either dark squares (males) or circles (females); homozygous pathogenic mutations are confirmed by Sanger sequencing in the affected individuals. **b** CLUSTAL Omega sequence alignment [39] shows the evolutionary conservation of *NAGLU* mutations across seven vertebrate species.

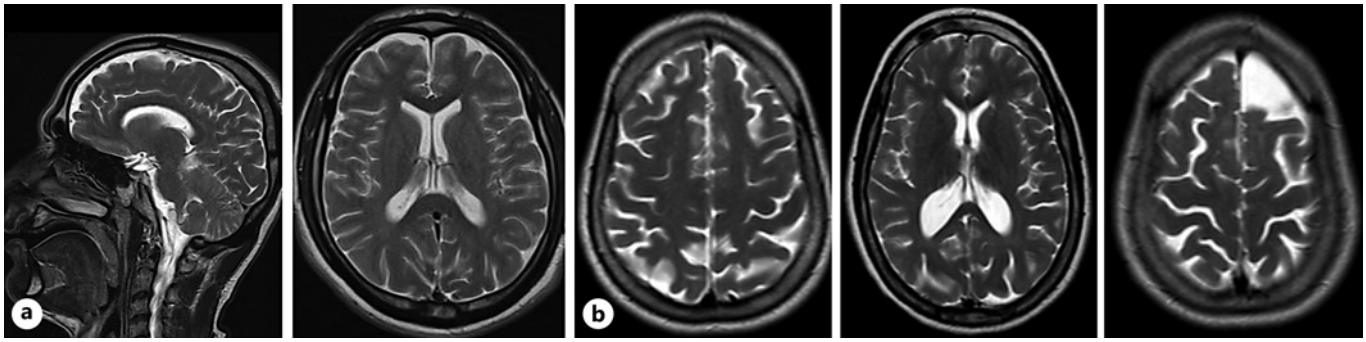

Fig. 2. The radiographic features of ITF1-1 and TURCK2-1. **a** Representative MRIs of individual ITF1-1 at 18 years of age. T2-weighted sagittal and axial MR images of the brain show no abnormality. **b** Representative MRIs of individual TURCK2-1 at 12 years of age. T2-weighted axial MRI of the brain shows widening of cortical sulci, mild dilation in the right ventricle, and an arachnoid cyst in left frontal lobe.

and hearing examinations were unremarkable. He has severe sleep problems and masturbates in public.

WES analysis identified a homozygous missense variant (NM_000263.4:c.700C>G (p.Arg234Gly)) in the ITF1 family in the *NAGLU* gene (Fig. 1a, online suppl. Fig. 1a). His lysosomal enzyme assay on the white blood cells showed decreased activity in α-N-acetylglucosaminidase (control >15, his level 0.3 nmol/h/mg protein), and his urinary analysis revealed increased heparan excretion. He has mild coarse facial features with thick lips and thick eyebrows together with long and wide palms. He does not have hepatosplenomegaly and no cardiomegaly. Because of his neurocognitive deterioration, he had MRI at the age of 18 and it was all normal (Fig. 2a). He did not have cerebral atrophy, hydrocephalus, hypomyelination, and thin corpus callosum which are commonly seen in MPS IIIB patients.

ITF1-5 is an 18-year-old male who is the cousin of ITF1-1. He was diagnosed with ASD and mild ID according to DSM-5 criteria at age of 10 and diagnosed with MPS IIIB at 11 years old. He was born via normal spontaneous vaginal delivery (NSVD) at term after an uncomplicated pregnancy. He sat and walked on time, and his first words came when he was 1 year old, but he never made sentences. He started to say fewer words after the age of 10. His hearing and ophthalmological testing were normal. He had ADHD and uncontrollable aggressive behavior. Over the years, his intellectual ability showed progressive deterioration, especially his language ability. He currently has echolalia and insists on carrying and sleeping with a specific bag, wearing shirts over sweater, and repetitive changing of TV channels. Following the genetic test, a lysosomal enzyme assay on white blood cells showed decreased activity of α-N-acetylglucosaminidase (control >15, patient's level 0.3 nmol/h/mg protein). Urinary analysis revealed increased heparan excretion. He did not have coarse facial features, hepatosplenomegaly, or cardiomegaly at the time of diagnosis. The rest of the physical examination was normal.

