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ARTICLE

De novo variants in *CNOT9* cause a neurodevelopmental disorder with or without epilepsy

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ABSTRACT

Purpose: The study aimed to clinically and molecularly characterize the neurodevelopmental disorder associated with heterozygous de novo variants in *CNOT9*.

Methods: Individuals were clinically examined. Variants were identified using exome or genome sequencing. These variants were evaluated using in silico predictions, and their functional relevance was further assessed by molecular models and research in the literature. The variants have been classified according to the criteria of the American College of Medical Genetics.

Results: We report on 7 individuals carrying de novo missense variants in *CNOT9*, p.(Arg46Gly), p.(Pro131Leu), and p.(Arg227His), and, recurrent in 4 unrelated individuals, p.(Arg292Trp). All affected persons have developmental delay/intellectual disability, with 5 of them showing seizures. Other symptoms include muscular hypotonia, facial dysmorphism, and behavioral abnormalities. Molecular modeling predicted that the variants are damaging and would lead to reduced protein stability or impaired recognition of interaction partners. Functional analyses in previous studies showed a pathogenic effect of p.(Pro131Leu) and p.(Arg227His).

Conclusion: We propose *CNOT9* as a novel gene for neurodevelopmental disorder and epilepsy.

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Introduction

The multicomplex CCR4-NOT (carbon catabolite repression 4-negative on TATA-less) is relevant for the regulation of gene expression by mediating deadenylation and ubiquitination of RNA. This is made possible by the interaction of the different components, of which 9 are known so far. The catalytic subunits are CNOT6/CNOT6L and CNOT7/CNOT8. CNOT4 has ubiquitin ligase activity and is loosely associated with the complex. CNOT2 and CNOT3 are regulatory subunits, and CNOT1 is the central scaffold of the complex. Not much is known about CNOT10 and CNOT11, but they likely support deadenylation by acquiring the nuclease module.[1]

CNOT9 is expressed in almost every tissue (as demonstrated by Genotype-Tissue Expression Project[2]) during fetal development in neurons and glia cells of the central nervous system (as demonstrated by the Descartes atlas[3]) and encodes the 299 amino acids long protein CNOT9. It consists of 6 armadillo repeats and has 2 binding sites. One can bind to the domain DUF3819 of CNOT1. This interaction is relevant to stimulate the nuclease module, which is formed of CNOT6/CNOT6L, CNOT7/CNOT8, and BTG2. Underlying mechanisms of the increase in activity of the nuclease module are discussed to be allosteric regulation by inducing a conformation with higher activity or affinity enhancement for the substrate by interaction with RNA.[4] On the opposite side of the CNOT1-binding site of CNOT9 is a positively charged cleft, which can interact with single- and double-stranded oligonucleotides.[5] In addition, CNOT9 contains 2 hydrophobic tryptophan-binding pockets,[6] which recognize protein partners (GW182, tristetetraprolin [TTP], and roquin) to influence the activity of deadenylation by the CCR4-NOT complex.[4] The interaction with TTP was investigated in more detail by Bulbrook et al.[7] For the degradation of inflammatory messenger RNA (mRNA) by the CCR4-NOT complex, the binding of TTP to the tryptophan-binding pockets of CNOT9 is essential. The amino acid changes in the tryptophan-binding pockets did not alter the overall structure of CNOT9, but TTP could no longer bind and RNA degradation was impeded.[7]

The relevance of *CNOT9* in embryonic development was previously demonstrated in *CNOT9* knockout mice. During gastrulation, the embryos showed developmental defects, such as small size, growth arrest, and vascularization defects, which led to embryonic death at E9.5 stage (Sarmah H, Ito K, Kaneko M, Abe T, Yamamoto T. Loss of CNOT9 begets impairment in gastrulation leading to embryonic lethality. Preprint. Posted online April 28, 2020. bioRxiv. https://doi.org/10.1101/2020.04.27.063172).

