CDC174, a novel component of the exon junction complex whose mutation underlies a syndrome of hypotonia and psychomotor developmental delay

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Abstract

Siblings of non-consanguineous Jewish-Ethiopian ancestry presented with congenital axial hypotonia, weakness of the abducens nerve, psychomotor developmental delay with brain ventriculomegaly, variable thinning of corpus callosum and cardiac septal defects. Homozygosity mapping identified a single disease-associated locus of 3.5 Mb on chromosome 3. Studies of a Bedouin consanguineous kindred affected with a similar recessive phenotype identified a single disease-associated 18 Mb homozygosity locus encompassing the entire 3.5 Mb locus. Whole exome sequencing demonstrated only two homozygous mutations within a shared identical haplotype of 0.6 Mb, common to both Bedouin and Jewish-Ethiopian affected individuals, suggesting an ancient common founder. Only one of the mutations segregated as expected in both kindreds and was not found in Bedouin and Jewish-Ethiopian controls: c.1404A>G, p.[*468Trpext*6] in CDC174. We showed that CDC174 is ubiquitous, restricted to the cell nucleus and co-localized with EIF4A3. In fact, yeast-two-hybrid assay demonstrated interaction of CDC174 with EIF4A3, a component of exon junction complex. Knockdown of the CDC174 ortholog in Xenopus laevis embryos resulted in poor neural fold closure at the neurula stage with later embryonic lethality. Knockdown embryos exhibited a sharp reduction in expression of n-tubulin, a marker for differentiating primary neurons, and of hindbrain markers krox20 and hoxb3. The Xenopus phenotype could be rescued by the human normal, yet not the mutant CDC174 transcripts. Moreover, overexpression of mutant but not normal CDC174 in neuroblastoma cells caused rapid apoptosis. In line with the hypotonia phenotype, the CDC174 mutation caused depletion of RYR1 and marked myopathic changes in skeletal muscle of affected individuals.
Introduction

Two siblings of non-consanguineous Jewish-Ethiopian kindred (Fig. 1A, P1) presented with a syndrome of congenital myopathy and psychomotor developmental delay: both were born at term, following normal pregnancies. Severe hypotonia of limbs and trunk was evident at birth, requiring ventilation as of 1 month of age and feeding through gastrostomy. Strabismus, with bilateral weakness of the abducens nerve, was evident, as were elongated face and open mouth reflecting low muscle tone. Undescended testes were found in the male and a small cardiac ventricular septal defect (VSD) in both siblings. No specific dysmorphism was seen. Mild psychomotor retardation, both gross and fine motor, was evident as was delay in speech acquisition. Blood creatine phosphokinase (CPK), lactate, ammonia, selenoprotein and amino acids, as well as urinary organic acids, were within normal limits. Karyotype, chromosomal microarray as well as molecular testing for DMPK mutations (myotonic dystrophy), Duchenne muscular dystrophy, Prader Willi and spinomuscular atrophy (SMA) were normal. Nerve conduction velocity testing was non-conclusive, suggesting a myopathy or a disease of the neuromuscular junction (NMJ). Single-fiber electromyography (SFEMG) suggested a myopathy or an NMJ defect as in myasthenia, yet tension testing was negative. Brain magnetic resonance imaging (MRI) at the age of 1 year demonstrated dilated lateral ventricles and thinning of corpus callosum with hypoplasia of uvula nodule vermis. Spinal cord MRI was normal. Growth in terms of height, weight and head circumference was normal. Muscle biopsy findings demonstrated random variability of muscle fiber size with no evidence of necrosis, no enhancement of endomysial fibrotic tissue or immune cell infiltrates. Immunohistochemistry staining for myosin demonstrated relative abundance of type I fibers. There was no evidence of glycogen storage or mitochondrial dysfunction. Staining for cox, desmin, spectrin, sarcoglycans (α, β, γ, δ), merosin (80 and 300 kD) and dystrophin 1–3 were normal. Electron microscopy (EM) studies demonstrated destruction of myofibrils with no evidence of regeneration or inflammation or glycogen storage (data not shown). Within the differential diagnosis, a possibility of merosin-positive muscular dystrophy was raised.

