

# Bi-allelic *ACBD6* variants lead to a neurodevelopmental syndrome with progressive and complex movement disorders

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## 1 Abstract

2 The acyl-CoA-binding domain-containing protein 6 (ACBD6) is ubiquitously expressed, plays a  
3 role in the acylation of lipids and proteins, and regulates the *N*-myristoylation of proteins via *N*-  
4 myristoyltransferase enzymes (NMTs). However, its precise function in cells is still unclear, as is  
5 the consequence of *ACBD6* defects on human pathophysiology. Utilizing exome sequencing and  
6 extensive international data sharing efforts, we identified 45 affected individuals from 28  
7 unrelated families (consanguinity 93%) with bi-allelic pathogenic, predominantly loss-of-  
8 function (18/20) variants in *ACBD6*. We generated zebrafish and *Xenopus tropicalis acbd6*  
9 knockouts by CRISPR/Cas9 and characterized the role of *ACBD6* on protein *N*-myristoylation  
10 with YnMyr chemical proteomics in the model organisms and human cells, with the latter also  
11 being subjected further to ACBD6 peroxisomal localization studies. The affected individuals (23  
12 males and 22 females), with ages ranging from 1 to 50 years old, typically present with a  
13 complex and progressive disease involving moderate-to-severe global developmental  
14 delay/intellectual disability (100%) with significant expressive language impairment (98%),  
15 movement disorders (97%), facial dysmorphism (95%), and mild cerebellar ataxia (85%)  
16 associated with gait impairment (94%), limb spasticity/hypertonia (76%), oculomotor (71%) and  
17 behavioural abnormalities (65%), overweight (59%), microcephaly (39%) and epilepsy (33%).  
18 The most conspicuous and common movement disorder was dystonia (94%), frequently leading  
19 to early-onset progressive postural deformities (97%), limb dystonia (55%), and cervical  
20 dystonia (31%). A jerky tremor in the upper limbs (63%), a mild head tremor (59%),  
21 parkinsonism/hypokinesia developing with advancing age (32%), and simple motor and vocal  
22 tics were among other frequent movement disorders. Midline brain malformations including  
23 corpus callosum abnormalities (70%), hypoplasia/agenesis of the anterior commissure (66%),  
24 short midbrain and small inferior cerebellar vermis (38% each), as well as hypertrophy of the  
25 clava (24%) were common neuroimaging findings. *acbd6*-deficient zebrafish and *Xenopus*  
26 models effectively recapitulated many clinical phenotypes reported in patients including  
27 movement disorders, progressive neuromotor impairment, seizures, microcephaly, craniofacial  
28 dysmorphism, and midbrain defects accompanied by developmental delay with increased  
29 mortality over time. Unlike ACBD5, ACBD6 did not show a peroxisomal localisation and  
30 ACBD6-deficiency was not associated with altered peroxisomal parameters in patient

1 fibroblasts. Significant differences in YnMyr-labelling were observed for 68 co- and 18 post-  
2 translationally *N*-myristoylated proteins in patient-derived fibroblasts. *N*-Myristoylation was  
3 similarly affected in *acbd6*-deficient zebrafish and *Xenopus tropicalis* models, including Fus,  
4 Marcks, and Chchd-related proteins implicated in neurological diseases. The present study  
5 provides evidence that bi-allelic pathogenic variants in *ACBD6* lead to a distinct  
6 neurodevelopmental syndrome accompanied by complex and progressive cognitive and  
7 movement disorders.

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8 **Running title:** *ACBD6*-related disease

9

10 **Keywords:** *ACBD6*; neurodegeneration; dystonia; ataxia; parkinsonism; *N*-myristoylation

11 **Abbreviations:** ACBDs=Acyl-CoA binding domain containing proteins; LCACoA=long-chain  
12 fatty acyl-CoA esters; ACBD6=Acyl-CoA-binding domain-containing protein 6; ACBD5=Acyl-  
13 CoA-binding domain-containing protein 5; ACB=Acyl-CoA binding; ANK=ankyrin repeat  
14 domain; dpf=days post-fertilization; hpf=hours post-fertilization; NMT=*N*-myristoyltransferase;  
15 Myr-CoA=myristoyl co-enzyme A; LOF=loss-of-function; PTZ=pentylentetrazole; VSR=visual  
16 startle response; WES=whole exome sequencing; WISH=whole-mount in situ hybridization;  
17 YnMyr=myristic acid alkyne; CRISPR =Clustered Regularly Interspaced Short Palindromic  
18 Repeats GDD=global developmental delay; ID=intellectual disability

19

## 20 **Introduction**

21 Acyl-CoA-binding domain-containing proteins (ACBDs) are a large multigene family of  
22 intracellular lipid-binding proteins, and in mammals, they include ACBD1-7. These proteins  
23 specifically bind long-chain fatty acyl-CoA esters (LCACoA) and control their intracellular  
24 concentration.<sup>1,2</sup> Various cellular functions have been ascribed to this protein family, ranging  
25 from lipid homeostasis, organelle formation, apoptotic responses, intracellular vesicle  
26 trafficking, as well as tethering between the peroxisome and endoplasmic reticulum.<sup>2</sup> ACBDs  
27 have been suggested to play a crucial role in brain development, via their strong proliferative



1 effects on the neural stem and progenitor cells.<sup>3</sup> To date, defects in only two members of ACBDs  
2 have been associated with Mendelian disorders in humans. *ACBD5* deficiency has been reported  
3 to cause a combination of retinal dystrophy with leukodystrophy and defects in peroxisomal very  
4 long-chain fatty acid metabolism in four families.<sup>4-7</sup> *ACBD6* has been suggested as a candidate  
5 gene for intellectual disability (ID) in two large-cohort gene discovery studies reporting limited  
6 phenotypic data<sup>8,9</sup> and in a case report describing 2 siblings with neurodevelopmental disorder,  
7 obesity, pancytopenia, diabetes mellitus, cirrhosis, and renal failure but with a limited  
8 neurological phenotype.<sup>10</sup>

9 The *ACBD6* protein has two domains: the N-terminal Acyl-CoA-binding (ACB) and the  
10 specialized C-terminal ankyrin repeat (ANK) domain. This protein is detected in the cytosol and  
11 nuclei of cells and modulates the acylation of lipids and proteins.<sup>11</sup> It has also been suggested  
12 that *ACBD6* is associated with *N*-myristoyltransferase enzymes (NMTs) in human cells by  
13 ligand binding and protein interaction, although direct evidence for this in cells is lacking.<sup>12</sup> In  
14 humans, NMT1 and NMT2 enzymatically catalyze the *N*-myristoylation of substrate proteins by  
15 transferring the myristate from myristoyl coenzyme A (Myr-CoA) onto the N-terminal glycine of  
16 nascent proteins (co-translationally, at the ribosome) or to internal glycines uncovered by  
17 protease cleavages during apoptosis (post-translationally).<sup>13</sup> The global *N*-myristoylated  
18 proteome consists of more than 200 co- and post-translationally *N*-myristoylated proteins in  
19 humans,<sup>14,15</sup> and *N*-myristoylation is important for the association of substrates with membranes  
20 and their interaction with other proteins.<sup>16</sup>

21 Here, we describe 45 affected individuals from 28 unrelated families with 18 bi-allelic predicted  
22 loss-of-function (LOF), 1 missense, and 1 in-frame insertion variants in *ACBD6* presenting a  
23 new and distinct neurodevelopmental syndrome with a complex and progressive movement  
24 disorder phenotype. We performed functional studies in zebrafish and *Xenopus tropicalis* (*X.*  
25 *tropicalis*) knockouts generated by CRISPR/Cas9 that recapitulate many clinical features  
26 reported in patients. We ruled out a major role for *ACBD6* in peroxisomes and investigated the  
27 deregulation of co- and post-translationally *N*-myristoylated proteins in *ACBD6* deficient human,  
28 zebrafish, and *X. tropicalis* cells, given its putative role in modulating NMT activity.

29

# 1 **Materials and methods**

## 2 **Patient identification and deep phenotyping**

3 By using the GeneMatcher platform,<sup>17</sup> extensive international data sharing, and screening the  
4 variant databases of several research and diagnostic laboratories worldwide, we identified 27  
5 families with bi-allelic variants in *ACBD6*. Follow-up details were obtained in the family  
6 reported by Yeetong *et al.* (2023).<sup>10</sup> For a comprehensive phenotypic characterization of the  
7 affected individuals, we obtained clinical details, including neurological examination, via a  
8 universally-adopted proforma (Supplementary Table 1). Where possible, video recordings and  
9 facial photographs of the affected individuals along with their brain MRIs were made available  
10 for review by a movement disorders specialist (K.B.), dysmorphologist (M.S.), and  
11 neuroradiologist (M.Sev.), respectively. Parents and legal guardians of all affected individuals  
12 consented to the publication of clinical and genetic information, including video and  
13 photographs, according to the Declaration of Helsinki, and the study was approved by the  
14 respective local Ethics Committees.

## 16 **Genetic analysis**

17 Using genomic DNA from whole blood samples of the probands from the 28 unrelated families,  
18 whole-exome sequencing (WES) was performed at 10 different centres worldwide with methods  
19 specified in Supplementary Table 1. WES data analysis and variant filtering and prioritization  
20 were performed using in-house implemented pipelines at different centres (Supplementary Table  
21 1 for methods). Genotyping and homozygosity mapping were done in families 1, 3, 5, 7, and 21  
22 according to standard procedures using the Automap software (<https://automap.iob.ch/>). Sanger  
23 sequencing was performed to confirm co-segregation in all available family members. mRNA  
24 expression analysis for *ACBD6* was performed by relative real-time PCR (Supplementary  
25 Method 1). RNA studies to assay the effect of the splice acceptor site variants were performed as  
26 previously described<sup>18,19</sup> (Supplementary Methods 2).

27  
28

1 **Functional studies in Zebrafish**

2 Zebrafish (*Danio rerio*) were raised and maintained under standard conditions in an AALAC  
3 accredited facility at the Oklahoma Medical Research Foundation (OMRF). All experiments  
4 were performed as per protocol 22-18 approved by the Institutional Animal Care Committee of  
5 OMRF (IACUC). Wild-type zebrafish strain (*NHGRI-1*)<sup>20</sup> or transgenic lines as described were  
6 used for all experiments. Detailed experimental procedures are described in Supplementary  
7 Method 3.

8  
9 **Functional studies in *Xenopus tropicalis***

10 *X. tropicalis* was used to test the gene-disease link for *ACBD6* further in a second animal model.  
11 The details of *X. tropicalis* care, generating F0 *acbd6* crispant animals, and phenotypic analysis  
12 of F0 *acbd6* crispant animals are provided in Supplementary Methods 4. All procedures were  
13 conducted in accordance with the Home Office Code of Practice, under PP4353452 with  
14 approval from the University of Portsmouth's Animal Welfare and Ethical Review Body.

15  
16 **Cell culture**

17 Human fibroblasts were obtained from skin biopsies of two unrelated affected individuals, F1:S1  
18 and F2:S1 with a single base change in *ACBD6* affecting the splice acceptor site in intron 5 and a  
19 frameshift variant c.82dupG; p.(Val28GlyfsTer6), respectively. A control cell line from the  
20 unaffected sibling from F1 (a homozygous wild-type sibling) was established (control 1). Wild-  
21 type human control (C109) skin fibroblasts were provided by H. Waterham (control 2). COS-7  
22 cells were cultured to perform immunofluorescence and microscopy analysis of *ACBD6*  
23 localization (Supplementary Methods 5).

## 1 **Analysis of peroxisomal VLCFA $\beta$ -oxidation parameters**

2 A D3-C22:0 loading test was performed by loading cells for 3 days with 30  $\mu$ M deuterated (D3)  
3 C22:0 followed by fatty acid analysis with tandem mass spectrometry, essentially as previously  
4 described.<sup>21</sup>

## 6 **YnMyr chemical proteomics, whole proteome analysis, and meta-** 7 **analyses**

8 Wild-type and ACBD6 deficient human fibroblasts, zebrafish, and *X. tropicalis* embryos were  
9 metabolically labelled in the presence or absence of YnMyr, followed by unbiased whole  
10 proteome analysis or chemical proteomics after YnMyr-enrichment to identify and quantify  
11 proteins *N*-myristoylated with YnMyr. Sample preparation, processing, and data analyses were  
12 performed as described previously, including the calculation of FDR adjusted P-values.<sup>22,23</sup>  
13 Meta-analysis for pathway- and disease-enrichment analysis were performed in Metacore  
14 (Clarivate). Detailed experimental procedures are described in Supplementary Methods 6.

