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SEMA6B variants cause intellectual disability and alter dendritic spine density and axon guidance

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Abbreviations: ACMG = American College of Medical Genetics and Genomics; CADD = Combined Annotation Dependent Depletion; DMEM = Dulbecco's modified Eagle's medium; EMEM = Eagle's Minimum Essential Medium; FBS = Fetal bovine serum; GnomAD = Genome Aggregation Database; HA = Human influenza hemagglutinin; HEK293T = Human Embryonic Kidney 293T cells; HRP = Horseradish peroxidase; HUGODIMS = Interregional Project of the Great West of France for the Exploration by Exome Approach of Molecular Causes of Moderate or Severe Intellectual Disability; ID = Intellectual Disability; INSERM = Institut National de la Santé Et de la Recherche Médicale; NCBI = National Center for Biotechnology Information; NMD = Nonsense Mediated Decay; ORF = Open Reading Frame; PBS = Phosphate Buffer Saline; PME = Progressive Myoclonic Epilepsy; RIPA = Radioimmunoprecipitation assay; SIFT = Sorting Intolerant From Tolerant; TBS = Tris buffer saline

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Abstract

Intellectual disability is a neurodevelopmental disorder frequently caused by monogenic defects. In this study, we collected 14 *SEMA6B* heterozygous variants in 16 unrelated patients referred for intellectual disability to different centres. Whereas until now *SEMA6B* variants have mainly been reported in patients with progressive myoclonic epilepsy, our study indicates that the clinical spectrum is wider, and also includes non-syndromic intellectual disability without epilepsy or myoclonus.

To assess the pathogenicity of these variants, selected mutated forms of *Sema6b* were overexpressed in HEK293T cells and in primary neuronal cultures. shRNAs targeting *Sema6b* were also used in neuronal cultures to measure the impact of the decreased *Sema6b* expression on morphogenesis and synaptogenesis.

The overexpression of some variants leads to a subcellular mislocalisation of SEMA6B protein in HEK293T cells and to a reduced spine density due to loss of mature spines in neuronal cultures. *Sema6b* knock-down also impairs spine density and spine maturation.

In addition, we conducted *in vivo* rescue experiments in chicken embryos with the selected mutated forms of *Sema6b* expressed in commissural neurons after knock-down of endogenous *SEMA6B*. We observed that expression of these variants in commissural neurons fails to rescue the normal axon pathway.

In conclusion, identification of *SEMA6B* variants in patients presenting with an overlapping phenotype with intellectual disability, and functional studies highlight the important role of SEMA6B in neuronal development, notably in spine formation and maturation, and in axon guidance. This study adds *SEMA6B* to the list of intellectual disability-related genes.

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Introduction

Intellectual disability (ID) is a neurodevelopmental disorder characterized by significant limitations in intellectual functioning and adaptive behaviour, affecting 2 to 3% of the general population. The recent advances in high-throughput sequencing technologies have allowed major progress in gene identification and have highlighted the role of *de novo* variants in the pathogenesis of intellectual disability.(1) Overall and considering all inheritance patterns, more than 1500 genes are known to be mutated in intellectual disability and almost as many candidate genes could be involved in this highly heterogeneous disorder (SysID database). In a research program dedicated to the identification of novel intellectual disability genes, HUGODIMS (« Inter-regional Project of the Great Western Exploration Approach for Exome Molecular Causes Severe Intellectual Disability Isolated or Syndromic »; Clinical Trials.gov registration no. NCT02136849), we detected a missense variant in the *SEMA6B* gene (semaphorin 6B) in a patient with severe intellectual disability, suggesting that this gene may belong to this long list of candidates.

SEMA6B maps to chromosome 19p13.3 and contains 17 exons with a full-length transcript of 3.8 kb (NM_032108.3). It encodes the semaphorin 6B (SEMA6B), a 95.3 kDa transmembrane protein of 888 amino acids that is a member of class 6 semaphorins. Until now, about 30 semaphorins have been described. They are divided into eight subfamilies and share a conserved cysteine-rich extracellular semaphorin domain.(2,3) These axon guidance molecules are secreted or membrane-associated glycoproteins able to orchestrate axonal growth in the central nervous system during embryogenesis. Widely expressed in the nervous system, many act as neural chemorepellents for various axon populations and also regulate cell morphology and motility by modelling the cytoskeleton, the organization of actin filaments and the microtubule network.(4) Importantly, class 6 semaphorins can act as

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ligands and receptors.(5,6) A crucial role of SEMA6B has thus been established in central nervous system development as this protein enables axons to find and connect to their targets.(7–9) However, its role in intellectual disability remains to be functionally characterized.

Until now, *SEMA6B* variants have been identified in individuals with epilepsy, including (1) 10 truncating variants in the last exon of *SEMA6B* in 10 patients with progressive myoclonic epilepsy (PME, MIM #618876),(10–14) and in one individual with global developmental delay and recurrent febrile seizures,(15) and (2) one missense variant in exon 14 in an individual with epilepsy without intellectual disability (Supplementary Table 1).(13) Here, we broaden the phenotypic spectrum and the role of *SEMA6B* by reporting 14 heterozygous variants, of which 11 are novel, identified in 16 unrelated patients with moderate to severe intellectual disability, as well as variable neurological features and seizures in some cases. *SEMA6B* variants were *de novo* in 13 out of 16 patients for whom parental DNA was available. Given the crucial role of SEMA6B in the formation of neural circuits, we hypothesized that *SEMA6B* variants could play a role in the pathophysiology of intellectual disability, and we conducted *in vitro* functional studies in HEK293T (Human Embryonic Kidney 293T) cells, in mouse primary hippocampal neurons, and *in vivo* in commissural neurons of chicken embryos. We report that overexpression of the variants and the loss of SEMA6B expression in neuronal cells impair dendritic spine density and maturation. In the absence of endogenous SEMA6B, expression of the *Sema6b* variants in commissural neurons fails to rescue the normal axonal pathway. Axons fail to turn rostrally along the longitudinal axis of the spinal cord. Taken together, these results confirm a role of the identified variants of *SEMA6B* in neural circuit formation that is consistent with the observed phenotypes of the patients.

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Results

Description of *SEMA6B* variants

In the present study, 14 *SEMA6B* variants, of which 11 were novel, were identified by exome sequencing in 16 patients with intellectual disability. Three of these variants were previously described, c.1976_1982del p.(Ala659Valfs*24) and c.1991del p.(Gly664Alafs*21) in Hamanaka *et al*.(14) and c.2066G>A p.(Trp689*) in Herzog *et al*.(10) A schematic view of the *SEMA6B* gene and the distribution of all variants are presented in Fig. 1. All variants reported in the present study were absent in the population database genome Aggregation Database (gnomAD v2.1.1, 141.456 individuals) except for the missense variant c.1589C>T p.(Ser530Leu) which was found only once out of 209,632 alleles. The nonsense variant c.1114C>T p.(Arg372*) was observed in three unaffected adults in GeneDx internal database. Recurrence was observed for two frameshift and two nonsense variants considering both the novel and previously described *SEMA6B* variants.

The variants described in the present study occurred *de novo* in thirteen patients. Paternal DNA was unavailable for three patients (I-3, I-4, I-15). Most of the variants (9/14) were nonsense (3/14) and frameshift (6/14), located in the last exon of *SEMA6B* (exon 17) and therefore susceptible to escape the Nonsense-Mediated Decay (NMD) pathway. These variants may lead to the production of a truncated protein lacking the cytoplasmic domain.
Four missense variants were found*:* two located in exon 7 coding part of the SEMA domain, one located in exon 14 corresponding to the extracellular domain, and one located in the last exon corresponding to the intracellular domain. They all were predicted to be pathogenic by most of *in silico* prediction programs (Supplementary Table 2).

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For the functional studies, we decided to test one loss-of-function variant in the last exon, escaping NMD (c.2066G>A p.(Trp689*)) which represents the most frequent gene defect described in *SEMA6B* and one missense variant as an example of a novel type of *SEMA6B* mutation (c.547G>C p.(Val183Leu)).

Since the p.(Arg372*) variant is located in exon 11, it probably produces an aberrant transcript degraded by NMD in the patient. However, we could not measure *SEMA6B* mRNA as it is barely detectable in tissues such as peripheral blood cells and fibroblasts (data not shown). We therefore modelled the impact of this variant by using shRNA transfection to knockdown endogenous *Sema6b* expression.

Clinical description (Table 1)

The 16 individuals (I-1 to I-16) were aged 6 years to 24 years at the time of reporting, and were sporadic cases with no consanguinity (15/15 patients), and of Caucasian origin (13/13) with one being partly Native American. The reason for referral was known in 15 cases and consisted in ten patients in global delay or intellectual disability - associated with epilepsy in four of them - and autism in one patient. Patients I-4 and I-15 were first seen for intractable epilepsy. Patients I-1 and I-3 were initially referred for growth retardation associated with minor congenital anomalies but I-1 had developmental delay at the first evaluation.

