


Biallelic *ELMO3* mutations and loss of function for DOCK-mediated RAC1 activation result in intellectual disability

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ABSTRACT

The engulfment and cell motility 3 (*ELMO3*) protein belongs to the *ELMO*-family of proteins. *ELMO* proteins form a tight complex with the *DOCK1-5* guanine nucleotide exchange factors that regulate *RAC1* spatiotemporal activation and signalling. *DOCK* proteins and *RAC1* are known to have fundamental roles in central nervous system development. Here, we searched for homozygous or compound heterozygous mutations in the *ELMO3* gene in 390 whole exomes sequenced *in trio* in individuals with neurodevelopmental disorders compatible with a genetic origin. We found a compound heterozygous mutation in *ELMO3* (c.1153A>T, p.Ser385Cys and c.1009 G > A, p.Val337Ile) in a 5 year old male child with autism spectrum disorder (ASD) and developmental delay. These mutations did not interfere with the formation of an *ELMO3/DOCK1* complex, but markedly impaired the ability of the complex to promote *RAC1*-GTP-loading. Consequently, cells expressing *DOCK1* and either of the *ELMO3* mutants displayed impaired migration and invasion. Collectively, our results suggest that biallelic loss-of-function mutations in *ELMO3* may cause a developmental delay and provide new insight into the role of *ELMO3* in neurodevelopmental as well as the pathological consequences of *ELMO3* mutations.

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Introduction

Neurodevelopmental disorders can lead to cognitive disabilities that affect both intellectual and adaptive functioning, and these can arise as a result of genetic mutations. As such, the discovery of novel mutations associated with such disorders might provide insight into the molecular and cellular defects that lead to cognitive disabilities.

DOCK-family proteins are *RAC*-specific guanine nucleotide exchange factors (GEFs) with established roles in neural differentiation, dendrite outgrowth, axon formation and axon-dendrite polarity. *DOCK1*, *DOCK2*, *DOCK3*, *DOCK4* and *DOCK5* can each form a complex with the Engulfment and Cell Motility (*ELMO*) family of scaffold proteins which includes *ELMO1*, *ELMO2* and *ELMO3* [1,2]. The *ELMO1-DOCK1*, *ELMO1-DOCK3* and *ELMO2-*

DOCK4 complexes participate in neurite and dendrite formation and the *ELMO/DOCK* pathway has been demonstrated to be involved in embryonic central nervous system (CNS) development [3–8]. While disturbance in these molecular functions likely explains the relation of *DOCK* and *RAC1* proteins with a number of neurodevelopmental disorders, the specific roles of *ELMO3* in CNS development or in neurodevelopmental disorders have yet to be investigated [9–11].

In the resting state, the *ELMO/DOCK* complex is auto-inhibited by intramolecular contacts (closed conformation) and exists in an open conformation when in complex with *RAC1* and other binding partners [12]. The open conformation *ELMO/DOCK* complex promotes localized GTP-loading of the small GTPase *RAC1* to promote actin cytoskeleton remodelling. The *ELMO/DOCK* complexes have been implicated in

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a number of cellular processes including migration/invasion, phagocytosis of apoptotic cells and myoblast fusion [1,2]. While ELMO1 and ELMO2 have been shown to function in cytoskeleton rearrangements during cell migration, phagocytosis and myoblast fusion, much less is known about the function of ELMO3 [1,13–15]. To date, ELMO3 has been linked to the migration and invasion of cancer cells and shown to contribute to stem cell renewal in the intestinal epithelium [16–19].

Here, we describe a patient with autism, developmental delay, and compound heterozygous mutations in *ELMO3*. Functionally, each of the *ELMO3* mutations carried by this patient impairs DOCK1-mediated RAC1 activation and cell migration and invasion. These findings support the notion that biallelic loss-of-function *ELMO3* mutations can cause a neurodevelopmental disorder characterized by developmental delay and intellectual disability.