## 16 ***Acbd6* expression studies in mouse brain**

17 Gene expression in the adult mouse brain was performed and visualized as described in  
18 Supplementary Method 7.

## 20 **Results**

21 Using WES and homozygosity mapping, we identified 20 homozygous variants in *ACBD6*  
22 across 28 unrelated families. Variants were subjected to familial segregation testing (Fig. 1A)  
23 and assessed using *in silico* analysis tools and genomic databases (Table 1 and Supplementary  
24 Tables 1 and 2). All variants are listed using the canonical transcript NM\_032360.4 (Fig. 1B).  
25 Family 1 showed a splice variant (c.574-2A>G) which has been shown to result in cryptic splice  
26 activation after 5 nucleotides in exon 6 (r.574\_578del, p.(Arg193SerfsTer7) (Fig. 1C,

1 Supplementary Fig. 1)). Families 12 (c.694+1G>A) and 13 (c.664-2A>G) each harboured novel  
2 splice variants that resulted in skipping of exon 7, leading to a frameshift and premature  
3 termination (r.664\_694del, p.(Asp222ProfsTer10). The (c.694+1G>A) variant showed additional  
4 evidence of activation of a cryptic splice donor site, also causing a frameshift and premature  
5 termination (r.694\_694+1ins23, p.(Ala232AspfsTer8) (Fig. 1C, Supplementary Figure 2).  
6 Families 7 and 27, 8, 14, and 28 carried homozygous frameshift variants including c.474delA,  
7 p.(Asp159ThrfsTer16) (Families 7 and 27 shared this *ACBD6* variant); c.285delA,  
8 p.(Lys95AsnfsTer23); c.719\_723delTTGTA, p.(Ile240ArgfsTer9); and c.360dupA,  
9 p.(Leu121ThrfsTer27), respectively. Families 2 and 21 shared the same homozygous frameshift  
10 *ACBD6* c.82dupG, p.(Val28GlyfsTer6) variant, as is the case with families 3, 16, and 17, who  
11 harboured the same homozygous frameshift *ACBD6* c.484\_488delATATT, p.(Ile162Ter) variant.  
12 Families 4, 9, 11, 18, 20, 22, and 26 carried homozygous nonsense variants including c.760C>T,  
13 p.(Arg254Ter); c.594G>A, p.(Trp198Ter); c.539C>A, p.(Ser180Ter); c.217A>T, p.(Lys73Ter);  
14 c.160C>T, p.(Gln54Ter); c.280 C>T, p.(Gln94Ter); and c.216C>A, p.(Tyr72Ter), respectively.  
15 Families 5 and 15 shared the same homozygous nonsense *ACBD6* c.187G>T, p.(Glu63Ter)  
16 variant. Likewise, families 20 and 25 shared the same homozygous nonsense *ACBD6* c.160C>T,  
17 p.(Gln54Ter) variant. Families 23 and 24 each harboured a large deletion variant (c.664-  
18 18556\_694+8366del, p.(?)) which spans 26,953bp of the *ACBD6* sequence and includes the  
19 complete deletion of exon 7. The affected individuals in family 6 carried an in-frame duplication  
20 variant c.654\_656dupTAA, p.(Asn219dup) in exon 6. Families 10 and 19 harboured the same  
21 homozygous predicted-deleterious missense *ACBD6* variant c.602A>G, p.(Asp201Gly) in exon  
22 6 (Fig. 1D). All variants were ultra-rare or absent in ~1.8 million alleles inspected through a  
23 number of large genetic variant databases listed in Supplementary Table 2. A detailed description  
24 of the variants is provided in Supplementary Tables 1 and 2.

25

## 26 **Clinical delineation of *ACBD6*-related disease**

27 The cohort includes 23 male and 22 female affected individuals whose current ages widely range  
28 between 1 and 50 years. While almost half of the individuals (21/45) were <10 years old (the 1st  
29 age group), 11/45 (25%) individuals were between the ages of 10-19 years (the 2<sup>nd</sup> age group),  
30 and 13/45 (29%) affected individuals were ≥20 years old (the 3<sup>rd</sup> age group) at the most recent

1 review. Two siblings from Family 28 died at the ages of 31 and 30 due to stage 5 chronic renal  
2 disease and aspiration, respectively. Almost all affected individuals are from consanguineous  
3 unions with diverse ethnic backgrounds populating South and Central Asia, the greater Middle  
4 East, Europe, and North and South America. Table 1 and Fig. 2A-D provide a summary of the  
5 core phenotypic features of 45 affected individuals from 28 unrelated families with bi-allelic  
6 *ACBD6* variants. A detailed phenotypic description is provided in Supplementary Table 1 and  
7 Supplementary case reports. Video recordings are available from 16 families (Supplementary  
8 videos 1-16).

9 Prenatal history was mostly unremarkable in the cohort and most of the affected individuals  
10 reached normal gestational age. Head circumference at birth was  $\leq$  3<sup>rd</sup> percentile in 7/21 (33%)  
11 affected individuals. Head circumference on the latest available review was  $\leq$  the 2<sup>nd</sup> percentile  
12 in 12/31 (39%) individuals and height was mostly below the age-adjusted average in the cohort  
13 (18/32, 56%). Current weight was  $>$  the 50<sup>th</sup> percentile in 20/34 (59%) affected individuals. All  
14 patients manifested a moderate-to-severe global developmental delay (GDD) involving all  
15 domains but predominantly affecting cognitive function and the acquisition of independent  
16 walking and expressive language. While 10 patients had failed to acquire independent  
17 ambulation by a mean age of  $9.4 \pm 5.0$  years (age range 4-20), the mean age of independent  
18 walking for the rest of the cohort was  $3.7 \pm 1.7$  years (age range 1.5-8).

19 Upon the latest available follow-up, moderate-to-severe GDD/ID (45/45, 100%) with significant  
20 expressive language impairment (40/41, 98% from non-verbal to a few words), movement  
21 disorders (33/34, 97%), facial dysmorphism (38/40, 95%), and mild cerebellar ataxia (35/41,  
22 85%) associated with limb spasticity/hypertonia (31/41, 76%) and gait abnormalities (33/35,  
23 94%) were among the cardinal clinical features observed in the *ACBD6* cohort (Fig. 2C).

24 The most conspicuous and common movement disorder present in all the 3 age groups was  
25 dystonia (30/32, 94%). This was frequently truncal dystonia leading to abnormal thoracic and/or  
26 thoracolumbar spinal flexion (camptocormia) (30/31, 97%) and mild-to-moderate lateral flexion  
27 of the trunk (Pisa syndrome, 22/32, 69%). Although the stooping of the body and its lateral  
28 flexion were equally common in the 2<sup>nd</sup> (9/9 and 8/9, respectively) and the 3<sup>rd</sup> (10/10 and 10/10,  
29 respectively) age groups, and were frequent in the 1<sup>st</sup> age group (10/12 and 4/12, respectively),  
30 the severity of truncal dystonia suggested an age-dependent progression (Fig. 2B). Additionally,

1 some affected individuals developed mild upper limb action-induced dystonia (6/15, 40%), lower  
2 limb dystonia (12/22, 55%), and cervical dystonia (8/26, 31%).

3 Another common hyperkinetic movement disorder in the cohort was a tremor. The upper limb  
4 jerky tremor at rest and/or intention tremor was present in 22/35 (63%) affected individuals and  
5 16/27 (59%) individuals had a mild head tremor. Dystonic head tremor, jerky tremor involving  
6 all limbs, and negative myoclonus were also seen in a small number of patients. With advancing  
7 age, parkinsonism/hypokinesia developed in 10/31 (32%) individuals, 6 of whom were over the  
8 age of 20 years, and 4 cases were between ages 10-20 years. A trial of levodopa was done in  
9 only 2 affected individuals with a minimal response. No other antiparkinsonian or anti dystonic  
10 medication has been tried in the cohort. Additionally, subtle perioral muscle twitching and  
11 stereotypic mouth dyskinesia were observed in the available video recordings of younger  
12 affected individuals. Remarkably, simple motor and vocal tics and tic-like vocalizations were  
13 detected in the video recordings of 7 young and adult cases (Supplementary Videos). Regarding  
14 eye movements, limited upgaze (12/26, 46%), impaired saccades (9/19, 47%), and strabismus  
15 (8/20, 40%) were frequent oculomotor abnormalities.

16 Lower limb spasticity, ascertained in 27/35 (77%) affected individuals, in combination with  
17 cerebellar ataxia led to gait abnormalities described as a spastic-ataxic gait in 14 patients, and  
18 clumsy/slow/broad-based or unstable gait in 17 individuals. Upper limb ataxia and spasticity  
19 were confirmed in 13 and 9 affected individuals, respectively. Tendon release surgery was done  
20 in 3 cohort members due to lower limb spasticity. Lower limb hypotonia was detected in single  
21 isolated cases.

22 A deterioration in motor and cognitive abilities was reported in 28/28 (100%) affected  
23 individuals suggesting a progressive disease course and underlying neurodegeneration. The  
24 oldest member of the cohort, currently aged 50 years, has lost his ability to walk independently  
25 and currently uses a wheelchair.

26 Complex partial, myoclonic, atonic, and generalized tonic-clonic seizures were reported in 13/39  
27 (33%) patients with the seizure debut from neonatal age to 35 years old. Seizures were often  
28 controlled with a combination of antiepileptic medications.

29 Younger affected individuals were reported to have hand stereotypies, and hyperactivity was  
30 present in 9/21 (43%) patients. Signs of premature aging were seen in 9/38 (24%) individuals

1 from five families. Forty-six percent of patients had autistic features (13/28), temper tantrums  
2 16/36 (44%), and aggressive behaviour (13/37, 35%) with a tendency to self-injury (6/34, 18%)  
3 were reported. Sleep disturbances (15/33, 45%) were common, and urinary incontinence was  
4 present in 15/24 (63%) individuals aged between 3-20 years.

5 Facial photographs were available from 32 affected individuals from 19 families (Fig. 2A). The  
6 analysis of 19 children revealed the most frequent dysmorphic features including full nasal tip  
7 (16/19), broad chin (14/19), bi-frontal/bi-temporal narrowing (12/19), hypertelorism (11/19), up-  
8 slanting palpebral fissures (9/19), and depressed nasal bridge (9/19). Assessment of 13 adult  
9 photos showed frequent signs such as a broad chin (11/13), full nasal tip (8/13), small mouth  
10 (7/13), high nasal ridge (5/13), thin upper lip (5/13) and full lower lip (5/13) (more details in  
11 Supplementary Tables 3 and 4).

12 Brain MRI scans were available for neuroradiological review in 29/45 subjects. The corpus  
13 callosum was abnormal in 20/29 subjects (70%): partial or complete callosal agenesis was  
14 observed in 7 individuals, while callosal hypoplasia with prevalent involvement of the posterior  
15 sections was noted in the remaining 13 individuals. In 19/29 patients (66%), there was marked  
16 hypoplasia/agenesis of the anterior commissure. Short midbrain and small inferior cerebellar  
17 vermis were each detected in 11/29 affected individuals (38%). Mild to moderate reduction of  
18 periventricular white matter with consequent ventriculomegaly was observed in 10/29 patients  
19 (34%). In 7/29 (24%) individuals, incomplete hippocampal inversion was found. Finally, mild  
20 hypertrophy of the Clava was noted in 5/21 (24%) individuals (Fig. 2D). Only 4/29 patients  
21 (14%) had normal neuroimaging findings.

22

### 23 ***Acbd6* expression studies in mouse brain**

24 *Acbd6* is expressed in nearly all regions of the adult mouse brain profiled by single-cell RNA  
25 sequencing (Supplementary Fig. 3).

26

27



## 1 **Characterization of *ACBD6* LOF using CRISPR/Cas9-mediated** 2 **zebrafish mutant and F<sub>0</sub> knockout models**

3 Zebrafish *Acbd6* protein is highly conserved across species and displayed similar tissue-specific  
4 expressions with humans (Supplementary Fig. 4). To examine the spatiotemporal expression  
5 pattern, we performed whole-mount *in situ* hybridization (WISH) analysis and results revealed  
6 that the *acbd6* mRNA was broadly expressed at 24 hours post-fertilization (hpf) and had elevated  
7 expression levels in the central nervous system, developing eyes, otic vesicle, and trunk muscles  
8 (Fig. 3A). Using CRISPR/Cas9 technology, we generated a genetic mutant of *acbd6*, and  
9 through RT-qPCR analysis, verified a significant notable decrease in *acbd6* mRNA expression in  
10 homozygous mutants (Supplementary Fig. 5A). During early embryonic developmental stages,  
11 we did not observe any visible morphological abnormalities in homozygous mutants. However,  
12 at the 6 days post-fertilization (dpf) stage, homozygous mutants (-/-) demonstrated a minor  
13 reduction in eye size (~3%, indicated by the red line in Fig. 3B) compared to wild-type (+/+) or  
14 heterozygous (+/-) animals (Fig. 3C). There was no significant change in head size (indicated by  
15 the blue line in Fig. 3B) (Fig. 3D). The visual startle response (VSR) analysis<sup>25</sup> indicated that  
16 reduced eye size impacts the visual function in *acbd6* -/- mutants (Fig. 3E). Furthermore, we  
17 performed locomotion behaviour tests on mutants at 6 and 12 dpf in 10-minute intervals of light-  
18 dark cycles (Fig. 3F-K and Supplementary Fig. 5-7, and detailed descriptions in Supplementary  
19 Result 3). In general, *acbd6*-/- mutants exhibited a gradual decline in locomotor activity in dark  
20 periods (Fig. 3F, G, I, J and Supplementary Fig. 7A, B, D, E) and an exaggerated response as  
21 soon as lights are turned off at 6 dpf (Figs. 3F, H, Supplementary Fig. 5C-D and Supplementary  
22 Fig. 7A, C), suggesting a hypertonia-like or spasticity behaviour.<sup>26</sup> As the larvae developed  
23 further at 12 dpf, the *acbd6*-/- mutants showed an increase in distance moved after light on and  
24 multiple locomotor bursts (Fig. 3I, K, Supplementary Fig. 6C and Supplementary Fig. 7D, F),  
25 indicating light-induced seizure-like behaviour.<sup>27</sup> The mutants also demonstrated increased  
26 mortality and severe developmental delay, with an overall reduction in brain size and disrupted  
27 muscular phenotype (Fig. 3L, M). Histological analysis showed a reduction in brain size (Fig.  
28 3N-W), particularly in regions such as the telencephalon, optic tectum, cerebellum, and retina  
29 (Fig. 3O-Q, Fig. 3R-T, Fig. 3U-W, Supplementary Fig. 8A-C). Furthermore, skeletal muscle  
30 fibres in *acbd6*-/- mutants displayed a disrupted phenotype, characterized by shortened and

