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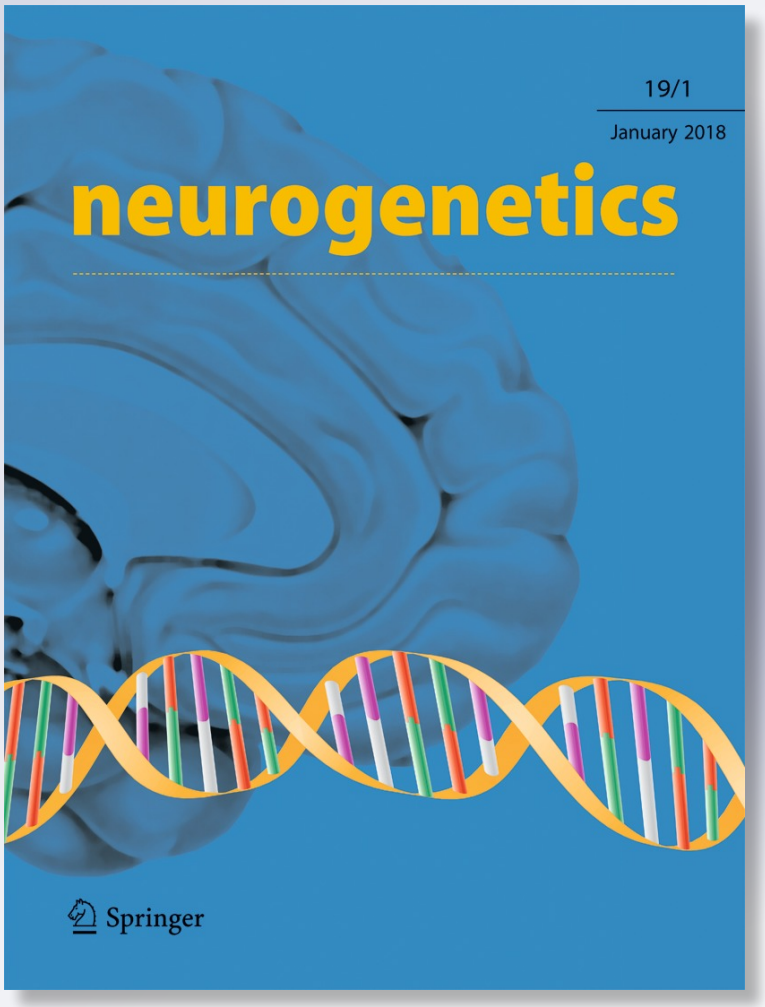

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ORIGINAL ARTICLE

The contribution of 7q33 copy number variations for intellectual disability

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Abstract

Copy number variations (CNVs) at the 7q33 cytoband are very rarely described in the literature, and almost all of the cases comprise large deletions affecting more than just the q33 segment. We report seven patients (two families with two siblings and their affected mother and one unrelated patient) with neurodevelopmental delay associated with CNVs in 7q33 alone. All the patients presented mild to moderate intellectual disability (ID), dysmorphic features, and a behavioral phenotype characterized by aggressiveness and disinhibition. One family presents a small duplication in *cis* affecting *CALD1* and *AGBL3* genes, while the other four patients carry two larger deletions encompassing *EXOC4*, *CALD1*, *AGBL3*, and *CNOT4*. This work helps to refine the phenotype and narrow the minimal critical region involved in 7q33 CNVs. Comparison with similar cases and functional studies should help us clarify the relevance of the deleted genes for ID and behavioral alterations.

Keywords 7q33 CNVs · *CALD1* · *AGBL3* · *EXOC4* · *CNOT4* · Duplication

Introduction

Interstitial CNVs in 7q are a rare event and, consequently, poorly characterized. Specifically, there are only 10 reports in the literature of interstitial deletions involving 7q33. Two cases are deletions (7.6 and 7 Mb) derived from chromosomal translocations [1–3]; one case is a small deletion (100 kb) affecting only two genes, *AKR1B1* and *SLC35B4*, in a patient with PHACE syndrome [4]; seven cases show large deletions ranging from cytoband 7q32 to 7q35 [5–11]. A deletion affecting 7q33 only was reported as an abstract but made no mention of the deletion size and genes affected [7]. The two most recent reports in the literature regarding interstitial 7q deletions describe genomic losses in a patient with intellectual disability (ID), language delay, and microcephaly [12] and in a patient with ID and dysmorphisms [11]. Not surprisingly, given the variable sizes of the deletions and duplications in all the reported cases, there is a widely variable phenotypic presentation, most likely due to the large number of genes involved in these variants. A summary of these reports is presented in Table 1.

It is possible to identify several interesting genes that could account for the ID/developmental delay (DD) phenotype associated with 7q33 CNVs, among which are *EXOC4* (exocyst complex component 4), *CNOT4* (CCR4-NOT transcription

Fátima Lopes and Fátima Torres contributed equally

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Table 1 Summary of literature reports of CNVs affecting the 7q33 cytoband

Publication	Affected cases (*n*)	CNV	Description	Size	Genes affected	Phenotype	Inheritance
Malmgren et al. 2005 [2]	3	Trans + del	ins(6;7)(p25;q33q34) Der(7) carriers: 7q33-q34 deletion	Del 7.4–7.6 Mb	Up to 68	DD/ID (variable degree), growth retardation, recurrent infections, facial dysmorphisms (long philtrum, thin upper lip, bulbous nose, large mouth, hypertelorism, dysmorphic ears)	Inherited (familial translocation leading to del/dup)
Malmgren et al. 2005 [2]	3	Trans + dup	Der(6) ins (6;7) carriers: 7q33-q34 duplication	Dup	Up to 68	Delay speech development, ID, difficulties in school	Inherited (familial translocation leading to del/dup)
Yue et al. 2005 [3]	1	Trans + del	t(7;10)(q33;q23) Der(7) carriers: 7q34-q35 deletion	Del 7 Mb	Up to 31; PTEN-EXOC4 gene fusion	DD, macrocephaly, hypotonia, scoliosis, feeding problems, recurrent infections, speech delay, eyes dysmorphisms	De novo
Nielsen et al. 1979 [10]	3	Trans + del	der(7)ins(13;7) (q32;q32q34) Der(7): 7q32-q34 deletion	ND	ND	ID, growth retardation, hypertelorism, facial dysmorphisms (bulbous nose, large mouth, large ears)	Inherited (familial translocation leading to del/dup)
Ponnala and Dalal 2011 [7]	1	Trans + del	t(7;14)(q33;q32.3) Der (7): 7q33-qter deletion	ND	ND	DD, absent speech, microcephaly, facial dysmorphisms (prominent eyes, arched eyebrows, malformed ears, bulbous nose)	Not maternal
Stallard and Juberg 1981 [9]	1	Del	7q31-q34 deletion	ND	ND	ID, growth retardation, facial dysmorphisms (long philtrum, thin upper lip, bulbous nose, dysmorphic ears)	De novo
Verma et al. 1992 [8]	1	Del	7q33-q35 deletion	ND	ND	ID, growth retardation, motor retardation, poor eye contact, recurrent infections, conductive deafness, cleft palate	De novo
Rossi et al. 2008 [5]	1	Del	7q33-q35 deletion	12 Mb	80	DD, autism, primary amenorrhea, neonatal seizures, sleep difficulties, poor language, truncal obesity, facial dysmorphisms (sunken eyes, hypertelorism, bulbous nose, long philtrum, large mouth)	De novo
Petrin et al. 2010 [6]	1	Del	7q33-q35 deletion	10 Mb	ND	DD, language delay, mild cerebellar and cerebral atrophy. Language: severe fluency disorder characterized by stuttering and cluttering.	De novo
Mitchell et al. 2012 [4]	1	Del	7q33 deletion	100 kb	SLC35B4	PHACE syndrome; brain anomalies (dysplastic right superior vermis, absent inferior vermis, hypoplastic right dural venous sinus, proliferating hemangiomas, aberrant circle of Willis), necrotizing enterocolitis (surgery required), non-viable small intestine, died at 2 months	ND