Because of the relevant role of the CCR4-NOT complex in regulating gene expression, it is not surprising that defective CCR4-NOT function has been associated with neurodevelopmental disorders (NDDs). Variants in *CNOT1*, which are leading to haploinsufficiency, are the cause of Vissers-Bodmer syndrome (MIM 619033), which causes intellectual disability (ID)/global developmental delay (DD), seizures, hypotonia, and behavioral problems. The *CNOT1* ortholog, *Not1* knockdown *Drosophila* models showed learning and memory defects, but the underlying pathomechanism is still unclear.[8] Haploinsufficiency of *CNOT2* has been associated with mild ID/DD, growth delay, seizures, skeletal anomalies, and dysmorphic facies (MIM 618608).[9,10] Individuals with heterozygous de novo variants in *CNOT3* have a similar phenotype, including ID/DD, hypotonia, small stature, and behavioral problems (MIM 618672). Functional studies concerning *CNOT3* to clarify the pathophysiological mechanism have not yet been performed.[11] Although rare de novo variants in *CNOT4*, *6L*, *7*, *8*, *9*, *10*, and *11* have been identified in individuals with an NDD (Deciphering Developmental Disorders [DDD] Study[12]), a related disorder for each gene has not yet been described.

Here, we describe 7 individuals with moderate to severe NDD in whom a de novo heterozygous variant in *CNOT9* was identified. We used structural in silico modeling and review of the literature to assess the possible impact of the variants on protein function.

Materials and Methods

Research cohort and identification of variants

All individuals underwent trio exome or genome sequencing using local protocols. Exome sequencing protocols have been previously described by Guillen Sacoto et al.[13] Maternity and paternity were confirmed for all individuals. All cases were evaluated in a clinical setting and no reportable variants were identified in a known disease gene (eg, by using the MorbidGenes panel[14]) (Supplemental Table 1). After families consented for research, evaluation of the data was continued in a scientific setting to identify novel gene disorder association. All variants were prioritized considering inheritance, allele frequency in Genome Aggregation Database (gnomAD),[15] impact on protein function (Supplemental Table 2), and involvement of candidate genes in neuronal processes. The procedures of identifying a gene may have some differences between the 7 contributing centers. For example, in Leipzig, these logic steps to evaluate variants in novel genes are integrated and trained in AutoCaSc,[16] an automated, web-based algorithm to prioritize novel candidate genes for NDD. The variant interpretation protocol used by GeneDx has been previously described by Retterer et al.[17] All other centers used a comparable approach. In total, around 60,000 trio exomes, exomes, or genomes from individuals with NDD were analyzed in a clinical or research setting. Each of the 7 probands was identified at a different medical center, and the cohort was assembled using GeneMatcher.[18] Phenotypic and genotypic information was obtained from the referring collaboration partners using a standardized clinical questionnaire (Supplemental Table 1). All variants in *CNOT9* are described with regard to the human reference genome version GRCh37 and to the transcript NM_005444.3 and have been

classified according to the criteria of the American College of Medical Genetics.[19]

Structural modeling

Structural analysis of the variants was performed based on the crystal structure of the CNOT9-CNOT1 complex (PDB: 4CRV[6]). For the p.(Arg292Trp) variant, ModLoop[20] was applied to explore the possibility of an intramolecular interaction, using the position of the tryptophan ligand in 4CRV as binding site. Amino acid changes were modeled with SwissModel,[21] and RasMol[22] was used for structure analysis and visualization.

Statistical analysis

To estimate a statistical enrichment of de novo variants in *CNOT9*, we applied the DenovolyzeR[23] in R (version 4.2.1).

Results

Clinical description

All 7 individuals had DD, with prominent speech delay. Four individuals showed severe DD, and 3 individuals showed moderate DD. Five individuals also have seizures. Behavioral disorders of the individuals included anxiety, impulsivity, and hyperactivity (Figure 1). In addition, clinicians described facial dysmorphisms for all individuals, but these did not suggest a specific pattern; no photographs are available for an objective evaluation by an experienced syndromologist or by software. Other symptoms present in individuals are described in detail below. An overview of the clinical symptoms is presented in Table 1 and Supplemental Table 1.

Individual 1 is an 18-year-old man. The pregnancy and birth were uneventful. He was able to walk at 23 months and is able to speak words and sentences. He presented with a mild ID with an IQ of 60. At the age of 1.5 years, he developed generalized seizures. Good control was achieved with valproate and lamotrigine and with lamotrigine and levetiracetam. Therapy with lamotrigine alone was not sufficient. Seizures returned after cessation of medication, whereupon carbamazepine was given. He is currently seizure-free under carbamazepine. At the age of 3 years, magnetic resonance imaging (MRI) was performed, which was unremarkable, as were the several long-lasting electroencephalograms (EEGs). Muscular hypotonia and ataxia were noted. He showed autistic features. He has peripheral hyperlaxity, flat feet, and extension deficit in elbows. Dysmorphic features as hypertelorism, upslanting palpebral fissures, high nasal bridge, short philtrum, and wide mouth were noted. Ophthalmological findings and further