A similar phenotype was observed in four affected individuals of a consanguineous Israeli Arab Bedouin kindred (Fig. 1A, P2). All four were born at term following uneventful pregnancies (reduced fetal movements were suggested in retrospect by the mother in some of the pregnancies). Severe axial hypotonia, psychomotor developmental delay (gross and fine motor) and weakness/palsy of the abducens nerve were evident. All four had undescended testes and cardiac VSD or atrial septal defects (ASD). Blood CPK, lactate, ammonia, amino acids, very long fatty acids, as well as urinary organic acids, were within normal limits. Molecular testing for SMA was normal. Nerve conduction velocity studies showed normal distal latencies, amplitudes and velocities of the examined nerves, while electromyography (EMG) demonstrated small sharp units, suggesting possible myopathy. There was no improvement with neostigmine. Brain MRI was normal. Growth in terms of height, weight and head circumference was normal. Muscle biopsy findings demonstrated random variability of muscle fiber size with no evidence of necrosis, no enhancement of endomysial fibrotic tissue or immune cell infiltrates. Immunohistochemistry staining for myosin demonstrated relative abundance of type I fibers. There was no evidence of glycogen storage or mitochondrial dysfunction. Staining for cox, desmin, spectrin, sarcoglycans (α, β, γ, δ), merosin (80 and 300 kD) and dystrophin 1–3 were normal. Electron microscopy (EM) studies demonstrated destruction of myofibrils with no evidence of regeneration or inflammation or glycogen storage (data not shown). Within the differential diagnosis, a possibility of merosin-positive muscular dystrophy was raised.

Results

Linkage analysis and identification of the disease-associated mutation

As the parents of the Jewish-Ethiopian siblings were non-consanguineous (tracing at least seven generations back) yet of the same ethnic origins and inbred community, we assumed possible homozygosity of a founder mutation in the affected children. Affymetrix 250 K single nucleotide polymorphism (SNP) arrays, testing all four family members, identified a single 3.5 Mb homozygosity locus on chromosome 3 (Fig. 1B) that was shared by both affected individuals (Fig. 1A, P1-II-1 and P1-II-2). Whole exome sequence analysis of the affected male identified within the 3.5 Mb locus only three homozygous mutations: in NUP210, GRIP2 and CCDC174 (C3orf19), encoding coiled-coil domain containing 174. The mutations were verified through Sanger sequencing (not shown). Illumina 6 K array analysis done for all four affected individuals in the Bedouin kindred and their parents as well as Affymetrix 250 K analysis done for individual P2:IV-6 (Fig. 1A), identified a single 18 Mb homozygosity locus that was shared by all affected individuals (Fig. 1B). Fine mapping using polymorphic markers within the 18 Mb locus, testing all available family members, narrowed the locus down to 13 Mb on chromosome 3, encompassing the entire 3.5 Mb locus of the Ethiopian family (Fig. 1A, P2 and B).

While GRIP2 and CCDC174 were within the locus shared by both families, NUP210 was not. Whole exome sequencing done for individual P2:IV-6 (Fig. 1A, P2) identified within the entire 13 Mb locus only two homozygous mutations: the same GRIP2 and CCDC174 mutations that were found in the Ethiopian family. Testing the available individuals of the Bedouin kindred for the GRIP2 mutation through Sanger sequencing identified a healthy individual (Fig. 1A, P2-IV-2) who was homozygous for the mutation. Thus, the only mutation common and unique to the affected individuals of both kindreds was c.1404A>G, p.(*468Trpext*6) in CCDC174, NM_016474.4(CCDC174_v001), termed also c3orf19 (Fig. 1C). The stop codon loss mutation is predicted to extend the CCDC174 protein by six additional amino acids. Restriction analysis for the CCDC174 mutation demonstrated full segregation within the entire tested kindred. The CCDC174 mutation was not found in 400 Bedouin non-related controls, while one carrier was found among 100 available Jewish-Ethiopian non-related controls. Interestingly, within the overlapping 0.6 Mb, the affected Ethiopian and Bedouin individuals were shown to have identical haplotypes, as evidenced by the 39 SNPs (Fig. 1B; Supplementary Material, Fig. S1) and two mutations within the shared locus.