Table 1 (continued)

Publication	Affected cases (*n*)	CNV	Description	Size	Genes affected	Phenotype	Inheritance
Dilzell et al. 2015 [11]	1	Del	7q33-q35 deletion	9.92 Mb	64	ID, recurrent infections, obesity, self-injury behavior, facial dysmorphisms (small ears, large mouth, smooth philtrum, thin upper lip, hypertelorism, bulbous nose, short neck)	Not maternal
Bartsch et al. 1990 [13]	2	Dup	7q33-qter duplication	ND	ND	DD, feeding difficulties (at birth), macrocephaly, chronic obstipation, facial dysmorphisms (high forehead, frontal bossing, deep nasal bridge, epicanthic folds, down-slanting palpebral fissures, microretrognathia), low-set ears, macroglossia, short neck, hypotonia, enlarged subarachnoid spaces and cisterns.	Inherited (maternal translocation leading to dup)

ND not described, *ID* intellectual disability, *DD* developmental delay, *Mb* megabase, *kb* kilobase, *Del* deletion, *Dup* duplication, *Trans* translocation

complex, subunit 4), *CALD1* (Caldesmon 1), and *AGBL3* (ATP/GTP binding protein-like 3). Genotype-phenotype correlations in patients can help define the most relevant genes in this perspective.

In this report, we describe the clinical and genetic findings of seven patients with 7q33 copy number variations (CNVs) and extend the phenotypic spectrum of 7q33 interstitial CNVs. We also propose that *CALD1* and *AGBL3* are major contributors for the ID phenotype of these patients.

Methods

Patients

Patients 1–3 and 5–7 were ascertained within a large study of neurodevelopmental disorders in Portugal, in which the enrollment of the patients and families was done by the referring doctor. Clinical information was gathered in an anonymous database authorized by the Portuguese Data Protection Authority (CNPD). The study was approved by the ethics committee of Center for Medical Genetics Dr. Jacinto Magalhães, National Health Institute Dr. Ricardo Jorge.

Written informed consent was obtained for all participants involved in this publication for the genetic and gene expression studies, blood collection, and for publication of results (including photos).

Molecular karyotyping

Genomic DNA was extracted from peripheral blood using Citogene® DNA isolation kit (Citomed, Portugal) for patients 1, 2, and 3 and QIAsymphony SP (QIAGEN GmbH, Germany) for patients 5, 6, and 7. The aCGH analysis was performed using aCGH Agilent 180 K custom array design, accessible through the gene expression omnibus GEO accession number GL15397, for patients 1, 2, and 3 (according to the previously published protocol and the across-array methodology [14, 15]; Agilent 44 K oligo for patient 4; Affymetrix CytoScan 750 K platform for patients 5 and 7. aCGH data was analyzed using Nexus Copy Number 5.0 software with FASST Segmentation algorithm for patients 1, 2, and 3; DNA Analytics v4.0.76 for patient 4; Analysis Suite (ChAS 3.0) software for patients 5 and 7.

Quantitative PCR confirmations

Primers for qPCR were designed using Primer3Plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and taking into account standard recommendations for qPCR primer development [16]. A set of primers was designed for exon 10 of the *CNOT4* gene (NM_001008225) and for exon 4 of the *CALD1* gene (NM_

033138). The reference genes used were *SDC4* (NM_002999) and *ZNF80* (NM_007136) localized in the 20q12-q13 and 3p12 regions, respectively. qPCR reactions were carried out in a 7500-FAST Real-Time PCR machine (Thermo Fisher Scientific, Waltham, MA, USA) using Power SYBR Green® (Thermo Fisher Scientific, Waltham, MA, USA). The specificity of each of the reactions was verified by the generation of a melting curve for each of the amplified fragments. The primer efficiency was calculated by the generation of a standard curve fitting the accepted normal efficiency percentage. Quantification was performed as described elsewhere [17]. Ct values obtained for each test were analyzed in DataAssist ™ software (Thermo Fisher Scientific, Waltham, MA, USA). First-strand complementary DNA (cDNA) was synthesized using SuperScript® III Reverse Transcriptase (RT) (Thermo Fisher Scientific, Waltham, MA, USA).

FISH analysis

FISH was performed in metaphase chromosome spreads from cultured peripheral blood cells from patient 6. The FISH probe was generated using the BAC clone RP11-615F13 (Empire Genomics, Buffalo, NY, USA) and labeled with Green 5-Fluorescein dUTP. Analysis was performed according to the manufacturer's indication, and the fluorescence signals were captured using an Isis Fluorescence Imaging System, MetaSystems (Altlussheim, Germany).

Gene fusion exploratory analysis

Total RNA isolation and cDNA synthesis was performed as described above. In order to determine the presence of a fusion gene at the breakpoints of the 7q33 duplication described in patients 5 and 6, a set of primers were designed for amplification and sequencing of possible gene fusions, namely those linking *CALD1* exon 4 with *AGBL3* exon 16 and *AGBL3* exon 16 with *CALD1* exon 4. The fragments were amplified by PCR and sequenced on an automated DNA-sequencer ABI 3730 XL DNA Analyzer (Thermo Fisher Scientific, Waltham, MA, USA).

Results

Clinical description

Patients 1, 2, and 3

The proband of the first family (patient 1) is a male who was evaluated at 12 years of age for psychomotor delay, ID, and dysmorphic features. Parents are non-consanguineous and the delivery was uncomplicated, with normal growth parameters. At the time of the first consultation, he had short stature, weight was in the 25th centile, and the occipitofrontal circumference (OFC) was in the 75th centile. Evaluation with the Wechsler Intelligence Scale for Children (third edition) [18] was performed at childhood and showed a full-scale IQ of 42, associated with behavioral changes such as aggressiveness, hyperactivity, and disinhibition. The patient is currently 24 years old. He is dysmorphic, with a bulbous and snub nose (with concave root of the nose), down-slanting palpebral fissures, epicanthic folds, deep set eyes, thin upper lip, poor dental implantation and narrow cleft palate, dysplastic and posteriorly rotated ears, and prognathism (Fig. 1a). Additionally, he also has bushy eyebrows, spiky hair with a frontal cowlick, and two hair whorls at the forehead. The hands present light membranous syndactyly of the second to third digits and feet with brachydactyly, sandal gap, and fetal pads. Brain magnetic resonance imaging (MRI) detected a perivascular space enlargement while the echocardiogram and abdominal ultrasound retrieved no abnormalities.