NG-3307 Family

NG-3307 is an 18-year-old male who was referred to the pediatric genetic clinic due to intellectual disability (ID) and ASD at age 10 (CARS: 35) and was diagnosed with MPS IIIB at 11 years old. He scored 54 at the CARS at age 14 during his follow-up visit for aggressive behavior, speech delay, and repetitive behaviors. He was born by NSVD at term. Growth parameters at birth and at first admission are not available for this patient. State that no brain MRI has been performed. He was late in developmental milestones, held his head at around 4 months, sat at 10 months, and walked at around 16 months, and his first words were around 14 months. Even though he is currently nonverbal, at the age of two and a half years old, he was able to make two-word sentences. He started to decline in speech and have behavioral problems around 4 years of age. Around age 10, his motor skills declined; he is ataxic and can only walk when handheld. He is toilet trained and can feed himself but needs help dressing and undressing. His facial coarse features were big lips, a bulbous nose, and thick eyebrows. He refused to have a physical exam and made a big tantrum which included shouting, crying, and hitting. He does not have epilepsy. He has limited eye contact and does not look when his name is called. He has repetitive behavior, such as clapping and turning lamps on and off. He also has routines such as organizing and aligning things, as well as grouping certain foods such as eggs. He

has self-harming behavior. He did not have MRI. His liver function tests, complete blood count, and thyroid function tests were normal. GAG excretion was not evaluated for this patient. We identified a homozygous missense variant (NM_000263.4:c.700C>G (p.Arg234Gly)) in *NAGLU* gene (Fig. 1a). There was a marked deficiency of plasma α-N-acetylglucosaminidase enzyme activity which was 0.69 umol/L/h (normal range 10–45). In urine analysis, heparin and heparan sulfate were increased, but a specific value was not available.

TURCK2 Family

The TURCK2 family has two affected children born via NSVD. Mother and father are first cousins. WES analysis identified a homozygous missense variant (NM_000263.4: c.934G>A (p.Asp312Asn)) in *NAGLU* gene, which was validated by Sanger sequencing in this family (Fig. 1a, online suppl. Fig. 1b).

TURCK2-1 was first referred to the clinic in 2012 at the age of 8 for speech delay and difficulty understanding commands. She was diagnosed and followed by ASD and ID. At the age of 17, she had severe symptoms of autism (CARS: 40.5) and a full-scale IQ score of 55 with the WISC-R. She displays severe deficits in social-emotional reciprocity, verbal-nonverbal communication, and eye contact. She has epilepsy, uncontrollable aggressive and self-injurious behavior, and stereotypies such as repeated hand-waving. Her physical examination reveals coarse facial features with thick lips, an enlarged tongue, and thick eyebrows. She has no hepatosplenomegaly and no cardiomegaly. Following the genetic analysis, lysosomal enzyme assay on white blood cells showed decreased activity in α-N-acetylglucosaminidase (control 5.92–6.23, her level 0.05 nmol/17 h/mg protein). MR imaging showed that she has mild cerebral atrophy and mild dilatation in the right ventricle, and an arachnoid cyst in the left frontal lobe at age 12 (Fig. 2b); EEG showed abnormality (irregular background rhythm) when she was 4 years old (no report on the type of seizures) and she was started on valproic acid (VPA) 400 mg. After 2 years, VPA was stopped due to miscompliance and risperidone was started. Risperidone was discounted after 4 years when she developed oculogyric crisis, and she was then started on quetiapine 25 mg. Later, VPA was re-introduced due to increasing aggressive behavior.

