CEP89 is required for mitochondrial metabolism and neuronal function in man and fly

Bregje W.M. van Bon1,2,5,†, Merel A.W. Oortveld1,2,6,†, Leo G. Nijtmans3, Michaela Fenckova1,2,6, Bonnie Nijhof1,2,6, Judith Besseling1,2,6, Melissa Vos7,8, Jamie M. Kramer1,2,6, Nicole de Leeuw1, Anna Castells-Nobau1,2,6, Lenke Asztalos9, Erika Viragh10, Mariken Ruiter1, Falko Hofmann1,2,6, Lillian Eshuis11, Licio Collavin12,13, Martijn A. Huynen4, Zoltan Asztalos8,10,14, Patrik Verstreken7,8, Richard J. Rodenburg3,5, Jan A. Smeitink3,5,∗, Bert B.A. de Vries1,3,6,†, and Annette Schenck1,2,6,

1Department of Human Genetics, 2Nijmegen Center for Molecular Life Sciences, 3Nijmegen Center for Mitochondrial Disorders, Department of Pediatrics and 4CMBI, Nijmegen Centre for Molecular Life Sciences, Radboud University Medical Centre, 6500 HB, Nijmegen, The Netherlands, 5Institute for Genetic and Metabolic Disease, Radboud University Medical Centre, 6500 HB, Nijmegen, The Netherlands, 6Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Centre, 6625 EN, Nijmegen, The Netherlands, 7VIB, Center for the Biology of Disease, 3000 Leuven, Belgium, 8KU Leuven, Center for Human Genetics & Leuven Institute for Neuroscience and Disease (LIND), Herestraat 49, 3000 Leuven, Belgium, 9Aktogen Ltd., Department of Genetics, University of Cambridge, CB2 SEH Cambridge, UK, 10Institute of Biochemistry, Biological Research Center, Hungarian Academy of Sciences, H-6726 Szeged, Hungary, 11Department of Pathology, Radboud University Nijmegen Medical Centre, 6525 GA, Nijmegen, The Netherlands, 12Laboratorio Nazionale Consorzio Interuniversitario per le Biotecnologie, Area Science Park, 34012 Trieste, Italy, 13Department of Life Sciences, University of Trieste, 34139 Trieste, Italy and 14Aktogen Hungary Ltd., Biological Research Center, Hungarian Academy of Sciences, 6726 Szeged, Hungary

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It is estimated that the human mitochondrial proteome consists of 1000–1500 distinct proteins. The majority of these support the various biochemical pathways that are active in these organelles. Individuals with an oxidative phosphorylation disorder of unknown cause provide a unique opportunity to identify novel genes implicated in mitochondrial biology. We identified a homozygous deletion of CEP89 in a patient with isolated complex IV deficiency, intellectual disability and multisystemic problems. CEP89 is a ubiquitously expressed and highly conserved gene of unknown function. Immunocytochemistry and cellular fractionation experiments showed that CEP89 is present both in the cytosol and in the mitochondrial intermembrane space. Furthermore, we ascertained in vitro that downregulation of CEP89 resulted in a severe decrease in complex IV in-gel activity and altered mobility, suggesting that the complex is aberrantly formed. Two-dimensional BN-SDS gel analysis revealed that CEP89 associates with a high-molecular weight complex. Together, these data confirm a role for CEP89 in mitochondrial metabolism. In addition, we modeled CEP89 loss of function in Drosophila. Ubiquitous knockdown of fly Cep89 decreased complex IV activity and resulted in complete lethality. Furthermore, Cep89 is required for mitochondrial integrity, membrane depolarization and synaptic transmission of photoreceptor neurons, and for (sub)synaptic organization of the larval neuromuscular junction. Finally, we tested neuronal Cep89 knockdown flies in the light-off jump reflex

†Shared first authors.
‡These authors jointly directed this work.

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habitation assay, which revealed its role in learning. We conclude that CEP89 proteins play an important role in mitochondrial metabolism, especially complex IV activity, and are required for neuronal and cognitive function across evolution.

**INTRODUCTION**

Oxidative phosphorylation (OXPHOS) disorders have an estimated incidence of ~1 in 10,000 live births and are clinically and genetically heterogeneous. They typically manifest in early infancy and predominantly affect tissues with high energy demands such as muscle, brain and heart (1–4). The OXPHOS system is located in the inner mitochondrial membrane and consists of five multi-protein enzyme complexes (complexes I–V) and two electron carriers. A large number of molecules are involved in the assembly and maintenance of this system, encoded by both mtDNA and nuclear DNA (5). The ATP synthase complex (complex V) uses the proton gradient produced by electron transport along the respiratory chain (complex I–IV) to generate ATP. Isolated complex IV deficiency is one of the most common respiratory chain defects in humans and often presents in the neonatal period (1,6). Complex IV comprises 13 different subunits of which the three largest are encoded by mtDNA and the remaining 10 by the nuclear genome. The latter are synthesized in the cytosol and thereafter transported to the mitochondrion. To form complex IV, all subunits have to be assembled in an active manner. Most known complex IV deficiencies are caused by mutations in nuclear genes that are involved in this process at the inner mitochondrial membrane (7). However, for the majority of individuals with isolated complex IV deficiency, the underlying genetic cause remains elusive, illustrating that our knowledge about crucial components of complex IV transport and/or assembly machinery is highly incomplete (6).

