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NDST1 Missense Mutations in Autosomal Recessive Intellectual Disability

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NDST1 was recently proposed as a candidate gene for autosomal recessive intellectual disability in two families. It encodes a bifunctional GlcNAc N-deacetylase/N-sulfotransferase with important functions in heparan sulfate biosynthesis. In mice, Ndst1 is crucial for embryonic development and homozygous null mutations are perinatally lethal. We now report on two additional unrelated families with homozygous missense *NDST1* mutations. All mutations described to date predict the substitution of conserved amino acids in the sulfotransferase domain, and mutation modeling predicts drastic alterations in the local protein conformation. Comparing the four families, we noticed significant overlap in the clinical features, including both demonstrated and apparent intellectual disability, muscular hypotonia, epilepsy, and postnatal growth deficiency. Furthermore, in *Drosophila*, knockdown of *sulfateless*, the *NDST* ortholog, impairs long-term memory, highlighting its function in cognition. Our data confirm *NDST1* mutations as a cause of autosomal recessive intellectual disability with a distinctive phenotype, and support an important function of NDST1 in human development.

Key words: autosomal recessive intellectual disability; heparan sulfate biosynthesis; NDST1; sulfateless

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INTRODUCTION

Intellectual disability (ID) comprises a large and heterogeneous group of neurodevelopmental disorders, characterized by significant early-onset cognitive impairment and limitations of learning, adaptive behavior and skills [Salvador-Carulla et al., 2011]. In outbred populations, the most frequent cause of severe sporadic ID is chromosomal abnormalities, de novo point mutations and small insertions/deletions [de Vries et al., 2005; Vissers et al., 2010]. Nonetheless, in affected children from consanguineous families, autosomal recessive inheritance is common [Musante and

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Ropers, 2014], and identifying the underlying genetic cause is an important issue in clinical genetics. By means of homozygosity mapping and next-generation sequencing approaches, a large number of candidate genes for autosomal recessive intellectual disability (ARID) have been identified, and although many of them share common pathways, there is a large degree of genetic heterogeneity [Najmabadi et al., 2007; Najmabadi et al., 2011]. Identifying multiple families with potentially pathogenic variants in the same gene is important to provide convincing evidence for disease causality of candidate genes and for delineating the associated phenotype.

NDST1 was previously suggested as a candidate gene for ARID based on a homozygous missense mutation in one family with ID [Najmabadi et al., 2011]. A further variant in this gene was reported in a second family in the supplementary material of this report although no interpretation was given. *NDST1* encodes the bifunctional GlcNAc N-deacetylase/N-sulfotransferase NDST1, which has an important function in the generation of sulfated ligand-binding sites during heparan sulfate biosynthesis [Esko and Selleck, 2002]. Heparan sulfate interacts with growth factors and morphogens and is crucial for embryonic differentiation and development. Mice lacking Ndst1 function die perinatally and exhibit variable developmental defects of the forebrain, forebrain-derived and facial structures [Ringvall et al., 2000; Grobe et al., 2005; Pallerla et al., 2007].

We report on two additional families with *NDST1* missense mutations in the sulfotransferase domain and provide protein modeling based evidence for the pathogenicity of all mutations. We also provide detailed clinical presentation in two previously published families [Najmabadi et al., 2011]. In addition, we show that brain specific knockdown of the *Drosophila NDST* ortholog, *sulfateless*, causes a long-term memory deficit in flies. Our data support the role of NDST1 in human development and provide insight into the clinical phenotype resulting from variants affecting the sulfotransferase domain.

PATIENTS AND METHODS

Patients

Patients were recruited either in Germany or in collaboration with local genetic counselors in Iran. Written informed consent was obtained from all participants or their respective guardians. The study was approved by the respective local ethic committees. Genomic DNA was extracted from blood samples using standard protocols. Tandem mass spectrometry screening for metabolic disorders was performed for at least one patient per family.

SNP Genotyping and Homozygosity Mapping

Molecular karyotyping using a high density Affymetrix Genome-Wide Human SNP Array was performed for selected individuals from each family and did not identify any relevant copy number variants. Homozygosity mapping using the computer program PLINK [Purcell et al., 2007] or parametric multipoint linkage analysis with MERLIN were performed assuming an autosomal recessive mode of inheritance and complete penetrance [Abecasis et al., 2002].

Exon Enrichment and High-Throughput Sequencing

For Patient 3.1, the SureSelect Human All Exon 50Mb Kit (Agilent Technologies) was used for enrichment. Paired-end sequencing (65/10 bp) was carried out on a SOLiD 5500xl instrument (Life Technologies/Applied Biosystems). For Patient 4.1, the so-called MPIMG-1-Test, a TruSeq Custom Enrichment Kit (Illumina Inc., San Diego, USA), targeting 1,222 genes linked to recessive childhood disorders or intellectual disability, was used for enrichment. 2×250 bp paired-end sequencing (Illumina MiSeq Reagent Kit v2) was carried out on an Illumina MiSeq system.

Sequence Alignment, Variant Calling, Annotation, and Verification

For Individual 3.1, read alignment to the human reference genome (hg 19, GRCH37) was performed with LifeScope version 2.5. Average target coverage was 74, while 82% of the target sequence was covered at least five times. Single-nucleotide variants and small indels were called with LifeScope version 2.5, GATK 2 [McKenna et al., 2010], and SAMtools/BCFtools [Li et al., 2009]. Data were compiled from a variety of public and in-house databases and variant annotation was performed with Annovar [Wang et al., 2010]. Assuming an autosomal recessive mode of inheritance, only variants in identical-by-descent segments were considered for further analysis. Computer-based analysis was performed with SIFT, PolyPhen-2, and MutationTaster. For Individual 4.1, read alignment was performed with SOAP version 2.2. More than 91% of the enriched exons were covered by at least ten reads. An improved version of the Medical Re-sequencing Analysis Pipeline (MERAP) developed by H. Hu (submitted) was used to check all detected variants against dbSNP137, the 1000 Genomes Project, the Exome Variant Server (NHLBI GO Exome Sequencing Project, http://evs.gs.washington.edu/EVS/), the OMIM catalog (http://www.ncbi.nlm.nih.gov/omim), and the Human Gene Mutation Database (http://www.hgmd.org/).

