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ARTICLE

A defect in the *CLIP1* gene (*CLIP-170*) can cause autosomal recessive intellectual disability

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In the context of a comprehensive research project, investigating novel autosomal recessive intellectual disability (ARID) genes, linkage analysis based on autozygosity mapping helped identify an intellectual disability locus on Chr.12q24, in an Iranian family (LOD score = 3.7). Next-generation sequencing (NGS) following exon enrichment in this novel interval, detected a nonsense mutation (p.Q1010*) in the *CLIP1* gene. CLIP1 encodes a member of microtubule (MT) plus-end tracking proteins, which specifically associates with the ends of growing MTs. These proteins regulate MT dynamic behavior and are important for MT-mediated transport over the length of axons and dendrites. As such, CLIP1 may have a role in neuronal development. We studied lymphoblastoid and skin fibroblast cell lines established from healthy and affected patients. RT-PCR and western blot analyses showed the absence of *CLIP1* transcript and protein in lymphoblastoid cells derived from affected patients. Furthermore, immunofluorescence analyses showed MT plus-end staining only in fibroblasts containing the wild-type (and not the mutant) CLIP1 protein. Collectively, our data suggest that defects in *CLIP1* may lead to ARID.
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INTRODUCTION

Lots of attention has been paid in recent years to a group of genetic disorders, whose major phenotypes include early-onset cognitive impairment or intellectual disability (ID). ID is characterized by an intelligence quotient below 70, and the presence or absence of certain clinical features. These disorders represent good genetic models for identifying genes involved in cognitive functions. Indeed, studies have shown that mutated genes in these disorders have critical roles in learning and memory and are also important for development of the central nervous system.[1]

With a prevalence of 1–3%, ID can be caused by both environmental and genetic factors. The latter may account for up to 50% of the ID cases, and even more of the severe forms.[1]

Considering its heterogeneity, it has been proposed that ID may be caused in large part by rare genetic variants. Linkage analysis based on homozygosity mapping can help identify such rare variants especially in families with consanguineous marriages and multiply affected siblings.[1]

In order to discover the genetics of autosomal recessive intellectual disability (ARID), our group has started a systematic strategy for mutation detection in large consanguineous Iranian families with the help of targeted exome sequencing. Up to now, we have reported several novel MRT loci (mental retardation loci) and genes for recessive cognitive disorders.[2–4]

Here, we present a single homozygous interval on chromosome 12q24, with LOD score >3, which is a new MRT locus for ARID. Targeted exome sequencing within this interval revealed a nonsense variant in the *CLIP1* gene (MIM 179838), which encodes a member of the microtubule (MT) plus-end tracking proteins.

MTs are one of the three types of cytoskeleton in eukaryotic cells. In neurons, most MTs lay along the length of axons and dendrites, where they are crucial for long range transport.[5] The dynamic behavior of MTs is largely controlled by a group of proteins called MT plus-end tracking proteins (+TIPs), which specifically associate with the ends of growing MTs. CLIP1 is the first reported member of the +TIPs. In non-neuronal cells, it contributes to kinetochore–MT attachments during mitosis,[6] and is an anti-catastrophe factor in mammalian cells in interphase.[7] Moreover, it has been shown to have an important role in spermatogenesis,[8] as well as in neuronal development.[9] CLIP1 and its relative CLIP2 appear to regulate neuronal polarization through MTs and growth cone dynamics.[10]

We show that the full-length CLIP1 protein is absent in patients' cell lines. Our data suggest that CLIP1 loss-of-function mutation can cause cognitive impairment and we propose *CLIP1* as a novel gene for ARID.