We identified a patient with a unique syndrome consisting of an isolated complex IV deficiency, intellectual disability (ID) and multisystemic problems that include cystinuria, cataract, broad-based walking pattern and deafness. Mutations in the mtDNA and the most frequently encountered nuclear genes involved in complex IV deficiency were excluded. By SNP array analysis, we identified the genetic defect that underlies these clinical features to be a homozygous deletion that uncovers two genes, SLC7A9 and CEP89. SLC7A9 is expressed at high levels exclusively in kidneys and small intestine and homozygous mutations are a well-known cause of cystinuria (8,9), whereas CEP89 is a protein of unknown function. A severe and complex phenotype such as in our proband has never been reported in complex IV deficiency patients who predominantly only display the cystinuria. Here we combine molecular methods and disease modeling in Drosophila to characterize the function of CEP89 on the molecular, cellular and organism level. Our results provide strong support that loss of CEP89 accounts for most of the clinical features seen in our proband.

**RESULTS**

**Complex IV deficiency in a female with homozygous deletion of CEP89**

This 9-year-old female was born after an uneventful pregnancy and delivery at 40 + 2 weeks of gestation with a weight of 3040 g (25th centile) and a length of 49 cm (20th centile). She was the first child of consanguineous Turkish parents. Early infancy was complicated by bilateral cataract, severe deafness, cystinuria, feeding problems and developmental delay.

At the age of 1 year and 6 months, a lactate day curve showed an elevation of serum lactate to 3.0 mmol/l (N < 2.1 mmol/l). Mitochondrial dysfunction was confirmed by muscle biopsy, showing a reduced ATP production rate, a reduced oxidation rate of 1-[14C]-pyruvate + malate, an isolated complex IV deficiency with complexes I and II around the lowest reference value and normal complex III activity (Table 1). Mutations in the mtDNA and the most frequently encountered nuclear genes involved in complex IV deficiency, SCO1 (MIM220110), SCO2 (MIM220110), SURF1 (MIM220110) and COX10 (MIM220110), were not identified. Morphologic microscopic and histochemical studies of the muscle and enzyme analyses in fibroblasts were also normal. Magnetic Resonance Imaging of the brain and electroencephalography at 3 years of age showed no abnormalities, whereas electromyography revealed signs of myopathy and auditory evoked potentials showed signs of peripheral conduction dysfunction.

At 12 years of age, she had severe intellectual disability without any speech development. Her height was 136.5 cm (<3rd centile), her weight 43 kg (>90th centile) and her OFC was 53.2 cm (40th centile). Echocardiography and electrocardiography were normal. Facial dysmorphisms included hypotelorism, small low-set ears, columella below alae nasi and micrognathia. In addition, she had a short broad neck, camptodactyly of fifth fingers and calcinosis cutis. She walked with a broad-based walking pattern with her legs in exorotation. For long distances she needed a wheelchair. Arms showed ataxic movements, but with relative intact fine motor movements and an active hand function.

Genome-wide 250 k SNP array analysis showed a homozygous deletion on chromosome 19q13.11 (SNP_A-2200372; SNP_A-2268111, 33.37–33.39 Mb) Hg19 (Fig. 1). The same deletion was confirmed in the heterozygous state in both parents. The homozygous deletion was validated and fine mapped by a qPCR. The deletion was 78 kb in size and comprised two genes, SLC7A9 (RefSeq accession number NM_014270, MIM 604144) and exons 15–19 of CEP89.
CEP89 localizes in the cytosol and in the mitochondrial intermembrane space

To verify the predictions of mitochondrial targeting prediction programs, we stably transfected HEK293 cells with an inducible C-terminally GFP-tagged CEP89 construct. Immunolabeling of these cells with an antibody against the mitochondrial marker ATP5A showed that CEP89-GFP partially co-localized with this marker and was also present in the cytoplasm (Fig. 2A). To confirm and obtain further information on CEP89-GFP (sub)mitochondrial localization, we performed a number of subcellular fractionation experiments. CEP89-GFP co-purified with both cytoplasmic marker citrate kinase b-type and mitochondrial outer membrane marker TOM20 (Fig. 2B, left). To reveal whether CEP89 is imported into the mitochondria or associates with the mitochondrial outer membrane, a mitochondrial fractionation in combination with a proteinase K protection assay was performed. In digitonin permeabilized mitochondria (mitochondria with a disrupted outer membrane), proteinase K treatment further discerns whether the protein is located in the inner membrane (IM), intermembrane space (IMS) or matrix. In whole mitochondria, the CEP89 signal was protected against proteinase K, indicating that the protein is imported and only susceptible for degradation after Triton X-mediated mitochondria lysis (Fig. 2B, right). In this situation, the outer membrane protein TOM 20 was degraded by proteinase K, whereas the matrix (SDHA) and the IMS (prohibitin 1) markers were protected. In the digitonin-treated mitochondria, CEP89-GFP was rapidly degraded by low concentrations of proteinase K. Together, this indicates that mitochondrial CEP89 resides in the IMS of the mitochondria. The matrix marker SDHA was still protected under this condition, whereas the IMS protein prohibitin 1 was strongly reduced by the proteinase K treatment. Of note, in the permeabilized mitochondria the IMS marker prohibitin 1 was strongly decreased upon proteinase K treatment; however, it did not completely disappear. This is explained by the fact that this protein highly folds and associates with the mitochondrial IM and is therefore less susceptible for degradation. Identical results were obtained in a fractionation using a CEP89-TAP tag expressing cell line (Supplementary Material, Fig. S1A). In summary, these data indicate that CEP89 localizes in the cytosol and in the mitochondrial intermembrane space.