For exclusion of technical artifacts and segregation testing of all variants, PCR and Sanger sequencing were performed according to standard protocols. Each variant was excluded in 100 to 340 chromosomes of ethnically matched controls.

Mutation Modeling

To further evaluate the effect of the amino acid substitutions on a molecular level, mutations were modeled *in silico* based on known crystal structures. The structure of the sulfotransferase domain of human NDST1 was retrieved from the protein data bank (PDB code: 1NST; [Kakuta et al., 1999]). The location of the substrate was deduced from the homologous structure of 3-O-sulfotransferase complexed with heparan sulfate (PDB code: 3UAN; [Moon et al., 2012]). A hexasaccharide was modeled into the large open substrate-binding site of NDST1 where it could be accommodated without any steric clashes. Mutations were modeled using Swiss-PDB Viewer [Guex et al., 1999] and the lowest-energy rotamer was selected for each mutated residue. RasMol [Sayle and Milner-White, 1995] was used for structural analysis and visualization.

Drosophila Behavioral Testing

The *uas-RNAi* lines (5070, 108109) were obtained from Vienna *Drosophila* RNAi center (http://stockcenter.vdrc.at/control/main). Flies were raised on semi-defined medium at 25°C in a 12 hr dark-light cycle. Virgin males were collected at eclosion and aged individually for five days before training. Canton-S premated females were aged for four days in groups of 50–100 with Canton-S males collected at the same time.

Males were assayed for courtship conditioning as described [Siwicki and Ladewski, 2003]. In brief, for training, individual males were placed in food chambers either with (trained) or without (naïve) a single premated female for seven hours. After training, each male was recovered, transferred to a fresh food chamber, kept in isolation and tested for long-term memory after 24 hr. Tests were performed in a 10 mm diameter courtship chamber and videotaped for 10 min (JVC handycam, 30 GB HD). Videos were scored with automated software (C. Schusterreiter, C. Machacek, B. Dickson, unpublished) for courtship index (CI), which is the fraction of time each male spent courting during the test. Mean CIs were used to calculate the learning index (LI): CI_{naive}-$CI_{trained}$/$CI_{naive} \times 100$. Data represent average values ± the standard error of the mean (SEM) of performance indices. Learning indices were analyzed using a MATLAB script by permutation test [Kamyshev et al., 1999]. Briefly, the entire set of courtship indices for both the naïve and trained flies were pooled and then randomly assorted into simulated naïve and trained sets of the same size as in the original data. A LI_p was calculated for each of 100,000 randomly permuted data sets, and *P*-values were estimated as the fraction for which $LI_p > LI$ (to test H_0, $LI = 0$).

RESULTS

Clinical Description

We describe eight patients from four unrelated families, who were initially referred for developmental delay or intellectual disability. Medical histories, diagnostic findings, and pedigrees were obtained in a clinical setting in Iran or Germany, and are summarized in Table I, Figure 1 and Figure 2.

Family 1

Patient 1.1 is the first child of consanguineous healthy parents from Eastern Iran. After an uneventful pregnancy, he was born at 38 weeks of gestation. Birth weight was unavailable, but said to be high for gestational age, length was not documented, and occipitofrontal circumference (OFC) was 34 cm (−0.2 SD). Psychomotor delay was noted in early childhood. At age 2.5 years, he started to walk and spoke first words. Fine motor development was not documented. Generalized seizures in childhood could be controlled by antiepileptic medication. Short stature and non-progressive ataxia were noted around age 15 years. The patient suffered from polyuria, but urine analysis and renal ultrasound were normal and further studies were not performed. At age 36 years, height was 153 cm (−3.7 SD) and OFC was 55 cm (−1.3 SD). Parental height was normal (−1 SD). The patient exhibited muscular hypotonia and showed good eye contact with his parents. He didn't have ability to communicate nor to follow simple commands. Testing of cognitive function using the Wechsler Adult Intelligence Scale (WAIS-IV) showed an intelligence quotient (IQ) of 30 in the range of severe intellectual disability. Aggressive social behavior, agitation, and sleep disturbances were confirmed by psychiatric assessment using the Diagnostic Assessment for the Severely Handicapped (DASH-II). Cranial magnetic resonance imaging (MRI) and electroencephalography (EEG) were normal, facial features are shown in Figure 2A.

Patient 1.2 is the younger sister of Patient 1.1. She was born at term after an uneventful pregnancy. Birth weight was unavailable, but said to be large for gestational age and OFC was 35.5 cm (+0.2 SD). She started to walk and spoke first words at age three years. She had a history of generalized seizures in childhood that could be controlled by antiepileptic medication. Short stature was noted from age 16 years. When evaluated at age 32 years, height was 145 cm (−3.1 SD) and OFC was 54 cm (−1 SD). Hearing and visual testing were normal except strabismus. IQ testing at age 32 years by WAIS-IV revealed an IQ of 26. The parents reported a generally quiet temperament with sometimes increased irritability, which was not formally tested.

Patient 1.3 is the youngest brother of patient 1.1. He was born at term after an uneventful pregnancy. Birth weight was again high for gestational age and OFC was 35 cm (+0.1 SD). He started to walk and spoke first words at age 2.5 years. Muscular hypotonia and flat feet were first described in adolescence, ataxia was not noted. At age 28 years, height was 150 cm (−4.1 SD) and OFC was 55 cm (−1.3 SD). An IQ of 26 was revealed by WAIS-IV. Aggressive and self-injurious behavior was noted by the family and was confirmed by psychiatric assessment using the DASH-II checklist. Hearing and vision were normal.