TURCK2-2 was first brought for the assessment of speech delay, difficulty understanding commands, hyperactivity, developmental delay, epilepsy and aggressive behavior in 2004 at the age of 4. He was diagnosed with ID and ASD. He currently has very limited verbal and nonverbal communication, has no eye contact, or interest in his surroundings, and displays repeated hand-waving. He was also diagnosed with ADHD according to the DSM-5. He scored 46 at the CARS and 47 with the WISC-R. At the age of 14, his lysosomal enzyme assay on white blood cells, which showed decreased activity in α-N-acetylglucosaminidase (control 5.92–6.23, his level 0.08 nmol/17 h/mg protein), was consistent with genetic analysis. Coarse facial features with thick lips, enlarged tongue, and thick eyebrows were detected on physical examination. He had no hepatosplenomegaly and no cardiomegaly. EEG showed a generalized paroxysmal abnormality (no more information), and the MRI at the age of 14 demonstrates a moderate dilatation of both lateral ventricles, a prominent appearance of the posterior horn of the left lateral ventricle, and a moderate thinning in the corpus callosum (image is not available). He currently (24 years old) has uncontrollable aggressive and self-injurious behavior and sleep problems.

NG1893 Family

The NG1893 has two affected children born via NSVD at term after an uncomplicated pregnancy. Birth measurements of weight, length, and head circumference were unremarkable according to parents. We identified a homozygous missense variant (NM_000263.4:c.934G>A (p.Asp312Asn)) in *NAGLU* gene, which was confirmed by Sanger sequencing in this family (Fig. 1a, online suppl. Fig. 1c).

NG1893-1 was brought for psychiatric evaluation due to developmental delay and hyperactivity at the age of 8 months. When she was 2 years old, she was diagnosed with global developmental delay and ASD according to DSM-5 criteria by the child psychiatrist. She was diagnosed with MPS IIIB when she was 16 years old. She repeats the same actions or movements repeatedly, such as opening-closing the door and running back and forth. She bites and chews on things. She can make eye contact. She is hyperactive, has an absence seizure, and is unable to learn colors and numbers. The course and onset of the psychiatric symptoms appeared more rapid, and the degree of delay is more severe than her sister's. The ophthalmologic and hearing examinations were normal bilaterally.

NG1893-4 is a 27-year-old female who was brought to the child psychiatry clinic with symptoms of destructive and aggressive behavior at the age of 4. She was diagnosed with atypical autism and ID according to DSM-5. She began speaking around 1.5 years old and had peer interactions until 4 years old. When she was 5 years old, she developed hyperactivity, trouble communicating with others, and sleep disturbances. She had seizure when she