Characterization of CCDC174

As there was no published information regarding CCDC174, we initiated its characterization: human CCDC174 is ubiquitously
Figure 1. Pedigrees of studied kindred and the CCDC174 mutation: (A) the affected Jewish-Ethiopian kindred (P1) and the affected Bedouin kindred (P2). Fine mapping demonstrates the disease-associated chromosome 3 locus. (B) Homozygosity at the shared locus. Black rectangles represent the homozygosity blocks per each pedigree. Gray is the identical haplotype shared by both P1 and P2. Physical positions (chromosome 3) and SNP rs IDs are given. (C) Sequence analysis demonstrating the CCDC174 mutation. Reference sequence from UCSC genome browser. Heterozygosity for the wild-type and the mutant allele and homozygosity for the mutant allele (black frame) in obligatory carrier and affected individual, respectively. (D) CCDC174 is ubiquitously expressed. PCR and electrophoresis of human cDNA panel. GAPDH expression was used as control.
Figure 2. CCDC174 protein knockdown in Xenopus embryos reduces neural folding and expression of neural markers and affects embryonic survival. (A) Neural folding and primary neuron marker expression. (a) In control embryos, normal neural folds were observed in 98% of the neurula stage embryos ($n = 40$). (b) In embryos injected with the CCDC174-MO (9 ng), an open neural folds phenotype was observed in 96% of the neurula stage embryos ($n = 52$), only 4% of the embryos had normal neural folding, like their siblings in (a). One representative embryo is shown for control and CCDC174-MO with a typical neural plate phenotype. (c) Whole-mount in situ hybridization to the \( n\)-\textit{tub} marker of primary neuron differentiation in the uninjected control group; \( n\)-\textit{tub} is expressed at normal levels in 88% of the embryos ($n = 16$). (d and e) Whole-mount in situ hybridization to the \( n\)-\textit{tub} marker in embryos injected at the one-cell stage with CCDC174-MO (9 ng). \( n\)-\textit{tub} expression levels are reduced in 93% of the embryos ($n = 14$). One representative embryo is shown for control and two representative embryos for CCDC174-MO. Note the more open neural plates in (d and e) versus (c). (B) CCDC174 protein knockdown reduces the expression of neural markers. Embryos were injected at one-cell stage with CCDC174-MO (9 ng) alone (Lane 3) or together with RNA (500 or 1000 pg) encoding the wild-type or mutant human ccdc174 proteins (Lanes 4–7). Total RNA was isolated from pools of seven embryos at neurula stage. Expression of the following neural marker genes was examined by sqRT-PCR: \( n\)-\textit{tub}, \textit{krox20}, \textit{hoxb3} and \textit{hoxb9}. Odc serves as a control for RNA levels in each sample. RT-PCR was performed on RNA isolated from control embryos (Lane 1). sqRT-PCR analysis of RNA isolated from un.injected control embryos (CE) serves as a positive control (Lane 2). (C) CCDC174 protein knockdown affects embryonic survival. Embryos injected at the one-cell stage with the CCDC174-MO (9 ng) alone ($n = 199$) or together with RNA (1000 pg) encoding wild-type ($n = 194$) or mutant ($n = 134$) human CCDC174 protein were scored at the tailbud stage for survival, in comparison with uninjected control embryos ($n = 162$). The bars represent the percentage of embryos surviving from neurula to tailbud stages in three independent experiments. (D) Myopathic changes represented by replacement of muscle fibers by fibrous tissue, marked variation in fiber size and rare fibers with internal nuclei (arrow) (hematoxylin & eosin, original magnification $\times200$). (E) Immunohistochemical staining of muscle biopsy sections with anti-RYR1 antibody. Duchenne muscular dystrophy biopsy served as control (original magnification $\times200$).
expressed (Fig. 1D). CCDC174 protein as well as its C-terminal end and stop codon are highly conserved among vertebrates (UCSC vertebrate MultiAln alignment and conservation browser, integrating phastCons and phyloP).