SUBJECTS AND METHODS

The study was approved by the Ethics Committee of University of Social Welfare and Rehabilitation Sciences, Tehran, Iran. Written informed consent was obtained from the living parent. The investigated family was from the southern part of Iran (Bushehr province), and being recruited to the Genetics Research Center (GRC). All ID patients resulted from normal deliveries following uneventful pregnancies, with maternal negative drug history and radiation exposure. Neonatal birth weights and measurements were normal. None of these individuals had psychomotor delay, including delayed walking or speech. Detailed history taking and clinical examination at the time of the study revealed moderate ID with additional features. The clinical measurements are depicted in Table 1. All patients had a history of seizure, which was

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controlled by antiepileptic drug therapy. Dermatological, cardiovascular and neurological examinations were normal. The physical examination of the eye showed strabismus in all patients. Funduscopic examination, which was available only for the male patient (IV: 6), showed non-specific changes in the retinal pigment epithelium. To exclude morphological abnormalities in brain structure, magnetic resonance imaging was performed for patient IV: 7, which revealed normal structure.

Urogenital system examinations were normal in female patients, but semen analysis and sperm tests showed azospermia in the male patient. Following repeated sperm analysis, testicular biopsy revealed complete spermatogenic hypoplasia. Peripheral blood samples were taken from all affected individuals, the mother and one healthy sibling (IV: 5). DNA extractions were done based on standard salting out protocol. Lymphoblastoid cell lines were established by Epstein–Barr virus from peripheral blood mononuclear cells for III: 5, IV: 5, IV: 6 and two unrelated normal individuals after getting written consent. The sample of anterior thigh punch skin biopsy in IV: 7 was used to derive fibroblast cell lines according to standard protocols.

SNP genotyping and linkage analysis

To calculate LOD scores for autozygous regions, whole-genome SNP genotyping using Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA, USA) was performed. We used Alohomora[11] software v0.32 for three steps of SNP array quality controls, as described previously.[12] We undertook multipoint parametric linkage analysis consistent with an autosomal recessive mode of inheritance, a disease allele frequency of 10^{-3} and complete peneterance, by Merlin[13] program. Our threshold for selecting linkage intervals was LOD score above three.[14]

Targeted NGS and data analysis

All the coding sequences and 60 base pairs of their nearby intronic sequences were enriched by Custom-made Agilent SureSelect DNA capture array (Agilent Technologies Inc, Santa Clara, CA, USA). NGS was performed using the Illumina Genome Analyzer II (Illumina, San Diego, CA, USA). The coverage of coding exons with at least $20\times$ is $>97\%$, and the average depth in the coding exons is $197\times$. Details of variant calling and filtering strategy are provided in Supplementary Document (NGS strategy). The final candidate variant was confirmed by Sanger sequencing. Details of the final variant can also be found in Leiden Open (source) Variation Database database.

RNA extraction and reverse transcription PCR

Total cellular RNA was isolated from lymphoblastoid and fibroblast cell lines using RNeasy Mini Kit (Qiagen, Hilden, Germany, cat. no. 74104), according to the manufacturer's procedure. After checking the quality and quantity, 1 μg of total RNA was used for first-strand cDNA synthesis using QuantiTect Rev. Transcription Kit (Qiagen, cat. no. 205311). The nonsense variant and the presence of *CLIP1* transcript variant 1 (NM_002956.2) were checked in lymphoblastoid cell lines with specific primers (Figure 1d shows the position of primers). Primers were designed across exon–exon junctions. The presence of RNA was controlled with the use of primers specific to the *GAPDH* transcript. Primer sequences are available upon request.

Table 1 Clinical characteristics of affected individuals in the family

Affected individual	*Sex*	*Age (years)*[a]	*Height (cm)*	*OFC (cm)*	*IQ score*
IV: 6	Male	23	171	55.3	55
IV: 7	Female	33	153.5	53	40
IV: 8	Female	28	159	54.4	45
IV: 9	Female	30	159	57	50
IV:10	Female	26	156	55	55

Abbreviations: IQ, intelligence quotient; OFC, occipitofrontal circumference.
[a]Age at the time of examination.