Patient 2 (patient 1's sister) was observed for the first time at 19 years of age. Pregnancy and delivery were uncomplicated. At the time of the clinical evaluation, she presented short stature, weight was in the 95th centile, and OFC was in the 75th centile. She presented several dysmorphic features, similar to the brother's: snub nose with a concave root, bushy eyebrows, spiky hair with a frontal cowlick and two hair whorls at forehead, deep set eyes, epicanthic folds, thin upper lip, and poor dental implantation (Fig. 1b). She also had a short neck; narrow palate; and small dysplastic ears, posteriorly rotated. Abnormalities of the hands and feet included light membranous syndactyly and brachydactyly, respectively. Computed tomography (CT) scanning, echocardiogram, and abdominal ultrasound showed no abnormalities. Evaluation with the Wechsler Intelligence Scale for Children (third edition) showed a full-scale IQ of 62. Currently, she is 29 years old. Concerning behavior, she presents aggressiveness (similar to her brother) and disinhibition.

Patient 3 is the mother of patients 1 and 2. She has some clinical features similar to the daughter, such as facial dysmorphic features (milder) and brachydactyly (Fig. 1c). She has mild ID, although no formal neuropsychological evaluation was performed; she did not complete the fourth grade of school but she has the ability to do household chores.

Patient 4

Patient 4 was born at term to unrelated parents that are phenotypically normal. He was noted to be dysmorphic at birth and was admitted to the hospital because of

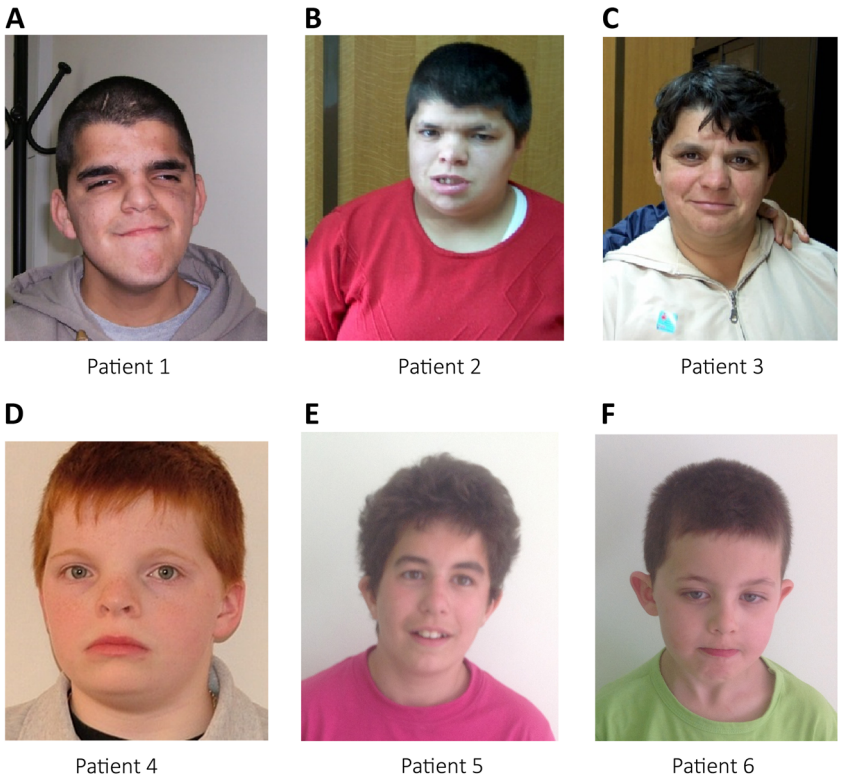

Fig. 1 Facial features of the patients

respiratory grunting. He had feeding problems early on. At 4 months of age, a right inguinal hernia was detected. He was noted to have a wide open anterior fontanelle at 8 months. Otitis media developed and a congenital meatal stenosis required meatoplasty at age 4 years. An evaluation at 10 years old revealed that he weighed 39.75 kg (centile 75) and had a height of 139.8 cm (centile 50) and an OFC of 57.2 cm (all within normal parameters) (Fig. 1d). Currently, he has hypertelorism and myopia. Behavioral issues were noticed at 4 years of age and he was referred to Child Psychiatry. His attention span was poor. He had aggressive outbursts, unpredictable behavior, and used bad language. He also presented a low frustration threshold, was impulsive, and with oppositional behavior. Currently, he has poor peer relationships (has no friends); he still has odd habits regarding feeding (concerns about bacteria on food) and is preoccupied with germs, death, bugs, and smells. He had a diagnosis of attention deficit and hyperactive disorder (ADHD) and developmental dyspraxia at age 11 years. He also has a tendency to be disinhibited.

Patients 5, 6, and 7

The proband of this family (patient 5) was evaluated at 11 years of age. Parents are non-consanguineous and delivery was uncomplicated, with normal somatometric parameters (at birth and now). He currently presents moderate ID (IQ = 54), associated with behavioral alterations (opposition, lack of attention, impulsiveness, and sexual disinhibition). He does not have significant facial dysmorphisms besides strabismus.

Patient 6 (patient 5's brother) is a 9-year-old boy with mild ID (IQ = 67) and aggressive behavior. He presents normal weight, height, and OFC (at birth and currently) and does not have significant facial dysmorphisms. He is short sighted (myopia).

Their parents were described as having learning difficulties at school. The mother (here referred as patient 7) has a documented ID (IQ < 60 at 20 years of age), a psychiatric disorder (emotional lability, obsessive behavior), and epilepsy. Although the father was not formally evaluated in the consultation by the responsible physician, he is described as healthy. Due to the mother's health condition, patients 5 and 6 currently live in an institution, since the mother does not have the intellectual and behavioral ability to take care of them. The facial appearance of patients 5 and 6 is presented in Fig. 1e, f.

A clinical comparison between the cases is presented in Table 2.