1 scattered fibres and gaps (Supplementary Fig. 8D-F). Interestingly, adult *acbd6*  $-/-$  mutant  
2 survivors (Supplementary Fig. 9 and Supplementary video 17, 18) exhibited behavior resembling  
3 that of individuals with an autism spectrum disorder.<sup>28</sup>

4 To verify the specificity of the *acbd6* mutant phenotype, we utilized the F<sub>0</sub> knockout (also known  
5 as F<sub>0</sub> crispant, or simply F<sub>0</sub>) method to induce bi-allelic mutations and observed similar  
6 morphological and molecular phenotypes in homozygous mutants and F<sub>0</sub> (Fig. 4A-D). We  
7 discovered that F<sub>0</sub> also exhibited several phenotypes previously reported in affected individuals,  
8 such as hypertelorism (Fig. 4E) and facial dysmorphism (broader chin; and wider lower jaw,  
9 Supplementary Fig. 10A-H). These phenotypes were restored upon co-injection of wild-type  
10 human mRNA, confirming the specificity of the phenotype. We also introduced the LOF  
11 p.(Glu63Ter) and missense variants p.(Asp201Gly) into *acbd6* cDNA and observed impaired  
12 protein function and failure to rescue the eye size phenotype (Fig. 4F) in F<sub>0</sub> knockouts. We  
13 further investigated *acbd6* F<sub>0</sub> behaviour in light-dark cycles at 6- and 12-dpf and observed that  
14 *acbd6* F<sub>0</sub> exhibited lower locomotor activity during dark periods (Fig. 4G, H) and an exaggerated  
15 response after lights off, like homozygous mutants (Fig. 4G, I, and Supplementary Fig. 11A, B).  
16 Additionally, *acbd6* F<sub>0</sub> exhibited light-induced seizure-like behaviour (red arrows in Fig. 4G and  
17 Supplementary Fig. 11C, D). By 12 dpf, *acbd6* F<sub>0</sub> demonstrated reduced locomotor activity in  
18 both light and dark periods (Supplementary Fig. 12A, B). These results suggest that *acbd6* F<sub>0</sub>  
19 larvae exhibit more severe abnormal locomotor behaviours than homozygous mutants. We  
20 hypothesized that the loss of *acbd6* might increase susceptibility to chemical-induced seizures, as  
21 observed in *acbd6*  $-/-$  mutants showing seizure-like behaviour.<sup>29,30</sup> To test this hypothesis, we  
22 exposed *acbd6* F<sub>0</sub> and control larvae to different doses of the anticonvulsant drug,  
23 pentylenetetrazole (PTZ), and discovered that *acbd6* F<sub>0</sub> larvae exhibited hyperexcited behaviour  
24 at higher doses (Fig. 4J). This suggests that downregulation of *acbd6* may contribute to the onset  
25 of epilepsy-like seizures. We examined the impact of *acbd6* F<sub>0</sub> on neuronal and skeletal muscle  
26 development and discovered excessive axonal arborizations (Fig. 4K-N and Supplementary Fig.  
27 13) and progressive degeneration of muscle fibres in *acbd6* F<sub>0</sub> larvae (Fig. 4O-T and  
28 Supplementary Fig. 14). We also observed an increase in myelin basic protein a (*mbpa*)  
29 expression in both *acbd6* F<sub>0</sub> and homozygous mutant (Supplementary Fig. 15A, B), which may  
30 explain the abnormal axonal development phenotype. A detailed description of our results can be  
31 found in the Supplementary Results 2. In summary, our zebrafish model replicated many of the

1 clinical features seen in individuals with bi-allelic variants in *ACBD6*, highlighting how these  
2 variants may contribute to the progressive disease course. Our extensive analysis of both mutant  
3 and F<sub>0</sub> in *acbd6* provides insight into the underlying mechanisms of the disease observed in  
4 affected individuals.

## 6 ***Xenopus ACBD6 F<sub>0</sub> knockout models***

7 *X. tropicalis* and humans have the same *acbd6* gene structures (Fig. 5A) and share 66% amino  
8 acid identity (Supplementary Fig. 16A). Crispant F<sub>0</sub> tadpoles were produced by injection of two  
9 non-overlapping sgRNAs targeting exon 1 of *acbd6* (Supplementary Fig. 16B). The effects were  
10 specific, since both sgRNAs produced the same phenotype and when embryos with a SNP in the  
11 PAM for one sgRNA were used, the embryos developed normally (Supplementary Fig. 17A, 3<sup>rd</sup>  
12 upper panel from the left). ICE analysis<sup>31</sup> of target amplicon sequences (Supplementary Fig.  
13 16C) showed that at the gastrula stage, 74% of alleles in the embryos had indels, and that 63.5%  
14 had a frameshift from a predominant 8bp deletion (sgRNA 68). For sgRNA71 the average was a  
15 53.6% KO from a mix of indels. The range of phenotypes at later stages was due to distinct  
16 levels of frameshift mutations among the groups (Supplementary Fig. 16D).

17 The first notable phenotype was gastrulation failure due to reduced cell movements (Fig. 5B and  
18 Supplementary Fig. 17A). This limits the analysis of phenotypes at later stages since the  
19 surviving embryos have been selected to have significantly greater mosaicism than normally  
20 produced in this type of experiment. At swimming tadpole stages more than half of the crispants  
21 had obvious craniofacial abnormalities (n = 36; Fig. 5C, Supplementary Fig. 17D, and Fig.17E)  
22 and head measurement showed a decrease in the average area from 2.07 +/- 0.36 mm<sup>2</sup> in controls  
23 to 1.52 +/- 0.27 mm<sup>2</sup> in crispants (t(34)=5.183, p<0.001; Fig. 5D). This was not a result of a  
24 defect in the structure of the head cartilage, although when *in situ* it did appear constrained by  
25 the overall head structure (compare Fig. 5E (control) and F (crispant)). The cartilage components  
26 were nonetheless present and morphologically normal upon dissection; however, the overall  
27 cartilage size was smaller than controls consistent with the observed microcephaly. To detect  
28 subtler changes in head structure, we compared control and crispant tadpoles by MicroCT (Fig.  
29 5G). Three things were apparent in the three embryos selected at random from the crispant  
30 group: first, the eyes (yellow arrows) were abnormal and displaced (e.g. the dorsal and frontal

1 views in the right-hand panel) and in one case there was anophthalmia, the latter was rare  
2 because it was not detected in the bright field images. Second, a muscle in the face was poorly  
3 developed or absent (red arrows). From comparison with *X. laevis* staining,<sup>32</sup> we tentatively  
4 identified this as the geniohyoidius, although the muscle seems more distant from the midline in  
5 *X. tropicalis* than in *X. laevis*. The brain also has obvious structural abnormalities which are most  
6 pronounced in the midbrain (blue arrows). Comparing the movement of control and *acbd6*  
7 crispant tadpoles showed that the crispants move less over a 10-minute period (average 2.37  
8 mm/s for controls and 1.01 mm/s for crispants, n = 50). The difference was statistically  
9 significant (t(78)=4.9, p<0.001) (Fig. 5H) (Supplementary videos 19-22). After the deaths at  
10 gastrulation, crispants and control embryos survived similarly until the feeding stage (Fig. 5I).

11

## 12 **Loss of ACBD6 does not impact peroxisome function**

13 Several members of the ACBD family have been linked to peroxisome function.<sup>2</sup> Furthermore,  
14 peroxisomal dysfunction is linked to developmental defects and neurological abnormalities.<sup>33</sup> To  
15 investigate if peroxisomal parameters were altered in *ACBD6* deficiency, patient (from F1:S1  
16 and F2:S1) and control (F1:II-2 unaffected sibling; wild type C109) fibroblasts were processed  
17 for immunofluorescence using antibodies against the peroxisomal membrane marker PEX14 and  
18 catalase, a peroxisomal matrix protein (Fig. 6 A, B). No alterations in peroxisome protein import,  
19 morphology, distribution, or number were observed when compared to control fibroblasts (Fig. 6  
20 A, B). In addition, expression of a Myc-ACBD6 construct in COS-7 cells confirmed a  
21 cytoplasmic and nuclear localisation of Myc-ACBD6,<sup>34</sup> but did not provide evidence for a  
22 peroxisomal localisation under standard culture conditions (Fig. 6C). In addition, fatty acid  
23 analysis after a D3-C22:0 loading test in cultured fibroblasts did not reveal any abnormalities of  
24 peroxisomal VLCFA  $\beta$ -oxidation (Supplementary Table 5) as observed in *ACBD5* deficiency.<sup>5,33</sup>  
25 Detailed results are described in Supplementary Results 3.

26

27

## 1 **Investigation of the effect of *ACBD6* deficiency on *N*-myristoylation** 2 **in patient-derived fibroblasts**

3 We employed a chemical proteomic approach to identify and quantify *N*-myristoylated proteins,  
4 by combining metabolic labelling of living cells with YnMyr,<sup>14</sup> an alkyne-containing myristic  
5 acid analogue, and Click chemistry-enabled enrichment of YnMyr-labelled proteins, coupled to  
6 mass spectrometry proteomics analysis.<sup>22</sup> Chemical proteomics revealed 68 known co-  
7 translationally, and 18 post-translationally *N*-myristoylated proteins expressed in the fibroblasts  
8 derived from F1:S3 and his healthy sibling (Supplementary Fig. 18A, B, respectively). As  
9 expected, the incorporation of YnMyr was markedly reduced by treatment with 100 nM IMP-  
10 1088, a selective and highly potent NMT inhibitor,<sup>23,35</sup> in both the fibroblasts of the healthy  
11 control and the patient, confirming the specificity of labelling (Supplementary Fig. 18C, D,  
12 respectively). Proteins co- and post-translationally *N*-myristoylated with YnMyr were detected in  
13 significantly higher abundance in the patient-derived fibroblasts compared to the healthy sibling  
14 (Supplementary Fig. 18E), suggesting *ACBD6* deficiency provokes increased incorporation of  
15 YnMyr, potentially through increased *N*-myristoylation or upregulation of lipid salvage  
16 pathways leading to increased YnMyr import (Fig. 6D). We hypothesise this may be caused by  
17 the differential interplay of the two *N*-myristoyltransferases (NMT1 and NMT2) with *ACBD6*  
18 for various substrates. Although fibroblasts from only one affected individual and a healthy  
19 sibling were compared, the apparent differential *N*-myristoylation warrants more detailed  
20 investigations of the role of *ACBD6* in *N*-myristoylation in cell types involved in the described  
21 human clinical phenotypes.

## 22 23 **Proteomics analyses of developing wild-type and *acbd6* crispant** 24 **zebrafish**

25 To shed light on the potential role of *acbd6* on *N*-myristoylation during zebrafish development,  
26 we employed metabolic labelling with YnMyr and chemical proteomics at different stages of  
27 development. At 72 and 120 hpf, both wild-type and *acbd6* crispant zebrafish express >32  
28 significantly enriched proteins, for each of which the human orthologue is a validated co-  
29 translationally *N*-myristoylated substrate (Supplementary Figs. 19A-D). In addition, significantly

1 enriched proteins also included >48 proteins with N-terminal glycine, thereby potentially *N*-  
2 myristoylated, but where unequivocal evidence of *N*-myristoylation is currently lacking the  
3 human orthologue, or where the human orthologue does not possess an N-terminal glycine. At 72  
4 hpf, an increased abundance of YnMyr-labelled proteins is observed in the *acbd6* crispant,  
5 including Marcks and Chchd-related proteins, known to be co-translationally *N*-myristoylated in  
6 humans (Fig. 7A). Of note, zebrafish express duplications of multiple proteins, including the  
7 aforementioned Marcks and Chchd-related proteins (Fig. 7A). Conversely, enrichment of several  
8 zebrafish proteins with N-terminal glycine in zebrafish but not found with a N-terminal glycine  
9 in humans was significantly reduced in *acbd6* crispants, including Sfpq, associated with brain  
10 development, CNS neuron axonogenesis and midbrain-hindbrain boundary initiation (Fig. 7A).  
11 Similar increases in Marcks and Chchd-related proteins are observed in the *acbd6* crispants at  
12 120 hpf, whereas Fus and Fusl are further decreased (Fig. 7B).

13 We next performed a whole proteomics analysis comparing wild-type and *acbd6* crispant at 72  
14 and 120 hpf (Supplementary Figs. 19E, F, respectively). In contrast to the significant increase of  
15 YnMyr labelling observed in *acbd6* crispants (Figure 7A, B), the abundance of *N*-myristoylated  
16 proteins such as Marcks and Chchd-related proteins is not significantly increased at the whole  
17 proteome level at 72 and 120 hpf, further indicating the role of *acbd6* on the process of *N*-  
18 myristoylation in zebrafish. Meta-analysis of up-regulated proteins in *acbd6* crispants revealed a  
19 significant involvement of translation- and metabolism-related pathways (Supplementary Fig.  
20 20A), and significant enrichments in disease networks including frontotemporal dementia and  
21 delayed speech and language development (Supplementary Fig. 20B). Down-regulated proteins  
22 are significantly involved in pathways related to nervous system development, as well as  
23 neurodegeneration (Fig. 7C). Enriched disease-related networks include dystonia muscle  
24 spasticity and movement disorders, a striking similarity with the observations in affected  
25 individuals (Supplementary Fig. 20C). At 120 hpf, a meta-analysis of the whole proteome data  
26 reveals similar enrichments (Supplementary Fig. 20D-G). Notably, upregulated proteins enrich  
27 in translation- and metabolism-related pathways, whereas downregulated proteins further enrich  
28 in pathways of neurological development and disease, including spasticity (Supplementary Fig.  
29 20G).