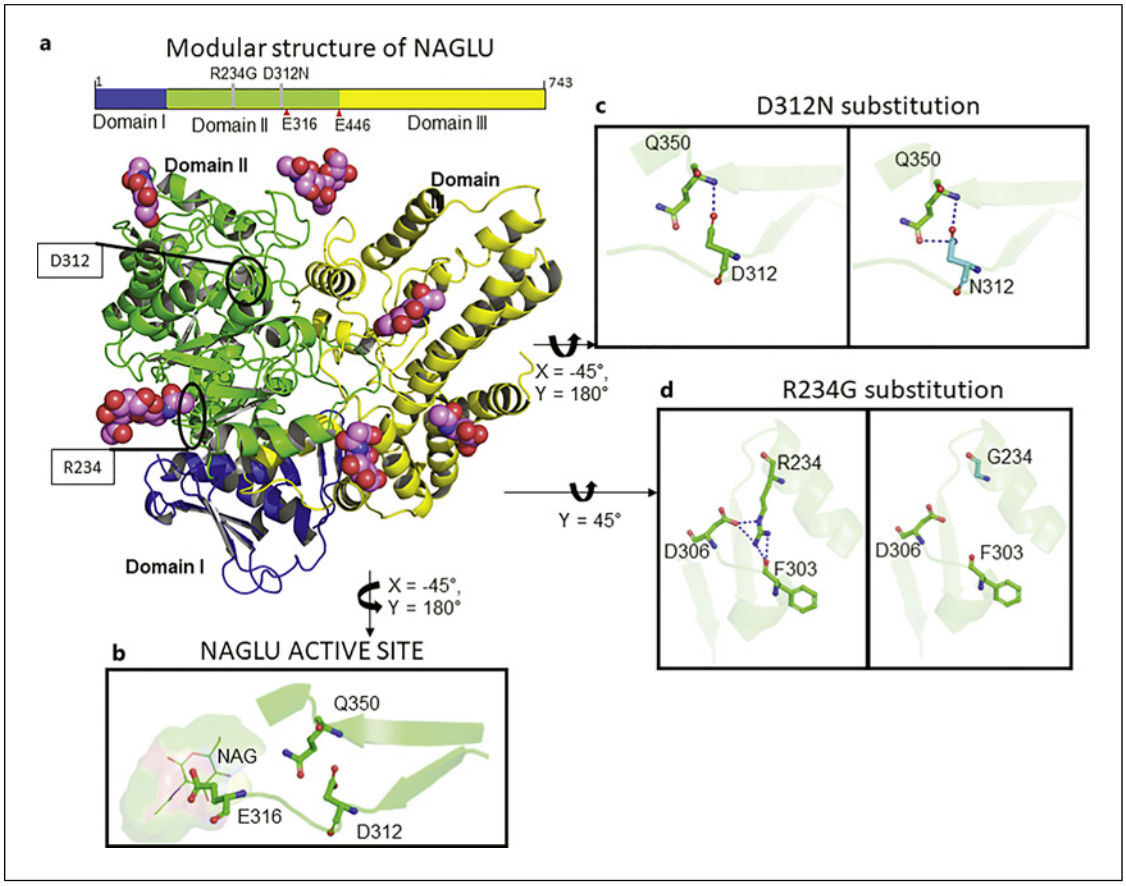

Fig. 3. Effect of MPS IIIB syndrome mutations on NAGLU structure. Sanfilippo syndrome mutations modeled on NAGLU crystal structure (PDB: 4XWH). **a** Modular structure of NAGLU. Blue, green, and yellow colors denote domains I, II, and III of the protein. Active site residues and key mutations are marked. **b** NAGLU active site. Active site glutamate (E316) is part of a flexible loop region critical for the activity of the enzyme. Substrate (NAG) binding pocket is lined by residues 312–316 on one side and Q350 on the other. **c** D312N substitution. Blue broken lines denote hydrogen bonds. While D312 only forms one hydrogen bond with the backbone carbonyl oxygen of Q350, the substitution of this residue with N allows an additional hydrogen bond between the side-chain amine group of asparagine and the side-chain carbonyl oxygen of glutamine 350 adding local rigidity to the active site loop. **d** R234G substitution. R234 forms hydrogen bonds with residues D306 and F303. Mutant protein with G234 loses the hydrogen bonds, causing local conformational flexibility in the protein. Blue broken lines denote hydrogen bonds.

was 12 years old. Her physical and psychiatric examination at 15 years old was notable for her inability to speak, autistic features, developmental delay, ataxic gait, and dysmorphic appearance which included coarse facies, thick alae nasi, and lips. Despite all these findings, she was not referred for genetic evaluation and she was diagnosed with MPS IIIB only when she was 20 years old. The enzyme activity assay from both patients is not available. Due to the cost of additional testing, urinary GAG analysis is not performed. There were no hepatosplenomegaly or skeletal findings. Ophthalmologic examination revealed no retinal or optic nerve abnormality, and the brainstem-evoked response audiometry tests were normal. Tandem mass spectrometry, plasma amino acid, and urinary organic acids were normal. Echocardiography, abdominal ultrasonography, brain MRI, and spectroscopy were within normal limits. Recently, she lost her ability to walk up the stairs.