Molecular findings

aCGH

aCGH in patient 1 revealed a maternally inherited 2.08 Mb deletion at chromosome region 7q33 (chr7:133,176,651–135,252,871, hg19) containing 15 genes (according to the DECIPHER database) [19]. A qPCR assay for the *CNOT4*

Table 2 Clinical summary of the patients

Clinical feature	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Gender	♂	♀	♀	♂	♂	♂	♀
Consanguinity	No	No	No	No	No	No	No
Age at presentation/evaluation	12y	19y	Adult	Neonate	11y	8y	Adult
ID	Moderate	Mild	Mild	Mild	Moderate	Mild	Mild
Stature	Short stature	Short stature	ND	Short stature	Normal	Normal	ND
EEG/seizures	Seizures (single episode)	No	No	No	No	No	No
Cerebral MRI	Enlargement of peri-vascular spaces	Normal CT scanning	NP	NP	NP	NP	NP
Hypotonia	Yes	No	ND	Yes	No	No	ND
Behavior phenotype	Aggressiveness, hyperactivity, and disinhibition	Aggressiveness, disinhibition	ND	Obsessive-compulsive disorder, emotional lability, aggressiveness, low frustration threshold, impulsiveness, disinhibition	Opposition behavior, lack of attention, impulsiveness, disinhibition	Aggressiveness	Emotional lability, obsessive behavior
Dysmorphisms	Yes	Yes	Yes	Yes	No	No	No
Eyes/ophtalmological examination	Strabismus, epicanthus, sunken eyes	Epicanthus, sunken eyes	ND	Down-slanting palpebral; hypertelorism; Normal vision	Strabismus	Myopia	ND
Nose, mouth, and teeth	Bulbous nose, thin upper lip, open mouth, poor and crowded dental implantation, high and thin palate	Bulbous nose, thin upper lip, open mouth, poor and crowded dental implantation	Bulbous nose; thin upper lip	Wide mouth	NA	NA	NA
Forehead, chin, and neck	Hair whorls at the forehead, prognathism	Hair whorls at the forehead, short neck	NA	Prominent forehead	NA	NA	NA
Ears/audition	Small, dysplastic	Small, unilateral hypoacusia	NA	Otitis media in infancy	NA	NA	NA
Hands and feet	Hands: light membranous syndactyly, feet: sandal gap and fetal pads	Hands: light membranous syndactyly, feet: sandal gap and fetal pads	ND	Small feet; arthrogryposis	NA	NA	NA
Abdomen and genitalia	Inguinal hernia	ND	ND	Right inguinal hernia; chordae of penis	NA	NA	ND
Family history	Family history of ID [maternal uncle with ID, dysmorphisms and epilepsy; second grade cousin (paternal) with ID]	Family history of ID [maternal uncle with ID, dysmorphisms and epilepsy; second grade cousin (paternal) with ID]	Family history of ID (brother with ID, dysmorphisms and epilepsy)	None	Family history of ID (brother and mother)	Family history of ID (brother and mother)	Mother with psychiatric disorder, although without formal assessment

NA not available, *ND* not described, *ID* intellectual disability, *DD* developmental delay

gene was designed and used for validation and determination of the copy number of the region in the sister and both parents, confirming the presence of only one copy of the segment in the patient, sister, and mother. The father presented two copies for the analyzed segment.

Patient 4 was found to carry a de novo 3.04 Mb deletion at chromosome region 7q33 (chr7:132,766,730–135,802,894, hg19) containing 21 genes (according to the DECIPHER database).

Patient 5 presented a 216 kb maternal duplication at 7q33 region (chr7:134,598,205–134,807,358, hg19) containing three genes (*CALD1*, *AGBL3*, and *C7orf49*). A qPCR assay for the *CALD1* gene was designed and used for validation of the copy number of the region in the patient and both parents and for the determination of the CNV in his brother, confirming the presence of three copies of the fragment in patients 5, 6, and 7 (mother). The father presented two copies for the analyzed fragment (a result concordant with the aCGH). Patient 5 also performed a targeted exome sequencing comprising 4813 genes associated with known clinical phenotypes based on the OMIM database, but no significant pathogenic variants were identified.

A comparison between the molecular alterations identified in the reported patients is presented in Fig. 2 and Table 3.

FISH results

FISH analysis in patient 6 revealed a signal in chromosome 7 that is indicative of the presence of the duplication in tandem, excluding a location in another chromosome (Fig. 3d).

Fusion transcript results

Considering that, according to Newman and colleagues, most of the duplications' CNVs are in tandem and could originate fusion genes at the breakpoints [20], we have designed a set of assays in order to test for the presence of such chimeric transcripts. A fusion transcript between *AGBL3* exon16 and *CALD1* exon4 was detected in patients 5 and 6 (Fig. 3e). This finding is in agreement with the FISH analysis and also indicates that the duplication is not inverted. This hypothesis was also reinforced by the fact that it was not possible to amplify any PCR products indicative for an inverted duplication in patient 5 (Fig. 3e). According to our analysis, the identified *AGBL3-CALD1* gene fusion transcript would lead to an out of frame protein from *CALD1* on (Fig. 3f).