Family 2

Patient 2.1 is the first child of healthy consanguineous parents (first cousins), originating from North Eastern Iran. The patient was born at term after an uneventful pregnancy. Birth weight was 4,200 g (+2.4 SD), length and OFC were not documented. She was breast-fed without problems for 12 months. She started to walk and spoke first words at age three years. Generalized seizures first occurred at age 12 years, and could be controlled by antiepileptic medication. No history of ataxia or muscular hypotonia was reported. Her body height at age 18 years was 155 cm (−1.4 SD), weight was 52 kg (BMI 22), and OFC was 55 cm (−0.4 SD). Facial appearance was non-dysmorphic and no physical malformation was observed. She had good eye contact with her parents, but showed very little interest in her environment and spoke less than 20 words. Speech perception was apparently better, and hearing was tested normal. Cognitive evaluation at age 18 years (WAIS-IV) yielded an IQ of 37 in the range of moderate intellectual disability. Aggressive behavior was assessed by the Achenbach Child Behavior Checklist (CBCL/6–18). A cranial MRI obtained at age 22 years showed normal brain anatomy.

Patient 2.2 is the younger brother of patient 2.1. He was born at term after an uneventful pregnancy. Birth weight was 4,000 g (+1.6 SD), length and OFC were not documented. He was breast-fed without problems for 11 months. Global developmental delay was noted in childhood, he started to walk and spoke first words at age 2.5 years. Generalized seizures and ataxic gait were

TABLE I. Summarized Clinical Findings in the Affected Individuals

		Patient 1.1*	Patient 1.2*	Patient 1.3*	Patient 2.1**	Patient 2.2**	Patient 3.1	Patient 4.1	Patient 4.2
NDST1 mutation (NM_001543)		c.2126G > A (p.Arg709Gln)	c.2126G > A (p.Arg709Gln)	c.2126G > A (p.Arg709Gln)	c.1926G > T (p.Glu642Asp)	c.1926G > T (p.Glu642Asp)	c.1918T > C (p.Phe640Leu)	c.1831G > A (p.Gly611Ser)	c.1831G > A (p.Gly611Ser)
Gender		male	female	male	female	male	female	male	female
Parental consanguinity		first cousins	first cousins	first cousins	first cousins	first cousins	first cousins	likely endogamy	likely endogamy
Ethnic origin		Iranian	Iranian	Iranian	Iranian	Iranian	Turkish	Turkish	Turkish
Birth		38w	40w	40w	38w	38w	41w, after preterm labor in the seventh month of pregnancy, cesarean section	at term, vacuum extraction with subsequent caput succedaneum and cephalhematoma	at term
	BW	high for gestational age	high for gestational age	high for gestational age	4200 g (+2.4 SD)	4000 g (+1.6 SD)	4390 g (+1.9 SD)	3850 g (+0.5 SD)	ND
	BL	ND	ND	ND	ND	ND	51 cm (−0.6 SD)	52 cm (−0.2 SD)	ND
	OFC	34 cm (−0.2 SD)	35 cm (+0.1 SD)	35 cm (+0.1 SD)	ND	ND	35.5 cm (+0.2 SD)	ND	ND
Age at last examination		36y	32y	28y	18y	12y	11y 9m	12y	4y 2m
Postnatal growth	Weight at last examination	normal for age and height	normal for age and height	normal for age and height	52 kg (BMI 22)	55 kg (BMI 34)	40 kg (BMI 18)	normal for age and height	22 kg (BMI 20)
	Height at last examination	153 cm (−3.7 SD)	145 cm (−3.1 SD)	150 cm (−4.1 SD)	155 cm (−1.4 SD)	126 cm (−3.1 SD)	149 cm (−0.1 SD)	147.5 cm (−1.2 SD)	105 cm (0.9 SD)
	OFC at last examination	55 cm (−1.3 SD)	54 cm (−1 SD)	55 cm (−1.3 SD)	55 cm (− 0.4 SD)	53 cm (−1 SD)	53.5 cm (−0.7 SD)	60.5 cm (+3.3 SD)	54 cm (+2.4 SD)
Psychomotor development	Gross motor development	delayed	delayed	delayed	delayed	delayed	mildly delayed	delayed	delayed
	Fine motor development	ND	ND	ND	delayed	delayed	delayed	delayed	delayed
	Language	expressive delayed	expressive delayed, slurred speech	expressive delayed	expressive delayed	expressive: delayed, perceptive: mildly delayed	expressive: delayed, perceptive: mildly delayed	profound expressive speech delay; speech perception better	severe expressive speech delay
	Social development	aggression (DASH-II)	increased irritability, generally quiet temperament (parent report)	aggression, self-injurious behavior (DASH-II)	aggression (CBCL/6-18)	aggression, self-injurious behavior (parent report)	aggression, self-injurious behavior, gender dysphoria (parent report)	low activity, quiet temperament (parent report)	well-balanced, kind temperament (parent report)
	Intellectual disability	severe (IQ 30; WAIS-IV)	severe (IQ 26; WAIS-IV)	severe (IQ 26; WAIS-IV)	moderate (IQ 37; WAIS-IV)	moderate-severe (estimated)	mild (IQ 64; HAWIK-IV)	severe (IQ 20–35; estimated)	moderate (estimated)
Epilepsy	Seizures (onset)	+ (childhood)	+ (childhood)	+ (childhood)	+ (14y)	+ (14y)	−	−	−
	Therapy response	+	+	+	+	+	−	−	−
Motor system	Ataxia	+	−	−	−	+	−	−	−
	Muscular hypotonia	+	−	+	−	−	+	+	+
Eyes	Visual function	normal	normal	normal	normal	myopia	normal	normal	normal
	Strabismus	−	+	−	−	+	−	−	not when rested, exotropia when tired
	Nystagmus	−	−	−	−	+	−	−	−
Hearing		normal	normal	normal	normal	normal	normal	normal	normal
Joints		normal	normal	normal	normal	normal	hypermobility of wrist and finger joints	normal	normal
Physical malformations		−	−	−	−	−	−	−	−
Facial dysmorphism		−	−	−	−	−	protruding chin, synophrys, gaps between teeth	large protruding ears	facial hypotonia, epicanthal folds, mild ptosis
MRI (age)		normal	ND	ND	normal (22y)	normal (10y)	normal (7.5y)	symmetrically enlarged lateral ventricles, less dilated 3rd ventricle, otherwise normal brain anatomy (8y)	ND
EEG		normal	ND	ND	ND	normal	normal	normal	ND
Other anomalies		sleep disturbance, polyuria		flat feet		sleep disturbance	sleep disturbance, frequent constipation	flat feet	

Abbreviations are as follows: y, years; m, months; w, weeks; ND, no data available; BW, birth weight; BL, birth length; OFC, occipito frontal head circumference; IQ, intelligence quotient.

