ABCC9-related Intellectual disability Myopathy Syndrome is a KATP channelopathy with loss-of-function mutations in ABCC9

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Mutations in genes encoding K$_{ATP}$ channel subunits have been reported for pancreatic disorders and Cantú syndrome. Here, we report a syndrome in six patients from two families with a consistent phenotype of mild intellectual disability, similar facies, myopathy, and cerebral white matter hyperintensities, with cardiac systolic dysfunction present in the two oldest patients. Patients are homozygous for a splice-site mutation in $ABCC9$ (c.1320 + 1 G > A), which encodes the sulfonylurea receptor 2 (SUR2) subunit of K$_{ATP}$ channels. This mutation results in an in-frame deletion of exon 8, which results in non-functional K$_{ATP}$ channels in recombinant assays. SUR2 loss-of-function causes fatigability and cardiac dysfunction in mice, and reduced activity, cardiac dysfunction and ventricular enlargement in zebrafish. We term this channelopathy resulting from loss-of-function of SUR2-containing K$_{ATP}$ channels $ABCC9$-related Intellectual disability Myopathy Syndrome (AIMS). The phenotype differs from Cantú syndrome, which is caused by gain-of-function $ABCC9$ mutations, reflecting the opposing consequences of K$_{ATP}$ loss- versus gain-of-function.
K<sub>ATP</sub> channels are nucleotide-gated potassium channels formed by the obligate co-assembly of pore-forming Kir6.x subunits and regulatory sulfonylurea receptors (SURx), which couple the membrane potential to metabolic state in multiple cell types<sup>1,2</sup>. In mammals, two Kir6.x isoforms are encoded by the paralogous KCNJ8 (Kir6.1; [OMIM: 600937]) and KCNJ11 (Kir6.2; [OMIM: 600935]) genes, which are each co-located with genes encoding two SUR isoforms, ABCC9 (SUR2; [OMIM: 601439]) and ABCC8 (SUR1; [OMIM: 600509]), respectively, on chromosomes 12 and 11. Molecular heterogeneity is further increased by alternative splicing of ABCC9 mRNA, yielding two major splice variants, SUR2A and SUR2B—while multiple other splice variants have also been reported<sup>3–8</sup>. Pancreatic and neuronal K<sub>ATP</sub> channels are predominantly formed by Kir6.2 and SUR1, smooth muscle K<sub>ATP</sub> channels are comprised of Kir6.1 and SUR2B, and the predominant combination in striated muscle is Kir6.2 and SUR2A<sup>3</sup>.

The causative role of gain-of-function (GoF) or loss-of-function (LoF) mutations in the Kir6.2/SUR1-dependent pancreatic K<sub>ATP</sub> channels in neonatal diabetes and congenital hyperinsulinism, respectively, was established nearly two decades ago<sup>9–12</sup>. Recently, it has been demonstrated that dominant GoF mutations in KCNJ8 and ABCC9 underlie Cantú Syndrome (CS [OMIM: 239850])<sup>13–15</sup>. CS is characterized by hypertrichosis, coarse facial features, and multiple cardiovascular abnormalities, including cardiomegaly and tortuous, dilated vasculature<sup>14–16</sup>. Behavioral problems and mild developmental delay have been reported in CS, but intellectual function is typically normal<sup>17</sup>.

The human consequences of LoF in Kir6.1 and SUR2 remain uncertain. In a single report, two heterozygous LoF mutations in an exon found only in SUR2A were associated with dilated cardiomyopathy (DCM [MIM: 608569])<sup>18</sup>. A missense mutation in the same exon was reported as predisposing to paroxysmal adrenergic atrial fibrillation (AF [MIM: 614050]), but only in one 53-year-old female patient<sup>19</sup>. The pathophysiological consequences of complete SUR2 LoF are unclear.

We report six patients from two non-consanguineous families from Northern Norway who exhibit a shared pathological constellation including similar facies, intellectual disability and developmental delay, anxiety, myopathy with hypotonia, muscle weakness, and ataxicity. Cardiac systolic dysfunction is found in the two oldest patients. All have cerebral white matter hyperintensities, and hyperreflexia is found in the oldest four. The families are investigated by comprehensive clinical exome sequencing, a powerful tool for identifying the genetic basis of rare and complex syndromes, both in patients with de novo mutations and in families with suspected recessive inheritance<sup>20,21</sup>.

We identify a homozygous ABCC9 splice site mutation (c.1320 + 1 G > A) in all affected individuals. We show that the mutation causes the in-frame deletion of exon 8, resulting in SUR2 protein lacking 52 amino acids, and loss of plasmalemmal K<sub>ATP</sub> function. Using CRISPR/Cas9 genome engineering, we introduce frameshift mutations into ABCC9 that result in premature protein truncation, in both zebrafish and mice. These animals lack functional SUR2 protein and myocyte K<sub>ATP</sub> channels and recapitulate the myopathy and cardiac dysfunction observed in patients. We conclude that SUR2 LoF results in a recessive syndrome: ABCC9-related Intellectual disability Myopathy Syndrome (AIMS).

Results

Patient descriptions. Four siblings from Family 1 (aged 12–21 years) and two siblings from Family 2 (aged 29, 33) (Fig. 1a) are described. The families are not known to be related, but are from the same area of Northern Norway. Genetic investigations had earlier been performed with normal results in several of the patients, including G-bandng, high-resolution Single-Nucleotide Polymorphism (SNP) array to look for genomic deletions and duplications, FMRI CGG repeat analysis, DMPK PCR, sequencing of multiple neuromuscular disease genes, screening for mitochondrial DNA sequence variants/deletions, and screening for inborn errors of metabolism. Clinical photographs of the patients are presented in Fig. 1b, c, MRI images are presented in Fig. 1d. Clinical features are summarized in Tables 1 and 2.

Family 1. Patient 1–1 is the first child of healthy parents from Northern Norway with probable Finnish ancestry. Pregnancy and birth were uneventful. Weight and length were below the 2.5 percentile in childhood. Hearing loss was reported in childhood, but a recent hearing evaluation was normal. Early psychomotor development was described as normal, but at age 2, in-toeing, toe-walking and reduced fine motor skills, generalized hypotonia, lumbar lordosis, and delayed development were noted. At age 11, neuropsychological testing identified mild intellectual disability. From age 15, she experienced episodes of tonic/tonic–clonic seizures, but epileptic activity was absent in repeated EEGs. Lamotrigine treatment was partly effective. She continues to report muscular pain, fatigue, and anxiety. Recently, hyperprolactinemia was found (720 mIU L<sup>−1</sup> (ref: 102–496 mIU L<sup>−1</sup>)). An MRI of the pituitary, however, did not disclose pituitary adenomas. She lives in her own home, but requires frequent supervision and help. For neuromuscular evaluation of all patients, including cerebral MRI with white matter changes, see Tables 1 and 2.

Patient 1–2 is the younger brother of 1–1. Pregnancy and birth were uneventful. Delayed psychomotor development and lumbar lordosis was noted early alongside eating difficulties and low body weight. In childhood, he displayed left lower extremity weakness, hyperreflexia, and limping. Muscle biopsy showed unspecific changes of mitochondrial aggregation and muscle fiber caliber variation. Mild intellectual disability was diagnosed by neuropsychological testing in teenage years.