Real-time quantitative PCR experiment

Real-time PCR experiments were performed in an ABI7500 (Applied Biosystems, Foster City, CA, USA), using Power SYBR Green PCR Master Mix (Applied Biosystems, cat. no. 4367659). The produced data files were analyzed using 7500 Software v2.0.1 (Applied Biosystems). Primers were the same in RT-PCR and qRT-PCR experiments.

Protein extraction and western blot analysis

Whole-cell lysates were extracted based on a standard protocol, using Tris-Triton lysis buffer supplemented with protease and phosphatase inhibitors. Proteins were electrophoresed on 6% SDS-polyacrylamide gels, and then transferred to a PVDF membrane (Roche Applied Science, Basel, Switzerland, cat. no. 03010040001).

CLIP1 was detected by using two rabbit polyclonal antibodies described previously: one against the C-terminus of CLIP1 (Ab 2360),[6] and another against the N-terminus of CLIP1 and CLIP2 (Ab 2221).[15] For normalization, we used a mouse monoclonal anti-beta Actin antibody (Abcam, Cambridge, England, mAbcam 8226). Detection was done by the BM Chemiluminescence Western Blotting Kit (Roche Applied Science, cat. no. 11520709001).

Immunofluorescence microscopy and localization studies

CLIP1 was detected with the antibodies described above. CLIP2 was detected by Ab 2221, and by a rabbit polyclonal antibody against its C-terminus (Ab 2238) that does not recognize CLIP1 and was described previously. Fibroblasts were seeded onto glass coverslips, grown for several days and subsequently fixed and stained with anti-CLIP antibodies as described previously.[16] Images were acquired using a Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

RESULTS

An Iranian consanguineous family, featuring moderate autosomal recessive ID, history of seizure and strabismus (pedigree Figure 1a), was screened for the underlying genetic cause of ID. Complete spermatogenic hypoplasia was observed in the male patient.

Genotyping results of seven DNA samples (Figure 1a) were used for homozygosity mapping and multipoint parametric linkage analysis (Figure 1b and Supplementary Figure 1). We identified two homozygous genomic intervals with LOD scores above 2. The first interval (about 11 Mb) was on chromosome 12q24, located between heterozygous SNP markers, rs4766991 and rs10846561 (GRCH37/ hg 19) (Figure 1c). The calculated parametric LOD score for this interval was 3.73, with a non-parametric *Z*-mean of 16.30. There was also another homozygous region (about 195 kb) with a LOD score, 2.7, on chromosome 19, defined by markers rs919275 and rs11667028. Haplotype construction showed homozygous SNP haplotypes for all affected members within these two intervals.

High-throughput sequencing, using IV: 6 genomic DNA sample, revealed a homozygous nonsense variant g.12:122812682G>A (GRCH37/ hg 19) on chromosome 12. The list of other excluded variants can be found in Supplementary Table 1. The substitution (NM_002956.2:c.3028C>T) affects the *CLIP1* gene (MIM 179838), which clearly co-segregated with the disease in the family (Figure 1a, and Supplementary Figure 2). It was predicted to code for a truncated form of CLIP1, lacking the C-terminus and part of the central coiled coil region.

The pathogenic effect of the mutation, chr12:g.122812682G>A, is unknown. Human *CLIP1* has at least three known isoforms (UCSC Genome Browser on Human Feb. 2009 (GRCh37/hg19) Assembly) and all include the exon which harbors the mutation (Figure 1c; NM_002956.2:c.3028C>T, NM_198240.1:c.2923C>T, NM_001247997.1:c.3061C>T).

