

# Mutations in *NSUN2* Cause Autosomal-Recessive Intellectual Disability

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With a prevalence between 1 and 3%, hereditary forms of intellectual disability (ID) are among the most important problems in health care. Particularly, autosomal-recessive forms of the disorder have a very heterogeneous molecular basis, and genes with an increased number of disease-causing mutations are not common. Here, we report on three different mutations (two nonsense mutations, c.679C>T [p.Gln227\*] and c.1114C>T [p.Gln372\*], as well as one splicing mutation, g.6622224A>C [p.Ile179Argfs\*192]) that cause a loss of the tRNA-methyltransferase-encoding *NSUN2* main transcript in homozygotes. We identified the mutations by sequencing exons and exon-intron boundaries within the genomic region where the linkage intervals of three independent consanguineous families of Iranian and Kurdish origin overlapped with the previously described *MRT5* locus. In order to gain further evidence concerning the effect of a loss of *NSUN2* on memory and learning, we constructed a *Drosophila* model by deleting the *NSUN2* ortholog, *CG6133*, and investigated the mutants by using molecular and behavioral approaches. When the *Drosophila melanogaster NSUN2* ortholog was deleted, severe short-term-memory (STM) deficits were observed; STM could be rescued by re-expression of the wild-type protein in the nervous system. The humans homozygous for *NSUN2* mutations showed an overlapping phenotype consisting of moderate to severe ID and facial dysmorphism (which includes a long face, characteristic eyebrows, a long nose, and a small chin), suggesting that mutations in this gene might even induce a syndromic form of ID. Moreover, our observations from the *Drosophila* model point toward an evolutionarily conserved role of RNA methylation in normal cognitive development.

## Introduction

Intellectual disability (ID) is characterized by mental-functioning limitations that manifest as deficiencies in conceptual, practical, and social skills and an intelligence quotient (IQ) below 70. This condition, which has an estimated prevalence ranging between 1% and 3% in the general population,<sup>1–4</sup> is a major problem in modern health care. A considerable number of cases are caused by genetic defects, which can be identified cytogenetically in about 15% of individuals with ID. Recently, submicroscopic chromosomal aberrations were also found to be of similar importance, yet the large remainder of ID cases with unclear etiology most likely carry single genetic defects (for an in-depth review of ID, see Ropers<sup>1</sup>). During the past decade, research into the genetic basis of recessive gene defects was mainly focused on familial forms of ID with an X-chromosomal mode of inheritance because autosomal-recessive disorders rarely occur in the generally small and exogamous families from the populations of industrialized countries. X-chromosomal genetic defects,

however, only account for about 10% of cases, whereas the large majority of ID-causing mutations are assumed to affect autosomal genes.<sup>2</sup> In order to investigate the causes of autosomal-recessive ID (ARID), we therefore started a large-scale study by performing linkage analysis followed by homozygosity mapping in large consanguineous families from Iran. During the course of this project, we identified numerous loci for ARID,<sup>3,4</sup> and mutation analysis led to the identification of several genes in which damaging or deleterious mutations caused cognitive dysfunction with or without additional clinical features in homozygous individuals.<sup>5–12</sup> In most cases, however, only one allelic disease-causing mutation was found, testament to the high degree of genetic heterogeneity of ARID. In this study, we resolve the underlying genetic defect of *MRT5*<sup>3</sup> by reporting on three different deleterious mutations in *NSUN2* (MIM 610916); we discovered these mutations in the original family,<sup>3</sup> in one additional independent consanguineous Iranian family in which several members suffer from a putatively syndromic form of ID, and in one Kurdish family recruited from Germany.<sup>4</sup>