Mitochondrial prediction programs support a role of CEP89 in mitochondria

CEP89 (FLJ14640/CCDC123) is a gene of unknown function. It is ubiquitously expressed and conserved across species (10). CEP89 encodes a 783 amino acid protein, which contains two coiled coil domains and has recently been isolated as a centrosomal-associated component (11,12). Nevertheless, the deficiency of complex IV in this female suggested a role of CEP89 in mitochondrial biology. This was supported by mitochondrial targeting prediction programs (MitoProt II, TargetP and Mitopred) that indicated a high probability for mitochondrial localization (89, 82 and 93%, respectively). MitoProt II also predicted a putatively cleavable, unusually long N-terminal mitochondrial target sequence of 78 amino acids (13).
Downregulation of CEP89 decreases activity and alters mobility of complex IV

Given the fact that muscle tissue of our patient showed a decrease of complex IV activity, we investigated the possible effect of CEP89 depletion on mitochondrial complex IV using a cellular system and three different siRNAs. siRNAs 1 and 2 efficiently knocked down the protein, while siRNA 3 had little or no effect. Blue native gel analysis showed that decreased expression of CEP89 resulted in a severe decrease of complex IV in-gel activity (Fig. 3A, top panel), in full agreement with complex IV deficiency in the patient. Moreover, western blot analysis of a duplicate blue native gel revealed that in the CEP89 knockdown cells, the complex IV immunoreactive signal is normal but shows a slightly increased mobility. This indicates that the less active complex IV is incomplete (either due to lack of CEP89 itself or due to other missing components) or has assembled into an incorrect conformation. Western blotting of denaturing SDS–PAGE gels demonstrated normal amounts of complex IV subunits I, II, IV and Va in CEP89 knockdown conditions (Fig. 3B), indicating that expression of these subunits is not affected.

Mutation analysis of CEP89 in patients with isolated complex IV deficiency

In an effort to identify further mutations in CEP89, we performed a qPCR and sequenced the coding exons of CEP89.
in 29 patients with an isolated complex IV deficiency. However, none of these individuals showed the complex combination of phenotypic features shown by our proband. We did not find any pathogenic mutation in any of these individuals (data not shown).

A *Drosophila* model for *CEP89* deficiency

Therefore, to determine whether *CEP89* deficiency can account for the systemic phenotypes presented by our patient, we established a *Drosophila* model for *CEP89* deficiency. *CEP89* contains mainly coiled coil and low complexity regions, and has no significant similarity (E < 0.001) to any protein domains represented in PFAM or SMART (14,15). A Blastp search of *CEP89* against the *Drosophila melanogaster* proteins in RefSeq retrieved only a single significant hit, CG8214 (E = 3e-05). Also a reciprocal search, with CG8214 against the human proteome, retrieved only *CEP89* as significantly similar (E = 2e-5), indicating a one-to-one orthology relation between *CEP89* and CG8214. Consistently, neither *CEP89* nor CG8214 are significantly similar to other proteins from the human and the fly proteome, respectively. Nevertheless, the E-value for the homology relation between *CEP89* and CG8214 is rather high and mainly based on the central regions of the proteins. To ensure that in our assignment of the one-to-one orthology, we did not miss paralogs in either species, we also employed a sequence-profile-based search in combination with best bidirectional hits (16) (see materials and methods), which further supported the one-to-one orthology of *CEP89* with the uncharacterized gene CG8214. We knocked down the expression of CG8214 (from here on referred to as Cep89) in the developing fly using the UAS-Gal4 system (17) and three inducible RNAi lines (UAS-RNAi) available from the Vienna *Drosophila* RNAi Center (VDRC) (18). In addition to its promoter-dependence, the efficiency of Gal4-induced knockdown depends on temperature, with stronger knockdown being achieved at higher breeding temperature (19). Knockdown can be further increased by co-expression of the endonuclease dicer2 (UAS-dicer2) under the same promoter (18). This makes the UAS-Gal4 system a highly tunable system.

**Cep89 is required for viability and complex IV activity in *Drosophila***

Induction of Cep89RNAi101132 with the ubiquitous act-Gal4 promoter in the presence of *UAS-dicer2* under stringent conditions (28°C) resulted in complete lethality at a late pupal stage. Induction of Cep89RNAi24240 and Cep89RNAi24241 under similar conditions caused partial lethality of 55% and 25%, respectively, at the same developmental stage (Supplementary Material, Table S1). To test whether the level of lethality induced by the three different RNAi lines is consistent with their

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**Figure 3.** Down regulation of *CEP89* results in a decreased activity and altered mobility of complex IV. Three different siRNAs were exploited to knockdown *CEP89*. Of these, siRNA #1 and #2 were effective and resulted in a decrease of *CEP89* mRNA. N: non-treated; M: Mock treated. (A) Blue native gel analysis shows that decreased expression of *CEP89* results in a severe decrease of complex IV in-gel activity (top panel). Western blot analysis using an antibody against COX4 of these blue native gels demonstrated that in the *CEP89* knockdown cells complex IV immunoreactive signal is normal but has a slightly decreased mobility (middle panel). A reference line at the height of control complex IV is shown. The 70 kDa subunit of complex II was used as a loading control (70 kDa, bottom panel). (B) Western blotting analysis of denaturing SDS–PAGE gels demonstrated normal amounts of complex IV subunits I, II, IV and Va (indicated as COX1, COX2, COX4 and COX5a) in the *CEP89* siRNA-treated cells. The 70 kDa subunit of complex II was used as a loading control (70 kDa).