*Najmabadi et al. [2011], Family M8600277.

**Najmabadi et al. [2011], Family M161.

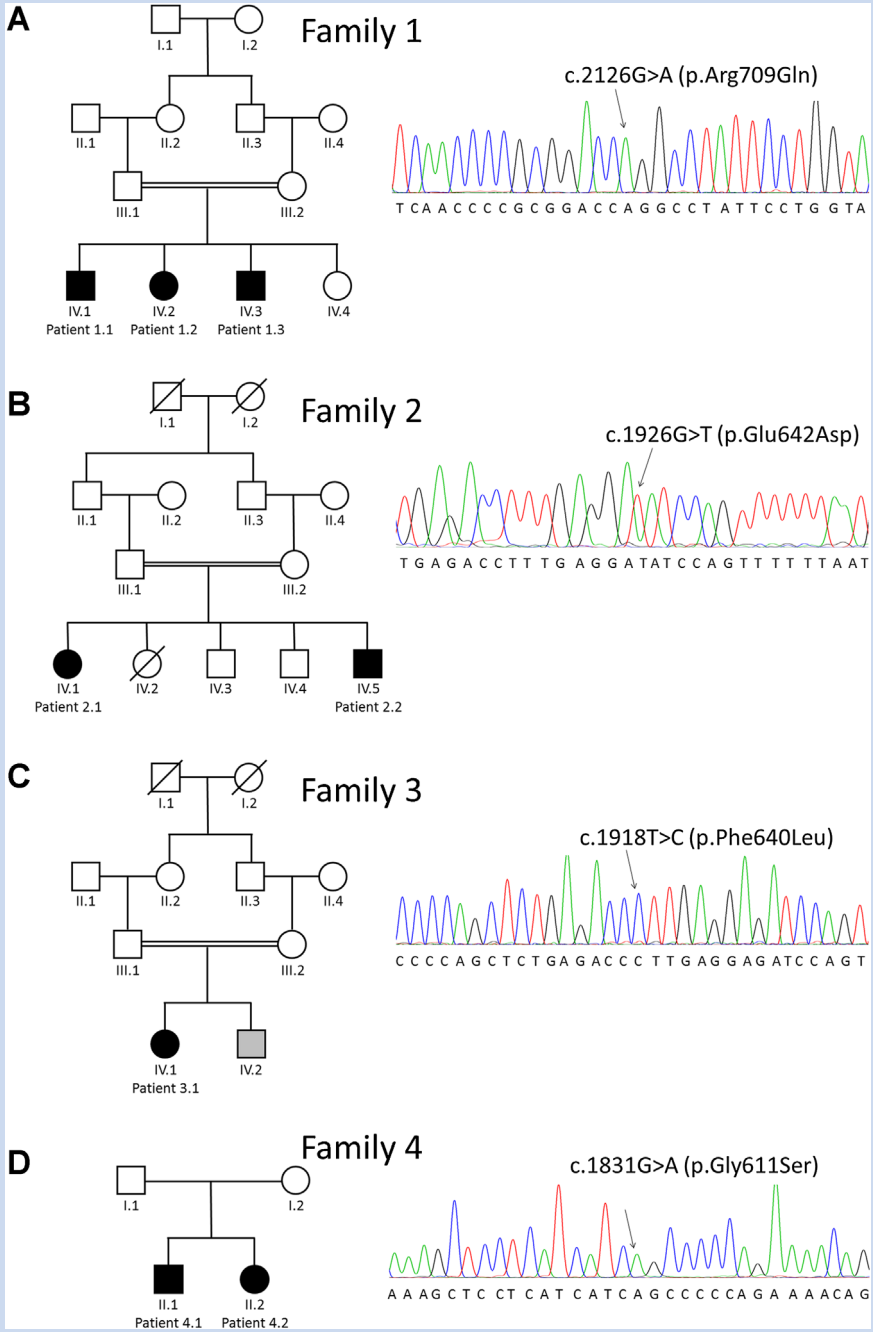

FIG. 1. Pedigrees and electropherograms of the four families. Black fill defines individuals with homozygous *NDST1* mutations, gray fill defines sibling with glycogen storage disease type 1b. (A) Family 1 (M8600277), homozygous *NDST1* mutation c.2126G > A (p.Arg709Gln). (B) Family 2 (M161), homozygous *NDST1* mutation c.1926G > T (p.Glu642Asp). (C) Family 3 (ER44462), homozygous *NDST1* mutation c.1918T > C (p.Phe640Leu). (D) Family 4 (MZ-778/12), homozygous *NDST1* mutation c.1831G > A (p.Gly611Ser).

noted at age 12 years. At this age, height was 126 cm (−3.1 SD), weight was 55 kg (BMI 35), and head circumference was 53.5 cm (−1 SD). IQ was not formally tested but he was unable to count money, nor was he able to memorize simple family names or degrees of relationships. The parents reported aggressive, self-injurious behavior, sleep disturbances, impaired verbal expression and communication problems. Visual testing showed myopia, strabismus, and horizontal nystagmus with no other abnormality. Facial appearance was normal and a cranial MRI obtained at age ten years was normal.