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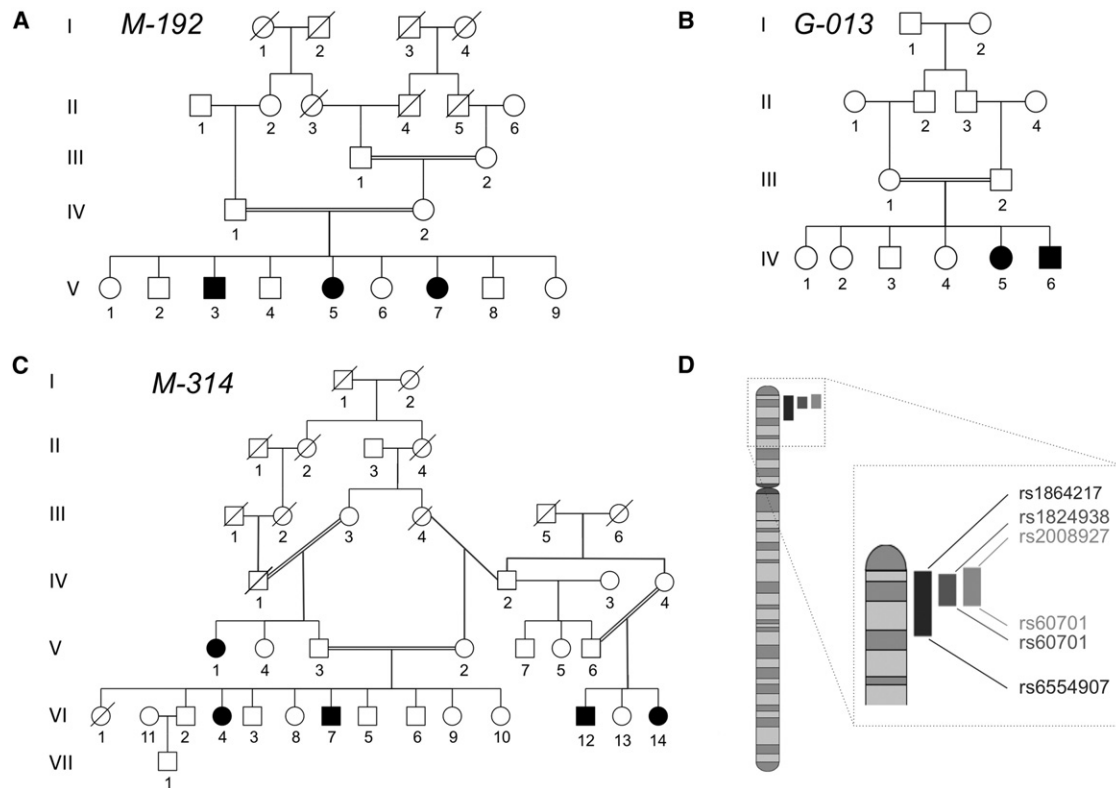
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**Figure 1. Family Pedigrees and Linkage Intervals**

Filled symbols indicate affected individuals (A–C). Bars alongside the idiogram of chromosome 5 shown in (D) represent the overlapping linkage intervals. The enlarged picture detail shows the flanking markers of the linkage intervals from the different families, distinguished by shades of gray (dark, G-013; medium, M-192; and light, M-314).

*NSUN2* encodes a methyltransferase, which catalyzes the intron-dependent formation of 5-methylcytosine at C34 of tRNA-leu(CAA).<sup>13</sup> All three mutations cause loss of *NSUN2* (UniProtKB/Swiss-Prot: Q08J23). In support of our hypothesis that defects in *NSUN2* lead to cognitive handicap, we show that the loss of the *Drosophila NSUN2* ortholog, *CG6133* (UniProtKB/Swiss-Prot: Q9W4M9), causes a short-term-memory deficit in *Drosophila*.

## Subjects and Methods

### Clinical Information

This study was performed in accordance with the ethical standards of the responsible institutional and national committees on human experimentation. The affected individuals and their families were recruited by the Genetic Research Center, Teheran, Iran, and by local clinical geneticists from Germany. The family pedigrees are shown in Figures 1A–1C, and facial aspects of affected individuals from families M-192 and G-013 are presented as part of the supplemental data (Figure S1, available online). The degree of ID in the affected family members ranges from moderate to severe (Table 1). In two families (M-314 and G-013), the affected individuals are microcephalic, and spasticity was reported in the older affected individuals of families M-192 and M-314. Short stature was found in about 50% of the affected individuals. Brain malformations were not observed. However, some facial characteristics, including a long face, characteristic eyebrows, a long nose,

and a small chin, were seen in all affected individuals (see also Table 1 and Figure S1).

After obtaining written informed consent from the parents of each family, blood was taken (according to standard protocols) from the affected individuals, their parents, and their healthy siblings for nucleic-acid isolation and, in some cases, for the establishment of Epstein–Barr virus (EBV)-transformed lymphoblastoid cell lines.

### Mutation Screening

For mutation screening, the exons and exon-intron boundaries of RefSeq genes within the respective linkage intervals were sequenced (primer sequences and PCR conditions are available upon request).

### RNA Experiments

RNA was isolated and RT-PCR experiments were performed as described previously.<sup>11</sup> Primer sequences are available upon request.

### *Drosophila* Genetics

Fly strains were reared under standard laboratory conditions<sup>15</sup> (25°C and a light/dark cycle of 12/12h). Transgenes were established in stock #9737 from the Bloomington *Drosophila* Stock Center. The FM7i, P{ActGFP}JID3 balancer was used for balancing, and *elav-GAL4*<sup>16</sup> was used for the re-expression experiment.