To begin to understand the pathophysiology of *CLIP1* mutation, we investigated mRNA and CLIP1 protein expression in cell lines derived

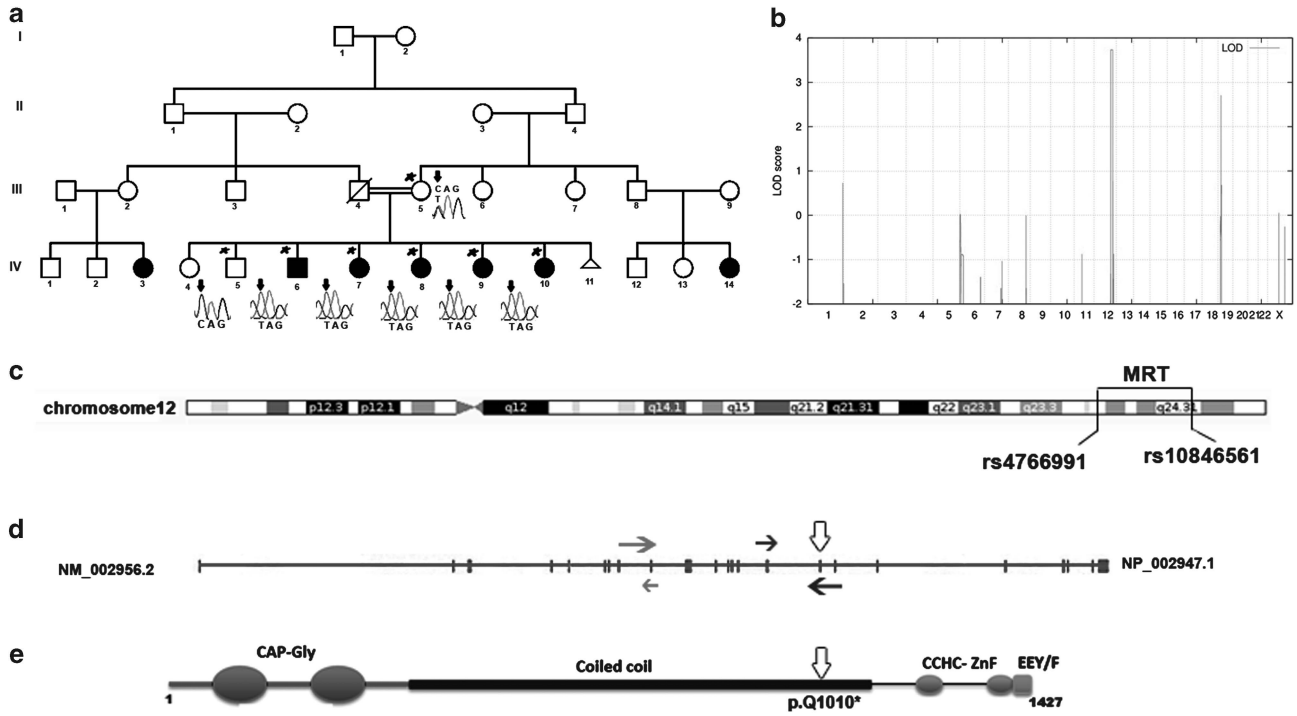

Figure 1 (**a**) Pedigree of the investigated family, *the genotyped samples. Filled symbols represent affected individuals. Co-segregation of the C>T transition in the family (arrows) has shown. This variant changes the Glutamine codon to a termination codon. The detailed chromatogram of all individuals can be found in Supplementary Figure 2. (**b**) Results of parametric linkage analysis, showing homozygous interval with LOD score above 3, on chromosome 12. (**c**) New genomic interval and its flanking SNPs. (**d**) Position of the nonsense variant (unfilled arrow) on *CLIP1* transcript (NM_002956.2). Red arrows indicate specific primers for amplification of NM_002956.2 and their covering exons. Blue (dark) arrows indicate specific primers for amplification of all transcripts in the region of nonsense variant and their covering exons. (**e**) CLIP1 protein (NP_002947.1) and its functional domains are shown. The N-terminal region of CLIP1 has two highly conserved CAP-Gly domains (Pfam: PF01302) that can be found in other +TIPs. They interact with EB1, alpha-tubulin and IQGAP1. The C-terminal part of CLIP1 consists of two zinc finger metal-binding motifs. The first motif interacts with the N-terminal domain. The second knuckle takes part in direct interaction with the dynactin subunit 1, DCTN1 ($P150^{Glued}$) of the dynein/dynactin complex. It can also bind to PAFAH1B1 (LIS1), which is a regulator for dynein function. The full colour version of this figure is available at *European Journal of Human Genetics* online.