Patient 1–3 is the younger brother of 1–1. Pregnancy and birth were unremarkable. He showed delayed early psychomotor development with generalized hypotonia and toe-walking. Hyperreflexia was noted in the lower extremities from age 5. Eating difficulties, nausea, abdominal pain, and low body weight were present from childhood. Neuropsychological testing at 5 years showed mild intellectual disability. Profound muscular pain and stiffness are reported after physical exercise. Mild bilateral high-frequency sensorineural hearing loss was found at age 12.

Patient 1–4 is the youngest brother of 1–1. Pregnancy and birth were unremarkable. Eating difficulties and low body weight were observed in early years, with weight and length at the 2.5 percentile. Delayed psychomotor development, in-toeing, lumbar lordosis, generalized muscular hypotonia, and strabismus were noted. At age 7, he experienced a cerebral episode with coma and tetraplegia preceded directly by repeated vomiting, and a few days earlier by high fever. Serum measurements showed a metabolic acidosis with S-lactate 6, normal S-potassium (measured after intravenous infusion), and elevated S-creatine kinase (738) at hospital admission. Cerebral MRI revealed multiple lesions in both hemispheres periventricularly, subcortically, in the pons and the basal ganglia, in both gray and white matter. Acute disseminated encephalomyelitis was discussed, and steroid treatment was given. He regained consciousness over the following few days. Re-evaluation of MRI findings concluded with an “inflammatory perivascular reaction”. MRI lesions were normalized a few weeks later, except for a white matter lesion by
the anterior horn of the left ventricle. He regained the same psychomotor level as before. Weight increased after that episode and is currently > 97.5 percentile. Neuropsychological testing at age 9 resulted in a diagnosis of mild intellectual disability.

Obstructive sleep apnea, probably related to being overweight, a large tongue and hanging soft palate were diagnosed at age 11, and operative treatment was recommended. Mild bilateral high-frequency hearing loss was found at age 11.

Individual II.3 was a female fetus, conceived inbetween patients 1–2 and 1–3. The parents elected to terminate pregnancy in Week 21.
pregnancy week 21, due to suspected thanatophoric dysplasia with micromelia and a narrow thorax.

**Family 2. Patient 2–1** is the first child of healthy parents. Pregnancy and birth were unremarkable. Delayed psychomotor development and muscular hypotonia were noted in toddler years. Due to similar findings in him and his sister, with a few depigmented skin patches and white matter lesions on cerebral computed tomography scan, a diagnosis of tuberous sclerosis (TS) was suggested, but later abandoned. The MRI pattern is not the same as in TS. Neither sibling has experienced epilepsy seizures. Feeding was difficult in childhood, with low body weight, but he gained weight in adulthood, and is now overweight, with short stature. Severe atopic eczema is currently managed by cyclosporine, and he has multiple food allergies.

Recent neuropsychological testing led to a diagnosis of mild intellectual disability. He has been medicated for anxiety with a selective serotonin reuptake inhibitor for the last few years. He reports frequent dizziness attacks. Cardiac ultrasound at age 33 revealed biventricular systolic dysfunction, moderate left ventricle dysfunction, left ventricle ejection fraction (EF) of 35–40%, and raised NT-proBNP. Left ventricle diameter was within the normal range, although, cardiac MRI shows left ventricle dilatation, without obvious pathology of the myocardium—compatible with early-stage dilated cardiomyopathy. Treatment with an ACE-antagonist and a beta-blocker was started. Hypertension was diagnosed before treatment initiation. A cardiac ultrasound in teenage years was normal. Cerebral MRIs at ages 16 and 33 showed widespread confluent white matter signal changes, described as similar to leucodystrophy. Lesions have increased significantly since the first investigation. MR angiography is normal, with normal cerebral vasculature calibers. Point lesions are found in the basal ganglia, pons, and white matter, representing possible mineral depositions. He lives in a sheltered home with daily help and supervision. He attends a sheltered work program, but is easily exhausted, and needs rest during the day. He is cheerful and social. Muscle strength is normal. Patient 2–2 is the younger sister of 2–1. Pregnancy and birth were unremarkable. She was treated for congenital hip dysplasia. Psychomotor development was delayed. Cerebral MRI in childhood showed periventricular white matter changes, and a diagnosis of tuberous sclerosis was considered (see above). Hypotonia, reduced muscle strength, and exhaustibility are reported since childhood. She has short stature and microcephaly. She had a thin build in childhood, but is now overweight. Recent neuropsychological testing indicates mild-to-moderate intellectual disability. She lives in her own flat with assistance. She has anxiety and has been followed by a local outpatient psychiatry service. Cardiac ultrasound at age 29 revealed biventricular systolic dysfunction, mildly reduced left ventricle ejection fraction (EF) of 48%, and a hypokinetic left ventricle with normal systolic dysfunction, left ventricle ejection fraction (EF) of 35–40%, and raised NT-proBNP. The MRI pattern is not the same as in TS. Both siblings have been medicated for anxiety with a selective serotonin reuptake inhibitor for the last few years. They report frequent dizziness attacks. Cardiac ultrasound at age 33 revealed biventricular systolic dysfunction, moderate left ventricle dysfunction, left ventricle ejection fraction (EF) of 35–40%, and raised NT-proBNP. Left ventricle diameter was within the normal range, although, cardiac MRI shows left ventricle dilatation, without obvious pathology of the myocardium—compatible with early-stage dilated cardiomyopathy. Treatment with an ACE-antagonist and a beta-blocker was started. Hypertension was diagnosed before treatment initiation. A cardiac ultrasound in teenage years was normal. Cerebral MRIs at ages 16 and 33 showed widespread confluent white matter signal changes, described as similar to leucodystrophy. Lesions have increased significantly since the first investigation. MR angiography is normal, with normal cerebral vasculature calibers. Point lesions are found in the basal ganglia, pons, and white matter, representing possible mineral depositions. They live in a sheltered home with daily help and supervision. They attend a sheltered work program, but is easily exhausted, and needs rest during the day. They are cheerful and social. Muscle strength is normal.