Materials and methods

Identification of mutations in the *ELMO3* gene

We analysed 390 patients suffering from neurodevelopmental disorders of probable genetic origin by whole exome sequencing for either homozygous or compound heterozygous mutations in the *ELMO3* gene (Engulfment and Cell Motility Gene 3, NM_024712.3). We identified a novel compound heterozygous mutation in *ELMO3* (c.1153A>T, p.Ser385Cys and c.1009 G > A, p.Val337Ile) in a young boy with autism. The study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and approved by the Local Ethics Committees (Madrid, Spain; Ref. 30,062,019). Informed consent was obtained from parents after a full explanation of the procedures.

Genetic study

Exome sequencing was performed using genomic DNA isolated (MagnaPure, Roche) from whole blood from proband and parents. Libraries were prepared using the Ion AmpliSeq™ Exome Kit (Life Technologies) and quantified by qPCR. The enriched libraries were prepared using Ion Chef™ and sequenced on PI™ Chip in the Ion Proton™ System (Life Technologies) to provide >90% of amplicons with at least 20X coverage. Signal processing, base calling, alignment and variant calling were performed on a Proton™ Torrent Server using the Torrent Suite™ Software. Variants were annotated using Ion Reporter™ Software, and pedigree analysis was performed using the Genetic Disease Screen (GDS) trio

workflow. Variant filtering and prioritization were performed with an in-house software and a local database. Candidate variants were visualized using Integrative Genomics Viewer (IGV) and were evaluated based on stringent assessments at both the gene and variant levels, taking into consideration both the patient's phenotype and the inheritance pattern. Variants were classified following the guidelines of the American College of Medical Genetics and Genomics (ACMG). A board of molecular clinical geneticists evaluated each variant classified as pathogenic, likely pathogenic, or variant of uncertain significance, and decided which, if any, had to be reported. In every case, causal variants were discussed with the referring physician and/or clinical geneticist. Identified variants were confirmed by Sanger sequencing.

Plasmids, cell culture and transfection

pcDNA3.1+/C-(K)DYK-*ELMO3* (obtained from Genscript) was used as a template, and the human *ELMO3* constructs were added to the pEGFP-C2 plasmid by Gibson Assembly (HiFi, NEB), to generate the following constructs: GFP-*ELMO3* WT, GFP-*ELMO3* Val337Ile and GFP-*ELMO3* Ser385Cys. The pCNX2-Flag-DOCK1 plasmid was a kind gift from M. Matsuda (Kyoto University, Japan). HeLa and HEK293 T cells were maintained in DMEM 10% FBS penicillin/streptomycin. HeLa cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific) and HEK293T cells by the calcium phosphate method as described in [13].

Co-Immunoprecipitation

Forty-eight hours following transfection with the indicated plasmids by calcium phosphate, HEK293T cells were lysed in 1% NP-40 lysis buffer (150 mM NaCl, 50 mM Tris pH7.5, 1% NP-40) supplemented with 1X Complete protease inhibitor cocktail (Roche). Following centrifugation to clarify cell lysates, 500 ug of total protein extracts were incubated with anti-Flag M2 affinity gel beads (Sigma) for 90 minutes at 4 °C with rotation. Beads containing the immune-complexes were washed three times with the lysis buffer and proteins were analysed by SDS-PAGE and immunoblotting, as previously described [13].

Purification of GST-PAK-PBD

BL21 bacterial cells harbouring the pGEX4T1-PAK-PBD plasmid were grown in 5 ml of LB+ampicillin

media. The next day, 45 ml of LB+ampicillin media was added to the cultured bacteria and induced at 37 °C for 3 hrs in the presence of IPTG. The cells were collected by centrifugation, resuspended in 1 ml of PBS/1% Triton and 1X Complete protease inhibitor cocktail (Roche), and sonicated. Clarified cell lysates were incubated with glutathione beads (Genscript) for 30 min at 4 °C with rotation. Beads were washed three times with PBS/0.1%Triton. The purified GST-PAK-PBD protein was separated by SDS-PAGE and quantified after Coomassie Blue staining of the gel and comparing against a range of concentrations of albumin (Bio-Rad) as a standard.