30

## 1 **Proteomics analyses of developing wild-type and *acbd6* crispant *X.*** 2 ***tropicalis***

3 Similar to the chemical proteomics analyses in zebrafish, we used YnMyr labelling to identify *N*-  
4 myristoylated substrates and the pathways affected by the loss of *acbd6* in developing *Xenopus*.  
5 Here, wild-type, crispant 68 or crispant 71 *X. tropicalis* were metabolically labelled from 1 hpf to  
6 18 hpf, due to the previously reported growth arrest. Chemical proteomics of YnMyr labelling  
7 revealed >10 *X. tropicalis* proteins the human orthologues of which are known to be co-  
8 translationally *N*-myristoylated, including a duplication of Marcks, as well as *X. tropicalis*  
9 proteins which share the N-terminal glycine with their orthologue in humans (Supplementary  
10 Fig. 21A-C). Comparing wild-type with *acbd6* crispant 68 and 71 (Figs. 7D and E, respectively)  
11 reveals a marked depletion of YnMyr-labelled proteins in the crispants, including all identified  
12 *X. tropicalis* proteins with co-translationally *N*-myristoylated human orthologues. Both crispants  
13 reveal prominent and significant reductions in proteins including Marcks, Ppm1b, Ppm1g. Whole  
14 proteome analysis revealed Marcks and Fus are markedly reduced in *acbd6* crispant 68, while  
15 Ppm1a is slightly increased, and in *acbd6* crispant 71, both Marcks and Ppm1b are reduced  
16 (Supplementary Figs 21D, E). Meta-analysis (Supplementary Fig. 22) revealed upregulated  
17 proteins in crispants 68 and 71 are significantly affected in pathways of translation and  
18 metabolism, and notably, the 'Parkinson-disease'-specific disease network was significantly  
19 enriched (Fig. 7F).

## 20 **Discussion**

### 21 **A neurodevelopmental syndrome with progressive movement** 22 **disorders characterizes *ACBD6*-related disease**

23 Despite their predicted roles in cellular lipid metabolism, the functions of many of the ACBDs  
24 still remain unclear, as is the consequence of ACBD protein defects on human pathophysiology.  
25 In this study, we identified novel and ultra-rare bi-allelic predicted LOF variants in *ACBD6* as  
26 the disease cause in 45 previously undiagnosed individuals from 28 unrelated families. A wide  
27 age range of the cohort members (1-50 years) delineated the age-related clinical spectrum and  
28 the natural history of the *ACBD6*-related disease. The disease had an invariably early-onset and

1 inevitably progressive course with significant motor and cognitive deterioration upon reaching  
2 adulthood, a course suggestive of underlying neurodegeneration. The phenotype is complex  
3 involving the constellation of extrapyramidal, pyramidal, and cerebellar ataxia symptoms  
4 associated with GDD/ID, microcephaly, and variable epilepsy. Impaired expressive language,  
5 delayed gait acquisition, and early-onset stooped posture with lateral trunk flexion (Pisa  
6 syndrome) were among the important pathognomonic features. Additionally, tics and tic-like  
7 vocalizations seen in 6 affected individuals are peculiar features associated with several  
8 neurological disorders, particularly with chorea-acanthocytosis.<sup>36</sup> Whilst the present cohort of  
9 patients with *ACBD6*-related disease did have facial dysmorphism, it did not suggest a  
10 recognizable facial "gestalt". The most common dysmorphic feature in the cohort was a broad  
11 chin. Of note, most of the patients had hypoplasia/agenesis of the corpus callosum and anterior  
12 commissure, suggesting a potential role for *ACBD6* in axonal pathfinding and corpus callosum  
13 development. Interestingly, claval hypertrophy observed in the present cohort is a well-  
14 documented neuroradiological sign of *PLA2G6*-associated neurodegeneration.<sup>37</sup>

15 Defects in numerous genes and pathways are known to present with the constellation of  
16 symptoms observed in *ACBD6*-related disease, particularly, with various combinations of  
17 dystonia, parkinsonism, ataxia, and spasticity.<sup>38-41</sup> Their clinical phenotypes are typically  
18 classified according to the predominating symptom; however, a later approach tends to define  
19 this with a spectrum of genetic dystonia-ataxia, parkinsonism-dystonia/ataxia, and ataxia-  
20 spasticity syndromes.<sup>38-41</sup> Thus, several forms of complicated hereditary spastic paraplegia,  
21 spastic ataxia, and young-onset dystonia-parkinsonism syndromes may overlap with *ACBD6*  
22 phenotypes. A suggested differential diagnosis with the disease pathways involved is given in  
23 Supplementary Table 7.

24 It has been suggested that neurodevelopmental abnormalities and neurodegeneration could share  
25 several molecular and cellular mechanisms. For instance, proteins, such as A $\beta$ , MAPT/tau, Rac1,  
26 Progranulin, Huntingtin, PINK, and Parkin, frequently implicated in Alzheimer's disease,  
27 Parkinson's disease, and Huntington's diseases are important for nervous system development.<sup>42</sup>  
28 A wide range of multisystem genetic disorders could present with a biphasic course where  
29 complex neurological phenotype gradually evolves on the background of a pre-existing  
30 neurodevelopmental disorder.<sup>43,44</sup> Therefore, we propose a likely clinical continuum associated



1 with *ACBD6*-related disease, characterized by a combination of neurodevelopmental  
2 abnormalities and neurodegeneration.

3

## 4 **Zebrafish *acbd6* knockouts recapitulate many features observed in** 5 **affected individuals**

6 In recent years, the zebrafish has gained significance as an animal model for investigating  
7 neurodevelopmental disorders, owing to its high physiological similarity to humans and its  
8 responsiveness to genetic and pharmacological interventions.<sup>45,46</sup> The acyl-CoA binding domain  
9 of human and zebrafish ACBD6 share significant identity and similarity in the ankyrin-repeat  
10 motif and acyl-CoA binding domain, with 80% identity and 95.4% similarity in the former and  
11 69.2% identity and 78.2% similarity in the latter. Zebrafish models for ACBD proteins do not  
12 currently exist; however, by generating *acbd6* knockouts in zebrafish, we observed similar  
13 phenotypes to those of affected individuals, such as movement disorders, seizures, and facial  
14 dysmorphism. Our findings suggest that *acbd6* is critical for animal development, as the loss of  
15 this protein results in severe global developmental delay and increased mortality over time, as  
16 evidenced by stunted growth and severe brain development impairment by 30 dpf. Moreover, we  
17 observed motor neuron over-branching during development and progressive muscle loss,  
18 suggesting a combination of muscle and neuronal degeneration leading to movement  
19 abnormalities. Furthermore, the knockout zebrafish demonstrated increased locomotor behaviour  
20 in the dark, potentially indicating seizure or anxiety-like behaviour, similar to that seen in  
21 affected patients. In summary, the *Acbd6* zebrafish models offer a promising tool for gaining  
22 deeper mechanistic insights into the role of *acbd6* and for screening potential therapeutic  
23 interventions, as zebrafish are ideal for high-throughput *in vivo* drug screening. As such, the  
24 *acbd6* model represents a valuable resource for drug discovery research.

25

26

1 ***X. tropicalis acbd6* knockouts have gastrulation failure, brain**  
 2 **defects, and reduced locomotion**

3 *X. tropicalis* is a diploid clawed frog with a genome that contains over 80% of identified human  
 4 disease genes and importantly is syntenic with over two-thirds of the human genome.<sup>47</sup> Features  
 5 of this animal include rapid external development and a transparent tadpole. A deep  
 6 understanding of *Xenopus* biology throughout the last 70 years,<sup>48</sup> combined with ease of genetic  
 7 manipulation<sup>49</sup>, makes it powerful for modeling disease genes<sup>50,51</sup> and understanding genetic  
 8 disease mechanisms.<sup>52-55</sup>

9 In the present study, the inactivation of *acbd6* in *X. tropicalis* caused severe cell movement  
 10 failures during gastrulation. Marcks requires *N*-myristoylation to act in early development<sup>56</sup> and  
 11 is required for normal cell movement during gastrulation in frog.<sup>57</sup> It is interesting to speculate  
 12 that the loss of *acbd6* may cause gastrulation defects due to a loss of Marcks *N*-myristoylation;  
 13 an aspect that requires future work. Due to embryo deaths at gastrula stages, a caveat for the  
 14 interpretation of the subsequent phenotype analysis is the unusually high mosaicism of these  
 15 crisant tadpoles. Despite this, there were clear phenotypic differences between the control and  
 16 crisant tadpoles. In some cases, the change predominated in one-half of the tadpole due to  
 17 mosaicism (on the L-R axis, see for example Fig. 5G second image from the left). These  
 18 differences included microcephaly, reduced movement, eye abnormalities, and brain structure  
 19 differences, data that strengthen the link between *ACBD6* variants and the pathology observed in  
 20 the patient cohort.

21  
 22 ***ACBD5* and *ACBD6* have different cellular localizations but both**  
 23 **genes exhibit a neurodegenerative phenotype**

24 *ACBD5* is a peroxisomal membrane protein. Although current knowledge of the phenotype  
 25 associated with *ACBD5* defects is limited to only four reported families,<sup>4-7</sup> similar to *ACBD6*,  
 26 individuals with defective *ACBD5* seem to exhibit a neurodegenerative disease, albeit with a  
 27 different range of associated symptoms. Our study on peroxisome morphology and function with  
 28 *ACBD6* deficient and control fibroblasts did not reveal significant alterations as has been

1 demonstrated in ACBD5 deficiency. Furthermore, Myc-ACBD6 did not localize to peroxisomes  
2 when expressed in COS-7 cells.

3

## 4 ***ACBD6* deficiency alters myristate probe YnMyr incorporation into** 5 **substrates of NMT**

6 Our chemical proteomics analysis comparing *N*-myristoylated proteins in fibroblasts, derived  
7 from a patient and their healthy sibling, revealed significant differences in YnMyr-labelled  
8 proteins. YnMyr incorporation was significantly higher in the *ACBD6*-deficient patient  
9 fibroblasts, spanning 68 known co- and 18 post-translationally *N*-myristoylated proteins.  
10 Interestingly, an apparent differential variation of YnMyr incorporation in the identified NMT  
11 substrates between healthy and patient fibroblasts was also observed. Our investigation in  
12 *ACBD6* deficient fibroblasts might indicate its role in *N*-myristoylation of a subset of NMT  
13 substrates, but further studies are required to investigate this putative role of *ACBD6*.  
14 Furthermore, to study the effect of *ACBD6* deficiency on *N*-myristoylation, future analyses will  
15 need to focus on cell types likely directly impacting the pathways involved in *e.g.* neurological  
16 development.

17 Both the zebrafish and *X. tropicalis* model systems recapitulate many of the identified human  
18 phenotypes, including movement disorders, seizures, facial dysmorphology, and developmental  
19 defects. This evidence for notable evolutionary conservation of *ACBD6* function across  
20 mammals, amphibia, and teleosts emphasizes the power of multiple, non-mammalian models as  
21 a method for rapid and cost-effective human gene analysis. Through our chemical proteomics  
22 analysis, coupled with whole proteome and meta-analysis, we identified that *acbd6*-deficiency in  
23 developing zebrafish embryos results in a prominent increase in metabolic labelling of known  
24 co- and post-translationally *N*-myristoylated proteins with YnMyr, suggesting *N*-myristoylation  
25 is dependent on a direct or indirect interaction between *acbd6* and the *N*-myristoyltransferases.  
26 Meta-analysis furthermore revealed that *acbd6* deficiency provokes an overall increase of  
27 proteins *N*-myristoylated with YnMyr, with a subset involved in the development of the eye,  
28 neuron and muscle, being reduced. Overall, this suggests that *ACBD6* is involved in the *N*-  
29 myristoylation of a subset of *N*-myristoyltransferase substrates that require modification, and

1 might indicate activation of a rescue mechanism in response to loss of *ACBD6*. Concomitantly,  
2 potentially compromised myristic acid-CoA binding and shuttling to *N*-myristoyltransferases in  
3 *ACBD6*-deficient cells may upregulate salvage pathways to increase cellular myristate  
4 concentration. For example, acyl-CoA synthases can activate exogenous lipids directly,  
5 potentially bypassing or compensating loss of *ACBD6*. In this case, YnMyr-CoA could more  
6 effectively compete with endogenous myristic acid-CoA for protein *N*-myristoylation than in  
7 healthy subjects, leading to the observed enhanced YnMyr labelling, suggesting exogenous  
8 myristic acid supplementation through dietary supplementation might have the potential to  
9 reduce the burden of *ACBD6* deficiency. The determined differences in protein *N*-myristoylation  
10 between the skin fibroblasts derived from a single patient and unaffected sibling align with the  
11 YnMyr chemical proteomics findings from our zebrafish study. Consequently, the YnMyr  
12 chemical proteomics data of the human patient and unaffected fibroblast serves only as  
13 qualitative confirmatory evidence of the zebrafish study. For this purpose, we did not seek  
14 further replication of these findings across multiple patient cell lines.

15 In contrast to *ACBD6* deficient human fibroblasts and zebrafish crispants, the *acbd6 X. tropicalis*  
16 crispants show a prominent and significant reduction of YnMyr labelling, including proteins  
17 such as Marcks, Chchd, Ppm1b, Ppm1g, and Fus, which are significantly involved in  
18 development. Meta-analysis indicates the upregulation of neurological and sight-related disease  
19 networks upon *acbd6* loss. The significant effect of *acbd6* loss on *X. tropicalis* development at  
20 the gastrula stage precludes a clear interpretation of the effect of *acbd6* knockout on *N*-  
21 myristoylation due to the presence of confounding changes in other processes during the period  
22 of metabolic labelling. The clearly non-viable state of the *X. tropicalis* embryos likely leads to  
23 defects in lipid metabolism, protein translation and related processes over this period which go  
24 beyond the direct impact of *acbd6* on cells.