**Table 1 General clinical features and dysmorphology of AIMS patients**

<table>
<thead>
<tr>
<th>Patient</th>
<th>1–1</th>
<th>1–2</th>
<th>1–3</th>
<th>1–4</th>
<th>2–1</th>
<th>2–2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age/sex</strong></td>
<td>21/female</td>
<td>17/male</td>
<td>13/male</td>
<td>11/male</td>
<td>33/male</td>
<td>29/female</td>
</tr>
<tr>
<td><strong>Cognitive function</strong></td>
<td>Mild ID</td>
<td>Mild ID</td>
<td>Mild ID</td>
<td>Mild ID</td>
<td>Mild ID</td>
<td>Moderate ID</td>
</tr>
<tr>
<td><strong>Hypotonia in childhood</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Fatigability</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Hearing</strong></td>
<td>Very mild sensorineural hearing loss</td>
<td>N</td>
<td>High-freq mild hearing loss</td>
<td>High-freq mild hearing loss</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td><strong>Sleep apnoea</strong></td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>+</td>
<td>NA</td>
</tr>
<tr>
<td><strong>Psychiatry</strong></td>
<td>Anxiety</td>
<td>Anxiety</td>
<td>Anxiety</td>
<td>Anxiety</td>
<td>Anxiety</td>
<td>Anxiety</td>
</tr>
<tr>
<td><strong>Skeletal</strong></td>
<td>Lumbal lordosis</td>
<td>Lumbal lordosis</td>
<td>Lumbal lordosis</td>
<td>Lumbal lordosis</td>
<td>Normal</td>
<td>Congenital hip dislocation</td>
</tr>
<tr>
<td><strong>Growth</strong></td>
<td>L:2.5-10p</td>
<td>L:10p</td>
<td>L:2.5p</td>
<td>L:10p</td>
<td>L:&lt; 2.5p (162 cm)</td>
<td>L:&lt; 2.5p (147 cm)</td>
</tr>
<tr>
<td><strong>Skin</strong></td>
<td>Marmoration</td>
<td>Marmoration</td>
<td>Marmoration</td>
<td>Marmoration</td>
<td>Marmoration</td>
<td>Atopic dermatitis</td>
</tr>
<tr>
<td><strong>Dysmorphology</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Prominent supraorbital ridges</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Hypotelorism</strong></td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Broad nasal tip</strong></td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><strong>Dental malocclusion</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Flat face</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Thin upper lip vermillion</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Macrodontia upper central incisors</strong></td>
<td>–</td>
<td>+</td>
<td>+, widely spaced</td>
<td>+, widely spaced</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td><strong>Food intolerance/allergies</strong></td>
<td>Milk protein intolerance</td>
<td>Lactose intolerance</td>
<td>N</td>
<td>Milk protein intolerance</td>
<td>Food allergies, anaphylaxia</td>
<td>Food allergies</td>
</tr>
</tbody>
</table>

| ID | intellectual disability, L | length, N | normal, NA | not assessed, OFC | occipitofrontal circumference, W | weight |

<table>
<thead>
<tr>
<th><strong>Additional clinical features</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Scoliosis</strong></td>
</tr>
<tr>
<td><strong>Dislocation</strong></td>
</tr>
<tr>
<td><strong>Elbow extension</strong></td>
</tr>
<tr>
<td><strong>Point lesions</strong></td>
</tr>
<tr>
<td><strong>Cafe au lait patch</strong></td>
</tr>
<tr>
<td><strong>Telangiectasia</strong></td>
</tr>
<tr>
<td><strong>Atopic dermatitis</strong></td>
</tr>
<tr>
<td><strong>Depigmented patches</strong></td>
</tr>
<tr>
<td><strong>Scoliosis</strong></td>
</tr>
<tr>
<td><strong>Elbow extension</strong></td>
</tr>
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<td><strong>Telangiectasia</strong></td>
</tr>
<tr>
<td><strong>Atopic dermatitis</strong></td>
</tr>
<tr>
<td><strong>Depigmented patches</strong></td>
</tr>
</tbody>
</table>
diameter. ProBNP is normal and there are no clinical signs of cardiac failure at this time. Cerebral MRI performed recently shows an increase in periventricular white matter signal changes compared with the last MRI at age 13. MR angiography of cerebral vessels is normal.

**Table 2 Neurological, electrophysiological, and cardiac features of AIMS patients**

<table>
<thead>
<tr>
<th>Table 2 Neurological, electrophysiological, and cardiac features of AIMS patients</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient</strong></td>
</tr>
<tr>
<td>Neurological examination</td>
</tr>
<tr>
<td>Cranial nerves</td>
</tr>
<tr>
<td>Muscle strength*</td>
</tr>
<tr>
<td>Hand grip</td>
</tr>
<tr>
<td>Truncal muscle weakness</td>
</tr>
<tr>
<td>Proximal upper limb</td>
</tr>
<tr>
<td>Proximal lower limb</td>
</tr>
<tr>
<td>Achilles contractures</td>
</tr>
<tr>
<td>Hyperreflexia</td>
</tr>
<tr>
<td>Balance (MiniBESTest)</td>
</tr>
<tr>
<td>Cardiac examination</td>
</tr>
<tr>
<td>6-min walk test</td>
</tr>
<tr>
<td>Electrophysiology</td>
</tr>
<tr>
<td>EMG</td>
</tr>
<tr>
<td>NCV</td>
</tr>
<tr>
<td>Repetitive nerve stimulation</td>
</tr>
<tr>
<td>EEG</td>
</tr>
<tr>
<td>Muscle biopsy</td>
</tr>
<tr>
<td>Cerebral MRI</td>
</tr>
<tr>
<td>Cardiac examination</td>
</tr>
<tr>
<td>Cardiac ultrasound</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
</tr>
</tbody>
</table>

*Muscle strength grading (0-5): 0 = paresis, 4 = 50% strength reduction, 4-+ = 25% strength reduction 5 = normal

**Identification of a splice site mutation in ABCC9.** We performed sequencing of one affected individual from each family (patients 1–2 and 2–1), plus their respective healthy parents, using a targeted panel of >4800 disease-associated genes. The samples were analyzed for recessive variants (homozygous and compound heterozygous) and non-Mendelian inheritance. The two trios were analyzed independently, and from a total of ~10,000 variants in each of the families, the homozygous variant ABCC9 c.1320 + 1 G > A was the only remaining causal candidate after filtering against quality, region of interest, coding effect, minor allele frequency, and manual review of ~20 variants in each of the two families. Next-generation sequencing (NGS) and subsequent Sanger sequencing revealed that all six patients were homozygous for ABCC9 c.1320 + 1 G > A. Analysis of DNA from the four parents and one unaffected sibling from family 2 showed that they were each heterozygous for the same mutation.
To exclude distant relatedness between the two families and thus exclude the possibility of the presence of more shared rare variants with an effect on protein function, we performed whole-genome sequencing on one affected individual from each family (patients 1–2 and 2–1), and determined a kinship coefficient of 0.0403. A kinship coefficient of ~0.05 is expected for unrelated samples, confirming non-relatedness between our families.

Since the original NGS analysis was performed on a gene panel, we analyzed the WGS data sets for candidate causal recessive and dominant variants. The focus of the analysis was the identification of shared variants or different variants in shared genes with possible damaging, but not identical variants leading to the same clinical phenotype, among the two cases. After the initial analysis, 67 shared variants were identified, of which 66 were heterozygous and 1 was homozygous (Supplementary Table 2). The single homozygous variant remaining after the filtering was the above variant in ABCC9:NC_000012.11:g.22063090 C>T; NM_020297.2:c.1320+1G>A (Supplementary Fig. 2). This variant lies in a shared homozygous block of 3.8 MB (chr12:18,326,590-22,176,010 hg19). Notably, gene interactions with the homozygous ABCC9 c.1320+1G>A variant cannot be excluded as participating in the syndrome.