A deletion mutant (*dNsun2<sup>ex1</sup>*) was constructed with the parental lines PBac{WH}dgt4(f06125) and PBac{RB}e02478

**Table 1. Features of Affected Individuals**

	M-192			M-314			G-013		Khan et al. <sup>a</sup>			Total
	V:3	V:5	V:7	V:1	VI:4	VI:7	IV:5	IV:6	II:3	II:4	II:5	
<b>Sex</b>	male	female	female	female	female	male	female	male	female	female	female	8 females and 3 males
<b>Age (years)</b>	29	28	17	61	27	22	9	6	14	13	6	
<b>Intellectual disability</b>	moderate	moderate	moderate	moderate	moderate	moderate	moderate	severe	moderate	moderate	moderate	moderate to severe
<b>Height (cm/SD)</b>	166/–1.7	147/–3.1	148/–2.9	152/–2.3	161/–0.9	168/–1.5	110/–4.1	99/–3.8	152/–1.4	136/–3.0	n.r.	
<b>OFC (cm/SD)</b>	53/–2.2	55/0	56/+0.8	45/–7.1	52/–2.1	51.5/–3.3	48/–2.9	47.5/–2.8	50/–2.9	49/–3.4	46/–3.5	
<b>Short stature</b>	–	+	+	+	–	–	+	+	+	+	n.r.	7/10
<b>Microcephaly</b>	+	–	–	+	+	+	+	+	+	+	n.r.	8/10
<b>Strabismus</b>	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	+	+	+	–	–	3/5
<b>Facial dysmorphism</b>	+	+	+	+	+	+	+	+	+	+	+	11/11
<b>Muscular hypertonia</b>	+	+	+	+	+	+	–	–	+	+	+	9/11
<b>Elevated CPK</b>	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	+	n.r.	–	1/2
<b>Others</b>	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	n.r.	crypt-orchidism and seizures	n.r.	n.r.	n.r.	

The following abbreviations are used: SD, standard deviation; OFC, occipitofrontal circumference; n.r., not reported; and CPK, creatine phosphokinase.

<sup>a</sup>See also Table 1 in Khan et al.<sup>14</sup>

(Exelixis at Harvard Medical School) according to Parks et al.<sup>17</sup> The deletion was validated by genomic PCR.

Before behavioral testing, all used chromosomes were out-crossed (on the basis of eye color) for more than five generations to *w*<sup>1118</sup>.

The genotypes used for the behavioral tests were the following: (1) *w*<sup>1118</sup>/*y, +/+*, *+/+*; (2) *dNsun2<sup>ex1</sup>/y, +/+*, *+/+*; (3) *dNsun2<sup>ex1</sup>/y, +/+*, *UAS-dNsun2<sup>+</sup>/+*; (4) *dNsun2<sup>ex1</sup>/y, +/+*, *elav-GAL4/+*; and (5) *dNsun2<sup>ex1</sup>/y, +/+*, *UAS-dNsun2<sup>+</sup>/elav-GAL4*.

Canton-S flies (CS) were used as an additional control group during the behavioral tests.

### **Drosophila Behavioral Testing**

3-octanol (OCT) and 4-methylcyclohexanol (MCH) were used as olfactory cues for the aversive olfactory experiments as previously described.<sup>18,19</sup> Flies were tested for odor memory 3 min after the training session (short-term memory [STM]).

Prior to the actual learning paradigm, the flies were tested for their innate odor and shock avoidance, a process which also involves elementary locomotion testing (Table S1). On the basis of the genetic situation, only male flies were taken into account for the statistical analysis.

### **Statistics**

Data were analyzed with IBM SPSS Statistics 17. LSD one-way ANOVA tests were performed, and asterisks are used to denote significance (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.005$ ; and ns,  $p > 0.05$ ). All data represent means  $\pm$  the standard error of the mean (SEM) of performance indices.

### **Molecular Cloning**

The *dNsun2* open reading frame was subcloned from a cDNA clone (GenBank accession number AY061496.1) into pENTR-D/TOPO (Invitrogen) with the use of the following primers: 5'-CAC CATGGGTCGAAACCAGAAGC-3' (forward) and 5'-ACTGGTGGC CACCACTTCG-3' (reverse).

The PhiC31 system with defined landing sites in the *Drosophila* genome was used for the generation of transgenic animals.<sup>20–22</sup> Therefore, pENTR-D *dNsun2<sup>+</sup>* was recombined with the pUAST-attB-rfA vector (kindly provided by C. Klämbt at the University of Münster) and yielded pUAST-attB-*dNsun2<sup>+</sup>*.

From pENTR-D *dNsun2<sup>+</sup>*, a 1,852 bp fragment, flanked by NotI and Sall, was subcloned into pBluescript II KS+ for in situ hybridization.

All sequences were validated by double-strand sequencing.