GST-PAK-PBD pull down assay

GST-PAK-PDB affinity precipitation was performed to analyse the GTP-loading status of RAC1 [20]. HEK293T cells were plated on a 10 cm tissue culture dish and transfected by calcium phosphate with pEGFP-C2 alone, Flag-DOCK1 and pEGFP-C2, or Flag-DOCK1 and GFP-ELMO3 WT or either of the ELMO3 mutants (with 1:2 ratio, with a total DNA of 12ug). Forty-eight hours after transfection, cells were lysed in lysis buffer (20 mM Tris pH7.2, 150 mM NaCl, 5 mM MgCl₂, 0.5% NP40, 5 mM β-glycerophosphate, 1 mM DTT; final pH = 7.4) and 1X Complete protease inhibitor cocktail (Roche). Lysates were incubated with 10ug of the purified GST-PAK-PDB proteins for 30 minutes at 4 °C with rotation. The beads were washed 3 times with lysis buffer. GTP-loaded RAC1 levels and total cell lysate (for normalization) were analysed by SDS-PAGE and immunoblotting. Quantifications were performed as follow: Pull-Down (GTP-loaded RAC1)/Input (total Rac1). The following antibodies were used: anti-Flag M2 (1:10,000) (Sigma), anti-GFP (B-2) (1:2000) (Santa Cruz), anti-RAC1 (1:3333) (Millipore) and anti-Tubulin (1:10,000) (Sigma).

Wound-Healing assays

HeLa cells were transfected in 6-well plates using Lipofectamine 2000 with 2ug of pEGFP-C2 alone, or Flag-DOCK1 together with constructs coding for either GFP-ELMO3 WT, GFP-ELMO3 Val337Ile or GFP-ELMO3 Ser385Cys for 24 h before being detached and re-plated in 12 well plates and allowed to form a monolayer for 24 h. About 80% of cells were positive for GFP, suggesting high transfection efficiency. After physically inducing a scratch using a pipet tip (200 ul), time-lapse movies were generated by taking pictures every 10 minutes for 6 h at 10X using a DM IRE2

microscope (Leica) equipped with an automated stage under controlled environment (37 °C, 5% CO₂, humidity; PECON). The Orca-ER Model C-4742 digital camera (Hamamatsu) was used to take the images. Wound closure was then measured and quantified at all time points using Image J software.

Boyden chamber invasion assay

HeLa cells were transfected in 6-well plates using Lipofectamine 2000 with 2ug of pEGFP-C2 alone, or Flag-DOCK1 together with constructs coding for either GFP-ELMO3 WT, GFP-ELMO3 Val337Ile or GFP-ELMO3 Ser385Cys for 48 h before performing the experiments. Boyden Chambers (24-well, 8 μm pore, Co-Star) coated with 100 ul of Matrigel (BD Biosciences) were used. The top chamber was seeded with 50 000 cells and their ability to migrate towards the bottom chamber containing 10% FBS media over a period of 16 h was measured. Cells in the upper chambers were physically removed using cotton swabs and cells on the bottom were fixed with 4% PFA. The membrane was then mounted on a glass slide using SlowFade Gold reagent (Invitrogen) and the average number of GFP positive migrating cells in five independent 10X microscope fields was evaluated.

Statistics

Data are presented as mean ± SEM from at least three independent experiments. Statistical analyses were performed with the GraphPad Prism Software using the Student's t test and p-values <0.05 were considered as significant (*p < 0.05, **p < 0.001, ***p < 0.0001).