A list of all shared variants including allele frequencies is provided in Supplementary Table 2. For a dominant mode of inheritance, we observed that, in 842 genes, one or more variants pass the filtering criteria in both cases. Again, the ABCC9 variant was the only variant seen in homozygous state in both samples (Supplementary Fig. 3). Compound heterozygous gene candidate analysis identified seven genes with two or more variants shared between the two cases. None of these genes are likely to contribute to the phenotype (Supplementary Fig. 4). Thus, after gene panel and WGS, we identified the homozygous ABCC9 c.1320+1G>A variant as the most likely causal variant. The variant is reported at very low allele frequency in the homozygous state in the European population (Finnish: 3/6586; European: (Non-Finnish) 4/66386, Exac, June 2018). According to gnomAD, the variant is reported at surprisingly high frequency in homozygous state in the Finnish population with an allele frequency of 0.0007 (18/24850). It is less common in non-Finnish Europeans (5/128232, allele frequency 0.00004) and absent in Asian or African populations (May 2019). Considering the probable Finnish ancestry of all patients, the syndrome might be more prevalent in the Finnish population than others. The homozygous state is absent in gnomAD.

The ABCC9 c.1320+1G>A variant is predicted to disrupt a splice donor site of exon 8 (Fig. 2a). To study the effect of the mutation, we performed cDNA analysis on fibroblasts from members of both families (Fig. 2b). Sequence analysis of the homozygous cDNA samples revealed that the sequence variant caused an in-frame deletion of exon 8 (r.1165_1320del) in SUR2 cDNA, and predicts a 52 amino acid (p.Ala389_Gln440del) deletion within the SUR2 protein. Thus, all patients are homozygous for a splice variant in ABCC9 which results in an in-frame deletion.

**The effect of exon 8 deletion on K\textsubscript{ATP} channel function.** Deletion of exon 8 is predicted to disrupt multiple transmembrane helices in SUR2 and thus have a profound effect on structure and function (Fig. 3a). We deleted exon 8 in SUR2 cDNA (SUR2AΔ8) and performed expression analyses on Cosm6 cells transiently transfected with Kir6.2 alongside either Flag-tagged SUR2A-WT or SUR2AΔ8. Western blot of whole-cell lysates shows that exon 8 deletion results in a ~50% decrease in SUR2 protein expression (Fig. 3b). We used a radioactive rubidium (\textsuperscript{86}Rb\textsuperscript{+}) efflux assay to determine effects on K\textsubscript{ATP} channel function. Cells expressing SUR2A-WT with Kir6.2 exhibited robust rubidium efflux rates when K\textsubscript{ATP} channels were activated by metabolic inhibition (Fig. 3c). In contrast, cells expressing Kir6.2/SUR2AΔ8 showed no rubidium efflux above the background levels observed in GFP-transfected cells. When Kir6.2 was co-expressed with a heteromeric 1:1 ratio of SUR2A-WT and SUR2AΔ8, K\textsubscript{ATP}-dependent efflux rate was similar to WT rates, indicating that SUR2AΔ8 has no marked dominant-negative effect on functional K\textsubscript{ATP} expression. The effect of the exon 8 deletion in SUR2Δ8 was also determined and, again, no significant efflux was observed in cells expressing SUR2BΔ8 (Fig. 3d).

A complete absence of functional K\textsubscript{ATP} channels was observed in inside-out patch clamp recordings from cells co-transfected with Kir6.2/SUR2AΔ8, in contrast to robust expression in cells transfected with SUR2A-WT or a 1:1 mix of SUR2A-WT and SUR2AΔ8 (Fig. 3e, f). Therefore, homomeric deletion of exon 8 results in a significant decrease in protein expression and complete loss of K\textsubscript{ATP} channel function. The decrease in functional expression of SUR2AΔ8 containing channels may arise due to either the absence of surface-expressed channels or the expression of non-functional channels, which requires more detailed study for elucidation. Co-expression of SUR2A-WT and SUR2AΔ8 did not affect channel regulation by ATP or pharmacological activation by pinacidil, suggesting that in the heterozygous context, the c.1320+1G>A mutation is without significant effect (Fig. 3g; Supplementary Fig. 5).

**Fatigability and cardiac dysfunction in SUR2-STOP mice.** To model the effects of SUR2 LoF in vivo, we used a mouse line in which a frameshift mutation, resulting in a premature stop codon at position Y1148 (SUR2-STOP), was introduced using CRISPR/Cas9 (Fig. 4a). Inside-out patch clamp recordings from ventricular myocytes and aortic smooth muscle cells showed that functional K\textsubscript{ATP} channels were essentially absent in homozygous SUR2-STOP mice (Fig. 4b, c; Supplementary Fig. 6), thus the SUR2-STOP mice recapitulate the key functional channel consequences of the exon 8 deletion.

SUR2-STOP mice and WT littermate controls were evaluated on a multiple-trial inverted screen test to assess strength and fatigability. SUR2-STOP and WT mice performed comparably in the first trial, suggesting no significant initial deficits in strength per se. However, in subsequent repeated trials, SUR2-STOP mice exhibited clear decreases in performance, whereas WT mice performance remained high (Fig. 4d). Significant genotype effects were observed, as well as genotype x trial and genotype x session interactions (Supplementary Table 3). These findings indicate that SUR2-STOP mice exhibited significant performance deficits, dependent on the specific session and trial. The total time the mice remained inverted across the six trials was calculated, and a significant decrease in performance was observed in SUR2-STOP mice (Fig. 4e). Thus, global loss of SUR2 results in decreased physical performance, suggestive of increased fatigability, which may be related to the clinically observed myopathy.

Echocardiographic assessment revealed that left ventricle fractional shortening was significantly decreased in SUR2-STOP mice (Fig. 4f, g), consistent with the findings in older AIMS patients, and in previously reported SUR2-deficient mice. A small, but statistically significant increase in left ventricular internal dimension in diastole (normalized to body length) was observed in SUR2-STOP mice, also mirroring the mild dilatation observed our eldest patient (patient 2–1), and the dilated cardiomyopathy previously associated with SUR2 mutations. Blood pressure was significantly increased in SUR2-STOP mice (Supplementary Fig. 6C, D), consistent with the known role of SUR2-containing vascular smooth muscle K\textsubscript{ATP} channels in the regulation of vascular tone.
Absence of cognitive or behavioral defects in SUR2-STOP mice. SUR2-STOP and WT mice were evaluated on a battery of cognitive and behavioral tests. During a 1-h locomotor activity test, SUR2-STOP mice displayed trends toward decreased total ambulations (whole-body movements) and verticalearing frequency, and increased rest time, but there were no significant overall genotype effects (Fig. 5a–c). No differences were observed in the distance traveled in the center or peripheral zones of the test field, indices of emotionality in mice (Fig. 5d, e). In addition, neither significant effects were found in a battery of sensorimotor measures designed to assess balance, coordination, strength, and speed of movement (ledge test, platform test, pole test, inclined screen test, Fig. 5f–i) nor in tests of spatial learning and memory, evaluated using a Morris water maze (Fig. 5j). Finally, an elevated plus maze was used to assess anxiety-like behaviors, which involves quantifying the reluctance of mice to move from the “protected areas” of the enclosed arms to open arms. No significant differences were observed between genotypes in terms of distance traveled, time spent or entries made into the open arms, or total distance traveled throughout the entire maze (Fig. 5m–p). Collectively, the behavioral findings suggest that the SUR2-STOP mice do not exhibit marked deficits in learning and memory nor show any obvious anxiety-like behaviors.