**Figure 4.** Knockdown of Cep89 results in decreased complex IV activity in *Drosophila*. Bar graph presenting the mean complex IV activity normalized against citrate synthase activity, with standard error of the mean, for control (act-Gal4+) and Cep89 knockdown pupae (act-Gal4/UAS-Cep89RNAi101132). *P = 0.017.
To determine whether knockdown of Cep89 in *Drosophila* results in decreased activity of complex IV similar to our patient, we prepared and measured mitochondrial fractions from pupae of the strongest knockdown condition. Complex IV activity was reduced to <50% compared to the genetic background control (Fig. 4). We conclude that Cep89 is required for complex IV activity in man and fly.

**Tissue-specific ablation of Cep89 in *Drosophila* muscles and neurons reveals functions related to its associated human pathology**

To determine whether aspects of the human pathology can be recapitulated and further investigated in flies, we turned to tissue-specific silencing. We targeted four different tissues/cell-types: muscles, neurons, wing and eye, for reasons outlined below (see also Table 2). We first knocked down Cep89 in muscle, a tissue with high energy demands and mitochondrial activity that likely underlies the myopathic phenotype of the patient. Induction of Cep89RNAi101132 using the *MEF2-Gal4* driver line in the presence and absence of *UAS-dicer2* resulted in complete lethality at the late pupal stage at temperatures ranging from 20 to 28°C. Muscle-specific knockdown using the weaker Cep89RNAi24240 allele was lethal under strongly induced conditions (28°C), whereas animals with mild induction of Cep89RNAi24240 (20°C, without *UAS-dicer2*) survived and showed no obvious alteration in locomotion and behavior. This suggests a certain threshold of Cep89 to be sufficient for muscle development and function. Next, we investigated Cep89 function in the nervous system, as ataxia and ID are major clinical features of our patient. Clearly, Cep89 is essential in neurons since panneuronal knockdown using Cep89RNAi101132 with the *elav-Gal4* promoter in the presence of *UAS-dicer2* at 28°C was mostly lethal. The few obtained survivors were weak and died shortly after pupal eclosion. Less stringent neuronal knockdown conditions (*elav-Gal4* at 26.5°C) resulted in a considerable amount of flies that eclosed. These neuron-specific knockdown flies showed striking motor defects (Supplementary Material, Movie S1), raising the possibility that in the patient neuronal in addition to muscular defects may contribute to the broad-based walking pattern and ataxic movements.

**Loss of Cep89 affects (sub)synaptic organization**

We further tested whether the observed abnormal motor coordination might result from abnormal synapse development and/or function of motor neurons. We analyzed synaptic and subsynaptic organization at the *Drosophila* larval neuromuscular junction (NMJ). The NMJ is a well-established synaptic model system that also shares important features with central excitatory synapses in the mammalian brain and was shown to be affected in a number of *Drosophila* models of ID disorders (20–22). Staining of these synapses with an antibody against discs large 1 (dlg1) highlights the overall morphology of the NMJ terminal. Computer-assisted quantitative measurements of the synaptic terminal area, its length and perimeter, the amount of synaptic boutons and branches revealed normal overall synapse morphology upon Cep89 knockdown (Fig. 5A and quantitative data not shown). We also quantified active zones, the presynaptic microdomains of neurotransmitter release, using an antibody against bruchpilot (brp). Cep89 knockdown resulted in a highly significant decrease in the amount of active zones in both RNAi conditions (81% and 90% of their genetic background controls, respectively, in Cep89RNAi101132 and Cep89RNAi24240) (Fig. 5A and B). Brp is an integral part of active zones and crucial for synaptic transmission and plasticity (23). Hence, the observed reduction of active zones suggests that loss of Cep89 affects (sub)synaptic organization and potentially function.

In our NMJ preparations, we also evaluated the size of the muscles where synapses are formed onto. The muscle size is significantly smaller in Cep89RNAi101132 animals compared with the control (Fig. 5C), which likely reflects an overall smaller size of Cep89 neuronal knockdown larvae.

**Loss of Cep89 results in growth defects**

A suitable system to support also a cell autonomous role of Cep89 in growth is the *Drosophila* wing. It develops from

### Table 2. Comparison of human- and tissue-specific fly phenotypes with CEP89 deletion and Cep89 knockdown respectively

<table>
<thead>
<tr>
<th>Individual with CEP89 deletion</th>
<th>Drosophila Cep89 knockdown</th>
<th>Tissue tested in Drosophila</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated complex IV deficiency</td>
<td>Reduced complex IV activity</td>
<td>Ubiquitous</td>
</tr>
<tr>
<td>Intellectual disability</td>
<td>Reduced viability</td>
<td>Panneuronal</td>
</tr>
<tr>
<td>Mitochondrial myopathy and complex IV deficiency</td>
<td>Decreased number of active zones at the NMJ</td>
<td>Panneuronal</td>
</tr>
<tr>
<td>Congenital cataract</td>
<td>Absence of depolarisation in ERG</td>
<td>Eye</td>
</tr>
<tr>
<td>Ataxia</td>
<td>Learning defect</td>
<td>Panneuronal</td>
</tr>
<tr>
<td>Short stature</td>
<td>Reduced viability</td>
<td>Muscle</td>
</tr>
<tr>
<td></td>
<td>Affected mitochondria</td>
<td>Eye</td>
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<td></td>
<td>Lens defect ommatidia</td>
<td>Eye</td>
</tr>
<tr>
<td></td>
<td>Defective motor coordination</td>
<td>Panneuronal*</td>
</tr>
<tr>
<td></td>
<td>Growth defects</td>
<td>Wing</td>
</tr>
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*Upon moderate panneuronal knockdown.
an ectodermal-derived epithelial pouch and is not essential for viability. Induction of Cep89 RNAi under control of the wing-specific MS1096-Gal4 promoter line produced flies with a dramatically reduced wing size, compared with MS1096-Gal4 control flies (Fig. 6A). These findings are in agreement with the growth defect of the patient.