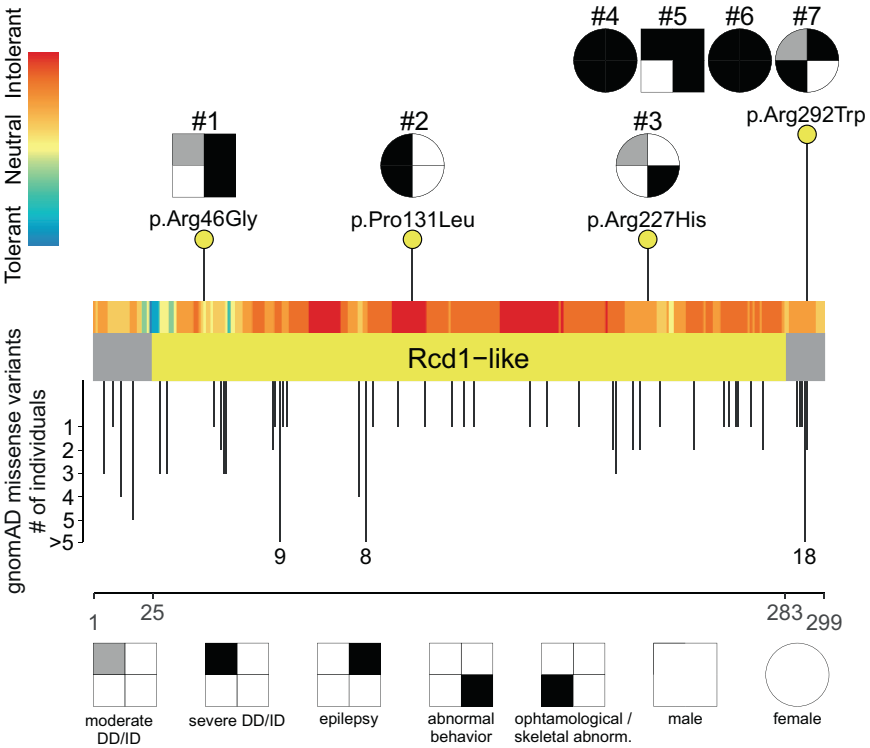

Figure 1 Overview of the variants in CNOT9. Location of missense variants in CNOT9 with respect to the domain structure of CNOT9 (GenBank: NM_005444.3). The x-axis represents the corresponding amino acid position of CNOT9. Variants reported in this study are labeled with the corresponding p-code. Missense variants in gnomAD[15] with allele count are shown below the protein scheme. The tolerance landscape (MetaDome[24]) is shown color coded above the protein scheme. The main characteristics of the phenotype of each individual is shown in the boxes. DD, developmental delay; ID, intellectual disability; *Rcd1*: radical-induced cell death 1.

Table 1 Summary of clinical symptoms of individuals with variants in *CNOT9*

Individual (Sex, Age)	# 1 (Male, 18 y)	# 2 (Female, 22 y)	# 3 (Female, 11 y)	# 4 (Female, 25 y)	# 5 (Male, 7 y)	# 6 (Female, 6 y)	# 7 (Female, 10 y)
CNOT9 variants	c.136C>G, p.(Arg46Gly)	c.392C>T, p.(Pro131Leu)	c.680G>A, p.(Arg227His)	c.874C>T, p.(Arg292Trp)			
Intellectual disability	Mild, IQ 60	Severe	Mild, IQ 52	Severe	Yes (not classified)	Severe	Mild
Speech and language development	Delayed, speaks words and sentences	Almost absent speech	Expressive language delay	Absent speech	Absent speech	Absent speech	Spontaneous words at 2 y
Motor development	Walked at 23 mo	Walked at 24 mo	Walked at 18 mo	Walked at 3.5 y	Stand with support at 4 y	Unknown	Walked at 22 mo
Muscular hypotonia	Yes	Not reported	Mild	Hypotonia in infancy	Yes	Not reported	Moderate
Seizures/seizure type	Yes, generalized	No	No	Yes, complex partial seizures	Yes, infrequent myoclonic events	Yes	Yes, complex febrile seizures
Medication/response to treatment	Good response to carbamazepine	-	-	Was ineffective, currently no medication	Good response to levetiracetam and ketogenic diet	Good response to vigabatrin	Previously levetiracetam and oxcarbazepine
EEG	Normal	Alteration of EEG organization, but no paroxysm abnormalities	Not performed	Multiple epileptiform discharges	Infrequent generalized spikes	Hypsarrhythmia at the age of 3 mo; mild slowing background activity later	Abnormal, potential epileptogenicity from the left temporal region
MRI	Normal at the age of 3 y	Cerebellar vermis hypoplasia, agenesis of anterior commissure	Not performed	Normal at the age of 20 y	Normal at the age of 4 mo	Normal at the ages of 16 mo and 3 y	Normal
Behavior	Autism spectrum disorder	Normal	Anxiety, impulsivity	Stereotypic and self-stimulating behavior, mood swings	Autism spectrum disorder	Anxiety, impulsivity, sleep disturbances, and hyperactivity	Normal
Ophthalmological findings	None	Strabismus, high myopia, dacryostenosis	None	Abnormal eye movements, random cases of dilation	None	Strabismus	Monocular vision, alternating esotropia with myopia
Skeletal findings	Distal hyperlaxity, extension deficit elbows, flat feet	Short stature, flexion contractures, pectus excavatum, hyperlordosis, camptodactyly V finger	None	Scoliosis	None	Clinodactyly, camptodactyly (bilateral)	Pes cavus, uses insert for left foot; hammer toes, high arches