Decreased motility in SUR2-STOP zebrafish. Assessment of a SUR2-STOP zebrafish model (Fig. 6a, b) also demonstrated clear phenotypic overlap with the clinical syndrome and mouse model. cDNA was analyzed by qPCR, to assess the effect of the S985 truncation, introduced via CRISPR/Cas9, on abcc9 mRNA expression in SUR2-STOP zebrafish larvae. This revealed ~four-fold abcc9 mRNA decrease in mutant fish compared with wild-type controls (Fig. 6c), consistent with aberrant mRNA being eliminated by nonsense mediated decay. Inside-out patch clamp recordings revealed the complete absence of functional K<sub>ATP</sub> channels in ventricular myocytes from SUR2-STOP zebrafish (Fig. 6d). Hence, the zebrafish model also recapitulates the key channel consequences of the exon 8 deletion.

Cardiac abnormalities in SUR2-STOP zebrafish. We performed high-speed video imaging<sup>24</sup> of the hearts of wild-type and SUR2-STOP larvae to examine cardiac function (Fig. 7a; Supplementary Movies 1, 2). Analysis revealed systolic dysfunction—resembling the cardiac phenotype in older patients. Both fractional shortening (FS) and ejection fraction (EF) are significantly reduced in SUR2-STOP mutants (FS: 29%, EF: 25%) (Fig. 7b, c). Consequently, cardiac output is dramatically lower (28%) (Fig. 7d) due to equivalently reduced stroke volume (Supplementary Fig. 7A). Supplementary Fig. 8 illustrates the assessment of ventricular contractility via high-speed video microscopy in zebrafish embryos. Ventricular end-diastolic volume (VEDV) and end-systolic volume (VESV) were unchanged in SUR2-STOP larvae (Fig. 7e).
Blood flow velocity of WT and SUR2-STOP larvae was assessed by high-speed video imaging of the cardinal vein. An increased velocity of red blood cells in SUR2-STOP fish (Fig. 7f) can be associated with high blood pressure found in SUR2-STOP mice.

No cardiac abnormalities were observed in larvae heterozygous for the SUR2-STOP mutation (Supplementary Fig. 9).

Adult zebrafish hearts were analyzed after sectioning and H&E staining (Fig. 7g). For assessment of ventricular size, tissue sections revealing the largest chamber area were selected. In five out of six SUR2-STOP fish, ventricular area was strikingly enlarged with abnormal morphology compared with control siblings. The atrial area shows similar morphology (Fig. 7f; Supplementary Fig. 10). We stained cryosections of WT and SUR2-STOP hearts with Acid Fuchsian Orange G (AFOG), which labels myocardium orange, collagen blue, and fibrin red. This revealed no visible fibrosis in SUR2-STOP hearts (Supplementary Fig. 11). TUNEL (TdT-mediated nick end labeling) analysis revealed very few apoptotic cells in WT hearts, while a sizable number of cells were TUNEL-positive in both cardiac chambers of SUR2-STOP fish (Fig. 7h). Myofiber structure in SUR2-STOP hearts is not different from WT as determined by immunohistochemistry staining for tropomyosin (Supplementary Fig. 12).

**Discussion**

The major clinical features observed in our patients consist of delayed psychomotor development with intellectual disability,
anxiety, muscle weakness and fatigability, and some shared dysmorphic features. Cerebral MRI revealed white matter abnormalities in all subjects. Cardiac systolic dysfunction is found in the two oldest, possibly as an early stage of dilated cardiomyopathy. All patients were found to be homozygous for a previously unreported splice site mutation in ABCC9 (c.1320 + 1 G > A), while unaffected parents are healthy heterozygous carriers of the variant (Fig. 2). No other shared recessive mutations were identified in the affected individuals using either next-generation gene panel analysis or whole-genome sequencing.

We show that this splice site mutation leads to the complete in-frame exclusion of exon 8 (SUR2Δ8) and consequent deletion of 52 amino acids within the TMD1 domain of the resultant SUR2 protein (Fig. 3). SUR2 is a regulatory subunit of K\textsubscript{ATP} channels expressed in various tissues, including striated and smooth muscle\textsuperscript{3-5}. Deletion of exon 8 results in complete loss of
Fig. 5 No major behavioral or cognitive deficits observed in SUR2-STOP mice. a Total time at rest b The total number of ambulations (whole-body movements), c the number of incidents of rearing, d distance traveled at edge of the observational field, and e distance traveled within center of observational field in 1 h locomotion observation, n = 11 for WT and 9 for SUR2-STOP for all behavioral tests. f Time mice spent on elevated ledge, g time mice remained on elevated circular platform, h time taken to descend during the pole test, and i time taken to ascend a wire-mesh screen maintained at 60° angle for WT and SUR2-STOP mice in sensorimotor battery. j The escape path length taken to find the platform by WT and SUR2-STOP mice in Morris Water Maze Cued trials. Note, no significant difference in swimming speed was observed between genotypes. k The mean escape path length (left) taken to find the platform, and average swimming speed (right) by WT and SUR2-STOP mice in Morris Water Maze Place trials. l Time spent within the target quadrant (the quadrant in which the platform had previously been positioned during Cued and Place trials) was measured. m Distance traveled within open arms, (n) time spent in open arms, o entries into open arms, and p total distance traveled in the Elevated Plus Maze test for WT and SUR2-STOP mice. The data were analyzed using a repeated measures (rm) ANOVA model that contained two between-subjects variable (genotype and sex) and one within-subjects variable (trial block). The data from individual experiments shown as dots alongside mean ± SEM. Source data are provided as a Source Data file.

Functional channel activity in recombinant SUR2/Kir6.2 K<sub>ATP</sub> channels, indicating that AIMS represents the human consequence of loss of SUR2 (Fig. 3). We show that key features of myopathy and cardiac dysfunction in AIMS are recapitulated in novel SUR2-STOP mouse (Figs. 4, 5) and zebrafish models (Figs. 6, 7). The animal models used in this study do not recapitulate the genetic defect identified in the AIMS patients, but were chosen as the functional effects of the frameshift mutations introduced into SUR2-STOP mice and fish mirror the functional effect of the SUR2Δ8 mutation. Future studies of CRISPR/Cas9 genome edited animal models in which human-disease-associated AIMS mutations are introduced may provide further insights into the severity and variety of phenotypes arising from specific mutations.

How loss of SUR2-dependent K<sub>ATP</sub> channel function may result in the complex pathophysiology observed in AIMS is discussed below.

Facial features: Affected individuals from the two families share some similar facial features, including prominent supraorbital ridges, flat face, and thin upper lip vermilion, as well as macrodontia and/or widely spaced upper central incisors and dental malocclusion. Since the affected individuals are from two
siblingships, more patient observations will be necessary in order to establish whether these facial features are consistent. Some of the individuals have hypotelorism, consistent with significantly decreased intraorbital distance found in SUR2-STOP zebrafish (Fig. 6f, g). This combination of facial features is markedly different from the acromegaloid facial features that characterize Cantú Syndrome and the associated conditions within the same spectrum: Acromegaloid Facial Features (AFF [MIM: 102150]) and hypertrichosis acromegaloid facial features disorder (HAFF), which all arise from GoF mutations in ABCC9. None of our patients have hypertrichosis, unlike Cantú syndrome patients. Therefore, Cantú syndrome and AIMS, arising from opposing molecular mechanisms, are both dysmorphologically and mechanistically distinct.

