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BRIEF COMMUNICATION

Loss of *ap4s1* in zebrafish leads to neurodevelopmental defects resembling spastic paraplegia 52

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Abstract

Autosomal recessive spastic paraplegia 52 is caused by biallelic mutations in *AP4S1* which encodes a subunit of the adaptor protein complex 4 (AP-4). Using next-generation sequencing, we identified three novel unrelated SPG52 patients from a cohort of patients with cerebral palsy. The discovered variants in *AP4S1* lead to reduced AP-4 complex formation in patient-derived fibroblasts. To further understand the role of *AP4S1* in neuronal development and homeostasis, we engineered the first zebrafish model of AP-4 deficiency using morpholino-mediated knockdown of *ap4s1*. In this model, we discovered several phenotypes mimicking SPG52, including altered CNS development, locomotor deficits, and abnormal neuronal excitability.

Introduction

The hereditary spastic paraplegias (HSP) are a group of rare and genetically heterogeneous neurodegenerative disorders characterized by progressive spasticity.[1,2] Autosomal recessive spastic paraplegia-52 (SPG52) is a form of childhood-onset complex hereditary spastic paraplegia characterized by early developmental delay, microcephaly, epilepsy, and progressive spasticity. Given the slowly progressive nature of this disease, patients are often diagnosed with cerebral palsy (CP) before genetic testing is pursued. SPG52 is caused by bi-allelic loss-of-function variants in *AP4S1*, which encodes the sigma subunit of the adaptor protein complex 4 (AP-4), and has been described in less than 10 individuals, thus far.[3] AP-4 role in endosome membrane trafficking was evaluated in a previous study.[4]

Here we screened a large cohort of patients with a diagnosis of CP for mutations in genes related to HSP and identified three novel unrelated individuals with

SPG52. To gain further insights into the role of mutant *AP4S1* in neuronal development and function, we developed the first model of AP-4 deficiency in zebrafish (*Danio rerio*) using morpholino-mediated knockdown of *ap4s1*, which recapitulate the main aspects of the patients' phenotype.

Materials and Methods

All participants, including relatives involved in the segregation studies, provided written informed consent in accordance with the Italian National Health Service guidelines and the Declaration of Helsinki. Ethics committee approval was available. Animal experiments were performed in accordance with the IACUC at the University of Pisa. Inclusion criteria were a clinical diagnosis of CP[5] and accurate recording of at least one neurologic examination and brain MRI. Variants in *AP4S1* (ENST00000216366.8) were detected using a targeted next-generation sequencing (NGS) panel approach designed to cover reported HSP and spastic ataxia genes[2] or, in 5 cases, using whole exome sequencing. Variants were confirmed by Sanger sequencing. Pathogenicity was scored in silico using CADD.[6] Fibroblasts were derived via diagnostic punch biopsies. However, qPCR, RT-PCR and Western blotting were carried out using published protocols. Adult male and female zebrafish AB and Tg (Neurod1-GcAMP6f) were maintained according to standard procedures.[7] Morpholino antisense oligonucleotides (MO) targeting transcription at the exon 3 acceptor splice site or ATG start codon and a standard scramble MO[8] were designed as reported in Data S1. Touch-evoked escape response was measured at 48 hpf on a semi-quantitative scale ranging from *severe* (=no movement), to *mild* (=flicker of movement but no swimming), or *normal* (=normal swimming).[9] Coiling frequency was evaluated at 30 hpf using the Leica M205FA microscope (Leica, Wetzlar, Germany) and Danio Scope software (Noldus, Wageningen, The Netherlands). Behavioral and movement data were acquired using Danio Vision at 120 hpf and analyzed using EthoVision software (Noldus). The transgenic NeuroD1 line was used to assess brain/head morphology after MO injection at 72 hpf. Electrophysiological forebrain recordings were performed at 120 hpf. Statistical analyses used GraphPad Prism v.7.1 with significance set at $P < 0.05$.