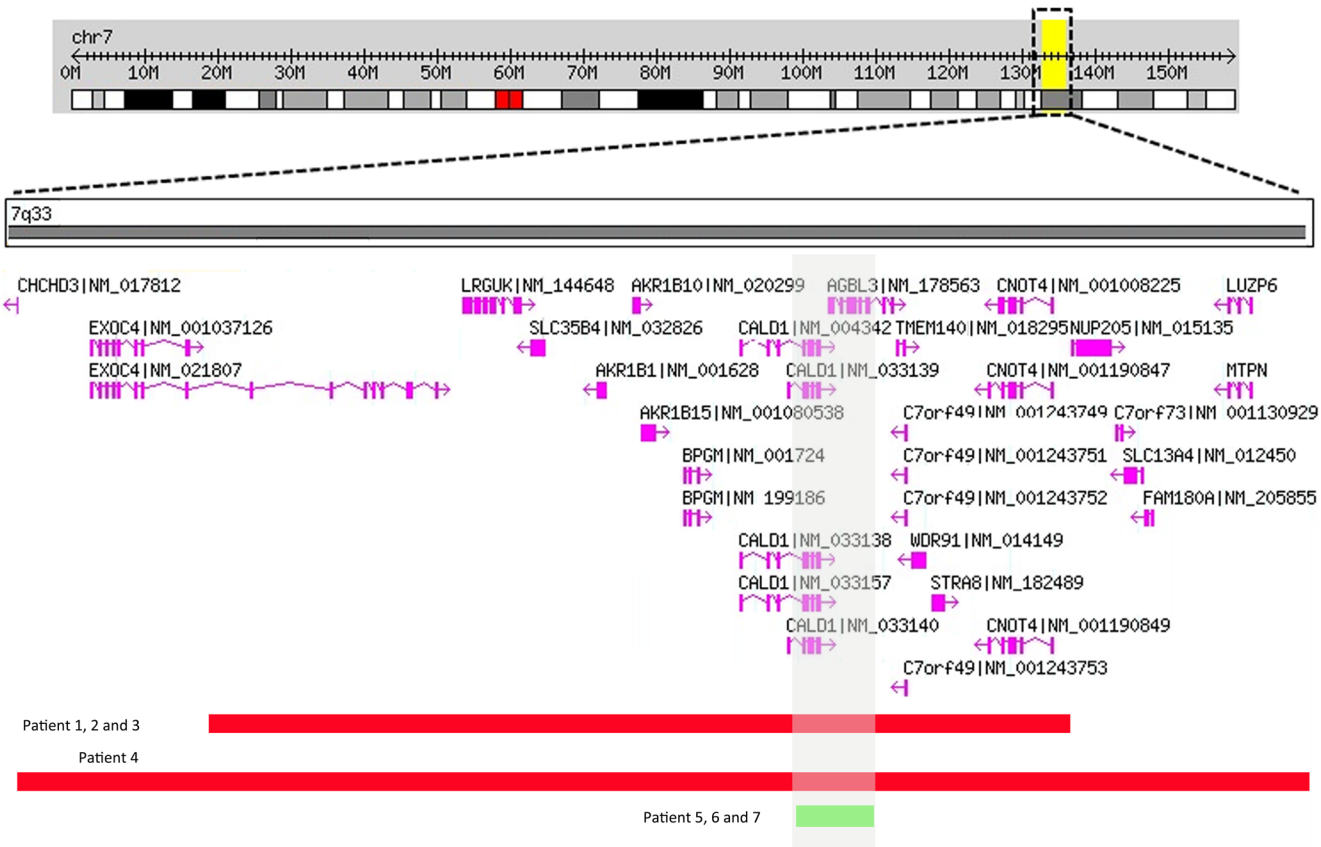

Fig. 2 Schematic representations and overlap of the CNVs found in the patients. A 3 Mb genomic portion of the cytoband 7q33 is shown. RefSeq genes present within the genomic region (in pink; transcriptional direction represented by the arrows) are shown. The overlapping deleted region for all the patients is shaded in gray. Individual red horizontal bars represent deletions. In each CNV, the corresponding patient is indicated

Table 3 Summary and comparison for the molecular findings present in the patients

Clinical feature	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7
Gender	♂	♀	♀	♂	♂	♂	♀
Consanguinity	No	No	No	No	No	No	No
Molecular karyotyping (aCGH)	Agilent 180 K	–	–	Agilent 44 K	CytoScan 750 K	–	CytoScan 750 K
Confirmation	qPCR (*CNOT4*)	qPCR (*CNOT4*)	qPCR (*CNOT4*)	NP	qPCR (*CALD1*); gene fusion	qPCR (*CALD1*); FISH (in *cis*); gene fusion	qPCR (*CALD1*)
CNV size	2.07 Mb	–	–	3,04 Mb	216 kb	–	209 kb
Interval coordinates (Hg19)	chr7:133,176,651–135,252,871	–	–	chr7:132,766,430–135,802,894	chr7:134,598,205–134,807,358	–	chr7:134,598,205–134,807,358
Inheritance	Maternal	Maternal	ND	de novo	Maternal	Maternal	ND
Genes affected	*AGBL3*, *AKR1B1*, *AKR1B10*, *AKR1B15*, *BPGM*, *C7orf49*, *CALD1*, *CNOT4*, *EXOC4*, *LRGUK*, *NUP205*, *SLC35B4*, *STRA8*, *TMEM140*, *WDR91*, *LOC653739*	*CNOT4* (performed by qPCR; presumably the same ones as patient 1)	*CNOT4* (confirmed by qPCR; presumably the same ones as patient 1)	*AGBL3*, *AKR1B1*, *AKR1B10*, *AKR1B15*, *BPGM*, *C7orf49*, *C7orf73*, *CALD1*, *CHCHD3*, *CNOT4*, *EXOC4*, *FAM180A*, *LRGUK*, *MTPN*, *NUP205*, *SLC13A4*, *LUZP6*, *STRA8*, *TMEM140*, *WDR91*, *SLC35B4*, *LOC653739*	*CALD1*, *AGBL3*, *C7orf49*, *LOC653739*	*CALD1* (confirmed by qPCR; presumably the same ones as patient 5)	*CALD1*, *AGBL3*, *C7orf49*, *LOC653739*

NP not performed, *qPCR* quantitative PCR

Constraint metrics

Several constraint metrics for all the 21 genes affected in patient 4 (with the larger CNV) are presented in Table 4. *EXOC4* and *CNOT4* are two of the genes with the highest ranks in the haploinsufficiency score (predicted probability of exhibiting haploinsufficiency); the data was retrieved from DECIPHER database, where the score was determined using the classification model published by Huang et al. [21]).

Discussion

While 7q33 CNVs are rare events, several interstitial deletions of chromosome 7q have been described in the recent past ranging from 7.6 to 13.8 Mb in size all [2, 5, 6, 11, 12]. In this work, we report seven patients (from three families) with 7q33 CNVs, all affecting at least the *CALD1* and *AGBL3* genes (Fig. 2 and Tables 2 and 3). Patients 5, 6, and 7 all present a small 216 kb duplication affecting the *CALD1*, *AGBL3*, and *C7orf49* genes, confirmed by FISH analysis to be in tandem and to lead to the formation of a fusion gene (Fig. 3e). This type of chimeric genes can be related to clinical phenotypes [20]. In fact, an enrichment of rare, brain-expressed chimeric genes was observed in individuals with schizophrenia, with functional studies suggesting a disrupting effect of these fusion genes in critical neuronal pathways [22]. Because both breakpoints occur in intronic regions, the genes are fused by *AGBL3* intron 15 and *CALD1* intron 3, leading to a fused transcript between *AGBL3* exon 16 and *CALD1* exon 4 without any apparent compromise of exonic regions (Fig. 3b, f). The variable amplification of the chimeric messenger RNA (mRNA) indicates that the fused transcript is likely to be degraded, though not completely, since we were able to amplify it in one of the samples collected from patient 5, but not in the other. Also, for patient 6, the fusion transcript was only possible to amplify in the first sample collected using a nested PCR protocol (Fig. 3e). The transcript was also not possible to detect in cultured blood cells for patient 6. This variability in the degradation of the product is not surprising, as it has been described before in a very similar study [23].

Nevertheless, and since the degradation of the *AGBL3-CALD1* chimeric gene does not appear to be complete, it is plausible that it might also contribute for the phenotype, since it could interfere with parent gene function [22].

Additionally, although the pathogenic contribution of the chimeric *AGBL3-CALD1* gene cannot be excluded, the detected rearrangement could also impair the individual expression of both the *AGBL3* and *CALD1* genes.