Neuromuscular manifestations: All patients report fatigability, present with generalized hypotonia, and muscle strength is below normal in all but one. Lumbar lordosis is found in all but one, and scoliosis in one. The miniBEST test shows moderate to severe deficits in balance in all patients, and walking distance in the 6-min walk test is reduced in all individuals. S-CK, B-lactate, nerve conduction velocities, and electromyography, including repetitive nerve stimulation, are normal in all individuals, except for discrete myopathic discharges in patient 2–2. In skeletal muscle, $I_{\text{KATP}}$ channels are typically closed at rest but open in response to metabolic stress or fatigue. Channel activation results in action potential shortening and stabilization of the resting membrane potential during the development of fatigue, which serves to reduce intracellular calcium, decrease resting
tension and protect myocytes from damage. Therefore, loss of K_{ATP} function might be expected to result in failure to recover from fatigue, myofiber degeneration, and excessive calcium influx, as reflected in the inverted screen tests in the mice. In addition, lower limb hyperreflexia was observed in the four oldest individuals, which might be caused by the white matter abnormalities and decreased inhibition or increased activation of upper motor neurons.

Although the expression pattern of Kir6.2 is not identical to that of SUR2, it is notable that previous studies of Kir6.2-null mice, as well as mice in which an internal deletion in SUR2 was engineered (resulting in loss of full length SUR2 but persistent expression of a mitochondria-limited short form), have reported that the consequent decrease of myocyte K_{ATP} activity results in impaired exercise capacity and response, and myofiber damage.22,28 Only relatively minor histological abnormalities were observed in a single-skeletal muscle biopsy from patient 1–2. This is consistent with previous reports which showed that SUR2 knockout mice only exhibit significant histological abnormalities when subjected to significant and chronic exercise.29 Here, we show that novel mouse and zebrafish models, in which CRISPR/Cas9 was used to introduce frameshift mutations resulting in premature stop codons and nonfunctional subunits, also exhibit decreased performance. Specifically, SUR2-STOP mice showed a diminished ability to hang upside down during the multiple-trial inverted screen test, suggesting decreased strength resulting from increased fatigability (Fig. 4d, e), while SUR2-STOP zebrafish show decreased total overall movement and swimming speed in tracking studies (Fig. 6i–l).

Intelectual disability and neurological abnormalities: Neuropsychological testing revealed mild-to-moderate intellectual disability in all affected individuals, and anxiety was also reported in several patients. It is not obvious how myocyte K_{ATP} dysfunction could explain the intellectual disability or anxiety, and there are no reports of cognitive impairment in previous SUR2 mutant animal models. Neuronal K_{ATP} channels are predominantly formed of Kir6.2 with SUR1 subunits, although transcripts for all K_{ATP} channel subunits have been identified in various neuronal populations26 and SUR2 is reportedly expressed in both central and peripheral neurons.33–35, where it has been implicated in hippocampal sclerosis in aging and amyotrophic lateral sclerosis.34,35–38

However, recent data demonstrate that SUR2-containing K_{ATP} channels play a critical role in regulation of cerebral vascular architecture.16 In Cantú syndrome, MRI reveals diffusely dilated and tortuous cerebral blood vessels and white matter changes as multiple T2 subcortical or scattered hyperintensities. Transient white matter changes suggestive of a reversible posterior encephalopathic syndrome are reported in one patient. Several Cantú syndrome patients have migraine, and some have epilepsy. Developmental delay is common, although seeming to improve with age.16 Since K_{ATP} channel GoF results in chronic vasodilation and altered neuro-vascular coupling,16 it is conceivable that K_{ATP} loss of function may impact the cerebral vasculature in a way that results in impaired dynamic coupling of blood flow to match neuronal metabolic demand.

Patient 1-1 has a diagnosis of epilepsy with episodes of unconsciousness and tonic-clonic seizures, but no definitive epileptic activity has been demonstrated on EEGs. Patient 1-4 experienced an episode of coma with transient widespread white and gray matter abnormalities, which is left unexplained. Possibly, these incidents could be caused by focal or generalized circulatory alterations. Interestingly, white matter hyperintensities are observed in both Cantú syndrome and AIMS. In both cases, they could result from ischemic events due to dysregulated cerebral blood flow, although the cognitive phenotype seems to be more definite in AIMS than in Cantú syndrome. Tests for cognitive deficits and anxiety did not reveal significant behavioral impairments in SUR2-STOP mice (Fig. 5). Additional testing for specific deficits (such as an assessment of working memory, fear conditioning, or other nonspatial forms of learning and memory) would be necessary to more fully characterize the behavioral phenotype of the SUR2-STOP mice. Notably, mild cognitive impairment, as seen in humans, can be difficult to recognize in animals. Whole-genome sequencing and copy number analysis did not identify other causes of intellectual disability in our patients.

Cardiovascular manifestations: While the younger patients (aged 11–21) displayed no clear cardiovascular abnormalities, the two oldest patients display cardiac biventricular systolic dysfunction, with only slightly decreased ejection fractions. No clinical signs of heart failure were observed in patient 2–2, but decreased ejection fraction and heart failure was observed in her brother, patient 2–1. Cardiac MRI in patient 2–1 showed dilated ventricles, and together with other clinical findings this may suggest early stages of dilated cardiomyopathy. In a previous study by BieneTEGR et al., two individuals with dilated cardiomyopathy (DCM) were found to have heterozygous, LoF, mis-sense mutations in exon 38 of ABCC9, an exon only included in the SUR2A splice variant, and not in SUR2B.18 In that case, K_{ATP} LoF would be expected in cardiomyocytes and skeletal muscle but not smooth muscle, where SUR2B is expressed. The DCM patients reported in that study were older than the patients we report and displayed drastic reductions in ejection fraction (to
15–23%). This may suggest a progressive cardiomyopathy which will require longitudinal analysis in AIMS.

In previously reported mouse models, SUR2 knockout resulted in cardiac hypertrophy with LV dilation and decreased fractional shortening\(^22,40\) Here, we show that SUR2-STOP mice also display decreased fractional shortening and LV dilation (Fig. 4f, g), while SUR2-STOP zebrafish show marked cardiac enlargement with reduced ejection fraction and cardiac output (Fig. 7b–d), again consistent with the observed clinical phenotype in the older AIMS patients. Significant cardiomegaly, with elevated cardiac output, is observed in Cantú Syndrome. This cardiac remodeling may arise as a secondary compensation to counter the vasodilatory effect of K\(_{ATP}\) GoF in vascular smooth muscle cells\(^41,42\). SUR2 LoF, in contrast, would be expected to increase vascular...
contractility and blood pressure. However, blood pressure was normal in AIMS patients, except for elevated systemic pressures in the oldest patient. SUR2 and Kir6.1-null mice are hypertensive\(^\text{43,44}\), which is again a feature of the SUR2-STOP mice, but in humans, effects on blood pressure may be more subtle and subject to tighter regulation through homeostatic feedback, or perhaps not yet manifesting in our still relatively young patients.