Results

Through NGS studies of 112 individuals with a clinical diagnosis of CP, we identified three children harboring biallelic variants in *AP4S1*. These variants are predicted to cause an early truncation of the protein (Fig. S1A) and segregate in the families (Fig. S2). Clinical, molecular and neuroimaging findings are in Table 1. All three patients presented with developmental delay and later intellectual disability as well as slowly progressive spasticity. Patient #3 required a wheelchair at the age of 9 years, whereas patients #1 and #2 are still ambulant (with aid) at age 18 and 4 years, respectively. Epilepsy was found in all three cases: patient #1 developed focal seizures at age 10 months; patient #2 had epileptic spasms at 14 months, and patient #3 experienced generalized seizures starting at 12 months. In all three cases, seizures were well controlled with standard antiepileptic drugs. Fibroblasts from patients #1 and #2 (Fig. 1A and B) showed reduced levels of AP4E1, consistent with the notion that a loss of the sigma subunit of AP-4 destabilizes the protein complex leading to reduced levels of other AP-4 subunits. Moreover, levels of ATG9A, the major cargo of AP-4, were significantly increased consistent with prior reports.[10] Zebrafish *ap4s1* shares 78% identity with the human gene and protein (Fig. S1C). In situ hybridization analysis (WISH) confirmed that *ap4s1* mRNA is ubiquitously expressed in 48 hpf embryos and 72 hpf larvae, with a high expression in the CNS (Fig. S1D). However, at earlier stages, WISH and qPCR demonstrated *ap4s1* mRNA expression mainly after 24 hpf (Fig. S1E and F). To knock-down gene expression, we designed two different MOs, ATG- and splice-MO; after testing both, we noticed a stronger effect using the splice-MO, whereas the ATG-MO induced a milder yet significant phenotype as compared to uninjected fish (Fig. S1G). MO-mediated knockdown of *ap4s1* (Fig. S1H and I) lead to several morphological defects including decreased head size, ventriculomegaly, altered eye development, cardiac edema, and a curved tail (Fig. 1C and D). Whilst Ap4e1 protein levels were reduced (Fig. 1E), we could not study ATG9A because commercial antibodies failed to recognize the fish protein (not shown).

Moreover, behavioral analysis revealed that at 30 hpf *ap4s1* morphant embryos showed an increased coiling frequency compared to uninjected ones (Fig. 1F; Videos S1 and S2). Touch evoked response was impaired in over 80% of injected morphants at 48 hpf (Fig. 1G). Rescue experiments were performed through co-injection of 200 pg of either human WT or zebrafish mRNA together with spliceMO at the same concentration used for the knockdown experiments (Fig. 1G). At 120 hpf *ap4s1*-deficient zebrafish larvae showed locomotor impairment (Fig. 1H and I). To further explore the nature of this motor deficit, we investigated the morphology of spinal motor neurons using the motor axon marker Znp1. Immunolabeling of spinal motor neuron axons in 48 hpf morphants and control embryos showed abnormal axon outgrowth and a reduction in axon length (Fig. 1J).

Table 1. Clinical features of three patients with SPG52.

Patient	Patient #1	Patient #2	Patient #3
Sex	Female	Female	Male
AP4S1 Variant[1]	c.47insT/c.234insG p.Ser17*/ p.Ala79Glyfs*4	c.234insG/c.234insG p.Ala79Glyfs*4/ p.Ala79Glyfs*4	c.138 + 2T>G/c.138 + 2T>G
CADD Score	28.9/28.1	28.1	25.9
Age at last evaluation	18 years	4 years, 10 months	14 years
Consanguinity	No	Yes	Unknown
Ethnicity	Caucasian	Caucasian	Caucasian
Spasticity	Spastic tetraplegia	Spastic tetraplegia	Spastic tetraplegia
Level of ambulation	Walks without support	Walks with support	Wheelchair-dependent
Developmental Delay/ Intellectual Disability	Severe	Severe	Moderate
Speech	Simple sentences	Nonverbal	Nonverbal
Short Statue	Yes	Yes	No
Microcephaly	Yes (postnatal)	Yes (postnatal)	No
Thin Corpus Callosum	Yes	Yes	Yes
Ventriculomegaly	No	No	Yes
Cerebral Atrophy	Yes	No	No
Cerebellar Atrophy	Yes	No	No
Seizures	Focal (onset at 10 years)	Epileptic spasms (onset at 14 months)	Focal and generalized (onset at 12 months)
EEG	Sharp waves on bilateral anterior regions	Generalized sharp waves	Diffuse epileptiform abnormalities

[1]*AP4S1* Reference Sequence ENST00000313566.