from the patients and controls. RT-PCR experiments showed that *CLIP1* mRNAs carrying a premature stop codon (r.3183c>u) (Supplementary Figure 3) are reduced in the patient's cell line (Figure 2a). Quantitative RT-PCR experiments corroborated this observation and showed only 25–30% of the mutated *CLIP1* transcript in the patient (Figure 2b).

To test if the mRNA carrying the mutation was translated, we investigated cell lysates from patient fibroblast cells (IV: 7), from lymphoblastoid cell lines derived from another patient (IV: 6), as well as from a heterozygous carrier (III: 5), a healthy sibling (IV: 5) and an unrelated normal individual. For CLIP1 detection, we used two different antibodies, one specific for the C-terminus (Ab 2360) and a second (Ab 2221) which recognized the N-terminus of both CLIP1 and CLIP2. Using antibodies against the C-terminus, we detected wild-type CLIP1 bands of expected size (about 160 kDa) in mother (III: 5), and healthy brother (IV: 5) cell lines. We could not detect CLIP1 in patients' cell lysates (IV: 6 and IV: 7) suggesting existence of either a truncated mutant protein without functional C-terminal domain or total absence of CLIP1. To investigate this hypothesis, we performed western blot experiments using the CLIP1 N-terminus antibody. As expected, we detected the wild-type CLIP1 in normal cell lysates (III: 5, IV: 5) as well as in an unrelated normal individual, but no CLIP1-truncated protein of predicted size (116 kDa) in IV: 6 and IV: 7 (Figure 3).

Results of immunofluorescence staining for mutant CLIP1 in the patient fibroblast cells (IV: 7) compared with normal control fibroblast cells were compatible with our immunoblotting results. With Ab 2221 (Figures 4a and b), MT plus-end staining was detected in the control and mutant fibroblasts, which represented CLIP1and CLIP2 in the control cells. In the mutant cells, it mainly represents CLIP2. Using Ab 2360, MT plus-end staining was seen only in the control but not in the mutant fibroblasts (Figures 4c and d). Using an anti-CLIP2 antibody (Ab 2238), MT plus-end staining was seen both in the control and mutant fibroblasts, confirming the presence of CLIP2 in these cells (Figures 4e and f).

DISCUSSION

We have delineated the underlying genetic cause of ARID in an Iranian family. Linkage analysis helped us to identify a single homozygous genomic interval with LOD score above 3 (3.73) on chromosome 12q24.

In our previous studies of over 250 consanguineous Iranian families with ARID, approximately 39 single linkage intervals with LOD scores above 3 had been identified.[2,4] Although the homozygous regions of some reported ARID genes (*MED13L, COQ5, ZCCHC8*) overlap with this locus,[3] to the best of our knowledge, the present locus has not been previously linked to ID. Up to now, 40 loci for ARID (*MRT1–40*) have been registered in the OMIM database. *MRT25* and *MRT34* have been reported on chromosome 12q, but our detected interval did not overlap with them. The above