### **In Situ Hybridizations and Image Acquisition**

Whole-mount embryonic in situ hybridizations were performed as described by the Berkeley *Drosophila* Genome Project (see Web Resources). For the *dNsun2* sense RNA probe, pBluescript KS+ *dNsun2* was cut with Sall and was transcribed in vitro with T7 polymerase. For antisense probes cut with NotI, T3 polymerase was used.

Light microscopy was performed on a Zeiss Axioplan microscope with a Jenoptik ProgRes C5 camera.

### **ClustalW and Phylogenetic Analyses**

Sequence alignments (ClustalW) and phylogenetic analyses were performed with the MacVector software 11.1.1 (MacVector). The phylogenetic tree was generated with the neighbor-joining method and shows the evolutionary relationship between *Drosophila*

NSUN2, the mouse and human orthologs, and other members of the evolutionarily conserved NOL1/NOP2/Sun domain family. The following UniProtKB/SwissProt entries were included in the analyses: NOP2 (mouse, Q922K7; human, P46087), NSUN2 (mouse, Q1HFZ0; human, Q08J23; *Drosophila*, Q9W4M9), NSUN3 (mouse, Q8CCT7; human, Q9H649), NSUN4 (mouse, Q9CZ57; human, Q96CB9), NSUN5 (mouse, Q8K4F6; human, Q96P11), NSUN6 (mouse, Q7TS68; human, Q8TEA1), and NSUN7 (mouse, Q14AW5; human, Q8NE18).

## **Results**

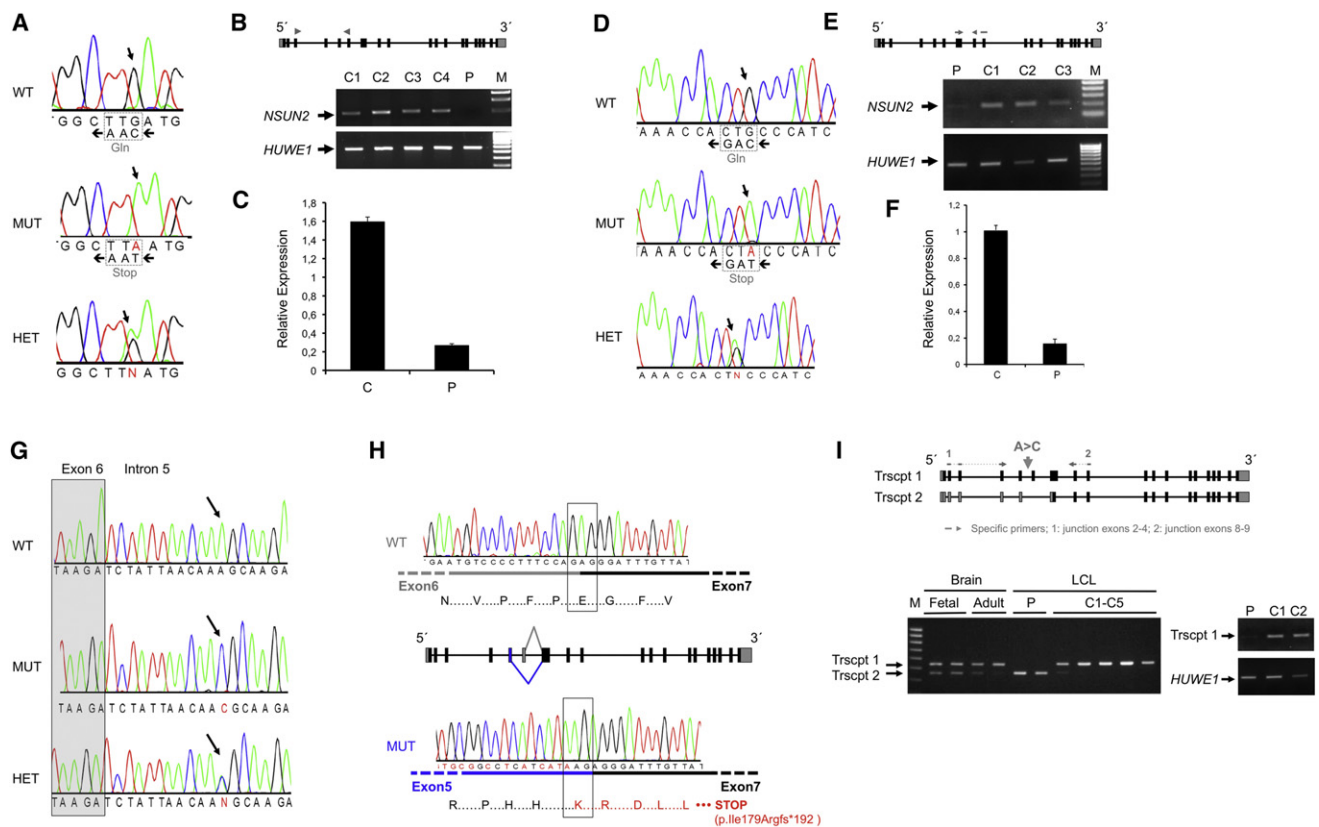
The pedigrees of the investigated families are presented in Figure 1, and the features of affected individuals are listed in Table 1 (facial aspects of the affected individuals are available as part of the Supplemental Data). As shown in Figure 1D, linkage analysis and homozygosity mapping in two independent families affected by ARID<sup>4</sup> led to the identification of linkage intervals (with a LOD score of 2.3 for family G-013 and 3.9 for family M-314) that overlapped with the previously described *MRT5* locus.<sup>3</sup> Mutation screening in the complete protein-coding and adjacent intronic regions within *MRT5* (family M-192) by Sanger-Sequencing revealed a nonsense mutation, c.679C>T (p.Gln227\*) (RefSeq NM\_017755.5), affecting *NSUN2* (Figures 2A–2C), which encodes four tRNA-methyltransferase transcripts that are expressed in the brain at various developmental stages (Figure S2). Subsequently, we screened this gene in the two families with overlapping linkage intervals. This screening revealed an additional nonsense mutation, c.1114C>T (p.Gln372\*) (NM\_017755.5), in family G-013 (Figures 2D–2F) and an intronic exchange of an adenine for a cytosine 11 nucleotides before exon 6 (g.6622224A>C [NC\_000005.9]) (Figure 2G) in family M-314.