Staining of acetylated α-tubulin confirmed truncated axons that appeared thinner than in controls and were hardly visible in the most severely affected embryos (Fig. 1K). Knockdown of *ap4s1* resulted in not only an alteration of ventrally projecting motor neurons at 48 hpf but also a strong reduction in axon distribution in the anterior region of the CNS. To further characterize the neurodevelopmental consequences of *ap4s1* depletion, we studied the pan-neural marker Sox3 and the postmitotic neuronal marker Huc/Hud (Fig. 1K). Morphants (24 hpf) showed a reduction in Sox3 staining suggesting an impairment in the maintenance of the neuronal progenitor pool (Fig. 1K). Huc/Hud staining was globally reduced indicating loss of postmitotic neurons, in particular at the level of diencephalon, telencephalon and in the optic tectum (Fig. 1K). Overall, these results suggested an impairment of primary neurogenesis in *ap4s1* zebrafish morphants. This corroborates the altered CNS morphology observed in 72 hpf *ap4s1*-depleted Neurod1-GFP embryos (Fig. 1D). To further explore the functional implications of loss of *ap4s1*, we measured local field potentials in the forebrain of control and *ap4s1*-depleted embryos at 120 hpf. We detected no difference at room temperature (23°C) (Fig. 1L). When the temperature was raised to 33°C, frequent bursts were observed in *ap4s1* morphant larvae (Fig. 1L), indicating a lower threshold for abnormal excitability in the setting of stressors, mimicking in part what is experienced by SPG52 children.[5]

Discussion

We here describe three additional cases of SPG52 discovered by genetic screening of a cohort of patients with a diagnosis of CP. Clinical and imaging features of these patients resemble those reported previously.[3,5,11–14] Interestingly, patient #1 presented with a relatively mild phenotype and preserved independent walking at the age of 18 years. On a cellular level, fibroblasts derived from two patients showed reduced levels of the AP4E1 subunit consistent with reduced stability of the AP-4 complex. In line with prior reports,[10] we also observed an accumulation of ATG9A, further corroborating the pathogenicity of the discovered SPG52 variants. In humans, *AP4S1* encodes a protein whose function is, only in part, understood, also because of the absence of in vivo models of SPG52, though initial iPSC studies[15] appear helpful to study axon outgrowth. To explore the consequence of loss of AP4S1 in vivo, we generated the first zebrafish model of AP-4-deficiency. Antisense morpholino-mediated knockdown of *ap4s1* resulted in a robust reduction in expression. Loss of formation of a functional AP-4 complex was confirmed by a reduction in protein levels of the Ap4e1 subunit, a surrogate for complex formation,[10] a finding already seen in SPG52 disease. Various neurodevelopmental brain malformations are found in patients with AP-4-associated hereditary spastic paraplegia (SPG47, SPG50, SPG51, and SPG52).[3] These include a thinning of the corpus