Results

Clinical case

Whole exome *in trio* analyses were performed from individuals suffering from a neurodevelopmental disorder and revealed two genetic variants in *ELMO3* in a young patient with autism spectrum disorder (ASD) (Figure 1a). A missense variant chr16:67,235,720 A/T, c.1153A>T, p.Ser385Cys and a missense variant chr16:67,235,477 G/A, c.1009 G > A, p.Val337Ile were identified (Figure 1a, b). Segregation analysis confirmed a compound heterozygous state, as one of the mutations is present in one allele of the mother and the other mutation is carried by the father, consistent with an autosomal recessive inheritance (Figure 1a). These mutations were further confirmed by Sanger sequencing.

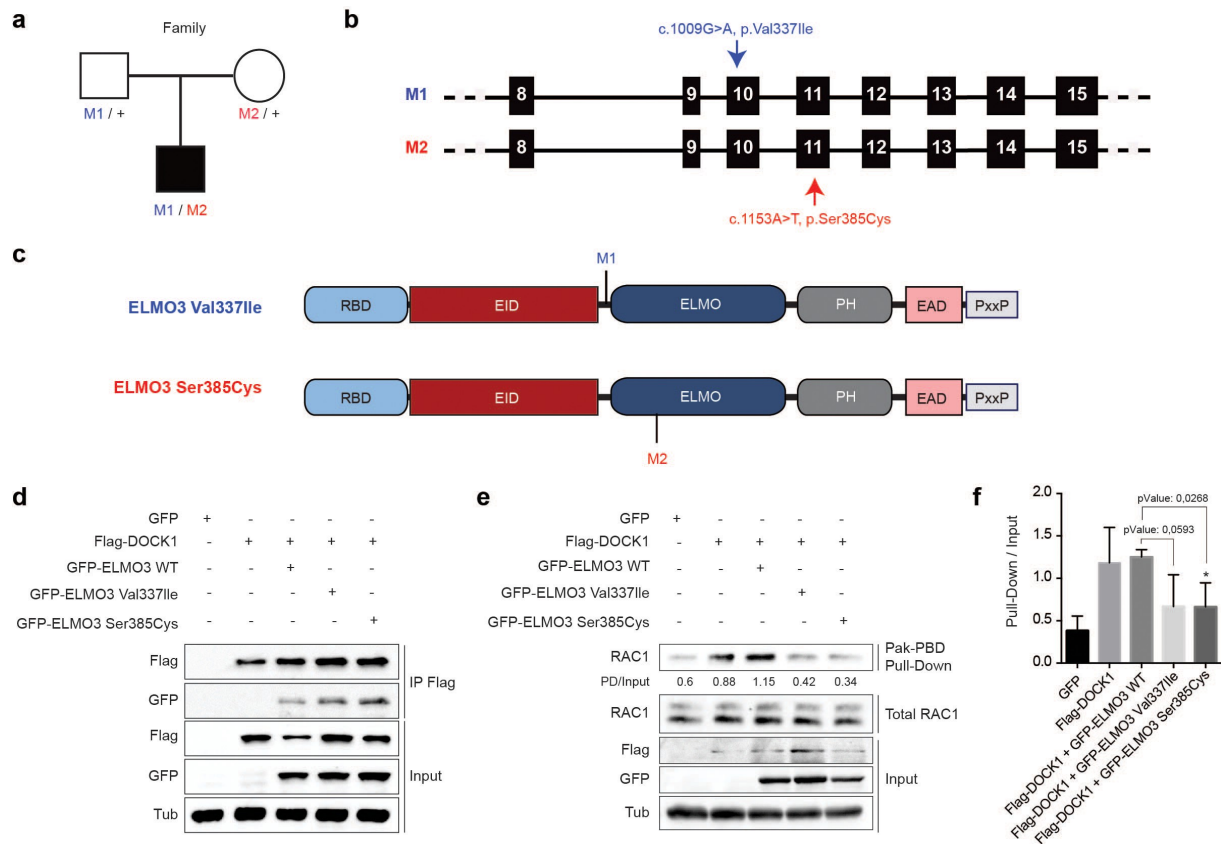


Figure 1. Two mutations in ELMO3, arising from biallelic heterozygosity in a patient, abrogate the GEF activity of the ELMO3/DOCK1 complex. **a)** Pedigree schematics. Filled symbol = affected individual. The mutation status is mentioned under each individual: + = WT allele, M1 and M2 = mutant alleles as defined in **b)**. **b)** Human *ELMO3* gene schematic with nucleotide position of M1 and M2 mutations pointed with an arrow. **c)** Schematic representation of ELMO3 and its functional domains: Ras-Binding Domain (RBD), ELMO inhibitory domain (EID), ELMO domain (ELMO), PH domain (PH), ELMO auto-regulatory domain (EAD) and proline-rich motif (PxxP). The mutations in ELMO3 reported in this study, Val337Ile and Ser385Cys, are found close or within the ELMO domain, and are each encoded by a single mutant allele of the biallelic heterozygosity state in the patient. **d, e)** Functional studies performed to characterize the biochemical properties of each of the ELMO3 mutants (each experiment was performed $n = 3$). **d)** The individual ELMO3 mutants co-immunoprecipitated with DOCK1 when co-expressed in HEK293T cells. **e)** Active RAC1 was analysed via a GST-PAK-PBD pull-down assay. **f)** Quantification of the assay done in **e)**. The expression of the ELMO3 mutants/DOCK1 complex led to less RAC1 activation in comparison to the exogenous expression of the wild-type ELMO3/DOCK1. Two-tailed unpaired t test was used; * $P < 0.05$.

The young patient with the two genetic variants in *ELMO3* is a young male child, who was referred to our clinic. At that time, he was 3 years old, the first child of non-consanguineous, apparently healthy parents of Spanish origin. He was the product of a 36-week pregnancy delivered via an uncomplicated C-section to his 40 years old mother. Apgar scores were 9–10 at 1 and 5 minutes, respectively. The birth weight was 2600 g (<5th centile), length 47 cm (5th centile), and the occipitofrontal circumference (OFC) was 34 cm (20th centile). The father of this child needed surgical treatment for an acoustic neuroma. Global developmental delays were noted in the first months of this child's life. He walked unsupported