(continued)

Table 1 Continued

Individual (Sex, Age)	# 1 (Male, 18 y)	# 2 (Female, 22 y)	# 3 (Female, 11 y)	# 4 (Female, 25 y)	# 5 (Male, 7 y)	# 6 (Female, 6 y)	# 7 (Female, 10 y)
Facial dysmorphology findings	Hypertelorism, upslanting palpebral fissures, high nasal bridge, short philtrum, wide mouth	Coarse facial dysmorphisms, gingival hypertrophy, dental malocclusion, microtia	Microretrognathia, hypertelorism, high and narrow palate, everted upper lip vermilion and wide nasal base	Deep-set eyes, short palpebral features	None	High sloping forehead, wide nasal base, everted upper lip vermilion, large ears, overfolded helix	Prominent nasal tip

EEG, electroencephalogram; *MRI*, magnetic resonance imaging.

symptoms were not described. Trio exome sequencing detected a heterozygous de novo variant in *CNOT9:* c.136C>G, p.(Arg46Gly). Variants in 2 other candidate genes were also identified in this individual: heterozygous de novo variant in *NAV2* (c.3674G>A, p.(Arg1225Gln)) and compound heterozygous variants in *SBTBN5* (c.7012C>G, p.(Arg2338Gly) paternal; c.6207+5G>A, p.? maternal). No additional clinically relevant de novo variants were identified in the other cases.

Individual 2 is a 22-year-old woman. The pregnancy was characterized by polyhydramnios and intrauterine growth retardation. Short limbs were noticed. The girl was born at 38 weeks of gestation. She learned to speak only a few words and showed severe ID. Seizures were not reported. The performed MRI showed cerebellar vermis hypoplasia and agenesis of anterior commissure. Muscular hypotonia was not noticed, and the behavior was described as normal. The ophthalmologic examination revealed strabismus, high myopia, and dacryostenosis. Skeletal abnormalities including flexion contractures, pectus excavatum, hyperlordosis, and camptodactyly V finger were evident. Coarse facial dysmorphisms, gingival hypertrophy, dental malocclusion, microtia, and a short neck (further details in Supplemental Table 1) were revealed. Macrocephaly was diagnosed with a head circumference of 61.4 cm (+6.37 SD). The head circumference at birth is not known. The individual showed a broad-based gait and had a short stature. The affected woman's other symptoms included recurrent otitis media and bronchitis, branchial fistula, gastroesophageal reflux disease, and a hoarse voice. Trio exome sequencing detected a heterozygous de novo variant in *CNOT9:* c.392C>T, p.(Pro131Leu).

Individual 3 is an 11-year-old girl. Pregnancy, birth, and infancy were unremarkable. The girl was born at 41 weeks of gestation. The parents noticed the DD from about the age of 3 years. In particular, an expressive language development disorder was conspicuous in the individual. Gross motor development was almost normal, but visuo- and grapho-motor skills were far below average. At the last examination at age 7 years, the IQ was determined to be 52. There is no history of epilepsy, and an MRI has not yet been performed. A low-grade muscular hypotonia was recognized. Additionally, significant anxiety, emotionality, impulsivity, and a tic disorder were evident. Ophthalmological and skeletal abnormalities were not described. The following facial dysmorphisms were recognized: microretrognathia, hypertelorism, high and narrow palate, everted upper lip vermilion, and wide nasal base. In addition, the following body characteristics stood out: slender build, single transverse palmar crease, and 2 to 4 toe syndactyly. Trio exome sequencing detected a heterozygous de novo variant in *CNOT9:*c.680G>A, p.(Arg227His).