All were born at term or near term. The neonatal period was normal except for patient I-I who had significant feeding difficulties. Birth length and post-natal height were in the normal range for 13/13 patients. Two patients (I-1 and I-3) had a low birth weight and a birth length in the low-normal values and I-1 still had growth retardation at -3 SD on last examination. A few patients had common digestive problems, mainly gastro-oesophageal reflux, but no

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serious medical problem was observed except for two cases of inflammatory bowel disease and one case of early puberty. A retinal dystrophy was suspected in I-1 but was not further documented. None of the patients had visceral or skeletal malformations but 7/13 had orthopaedic problems as a consequence of hypotonia, joint laxity or spasticity. Variable dysmorphic features were reported in 11/16 patients, but no consistent morphological phenotype seems to be recognizable.

Fourteen patients had hypotonia in infancy and early childhood and had delayed development. Independent walking was acquired after 18 months in half of the patients and was difficult with frequent falls in three, and one lost ability to walk. Two were not able to walk without assistance. Fine motor problems were observed in 14/14 patients and 5/14 had regression of their motor abilities. Language was delayed in 10/12 and on last examination, six were non-verbal or had a few words, four were able to do short sentences and six had simple conversation or were fluent. Only 9/14 were fully toilet-trained (above the age of 4 years in six). Intellectual disability of variable degree was observed in 13/14 patients (one mild, five moderate or moderate to severe, six severe and one profound), and one patient was reported as having learning difficulties and followed online school. Autism or autism spectrum disorder (ASD) was diagnosed in 4/15 patients, but three others had stereotypies. Behavioural problems were reported in 9/15 patients (including the four with ASD) and anxiety in 6/13. Abnormal movements, such as dyskinesia, chorea-like movements, dystonic posturing, were reported in three patients and 10/16 had ataxia or unsteady gait. Tremor and myoclonus were present in 5/12 and 4/11 patients respectively, but each resolved over time in one patient. Half of the patients had pyramidal signs described as spasticity and/or increased/brisk tendon reflexes.

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Epilepsy occurred in 13/16 patients, with an age at onset known in ten cases ranging from 11 months to 5 years (mean = 36 months). Epilepsy consisted in complex partial seizures in three patients, a combination of various types of seizures (myoclonic, atonic, tonic-clonic, tonic and atypical absences) refractory to treatment in four, and three patients received a diagnosis of Lennox-Gastaut syndrome. In one of the latter the diagnosis later changed for Dravet syndrome and then epileptic encephalopathy. In addition, three patients had non-epileptic drop-attacks. Following the results of exome sequencing compared to their medical history, two patients are now considered as having progressive myoclonic epilepsy. Epilepsy resolved by the age of 4 ½ years in the patient with the nonsense variant p.(Arg372*) located in exon 11. It is worthwhile to note that neither of the two patients (both already aged 16 years) with a missense variant in exon 7 had seizures, but one patient with a frameshift variant in exon 17 (p.(Arg625Valfs*25), nearly aged 9 years, did not suffer from epilepsy either.

Head circumference (HC) at birth was in the normal range, mostly in the low-normal values, in 7/8 patients and above the 95th percentile for one patient. On last examination 13/14 patients had a HC in the low-normal values (mean at -0.85 SD), with four of them being around -2 SD, whereas one was at +2.59 SD.

Brain MRI abnormalities were reported in 5/14 patients and consisted in arachnoid cyst, increased extrafluid spaces and/or cerebral ventricles probably related to some degree of cortical atrophy, cerebellar atrophy, thin or short corpus callosum and severely stretched out pituitary stalk in one patient with growth hormone deficiency.

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Expression and subcellular localisation of wild-type and SEMA6B mutants

Wild-type and mutant forms of S*ema6b* were overexpressed first in HEK293T cells in order to assess whether these variants could disturb *Sema6b* expression, stability or its subcellular localisation. Wild-type and mutant forms of *Sema6b* were all expressed at the RNA level and at the protein level (Supplementary Fig. 1). Proteins seemed to be present in equal quantity (P=0.3918) in HEK293T cells (Supplementary Fig. 1), suggesting that the mutant proteins are produced and stable. The nonsense variant leads to the production of truncated proteins with the predicted size of 75 kDa. As expected for a transmembrane protein, the wild-type protein when overexpressed was located at the plasma membrane and more precisely at protrusions or pseudopods of the cells. The mutant form of SEMA6B overexpressing the missense variant p.(Val183Leu) presented the same localisation. Surprisingly, the mutant form of SEMA6B overexpressing the nonsense variant p.(Trp689*), expected to be present at the plasma membrane as the truncation appears downstream of the transmembrane domain, was only partially present at the plasma membrane and was also partially retained in the cell (Fig. 2). Co-localisation of SEMA6B with a marker of the plasma membrane, a sodium potassium ATPase, was decreased with overexpression of the nonsense variant (Fig. 2).

The wild-type and mutant forms of *Sema6b* were next overexpressed in primary hippocampal neuronal cultures. SEMA6B was located in the three main parts of the neurons (dendrites, axon and soma) whatever the condition (overexpression of the wild-type form or mutant forms of SEMA6B) (Supplementary Fig. 2).

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Spine density and spine maturation are reduced with overexpression of *Sema6*b variants

The impact of the wild-type and mutant forms of *Sema6b* on neuronal development and morphology was assessed by measuring the branching complexity and the spine density. Overexpression of the different variants did not affect neuronal morphology, dendritic growth and branching complexity (data not shown) but had an impact on spine density. The variants p.(Val183Leu) and p.(Trp689*) led to a decrease of the total spine number compared to the wild type. They also induced a decrease in the number of mature spines and a change in the ratio between mature and immature spines (Fig. 3A and 3B). Taken together, these findings suggest that SEMA6B plays an important role in the formation and the maturation of dendritic spines.

Spine density and spine maturation are reduced after down-regulation of *Sema6b* expression

The transfection of the effective shRNA in Neuro-2a cells resulted in a 54% reduction of *Sema6b* mRNA expression level (data not shown). Effective shRNAs were then transfected in neuronal primary cultures to assess the impact of decreased *Sema6b* expression on spine density. Removing SEMA6B from hippocampal neurons *in vitro* resulted in a reduction of the number of spines (Fig. 4A, 4B). This reduction was mainly due to a loss of mature spines, as the number of immature spines was not different from controls.

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The *SEMA6B* variants are unable to support axonal navigation during neural circuit formation

Sema6B was previously shown to be required for axon guidance across the midline.(9) Therefore, we analysed whether the truncating mutants or the p.(Val183Leu) mutant were able to rescue axonal pathfinding. To this end, we silenced endogenous chicken SEMA6B with dsRNA derived from the 3'UTR. As expected and shown previously,(9) absence of SEMA6B interfered with the navigation of commissural axons across the floorplate, the ventral midline of the spinal cord (Fig. 5A), as only 30.4 ± 7.6% of the DiI injection sites showed normal axonal trajectories after silencing *SEMA6B*. In control embryos, injected and electroporated with the EGFP-encoding plasmid only, 74.0 ± 7.0% of the DiI injection sites showed normal axonal trajectories. Co-injection and electroporation of a plasmid encoding wild-type mouse SEMA6B rescued axonal pathfinding, as under these conditions 83.7 ± 4.9% of the DiI injection sites exhibited normal trajectories. This was not different from the values for the control group. However, none of the variants was able to rescue aberrant axon guidance, as we found only 24.2 ± 12.1% DiI injection sites with normal trajectories after co-injection of p.(Val183Leu). Similarly, the values for p.(Trp689*) were 21.9 ± 9.3%. There was no qualitative difference between the phenotypes (Fig. 5B and Supplementary Fig. 3). None of the variants was able to fully or partially rescue the aberrant axonal pathfinding seen after silencing SEMA6B. In contrast to axonal trajectories in control embryos (Fig. 5B), where axons cross the midline and turn rostrally along the contralateral floorplate border, axons failed to cross the floorplate and to turn rostrally in the absence of SEMA6B. The aberrant phenotypes were still found after co-injection of plasmids encoding the *Sema6B* variants, but not after co-injection and electroporation of wild-type *Sema6B*.

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Discussion

With 14 variants, of which 11 are novel, added to those already reported in the literature, (10–15) our study leads to a total of 22 heterozygous variants currently identified in *SEMA6B* in 28 unrelated patients. In all patients for whom DNA of the two parents was available (22/28), these variants had occurred *de novo*. The vast majority consists of truncating variants (17/22), mostly frameshift (12) or nonsense (5), compared to only 5 missense (Fig. 1). Four of them were recurrent and observed in different ethnic backgrounds (Supplementary Table 1). In previous reports, all but one were truncating variants located in the last exon, which encodes the intracellular domain of the protein, whereas the sole missense variant was located in exon 14. This could have suggested a particular molecular mechanism leading to a preferential type of pathogenic variants in this region. However, we also found a missense in exon 17 and a nonsense in exon 11. The high proportion of pathogenic variants in the last exon (17/22) is probably not only related to the size of this exon, which is the largest of the gene (926 bp, i.e. 34% of the coding sequence).