Both nonsense mutations cause a complete loss of *NSUN2* transcripts in the homozygous affected individuals (Figures 2B, 2C, 2E, and 2F). The intronic nucleotide change causes exon 6 to be skipped during splicing, which leads to a change of reading frame (Figure 2H) and a stop codon starting at nucleotide 71 of exon 7 (p.Ile179Argfs\*192). This stop codon results in the loss of the main transcript in tissues from affected individuals (Figure 2I).

Each mutation cosegregated faithfully with the phenotype observed in affected individuals and did not appear in 384 chromosomes from ethnically matched controls or 540 chromosomes from German controls. Furthermore, the mutations were not observed in the published genomes of 185 individuals from the 11000 Genomes Project<sup>23</sup> or in the exomes of 200 Danish individuals.<sup>24</sup>

All together, these molecular findings suggest that a deficiency in *NSUN2* function causes ID in individuals homozygous for these mutations.

Ideally, *NSUN2* function would be tested in a genetically accessible model system. ClustalW and subsequent phylogenetic analysis showed that *Drosophila melanogaster* has a clear *NSUN2* ortholog (Figure 3A). Regarding the region



**Figure 2. Mutations**

(A) Sequence chromatograms of heterozygous carriers (“HET”) and individuals homozygous (“MUT”) for c.679C>T (Gln227\*) (NM\_017755.5) are compared to the normal sequence (“WT”).

(B) RT-PCR results and (C) qPCR results with primers specific to exons 3 and 6 (indicated by arrowheads in the schematic on top) show loss of *NSUN2* transcription in an individual homozygous for Gln227\* (“P”) and in controls (“C1”–“C4”). Relative expression was determined by comparison to *GAPDH* expression levels. The presence of RNA was controlled for with the use of primers specific to the X-chromosomal *HUWE1* transcript. Size marker (“M”): HyperLadder IV (Bioline.) Error bars represent the standard deviation (n = 3).

(D) Sequence chromatograms of individuals heterozygous (“HET”) and homozygous (“MUT”) for c.1114C>T (Gln372\*) (NM\_017755.5) are compared to the normal sequence (“WT”).

(E) RT-PCR results and (F) qPCR results with primers specific to exons 7–9 (indicated by arrows in the schematic on top) show loss of *NSUN2* transcription in an individual homozygous for Gln372\* (“P”) and in controls (“C1” – “C3”). Relative expression was determined by comparison to *GAPDH* expression levels. The presence of RNA was controlled for with the use of primers specific to the X-chromosomal *HUWE1* transcript. Size marker (“M”): HyperLadder IV (Bioline.) Error bars represent the standard deviation (n = 3).

(G) Sequence chromatograms of individuals heterozygous (“HET”) and homozygous (“MUT”) for g.6622224A>C (NC\_000005.9) (hg19) are compared to the normal sequence (“WT”).