Individual 4 is a 25-year-old woman. The pregnancy and delivery were normal. The girl was born at full term. She learned to walk at 3.5 years and never learned to speak. She was assessed with severe ID. Subsequently, she developed complex partial seizures that were unresponsive to medication, although ketogenic diet showed some success. Since

the age of 15 years, she has not experienced seizures. A recently performed EEG showed multiple epileptiform discharges. Her MRI was normal at 20 years of age. In infancy, she presented with muscular hypotonia. Her behavior was conspicuous by stereotypical movements, staring at light, smelling everything, and self-injurious and self-stimulating behavior, such as constant rocking. In addition, frequent bouts of unexplained crying and mood swings occurred. Abnormal eye movements and random instances of dilation appeared, and she was affected by scoliosis. Deep-set eyes and short palpebral features were noticeable. In addition, she exhibited unexplained dentition changes, kidney stones, and frequent constipation. Trio genome sequencing detected a heterozygous de novo variant in *CNOT9*: c.874C>T, p.(Arg292Trp). This variant also occurs with individuals 5, 6, and 7.

Individual 5 is a 7-year-old boy. The pregnancy and delivery were uneventful. The boy was born at term. He walked independently at 4 years and uses single words at 7 years. At 3 months of age, he developed myoclonic seizures, which continued to occur infrequently, especially during febrile illnesses. Antiepileptic therapy consisted of levetiracetam and ketogenic diet, which was able to elicit a good response. A performed EEG showed infrequent generalized spikes. An MRI was performed at 4 months of age and revealed unremarkable findings. Muscular hypotonia was described, and he was diagnosed with autism spectrum disorder at 5 years of age. Trio exome sequencing detected a heterozygous de novo variant in *CNOT9*: c.874C>T, p.(Arg292Trp).

Individual 6 is a 6-year-old girl. The pregnancy and delivery were uneventful. She reached independent gait at the age of 2.5 years. Language development was severely delayed. At the age of 3 months, she started to have epileptic spasms associated with psychomotor regression, which was suggestive of West syndrome. Subsequently, she also developed focal seizures. Epileptic spasms were successfully treated with steroids, whereas focal seizures were treated with phenobarbital and zonisamid but with poor response. The introduction of vigabatrin gave seizure remission at the age of 12 months. EEG was characterized by hypsarrhythmia during the first months of life associated with epileptic spasms. Later, EEG showed a mild slowing background activity without epileptic abnormalities. Brain MRI was normal (age of 16 months and 3 years). Visual evoked potential and electroretinogram were normal. Muscular hypotonia was not reported. Anxiety, impulsivity, hyperactivity, and sleep disturbances occurred as behavioral problems. Strabismus was evident in ophthalmologic examination. Skeletal abnormalities included bilateral clinodactyly and camptodactyly. Mild facial dysmorphic features were high sloping forehead, wide nasal base, everted upper lip vermilion, large ears, and overfolded helix. Trio exome sequencing detected a heterozygous de novo variant in *CNOT9*: c.874C>T, p.(Arg292Trp).

Individual 7 is a 10-year-old girl. The pregnancy and delivery were normal. She mimicked words at 18 months, and spontaneous words were spoken at 2 years; she learned to walk at 22 months. She was diagnosed with mild ID. She developed complex febrile seizures. The previous seizure medication was levetiracetam and oxcarbazepine. An EEG in 2015 showed potential epileptogenicity from the left temporal region, an EEG performed in 2016 was normal, and MRI was reported as normal. Her muscle tone was described as moderately low. Her behavior was reported to be normal. Ophthalmologic examination revealed monocular vision and variable esotropia with myopia. Skeletal abnormalities were pes cavus, hammer toes, and high arch of the foot. The face was described as largely nondysmorphic, except for a prominent nasal tip. Cardiac malformations included a bicuspid aortic valve without stenosis, mild bicuspid regurgitation, and a small patent ductus arteriosus. In addition, hypoplastic teeth were noted. Trio exome sequencing detected a heterozygous de novo variant in *CNOT9*: c.874C>T, p.(Arg292Trp).