late at 17 months. At 3 years of age, he was speech impaired and showed no interest in interacting with other children, always played alone and avoided social eye contact. Clinical examinations disclosed a weight of 15 kg (50th centile), a height of 98 cm (70th centile), and an OFC of 51 cm (50th centile), without dysmorphic features. Observation of the patient revealed verbal and nonverbal communication deficits, echolalia, neologisms, stereotyped and idiosyncratic language. Routine laboratory screening including thyroid function and neurometabolic tests were within normal range. Sleep video-EEG test and auditory evoked potentials displayed normal results. Brain 3T MRI did not reveal any significant

structural malformations. Conventional genetic studies (karyotype and array comparative genomic hybridization analysis) also revealed no abnormalities.

At the age of 5 years, this child still displayed severe verbal and nonverbal communication deficits. At this age, because of additional concerns regarding social communication and repetitive behaviours, further evaluation was performed using the Autism Diagnostic Interview-Revised (ADI-R) and the Autism Diagnostic Observation Scale (ADOS). He met the criteria for autism on both instruments. Battelle Developmental Inventory revealed scaled scores for each of the five domains (adaptive, social, communication, motor, and cognitive domains) below the 5th centile (equivalent age of 2 years).

Functional results

ELMO3, similar to other ELMO proteins, is composed of an N-Terminal Ras-Binding Domain (RBD), an ELMO inhibitory domain (EID), an ELMO domain, an atypical PH domain, an ELMO auto-regulatory domain (EAD) and a C-Terminal proline-rich motif (Figure 1c). Here, we identified a compound heterozygous mutation in *ELMO3*: c.1153A>T, p.Ser385Cys and c.1009 G > A, p.Val337Ile in the aforementioned patient. Interestingly, one of these mutations (Val337Ile) is found immediately preceding the ELMO domain, while the other one (Ser385Cys) lies within this domain (Figure 1c). Val337 is conserved among the three human ELMO proteins while Ser385 is not. Notably, no function has yet been ascribed to the ELMO domain of ELMO1-3 proteins.