(H) Sequence chromatograms of the normal junction between exons 6 and 7 (“WT”) and the junction between exons 5 and 7 (“MUT”) caused by mutation g.6622224A>C (NC\_000005.9) (hg19). The resulting protein sequences are indicated; the frameshift in the mutant leads to a downstream stop codon starting at nucleotide 71 of exon 7 (p.Ile179Argfs\*192).

(I) RT-PCR results with primers specific to the junctions of exons 2 and 3 and exons 8 and 9 (positions are indicated in the schematic representations of *NSUN2* transcripts 1 and 2 presented on top) and commercially available RNA from fetal and adult brains. RNA prepared from lymphoblasts (“LCL”) of affected individuals (“P”) and controls (“C1” – “C5”) show loss of the main transcript (*Trscpt 1*) in lymphoblasts (“LCL”) of both affected individuals. The presence of RNA was controlled for with the use of primers specific to the X-chromosomal *HUWE1* transcript (the right panel shows the results from one affected individual and controls). Size marker (“M”): HyperLadder IV (Bioline.).

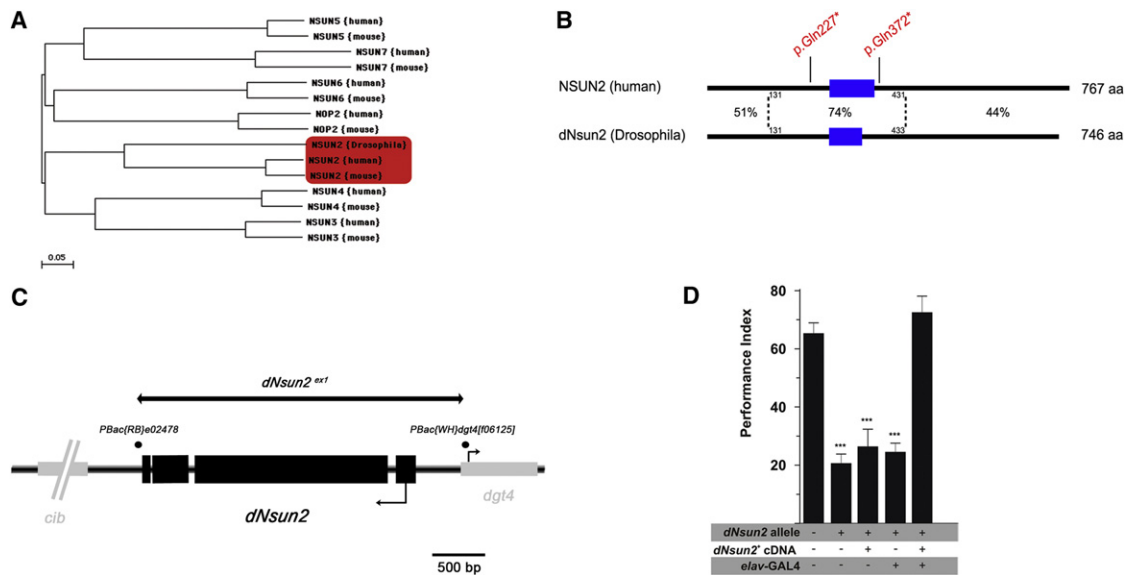
between positions 131 and 431 in *NSUN2*, this ortholog has 74% sequence similarity and 59% identical amino acids (Figure 3B and Figure S3). Thus, from here on, we refer to *Drosophila CG6133* as *Drosophila Nsun2* (*dNsun2*). *dNsun2* contains four exons and spans 2,578 bp on the X chromosome. So far, a single transcript encoding a 746 amino acid polypeptide is reported.<sup>25</sup>

Our in situ hybridization experiments showed a rather uniform *dNsun2* mRNA expression throughout *Drosophila* embryos; some mRNA enrichment was found in the pro-

ventriculus area of the foregut and in the hindgut (Figure S4). For later developmental stages, an upregulation of expression has been reported for the larval salivary gland and the CNS but not for the adult brain or head.<sup>26</sup> In general, the mRNA for *dNsun2* is not restricted to nervous tissue but is rather ubiquitously expressed.

To elucidate the function of *NSUN2* in *Drosophila*, we deleted *dNsun2* from the *Drosophila* genome by the Flippase-mediated transdeletion of Flippase recognition target-site-containing transposon lines<sup>17</sup> flanking *dNsun2*





### Figure 3. Mutants of the *Drosophila* Ortholog of NSUN2 Show an STM-Specific Defect

(A) A phylogenetic reconstruction by the neighbor-joining method is based on the amino acid sequences of *Drosophila* NSUN2 and mouse and human methyltransferases of the NOL1/NOP2/Sun domain family. The scale bar indicates phylogenetic distance, and the tree has been rooted with the midpoint method. Note that the tree shows a distinct clade that clusters the *Drosophila*, human, and mouse NSUN2 (red box).