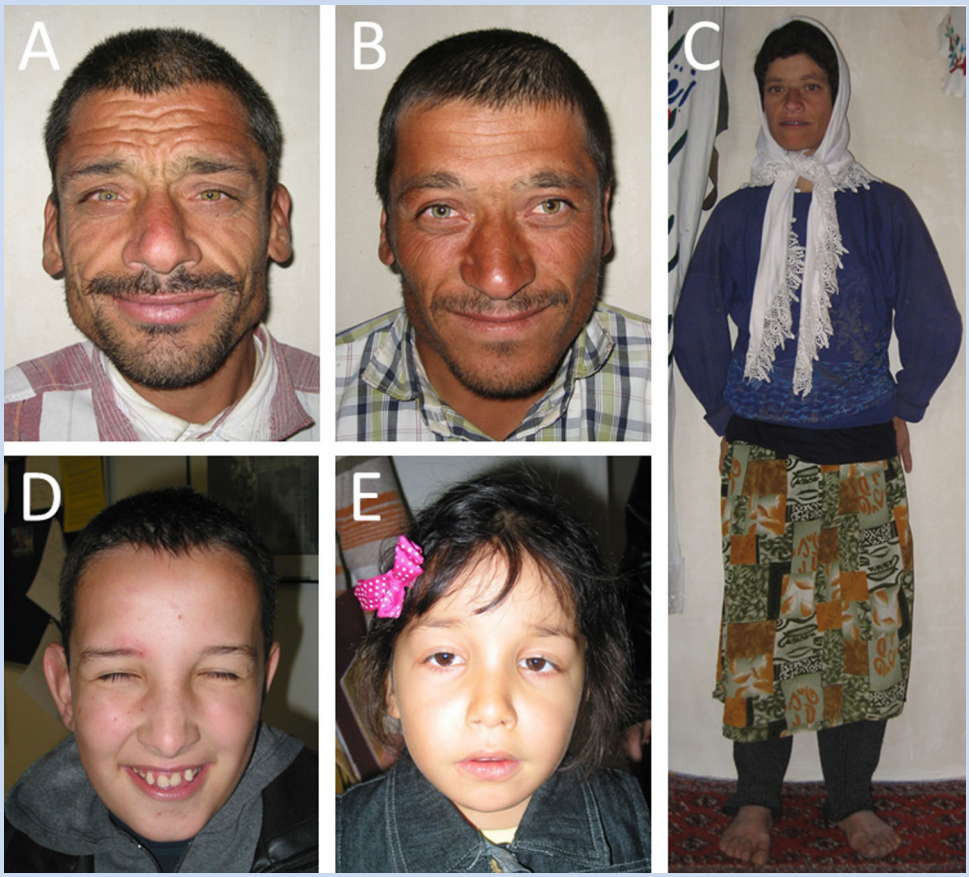

FIG. 2. Photographs of affected individuals from families 1 and 4. (A) Patient 1.1. (B) Patient 1.3. (C) Patient 1.2. (D) Patient 4.1 aged 12 years, exhibiting large protruding ears. (E) Patient 4.2 aged 4 years 3 months, exhibiting mild ptosis of eyelids, epicanthal folds, and facial hypotonia.

Family 3

Patient 3.1 is the first child of healthy consanguineous parents (first cousins), originating from Turkey. The pregnancy was uneventful except preterm labor in the seventh month of pregnancy. The girl was born by cesarean at 41 weeks of gestation. Her weight was 4,390 g (+1.9 SD), length 51 cm (−0.6 SD), OFC 35.5 cm (−0.4 SD), APGAR 10/10, umbilical cord pH 7.28. Development initially appeared normal; she walked and spoke first words at age 12 months. However, in follow-up examinations global developmental delay was noted, with building of two-word phrases at age three years, delayed fine motor development, and muscular hypotonia. Formal developmental testing at age 7.5 years by Wechsler Intelligence Scale for Children (HAWIK-IV) showed an IQ of 64. The patient attended a special needs school. When last seen at age 11 years 9 months, the parents reported difficulties concentrating, hyperactivity, reduced memorization and social interaction problems (aggression, self-injurious behavior, gender dysphoria), as well as enuresis, encopresis, sleep disturbances, and frequent constipation. No history of seizures or deterioration in neurological functions was reported. Growth parameters were all normal at age 11 years 9 months. Her height was 149 cm (−0.1 SD), weight 40 kg (BMI 18), OFC 53.5 cm (−0.7 SD). She spoke only short sentences with limited vocabulary (Turkish and German), but receptive language appeared better. Balance, coordination, and deep tendon reflexes were normal. Hypermobility of the wrist and finger joints resulted in a Beighton score of 4/9. Minor dysmorphic features included a prominent protruding chin, synophrys, wide gaps between the teeth, and a compact trunk. Ophthalmologic and audiometric examinations were normal. Cranial MRI and EEG examinations at age 7.5 years were normal.

Family 4

Patient 4.1 is the first child of a healthy 27-year-old mother and 29-year-old father. His parents were of Turkish origin and not known to be consanguineous, but likely endogamic originating from the same religiously isolated village in Bulgaria. After an uneventful pregnancy, he was born at term by vacuum extraction, with subsequent caput succedaneum, and cephalhematoma. His weight was 3,850 g (+0.5 SD), length 52 cm (−0.2 SD), but his OFC was not documented. Developmental delay and muscular hypotonia were noted in the first year of life; he walked and spoke first words at

age three years. A full neuropediatric evaluation was performed in Germany at age eight years and five months. He had good eye contact with his parents, but showed very little interest in his environment, played stereotypically, and spoke no word. Parents reported a total vocabulary of less than 10–20 words. Receptive language appeared better, and hearing loss was excluded. Cognitive testing at age eight years and five months, using the Snijders-Oomen non-verbal intelligence test (SON-R 2 1/2–7) was not possible due to lack of participation. IQ was estimated within range 20–35. At age 12 years, parents still reported a total vocabulary of maximum 10–20 words and a quiet temperament. He attended a special needs school for the severely intellectually disabled. Growth parameters at 8.5 years were: height 125 cm (−1 SD), weight 31 kg (BMI 20), OFC 59 cm (+3.1 SD); 12 years: height 147.5 cm (−1.2 SD), OFC 60.5 cm (+3.3 SD); parental OFCs were within normal ranges. Minor dysmorphic features included large protruding ears (Fig. 2D) and broad, flat feet. A cranial MRI obtained at age 8 years showed symmetrically enlarged lateral ventricles and a less dilated third ventricle, but otherwise normal brain anatomy.