Pathogenic variants in *SEMA6B* have been first identified in patients with epilepsy, mainly progressive myoclonic epilepsy (PME),(14) a condition characterized by myoclonus, epilepsy, motor and cognitive decline, and other neurological signs such as ataxia, dysarthria, tremor, spasticity. Our study shows that the *SEMA6B*-related phenotype is wider than previously recognized as none of the 15 patients for whom we had the information was referred with this diagnosis. PME has been finally considered or recognized after the results of exome sequencing in only two patients, although several patients with intellectual disability and epilepsy had various neurological signs in common with PME, mainly ataxia or spasticity, or regression of their motor and cognitive abilities. Other patients presented with

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intellectual disability and refractory epilepsy suggesting that *SEMA6B* should be added to the panel of epileptic encephalopathy genes. In contrast, epilepsy was not observed in three patients, although we cannot totally exclude that it will occur later in life. This observation and the fact that the reason for referral in our series was mostly global delay or intellectual disability, indicate that *SEMA6B* is a gene to consider not only when dealing with severe epilepsy but more generally in neurodevelopment disorders. Previous reports suggested a strong genotype-phenotype correlation, as all truncating variants in the last exon were associated with a severe phenotype, mainly PME, observed in nine of the eleven patients for whom clinical data were available, or severe intellectual disability with early onset recurrent febrile seizures in one patient, whereas the missense variant in exon 14 was seen in a patient with epilepsy but normal development (Supplementary Table 1). Although the number of patients with variants outside exon 17 is still limited, our results seem to confirm that patients with truncating variants in the last exon are the most susceptible to manifest PME or refractory epilepsy. In particular, none of the two patients with a missense in exon 7 had epilepsy at the age of 16 years. However, two patients with a missense variant, one in exon 17 but the other in exon 14, also had severe epilepsy. Finally, we did not see significant clinical differences between patients bearing the same recurrent variant.

To assess the involvement of the *SEMA6B* variations in these phenotypes, we performed several functional analyses, notably in primary hippocampal neurons from mouse embryos and in commissural neurons of chicken embryos.

Our *in vitro* data indicate first that overexpression of the variants tested, p.(Val183leu), and p.(Trp689*), do not disturb SEMA6B synthesis and stability in HEK293T cells. However, the nonsense variant seems to partially prevent the truncated SEMA6B protein from reaching the

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plasma membrane whereas the missense variant does not change SEMA6B subcellular distribution. These results are in agreement with the work of Xiaozhen *et al*. (13) showing that another SEMA6B nonsense variant (p.Gln686*) produced a truncated protein which also failed to reach the plasma membrane in HEK293T cells.

Our functional studies validate the pathogenicity of the two variants p.(Val183Leu) and p.(Trp689*) as their overexpression affects spine density and spine maturation of neurons. Moreover, these two variants are also unable to rescue the aberrant axonal pathfinding seen after silencing SEMA6B in commissural neurons of chicken. In line with the finding that SEMA6B acts as a receptor in neural circuit formation, mutations resulting in truncation of the cytoplasmic domain could prevent binding of signalling molecules to SEMA6B. The variant p.(Val183Leu) has a transmembrane domain and a normal cytoplasmic domain, and therefore, would not necessarily be non-functional. However, the variant is located in an area of the protein that is required for the interaction with PlexinA2.(16,17) Because the interaction between PlexinA2 and class-6 semaphorins in cis, that is in the plane of the same membrane, has been demonstrated to be crucial for axonal targeting(5,9) and, at least indirectly, for spine formation,(3,4) the ability of p.(Val183Leu) to form cis-interactions could be modified in hippocampal neurons. Our study demonstrates a role of SEMA6B in dendritic spine development and maturation. Several observations indicate that semaphorins are, in general, known contributors of formation,(18–20) maturation,(21,22) regulation(23,24) and renewal of spines both in development and mature brain.(25) Semaphorins also contain a PDZ-binding motif which allows them to interact with proteins from the postsynaptic density such as PSD-95.(26–28) This interaction enables the semaphorins and their receptors to localise at synapses.(29) Effects of semaphorins on dendritic spines seem to be specific to each class or subclass of semaphorin, to neuronal type and depend on the endogenous action of the semaphorin, if it is expressed in the neurons, or

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on their external action if they are found in the media surrounding the neurons. A disruption of one of these pathways mediated by the semaphorins may lead to a default in circuit formation and/or in synaptic transmission or network excitability, associated to neurodevelopmental diseases.

Interpretation of the nonsense heterozygous variant, c. 1114C>T p.(Arg372*), located far upstream of the last exon-exon junction remains challenging. Indeed, the phenotype of the patient is nonspecific and less severe. This patient (I-3) is the only one who had learning difficulties but was not formally considered as having ID, and epilepsy resolved by the age of 4-5 years. Moreover, the DNA of the father was unavailable for this patient and this variant was found three times in GeneDx internal database. This variant is predicted to induce NMD leading to the loss of the corresponding truncated transcript. However, even if our *in vitro* analyses suggest that a decreased SEMA6B level impairs dendritic spine density in neuronal cells, these findings remain difficult to interpret in light of what happens in humans. On the one hand, a single copy of *SEMA6B* could be sufficient to maintain SEMA6B function especially as *SEMA6B* has a low probability of being loss of function intolerant (pLI = 0.06 in GnomAD v2.1.1). On the other hand, a mechanism of genetic compensation cannot be excluded. Through this mechanism, nonsense mutation might lead to upregulation of related genes which can themselves assume the function of the mutated gene (30). This dosage compensation mechanism which is not observe *in vitro* after gene knockdown (31) could explain, in humans, mild or non-penetrant phenotype associated with truncating variants located in the NMD-sensitive region. Moreover, it is very likely that truncating variants of the last exon, as they do not induce NMD, lead to a dominant negative effect which can explain the more severe and homogeneous phenotype observed with these variants compared to the milder phenotype associated with the nonsense p.(Arg372*) variant.

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In conclusion, our study expands the mutational and clinical spectrum associated with *SEMA6B* variations. This study confirms that loss-of-function variants in the last exon confer pathogenicity and shows that missense mutations also exert deleterious effects. Finally, SEMA6B plays an essential role in neuronal development notably in spine formation and maturation in addition to its well-known role in axon guidance.

Materials and methods

Inclusion of patients and genetic analyses

Affected individuals included in the present study were enrolled together with their healthy biological parents in different programs or centres investigating the molecular basis of developmental disorders in a research or clinical setting, and gathered through the GeneMatcher platform.(32) If done in a research setting, the studies were approved by local institutional review boards. Written informed consent was obtained from the patient's legal guardians. Clinical information was obtained by review of medical records and affected individuals' examination. Routine clinical genetic and metabolic screenings performed during initial workup were negative in each case, which allowed further investigation and exome sequencing.

Rare heterozygous variants were selected according to their *de novo* occurrence if known, their absence in the population database Genome Aggregation Database (gnomAD v2.1.1) and their predicted deleterious effect on the protein.

In silico prediction was performed with Sorting Intolerant From Tolerant (SIFT4G),(33) PolyPhen-2 HumVar,(34) MutationTaster,(35) Combined Annotation Dependent Depletion

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(CADD).(36) Interpretation of the variants was done according to the recommendations of the American College of Medical Genetics and Genomics (ACMG).(37)

The variants described in this study were submitted to the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/).

Sema6b expression plasmids and site-directed mutagenesis

Experiments were performed using a pCAG expression plasmid containing the full-length *Sema6b* mouse cDNA sequence (NM_032108.3) fused to an HA (human influenza hemagglutinin) tag. Human and mouse *SEMA6B* cDNA sequences were compared using nBLAST tool to check the conservation between these two sequences. 82% of identity was obtained between the two sequences. Amino acids and nucleotides modified by our *SEMA6B* variations are conserved between human and mouse. *Sema6b* cDNA was amplified by PCR and inserted into a pCR-Blunt II-TOPO (Zero Blunt TOPO PCR Cloning Kit, Invitrogen) cloning vector. The missense variant p.(Val183Leu) was generated by site-directed mutagenesis with the Q5 Site-Directed Mutagenesis kit (New England Biolabs). The oligonucleotides used to generate this missense variant were designed using NEBase Changer and included the following sequences: F-5'ACATGCCAATcTCGCCCTCTTC, R-5'TTGGGGTCATAGGGGCAG. The sequences of all constructs were confirmed by automated DNA Sanger sequencing. Then, the original pCAG expression plasmid and the TOPO plasmids containing the mutated cDNA were cut at the same restriction sites BglII and XbaI to clone back the mutated cDNA insert into the pCAG vector. In order to generate the *Sema6b* constructs containing the nonsense variants p.(Arg372*) or p.(Trp689*), the cDNA located upstream of the variants was amplified by PCR and inserted into the pCAG vector previously cut at BglII and XbaI cloning sites to remove the original *Sema6b* cDNA.