(B) Human NSUN2 and the *Drosophila* ortholog (*dNsun2*) show a high degree of similarity. A region of about 300 aa in the center of the protein shares 74% sequence similarity and 59% identical amino acid (for a complete alignment, see Figure S3). The locations of the two nonsense mutations are indicated. Amino acid positions bordering the region of highest similarity are given, and blue boxes indicate SUN domains.

(C) Genomic location of *dNsun2* on the X chromosome at 4A5–4A6. *dNsun2*-deficient animals were constructed with *Drosophila* lines carrying transposon-mediated flippase recognition target sites<sup>17</sup> that neighbored *dNsun2* (black boxes indicate *dNsun2* exons, and gray boxes indicate neighboring loci). The obtained deficiency (black line with arrowheads) was confirmed by PCR. In addition to the entire *dNsun2*, the 5' *dgt4* was also excised.

(D) Behavioral tests for STM performance demonstrate a requirement for dNsun2. In contrast to a performance index (PI) of  $65.30 \pm 3.58$  ( $n = 11$ ) for wild-type flies ( $w^{1118}$  males), the mutant flies only show a PI of  $20.71 \pm 3.15$  ( $n = 11$ ). Expressing the *dNsun2<sup>+</sup>* cDNA or the driver line alone into the mutant background does not rescue the phenotype ( $26.51 \pm 5.93$ ,  $n = 7$  and  $24.5 \pm 2.97$ ,  $n = 6$ , respectively). The impaired STM performance in *dNsun2<sup>ex1</sup>* flies is only rescued by pan-neuronal (*elav-GAL4*) re-expression of the *dNsun2<sup>+</sup>* cDNA ( $72.64 \pm 5.55$ ,  $n = 16$ ). All data represent means  $\pm$  SEM. Asterisks indicate  $p < 0.005$ .

(Figure 3C). This *dNsun2*-deficient line (*dNsun2<sup>ex1</sup>*) was confirmed by genomic PCR. Of note, *dgt4* is also partially deleted here.

Because *dNsun2* is located on the X chromosome, male adults hemizygous for the *dNsun2* deletion (*dNsun2<sup>ex1</sup>/y*), were used for further analysis. Notably, these males hatched in expected numbers, were fertile, and appeared morphologically normal and fully viable (interestingly, the equally viable female mutants showed markedly reduced fertility). Consistently, initial characterization with the use of confocal microscopic scans of a *dNsun2<sup>ex1</sup>/y* brain stained with the monoclonal antibody Nc82 did not reveal major deficits in *dNsun2*-mutant males (not shown). Thus, in keeping with the phenotype observed in affected human individuals, this locus does not seem essential for the morphological development of the nervous system.

Given that the loss of NSUN2 function had no major impact on survival and elementary nervous-system functioning, we were able to investigate learning as a higher cognitive function.<sup>27</sup> We used an aversive olfactory conditioning paradigm in which we applied an electric shock as an aversive unconditioned stimulus to obtain a measure of

olfactory STM.<sup>28,29</sup> Importantly, *dNsun2* mutants showed normal innate odor reaction and shock avoidance, which is also consistent with the fact that their principal nervous-system functioning is normal. In clear contrast, however, *dNsun2* mutants suffered from a drastic lack of olfactory STM within 3 min after a single training (Figure 3D). Although control animals ( $w^{1118}$ , wild-type) showed a mean performance index of  $65.30 \pm 3.58$  SEM, the mean index for *dNsun2* mutants was only  $20.71 \pm 3.15$  SEM. Notably, this is in the range of well-established “severe” memory mutants, such as *dunce* or *rutabaga*.<sup>30,31</sup>

Because *dgt4* is also partially deleted in our model, we confirmed that the cognitive phenotype of *dNsun2<sup>ex1</sup>* mutants is solely attributable to a disruption of dNsun2 function. We confirmed this by using a transgene containing full-length *Drosophila* *Nsun2* (UAS-*dNsun2<sup>+</sup>* cDNA) in the *dNsun2<sup>ex1</sup>*-mutant background with the *elav-GAL4* driver,<sup>16</sup> which promotes expression in a pan-neuronal fashion that is not outside the nervous system.

Pan-neuronal expression of dNsun2 in all neurons fully rescued the STM phenotype of *dNsun2<sup>ex1</sup>* mutants (mean performance index of  $72.64 \pm 5.55$  SEM). This result

confirms that *dNsun2* is essential for proper STM in *Drosophila* (Figure 3C) and excludes a role of *dgt4* for the *dNsun2<sup>ex1</sup>* STM phenotype.

All together, both our clinical findings and the results from our model system analysis confirm that the highly conserved *NSUN2* is not essential for survival, gross nervous-system function, or elementary behavior such as locomotion, whereas in both contexts, *NSUN2* is apparently crucial for higher cognitive functioning.