The *AGBL3* (ATP/GTP binding protein-like 3) gene encodes a cytosolic carboxypeptidase (CCP3) that is able to mediate both the deglutamylation and deaspartylation of tubulin [24]. The deglutamylation of tubulin plays an important

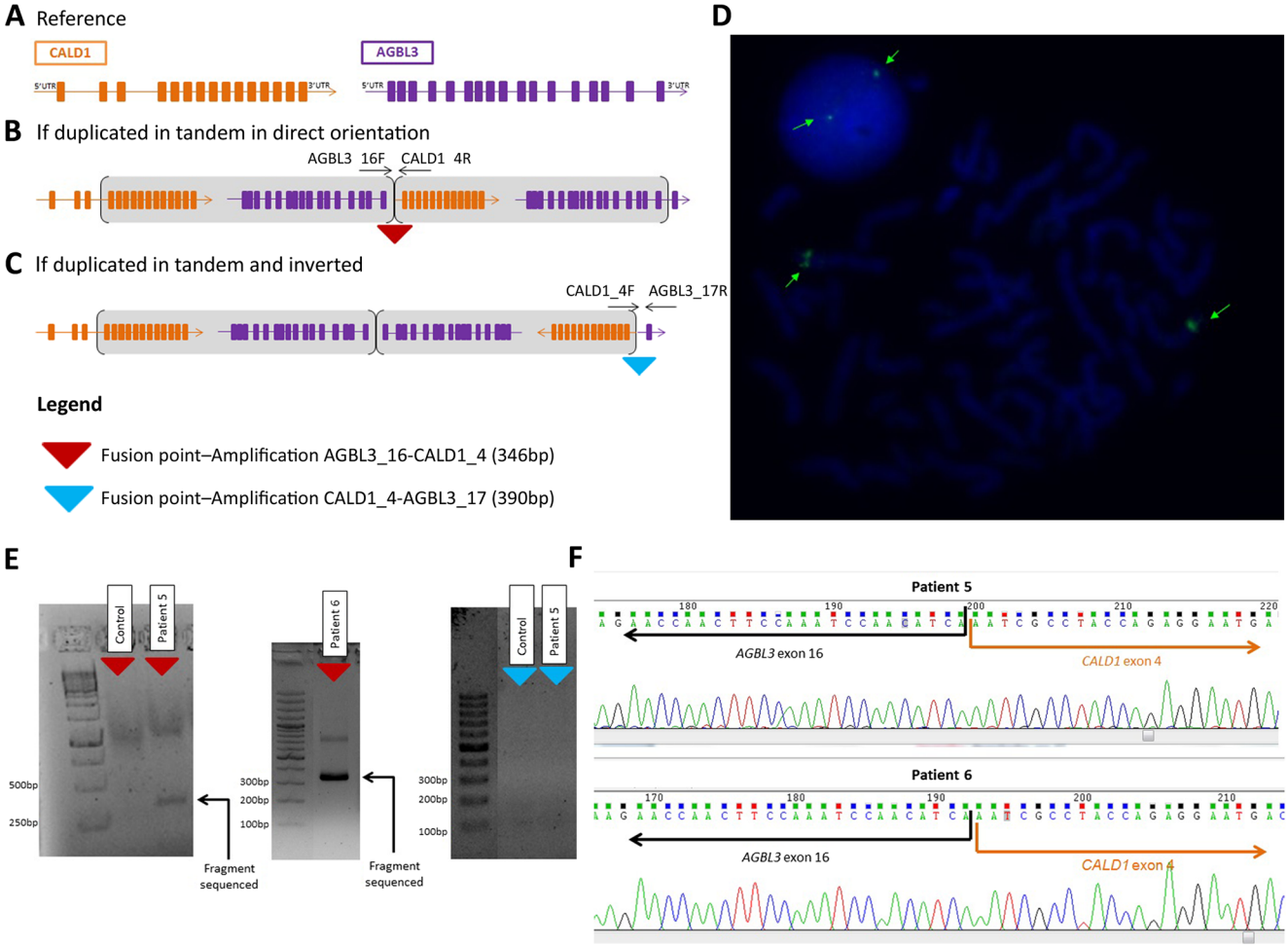

Fig. 3 Schematic representation of *CADL1* and *AGBL3* genes at the 7q33 cytoband. **a** Schematic representation of the normal location of *CADL1* and *AGBL3* genes. **b** The hypothesis that the duplicated region (highlighted in gray) is located in tandem and not inverted is represented, which would hypothetically lead to the formation of a gene fusion between *AGBL3* and *CALD1*. **c** The hypothesis that the duplicated region (highlighted in gray) is located in tandem and inverted is represented, which would hypothetically lead to the formation of a gene fusion between *AGBL3* and *AGBL3* and between *CALD1* and *AGBL3*. The red triangle represents the possible fusion between *AGBL3* and *CALD1* (in the "in tandem not inverted" scenario), the green triangle the possible fusion between *AGBL3* and *AGBL3*, and the blue triangle the possible fusion between *CALD1* and *AGBL3* (in the "in tandem inverted" scenario). **d** FISH analysis for the duplicated region using the BAC clone FISH probe RP11-615F13 located in *CALD1* gene where it is possible to observe a signal indicative of the presence of the duplication in tandem (arrow). **e** PCR amplification of potential fusion products from patients' and controls' cDNA. Only the PCR product corresponding to the *AGBL3* and *CALD1* fusion transcript was possible to amplify (indicated by the arrow) in patients 5 and 6. The absence of a PCR product for both control and patient 5's cDNA on the right (blue triangle) is not in support of the presence of this fusion product ("duplication inverted"). **f** Sanger sequencing of the PCR fragment amplified by AGBL3_16F and CALD1_4R revealed that *AGBL3* exon 16 and *CALD1* exon 4 were fused at the cDNA pf patient 5

role in regulation of the microtubule cytoskeleton, of known relevance for neurons; in fact, the control of the length of the polyglutamate side chains linked to tubulin was shown to be critical for neuronal survival [25], which would make this gene a possible contributor to the patients' phenotype. However, and although tubulin is a key protein in regulation of the microtubule cytoskeleton and this is of known relevance for neurons [25], there is not enough evidence that *AGBL3* does have a function in cytoskeleton regulation in neurons. In fact, according to the GTEx portal [26], *AGBL3* has very low expression in most of the tissues in human, with only the testis presenting a slightly higher expression at the mRNA level [24, 26]. Therefore, the contribution of this gene to the ID phenotype is actually unclear.

The other gene affected by this rearrangement is *CALD1* (Caldesmon1) which encodes for the caldesmon protein and is widely expressed, including in the nervous system. Caldesmon is an actin-linked regulatory protein that binds and stabilizes actin filaments and regulates actin-myosin interaction playing an important role in cell motility regulation [27]. Since caldesmon has numerous functions in cell motility (such as migration, invasion, and proliferation), executed through the reorganization of the actin cytoskeleton [28], its alteration is likely to have a functional contribution for ID

Table 4 OMIM entrance, haploinsufficiency score, and constraint metrics for the genes deleted in patient 4 (the largest deletion)