25 In summary, we have shown that bi-allelic pathogenic variants in *ACBD6* are associated with a  
26 new and distinct neurodevelopmental disease with a complex and progressive dystonia-  
27 parkinsonism-ataxia phenotype. Zebrafish and *X. tropicalis* crispants recapitulate the main  
28 clinical features of the cohort with affected pathways underlying translation, metabolism,  
29 neurological development, and neurological diseases. Further studies are warranted to delineate  
30 the clinical phenotype and understand the pathomorphological presentation and molecular  
31 mechanisms of the *ACBD6*-related disease. This includes elucidating the molecular interplay

1 between ACBD6 and *N*-myristoyltransferases involved in the co- and post-translational  
2 modification of nascent protein chains, how ACBD6 affects lipid metabolism and the  
3 identification of treatments of ACBD6 deficiency.

4

## 5 **Data availability**

6 The data that support the findings of this study are available from the corresponding author, upon  
7 reasonable request. The mass spectrometry proteomics data have been deposited to the  
8 ProteomeXchange Consortium via the PRIDE<sup>24</sup> partner repository with the dataset identifiers  
9 PXD024957 (YnMyr chemical proteomics in human cells), PXD043676 (YnMyr chemical  
10 proteomics in zebrafish), PXD043679 (zebrafish whole proteome), PXD043677 (YnMyr  
11 chemical proteomics in *X. tropicalis*) and PXD043680 (*X. tropicalis* whole proteome).

12

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14

## 15 **Competing interests**

16 E.W.T. is a director and shareholder of Myricx Pharma Ltd, and an inventor on a patent  
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18 RJ; Hutton, JA; Brannigan, JA, "Compounds and their use as inhibitors of *N*-myristoyl  
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20 J.R.L. holds stock in 23andMe and is a consultant for Genome International. Y.J. is an employee  
21 of Novo Nordisk.

22

## 23 **Supplementary material**

24 Supplementary material is available at *Brain* online.

25

26

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28

## 1 **Figure legends**

2 **Figure 1 Family pedigrees, schematic variants' representation, conserved regions of**  
 3 **substitution variants in *ACBD6*, and splicing effects. (A)** Pedigrees and segregation results for  
 4 the 28 unrelated families. Double lines between individuals represent consanguinity. The 45  
 5 affected individuals recruited for the study are shaded and indicated with their respective subject  
 6 (S) number (S1, S2 or S3). The segregation data for all individuals tested via Sanger sequencing is  
 7 shown with the presence of the *ACBD6* variant (red) and/or the reference allele (black), two  
 8 red/black texts indicate a homozygous state, and one red + one black text indicates a heterozygous  
 9 state. The genotyping is based on the coding DNA sequence. WT refers to wild type, DEL refers  
 10 to deletion and DUP refers to duplication. **(B)** Schematic representation of the gene and protein  
 11 positions of detected variants in *ACBD6*. *ACBD6* is located on chromosome 1 at cytogenetic  
 12 position q25.2q25.3 (upper panel). The middle panel shows the genetic variants mapped to the  
 13 NM\_032360.4 transcript of *ACBD6*. The lower panel shows *ACBD6* variants mapped on the  
 14 protein level. Three variants including p.(Gly22fs), p.(Leu121ThrfsTer27), and a 30kb deletion in  
 15 the C-terminus have been reported previously.<sup>6, 7,10</sup> Recurrent variants are labelled by family  
 16 codes. **(C)** Splicing schematic for the c.574-2A>G variant in *ACBD6* showing cryptic acceptor  
 17 splice site activation in exon 6 (upper panel). The c.664-2A>G (middle panel) and c.694+1G>A  
 18 (lower panel) variants affect splicing of exon 7 both show exon skipping. Additionally, the  
 19 c.694+1G>A variant activates a cryptic donor splice site. **(D)** Interspecies alignment performed  
 20 with Clustal Omega showing the complete conservation down to invertebrates of the amino acid  
 21 residues affected by a missense variant leading to an amino acid substitution p.(Asp201Gly) and  
 22 an in-frame duplication p.(Asn219dup).

23  
 24 **Figure 2 Clinical features of the affected individuals with bi-allelic *ACBD6* variants. (A) i-**  
 25 **xiii:** Representative photographs demonstrating facial features of the affected individuals in  
 26 adulthood. F1:S1 (i), F1:S2 (ii), F1:S3 (iii), F3:S2 (iv), F3:S1 (v), F6:S1 (vi), F7:S2 (vii), F7:S1  
 27 (viii), F11:S1 (ix), F17:S1 (x), F17:S2 (xi), F20:S1 (xii), F16:S1 (xiii). xiv-xxxi: Representative  
 28 photographs demonstrating facial features of the affected individuals in childhood. F3:S2 (xiv) at  
 29 5 years old, F3:S1 (xv) at 12 years old, F6:S2 (xvi), F8:S1 (xvii), F8:S2 (xviii), F10:S1 (xix),  
 30 F10:S2 (xx), and F11:S1 (xxi) at younger age, F13:S1 (xxii), F14:S1 (xxiii), F15:S1 (xxiv),

1 F16:S1 (xxv) at 2 years old, F16:S1(xxvi) at 4 years old, F19:S1 (xxvii), F19:S2 (xxviii), F20:S2  
 2 (xxix), F12:S1 (xxx), F12:S2 (xxxi). The most frequently seen facial dysmorphologies in adults  
 3 are high nasal ridge, full nasal tip, small mouth, thin upper lip, and broad chin. The most  
 4 frequently seen facial dysmorphologies in children are bi-frontal/bi-temporal narrowing, arched  
 5 eyebrows, hypertelorism, up-slanting palpebral fissures, depressed nasal bridge, full nasal tip,  
 6 thin upper lip, full lower lip, and broad chin. **(B)** Representative photographs demonstrating  
 7 postural abnormalities seen in the affected individuals. A stooped body posture and lateral  
 8 flexion of the trunk can be seen in the individuals including F1:S1 (i), F1:S2 (ii), F1:S3 (iii),  
 9 F3:S1 (iv), F3:S2 (v), F6:S1 (vi), F6:S2 (vii), and F8:S2 (viii), F13:S1 (ix). **(C)** Bar graph  
 10 summarizing proportions of various clinical findings in the *ACBD6* cohort. Blue – affected,  
 11 orange – unaffected, grey – not ascertained/not applicable. GDD, Global developmental delay.  
 12 ID, Intellectual disability. **(D)** Representative neuroimaging features of the affected individuals.  
 13 Brain MRI, midline sagittal images of the affected individuals F1:S1 (i), F1:S2 (ii), F5:S1(iii),  
 14 F56:S2 (iv), F7:S1 (v), F7:S2 (vi), F10:S1 (vii), F10:S2 (viii), F11:S1 (ix), F13:S1 (x), F14:S1  
 15 (xi), and F19:S1 (xii). Most of the affected individuals have corpus callosum agenesis or  
 16 hypoplasia with prevalent involvement of the posterior sections (empty arrows), variably  
 17 associated with short midbrain (thin arrows) and small inferior cerebellar vermis (arrowheads).  
 18 In addition, mild hypertrophy of the clava was noted in some subjects (dotted arrows). Note that  
 19 the anterior commissure was markedly hypoplastic or absent in all affected individuals.

20  
 21 **Figure 3 CRISPR/Cas9 mutation of zebrafish *acbd6* causes smaller eyes, impaired vision,**  
 22 **abnormal locomotion, developmental delay, and increased mortality. (A)** Whole-mount *in*  
 23 *situ* hybridization for detecting *acbd6* mRNA expression pattern in zebrafish embryo at 24 hpf.  
 24 Forebrain (fb), midbrain (mb), midbrain and hindbrain boundary (MHB), hindbrain (hb), otic  
 25 vesicle (ov). Dorsal view, anterior to the left. **(B)** Representative images of wild-type  
 26 (*acbd6*+/+), heterozygous (*acbd6*+/-) and homozygous (*acbd6*-/-) mutant larva at 6 dpf. Head  
 27 size and eye size are indicated by blue and red lines, respectively. Anterior to the left and dorsal  
 28 to the top. Scale bar = 200  $\mu$ m. **(C, D)** Quantification of eye and head size as indicated in Fig.  
 29 3B. +/+ (n = 26 larvae), +/- (n = 114 larvae) and -/- (n = 47 larvae). Each symbol represents one  
 30 larva. Values are calculated as a percentage of the mean value of +/+ larvae. Error bars = mean  $\pm$

1 standard deviation (SD). **(E)** The result of VSR analysis performed on  $+/+$  ( $n = 43$  larvae),  $+/-$  ( $n$   
2  $= 99$  larvae), and  $-/-$  ( $n = 48$  larvae) zebrafish larvae at 6 dpf. Each symbol represents one larva.  
3 The number of responses for 5 stimuli of each larva is calculated as a percentage of responses.  
4 Error bars = mean  $\pm$  standard error of the mean (SEM). **(F)** Locomotor activities of zebrafish  
5 larvae in light and dark periods at 6 dpf.  $+/+$  ( $n = 42$  larvae),  $+/-$  ( $n = 99$  larvae), and  $-/-$  ( $n = 48$   
6 larvae) zebrafish larvae were habituated in the dark for 30 minutes, followed by three cycles of  
7 10-minute time bins of light and dark periods. Error bars = mean  $\pm$  SEM. The dark period (D)  
8 and light period (L) are labelled. Black arrows indicate the increased movement of homozygous  
9 mutants at the first minute in the dark. **(G)** Average cumulative distance traveled of each larva  
10 from three cycles of either light or dark periods in Fig. 3F. Error bars = mean  $\pm$  SD. **(H)** Average  
11 cumulative distance traveled of each larva during the first minute of the dark period across three  
12 cycles as indicated by black arrows in Fig. 3F. Error bars = mean  $\pm$  SD. **(I)** Locomotor activities  
13 of zebrafish larvae in light and dark conditions at 12 dpf.  $+/+$  ( $n = 39$  larvae),  $+/-$  ( $n = 71$  larvae)  
14 and  $-/-$  ( $n = 29$  larvae). Error bars = mean  $\pm$  SEM. Red arrow indicates increased movement of  
15 homozygous mutants at the first minute after light on. Red arrowhead indicates increased  
16 movement of homozygous mutants at the second minute after light on. **(J)** Average cumulative  
17 distance traveled by each larva during three cycles of either light or dark periods in Fig. 3I. Error  
18 bars = mean  $\pm$  SD. **(K)** Average cumulative distance traveled by each larva during the first cycle  
19 of the first minute of the light period as indicated by red arrow in Fig. 3I. Error bars = mean  $\pm$   
20 SD. **(L)** Genotyping results of zebrafish at 6 dpf ( $n = 191$  larvae), 12 dpf ( $n = 196$  larvae) and 30  
21 dpf ( $n = 118$  juveniles) stages from *acbd6* $+/+$  intercross. **(M)** Representative images of  
22 morphological phenotype from *acbd6* $+/+$ , *acbd6* $+/-$  and *acbd6* $-/-$  at 30 dpf. Anterior to the left  
23 and dorsal to the top. **(N)** Sagittal section of *acbd6* $+/+$  brain at 30 dpf. Anterior to the left and  
24 dorsal to the top. Olfactory bulb (Ob), periventricular gray zone of optic tectum (PGZ), medulla  
25 oblongata (MO). **(O-W)** Representative images of transverse sections of telencephalon (O-Q),  
26 optic tectum (R-T) and cerebellum (U-W) from *acbd6* $+/+$ , *acbd6* $+/-$  and *acbd6* $-/-$  juvenile as  
27 indicated in Fig. 3N. In (C, D), one-way ANOVA with Tukey's multiple comparisons test. In (E,  
28 G, H, J, K), one-way ANOVA with Dunnett's T3 multiple comparisons test. ns, not significant  $p$   
29  $\geq 0.05$ ,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$  and  $****p < 0.0001$ .

30



1 **Figure 4 Zebrafish *acbd6* F<sub>0</sub> knockouts exhibit increased susceptibility to chemical-induced**  
2 **seizures, excessive motor neuron branching, and skeletal muscle degeneration. (A)**  
3 Representative images of uninjected control and *acbd6* F<sub>0</sub> larvae at 6 dpf. Left column, ventral  
4 view, anterior to the left. Right column, dorsal view, anterior to the left. Blue line indicates head  
5 size, magenta line indicates eye size, and cyan line indicates eye distance. Scale bar = 0.2 mm.  
6 **(B, C)** Quantification of the head and eye size (n = 30 larvae for each group) of uninjected  
7 control ( uninj.), *acbd6* F<sub>0</sub> knockout (F<sub>0</sub>), F<sub>0</sub> + zebrafish wild-type *acbd6* mRNA (+*acbd6*), and F<sub>0</sub>  
8 + human wild-type *ACBD6* mRNA (+*ACBD6*) as indicate in Fig. 4B. **(D)** The VSR analysis  
9 after mRNA rescue at 6 dpf. n = 36 larvae for each group. Each symbol represents one larva. The  
10 number of responses to 5 stimuli of each larva was calculated as a percentage of responses. Error  
11 bars = mean ± SEM. **(E)** Quantification of the eyes distance (n = 20 larvae for each group) as  
12 indicate in Fig. 4B. **(F)** Quantification of the eye size of F<sub>0</sub> knockout rescued with mRNA of  
13 human p.Glu63Ter (+E63\*) or p.Asp201Gly (+D201G) variant. n = 25 larvae for each group.  
14 **(G)** Locomotor activities of zebrafish larvae in light and dark conditions at 6 dpf. n = 64 larvae  
15 for each group. The larvae were habituated in the dark for 30 minutes, followed by three cycles  
16 of 10-minute periods of light and dark. Error bars represent the mean ± SEM. Dark period (D),  
17 light period (L). Red arrows indicating increased movement of F<sub>0</sub> one minute after light on, and  
18 black arrows indicate increased movement one minute after light off. **(H)** Average cumulative  
19 distance traveled by each larva during three cycles of either light or dark periods in Fig. 4G.  
20 Error bars = mean ± SD. **(I)** The average cumulative distance traveled by each larva during the  
21 first minute of the dark period was measured over three cycles, as shown by the black arrow in  
22 Fig. 4G. Error bars represent the mean ± SD. **(J)** The average cumulative distance traveled by the  
23 larvae was measured for each group after being treated with different doses of pentylenetetrazole  
24 (PTZ) at 5 dpf. n = 16 larvae for each group. **(K-N)** Confocal images of  
25 *Tg(mnx1:GFP;olig2:DsRed)* larva at 12 dpf are shown, with transgenic larvae injected with  
26 *slc45a2* sgRNA used as a control and those injected with *acbd6* + *slc45a2* sgRNAs shown as  
27 *acbd6* F<sub>0</sub>. (L' and N') Enlarged images from white boxes are shown in L' and N', with red  
28 asterisks indicating autofluorescence from remaining pigment cells. GFP and DsRed are  
29 displayed in cyan and magenta, respectively, with magenta arrowheads indicating excess axonal  
30 arborizations. The images are presented in a lateral view, with anterior to the left and dorsal to  
31 the top. Additional motor neuron phenotypes at 6 and 12 dpf can be found in Supplementary Fig.