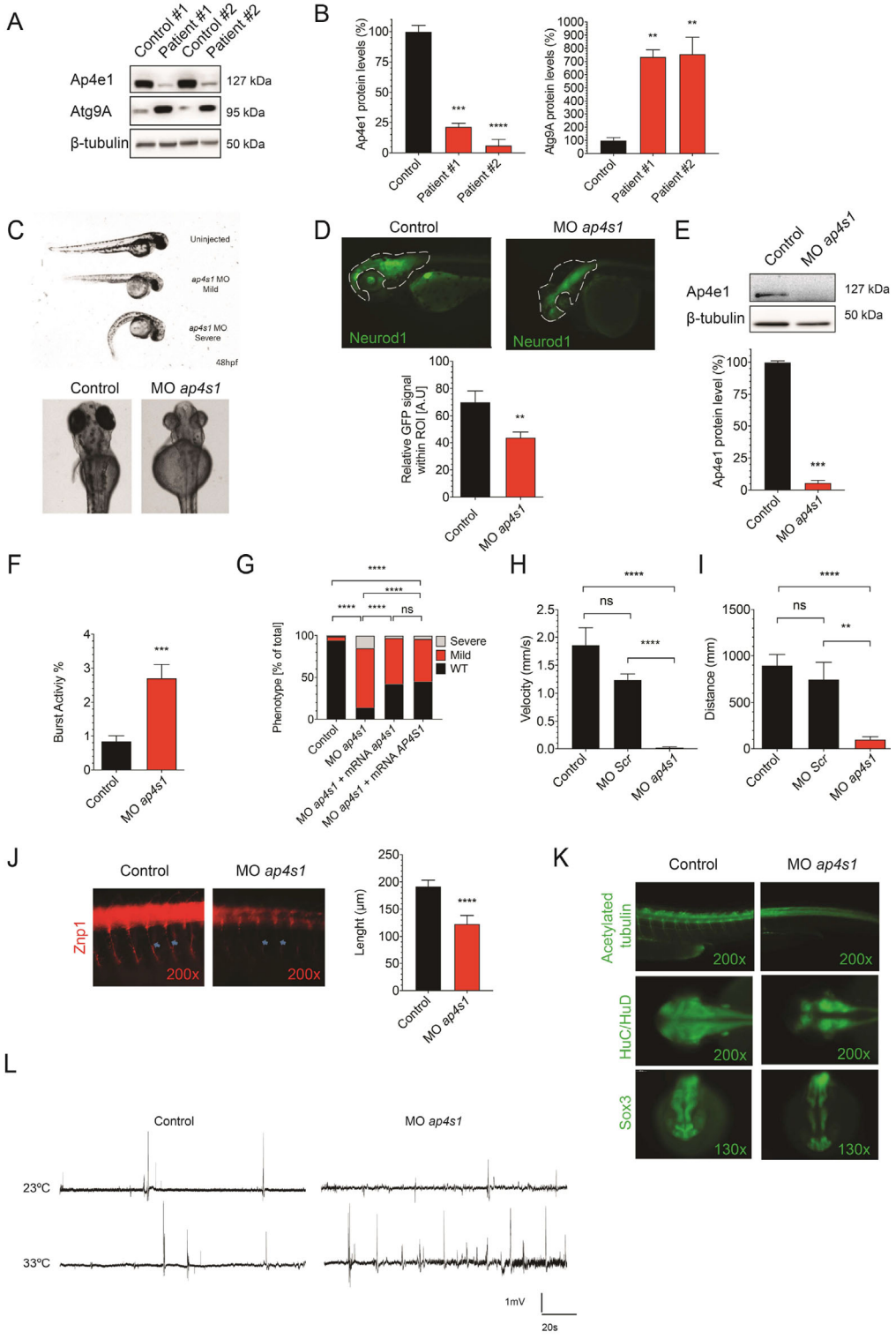
A
Control #1
Patient #1
Control #2
Patient #2
Ap4e1
Atg9A
β-tubulin
127 kDa
95 kDa
50 kDa
B
Ap4e1 protein levels (%)
Atg9A protein levels (%)
Control
Patient #1
Patient #2
C
Uninjected
ap4s1 MO Mild
ap4s1 MO Severe
48hpf
Control
MO ap4s1
D
Control
MO ap4s1
Neurod1
Relative GFP signal within ROI [A.U]
E
Control
MO ap4s1
Ap4e1
β-tubulin
127 kDa
50 kDa
Ap4e1 protein level (%)
F
Burst Activiy %
G
Phenotype [% of total]
Severe
Mild
WT
MO ap4s1 + mRNA ap4s1
MO ap4s1 + mRNA AP4S1
H
Velocity (mm/s)
MO Scr
I
Distance (mm)
J
Znp1
200x
Lenght (μm)
K
Acetylated tubulin
HuC/HuD
Sox3
200x
130x
L
23°C
33°C
1mV
20s