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Cell cultures

HEK 293T cell lines (ATCC (American Type Culture Collection), CRL-3216) were cultured and maintained with Dulbecco's Modified Eagle Medium (DMEM) (Gibco, ref 31966047) medium and 10% of fetal bovine serum (FBS; Eurobio, CVFSVF06-01). Neuro-2a cell lines (ATCC, CCL-131) were cultured and maintained with Eagle's Minimum Essential Medium (EMEM) medium (ATCC, 30-2003) and 10% of FBS.

For primary neuronal cultures, all mouse experiments were performed according to protocols approved by the University of Tours and Institut National de la Santé Et de la Recherche Médicale (INSERM). Hippocampi were dissected from embryonic day 17 C57BL/6J mouse embryos (Janvier-Labs). The primary neuronal cultures were prepared as previously described.(38) Dissociated cells were then plated onto glass coverslips coated in poly-D-lysine (Merk) and laminin (Cat#23017-015, Invitrogen) at a density of 210 cells per mm^2. The cultures were kept in Neurobasal/B-27, and half of the medium was changed twice a week.

RT-PCR and Western Blot analyses in HEK293T cells

Expression plasmids (wild-type and mutated forms of *Sema6b*) were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen). RNA was extracted 48h after transfection using the Direct-Zol RNA Miniprep Plus kit (Zymo Research). Complementary DNA was obtained from 100 ng of mRNA using the sensiFAST cDNA synthesis kit (Meridian Bioscience). Primers were designed with Primer3 (sequences available upon request).

Proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer from Invitrogen. Protein samples were processed for SDS-PAGE at 190V for 30 minutes. SDS-

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PAGE gels were blotted on Nitrocellulose membrane using TransBlot Turbo (Bio-Rad) for 7 minutes (1.3A, 25V). The membrane was blocked for 1 hour with 5% milk diluted in Tris buffer saline (TBS)-Tween (1% Tween 20). The membrane was incubated overnight in primary antibody for HA tag (1/500 Cat# 11867423001, Merck) diluted in 5% milk in TBS-Tween buffer. After three 10-minutes washes with TBS-Tween, the membrane was incubated with a secondary goat horseradish peroxidase (HRP)-coupled anti-rat antibody (1/10000 Cat#629520, Invitrogen) diluted in 5% milk in TBS-Tween buffer for 1 hour at room temperature. The chemiluminescence detection was done using Clarity Western ECL Substrate kit (cat# 1705060, Bio-rad) and the membrane was visualized in a Chemidoc Touch imaging system (Bio-rad). The membrane was then washed three times for 10 minutes in TBS-Tween and then secondary antibodies were removed by 2 washes of 10 minutes with a stripping buffer (15g glycine, 1g SDS, 10mL Tween20, 1L Water, pH 2.2), washed again with TBS-Tween and then blocked for 1 hour with 5% milk diluted in TBS-Tween. The membrane was incubated 1h with a mouse antibody targeting GAPDH coupled to HRP (Cat#G9295, Merck) diluted in 5% milk in TBS-Tween buffer, washed three times with TBS-Tween and detection was performed as previously described.

Immunocytochemistry and image analysis in HEK293T cells

Expression plasmids (wild-type and mutated forms of *Sema6b*) were transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) and HEK293T cells were fixed 48 hours later with a solution containing 4% paraformaldehyde / 4% sucrose in PBS. Cultures were then incubated with a blocking buffer (10% donkey serum / 0.2% Triton X-100 in PBS) for 1 hour, washed with PBS and incubated for 1 hour with the following primary antibodies diluted in 3% donkey serum / 0.2% Triton X-100 in PBS buffer: rat anti-HA monoclonal antibody (1/200 Cat# 11867423001, Merck) and recombinant rabbit anti-Sodium

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Potassium ATPase antibody (1/500 Cat#ab76020, Abcam). After several washes in PBS, neurons were incubated for 45 minutes with the following secondary antibodies diluted in 3% donkey serum/0.2% Triton X-100 in PBS buffer: FluoProbes 488 goat anti-rat Ab (1/500, Cat# A-11006, ThermoFisher) and FluoProbes 594 donkey anti-rabbit Ab (1/500, Cat#FP-SD5110, Interchim). After three washes in PBS, the fixed and stained HEK293T cells were mounted in ProLong Diamond Antifade reagent (Cat# P36391, Invitrogen). Sequential acquisitions were made, and high-resolution z stack images of cells were taken with the x63 objective of a laser-scanning confocal microscope SP-8 (Leica) with optical section separation (z interval) of 0.3 µm. Images were generated by the in-built Leica Application Suite X (Leica). Median projections were made for image analysis. Co-localisation quantification was performed using the JACoP plugin of the Fiji software (Wayne Rasband, Bethesda NIH) to obtain Pearson's and Mander's coefficients.

Immunocytochemistry and image analysis of primary neuronal cultures

After transfection with *Sema6b* expression plasmids (wild type or mutants), neurons were fixed at DIV 6 for subcellular localisation analyses and neuronal arborisation measurements or at DIV13 for spine density assessment with a solution containing 4% paraformaldehyde/4% sucrose in PBS. After incubation with blocking buffer, fixed neurons were incubated with the monoclonal rat anti-HA antibody (1/200 Cat# 11867423001, Merck) and the mouse anti-PSD95 antibody (1/200, Cat#MA1-046, ThermoFisher) diluted in 3% donkey serum / 0.2% Triton X-100 in PBS buffer. After the washes, they were incubated with the secondary antibodies FluoProbes 594 goat anti-rat (1/500, Cat#A110007, Life Technologies) and FluoProbes 680 goat anti-mouse (1/500, Cat#A21057, ThermoFisher) diluted in 3% donkey serum/0.2% Triton X-100 in PBS buffer. After several washes in PBS,

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the fixed and stained neurons were mounted in ProLong Diamond Antifade reagent (Cat# P36391, Invitrogen). Sequential acquisitions were made, and high-resolution z stack images of neurons were taken with the x63 objective of a laser-scanning confocal microscope SP-8 (Leica) with optical section separation (z interval) of 0.3 µm. SEMA6B transfection of every neuron has been checked before image generation. Images were generated by the in-built Leica Application Suite X (Leica). Maximal projections were made for image production, EGFP labelling was used as a tracer of neuronal and spine morphology and PSD95 as a marker of post-synaptic density. The spine density measurements were performed using the Fiji software (Wayne Rasband, Bethesda NIH). Mature spines and immature spines were counted based on their head diameter. Mature spines presented a head diameter of more than 0.6 µm. Immature spines presented a head diameter of less than 0.6 µm. Three segments from different dendrites were analysed for each cell. Quantification was based on three independent experiments with more than 16 cells of each type analysed. To measure neuronal arborisation, EGFP was used as a tracer of neuronal morphology. Images were analysed with the filament tracer plugin of the IMARIS software. Quantification was based on three independent experiments with more than 20 neurons of each type that were analysed.

Knock-down experiments

In vitro

Plasmids containing a small hairpin RNA (shRNA) targeting *Sema6b* mouse transcripts as well as plasmids containing a scrambled shRNA were used (cat# MSH073168-CU6 GeneCopoeia). The expression of the shRNA was driven by a U6 promoter. These constructs contained the puromycin resistance gene and an EGFP gene which expresses EGFP protein constitutively in mammalian cells. Both effective shRNA and non-effective shRNA were first transfected in Neuro-2a cells to assess their efficiency to repress the expression of

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endogenous *Sema6b*. shRNAs were transfected (Lipofectamine 2000, Invitrogen) in Neuro-2a cells 24h after seeding, and RNA extraction was performed 48h after transfection using the Direct-Zol RNA Miniprep Plus commercial kit (Zymo Research). Complementary DNA was obtained from 400 ng of mRNA using the PrimeScript RT Reagent Kit (Perfect Real Time) (cat# RR037A, Takara). qPCR experiments were run in duplicates with 500 ng of cDNA on the LightCycler 480 (Roche Diagnostics, Meylan, France) using the SYBR Green Master Mix commercial kit (Applied Biosystem, Foster City, CA, USA). Relative expression was assessed using the advanced E-method from the LightCycler software (Roche Diagnostics). *Hprt*, *Gapdh* and Beta-actin were used as reference genes for normalization. To measure the expression levels of *Sema6b* in each condition, RT-qPCR analyses were performed on three independent transfections.

Neurons were transfected (Lipofectamine 2000, Invitrogen) at DIV 10 with non-effective or effective shRNA. They were fixed at DIV 13 and spine density measurements were performed as described previously. The EGFP protein was used to show spine morphology. Quantification was based on three independent experiments leading to more than 17 cells analysed for each condition.