7q33	List of all the genes affected in P4	*AGBL3*, *AKR1B1*, *AKR1B10*, *AKR1B15*, *BPGM*, *C7orf49*, *C7orf73*, *CALD1*, *CHCHD3*, *CNOT4*, *EXOC4*, *FAM180A*, *LRGUK*, *MTPN*, *NUP205*, *SLC13A4*, *LUZP6*, *STRA8*, *TMEM140*, *WDR91*, *SLC35B4*							
Gene	Morbid gene	OMIM	HI score %	DDG2P	ClinVar	Constraint metrics			
						Synonymous (z)	Missense (z)	LoF (pLI)	CNV (z)
AGBL3	No	–	64.11%	–	10dels/11dups	–	–	–	–
AKR1B1	No	–	25.64%	–	9dels/10dups	− 0.32	0.27	0	− 2.25
AKR1B10	No	–	77.14%	–	9dels/11dups	− 0.28	− 0.27	0	− 4.12
AKR1B15	No	–	85.40%	–	9dels/11dups	0.02	− 1.03	0	− 3.36
BPGM	Yes	222800, erythrocytosis due to bisphosphoglycerate mutase deficiency, AR	22.09%	–	11dels/11dups/1SNV	0.16	0.77	0.13	0.5
C7orf49	No	–	80.37%	–	10dels/11dups	− 0.13	− 0.36	0.34	0.56
C7orf73	No	–	24.71%	–	11dels/11dups	–	–	–	–
CALD1	No	–	20.28%	–	10dels/11dups	1.02	− 0.14	1	0.73
CHCHD3	No	–	6.30%	–	9dels/13dups	0.18	0.15	0.04	− 0.13
CNOT4	No	–	6.19%	–	11dels/12dups	0.14	3.38	1	0.81
EXOC4	No	–	4.22%	–	18dels/18dups/1SNV	− 0.09	− 0.27	0	− 1.74
FAM180A	No	–	63.78%	–	11dels/11dups	− 0.26	− 0.33	0.34	1.16
LRGUK	No	–	71.82%	–	10dels/12dups	0.6	− 1.63	0	− 1.4
MTPN	No	–	15.71%	–	11dels/11dups	0.57	2.05	0.75	0.98
NUP205	Yes	616893, nephrotic syndrome, type 13	11.40%	–	11dels/12dups/1SNV	− 0.77	0.87	1	0.18
SLC13A4	No	–	40.17%	–	11dels/11dups	0.64	2.16	0.92	− 0.96
LUZP6	No	–	86.19%	–	11dels/11dups	–	–	–	–
STRA8	No	–	56.99%	–	10dels/11dups	1.42	0.74	0	0.51
TMEM140	No	–	83.19%	–	10dels/11dups	− 0.01	− 0.05	0.04	–
WDR91	No	–	46.24%	–	10dels/11dups	0.7	1.12	0	0.51
SLC35B4	No	–	21.16%	–	9dels/12dups	− 1.1	0.44	0	0.04

OMIM Online Mendelian Inheritance in Man, *HI score* Haploinsufficiency Score index—high ranks (e.g., 0–10%) indicate that a gene is more likely to exhibit haploinsufficiency, and low ranks (e.g., 90–100%) indicate that a gene is more likely NOT to exhibit haploinsufficiency (retrieved from DECIPHER), *LoF* loss of function, *CNVs* copy number variations, *z* Z score is the deviation of observed counts from the expected number for one gene (positive Z scores = gene intolerance to variation, negative Z scores = gene tolerant to variation) (retrieved from ExAC), *pLI* probability that a given gene is intolerant of loss-of-function variation (pLI closer to one = more intolerant the gene is to LoF variants, pLI ≥ 0.9 is extremely LoF intolerant) (retrieved from ExAC), *del* deletion, *dup* duplication, *SNV* single nucleotide variant, *ins* insertion, *indel* insertion/deletion

pathogenesis, as this is a common biological theme linking many ID-causative genes. Caldesmon overexpression induced by excess glucocorticoids was described to lead to altered patterns of neuronal radial migration through the reorganization of the cytoskeleton and impact on nervous system structure and function [29, 30]. Caldesmon is an important regulator of axon development [31] and may also play a role in synaptogenesis, synaptic plasticity, and dendritic arborization [32].

Four of the patients presented larger deletions also affecting the *EXOC4* and *CNOT4* genes. Considering that patients 1 and 2 are siblings and present the same deletion and very similar phenotypes, the main comparison should be made with patient 4. Concerning the behavioral phenotype, patients 1, 2, and 4 display aggressive behavior, disinhibition, and hyperactivity. Patients 1 and 2 also present some overlapping facial dysmorphisms with those of a patient previously described by Dilzell and colleagues—bulbous nose, thin upper lip, philtrum anomalies, small ears, and low posterior hairline [11]. The deletions' overlap for these four patients is defined by the deletion of patients 1, 2, and 3, resulting in a 2.08-Mb region that includes 15 genes. *EXOC4* (EXOCYST COMPLEX COMPONENT 4) is one of the common genes deleted among the first four patients. *EXOC4* is the human homolog of Sec8 in yeast. *EXOC4*/Sec8 encodes a member of the exocyst complex, broadly expressed in rat brain, localized in the synapses, and which plays a role in neurotransmitter release [33]. Sec8 was described to be involved in the directional movement of

Table 5 Summary of the DECIPHER patients with relatively small (< 2.6 Mb) and overlapping CNVs in 7q33

DECIPHER number	CNV	Size	Genes affected[a]	Inheritance	Pathogenicity	Index phenotype	Parent phenotype
280,233	del	178 kb	*EXOC4*	paternal	ND	ID	ND
253,613	del	45 kb	*EXOC4*	ND	ND	ID, autism, speech delay, hypotonia, obesity, puberty delay, limb abnormalities (short foot and tapered finger)	ND
262,735	del	259 kb	*EXOC4*	ND	ND	ID, behavioral abnormalities, hypotonia, atopic dermatitis	ND
271,567	del	160 kb	*EXOC4*	ND	ND	ND	ND
273,272	del	139 kb	*AKR1B1*	ND	ND	ID	ND
333,171	del	121 kb	*EXOC4*	ND	ND	Behavioral abnormality, language impairment	ND
338,702	del	468 kb	*EXOC4*	ND	ND	Behavioral abnormality, delayed speech and language development	ND
331,287	del	585 kb	*EXOC4*	maternal	Likely pathogenic	Developmental delay	ND
267,399	del	123 kb	*EXOC4*, *LRGUK*	ND	ND	ND	ND
328,659	del	2.6 Mb	*AGBL3*, *AKR1B1*, *AKR1B10*, *AKR1B15*, *BPGM*, *C7orf49*, *C7orf73*, *CALD1*, *CHRM2*, *CNOT4*, *FAM180A*, *LUZP6*, *MTPN*, *NUP205*, *SLC13A4*, *STRA8*, *TMEM140*, *WDR91*	De novo	Likely pathogenic (partially explaining part of the phenotype)	ID, psychosis	ND
282,285	dup	487 kb	*EXOC4*, *LRGUK*, *SLC35B4*	maternal	Uncertain (has a larger de novo pathogenic del in chr9)	Autism	ND
305,865	dup	346Kb	*EXOC4*, *LRGUK*, *SLC35B4*	ND	Uncertain	Autism, global developmental delay	ND
255,520	dup	719 kb	*CHCHD3*, *EXOC4*	Inherited from normal parent	ND	ND	Healthy
251,768	dup	828 kb	*AKR1B1*, *AKR1B10*, *AKR1B15*, *BPGM*, *EXOC4*, *LRGUK*, *SLC35B4*	Inherited from normal parent	ND	ID, hypotonia, brachydactyly, sparse hair, synophrys, abnormal dental morphology, high and narrow palate, open mouth, microcephaly, strabismus, large hears, heart defects (atrial and ventricular septal defect, coarctation of aorta)	Healthy
256,271	dup	1 Mb	*CHCHD3*, *EXOC4*, *PLXNA4*	De novo	ND	ND	ND

ND not described, *del* deletion, *dup* duplication, *ID* intellectual disability

[a] Genes affected in the DECIPHER patient

AMPA-type glutamate receptors towards synapses, promoting the membrane communication between polarized cells, as well as in the delivery of NMDA (*N*-methyl-D-aspartate) receptors to the cell surface in neurons through the interaction of Sec8 PZD domain with synapse-associated protein 102 (SAP102) [34, 35]. Sec8 was also described to bind to postsynaptic density protein-95 (PSD-95), essential for synaptic function [36].