1 13. **(O-T)** Confocal images of stained skeletal muscle fibers with phalloidin are presented,  
 2 including images from *slc45a2* sgRNA-injected control (O-Q) and *acbd6* + *slc45a2* sgRNAs-  
 3 injected (R-S) larvae at 12 dpf. Orthogonal views generated from (P and S) using the Orthogonal  
 4 views tool in ImageJ are also displayed. Degenerated muscles are indicated by (#), while white  
 5 arrowheads and a white arrow indicate Z-discs and the thickness of the myotube, respectively.  
 6 Supplementary Fig. 14 provides additional muscle phenotypes at 6 and 12 dpf. In (B-D and F),  
 7 one-way ANOVA with Tukey's multiple comparisons test. In (E and H-J), one-way ANOVA  
 8 with Dunnett's T3 multiple comparisons test. ns, not significant  $p \geq 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ ,  
 9 \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$ .

10

11 **Figure 5 *acbd6* *X. tropicalis* crispants have gastrulation, movement, craniofacial, brain and**  
 12 **eye defects together with microcephaly. (A)** The gene structure of human (*ACBD6*) and *X*  
 13 *.tropicalis* (*acbd6*) reveals 8 exons. **(B)** Gastrulation defects, including failure of blastopore  
 14 closure and anterior posterior defects, were observed in F<sub>0</sub> *X. tropicalis* embryos injected with  
 15 two different CRISPR/Cas9 constructs (sgRNA-68 and sgRNA-71) disrupting exon 1 of *acbd6*.  
 16 **(C)** Those animals surviving to free-feeding stages presented with microcephaly, craniofacial  
 17 dysmorphism and eye abnormalities. **(D)** The differences in head size between the uninjected  
 18 control (2.07 +/- 0.36mm) and *acbd6* crispant tadpoles (1.52 +/- 0.27mm, sgRNA-68) were  
 19 found to be significant,  $t(34)=5.183$ ,  $p<0.001$ . **(E-F)** Alcian Blue staining marking the  
 20 cartilaginous structures in the head and neck show equivalent structures between control **(E)** and  
 21 *acbd6* crispant tadpoles **(F)** revealing no gross morphological abnormalities. **(G)** Detailed  
 22 structural analysis in higher resolution MicroCT imaging (1% phosphotungstic acid contrast  
 23 stain) revealed significant structural abnormalities in the facial musculature (Red arrows, G),  
 24 abnormalities of the eye (microphthalmia, anophthalmia – Yellow arrows, G) and structural  
 25 abnormalities in the brain most pronounced in the midbrain regions (Blue arrow, G). **(H)**  
 26 Locomotion analysis at NF44/45 revealed that crispants moved significantly less than control  
 27 tadpoles. **(I)** The Kaplan-Meier survival analysis of 65 control and crispant tadpoles shows two  
 28 periods of crispant-specific decline, the first at gastrula stages (day 0-1) and the second with  
 29 post-feeding (day 8, NF stage 47).

30

1 **Figure 6 Morphological characteristics of peroxisomes in *ACBD6*-deficient patient cells are**  
 2 **not altered and chemical proteomic profiling of *N*-myristoylation in human fibroblasts. (A)**  
 3 Patient fibroblasts and controls were processed for immunofluorescence microscopy using  
 4 antibodies against the peroxisomal membrane marker PEX14, the matrix marker catalase, or  
 5 mitochondrial ATP synthetase B (ATPB). Peroxisomal localisation of PEX14 and catalase  
 6 indicate that peroxisomal membrane and PTS1-dependent matrix import are normal. Note that  
 7 the morphology of mitochondria, which are elongated in fibroblasts, was also not altered when  
 8 compared to controls. **(B)** Quantification of peroxisome number based on immunofluorescence  
 9 images (see A for representative images) (n = 29-36 cells). Data are from three independent  
 10 experiments. ns, not significant; Kruskal-Wallis ANOVA test with Dunn's multiple  
 11 comparisons. **(C)** COS-7 cells were transfected with plasmids encoding Myc-ACBD5 or Myc-  
 12 ACBD6 and processed for immunofluorescence microscopy using antibodies against Myc and  
 13 PEX14. Note that Myc-ACBD5 localises to peroxisomes, whereas Myc-ACBD6 localises to the  
 14 nucleus and the cytoplasm in COS-7 cells. Scale bars, 10  $\mu$ m. **(D)** Ranked plot of YnMyr-  
 15 labelled, known co- and post-translationally *N*-myristoylated proteins, as identified in  
 16 (Supplementary Figure 18E). Position on the left equals lower abundance in *ACBD6* deficient  
 17 fibroblasts, position on the right equals higher abundance in *ACBD6* deficient fibroblasts.

18  
 19 **Figure 7 Chemical and whole proteome analysis of *acbd6* wild-type and crisprant zebrafish**  
 20 **and *X. tropicalis* model systems. (A)** Volcano plot comparing YnMyr labelling of proteins in  
 21 wild-type and *acbd6* crisprant zebrafish at 72 hpf. Horizontal dotted line: significance threshold  
 22 (p-value = 0.05). FC: fold-change. Position on the left equals reduced in crisprant, position on the  
 23 right equals increased in crisprant. **(B)** Comparing YnMyr labelling of proteins in wild-type and  
 24 *acbd6* crisprant zebrafish at 120 hpf. Further description as in (A). **(C)** Top-20 of biological  
 25 processes most significantly enriched in proteins down-regulated in *acbd6* crisprant zebrafish at  
 26 72 hpf. The most significantly enriched process at the top. Colour indicates Q-value as secondary  
 27 significance indicator. Size of circles indicates a number of proteins enriched in the depicted  
 28 process. Dr: *Danio rerio*. Hs: *Homo sapiens*. **(D)** Volcano plot comparing YnMyr labelling of  
 29 proteins in wild-type and *acbd6* crisprant 68 *X. tropicalis*. Horizontal dotted line: significance  
 30 threshold (p-value = 0.05). FC: fold-change. Position on the left equals reduced in crisprant,  
 31 position on the right equals increased in crisprant. **(E)** Comparing YnMyr labelling of proteins in

1 wild-type and *acbd6* crisprant 71 *X. tropicalis*. Further description as in (D). (F) Top-20 of  
2 biological processes most significantly enriched in proteins up-regulated in *acbd6* crisprant 68 *X.*  
3 *tropicalis*. Most significantly enriched processes are at the top. Colour indicates Q-value as  
4 secondary significance indicator. Size of circles indicates a number of proteins enriched in the  
5 depicted process. Xt: *Xenopus tropicalis*. Hs: *Homo sapiens*.

6

7

8

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**1 Table I Main clinical features of the affected individuals with homozygous ACBD6 variants**

Family ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
N of affected	3	1	2	1	2	2	2	2	1	2	1	2	1	1	1	1	2	1	2	2	1	2	2	1	1	2	2	2
GDD/ID	3+	+	2+	+	2+	2+	2+	2+	+	2+	+	2+	+	+	+	+	2+	+	2+	2+	+	2+	2+	+	+	2+	2+	2+
Progressive disease course	3+	X	2+	X	2+	2+	2+	2+	X	2+	+	2X	+	+	+	X	2+	X	2X	2X	+	2X	2+	+	X	2X	2+	2+
Microcephaly	1+	-	2-	+	2-	1+	1+	2+	X	2-	-	2-	+	-	-	+	2X	-	2+	2X	-	2X	2-	+	X	2X	1+	2X
Short stature	3-	+	1+	X	2-	2+	2+	2+	-	2-	+	2-	+	+	-	X	2X	X	2-	2+	+	2X	1- = 1X	+	X	2X	2X	2X
Facial dysmorphism	3+	+	2+	X	2+	2+	2+	2+	+	2+	+	2+	+	+	+	+	2+	X	2+	2+	+	2X	2-	+	X	2X	2+	2+
Oculomotor abnormalities	3X	X	2+		2+	2+	2X	1+	X	2+	-	2+	+	-	+	+	2X	+	2-	1+	+	2X	2-	X	X	2X	2+	2X
Cerebellar ataxia	3+	+	1+	+	2+	2+	2+	2+	-	2+	+	2+	+	+	+	+	2+	X	2+	2+	-	2X	2-	-	X	2+	2+	2+
Limb spasticity/hypertonia	3+	+	2+	+	2+	1+	2+	2+	-	2+	-	2-	+/-	+	+/-	+	2+	X	2+	2-	-	2X	1+ = 1-	-	X	2+	2+	2+
Gait abnormalities	1+	+	2+	+	2+	2+	2+	2+	-	2+	+	2+	+	X	+	+	2+	+	2+	2X <sub>p</sub>	X	2X	1+ = 1X	X	X	2+	2+	2+
No independent gait	1+	+	2-	X	2-	2-	2-	2-	-	1+	-	2-	-	+	-	-	1+	-	2+	2+	+	2X	1- = 1X	+	+	2X	2-	2-
Hypokinesia/Parkinsonism	3+	X	1+	X	2-	2+	2X	2-	X	2X	X	2-	-	-	-	+	1+	X	2-	2-	-	2X	2-	-	X	2X	1+	1+
Truncal/limb dystonia	3+	X	2+	X	2+	2+	2X	2+	X	2+	+	1+ = 1-	+	+	+	+	2+	X	2+	2+	+	2X	2X	-	X	2X	2+	2+
Upper limb/head tremor	3X	+	2+	+	2+	2+	2-	2-	-	2+	+	2-	+	+	+	+	2+	+	2+	2-	-	2X	2-	-	X	2X	2+	2X
Tics and TLV	1+	X	1+	X	2X	2X	2X	2+	X	2-	-	2X	+	X	X	X	2X	-	2X	1+	-	2X	2-	-	X	2X	1+	2X
Postural instability	2+	X	2-	X	2+	2X	2X	2+	X	2+	+	2-	+	+	+	+	2X	X	2X	2X	X	2X	1- = 1X	X	X	2X	2+	2+
Epileptic seizures	2+	+	2-	+	2+	2-	2-	2-	+	2-	-	2-	+	-	-	+	2X	-	2-	2-	+	2X	1+ = 1-	-	+	2X	2-	2-
Behavioural problem	3+	-	2-	-	2+	2+	2+	2-	-	2+	-	2-	+	+	+	+	2X	X	2+	2+	-	2X	1+ = 1-	-	X	2X	2+	2+
Premature aging	3+	-	2-	-	2-	1+	1+	2-	-	2-	-	2-	-	-	-	-	2+	-	2-	2-	-	2X	2-	-	X	2X	2-	2+
Reduction of PWM	2+ <sup>a</sup>	X	1+	X	1+	1+	1+	1- <sup>a</sup>	X	2+	-	2X	+	-	-	X	2X	X	2-	2-	X	2X	2X	X	X	2X	2-	2X
CC hypoplasia/agenesis	2+ <sup>a</sup>	+	1+	X	1+	1+	2+	1- <sup>a</sup>	X	2+	+	2X	-	+	-	+	2+	X	2+	2-	+	2X	2X	X	X	2X	2+	2X
AC Hypoplasia/agenesis	2+ <sup>a</sup>	X	2-	X	2+	2+	2+	1+ <sup>a</sup>	X	2+	+	2X	+	+	+	X	2X	X	2+	2-	X	2X	2X	X	X	2X	2+	2X
Short midbrain	2+ <sup>a</sup>	X	2-	X	1+	2+	1+	1+ <sup>a</sup>	X	2+	+	2X	-	+	-	X	2X	X	2-	2-	X	X	2X	X	X	2X	2-	2X

ICV hypoplasia	2 <sup>+</sup> <sup>a</sup>	X	2-	X	1+	2+	1+	1 <sup>+</sup> <sup>a</sup>	X	1+	-	2X	+	+	-	X	2X	X	2-	2-	+	2X	2X	X	X	2X	2-	2X
Hypertrophy of the clava	2 <sup>-</sup> <sup>a</sup>	X	2-	X	2-	1+	1-	1 <sup>-</sup> <sup>a</sup>	X	2-	-	2X	+	+	-	X	2X	X	2+	2-	X	2X	2X	X	X	2X	2-	2X