In vivo

Silencing of SEMA6B in chicken embryos was done by *in ovo* RNAi, as described previously.(39) In brief, fertilized chicken eggs were obtained from a local hatchery (Brüterei Stoeckli, Ohmstal). All experiments including chicken embryos were carried out in accordance with Swiss law on animal experimentation and approved by the cantonal veterinary office of the Canton of Zurich. After 2.5-3 days of incubation at 39°C, eggs were windowed to gain access to the developing embryos for injection and electroporation of

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dsRNA and/or plasmids. A mix of 350 ng/µl of dsRNA derived from the 3'untranslated region (UTR) or the Open Reading Frame (ORF) of SEMA6B and 20 ng/µl βactin-EGFP for transfection control were injected into the central canal of the spinal cord prior to electroporation with 5 pulses of 50 ms duration at 25 V, as described previously.(9,39) The generation of dsRNA was done as described previously.(40) For rescue experiments, 300 ng/µl of the respective plasmid (see above) were used for co-injections with the dsRNA and the EGFP plasmid.

We demonstrated that the dsRNA derived from the 3'UTR of SEMA6B was as effective as the dsRNA derived from the ORF that we used in our previous experiments (Andermatt et al.(9)).We also verified the suitability of our approach by using dsRNA from the ORF of SEMA6B together with the ORF of wild-type mouse Sema6B. Under these conditions, there was no rescue of the aberrant axon guidance phenotype, as the dsRNA derived from the ORF of chicken SEMA6B did also destroy the mRNA derived from mouse *Sema6B*.

Post-commissural neuron phenotype analysis

Chicken embryos were sacrificed at HH25-26 and the spinal cords were dissected as open-book preparations to label dI1 commissural neurons with the lipophilic dye Fast-DiI (5 mg/ml in ethanol, Molecular Probes) as described previously.(39) DiI injection sites were then analysed by fluorescent microscopy (Olympus DSU coupled to BX61 microscope). An injection site was considered to exhibit a 'floorplate stalling' phenotype, when more than 50% of the axons failed to cross the floorplate and stalled before the exit site. An injection site was considered to show a 'no turn' phenotype, when at least 50% of the axons reaching the exit site failed to turn into the longitudinal axis. Note that the 'no turn' phenotype is not independent of the 'floorplate stalling' phenotype, because we did not consider injection

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sites, where less than 10 fibres reached the exit site. Thus, a strong 'floorplate stalling' phenotype prevented the analysis of the turning phenotype. For this reason, we only compared the DiI injection sites with normal trajectories in our statistical analysis. Images were taken with an Olympus BX61 microscope equipped with a spinning disc unit.

Statistical analysis

To compare variables between subgroups, a Kruskal-Wallis test followed by a Dunn's multiple comparison test were used for *in vitro* studies on HEK293T cells and on primary hippocampal neurons or a One-way ANOVA followed by Tukey's posthoc test were used for *in vivo* studies on chicken embryos. A P-value < 0.05 was considered significant. Statistical analyses were performed using Prism v8 software (GraphPad).

Supplementary material

Supplementary material is available at Human Molecular Genetics online.

Data availability

The authors confirm that the data supporting the findings of this study are available within this article and its supplementary material, or available upon request without undue reservation except for exome sequencing data due to privacy and ethical restrictions.

Web resources

GeneMatcher, https://genematcher.org/

GnomAD Browser v2.1.1, https://gnomad.broadinstitute.org/

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OMIM, https://www.omim.org/

SysID database, https://www.sysid.dbmr.unibe.ch/

NBLAST, https://blast.ncbi.nlm.nih.gov/

Nebase Changer, https://nebasechanger.neb.com

ClinVar, https://www.ncbi.nlm.nih.gov/clinvar/

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Conflict of interests

ET and EAN are employees of GeneDx, Inc. The other authors report no competing interests.

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Figure legends

Figure 1 Distribution of *SEMA6B* heterozygous variants. The genomic structure of the *SEMA6B* gene including 17 exons is represented. cDNA numbering above *SEMA6B* gene is according to NM_032108.3. The SEMA6B protein structure is represented below the gene. Amino acid numbers are indicated above the protein. Variants are displayed as changes at DNA (c.) and protein levels (p.). Variants identified in our study are written in bold. The others were described in previous publications (*Hamanaka et al. 2020, Li et al. 2021, Courage and al.2021, Herzog et al. 2021, Shu et al., 2021 and Xiaozhen et al. 2021)*. For recurrent variants, the number of patients reported is indicated in parentheses next to the variant. Triangles and black circles indicate missense and truncating variants (frameshift or nonsense variants) respectively. Variants for which functional studies have been conducted, are underlined. TM: Transmembrane Domain.

Figure 2 Subcellular localisation of wild-type or mutants forms of SEMA6B protein overexpressed in HEK293T cells. (A) Confocal images showing SEMA6B localisation (magenta) in HEK293T cells after overexpression of SEMA6B variants or wild-type forms. Plasma membrane is shown in green. **(B)** and **(C)** Co-localisation was quantified on median projections using JaCop plugin from the ImageJ software based on Pearson's correlation coefficient **(B)** and Mander's coefficient **(C)**. Data shown are median, first and third quartiles, minimal and maximal values. More than 23 cells from two independent experiments were

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analysed. Statistical analysis was performed with the GraphPad Prism 8.0 software (La Jolla, CA, USA) by using a Kruskal-Wallis test; ****p<0.0001.

Figure 3 Altered spine density of primary hippocampal neurons after overexpression of *Sema6b* variants. (A) The EGFP protein was used to show spine morphology (x63 objective, Scale bar: 10µm). PSD95 in magenta is used to show post-synaptic density of dendritic spines. SEMA6B transfection of every neuron has been checked before image generation. **(B)** Quantification of spine number per 10µm of dendrite for each condition. Mature and immature spines were differentiated according to their head diameter (>0.6µm for mature spines and <0.6µm for immature spines). Three independent experiments were conducted leading to the analysis of more than 16 neurons for each condition. Means +/- SD are represented on the graph presenting the ratio of mature and immature spines. Median, first and third quartiles, minimal and maximal values are represented on the other graphs. Statistical analysis was done by using a Kruskal-Wallis test with the GraphPad Prism 8.0 software (La Jolla, CA, USA): *p<0.05, **p<0.01, ***p<0.001.

Figure 4 Altered spine density of primary hippocampal neurons after inhibition of *Sema6b* expression. (A) The EGFP protein was used to show spine morphology (x63 objective, Scale bar: 10µm). PSD95 in purple is used to show post-synaptic density of dendritic spines. **(B)** Quantification of spine number per 10µm of dendrite for each condition. Mature and immature spines were differentiated according to their head diameter (>0.6µm for mature spines and <0.6µm for immature spines). Three independent experiments have been done leading to the analysis of more than 17 neurons for each condition. Means +/- SD are represented on the graphs. Statistical analysis was done by using a Kruskal-Wallis test with the GraphPad Prism 8.0 software (La Jolla, CA, USA): *p<0.05, **p<0.01, ***p<0.001.

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Figure 5 None of the mutant SEMA6B variants can rescue axon guidance. (A) Axonal trajectories of dorsal commissural axons were analysed in control-treated and experimental embryos after silencing SEMA6B using the lipophilic dye DiI to visualize axons in spinal cord whole-mounts. In control-treated embryos, injected only with the EGFP-encoding plasmid, normal trajectories of commissural axons were seen at 74.0 ± 7.0% of the DiI sites (*N*= 9 embryos with a total of *n*=95 DiI injection sites). After silencing SEMA6B with dsRNA derived from the 3'UTR, normal trajectories were only seen at 30.4 ± 7.6% of the DiI injection sites (*N*=10 ; *n*=82). Axon guidance was restored to normal levels when a plasmid encoding wild-type *Sema6B* was co-injected with the dsRNA (83.7 ± 4.9%; *N*=10; *n*=101). Neither co-injection of the p.(Val183Leu) variant (24.2 ± 12.1%; *N*=10; *n*=92), nor the co-injection of the variant p.(Trp689*) (21.9 ± 9.3%; *N*=9 ; *n*=64) was able to restore normal axon pathfinding. **(B)** Dorsal commissural axons in control-treated embryos crossed the floorplate (indicated by dashed lines) and turned rostrally along the contralateral floorplate border (black arrowhead). After silencing *SEMA6B* with dsRNA derived from the 3'UTR, axons stalled in the floorplate (blue arrowhead) or failed to turn along the contralateral floorplate border (green arrowhead). This aberrant axonal pathfinding behavior was rescued by co-injection and electroporation of the *SEMA6B* ORF, which was not sensitive to the dsRNA from the 3'UTR. None of the variants was able to rescue aberrant axon guidance.