We carried out functional studies to evaluate the impact of each of these mutations on the formation of an ELMO3/DOCK1 complex. Since ELMO3 complexes with DOCK1, DOCK3 and DOCK4 in neurons, it was considered possible that the mutations that are located near or in the ELMO domain of ELMO3 might cause structural aberrations that would affect the interaction with DOCK proteins, which is mediated by the adjacent atypical PH domain [3–7]. However, we found that each of the GFP-ELMO3 mutants displayed a robust interaction with Flag-DOCK1, similar to the wild-type GFP-ELMO3 (Figure 1d). This finding ruled out the possibility that the phenotype was due to abnormal ELMO3/DOCK interactions.

To determine if the ELMO3 mutations affected DOCK1-mediated RAC1 activation, we co-expressed Flag-DOCK1 with either wild-type ELMO3 (GFP-ELMO3 WT) or with each of the mutants in HEK293T and assessed RAC1 activation by PAK-PBD

pull-down assays. As expected, these experiments revealed that wild-type ELMO3/DOCK1 robustly promoted RAC1 GTP-loading in comparison to cells transfected with a control plasmid expressing GFP alone (Figure 1e, f). In contrast, co-expression of Flag-DOCK1 with either of the GFP-ELMO3 mutants resulted in greatly decreased activation of RAC1 (Figure 1e, f). Collectively, these experiments strongly suggest that both of the mutations in ELMO3 affect the ability of the ELMO3/DOCK1 complex to stimulate RAC1 GTP-loading.

We next explored whether the defects in RAC1 activation seen with the mutant ELMO3/DOCK1 complexes affected cellular migration and invasion. As expected, expression of wild-type GFP-ELMO3 with Flag-DOCK1 significantly increased cell migration in a wound healing assay in HeLa cells as compared to cells expressing GFP alone (Figure 2a, b). In contrast, when Flag-DOCK1 was co-expressed with either of the GFP-ELMO3 mutants, cell migration in the wound healing assay was significantly diminished (Figure 2a, b). Identical results were obtained when these cells were subjected to Boyden chamber Matrigel invasion assays (Figure 2c). Together, these results demonstrate that while neither of the ELMO3 mutations identified in this patient prevented the formation of a complex with DOCK1, they both profoundly inhibited the ability of the resulting ELMO3/DOCK1 complex to promote RAC1 activation as well as cell migration and invasion, thereby implicating the signalling activity of this axis in the development of the CNS.

Discussion

Here we describe the first case of intellectual disability and developmental delay associated with compound heterozygous *ELMO3* mutations. Each of the ELMO3 mutations described in this patient interfered with RAC1 activation and cell migration and invasion processes, but the mutant proteins ability to bind DOCK1 was not altered. According to these results, we hypothesize that a lower activity of the ELMO3/DOCK complex could alter the development of the CNS in these cases. Indeed, the involvement of the ELMO1 and ELMO2 proteins with the different DOCK proteins has an important role in neuronal development, dendritic and axonal formation [3–7]. In our study, we propose that the lower activity of RAC1 as well as the alteration of the cell migration process demonstrated in cells expressing ELMO3 mutants could result in neuronal dysfunction [9–11].