Patient 4.2, the younger sister of patient 4.1, was born at term after an uneventful pregnancy. Developmental delay and muscular hypotonia became obvious in the second year of life. She walked at age 18–24 months and expressive speech was delayed. Non-verbal cognitive testing using the SON-R 2 1/2 - 7 was attempted at age four years and two months, but she was unable to follow the instructions, neither in German language nor with a Turkish interpreter. Moderate intellectual disability was diagnosed and her developmental age was estimated at less than two years. She attended a special needs kindergarten. The parents described a happy, well-balanced, and kind temperament. At age four years two months, weight and OFC exceeded normal values. Her height was 105 cm (0.9 SD), weight 22 kg (BMI 20), OFC 54 cm (+2.4 SD). Minor dysmorphic features included frontal bossing, facial hypotonia, mild ptosis, epicanthal folds, a single crease on the right hand, short fingers, and a sacral dimple. Hearing tests were repeatedly performed with likely normal results.

Mutations

Using homozygosity mapping and next-generation sequencing approaches, missense mutations in the *NDST1* gene (MIM*600853) could be identified in two further unrelated families with intellectual disability. In patient 3.1, an apparently homozygous mutation c.1918T > C (NM_001543; chr5:149922481T > C; p.Phe640Leu) was identified by exome sequencing, and in patient 4.1, an apparently homozygous mutation c.1831G > A (NM_001543; chr5:149921213G > A; p.Gly611Ser) was identified by custom-designed targeted sequencing (Fig. 1C and D). Direct Sanger sequencing analysis was performed for all available family members, and demonstrated segregation in agreement with a recessive mode of inheritance. The mutations have not been reported in dbSNP 137, 1000 Genomes and the NHLBI Exome Variant Server, were absent in 100 to 340 chromosomes of ethnically matched controls, and affected highly conserved amino acids. Analyses with SIFT, PolyPhen-2, and MutationTaster predicted the amino acid substitutions to be damaging.

In silico mutation modeling suggested that all four mutations alter the sulfotransferase domain of NDST1. Phenylalanine and glutamic acid at positions 640 and 642, respectively, are located in a loop of the substrate-binding site and establish tight contacts with the substrate (Fig. 3A and C). These interactions are lost, when the respective residue is substituted by an amino acid with a shorter side chain (leucine or aspartic acid) (Fig. 3B and D). Arginine at position 709 forms direct interactions with cysteine at position 751, as well as water-mediated interactions with glutamine at position 613 and arginine at position 750 (Fig. 3E). These interactions cannot be maintained by the shorter and uncharged glutamine at position 709 in the mutant (Fig. 3F), which is predicted to cause a drastic destabilization of the three-dimensional fold of NDST1. Glycine at position 611 is located in a loop in spatial proximity to the 3′-phosphoadenosine 5′-phosphate (PAP) binding site (Fig. 3G). It adopts an unusual backbone conformation ($\Phi = 97^\circ$, $\Psi = -147^\circ$), exclusively feasible for the small amino acid glycine lacking a side chain. Consequently, a replacement by the larger serine results in steric clashes with adjacent amino acids, in particular proline at position 612 (Fig. 3H). These clashes will result in a destabilization of the structure and conformational rearrangement of the loop, which most likely also affects PAP binding. In summary, all four amino acid substitutions are predicted to change the substrate–binding capability and/or three-dimensional structure of the sulfotransferase domain of NDST1.

Drosophila Behavioral Testing

In order to evaluate NDST1 function in the brain, the *Drosophila* ortholog of the NDST family, *sulfateless* (*sfl*) [Aikawa et al., 2001], was knocked down pan-neuronally by driving the RNAi expression with elev-Gal4. To strengthen the knockdown efficiency, we co-expressed dicer2 [Dietzl et al., 2007]. Given the fact that brain knockdown had no impact on survival or morphology, and locomotion appeared normal in negative geotaxis assay (data not shown), we investigated long-term memory as a higher cognitive function by the courtship-conditioning paradigm [Siwicki and Ladewski, 2003]. In this paradigm, male flies learn to adapt their courtship behavior. In response to rejection by a previously mated unreceptive female during training, the male fly will reduce its courtship attempts when exposed again to another unreceptive female during the test phase. This was shown for control flies (control-RNAi/elav-Gal4 and uas-sfl^{RNAi}/+) by significantly different courtship indices CI (Fig. 4A) and high learning indices LI (Fig. 4B). In contrast, CI of naïve and trained *sfl* knockdown flies were not significantly different (Fig. 4A), resulting in a severely reduced LI of 5% (Fig. 4B), indicating that long-term memory of *sfl* knockdown flies was severely impaired. Levels of naïve courtship however were similar for all genotypes indicating that sensorimotor functions did not influence the memory phenotype observed in *sfl* knockdown (Fig. 4A).

DISCUSSION

Autosomal recessive intellectual disability is extremely heterogeneous in terms of its underlying genetic causes, and the total