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Table 1 Clinical features of the patients carrying a *SEMA6B* variant

Individuals	**I-1**	**I-2**	**I-3**	**I-4**
Gender / Age at last examination	M / 16 y	M / 16 y 3 mo	F / 13 y 9 mo	F / 9 y
Reason for referral	Growth delay, hypotonia, congenital anomalies	Global delay, ID	Short stature, mild dysmorphic features	Lennox Gastaut syndrome
cDNA variant (NM_032108.3) / Exon	c.481C>G / exon 7	c.547G>C / exon 7	c.1114C>T / exon 11	c.1589C>T / exon 14
Protein variant	p.(Leu161Val)	p.(Val183Leu)	p.(Arg372*)	p.(Ser530Leu)
Inheritance	*De novo*	*De novo*	Not inherited from the mother	Not inherited from the mother
Delayed development	Y	Y	N	Y
Global / Fine motor problems	Y / Y	Y / Y	N / Y	Y / Y
Language abilities / Age at first words	Delay / 4 y	≈ 10 words	Sentences / 18 mo	2-3 word sentences
Motor regression	Y (wheelchair)	N	N	N
ID degree	Severe	Severe	Learning difficulties	Severe
ASD / Stereotypic movements	N / N	N / Y (hand flapping)	N / N	N / N
Behavioural problems / Anxiety	N / N	N / Y	N / Y	N / N
Ataxia	Y	Y (not cerebellar)	N	N
Tremor / Myoclonus	N / N	N / N	N / N	N / N
Pyramidal signs	Brisk reflexes, spasticity	Brisk reflexes and spasticity in LL	N	Brisk reflexes
Epilepsy / Age at onset / Type	N	N	Y / from 18 mo to 4.5 y	Y / 2 y / Myoclonic, atypical absences, atonic seizures
Brain MRI anomalies	Cerebellar atrophy, thin corpus callosum, large ventricles, cortical atrophy	Arachnoid cyst, severely stretched out pituitary stalk, mildly short corpus callosum	N	N
Final Diagnosis	ID + neurological signs	ID + neurological signs	Learning disability + resolving epilepsy	ID + epilepsy (Lennox Gastaut)

ASD: autism spectrum disorder; GTC: generalized tonic-clonic; ID: intellectual disability; LL: lower limbs; mo: months; NA: not available; N: no; PME: progressive myoclonic epilepsy; y: years; Y: yes

Table 1 (continued) Clinical features of the patients carrying a *SEMA6B* variant

Individuals	**I-5**	**I-6**	**I-7**	**I-8**
Gender / Age at last examination	M / 8 y 11 mo	F / 14 y 6 m	M / 21 y	F / 15 y
Reason for referral	Autism	Global delay, regression, movement disorder	Global delay, multiple congenital anomalies	Developmental delay
cDNA variant (NM_032108.3) / Exon	c.1873del / exon 17	c.1930del / exon 17	c.1958del / exon 17	c.1966G>T / exon 17
Protein variant	p.(Arg625Valfs*25)	p.(Ala644Argfs*6)	p.(Arg653Profs*32)	p.(Glu656*)
Inheritance	*De novo*	*De novo*	*De novo*	*De novo*
Delayed development	Y	Y	y	Y
Global / Fine motor problems	Y / Y	Y / NA	Y / Y	Y / Y
Language abilities / Age at first words	Delay / 13 mo	2-3 word sentences, dysarthria	Simple conversation	Short sentences
Motor regression	N	Y	N	Y
ID degree	NA	NA	Profound	Severe
ASD / Stereotypic movements	Y / Y (hand flapping)	N / N	N / N	Y / N
Behavioural problems / Anxiety	Y / Y	Y / N	N / Y	Y / N
Ataxia	N	Y	N	N
Tremor / Myoclonus	N / N	Y (resolved) / NA	N / N	Y / Y
Pyramidal signs	N	Brisk reflexes, spasticity	Brisk reflexes, spasticity	N
Epilepsy / Age at onset / Type	N	Y / 8 y / Partial epilepsy	Y / Childhood / Complex partial seizures	Y / 5 y / Complex partial seizures
Brain MRI anomalies	NA	N	NA	N
Final Diagnosis	Autism + ID	ID + epilepsy + neurological signs	ID + epilepsy + neurological signs	ID + epilepsy + neurological signs

ASD: autism spectrum disorder; GTC: generalized tonic-clonic; ID: intellectual disability; LL: lower limbs; mo: months; NA: not available; N: no; PME: progressive myoclonic epilepsy; y: years; Y: yes

Table 1 (continued) Clinical features of the patients carrying a *SEMA6B* variant

Individuals	**I-9**	**I-10**	**I-11**	**I-12**
Gender / Age at last examination	F / 20 y	M / 21 y	F / 16 y	F / 6 y
Reason for referral	Global delay	ID, seizures	Severe ID, seizures	NA
cDNA variant (NM_032108.3) / Exon	c.1966G>T / exon 17	c.1976_1982del / exon 17	c.1978del / exon 17	c.1991del / exon 17
Protein variant	p.(Glu656*)	p.(Ala659Valfs*24)	p.(Gln660Argfs*25)	p.(Gly664Alafs*21)
Inheritance	*De novo*	*De novo*	*De novo*	*De novo*
Delayed development	Y	Y	Y	Y
Global / Fine motor problems	Y / Y	Y / Y	Y / NA	Y / Y
Language abilities / Age at first words	Simple conversation	Non verbal	≈ 10 words / 3 y	Delay, a few words / 2 y
Motor regression	N	Y	N	N
ID degree	Moderate	Severe	Severe	Mild to moderate
ASD / Stereotypic movements	N / N	NA / Y	N / NA	Y / Y (hand flapping and clapping)
Behavioural problems / Anxiety	Y / Y	Y / NA	NA / NA	ADHD / sensitive to noise
Ataxia	Y	Y	Y	Y
Tremor / Myoclonus	N / Y	Y / N	NA	NA
Pyramidal signs	Increased LL reflexes, spasticity	Brisk reflexes	NA	N
Epilepsy / Age at onset / Type	Y	Y / 3.5 y	Y / 11 mo	Y
Brain MRI anomalies	N	Mild cerebellar atrophy	N	Slightly prominent extra-axial spaces, widened Sylvian fissure
Final Diagnosis	PME (after ES)	ID + epilepsy + neurological signs	ID + epilepsy	ID + epilepsy (Lennox Gastaut)

ASD: autism spectrum disorder; GTC: generalized tonic-clonic; ID: intellectual disability; LL: lower limbs; mo: months; NA: not available; N: no; PME: progressive myoclonic epilepsy; y: years; Y: yes

Table 1 (continued) Clinical features of the patients carrying a *SEMA6B* variant

Individuals	**I-13**	**I-14**	**I-15**	**I-16**
Gender / Age at last examination	F / 15 y	F / 16 y	M / 15 y 10 mo	F / 10 y 8 mo
Reason for referral	ID	Epilepsy, developmental delay	Intractable epileptic encephalopathy	Epilepsy, ID, poor fine motor skills
cDNA variant (NM_032108.3) / Exon	c.2066G>A / exon 17	c.2066G>A (mosaic) / exon 17	c.2131G>T / exon 17	c.2138C>T / exon 17
Protein variant	p.(Trp689*)	p.(Trp689*)	p.(Glu711*)	p.(Thr713Met)
Inheritance	*De novo*	*De novo*	Not inherited from the mother	*De novo*
Delayed development	Y	Y	Y	N
Global / Fine motor problems	Y / Y	Y / Y	Y / Y	N / Y
Language abilities / Age at first words	Short sentences / 2.5 y	Fluent / 20 mo	A few words	Fluent / 10 mo
Motor regression	Y	NA	NA	N
ID degree	Moderate to severe	Moderate	Moderate to severe	Mild
ASD / Stereotypic movements	N / N	N / Y (repetitive vocalizations, skin picking)	Y / NA	N / N
Behavioural problems / Anxiety	N / N	Hyperphagia / N	Y (aggressive, head banging) / NA	ADHD / Y
Ataxia	Y	Y	Y	N
Tremor / Myoclonus	Y / Y	NA	NA	Y / Y
Pyramidal signs	Increased LL reflexes	NA	NA	N
Epilepsy / Age at onset / Type	Y	Y / 4.5 y / Myoclonic, tonic, tonic-clonic, atonic seizures, medically refractory	Y / 2 y / Refractory GTC, myoclonic and focal seizures	Y / 5y / Refractory myoclonic-atonic, absence,GTC and tonic seizures
Brain MRI anomalies	N	N	N	Arachnoid cyst
Final Diagnosis	PME (when awaiting results of ES)	ID + epilepsy + neurological signs	Epileptic encephalopathy	ID + myoclonic-astatic epilepsy + neurological signs

ASD: autism spectrum disorder; GTC: generalized tonic-clonic; ID: intellectual disability; LL: lower limbs; mo: months; NA: not available; N: no; PME: progressive myoclonic epilepsy; y: years; Y: yes

Abbreviations: ACMG = American College of Medical Genetics and Genomics; CADD = Combined Annotation Dependent Depletion; DMEM = Dulbecco's modified Eagle's medium; EMEM = Eagle's Minimum Essential Medium; FBS = Fetal bovine serum; GnomAD = Genome Aggregation Database; HA = Human influenza hemagglutinin; HEK293T = Human Embryonic Kidney 293T cells; HRP = Horseradish peroxidase; HUGODIMS = Interregional Project of the Great West of France for the Exploration by Exome Approach of Molecular Causes of Moderate or Severe Intellectual Disability; ID = Intellectual Disability; INSERM = Institut National de la Santé Et de la Recherche Médicale; NCBI = National Center for Biotechnology Information; NMD = Nonsense Mediated Decay; ORF = Open Reading Frame; PBS = Phosphate Buffer Saline; PME = Progressive Myoclonic Epilepsy; RIPA = Radioimmunoprecipitation assay; SIFT = Sorting Intolerant From Tolerant; TBS = Tris buffer saline