This study is the first report of a function of the ELMO domain in any ELMO-family proteins. Both mutations identified fall in or very close to this evolutionarily

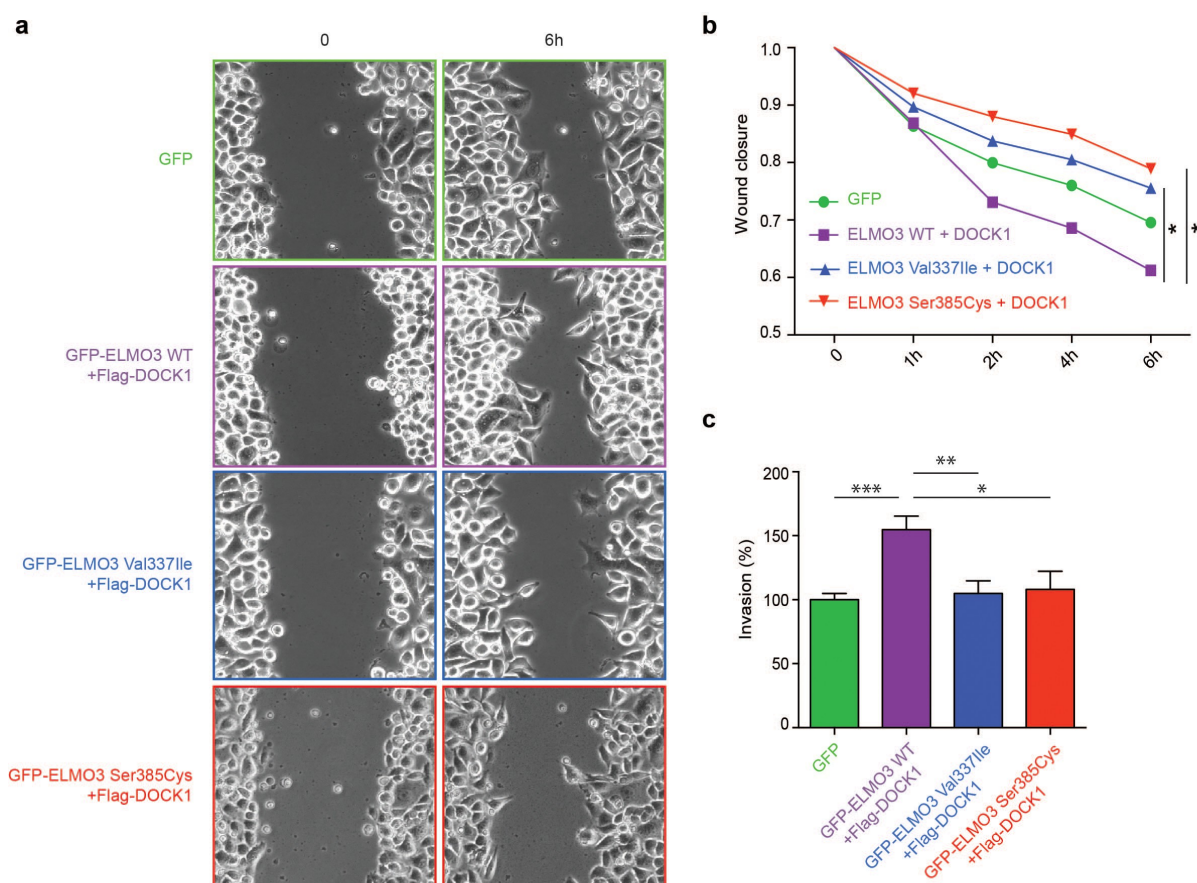


Figure 2. The individual patient-derived ELMO3 mutations impair cell migration and invasion. **a**) HeLa cells expressing Flag-DOCK1 with wild-type GFP-ELMO3 showed increased cell migration in comparison to cells expressing GFP alone, while Flag-DOCK1 co-expressed with either of the ELMO3 mutants showed less cell migration in a wound healing assay ($n = 3$, $*p = 0.0319$ and $***p = 0.0019$). **b**) Quantification of the wound closures shown in (a). **c**) Boyden chamber Matrigel invasion assays were performed with HeLa cells expressing wild-type or ELMO3 mutants together with DOCK1, or as a control, GFP alone. DOCK1/ELMO3 mutants showed an impaired ability to promote invasion in comparison to DOCK1/ELMO3 WT ($n = 3$, $***p < 0.0001$, $**p = 0.0017$ and $*p = 0.0134$).

conserved domain and result in decreased RAC1 GTP-loading. Exactly how the ELMO domain contributes to RAC1 activation and signalling remains an important area of investigation. Because each of the mutations represents a loss of biochemical function, in the biallelic heterozygosity state in the patient the two variant ELMO3/DOCK complexes are likely to be unable to carry out their main functions of activating RAC1. Since neither parent exhibited phenotypes, a single copy coding for the mutant ELMO3 protein is unlikely to be sufficient to cause developmental defects.