Results of sequencing and description of genetic variants

Six trio exome and 1 trio genome sequencing were performed with all above mentioned individuals. In all individuals, heterozygous de novo missense variants were found in the gene *CNOT9*. By applying the DenovolyzeR model,[23] we found a significant enrichment of de novo missense variants in *CNOT9* in individuals with DD in our cohort (n = 60,000; observed/expected de novo variants = 4.89; P = .000708).

Individuals 4 to 7 carry the same de novo variant (NM_005444.3:c.874C>T, p.(Arg292Trp)). Based on the missense mutation rate for *CNOT9* ($P = 8.731724 \times 10^{-6}$, calculated from the gnomAD data by Samocha et al[25]), the probability that 3 further individuals carry the same nucleotide variant in the *CNOT9* gene (900 coding nucleotides) can be calculated approximately as follows: $P = 8.73 \times 10^{-6} \times (1/900/4)^3 = 1.871511 \times 10^{-17}$.

The variants that we have identified are evolutionarily highly conserved. *CNOT9* is neither tolerant to missense nor null variants (Z-score = 3.28, observed/expected ratio = 0.29 [0.23-0.36], and pLI (probability of being loss-of-function intolerant) score = 0.99 observed/expected ratio = 0.06 [0.02-0.28])[15] (Supplemental Table 3). According to the ClinGen recommendation for computational predictors,[26] based on CADD and REVEL, the variant p.(Arg227His) (REVEL: 0.761; CADD: 32) would reach moderate evidence for pathogenicity for both in silico scores, whereas only REVEL predicts a damaging effect for variant p.(Arg46Gly) (REVEL: 0.701; CADD: 24.3) and CADD only for the variant p.(Pro131Leu) (REVEL: 0.599; CADD: 28.7) (Supplemental Table 2). p.(Arg292Trp) was not predicted to be deleterious either by REVEL or CADD (Supplemental Table 2).

The 4 de novo missense variants are all absent from in-house databases of the specific centers and the general population based on gnomAD.[15]

The individual variants are examined in more detail below, including the results of the structural analysis we performed (Figure 2).

p.(Arg46Gly) is not described in gnomAD, ClinVar or any other variant databases. Garces et al have studied the crystal structure of human CNOT9 (formerly known as Rcd-1 or RQCD1) and showed that Arg46 is involved in the formation of the cleft structure important for nucleic acid binding.[5] The loss of a positive charge in this region is therefore predicted to hamper the interaction with negatively charged oligonucleotides. In addition, molecular modeling revealed a loss of stabilizing intramolecular interactions of the arginine side chain with Ser43.

p.(Pro131Leu) has been analyzed by Pavanello et al because it has been identified in malignant melanomas. Functional analysis showed that the stability of the protein is reduced by the p.(Pro131Leu) variant. The measured deadenylation activity was not significantly decreased by the p.(Pro131Leu) variant compared with the wild type. Reduced protein levels because of decreased cellular stability or specific protein-protein interactions are discussed as a possible pathomechanism.[4] Molecular modeling revealed that intramolecular steric clashes with residues Leu82, Thr83, and Ser87 arise from the p.(Pro131Leu) exchange, which are suggested to cause destabilization of CNOT9.

Garces et al analyzed the position Arg227 in detail as they studied the cleft structure of CNOT9 and the most important residues for the interaction and function of the protein. The positively charged cleft is used for interaction with oligonucleotides, which is base-selective as poly(G), poly(C), and poly(T) are preferred over poly(A). Garces et al found that Arg227 is highly relevant for the electrostatic potential of the cleft region. They modified this residue to glutamic acid and found that the ability to bind oligonucleotide was abolished. As a conclusion, it was deduced that the modification of arginine residues in the cleft region leads to reduced binding of oligonucleotide, but the overall protein structure remains similar.[5] In addition to weakened oligonucleotide binding, molecular modeling of p.(Arg227His) indicates a loss of the stabilizing intramolecular interactions of the arginine side chain with Glu176.

Interestingly, this variant (c.680G>A, p.(Arg227His)) was also identified de novo in the DDD study.[12] Besides a DD and growth abnormalities, no clinical information was available for this individual (DDD Study 13k.03498).

This variant has not been described in public databases or studied in the literature so far. It is a less conserved position,[27] and sequence-based predictions estimate the pathogenicity of the variant to be less severe (Supplemental Table 2). In the database gnomAD, a change to glutamine has been reported twice.[15] Residue Arg292 is located in the unstructured region (residues 285-299) of the CNOT9 C terminus, which is close to one of the tryptophan-binding pockets of CNOT9 (Figure 2A). Residue Trp292 present in the variant creates a binding motif that fits into in the tryptophan-binding pocket (Figure 2B). The tryptophan thus blocks intramolecularly the binding pocket, which is otherwise used for intermolecular interactions with binding partners of CNOT9.