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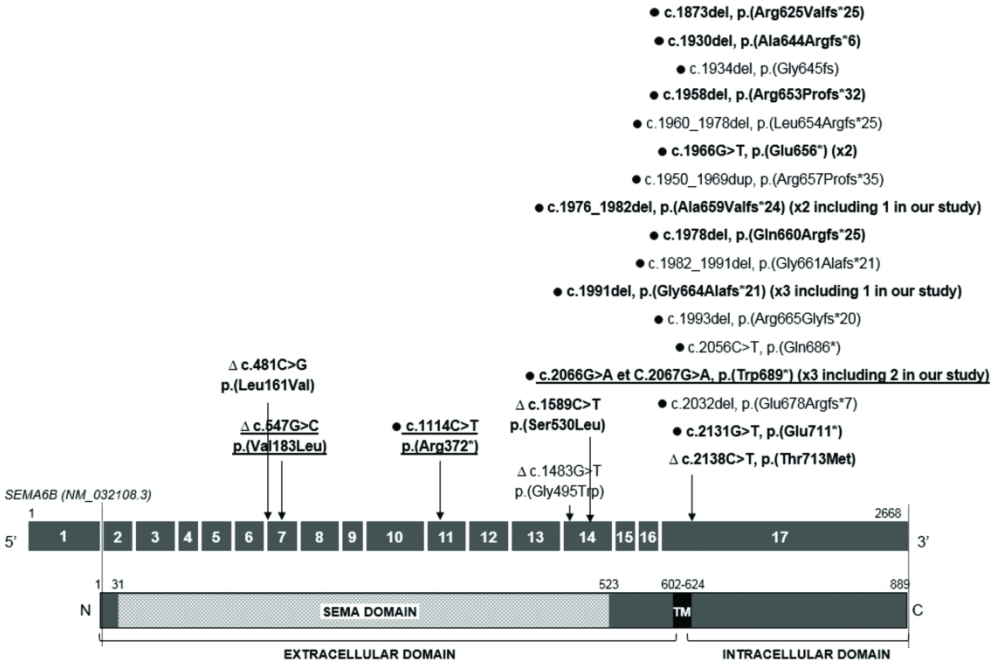


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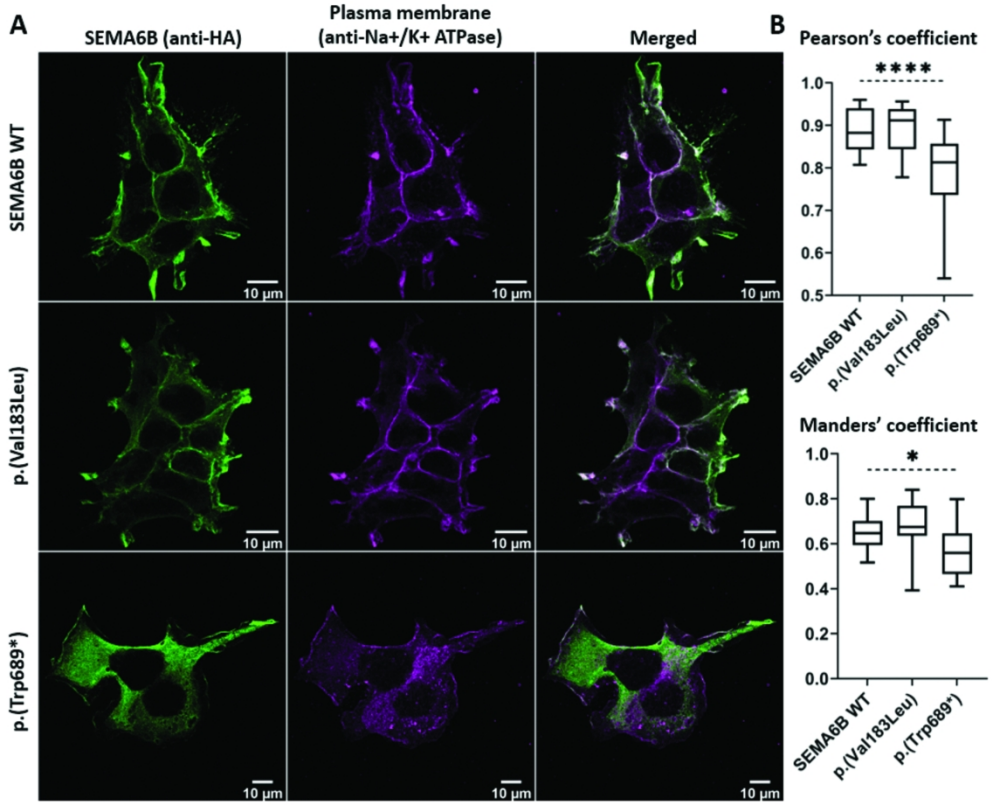


Figure 2 Subcellular localisation of wild-type or mutants forms of SEMA6B protein overexpressed in HEK293T cells. (A) Confocal images showing SEMA6B localisation (magenta) in HEK293T cells after overexpression of SEMA6B variants or wild-type forms. Plasma membrane is shown in green. (B) and (C) Co-localisation was quantified on median projections using JaCop plugin from the ImageJ software based on Pearson's correlation coefficient (B) and Mander's coefficient (C). Data shown are median, first and third quartiles, minimal and maximal values. More than 23 cells from two independent experiments were analysed. Statistical analysis was performed with the GraphPad Prism 8.0 software (La Jolla, CA, USA) by using a Kruskal-Wallis test; ****p<0.0001.

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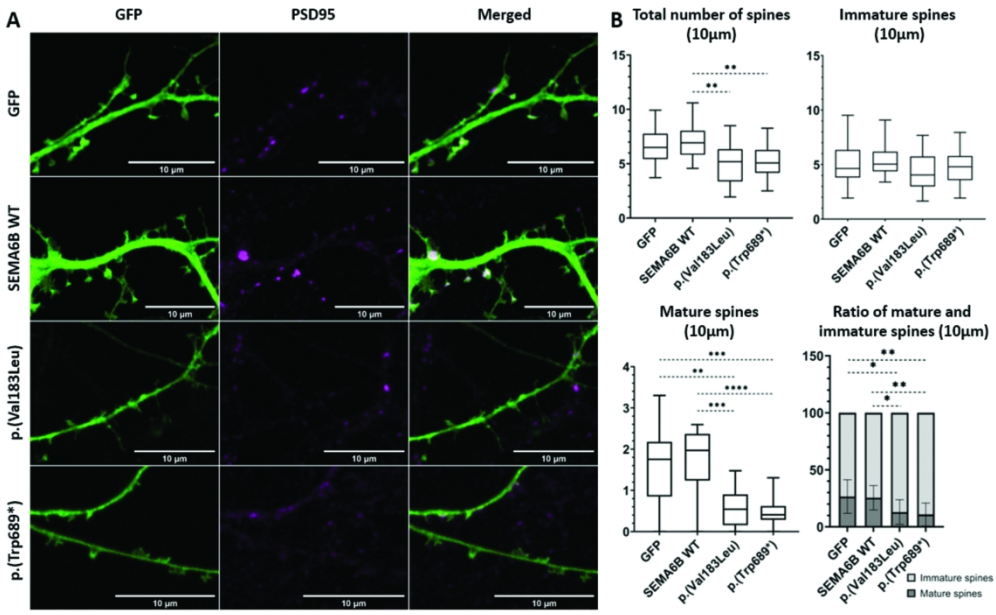


Figure 3 Altered spine density of primary hippocampal neurons after overexpression of Sema6b variants. (A) The EGFP protein was used to show spine morphology (x63 objective, Scale bar: 10µm). PSD95 in magenta is used to show post-synaptic density of dendritic spines. SEMA6B transfection of every neuron has been checked before image generation. (B) Quantification of spine number per 10µm of dendrite for each condition. Mature and immature spines were differentiated according to their head diameter (>0.6µm for mature spines and <0.6µm for immature spines). Three independent experiments were conducted leading to the analysis of more than 16 neurons for each condition. Means +/- SD are represented on the graph presenting the ratio of mature and immature spines. Median, first and third quartiles, minimal and maximal values are represented on the other graphs. Statistical analysis was done by using a Kruskal-Wallis test with the GraphPad Prism 8.0 software (La Jolla, CA, USA): *p<0.05, **p<0.01, ***p<0.001.