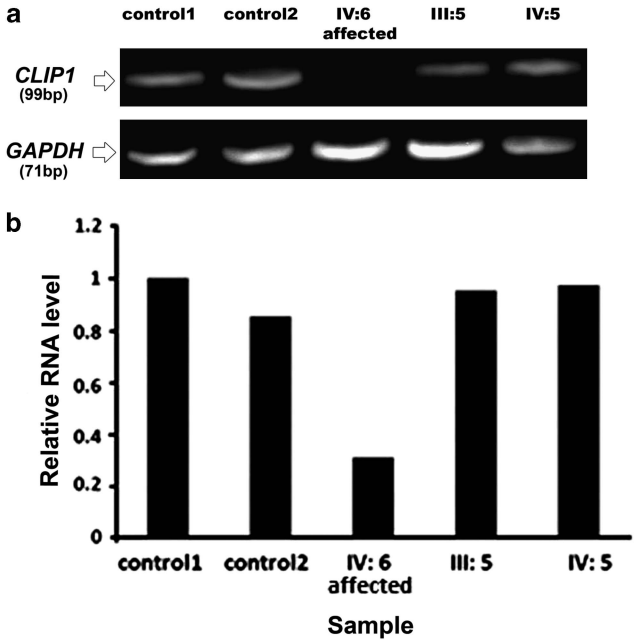

Figure 2 (**a**) RT-PCR results and (**b**) qPCR results with primers specific to NM_002956.2, show loss of *CLIP1* transcription in an individual homozygous for Q1010*. Relative RNA levels were determined by comparison to *GAPDH* expression levels. The presence of RNA was controlled with the use of primers specific to the *GAPDH* transcript.

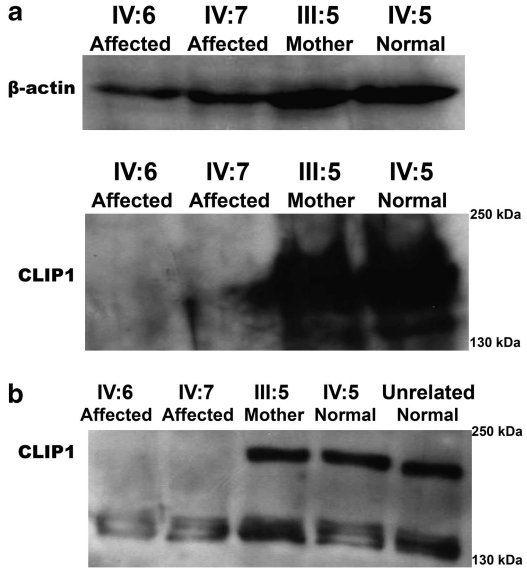

Figure 3 (**a**) Results of western blotting using Ab 2360, specific for CLIP1 C-terminus. The wild-type CLIP1 is absent in affected individuals. (**b**) Results of western blotting using Ab 2221 antibody, specific for CLIP1 and CLIP2 N-terminus. No full-length CLIP1 is detected in patients, whereas in normal controls the wild-type protein (160 kDa) is present. Ab 2221 also detects a protein of approximately 130 kDa in control and patient fibroblasts. No other protein is detected in this region of the blot. This protein very likely represents CLIP2, although the size of the protein is higher than expected based on the molecular weight of CLIP2 (115 kDa). We note that also in mouse CLIP2 migrates at a higher position than expected. For a comparable western blot analysis of CLIP2 in mouse fibroblasts, please see Figure 2c in Akhmanova *et al*.[8] Normalization was done with β-actin antibody.

data indicate that we have identified a new locus for ARID. These results are consistent with the great heterogeneity of ARID.

By appropriate filtrations after targeted exome sequencing, we could narrow down the variants to a single homozygous nonsense variant in *CLIP1*, a gene, which has never been reported in association with cognitive impairments. The detected nonsense variant in *CLIP1* gene causes a PTC in *CLIP1* mRNA. The mutation is predicted to result in a truncated form of CLIP1 (without C-terminal metal-binding motifs, and part of the central coiled coil regions). However, the location of PTC that is far upstream of the last exon, making the transcripts prone to nonsense-mediated decay, a protective mechanism leading to degradation of mRNAs containing PTCs.[17] RNA experiments showed a significant reduction of *CLIP1* transcript in the male patient in comparison with normal individuals (III: 5 heterozygous and IV: 5 wild type). The results of immunoblotting and immunostaining with two different anti-CLIP1 antibodies were also in support of nonsense-mediated decay for *CLIP1* mRNA, as we could not detect any CLIP1 protein (full length or truncated) in the patients.