Yue and colleagues reported a patient with DD and macrocephaly who presented a de novo translocation t(7;10)(q33;q23), together with a paternal 7-Mb deletion at 7q33. The authors hypothesized that the phenotype might arise due to the resulting EXOC4-PTEN fusion protein and/or haploinsufficiency of the disrupted genes [3]. The patient had some clinical features in common with the four patients reported here: he also presented ID, delayed speech, hypotonia, and facial dysmorphisms. Unfortunately, a picture is not available in order to allow a comparison with the present cases (Thomas Haaf and Susan Holder, personal communication).

The heterozygous deletion of this gene is thus common to four of the patients here described and to the patient reported by Yue and colleagues. At this point, we can only hypothesize that *EXOC4* haploinsufficiency can result in neurotransmission and synaptic impairment, and thus contribute to ID in these patients. However, we cannot disregard that the deletions present in patients 1, 2, 3, and 4 encompass other interesting genes.

One of those is the *CNOT4* (CCR4-NOT transcription factor complex, subunit 4) gene which encodes a protein that belongs to the conserved Ccr4-Not complex, involved in biological processes such as transcription regulation, mRNA degradation, histone methylation, and DNA repair [37–39]. The disruption of the proper methylation state of several genes has been shown to be associated with several neurodevelopmental disorders (see [40] for revision). In yeast, the *CNOT4* homolog Not4 functions as an E3 ubiquitin ligase and controls the level of Jhd2, the yeast ortholog of *JARID1C* [41]. This is interesting since mutations in *JARID1C* (lysine-specific demethylase 5C) were reported in patients with X-linked ID, revealing that the correct expression of this protein is essential for correct neuronal function [42–44]. Mersman and colleagues demonstrated that in the yeast, JARID1C homolog protein (Jhd2) levels are also regulated by *CNOT4* via a polyubiquitin-mediated degradation process [41]. More recently, Not4 was also described to be involved in the regulation of JAK/STAT pathway-dependent gene expression, an important pathway involved in organogenesis and immune and stress response in *Drosophila* [39]. The International Mouse Phenotyping Consortium [45] reports that mice carrying a homozygous intragenic deletion in Cnot4 present preweaning lethality (with complete penetrance), while the heterozygous mice have an abnormal caudal vertebrae morphology, hematopoiesis, and immune system defects [46]. No mention is made to central nervous system (CNS) or cognitive deficits, or craniofacial features in these mice. However, the literature reports its E3 ubiquitin ligase activity (UPS function being a common theme in neurodevelopmental genetics) and the functional connection to other known ID-causative genes further reinforces the possible contribution of *CNOT4* for the phenotype in patients 1, 2, 3, and 4.

Besides the analysis of the candidate genes in the 7q33 affected region, it is also important to take into account the patients described in DECIPHER database [19], with deletions and duplications that partially overlap the 7q33 affected region, summarized in Tables 4 and 5. Regarding the deletions, there are two patients (DECIPHER 280233 and 331287) with small inherited deletions affecting only the *EXOC4* gene. Even though for patient 331287 the submitters classified it as likely pathogenic, the phenotypic description of the transmitting progenitor is not provided. Additionally, we became aware of the existence of at least two more patients (unrelated, one with speech delay and the other with ID and hypotonia) carrying small deletions affecting only *EXOC4* gene that are inherited from the presumably healthy parents (personal communication by Audrey Briand-Suleau, Cochin Hospital, Paris, France). Concerning the duplications, there are two DECIPHER patients (255520 and 251768) carrying duplications affecting *EXOC4*, inherited from normal parents. As mentioned before, in these cases, it is important to determine if the duplicated region is located in tandem or not, in order to fully understand the impact of the duplication in the expression of the contained genes. For this reason, the inherited duplications in DECIPHER cases 255520 and 251768 must be interpreted with caution. In the literature, there are few reports of duplication affecting the 7q33 cytoband [2, 13]. Although their size is significantly larger than that of the duplication in patients 5, 6, and 7, the patients with duplications in this region reported by Malmgren and colleagues appear to have a lighter phenotype than those with the corresponding deletion. As for the report of Bartsch and colleagues, both reported patients have a very severe presentation, which might be due to the duplicated region being very large, encompassing the entire genomic region from 7q33 until the telomere. The difference in size makes the cases reported in these two publications very difficult to compare with patients 5, 6, and 7.

In the Database of Genomic Variants (DGVs), there are no deletions as large as the one present in patient 4. As for the duplicated region, there are no similar duplications in DGV. There are three small deletions in this region (affecting *AGBL3*, *CALD1*, and *TMEM140* genes); however, the presence of these deletions should be interpreted with care, as many of these large studies of control populations might have false calls and/or affected individuals as controls and they cannot be the basis of exclusion of a candidate alteration, especially in the light of other genetic and functional evidence supporting its relevance.

Nevertheless, these six cases raise doubts about the straightforward contribution of *EXOC4* for the NDD phenotype, leaving *AGBL3*, *CNOT4*, and *CALD1* as the more promising candidates.

In summary, this work presents seven patients with interstitial 7q33 CNVs and suggests that *EXOC4*, *CNOT4*, *AGBL3*, and *CALD1* genes are likely contributing for ID and a behavioral phenotype, characterized by aggressiveness and disinhibition. CNVs could impact the phenotype observed in these patients not only by means of haploinsufficiency but also due to the formation of chimeric genes, as the one observed in the patients with the duplication. Chimeras may disrupt critical brain processes, including neurogenesis, neuronal differentiation, and synapse formation, supporting the idea that chimeric genes play a role in the illness, at least in a small number of affected individuals, as recent publications have illustrated [22, 47]. Further studies need to be performed in order to better understand the contribution of each gene within this region to the phenotype.

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Author contribution FL, FT, SS, and PR performed the molecular studies and analyzed the molecular data. AMF, SAL, AJ, and JS collected clinical data. FL, FT, and PM drafted the paper. PM obtained funding for this study. The study was performed under the direction of PM.

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Compliance with ethical standards

Competing interests The authors declare that they have no conflict of interest.

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