Figure 1. (A and B) Western blot analysis from whole cell lysates of patient #1 and patient #2 as well as two healthy, unrelated controls. Levels of AP4E1 are reduced, consistent with the notion that loss of AP4S1 destabilizes the AP-4 complex and lowers levels of the other subunits. ATG9A, the main cargo of AP-4, is increased in fibroblasts from patient #1 and patient #2, consistent with prior reports. This indicates a loss of AP-4 function (one-way ANOVA with multiple comparison, n = 2–6 samples per condition per experiment, P-value: ****<0.0001; ***0.0002; **0.0008). (C) Morphology of zebrafish embryos injected with MO^{ap4s1} at 48 hpf. Mild and severe phenotypes are found. Several dysmorphic features are present, including an abnormal eye shape, smaller head size, cardiac edema and curved tail. (D) CNS morphology as assessed by GFP fluorescence in NeuroD1-GFP zebrafish at 72 hpf. Dotted line outlines head area (ROI). GFP-fluorescence within the ROI is significantly reduced (Mann–Whitney test, n = 10; P-value: **0.0052). (E) Western blot analysis from lysates of *ap4s1*-depleted zebrafish at 72 hpf shows reduced Ap4e1 expression (unpaired t-test, n = 2; P-value: ***0.0004). (F) Coiling frequency in zebrafish embryos at 30 hpf is increased in *ap4s1*-depleted zebrafish (Mann–Whitney test, n = 80 in 4 independent experiments; P-value: ***0.0002). (G) Touch-evoked escape response was measured at 48 hpf on a semi-quantitative scale ranging from *severe* (=no movement), to *mild* (=flicker of movement but no swimming), or *normal* (=normal swimming). About 80% of *ap4s1*-depleted zebrafish show a mild or severe impairment and about 40% of morphants showed partial rescued phenotypes, after co-injection with either human or zebrafish mRNA (Chi Square Test, n = >100 in 2 independent experiments; P value: ****<0.0001). (H and I) Automated analysis of spontaneous motor activity revealed a reduction in swim distance and velocity in *ap4s1*-depleted zebrafish at 120 hpf (Mann–Whitney test, n = 37 per condition; P-value: ****=<0.0001; **=0.0011). (J) Immunocytochemistry with motor neuron marker znp1 demonstrates a reduction in axon length of spinal motor neurons in *ap4s1*-depleted zebrafish at 48 hpf (Mann-Whitney test, n = 20; P-value: ****=<0.0001). (K) Immunocytochemistry using the pan-neural marker sox3 and the postmitotic neuronal marker Huc/Hud in 24 hpf embryos. A reduction in sox3 staining, suggesting an impairment in the maintenance of the neuronal progenitor pool, is found in *ap4s1*-depleted zebrafish. Huc/Hud staining was globally reduced in *ap4s1*-depleted zebrafish indicating loss of postmitotic neurons, in particular at the level of diencephalon, telencephalon and in the optic tectum. (L) Local field potentials recorded from 120 hpf zebrafish. At an ambient temperature of 23°C there was no difference observed. When the temperature was raised to 33°C frequent bursts were found in *ap4s1*-depleted zebrafish, indicating abnormal excitability and a lower threshold for epileptiform activity in the setting of stressors.

callosum, loss of periventricular white matter with resulting ventriculomegaly, as well as polymicrogyria in some. In *ap4s1*-depleted zebrafish, we observed a reduction in head size and a complex CNS malformation. In combination with the finding of reduced staining for neuronal progenitors and postmitotic neurons, these results demonstrate the crucial role of AP-4 for neuronal development. Mimicking the human phenotype, we found locomotor deficits in *ap4s1*-depleted zebrafish. Behavioral assays in morphant larvae revealed significant defects inverall mobility, suggesting that *ap4s1*-related motor neuron development and function is required for normal motility in zebrafish. We next examined spinal motor axons and found that morphants exhibited shorter axonal length, indicating that SPG52 may be important for the outgrowth of motor axons. This is consistent with impaired neuron outgrowth found in cultured neurons from *Ap4e1* knockout mice[16,17] and similar to results seen in several zebrafish models of HSP including spastin,[18] *atl1*,[19] and *spatacsin*.[20] Seizures are found in about two-thirds of AP-4-HSP patients and febrile seizures are common early in life.[3] Local field potentials recordings of forebrain in *ap4s1*-depleted zebrafish revealed no spontaneous seizures at basal temperature. With hyperthermia, however, burst of abnormal activity were induced, suggesting the possibility for a lower threshold for epileptiform activity in the setting of stressors.

Summarizing, *ap4s1* knockdown in zebrafish leads to several phenotypes that resemble *AP4S1*-associated hereditary spastic paraplegia. Thus, this first zebrafish model of AP-4 deficiency represents a new tool for dissecting the role of AP-4 in neurodevelopment and neurodegeneration and could be the starter point for future drug screening, along with a KO model.

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Conflict of Interest

Ebrahimi-Fakhari reports grants from Thrasher Research Fund, grants from Spastic Paraplegia Foundation, grants from CureSPG47 Foundation, during the conduct of the study. All other authors declare no conflict of interest related to this study.

Author Contribution

Conception and design of the study: A.D., F.G., F.M.S. Acquisition and analysis of data: A.D., A.T., V.N., G.F., D.G., M.M., S.M., G.B., A.C., M.T.B., F.S., J.B., R.R., R.B., I.R., A.R., and D.E.F. Drafting a significant portion of the manuscript or figures: A.D., J.H., M.M., D.E.F, M.S., and F.M.S.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. (A) Three-dimensional model of human *AP4S1* (qmean4 global score: 0.710).
Figure S2. Family tree and segregation of mutations in the kindred. Circles and squares are women and men, respectively. Filled symbols are affected individuals.
Data S1. Additional methods.
Video S1. Coiling frequency in uninjected zebrafish embryos at 30 hpf.
Video S2. Coiling frequency in zebrafish embryos injected withMO^{ap4s1} at 30 hpf.