Protein Structure Modeling to Understand the Effect of the Mutations on the Structure and Function of NAGLU *Enzyme*

NAGLU consists of three structural domains (domain I–III). Domain I (blue) located at the N-terminus of the protein lines the bottom of the catalytic pocket, and a β-sheet from this domain is inserted between domain II (green) and domain III (yellow) (Fig. 3a). Domain III is the catalytic domain of the enzyme with two glutamate

residues E316 and E446, marking two sides of the catalytic (NAG-binding) pocket. To extract the substrate, NAG, bound in the enzyme active site, we superposed NAGLU (PDB: 4XWH) onto bacterial NAGLU (PDB: 2VCA) and saved NAG and human NAGLU as a single PDB file. As shown in Figure 3b, residue 312 is critical for the maintenance of the flexible conformation of the catalytic loop, which contains active site glutamate E316. The substitution of aspartate at this position with asparagine introduces an additional hydrogen bond with glutamine Q350, which lies adjacent to the substrate (NAG)-binding pocket (Fig. 3b, c). The additional hydrogen bond is predicted to introduce rigidity in the catalytic loop, which will adversely affect the activity of the enzyme. The analysis of the effect of the Asp312Asn substitution on the stability and conformational dynamics of the protein using DynaMut web server also suggested that N234 destabilizes this protein (ΔΔG: −0.618 kcal/mol; a negative value of ΔΔG indicates the mutation destabilizes the protein) [24]. Arg234Gly variant, although away from the active site, introduces a major change in the hydrogen bonding network involving residues 234, 303, and 306 (Fig. 3d) in the catalytic domain II. Notwithstanding the negative ΔΔG (ΔΔG: −1.461 kcal/mol) in DynaMut analysis, which indicates the mutation would destabilize the protein, it affects the local conformation of the protein and is predicted to alter the interaction between domain I and domain III. Importantly, the loss of a hydrogen bond between residues 234, 303, and 306 due to glycine substitution could alter the stability of a *NAGLU* trimer, as K301, and E302, residues adjacent to these residues are known to participate in hydrogen bonding with another NAGLU molecule, which is critical for the stability of a trimer, a feature essential for the maintenance of the activity of the enzyme [25].

Discussion

It has been previously documented that symptoms of ASD in the domains of language, communication, anxiety, and social difficulties with little evidence of restricted/repetitive behavior are highly prevalent in MPS IIIB syndrome [11, 14, 15]. Patients presented in this study with *NAGLU* gene variants showed a similar pattern of disease progression as observed in patients with the MPS IIIB [15, 40]. However, even though our patients were seen by a neurologist/psychiatrist for ID, behavioral problems, and delayed speech at a median age of 4 years (range 8 months–8 years), they were not diagnosed with ASD until a median age of 8 years (range 2–11 years) due to the delayed appearance of core ASD symptoms and delayed diagnosis of MPS IIIB until a median age of 13.4 years (range 10–20 years) due to unrecognized dysmorphological findings and not referring patients to medical genetics department.

Our patients exhibited delayed development of verbal and nonverbal skills as well as progressively increasing sleep disorders, aggressive behavior, hyperactivity, cognitive and physical deterioration. Despite the neurodegenerative nature of this disease, ITF1-1 (Fig. 2a) and NG1893-3 had normal MRI results, and TURCK2-1 (Fig. 2b) and TURCK2-2 had mild dilatation in lateral ventricles and moderate thinning in the corpus callosum. Three patients did not have MRI.

Although large spectrum of findings have been observed, common features of brain MRI in patients with MPS include brain atrophy, the abnormal signal intensity in the white matter, and dilatation of periventricular spaces and third ventricle [41]. Although we do not expect to see a normal MRI with a progressed MPS disease, our findings and a literature report highlight the absence of a consistent association between MRI findings and the clinical status of the disease. As hypothesized by Nicolas-Jilwan et al. [42], the correlation between neuroimaging features and disease severity is controversial, without well-established imaging biomarkers. Additional pathophysiological mechanisms related to neurodegeneration require further investigation. Also, there may be more cases with normal MRI results despite disease progression. However, due to the difficulty of obtaining an MRI for these patients, such cases may not be reported in the literature. For example, in a recent series of 19 Brazilian patients with MPS IIIB, none of them had MRI [43]. Still, neuroimaging is an important tool to identify craniocervical junction stenosis, cord compression, and hydrocephalus, so that timely intervention can be made before permanent damage occurs [42].