Although DOCK-family and RAC1 proteins have been related to different neurodevelopmental disorders, this is the first case that suggests the relation between ELMO proteins and developmental retardation. DOCK proteins, with known functions in neurite differentiation, dendrite development and axonal growth, have been implicated in different neurological disorders, including autism,

psychosis, Alzheimer's disease, intellectual disability or attention deficit hyperactivity disorder [9,21]. Furthermore, homozygous or compound heterozygous loss-of-functions of DOCK proteins have been proposed as a cause of neurodevelopmental delay [22–24].

Several neurological diseases associated with cognitive disability presenting aberrant spine formation associated with altered active cytoskeleton dynamics have been described. RAC1 is undoubtedly involved in this process and has critical roles in neuronal polarization, axonal development and cell differentiation. Indeed, RAC1 appears to be dysregulated in several neurodevelopmental disorders [10,25]. Additionally, heterozygous RAC1 missense mutations have been related to intellectual disability and abnormal cranial growth patterns [26].

The implication of DOCK-family and RAC1 proteins in other very different neurodevelopmental

disorders, such as attention deficit hyperactivity disorder (ADHD) or dyslexia, has also been described [9,27–29]. Dysfunction of DOCK proteins and their impact on neuronal migration or regulation of BDNF functions have been proposed as a possible cause of ADHD in some patients [30]. Indeed, a pericentric inversion breakpoint in the DOCK3 gene has been described in ADHD patients [31]. The dysregulation of cortical and hippocampal GABAergic interneuron maturation, its functional GIT1 association and glutamate signalling have been described as possible mechanisms to explain the relation of RAC1 with ADHD [27,32,33].

Although ELMO1 has been previously associated with autism, our case is the first one that relates ELMO3 with this disorder [34,35]. However, no relationship has been previously described between ELMO proteins and other neurodevelopmental disorders. Because of the low haploinsufficiency score of ELMO3 gene, the dysfunction of ELMO3-DOCK1 and its secondary impact on brain development should only be observed in biallelic ELMO3 mutations. More studies are needed to understand the possible participation of the ELMO family in neurodevelopmental disorders that cause autism or intellectual disability. The implication of the DOCK-family and the RAC1 proteins in ADHD raises the possibility that the ELMO proteins could also be involved in other disorders. Indeed, heterozygous loss-of-function mutations of ELMO genes could combine with decreased function in other signalling genes to explain the polygenicity of certain neuronal disorders.

Neurodevelopmental disorders are aetiologically extremely heterogeneous. In recent years, the findings revealed by large-scale next-generation sequencing and heritability studies support the high causal role of genetics. Sequencing studies have identified several mutations that lead to the brain dysfunction resulting from these neurodevelopmental disorders. Although 60% of these gene mutations follow autosomal-recessive inheritance, *de novo* heterozygous mutations and copy number changes explain the majority of cases with intellectual disability or autism in non-consanguineous populations [36–38]. Rare disorders caused by non-recurrent mutations or infrequent biallelic mutations can provide evidence for the identity of biochemical pathways that can affect neuronal development and function and provide important insight into future avenues for clinical and functional studies.

Contributors

MM and SA provided molecular genetic data; AJDD, PT, BCP and AFJ provided clinical data and reviewed the manuscript; VT and MAG provided functional data and reviewed

the manuscript; VT, SA, JFC and AFJ supervised the study and wrote the paper.

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Disclosure statement

The authors declare no conflict of interests.

Patient Consent

Obtained

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