CLIP1 encodes the CAP-Gly domain-containing linker protein 1 (also known as CLIP-170).[18] It is a MT rescue factor residing on growing (plus) ends of MTs.[5] At prometaphase of mitosis, CLIP1 links the outer part of unattached kinetochores to MT plus ends. This attachment is regulated through the interaction of CLIP1 with the dynein/dynactin complex.[19] CLIP1 is ubiquitously expressed and very conserved across species.[20,21] In *Drosophila melanogaster*, D-CLIP-190 in complex with 95 F myosin is colocalized in vesicles, and has a role in the axonal process of neurons.[22]

A close homolog of CLIP1 is CLIP2 (also known as CLIP-115), which is mainly expressed in the nervous system. CLIP2, a brain-specific cytoplasmic linker protein, has the same structure as CLIP1 but lacks the C-terminal metal-binding motifs.[23] Although the exact function of CLIP2 is unknown, the protein is involved in regulating MT dynamics in neurons[24] and in nervous system development and function.[16] In human, the *CLIP2* gene is located in the region that is commonly deleted in Williams–Beuren syndrome (MIM 194050) patients.[25]

In mice, CLIP1 was shown to have a role in spermatid differentiation. Loss of CLIP1 results in subfertility in male mice because of abnormal sperm head shape.[8] Putative cognitive impairments have not yet been studied in the *Clip1* knockout mice. CLIP1 was also shown to be involved in the maintenance of a normal neuromuscular phenotype.[26]

The N-terminal region of CLIP1 interacts with EB1 and alpha-tubulin, leading to CLIP1 localization on MT plus ends.[27] CLIP1 also interacts with IQGAP1 (IQ Motif Containing GTPase Activating Protein 1), which together with CLIP1, is part of a complex that has a role in linking the actin cytoskeleton to MTs during cell migration.[28] In rat neurons, CLIP1 together with IQGAP1 controls dendrite morphology, again possibly by regulating the interaction between MTs and the actin cytoskeleton.[9]

The second zinc knuckle in the C-terminal domain of CLIP1 takes part in direct interaction with the dynactin subunit 1, DCTN1 ($P150^{Glued}$) of the dynein/dynactin complex.[29] Dynactin (dynein activator) is essential for every cellular function of cytoplasmic dynein. It is responsible for targeting dynein to MT plus ends; helps to link dynein to cargos and to increase dynein processivity.[29] DCTN1 is the largest accessory subunit of dynactin.[30] Mutations in DCTN1 have been observed in amyotrophic lateral sclerosis (MIM 105400), and distal hereditary motor neuropathy 7B (MIM 607641),[5] showing the effect of impaired dynein pathway in disorders of the nervous system.

Dynein is responsible for retrograde (MT minus end directed) axoplasmic transportation. For example, dynein brings back