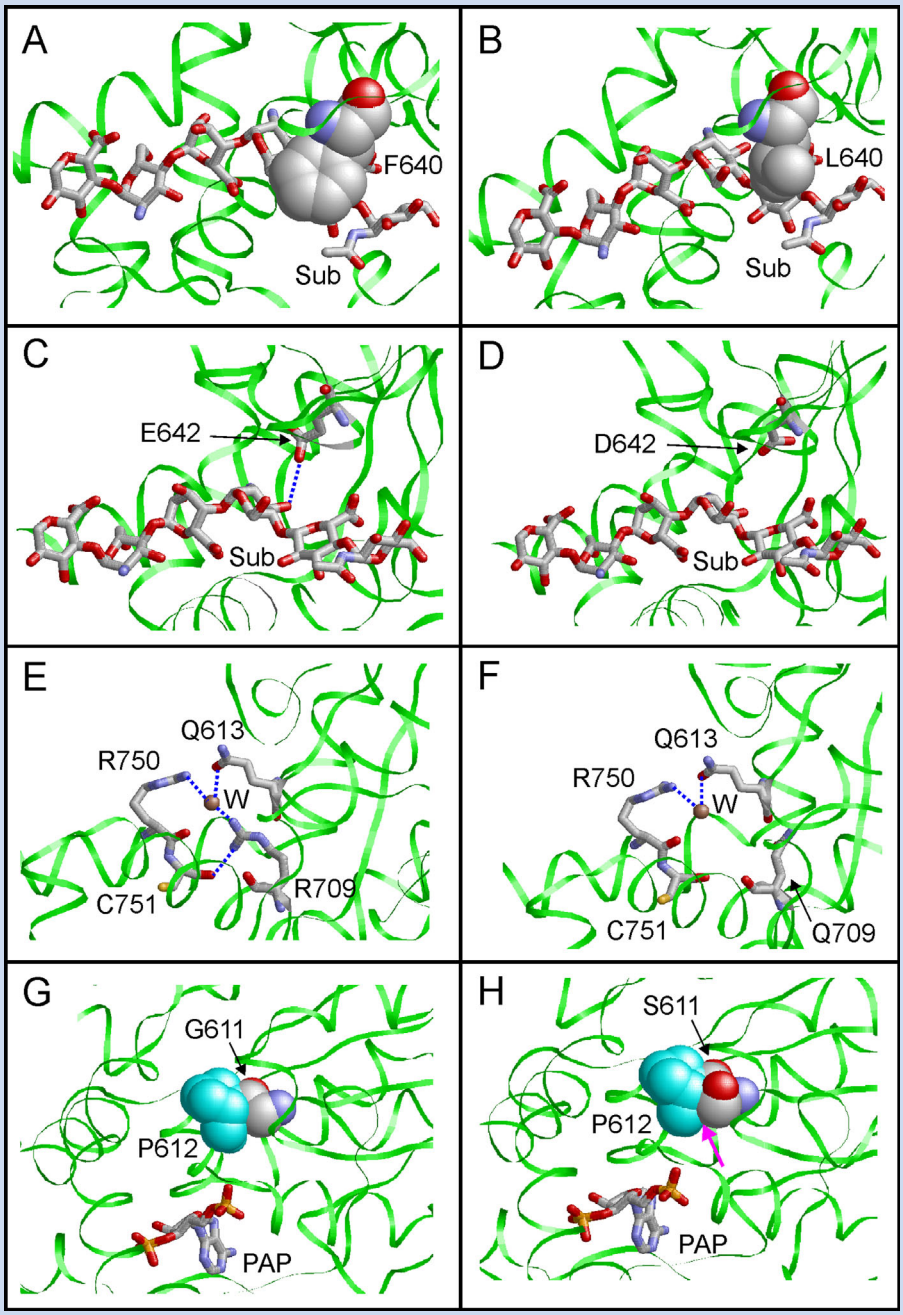

FIG. 3. Structural effect of the mutations in NDST1. (A) Location of F640 (space-filled) in the substrate binding site of NDST1. A hexasaccharide substrate (Sub) is shown in stick presentation and colored according to the atom type. The backbone of NDST1 is indicated as green ribbon. (B) Effect of the F640L mutation revealing that the lid formed by F640 on the substrate cannot be formed by the smaller leucine sidechain. (C) Location of E642 (stick presentation) in the substrate-binding site of NDST1. A hydrogen bond formed with a hydroxyl group of one N-acetylglucosamine unit is indicated as blue dotted line. (D) The hydrogen bond cannot be formed by the shorter sidechain in the E642D mutation. (E) R709 forms hydrogen bonds (blue dotted lines) with Q613, R750, and C751. Two of these interactions are mediated by a water molecule (W; shown as brown ball). (F) Effect of the R709Q mutation, showing that several stabilizing interactions are lost compared to the wildtype. (G) G611 is located in a turn close to the PAP binding site (stick presentation). G611 and P612 (cyan) are shown in space-filled representation. G611 and PAP are colored according to the atom type. (H) Effect of the G611S mutation resulting in steric clashes between S611 and P612 (magenta arrow).