Loss of Cep89 disturbs ommatidia organization and corrupts integrity of mitochondria

Next, we investigated Cep89 function in the Drosophila eye. Induction of Cep89 RNAi in the developing eye disturbed the highly organized array of facets (called ommatidia) and bristles (Fig. 6B). Scanning electron microscopy (SEM) revealed the surface of Cep89 ommatidia to be collapsed (Fig. 6B, right panel), likely due to a problem in the lens or pseudocone that are located directly underneath the ommatidia surface (24).

To analyze structural integrity of photoreceptor neurons and their mitochondria, we performed histological semi- and ultrathin sectioning of Cep89 knockdown eyes. The former showed intact albeit affected photoreceptors, characterized by vacuolar structures and reduced volume of rhabdomeres (the photosensitive apical membrane compartment of photoreceptors) (Fig. 6C). Importantly, electron microscopy (EM) of photoreceptor neurons revealed corrupted integrity of mitochondria (Fig. 6D), in agreement with the role of Cep89 in mitochondria biology and function.

Loss of Cep89 affects neuronal function

We took advantage of the high experimental accessibility of the fly eye to provide direct evidence that loss of Cep89 affects neuronal function. We recorded electroretinograms (ERGs). ERGs are extracellular field recordings that measure photoreceptor de- and repolarization and the synaptic response that arises in the postsynaptic circuit, the latter highlighted by ‘on’ and ‘off’ transients, in response to light (25). Compared with controls, ‘on’ and ‘off’ transients in Cep89 knockdown eyes were strongly reduced and membrane depolarization was nearly absent, demonstrating a dramatic defect in basal neurotransmission (Fig. 6E).

Cep89 is required for non-associative learning in Drosophila

Finally, we addressed the role of Cep89 in Drosophila cognitive processes, using the light-off jump reflex habituation paradigm. Habituation is a form of non-associative learning where an initial response to a repeated stimulus gradually wanes (26). In this assay, individual flies were exposed to repeated short
light-off stimuli (trials; 100 trials at 1s inter-trial interval) and the jump responses were recorded. Flies were deemed to have habituated when they failed to jump in five consecutive trials (no-jump criterion). Both genotypes showed a healthy, high initial jump response. However, whereas control flies quickly habituate to the light-off stimuli and, consequently, quickly reduce their jump response, we found that Cep89RNAi101132 flies habituate slower and maintain a higher jump response throughout the entire course of the experiment (Fig. 7A). This can be quantitatively described using the trails to criterion (TTC) parameter (the mean number of trials required to reach the no-jump criterion). The TTC of Cep89RNAi101132 conditions was significantly higher (1.5-fold) compared with their genetic background controls (Fig. 7B, P = 0.026), establishing a role for Cep89 in learning.

**DISCUSSION**

We identified a homozygous deletion of CEP89 and SLC7A9 associated with isolated complex IV deficiency, intellectual disability, cataract, deafness, ataxia and cystinuria in human. Whereas cystinuria is a known consequence of SLC7A9 deficiency, we have shown here that loss of CEP89 leads to complex IV deficiency and accounts with high likelihood for the additional phenotypic features, as supported by a number of Drosophila knockdown phenotypes. The results of this study provide several lines of evidence that point toward an important role for CEP89 in mitochondrial metabolism and nervous system function, including learning.

CEP89 is a protein of unknown function. Recently, it has been characterized as a candidate centrosomal-associating
protein (11,12). In addition, mitochondrial localization was predicted by mitochondrial targeting prediction programs. However, so far no studies have reported mitochondrial localization or function for CEP89. We showed that CEP89 localizes in the cytosol and in the mitochondrial intermembrane space, possibly pointing toward a dual function of the protein. As CEP89 must transit the cytosol to reach the mitochondrial intermembrane space, it may participate in other tasks in the cell, outside of the mitochondria, including a possible structural or enzymatic function at centrosomes. Furthermore, in concordance with the isolated complex IV deficiency observed in the patient with homozygous CEP89 deletion, we showed that down regulation of CEP89 in Drosophila larval NMJ and showed that CEP89 knockdown resulted in a highly significant decrease in the amount of active zones, which are crucial for synaptic transmission and plasticity (23). Furthermore, dramatically decreased depolarization of the photoreceptors and a nearly absent membrane depolarization in ERGs of Cep89 knockdown eyes provide direct evidence that loss of Cep89 affects neuronal function. This ERG phenotype resembles phenotypes of several neurodegenerative mutants and has also been reported in several Drosophila models of mitochondrial disease (29–31). The exact mechanisms by which mitochondria influence neurotransmission remain unknown, but mitochondria are highly abundant at synapses and synaptic transmission is a very energy demanding process (32). Furthermore, decreased amounts of synaptic mitochondria have been found in several Drosophila mutants that were identified in screens for aberrant synaptic function (33–35).

Third, we addressed the role of Cep89 in growth, by studying the Drosophila wing. In agreement with the growth defect of the patient, Cep89 knockdown in wing produced flies with a dramatically reduced wing size. These findings are also in agreement with impaired mitochondrial function, which is known to affect the tissue size in Drosophila (36,37).