Other features: Mild hearing loss was found in 3/6 patients. This is not straightforward to explain, as there is no known function for SUR2 in auditory neurons. One explanation might be altered vascular tone and ischemic stress of these neurons. Strabismus and myopia were found in several individuals while one also displayed nystagmus. Sensory assessment was not performed in our animal models but should be the subject of future study. Feeding difficulties in childhood were reported in all individuals. K\(_{\text{ATP}}\) channels are expressed in gastrointestinal smooth muscle\(^\text{45}\), and thus LoF may affect GI contractility and motility. The two patients in family 2 have short stature and microcephaly. In Cantú syndrome, there is a range of skeletal manifestations. Our patients are not thoroughly evaluated radiologically, but do not display obvious skeletal changes, except for scoliosis, congenital hip dysplasia, and elbow extension deficits in patient 2–2. Lastly, patient 1–1 presents with hyperprolactinemia lacking pituitary adenomas. Interestingly, pituitary adenomas and elevated levels of prolactin are recently reported in a family with Cantú syndrome\(^\text{46}\).

Disease progression: As discussed above, the two oldest patients exhibit decreased myocardial contractility, which may reflect the onset of a progressive cardiomyopathy, and hypertension is found in the oldest patient. It will be important to chronicle the long-term effects of K\(_{\text{ATP}}\) LoF in all the patients to elucidate the natural history of AIMS, which may also be informed by longitudinal studies in the animal models presented here. White matter hyperintensities were found to have increased with age in the two oldest patients, where MRIs have been repeated.

Heterozygous carriers: Lack of phenotype in the healthy heterozygous carriers of the ABCC9 c.1320 + 1G > A variant is consistent with functional studies of recombinant K\(_{\text{ATP}}\) channels reported here, which show that SUR2Δ8 subunits do not exert obvious dominant-negative effects in heterozygous expression with WT subunits. Indeed, essentially normal Rb fluxes in this case may reflect a rescue by WT subunits (Fig. 3c).

Possible additional molecular consequences: In addition to its canonical role as a regulatory subunit of plasmaemimal K\(_{\text{ATP}}\) channels, short-form variants of SUR2 have been identified in the mitochondria and reported to form functional K\(_{\text{ATP}}\) channels\(^\text{47}\), although the role of these truncated channel complexes remain poorly understood. It is also conceivable that SUR2 proteins play additional noncanonical (i.e., non-channel regulatory) roles, which may or may not be impaired by the exon 8 deletion. Importantly, the animal models employed here, in which premature stop codons were engineered into ABCC9, are not a bona fide recapitulation of the human genetics of the AIMS patients, and accordingly may not reiterate specific novel consequences of the truncated SUR2Δ8 protein.

Potential pharmacotherapy: In principle, K\(_{\text{ATP}}\) channel LoF could be countered using K\(_{\text{ATP}}\) channel activators, such as cromakalin, pinacidil, diazoxide or minoxidil, which are used clinically as vasodilatory agents\(^\text{47}\). In the case of the AIMS patients presented here, however, where the exon 8 deletion results in a complete loss of functional channel expression, these drugs would likely be ineffective. Interestingly, in each of the myocyte tissues in which SUR2 is expressed, K\(_{\text{ATP}}\) forms part of an electrical “brake”, where K\(_{\text{ATP}}\) activation results in membrane potential hyperpolarization, decreased intracellular calcium, and promotes myocyte relaxation. Loss of K\(_{\text{ATP}}\) function is therefore expected to increase intracellular calcium and modulating myocyte calcium handling might prove beneficial.

In the future, it is expected that AIMS patients will be identified with additional mutations in ABCC9. Such mutations may prove to be autosomal dominant or may exhibit only partial LoF, as is observed in the analogous pancreatic K\(_{\text{ATP}}\) disorder, congenital hyperinsulismus (CHI [MIM: 256450]), which arises from LoF mutations in ABCC8\(^\text{48}\). Depending on the mutational consequence, some CHI patients are responsive to treatment by the potassium channel opener diazoxide, and K\(_{\text{ATP}}\)-openers may indeed prove beneficial in some AIMS patients.

Methods

Patients. Four siblings from Family 1 and two siblings from Family 2 (Fig. 1a, pedigrees) were initially referred for assessment of developmental delay and/or neuromuscular impairment, and were investigated in a clinical diagnostic setting. Written informed consent for Next-Generation-Sequencing in both a diagnostic and research setting was obtained from the patients’ parents. The authors affirm that human research participants provided informed consent for publication of the images in Fig. 1b–d. Whole-genome sequencing on patients was approved by the Medical Ethical Committee of the University Medical Center Utrecht. All study participants, or their legal guardian, provided informed written consent prior to publication.

Clinical evaluation by the Neuromuscular Centre team. The clinical diagnostic evaluation included patient history, a structured neurological examination and physiotherapist examinations including the mini-Balance Evaluation System Test (miniBEST)\(^\text{49}\) and 6-min walk test. The miniBEST is an assessment of dynamic balance and postural control. There are no normative values for children or people with intellectual disability, but the test was modified for our patients. The 6-min walking test- 6MWD\(^\text{50,51}\) assesses distance walked over 6 min as a sub-maximal test of aerobic capacity/endurance. For adults, normative values can be calculated by an equation developed by Enright and Sherill (1998)\(^\text{52}\). The calculations are: men: 6MWD = (7.57 × height cm) − (5.02 × age) − (1.76 × weight kg) − 309; Women: 6MWD = (2.11 × height cm) − (2.29 × weight kg) − (5.78 × age) + 667). Normative values for children and adolescents are described by Geiger et al.\(^\text{53}\),
Normative values are not given for intellectually disabled people. The neurological examination comprised the following: cranial nerve examination, motor function (strength, tempo, tone), deep tendon reflexes (brachial, triceps, patella, achilles) and plantar reflexes, superficial skin sensation, vibration, proprioception, cerebellar function tests, and gait. In addition, the following supplementary electrophysiologic investigations were performed: electromyography (EMG), nerve conduction studies (NCS), repetitive nerve stimulation. All patients had previously undergone cerebral magnetic resonance imaging (MRI). Blood samples were analyzed for serum levels of CK and lactate. In addition, all patients had audiometry, electrocardiograms (ECG), and electrocardiography done.

**Illumina TruSight One gene panel analysis.** DNA libraries for sequencing of 4813 genes with known associated clinical phenotypes were generated for patients 1–2 and 2–1 (Fig. 1) with their respective parents for trio analyses. Genomic DNA was extracted from whole blood using MagExtractor DNA midi kit (Qiagen) on a Biorobot M48 (Qiagen) according to the manufacturer’s protocol. Sequencing libraries were prepared from 50 ng of gDNA using the TruSight One Sequencing Panel (Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. Paired-end 150 bp sequencing was performed on a MiSeq (Illumina). Desktop Sequencer, targeting a mean region coverage depth >100x and >97% of the region at >10x. Analysis of sequencing data was performed by applying a MiSeq Reporter Enrichment Workflow (v.2.4.6.60.8) including a Burrows–Wheeler Aligner (v.0.6.1-r104-tpx) and a Genome Analysis Toolkit (v.1.6-12-gec87b6), with the target region defined as the exon part with flanking 10 bp of 4813 disease-associated genes as defined by Illumina TruSight One Sequencing panel (http://www.illumina.com/products/tru Insight-one-sequencing-panel.html).