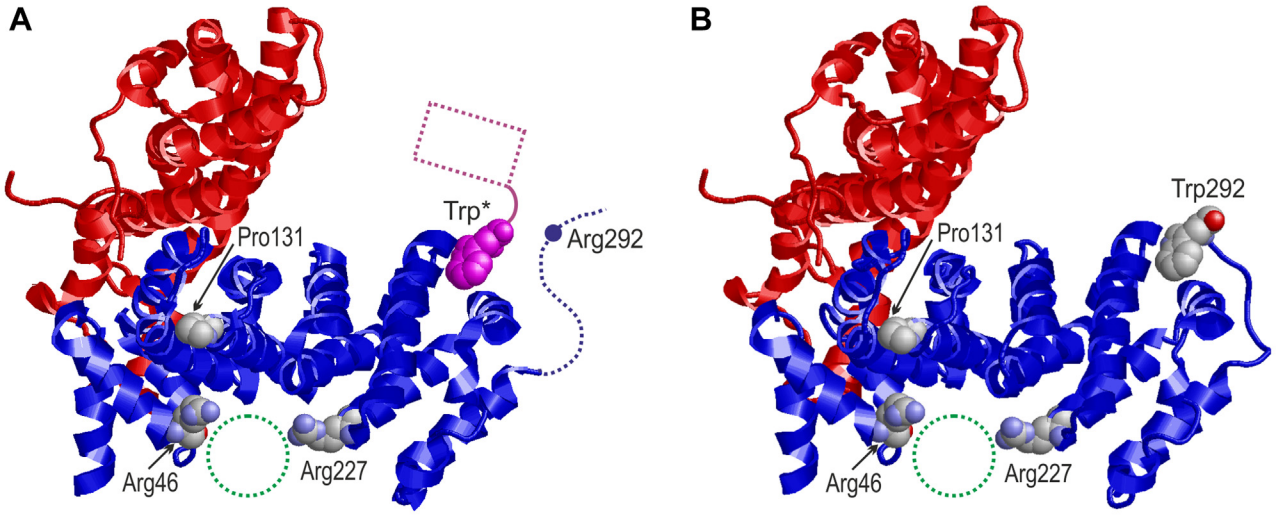

Figure 2 Structural analysis of the variants in CNOT9. A. Crystal structure of CNOT9 (blue) in complex with CNOT1 (red). The oligonucleotide binding site mapped from experimental data[5] is indicated by a green dotted circle. For protein ligands binding to the tryptophan-binding pocket only, the position of the tryptophan (Trp*) is known from the experiment[6]; the remaining parts are indicated as dotted rectangle (colored in magenta). Sequence positions affected by amino acid exchange are shown in space-filled presentation and colored according to the atom type. Arg46 and Arg227 are close to oligonucleotide binding site, whereas P131 is buried in the CNOT9 structure. Arg292 is located in the flexible C-terminus of CNOT9 (blue dotted line). B. Effect of the p.(Arg292Trp) variant in CNOT9. Because of the spatial proximity, Trp292 will be able to interact with the tryptophan-binding pocket, thereby hampering the recognition of physiological CNOT9 interaction partners.

Discussion

We present 7 individuals with 4 different de novo missense variants in *CNOT9*. The affected individuals exhibited NDD, moderate to severe DD/ID, seizures, and behavioral disturbances (Figure 1).

Although the first 3 variants were found to be deleterious in silico (Supplemental Table 2), the variant p.(Arg292Trp) is not striking. The molecular modeling offers a possible explanation for this finding: unlike most disease-causing variants, in which the wild-type residue plays an important structural or functional role, Arg292 seems to be rather dispensable for CNOT9 structure (Figure 2A). Thus, most types of amino acid exchanges are tolerated at this position; only the exchange to tryptophan results in a novel binding motif that blocks a protein-protein interaction site (Figure 2B). Consequently, in silico methods that strongly rely on the detection of sequence conservation may fail to correctly classify of such types of exchanges. This blockade of the binding site can then potentially lead to reduced degradation of mRNA because the activity of the deadenylase is not fully used. However, functional analysis has yet to confirm this hypothesis. Of note, other obvious disease-causing variants have been described, despite in silico or even functional analyses indicating no effect on protein function (eg, see the variant p.(Arg1146Cys) in *MAPK8I3*[28]). Additional independent evidence for the pathogenicity of p.(Arg292Trp) comes from the fact that it appeared 4 times in individuals with strong phenotype overlapping (similar DD, ID, and epilepsy).