Finally, we established a role of Cep89 in Drosophila non-associative learning. In the light-off jump reflex habituation paradigm, flies are exposed to a sudden light-off pulse, in response to which they jump, which can be quantitatively measured. This behavioral response is mediated by the giant fiber interneurons, which receive sensory input from the visual system in the brain and relay this information through the thoracic ganglion, where efferent neurons descending from the giant fiber to thoracic muscles are stimulated. Interestingly, classic Drosophila learning mutants such as rutabaga are affected in this assay (38). Moreover, first orthologs of genes causing ID in human have also been reported with habituation defects (39). To our knowledge, Cep89 is the first evidence for a role of Cep89 in mitochondrial biology and function, and validate our model. As to the function of Cep89 in mitochondria, it is interesting to note that the Drosophila protein is poorly conserved at its N-terminus and no mitochondrial target sequence was predicted. However, with 78 amino acids also the putative human target sequence is unusually long and no consistent indications of N-terminal cleavage were found in our experiments. Mitochondrial proteins of the intermembrane space, like CEP89, often do not contain a cleavable leader sequence. Some of these are known to be imported by the MIA import pathway. It is also worth mentioning that Cep89 was recently identified as an interactor of Drosophila p53 (27). Drosophila p53 is the sole representative of the human p53, p63 and p73 family, and shares a number of p53 functions. Recent work suggests that p53 also plays an important role in mitochondrial apoptosis (28).

In agreement with the patient’s movement disorder, we investigated whether the observed abnormal motor coordination results from abnormal synapse development and/or function of motor neurons, we analyzed synaptic and sub-synaptic organization at the Drosophila larval NMJ and showed that Cep89 knockdown resulted in a highly significant decrease in the amount of active zones, which are crucial for synaptic transmission and plasticity (23). Furthermore, dramatically reduced depolarization of the photoreceptors and a nearly absent membrane depolarization in ERGs of Cep89 knockdown eyes provide direct evidence that loss of Cep89 affects neuronal function. This ERG phenotype resembles phenotypes of several neurodegenerative mutants and has also been reported in several Drosophila models of mitochondrial disease (29–31). The exact mechanisms by which mitochondria influence neurotransmission remain unknown, but mitochondria are highly abundant at synapses and synaptic transmission is a very energy demanding process (32). Furthermore, decreased amounts of synaptic mitochondria have been found in several Drosophila mutants that were identified in screens for aberrant synaptic function (33–35).

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mitochondrial gene that has been implicated in *Drosophila* habituation and learning.

In conclusion, the combination of human and fly phenotypes complemented by additional molecular studies revealed that CEP89 is required for normal assembly of complex IV, mitochondria activity and integrity, and plays a crucial role in neuronal function and learning across evolution. Our study identified a novel player required, directly or indirectly, for mitochondrial oxidative phosphorylation in health and disease, adding one more crucial piece to the complex mitochondrial machinery.

### MATERIALS AND METHODS

#### Molecular characterization deletion

DNA was isolated according to the standard procedures. *Array analysis*: 250 k SNP array analysis was performed according to the standard Affymetrix GeneChip protocol (Affymetrix Inc., Santa Clara, CA, USA) and analyzed (CNAG 2.0) as previously described (40). The positions of the array targets were mapped to the hg17 assembly of the human genome and converted to the latest version, hg19. *Genomic real-time quantitative PCR analysis*: SYBR Green-based genomic real-time quantitative PCR (qPCR) was performed on a 7500 Fast Real-Time PCR System by using a Power SYBR Green PCR Master Mix (both Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Primers were developed by the primer3 program (41) (primer sequences available upon request). Copy numbers were measured relative to one reference gene, *CFTR*.

#### Complex measurements in human

The activity of mitochondrial enzymes in human muscle was determined in mitochondria-enriched fractions, prepared as follows. The samples were resuspended at 10% (w/v) in 0.25 M sucrose, 2 mM K-EDTA, 10 mM Tris-Cl pH 7.4 and 5.104 U/l heparin and homogenized by a Potter-Elvehjem homogenizer. Cell debris was removed by centrifugation at 14 000 g. The homogenate was resuspended in 10 mM Tris-Cl, pH 7.6 and used for subsequent enzyme activity measurements. The activities of complexes I, II, III, IV and V, and citrate synthase were determined following the established protocols (42–44).

#### Generation of inducible cell lines and cell culture

CEP89 (NM_032816) ORF sequences (without stop codon) flanked by Gateway AttB sites (Invitrogen) were created by a PCR, in accordance with the manufacturer’s instruction, and cloned into pDONR201 with the use of a Gateway BP Clonase II enzyme mix (Invitrogen). For inducible CEP89-TAP and -GFP vectors, the pDONR201 vector containing CEP89 was recombined with the TAP and GFP destination vectors via the Gateway LR Clonase II Enzyme mix (Invitrogen). All constructs were transfected into Flp-In T-REx293 cells (Invitrogen) with SuperFect Transfection reagent (Qiagen), in accordance with the manufacturer’s protocols. Flp-In T-REx293 cells and HEK293 cells were cultured in DMEM (Biowhitaker) supplemented with 10% FCS (v/v) and 1% penicillin/streptomycin (Gibco). Inducible cell lines were selected on 5 mg/ml blasticidin (Invivogen) and 200 mg/ml hygromycin (Invivogen). The inducible cell lines were treated with 1 µg/ml doxycycline (48 h, Sigma Aldrich) for expression of the transgene. Patient skin fibroblasts were cultured in medium 199 supplemented with 10% FCS (v/v) and 1% penicillin/streptomycin (Gibco).