- 1 - = negative for the feature of interest; + = positive for the feature of interest; +/- = mild hypertonia; AC = anterior commissure; CC = corpus callosum; F = family; GDD = global developmental delay; ICV = hypoplasia of the inferior cerebellar vermis; ID = intellectual disability; N = number; PWM = periventricular white matter; TLV = tic like vocalizations; X = not available/not applicable.
- 2 The numbers preceding the symbols “+” = “-” = and “X” indicate the number of siblings who are positive, negative or don’t have data on the feature of interest in families with multiple affected.
- 3 <sup>a</sup>Brain MRI scans are not available from another affected sibling.
- 4
- 5

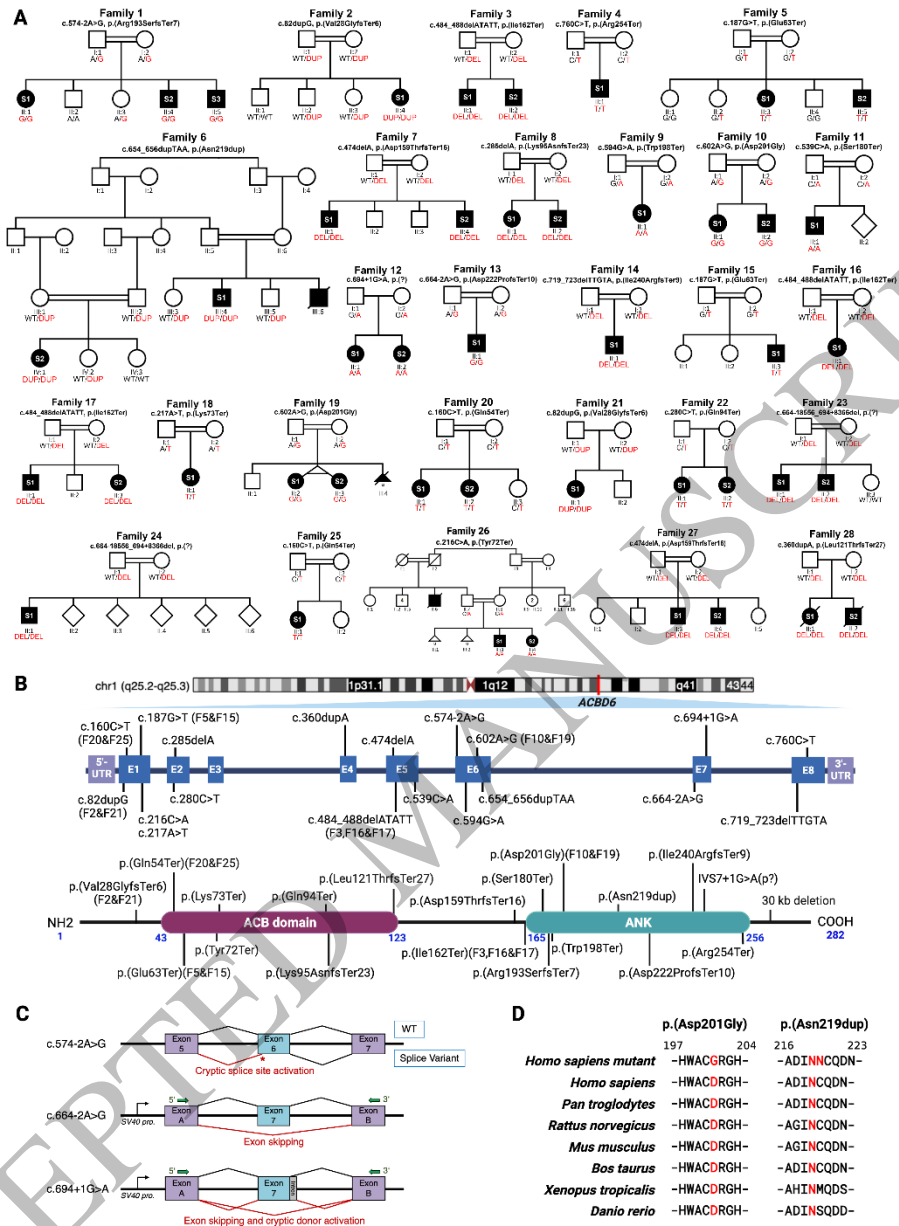


Figure 1  
246x336 mm ( x DPI)

1  
2  
3  
4

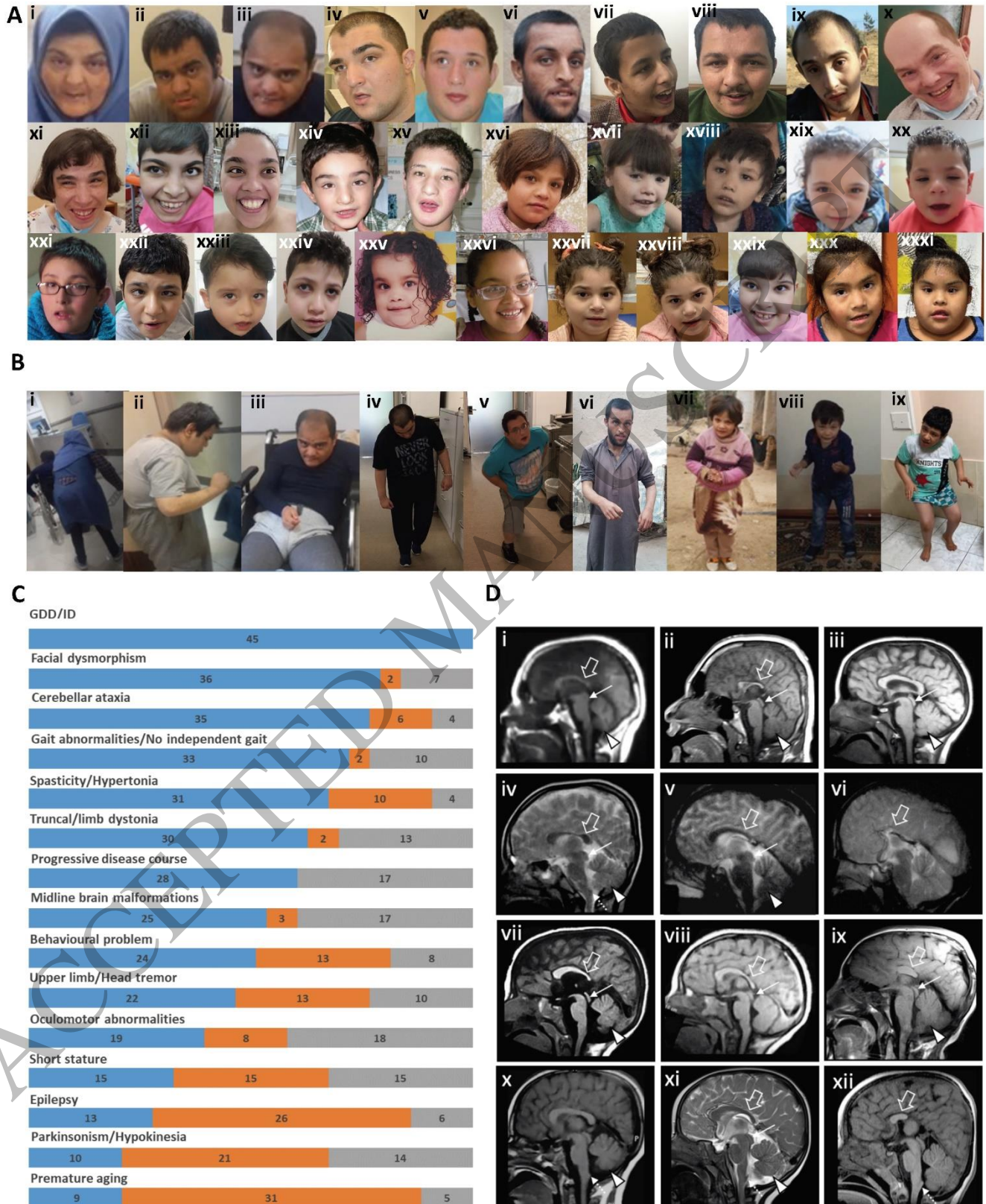


Figure 2  
185x230 mm ( x DPI)

1  
2  
3



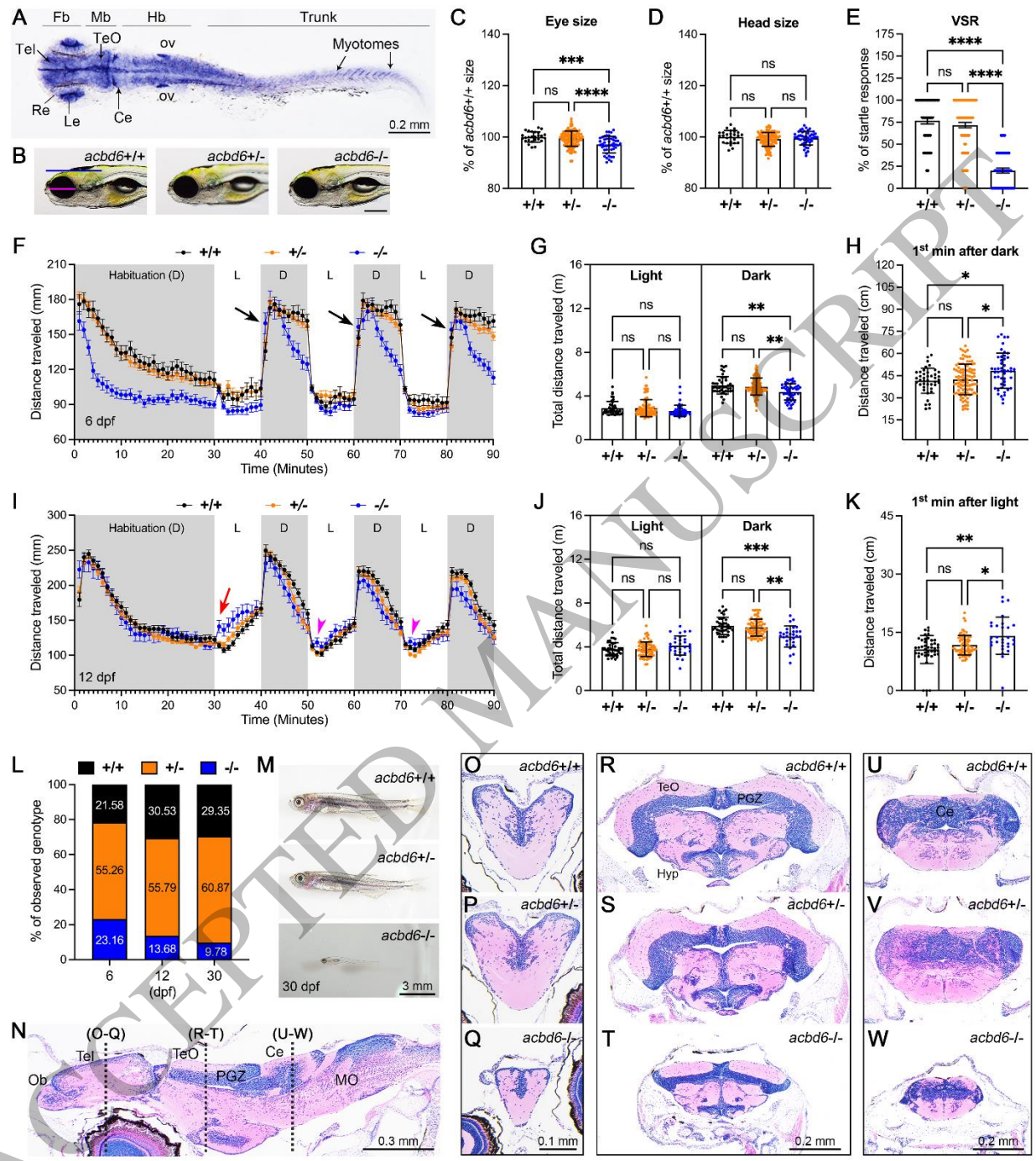


Figure 3  
305x344 mm ( x DPI)