To better understand the functional significance of CCDC174 and the impact of the mutation found, we studied its Xenopus ortholog, cdc174. At neurula stages, cdc174-MO injected embryos exhibited poor neural folds closure versus uninjected controls (Fig. 2A, a and b). By whole-mount in situ hybridization, these same embryos with poor neural folding also had a sharp reduction in n-tubulin (n-tub) gene expression (Fig. 2A, c and d); n-tub is a marker for differentiating primary neurons. Pools of cdc174 morphant versus control embryos were examined by semi-quantitative reverse transcription PCR (sqRT-PCR) for expression of additional neural markers (Fig. 2B). In cdc174 knockdown embryos, there was also a strong reduction in hindbrain marker (krox20 and hoxb9) gene expression (Fig. 2B, Lanes 2 and 3). In contrast, spinal cord (hoxb9) and neural crest (not shown) marker gene expression was not significantly modulated (Fig. 2B, Lanes 2 and 3). These results show that some aspect of early neural plate patterning and neuron formation is disrupted by ccdc174 knockdown. The cdc174 morphant embryos had neural folds defects but were still alive at neurula stages. However, by later tailbud stages, survival was only 10%, reduced 8-fold versus control embryos (Fig. 2C). Thus, the cdc174 knockdown phenotype in Xenopus is embryonic lethal at later stages.

Functional studies of the human CCDC174 mutation

To assay function of wild-type versus mutant human CCDC174 protein in Xenopus embryos, in vitro transcribed mRNAs encoding these proteins were separately injected into cdc174-MO embryos. Ectopic expression of the wild-type human CCDC174 protein rescued the cdc174-MO phenotype, leading to an increased control-like expression of the n-tub, krox20 and hoxb3 genes (Fig. 2B, Lanes 2–5). Unlike the wild-type protein, the mutant protein did not rescue neural marker expression (Fig. 2B, Lanes 2 and 3, 6 and 7). Moreover, the mutant human CCDC174 appeared to slightly enhance the inhibited expression of neural genes, even more than in the cdc174-MO group alone (Fig. 2B, Lanes 3, 6 and 7).

Sharp differences in rescue were detected between the wild-type and mutant human CCDC174 proteins in the survival assay. In wild-type CCDC174/cdc174-MO co-injected tailbud stage embryos, survival was highly enhanced, increasing over 4-fold versus the cdc174-MO group (Fig. 2C). However, in the mutant CCDC174/cdc174-MO co-injected embryos, survival was almost 3-fold lower than in the cdc174-MO group, and over 10-fold lower than the wild-type rescued group (Fig. 2C). This result shows that unlike the wild-type protein, the mutant CCDC174 protein does not functionally replace endogenous Xenopus ccdc174 protein. Ectopic expression of either normal or mutant CCDC174 protein into normal embryos expressing endogenous Xenopus ccdc174 protein gave no significant phenotypes (not shown).