#### RNA interference in HEK cells

HEK293 cells (2 × 105) were seeded in 2 ml DMEM supplemented with 10% FCS (without antibiotics) per well in six-well plates a day preceding the siRNA transfection. The cells were transfected with an siRNA duplex in the presence of 4 µl DharmaFECT 1 (Thermo Fisher Scientific) and opti-Mem 1 (Invitrogen) to achieve a final concentration of 100 nm in a total volume of 2.4 ml. CEP89 #1 antisense strand: 50-UUCUUCUCAACAGCAGG dTdT-30, CEP89 #2 antisense strand: 50-UUCUUCUCAACAGCAGG dTdT-30, CEP89 #3 antisense strand 50-AUCUAAACGCAUGGAGACAGC TdTdT-30. The cells were incubated at 37°C in a CO2 incubator for 72 h, and a second cycle of transfection was performed.

#### Blue native, SDS–PAGE and in-gel activity assays

Blue native gradient (5–15%) gels and one-dimensional 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) were performed as described previously (45). Lanes were loaded with 40 (SDS analysis) or 80 µg (BN-SDS analysis) of solubilized mitochondrial protein. After electrophoresis, gels were processed further for the in-gel complex IV activity assay, in-gel fluorescence detection, immunoblotting or two-dimensional 10% SDS–PAGE (46). For blotting, proteins were transferred to a PROTAN nitrocellulose membrane (Schleicher & Schuell).

#### Cellular fractionation and proteinase K protection assay

Cellular fractionation was carried out as described before (47). Permeabilization of mitochondria with digitonin followed by a proteinase K protection assay was performed as before (48). Susceptibility of proteins to degradation by proteinase K was assayed with SDS–PAGE followed by western blotting and probing the membranes with specific antibodies.

#### Antibodies and ECL detection

The GFP antibody was kindly provided by Frank van Kuppeveld, Nijmegen, The Netherlands. A CBP antibody was obtained from GenScript. Other antibodies used were...
Conservation of CEP89

In order to establish orthologs outside the vertebrates, we employed a profile-based sequence search in combination with a best reciprocal hits approach. A PSI-Blast search in the refseq database starting with the CEP89 sequence retrieves the *Drosophila melanogaster* protein CG8214 at the first iteration \( (E = 1 \times 10^{-22}) \) as best hit. Given the coiled coil structure of the protein, it is not surprising that also other (likely coiled coil) *D. melanogaster* proteins are also retrieved, these however have a much higher \( E \)-value \( (E > 1 \times 10^{-11}) \), just like other *H. sapiens* proteins that were retrieved in this iteration \( (E > 1 \times 10^{-14}) \). For the reciprocal search, we first selected the insect homologs of CG8214 in PSI-Blast to generate a sequence profile. The *CEP89* protein is subsequently retrieved as the most similar human protein \( (E = 3 \times 10^{-18}) \) followed by less similar human proteins \( (E > 8 \times 10^{-6}) \) and less similar *D. melanogaster* proteins \( (E > 0.001) \). Homology detection in coiled coil proteins is generally hampered by their biased amino acid composition. Reciprocal-best hit methods using profile-based methods have been successfully applied to identify orthologs with a biased amino acid composition, including transmembrane proteins of oxidative phosphorylation \( (16) \).

Immunocytochemistry

Hek293 cells were grown on coverslips in 6-well plates and CEP89 expression was induced with 1 \( \mu \)g/ml doxycycline for 1 day. Cells were fixed with 1% \( w/v \) paraformaldehyde in PBS and permeabilized with 0.4% Triton X-100 in PBS containing 3% bovine serum albumin (BSA). After permeabilization, the samples were blocked with 3% BSA in PBS and incubated with anti-ATP5A (dilution 1:1000; MitoSciences) followed by incubation with a secondary Alexa-594-conjugated anti-rabbit antibody (dilution: 1:1000; Invitrogen). Finally, cover slips were mounted using ProLong Gold anti-fade with DAPI (4',6-diamidino-2-phenylindole; Invitrogen). Images were acquired using a Zeiss Apotome microscope.

Tandem affinity purification

The tandem affinity purification was performed according to the protocol for the InterPlay Mammalian TAP System (Stratagene). \( 2 \times 10^7 \) cells were used per purification. Proteins were eluted by incubating in 50 \( m \)M \( H_3\text{PO}_4 \) (pH 8.0) at 95°C for 5 min. Eluates were analyzed on SDS–PAGE gels.

Fly stocks and genetics

CG8214/Cep89 RNAi lines (stocks 101132, 24240 and 24241), genetic background controls (60000, 60100) and UAS-dicer2 (60008, 60009) were obtained from the VDRC.

Imaging

A Zeiss Lumar V12 microscope equipped with an AxioCamm camera was used to acquire pictures/movies of fly and fly wings. Detached wings were transferred to isopropanol, processed through a series of mounting solutions (1. 30% glycerol, 10% Isopropanol) and mounted on glass slides for further inspection on a Zeiss Axio Imager Z1, using a 40× objective.

Survival assay

For Cep89 knockdown, heterozygous act-Gal4/CyO GFP: UAS-dicer2 virgin females were mated to homozygous UAS-Cep89RNAi101132, homozygous UAS-Cep89RNAi24241, or heterozygous UAS-Cep89RNAi24241/CyO males yielding progenies of two or three genotypes, respectively, the expected ratios of which provided the basis for the evaluation of lethality.