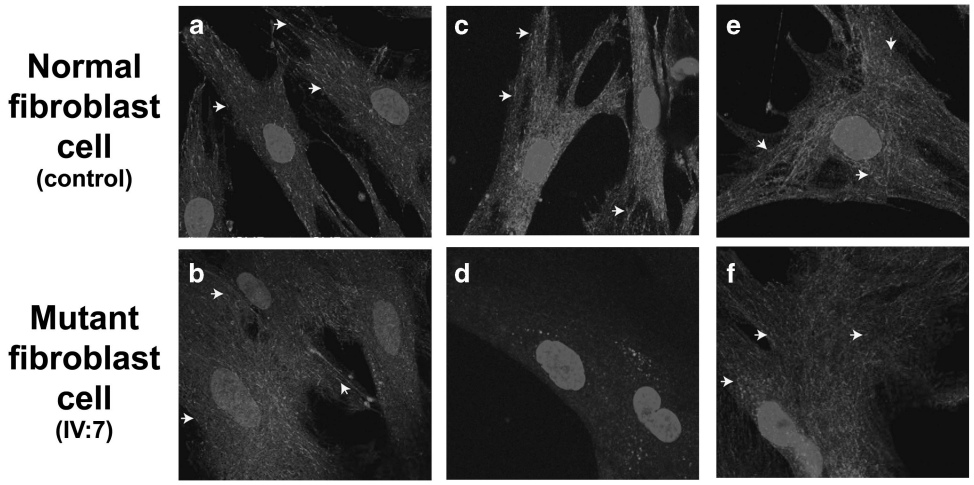

Figure 4 (**a** and **b**) Immunostaining with Ab 2221, detecting CLIP1 (CLIP-170) and CLIP2 (CLIP-115) N-terminus. In normal cells, the intracellular distribution represents both CLIP1 and CLIP2. In the mutants, it shows CLIP2 only. (**c** and **d**) Immunostaining with Ab 2360 antibody, detecting the CLIP1 C-terminus. No staining in mutant fibroblasts was detected because of lack of full-length CLIP1. (**e** and **f**) Immunostaining with Ab 2238 antibody, detecting the CLIP2 C-terminus. Both in normal and mutant cells CLIP2 staining are detected. Arrows indicate examples of plus-end staining of microtubules. The full colour version of this figure is available at *European Journal of Human Genetics* online.

misfolded proteins to the body of the cell, allowing them to assemble into aggresomes for subsequent degradation.[31] Dynein also has a role in regulation of gene expression in neuronal cells, by binding and moving signaling endosomes from axons to cell bodies. Mutations in cytoplasmic dynein can cause defects in axonal retrograde transport, affecting survival of the neurons, which has a role in neurodegenerative disorders.[32] Furthermore, in neurons, the abrogated interaction between dynactin (which mediates dynein function) and CLIP1 may result in hampered vesicle trafficking, and result in abnormal dendritic spines.

The second zinc finger motif of CLIP1 can also bind to PAFAH1B1 (LIS1), which is another regulator of dynein function.[6] PAFAH1B1 is important for correct migration of cortical and cerebellar neurons and also for transport of GABA-containing vesicles. Mutations in this gene can lead to type I lissencephaly.[33–35]

The above data in accordance with clinical manifestations, and the results of RNA and protein experiments, have greatly strengthened our primary hypothesis for the role of CLIP1 in ID, and also suggest an association between the impaired regulation of the MT cytoskeleton and a human neurodevelopmental disorder. Absence of CLIP1 abolishes its interactions with IQGAP1 and the dynein/dynactin complex, possibly affecting dendrite morphology and axoplasmic transport. We also hypothesize that CLIP1 might be involved in neurotransmitter and GABA-containing vesicle trafficking. Loss of interaction between PAFAH1B1 and CLIP1, and the normal brain magnetic resonance imaging in one of the patients, cannot exclude the role of PAFAH1B1 in neurological phenotypes, like seizure. It seems that CLIP1 deficiency may lead to a phenotype different from PAFAH1B1 mutations.

It is of note that our male patient had complete spermatogenic hypoplasia, confirming that CLIP1 also has a role in human spermatogenesis. Altogether, our data support the hypothesis that defects in CLIP1 can be the underlying etiology of ID plus male infertility.

CONCLUSION

Our data present a novel locus for ARID. By demonstrating the lack of the CLIP1, we provide the first evidence for the probable role of CLIP1 in human cognitive function. These data also could support the important interaction between MTs and IQGAP1, and dynein/dynactin complex in neuronal function and development. More investigations should be done to understand by which molecular mechanism defects in CLIP1 can affect cognitive functions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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