Interaction of wild-type and mutant CCDC174 with EIF4A3

Possible interaction between CCDC174 and EIF4A3 was previously suggested based on a massive protein-protein interaction screen (1). EIF4A3 is a core component of the exon junction complex (EJC), which plays a critical role in processing of RNA, nonsense-mediated decay and translation (2). To get further insight regarding the CCDC174-EIF4A3 interaction and the effect of the *468Trpext*6 mutation, we performed transfections of EIF4A3, CCDC174-WT and CCDC174-MUT expression vectors into a neuroblastoma cell line. Transfection of EIF4A3 and CCDC174-WT separately showed that both proteins are nuclear (Fig. 3A, a and b, respectively). While some cells showed also cytosolic EIF4A3 (not shown), CCDC174 was exclusively restricted to the nucleus. Co-transfection of EIF4A3 with either normal or mutant...
CCDC174 showed marked co-localization in the nucleus (Fig. 3B). This was further validated by yeast-two-hybrid assay, which showed interaction of EIF4A3 with both wild-type and mutant CCDC174 protein (Fig. 3E, a and b, respectively; Supplementary Material, Fig. S2). Interestingly, overexpression of the mutant (CCDC174-MUT) but not the normal (CCDC174-WT) protein resulted in rapid and massive apoptosis of cells (Fig. 3C, b and a, respectively; Fig. 3D, b) while aggregation of CCDC174-MUT in the nucleus was evident (Fig. 3D, a).

**Effect of the CCDC174 mutation on RYR1**

Knockdown of eif4a3 in Xenopus results in full-body paralysis of embryos due to downregulation and improper splicing at the 3′ end of ryr1 transcripts (3). To evaluate the effect of the CCDC174 mutation on RYR1, we used anti-RYR1 antibody for immunohistochemical staining of muscle biopsy sections derived from an affected individual (Fig. 1A, P2-IV6). While cytoplasmic immunoreactivity was clearly evident in the control case, no immunoreactivity was observed in the case biopsy (Fig. 2E).

**Discussion**

We demonstrated that CCDC174 is a ubiquitously expressed nuclear protein. It interacts with EIF4A3, as demonstrated both through yeast-two-hybrid data and through co-localization experiments in neuroblastoma cells. Thus, CCDC174 is part of the EJC, some of whose components have been shown to take part in neuron-related processes such as neural stem cell division (4), as well as impacting synaptic plasticity and behavior (5). Moreover, mutations in EJC genes have been shown to cause mental retardation as well as behavior and muscular phenotypes (3–5).

The human and Xenopus data put together demonstrate that CCDC174 is essential for neuronal differentiation and that its founder mutation, common to a Jewish-Ethiopian family and a Bedouin kindred, underlies a severe autosomal recessive syndrome of hypotonia, psychomotor developmental delay and abducens nerve palsy. The CCDC174 mutation found in the patients does not affect the nuclear localization of the protein or its interaction with EIF4A3. When overexpressed in neuroblastoma cells, the mutated protein does, however, cause apoptosis of neuroblastoma cells in vitro, preceded by formation of mutant CCDC174 nuclear aggregates.

In the Xenopus experiments, while the wild-type human CCDC174 rescued the cdc174-silencing phenotype, the mutant CCDC174 not only failed to rescue the silencing phenotype, but rather induced an even somewhat more severe phenotype as demonstrated in the survival rates (Fig. 2C). This enhanced severity of the Xenopus phenotype seen with the mutant CCDC174 could imply co-dominance or toxic gain of function, possibly due to unintended interactions between massive amounts of the human mutant protein with endogenous Xenopus proteins. In line with the recessive heredity of the human disease, ectopic expression of either normal or mutant CCDC174 protein into normal embryos expressing endogenous Xenopus cdc174 protein gave no significant phenotypes. Regarding the hypotonia phenotype of the affected individuals, we showed that no RYR1 could be detected in a muscle biopsy of an affected individual, in line with the downregulation of ryr1 caused by eif4a3 knockdown in Xenopus.

While the Ethiopian-Jewish community is inbred, consanguineous marriages have been carefully avoided, with a tradition of verifying lack of consanguinity seven generations back in every marriage. We have recently demonstrated that social structure of an inbred community can be deciphered through genomic data (6). Specifically, we showed that while genomic similarities due to recent intermarriages between social groups are reflected in shared large loci, common ‘old’ founder loci are reflected in sharing of small loci (6). The above is well demonstrated in that the founder mutation of the Ethiopian family lies within a single small 3.5 Mb locus of homozygosity in the entire genome.