## Discussion

On the basis of our clinical observations, we conclude that there is an overlapping phenotype in individuals with *NSUN2* mutations; this phenotype consists of moderate to severe ID and facial dysmorphism, which includes a long face, characteristic eyebrows, a long nose, and a small chin, in all affected individuals. Muscular hypertonia was present in all older affected individuals (9/11 individuals) but seems to be lacking in early childhood. Microcephaly (8/10 individuals) and short stature (7/10 individuals) are common features. In two *Nsun2* knockout lines that have only recently been reported by Blanco et al.<sup>32</sup> and the Wellcome Trust Sanger Institute (Mouse Resources Portal of the Wellcome Trust Sanger Institute), *Nsun2<sup>-/-</sup>* mice showed a significant reduction in size; this finding matches the short stature observed in several affected individuals from our study and the report by Khan et al.<sup>14</sup> However, neurocognitive or behavioral anomalies were not reported, but this is probably because both models do not seem to have been evaluated with a particular focus on cognitive features.

*NSUN2* was the first SUN-domain-containing protein to be characterized in vertebrates, and it is strongly conserved from bacteria to humans. It is the human ortholog of the yeast *Trmt4* protein and was identified by Frye and Watt<sup>33</sup> as a mediator of Myc-induced proliferation. Furthermore, they found that it has methyltransferase activity in vitro against rRNA, tRNA, and hemimethylated DNA. Independently, Brzezicha et al.<sup>13</sup> showed that it catalyzes the intron-dependent formation of 5-methylcytosine at C34 of tRNA-leu(CAA).

Modification of tRNAs by methylation and/or other alterations has effects on their structural integrity, translational efficiency, and/or fidelity.<sup>33</sup> *NSUN2* modifies the nucleotide in the wobble position of tRNA-leu(CAA). Therefore, it is conceivable that loss of *NSUN2* function might lead to the absence of tRNA-leu(CAA) and, as a consequence, cause changes in tissue-specific protein expression, e.g., through alterations in codon usage. In mammals, codon usage is known to have dramatic effects on translation rate, especially during cell differentiation.<sup>34</sup> In view of the *NSUN2* expression that we observed in the fetal brain, it is therefore conceivable that the phenotype of affected individuals is the result of proteomic shifts caused by the absence of *NSUN2* at critical stages during brain development. *NSUN2* might therefore play a role

in translational regulations needed for proper synaptic plasticity and thus learning and memory.<sup>35</sup>

Another conceivable disease mechanism might involve impaired methylation of hemimethylated DNA, which is also one of the targets of *NSUN2* activity. It is a well-established fact that alterations in DNA methylation patterns can lead to changes in gene transcription patterns and can also promote mutational events.<sup>36,37</sup> Such epigenetic modifications can also cause specific changes in brain functions,<sup>38–40</sup> which is of particular interest with respect to the cognitive phenotype observed in our affected individuals. For instance, both the metabotropic (GRM1–7 [MIM 604473, 604099, 601115, 604100, 604102, 604096, 604101]) and ionotropic (e.g., NMDA, AMPA, and kainate) glutamate-receptor-encoding genes undergo dynamic, region-specific, and cell-specific changes in expression during the course of brain development.<sup>41</sup>

Additional work will be required, but with our *dNsun2<sup>ex1</sup>* *Drosophila* fly line, we do have a model system in our hands. This model system will help us to further investigate and understand the role of this methyltransferase in the developing brain.<sup>32</sup>

Interestingly, *NSUN2* is not the only RNA-methyltransferase that seems to play a role in the development of higher cognitive functions. We and others, for example, have shown that inactivation of the X-linked *FTS1* (MIM 300499), another RNA methyltransferase, gives rise to ID<sup>42,43</sup> as well, and most recently, we have identified mutations in *TRMT1* (MIM 611669) in individuals with ARID.<sup>12</sup> *TRMT1* dimethylates a single guanine residue at position 26 of most tRNAs.

All together, our findings provide strong evidence that loss of *NSUN2* causes ARID and thus implicate *NSUN2* in the development of higher cognitive functions.

## Supplemental Data

Supplemental Data include four figures and one table and can be found with this article online at <http://www.cell.com/AJHG>.

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## Web Resources

The URLs for data presented herein are as follows:

Berkeley *Drosophila* Genome Project, <http://www.fruitfly.org/>  
Mouse Resources Portal of the Wellcome Trust Sanger Institute, [www.sanger.ac.uk/mouseportal](http://www.sanger.ac.uk/mouseportal)  
Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org>  
UniprotKB, <http://www.uniprot.org/>

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