When investigating the genetics of MPS IIIB, it has been demonstrated that the same variants in the *NAGLU* gene can lead to a different clinical spectrum in individuals [44]. The likely pathogenic variant, p.Arg234Gly, has been reported in another Turkish patient with neurodevelopmental delay, ASD, and macrocephaly [45]. Our 3 patients with the same variant do not have macrocephaly. Additionally, while NG-3307 is ataxic and can only walk with assistance, the siblings from ITF1 family had a normal physical exam. The variant p.Asp312Asn in *NAGLU* classified with conflicting classifications of pathogenicity by the ClinVar database was previously reported in three

affected siblings with severe ID accompanied by epileptic seizures without ASD and behavioral abnormalities [46]. However, our patients with p.Asp312Asn variant presented with autism and severe behavioral changes including hyperactivity, destructive and aggressive behavior in addition to ID, and seizure. We also observed phenotypic variability even between siblings who carry the same variant in terms of progress and severity of the symptoms. Therefore, it is crucial to elucidate how variants in *NAGLU* lead to both neurodevelopmental abnormalities such as autism-like behaviors and neurodegenerative disorders with different severities in each patient.

In summary, each variant adversely affects the stability of the enzyme and causes a loss of activity by reducing either the level of the intact enzyme or the activity of the enzyme by altering its active site. Considering the genetic and phenotypic variability of MPS IIIB, it is important to understand the causal mechanism of ASD development in MPS IIIB patients to be able to discover the common molecular pathways between syndromic and non-syndromic ASDs. Our findings suggest that molecular and cellular mechanisms controlled by the genes positively regulated with *NAGLU* expression have promised to develop the potential treatment for neurodevelopment and neurodegeneration in patients with MPS IIIB and autism. Our study also shows the importance of genetic counseling in patients with ASD and ID for early diagnosis of genetic disorders which the symptoms may not be obvious and family planning.

So far, enzyme replacement and gene therapies have not resulted in marked improvement in disease course [18, 47]. Consensus guideline for the management of these patients has been developed recently by Muschol et al. [6]. Further studies will be required to model these convergent genes in 3-D brain organoid models that will be valuable to screen compounds for drug development.

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Statement of Ethics

This study protocol was reviewed and approved by Yale University's Human Investigation Committee and Human Research Protection Program with IRB protocol ID: 1503015527. The Institutional Review Board of Istanbul University, Istanbul Medical School, Turkey, approved this study (2015/784). Written informed consent was obtained from participants.

Conflict of Interest Statement

The authors declare no conflict of interests. Devendra K. Rai is currently affiliated with Pfizer, NY.

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Author Contributions

A.G.E.-S. and E.Z.E.-O. conducted genome analysis. D.K.R. analyzed the structural impact of the variants on *NAGLU* gene. H.K.C., I.K., S.E., M.C.U., D.O., H.G., H.P., S.C., K.B., A.K., and A.O.C. contributed to patient recruitment and phenotyping. I.B. performed PCR analysis. N.A. reviewed the MRIs. A.G.E.-S., K.M.G., T.B., D.K.R., H.K.C., and A.K. contributed to the writing of the initial draft of the manuscript. All authors contributed to revising the manuscript and reviewing the final draft.

Data Availability Statement

Each variant in Table 1 is annotated with information regarding amino acid change, CADD score, SIFT, PolyPhen, LRT, MutationTaster, MetaSVM, MetaLR, GERP score for deleteriousness, and allele frequency in gnomAD. Online supplementary Table 3 provides individual-level phenotypic data from patients with *NAGLU* variants. Whole-exome data (BAM file) of each patient are available upon request. The crystal structure of NAGLU (Protein Data Bank [PDB]: 4XWH) was used to generate a structural model.

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