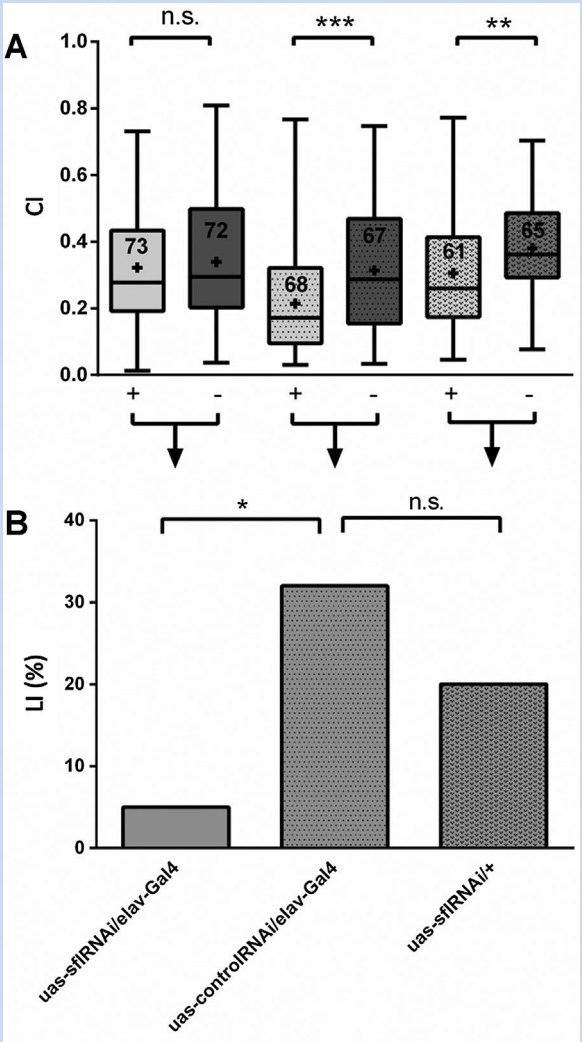

FIG. 4. Brain knockdown of *sulfateless* results in long-term memory deficit in *Drosophila*. (A) Mean courtship indices (CIs) of trained (+) and naïve (−) flies are displayed. Learning ability was assayed using a non-parametric permutation (randomization) test. *P*-values are indicated for H_0, LI = 0 and thus show the probability that flies did not learn-a low *P*-value signifies that flies learned. Naïve and trained control flies (uas-controlRNAi/elav-Gal4 and uas-sfl^{RNAi}/+) show a significant difference (*, $P = 0.00051$ and **, $P = 0.00388$), whereas (+) and (−) Sfl-KD (uas-sfl^{RNAi}/elav-Gal4) flies show no difference (NS, $P = 0.29232$), indicating impairment in learning ability. Numbers in the boxplot depict sample sizes (n) for each genotype and condition. (B) The relative difference between naïve and trained CIs, called learning index (LI), is shown. LI of sfl-KD was significantly lower than LI of control (uas-controlRNAi/elav-Gal4) (**, $P = 0.03228$), while the two controls were not significantly different. Asterisks denote significance (*, $P < 0.05$; **, $P < 0.01$; ***, $p < 0.001$).**

number of ARID genes is estimated to run into the thousands [Musante and Ropers, 2014]. Complicating the identification of novel disease-related genes is the fact, that offspring of consanguineous parents typically carry numerous rare and difficult-to-assess homozygous variants. A detection of mutations in the same gene in unrelated patients is therefore important to provide further evidence for its disease causality.

Two missense mutations in *NDST1* were initially reported by Najmabadi et al., [2011], each in one consanguineous family with autosomal recessive intellectual disability. We now identified two additional families with *NDST1* mutations and provide a first characterization of the human phenotype, in a total of eight patients from four unrelated families, including two families previously reported by Najmabadi et al. [2011]. Their clinical features exhibited remarkable overlap (Table I), supporting the pathogenic impact of the described *NDST1* missense mutations. The main symptom was an impairment of motor and cognitive functions. In addition, there was evidence for a frequent occurrence of muscular hypotonia and postnatal growth deficiency, affecting particularly adult height, but not OFC. Some patients developed seizures in childhood or adolescence. Malformations of the brain or other organs were not detectable (apart from enlarged lateral ventricles in one patient). In addition, mutation modeling performed for all four mutations also argues for a functional relevance of the amino acid substitutions.

In mouse models and murine cells, the role of Ndst1 in differentiation and development is well established. Ndst1 is one in a family of four bifunctional GlcNAc N-deacetylase/N-sulfotransferase enzymes (Ndsts), catalyzing the first and essential modifying steps (N-deacetylation/N-sulfation of GlcNAc residues) in heparan sulfate (HS) biosynthesis. Different forms of HS are present on most cell membranes and in extracellular matrix proteoglycans, and by interaction with growth factors and morphogens are crucial for embryonic differentiation and development [Bernfield et al., 1999; Esko and Lindahl, 2001]. An important role of HS in the development and maintenance of the central nervous system is indicated by studies in conditional knockout mice and cell cultures [Inatani et al., 2003; Irie et al., 2012].

The expression of HS sulfotransferase genes is spatiotemporally regulated [Yabe et al., 2005]. Ndst1 is expressed ubiquitously, and purified protein has higher N-sulfotransferase activity compared to the other three isozymes [Aikawa et al., 2001]. Mice lacking Ndst1 function die perinatally and exhibit variable developmental defects of the forebrain- and neural crest-derived structures (telencephalon, diencephalon, eyes, neuro- and viscerocranium) and, to a lower extent, the neural tube [Grobe et al., 2005; Pallerla et al., 2007]. The developmental defects were attributed to impaired Wnt, sonic hedgehog and fibroblast growth factor signaling [Pallerla et al., 2007]. Mouse embryonic stem cells deficient of both Ndst1 and Ndst2 resulted in undersulfation of heparan sulfate and a differentiation block at the primitive ectoderm/endoderm stage, while mesodermal differentiation appeared to be normal [Forsberg et al., 2012].

Heparan sulfate also exhibits crucial functions in *Drosophila* development [Perrimon and Bernfield, 2000] and the *NDST* ortholog *sulfateless* is required for the development of the stomatogastric nervous system [Lin and Perrimon, 1999]. Genes,

pathways and regulatory networks are well conserved between human and *Drosophila* [Bellen et al., 2010]. Increasing evidence indicates that flies represent a powerful animal model to gain insights into ID/ARID gene functions [Abbasi-Moheb et al., 2012; Gatto and Broadie, 2011]. Our data indicate that *sfl* brain-specific knockdown results in long-term memory deficits in flies, strongly suggesting a crucial role for *sfl* in higher cognitive functions. We speculate, that a regulatory role of sfl in the Wnt receptor signaling pathway [Kamimura et al., 2011] might be responsible for the observed long-term memory phenotype, as Wnt signaling has recently been shown to be required for long-term olfactory memory formation in *Drosophila* [Tan et al., 2013].

Our data support an important function of NDST1 also in human development. Sibs carrying the same missense mutation in *NDST1* exhibited a strikingly similar clinical phenotype, whereas certain differences were apparent between the families. It is unclear at this stage, whether the variability is attributable to the different *NDST1* mutations or to additional genetic or non-genetic factors. All so far described homozygous mutations are missense mutations clustering in the sulfotransferase domain, and are predicted to incapacitate the enzymatic function. Whether amino acid substitutions in other protein domains cause a similar phenotype, and if a complete loss of the enzymatic activity would be compatible with life in humans, remains unclear at this point. Descriptions of further patients will be necessary to elucidate correlations between particular mutant alleles and consistent clinical phenotypes. In addition, further analyses regarding NDST1 activity and the extent and pattern of sulfation in patients' cell lines will be required to test the functional consequences of specific missense mutations.

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