The variants p.(Arg46Gly) and p.(Arg227His) result in the loss of positively charged arginine in the nucleic acid binding cleft of CNOT9. Modification of the arginine residue at Arg227 has also already been shown to undergo changes in the electrostatic potential of CNOT9, resulting in a reduction in the ability to bind oligonucleotides.[5] This suggests that the recruitment of, for example, mRNA to the CCR4-NOT complex could be prevented, thus negatively affecting RNA metabolism. Unfortunately, such detailed studies lacking for Arg46 but because this residue was also involved in the formation of the cleft structure,[5] one could speculate that missense variation causes a comparable pathomechanism, similar to Arg227. On the contrary, a destabilizing effect is predicted for p.(Pro131Leu), which has also been previously reported by Pavanello et al. However, a resulting reduced activity of the deadenylase could not be shown. Therefore, this variant may be causing a different, still unknown pathomechanism. Pavanello et al suspected reduced protein levels or altered protein-protein interaction mechanisms.[4]

All individuals presented with ID/DD. Particularly striking in the phenotype is the lack of language development in 4 of the 7 individuals. Motor development is also affected in all individuals because they learned to walk late or never did. The second major symptom that stood out was epilepsy, which also occurred in 5 of 7 individuals. Furthermore, all individuals had behavioral disorders, facial dysmorphia, and ophthalmological or skeletal abnormalities, but no specific symptoms could be identified in detail. Because the total number of individuals is low, further studies are needed to better understand the phenotypic spectrum of this disorder. Furthermore, the spectrum of severity may be biased as is often the case for novel disorders. Nevertheless, it is noticeable that individuals 4 to 7 (p.(Arg292Trp) tend to have a more severe phenotype compared with individuals 1 to 3 (75% and 25% with severe ID/DD, respectively). Therefore, a genotype-phenotype correlation based on the functional impact of each variant seems plausible.

Using the PanelApp Gene Panel guidelines,[29] the present evidence would meet the criteria for diagnostic grade (green). Specifically, we identified a plausible disease-causing variant with a Mendelian pattern of causation in 3 or more cases/families. Furthermore, the identified disease-causing variants are plausible because they occurred recurred de novo or affect gene's function. The variants were not detected in controls (gnomAD) and cause a fully penetrant rare disease.

It is notable that the symptoms ID/DD, hypotonia, behavioral abnormalities, and partial ophthalmic and skeletal abnormalities of individuals present in this study are similar to those of individuals with variants in *CNOT1*, *CNOT2*, and *CNOT3* (Supplemental Figure 1).[8,9,11] Although a large proportion of individuals with a variant in *CNOT1*, *CNOT2*, *CNOT3*, and *CNOT9* showed facial dysmorphic findings, no specific overlapping facial features were found (Supplemental Table 4). The overlapping symptoms suggest that a similar pathomechanism may lay behind all these diseases, which are caused by variants in parts of the CCR4-NOT complex. This suggests that missense or null de novo variants in other components of this complex may also lead to ID/DD as 6 of the genes encoding proteins in this complex have high Z-scores and pLI scores (Supplemental Table 3). With the current knowledge, we cannot make a final conclusion on that, but characterizing the identified *CNOT9* variants and other genes of the CRR4-NOT complex genes needs to be addressed in an independent study to test the hypothesis of a common pathomechanism.

In summary, the overlapping phenotype of the 7 individuals and those of the known *CNOT1-3*–related disorders, the genetic findings, the in silico data, and the structural modeling suggest that variants in *CNOT9* lead to a novel monogenetic form of NDD.

Data Availability

All data concerning this work are included in the manuscript and its supplement.

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Ethics Declaration

The study was approved by the ethics committee of the University of Leipzig, Germany (402/16-ek). All families provided informed consent for clinical phenotyping, genetic testing, and publication. Testing and research analysis was approved by local ethics committees in the respective institutions.

Conflict of Interest

Teresa Santiago-Sim is an employee of GeneDx, LLC. All other authors declare no conflicts of interest.

Additional Information

The online version of this article (https://doi.org/10.1016/j.gim.2023.100859) contains supplementary material, which is available to authorized users.

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