Of unique interest is the fact that the mutation in the Jewish-Ethiopian family was found also in a Bedouin kindred, within a shared identical 0.6 Mb genomic segment. The origins of Israeli Bedouins are diverse (6), and the specific Bedouin kindred originates from Egypt. The Jewish-Ethiopian community has been shown to cluster with its neighboring autochthonous population in Ethiopia (7). Mitochondrial DNA studies of Egyptians of the Gurma region near Thebes, considered to be descendants of ancient Egyptians, demonstrated unique similarities specifically to the Ethiopian population (8). Thus, it is perhaps not surprising that the Bedouin-Egyptian and Jewish-Ethiopian families share an ancient founder mutation.

**Materials and Methods**

**Genetic studies**

SNP arrays were done as previously described (9). Whole exome sequencing was performed as previously described (9). After filtering for known variants (SNP database http://www.ncbi.nlm.nih.gov/projects/SNP, Seattle, WA, http://evs.gs.washington.edu/EVS, accessed December 2011), sequence variants that were not annotated in any of the database SNP or 1000 genomes databases were prioritized for further analysis. Variants identified within the disease-associated locus through whole exome sequencing were verified using Sanger sequencing. Analysis for the CCDC174 mutation was done through polymerase chain reaction (PCR) (forward primer 5′AGCCCTGAACATACGTCACC3′, reverse 5′AATCCAAACCGAACTGTGTG3′) followed by DpnII restriction (wild-type 109 and 135 bp fragments; mutant 244 bp fragment).

**Xenopus studies**

Xenopus ovulation, in vitro fertilization, culture and staging were done as described (10). To knockdown Xenopus ccdc174 protein, one-cell stage embryos were injected with 9 ng of the translation-blocking cdc174 antisense translational blocking morpholino oligonucleotide (MO; Gene Tools). The ccdc174-MO sequence is 5′GCAGCTTCTCTCTGTGTTGATGTG3′. In rescue experiments, capped in vitro transcribed RNAs (500–1000 pg) encoding either the wild-type or mutant human CCDC174 proteins were separately co-injected into the ccdc174-MO embryos. Cultures were cultured to neurula stages, and total RNA was isolated for semi-quantitative qRT-PCR analysis (11). Expression of the following genes was examined by qRT-PCR: ODC (housekeeping positive control for RNA loading), n-tubulin (n-tub—primary neuron marker), krox20 and hoxb3 (hindbrain markers) and hoxb9 (spinal cord marker). Each experiment was repeated at least three times, and individual samples are typically assayed three times for each marker. Whole-mount in situ hybridization was performed (12) with the n-tubulin probe (13).

**In vitro studies**

Constructs were generated for transient transfections: EIF4A3 was fused to RFP, while wild-type or mutant CCDC174 was
fused to GFP (pEGFP-C2 expression vector). Neuroblastoma cell line was cultured on glass coverslips to 50–60% confluence and transiently transfected with 1 µg DNA of either expression vector or both using lipofectamin2000 (Invitrogen) following the manufacturer’s instructions. Transfected cells were examined under an Olympus Fluoview FV1000 confocal laser scanning microscope. Co-transfection of an empty vector expressing a different fluorescent marker was performed for transfection internal control and background cytosolic fluorescence. Yeast-two-hybrid assay was performed using the commercial GAL4-based Two-Hybrid Phagemid Vector Kit (Stratagene) following the manufacturer’s instructions.

Supplementary Material
Supplementary Material is available at HMG online.

Conflict of Interest statement. None declared.

Funding
This work was supported by Teva Pharmaceutical Industries Ltd under the Israeli National Network of Excellence in Neuroscience (NNE) established by Teva; by the Legacy Heritage Bio-Medical Program of the Israel Science Foundation (Grant No. 1814/13); and through the Kahn Family Foundation. D.F. was supported by a grant from the Israel Science Foundation (658/09).

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