1  
2  
3  
4

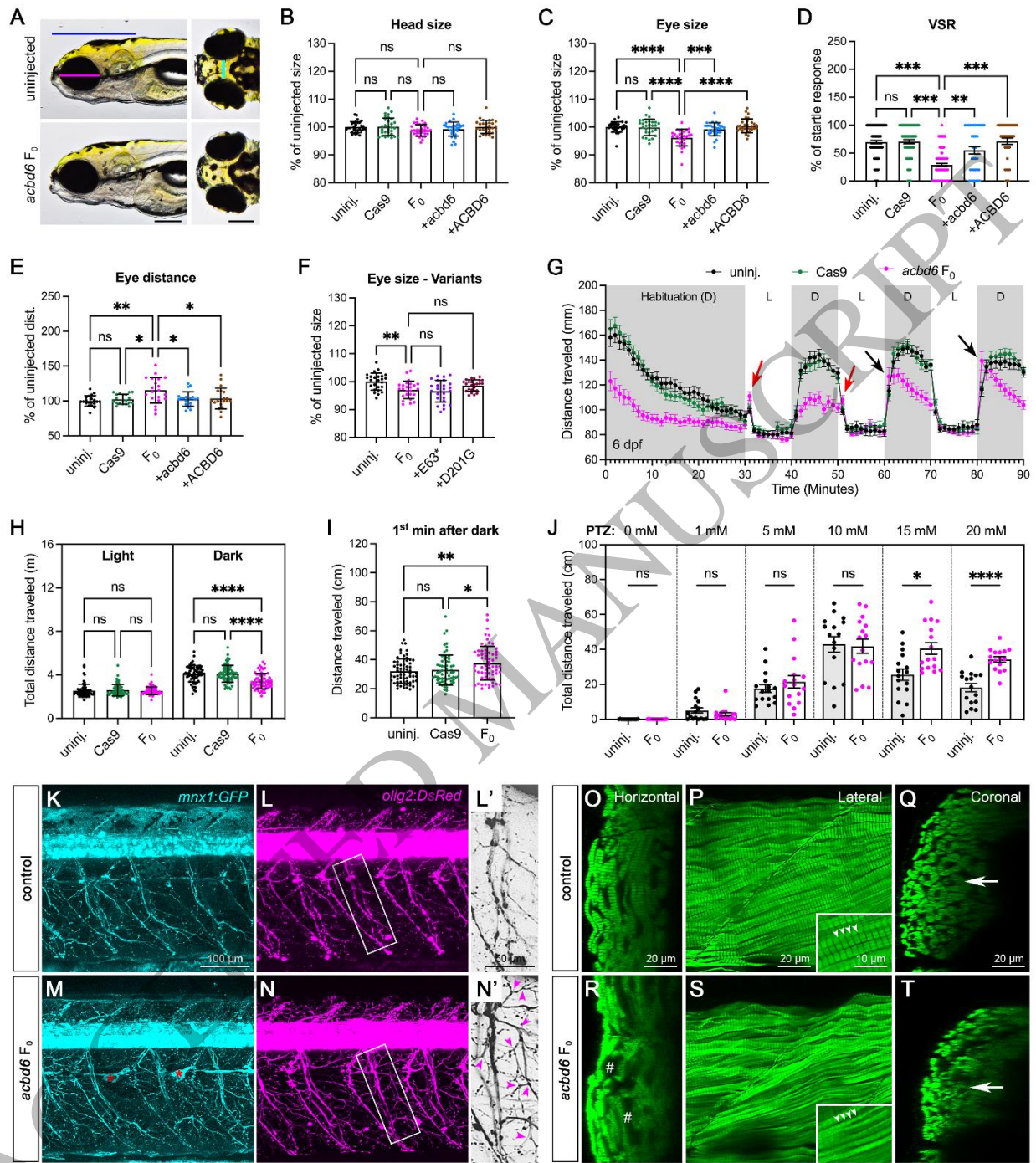
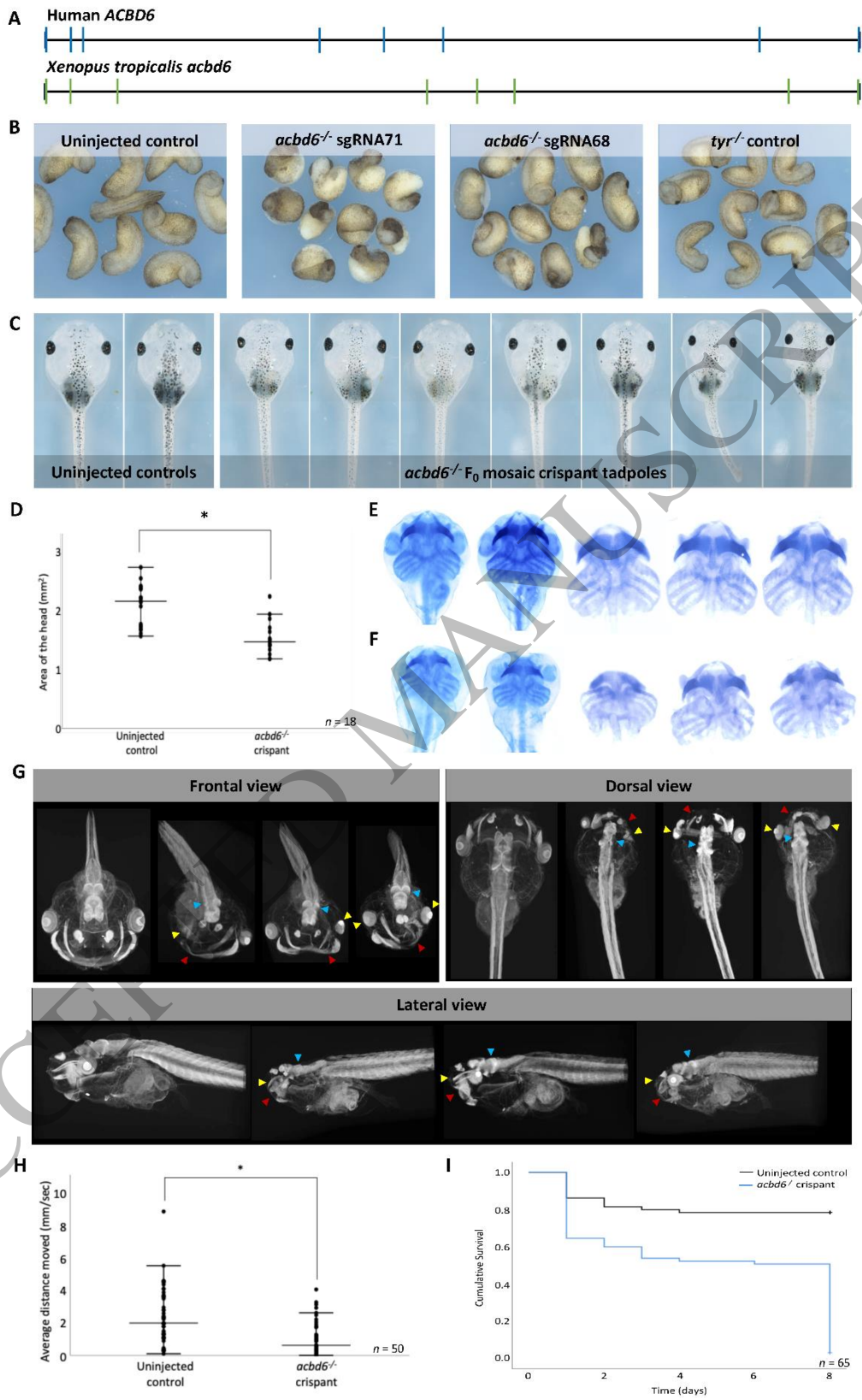


Figure 4  
 305x345 mm ( x DPI)

1  
 2  
 3  
 4



1  
 2  
 3

Figure 5  
 176x275 mm (x DPI)

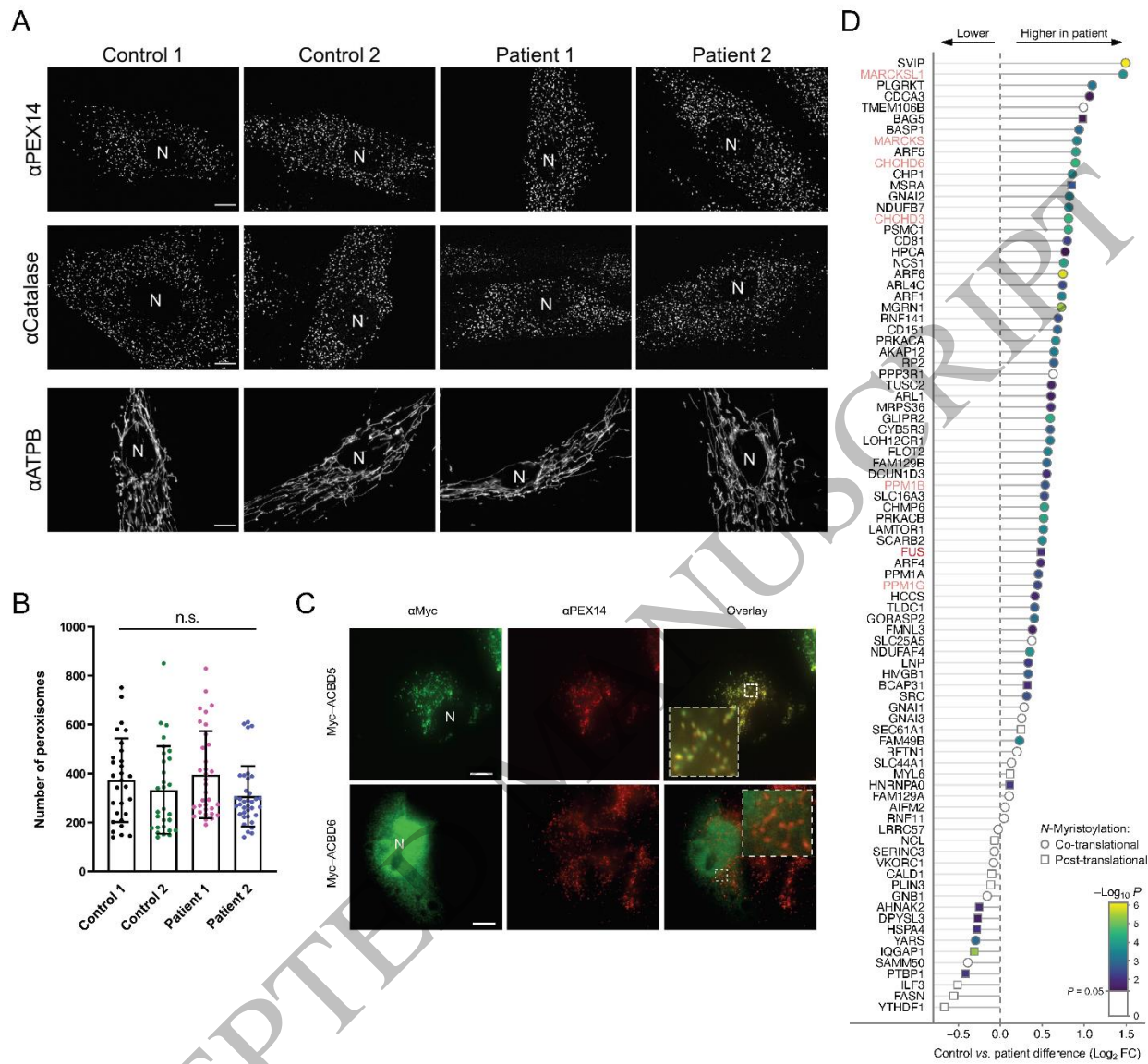
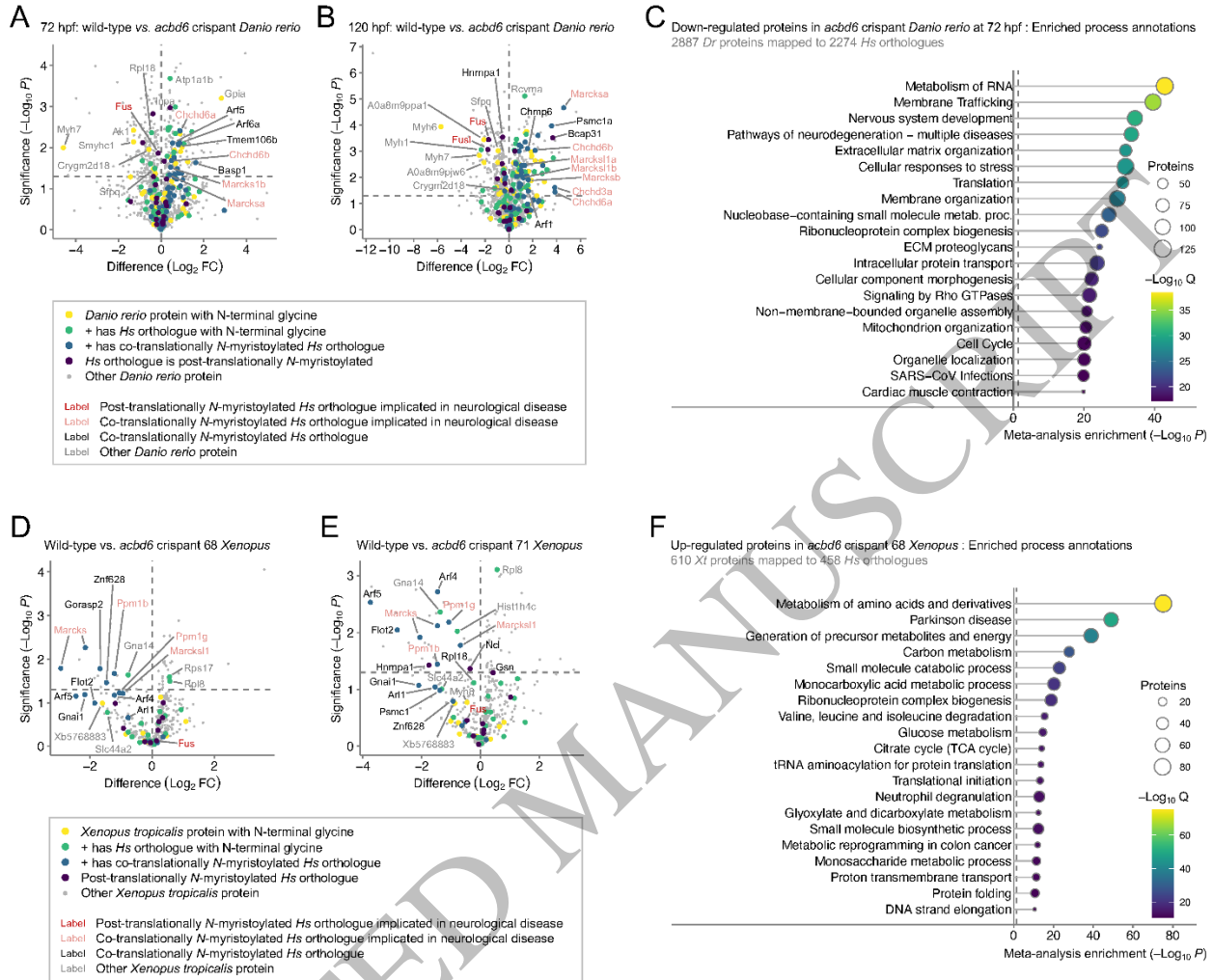


Figure 6  
339x316 mm (x DPI)

1  
2  
3  
4  
5



1  
2  
3

Figure 7  
339x283 mm ( x DPI)