Complex measurements in *Drosophila*

The following fly stocks were obtained from the Bloomington *Drosophila* stock center (Indiana University): MEF2-Gal4 (27390), UAS-dicer2; MEF2-Gal4 (25756), GMR-Gal4 (1104), elav-Gal4 (8760), MS1096-Gal4 (8696), MS1096-Gal4; UAS-dicer2 (25706), and act-Gal4/CyO (4414). In house assembled drivers were GMR-Gal4; UAS-dicer2, act-Gal4/CyO-GFP and UAS-dicer2; elav-Gal4 and the 2xGMR-wIR; elav-Gal4, UAS-Dicer2 line that was used for habituation experiments. The latter was prepared by combining two copies of GMR-wIR (RNAi against white gene under an eye-specific promotor) with elav-Gal4 and UAS-Dicer2. Flies were cultured according to standard procedures and raised at 28°C, unless indicated otherwise.

Quantitative real-time PCR

Conditional knockdown of Cep89 was achieved using the UAS/GAL4 system in combination with UAS-RNAi constructs \( (18) \), as indicated. In order to evaluate potency of Cep89 RNAi lines, RNAi lines and genetic background controls were crossed to the ubiquitous act-Gal4/CyO-GFP driver at 25°C and GFP-negative L3 larvae were selected for quantitative real-time PCR (qPCR). The extraction of mRNA from larvae, cDNA synthesis and qPCR were performed as described \( (49) \). For each genotype, three biological and two technical replicates were performed. RNA was subjected to an extra DNAse treatment using the DNA-free kit (Ambion) before cDNA synthesis. \( \beta \)-cop was used as reference gene.
Immunohistochemistry

Type 1b neuromuscular junctions (NMJs) of muscle 4 were analysed after dissection of wandering L3 larvae and fixation in 3.7% PFA for 30 min. Discs large 1 and Bruchpilot were co-stained using the Zenon Alexa Fluor 568 Mouse IgG1 labeling kit (Invitrogen) and the primary antibody ncl82 (1:125) (DSHB), applied overnight at 4°C, respectively. The Alexa 488-labeled antibody goat-anti-mouse was used as a secondary antibody (1:500) (Invitrogen). NMJ pictures were obtained using a Leica automated brightfield multi-color epifluorescent microscope. To ensure accurate and objective evaluation, images were automatically processed and measured by an advanced self-made ImageJ/Fiji macro, measuring the following parameters: NMJ area, perimeter, NMJ length, branching pattern and number of active zones. Muscle area and bouton number were manually counted. A total of 450 control NMJs and 115 Cep89 knockdown NMJs were included. NMJs from three independent experiments were counted.

Histology and electron microscopy

Cep89RNAi101132 and control lines were crossed to GMR-Gal4 virgins and raised at 25°C. Bisected Heads from 3 to 4 days old females were prefixed in 2% glutaraldehyde buffered with 0.1 M sodium cacodylate pH 7.4, after 30 min heads were postfixed for 1 h in 1% osmium teroxide in Paladebuffer pH 7.4 with 1% kaliumhexacyanonferrat(III)-Trihydrat, dehydrated in ethanol and propyleenoxide and embedded in a single drop of Epon. Semithin, 1 μm thick transverse and longitudinal sections were stained with 1% Toluidine Blue. Ultrathin sections were stained with Uranyl acetate and Lead citrate and examined in a JEOL 1200.

Scanning electron microscopy

Cep89RNAi101132 and control males were crossed to GMR-Gal4; UAS-dicer2 virgins and raised at 28°C. Bisected Heads from 3 to 4 days old females were prefixed in 2% glutaraldehyde buffered with 0.1 M sodium cacodylate pH 7.4, after 30 min heads were taken out, bisected and then put back into the fixative, which was continued for 24 h. Subsequently the bisected heads were postfixed for 1 h in 1% osmium tetroxide in Paladebuffer pH 7.4 with 1% kaliumhexacyanoferat(III)-Trihydrat, dehydrated in ethanol and propyleenoxide and embedded in a single drop of Epon. Semithin, 1 μm thick transverse and longitudinal sections were stained with 1% Toluidine Blue. Ultrathin sections were stained with Uranyl acetate and Lead citrate and examined in a JEOL 6310 SEM.

Electroretinograms

Cep89RNAi101132, Cep89RNAi24240 and control lines were crossed to GMR-Gal4; UAS-dicer2 virgins and raised at 28°C. Female progeny were selected at 3 to 4 days old and sequentially fixed in 1% glutaraldehyde, dehydrated in ethanol, critically-point dried and mounted on aluminum stubs. Samples were coated in gold by sputter coating before photography with a JEOL 6310 SEM.

Light-off jump reflex habituation

Male flies were collected shortly after eclosion. They were kept in groups of 15 in small food vials for 3 to 5 days and then tested in the habituation assay. In the high-throughput light-off jump reflex habituation system, 32 flies were tested in parallel using two independent 16-chamber light-off settings. Each male fly was kept in an individual semi-transparent plastic chamber in which it received 100 light-off stimuli with a 1 s time interval between each stimulus (the inter-trial interval). The wing vibration following every jump response was recorded by two microphones placed at both the ends of each chamber, amplified and analyzed by custom-made LabView software (National Instruments). A response was recorded when the fly jumped during or within 500 ms after a 15-ms light-off stimulus. Both genotypes had a high initial jump response, which decreased upon repeated presentation of the stimulus (habitation). Flies were supposed to have habituated when they failed to jump in five consecutive trials (no-jump criterion). Habituation was scored as the number of trials required to reach the no-jump criterion (Habitation Index = HI). We calculated the mean HI for 16 flies (of the same genotype) tested in one independent system and repeated this with 8 independent groups of 16 (total number of flies per genotype = 128). 8 log-transformed HI mean values of two genotypes (Cep89 knockdown versus control) were compared with ANOVA and adjusted for day and system effects in the SPSS software.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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