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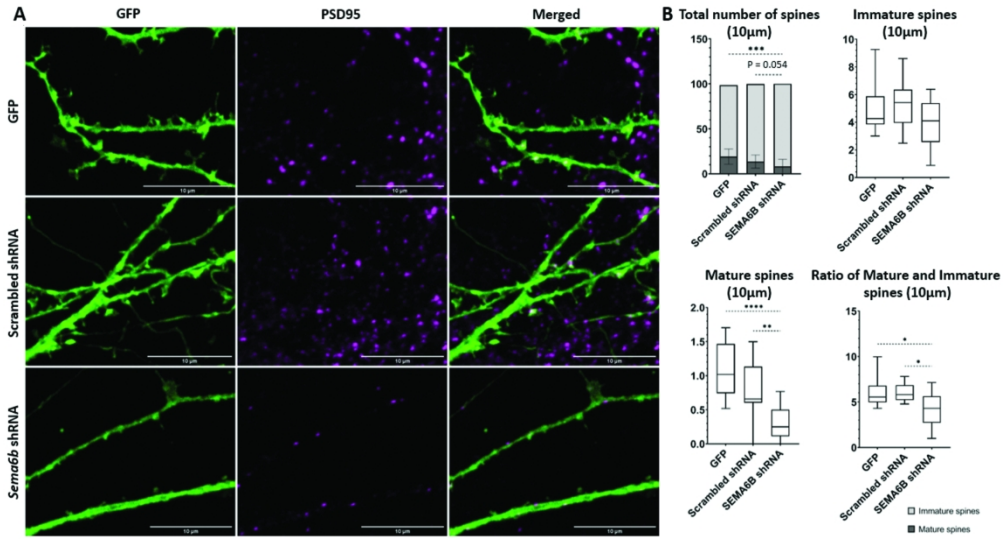


Figure 4 Altered spine density of primary hippocampal neurons after inhibition of Sema6b expression. (A) The EGFP protein was used to show spine morphology (x63 objective, Scale bar: 10µm). PSD95 in purple is used to show post-synaptic density of dendritic spines. (B) Quantification of spine number per 10µm of dendrite for each condition. Mature and immature spines were differentiated according to their head diameter (>0.6µm for mature spines and <0.6µm for immature spines). Three independent experiments have been done leading to the analysis of more than 17 neurons for each condition. Means +/- SD are represented on the graphs. Statistical analysis was done by using a Kruskal-Wallis test with the GraphPad Prism 8.0 software (La Jolla, CA, USA): *p<0.05, **p<0.01, ***p<0.001.

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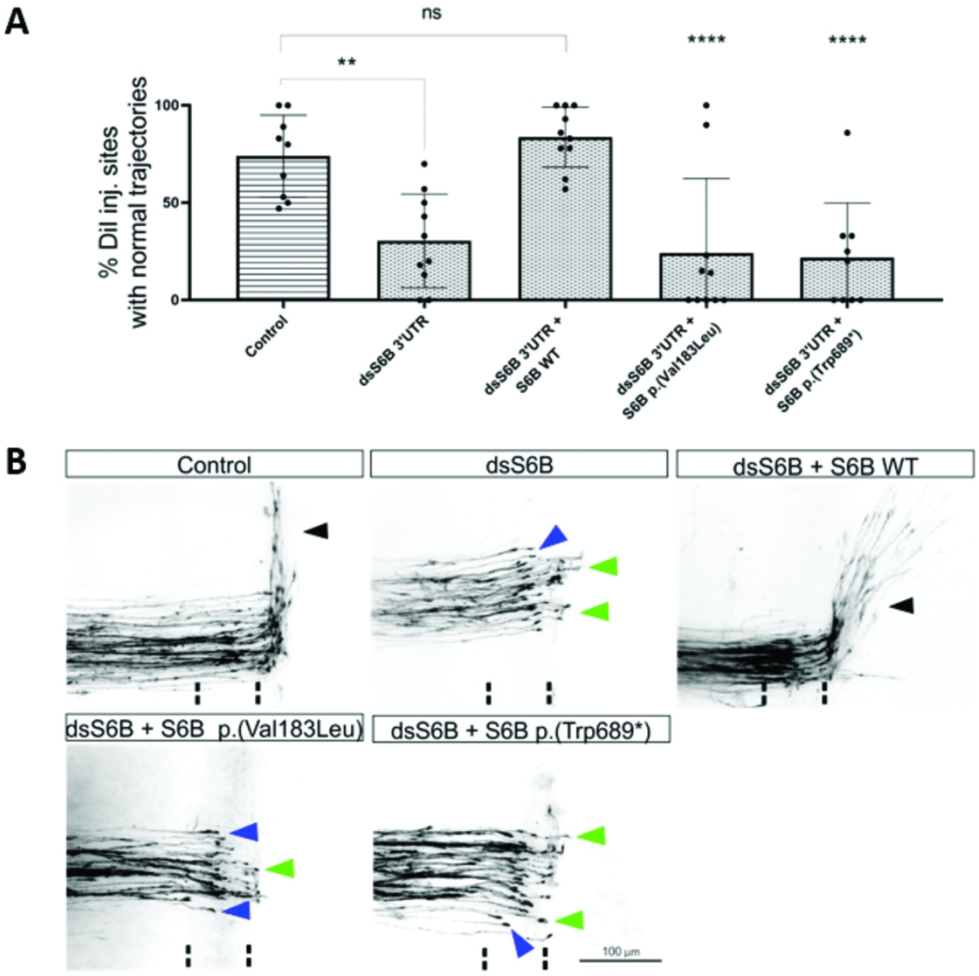
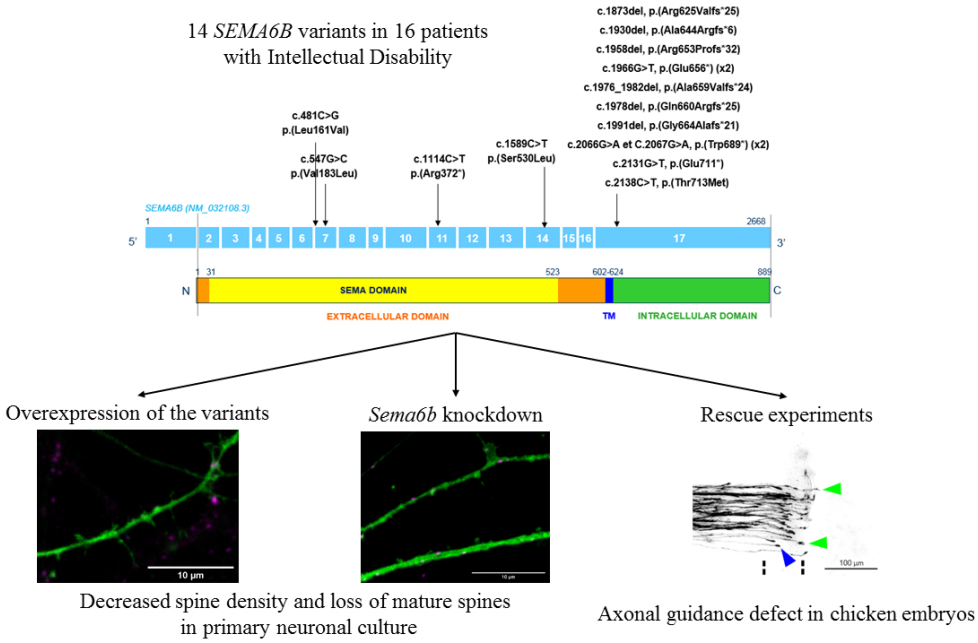


Figure 5 None of the mutant SEMA6B variants can rescue axon guidance. (A) Axonal trajectories of dorsal commissural axons were analysed in control-treated and experimental embryos after silencing SEMA6B using the lipophilic dye DiI to visualize axons in spinal cord whole-mounts. In control-treated embryos, injected only with the EGFP-encoding plasmid, normal trajectories of commissural axons were seen at 74.0 ± 7.0% of the DiI sites (N= 9 embryos with a total of n=95 DiI injection sites). After silencing SEMA6B with dsRNA derived from the 3'UTR, normal trajectories were only seen at 30.4 ± 7.6% of the DiI injection sites (N=10 ; n=82). Axon guidance was restored to normal levels when a plasmid encoding wild-type Sema6B was co-injected with the dsRNA (83.7 ± 4.9%; N=10; n=101). Neither co-injection of the p.(Val183Leu) variant (24.2 ± 12.1%; N=10; n=92), nor the co-injection of the variant p.(Trp689*) (21.9 ± 9.3%; N=9 ; n=64) was able to restore normal axon pathfinding. (B) Dorsal commissural axons in control-treated embryos crossed the floorplate (indicated by dashed lines) and turned rostrally along the contralateral floorplate border (black arrowhead). After silencing SEMA6B with dsRNA derived from the 3'UTR, axons stalled in the floorplate (blue arrowhead) or failed to turn along the contralateral floorplate border (green arrowhead). This aberrant axonal pathfinding behavior was rescued by co-injection and electroporation of the SEMA6B ORF, which was not sensitive to the dsRNA from the 3'UTR. None of the variants was able to rescue aberrant axon guidance.

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