Cartagenia Bench Lab NGS (v.4) was used for variant annotation and prioritization, considering a recessive inheritance model using Cartagenia Inheritance Analysis, with the index patients defined as affected and the parents as unaffected. To focus the analysis on homoyxous or compound heterozygous variants inherited in a recessive pattern from both the parents, or variants showing non-Mendelian inheritance, indicating a de novo event. Variants were filtered based on quality criteria (LowGQ<10, LowVariantFreq <0.20, LowGQ < 30.00, R8 > 8, SB > -10, LowDP < 20) and their presence in population frequency databases with a maximum minor allele frequency <0.20, LowDP < 20. WGS statistics are provided in Supplementary Table 1.

**Whole-genome sequencing.** DNA libraries for Illumina sequencing were generated for patients 1–2 and 2–1 using standard protocols (Illumina) from 200–1 µg of genomic DNA. The libraries were sequenced with paired-end (2×150 bp) runs using Illumina HiSeq X Ten sequencers with a target depth of 30 x base coverage. The Illumina data were processed with our in-house developed pipeline v 2.5.1 (https://github.com/UCMC/Genetics/IAAP) including Genome Analysis Toolkit (https://software.broadinstitute.org/gatk/). Alignments were flagged as PASS only if they do not meet the following criteria: QD < 2.0, MQ > 40.0, FS > 60.0, HaploTypoScore > 13.0, MQRankSum > -12.5, ReadPosRankSum > -8.0, snpclusters > 3 in 35 bp. For indels: QD > 2.0, FS > 20.0, ReadPosRankSum > -20.0. WGS statistics are provided in Supplementary Table 1. Full analysis was performed on a merge of the reference coding regions obtained from Ensembl (v.75) (http://www.ensembl.org/) and UCSC (https://genome.ucsc.edu/) with ≥100 bp flanks. Cartagenia Bench Lab NGS v.4.3.5 was used for variant interpretation and prioritization. Variant analyses were made considering a recessive inheritance model using the Cartagenia cohort analysis tool. The focus of the analysis was to identify shared variants or shared genes with possible damaging, but not identical variants, amongst the two cases. Lenient prioritization was used considering multiple internal replicates (N≥3, n ≥ 4). The Mann–Whitney U test was used to determine statistical significance at a p-value < 0.05.

**Rudibium (85Rb) efflux assay.** Cosm6 cells were plated and transfected in 12-well plates at sufficient density to allow for ~75% confluence on the day of experimentation. Cells were loaded with radioactive 85Rb by incubation (≥6 h) with DMEM media spiked with 1 µCi ml-1 85RbCl (PerkinElmer) at 37 °C/5% CO₂. After isotope loading, cells were washed in Ringer’s solution which contained (in mM): 118 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 2.5 CaCl₂, and 1.6 MgSO₄, and was supplemented with 2.5 mg/ml oligomycin and 1 mM 2-deoxy-D-glucose to induce metabolic inhibition (MI), and incubated at room temperature for a further 10 min. Cells were washed and Ringer’s solution was added to each well, before being collected and replaced at the defined time points (2.5, 5, 12.5, 22.5, and 37.5 min). Cells were then lysed with 2% SDS to liberate remaining intracellular 85Rb and sample radioactivity was determined by scintillation counting.

Cumulative 85Rb efflux was calculated from the total counts from each time point. A time-dependent divergence from a mono-exponential efflux was observed, as noted previously58, and thus rate constants were derived from exponential functions fit to early time points only (2.5–12.5 min). kₐ and kₜₐp-independent efflux rate constants (kₑ) were obtained from GFP-only transfected cells using the equations:

\[
\text{Efflux} = 1 - e^{-k_e t},
\]

where kₑ was obtained from GFP-only transfected cells (Eq. (1)), and the number of active channels was assumed to be proportional to kₑ. Efflux-time data shown represent the mean ± SEM from at least three independent experiments with multiple internal replicates (N≥3, n ≥ 4). The Mann–Whitney U test was used to determine statistical significance at a p-value < 0.05.

**Patch clamp recording of recombinant channels.** Cosm6 cells were transfected with WT Kir6.2 (0.6 µg) and either WT SUR2A (1 µg), SUR2AA8 (1 µg), or a 1:1 ratio WT SUR2AA8, SUR2A (0.5 µg each) plasmid (Qiagen, katalognr 951356) on a Biorobot M48 (Qiagen) according to the manufacturer’s protocol. cDNA was produced using SuperScript™ VILO™ cDNA Synthesis Kit (Invitrogen). Two microliters of cDNA were used in a PCR reaction amplifying AARC9 exon 6–9 with primers as described in the Supplementary Methods. PCR products were subsequently verified by Sanger sequencing.

**Molecular biology and recombinant expression.** For recombinant protein expression and functional characterization, a 52 amino acid deletion (Ala386 to Gln437; Δ8) equivalent to the human exon 8 coding region was engineered into either a N-terminal Flag-tagged SUR2A construct from Cosm6 cells (Fl-F, GenBank accession no. D83958.1), a wild-type rat SUR2A construct (pCMV_SUR2A-WT; GenBank accession no. D83958.1) or a wild-type rat SUR2B construct (pCDNA, rsUR2B-WT; GenBank accession no. AF019628.1). The rat SUR2 sequence is 97% identical to human SUR2 and was used to allow for comparison with an extensive number of previous studies of recombinant SUR2. SUR2 cDNA (1 µg) was transiently transfected into Cosm6 cells (RRID: CVCL_8561) using Fugene 6 (Roche) alongside wild-type Kir6.2 (0.6 µg; pCDNA3.1, mkKir6.2; GenBank accession no. D50581.1). To mimic heterozygous expression (0.5 µg/0.5 µg), Western blot analysis and radioactive rudibium efflux assays were performed 48–72 h after transfection.

**Western blot analysis of recombinant SUR2.** Cosm6 cells were transfected with GFP alone, Kir6.2 with Flag-tagged SUR2A-WT or Kir6.2 with Flag-tagged SUR2AA8 (N-terminal Flag tag with amino acid sequence MDYKDDDDKGP was inserted prior to the SUR2A start codon). After 48 h, cells were harvested in phenol red-free paraformaldehyde fixed saline (PFA) (1:10), pelleted, and lysed in 50 µl of PBS containing 1% Triton X-100 (Sigma) by mixing for 1 h at 4 °C. Cell lysate was mixed with 2x SDS loading buffer, and was separated by electrophoresis using a precast 4–12% Bis-Tris Gel (NuPage). Gel contents were transferred to membranes using an iBlot 2 (Thermo-Fisher). Membranes were first incubated in a blocking solution (1% BSA in 0.1% PBST) for 1 h at room temperature (RT) with gentle shaking, before being washed (3x using PBS/TWEEN) and incubated in the primary horseradish peroxidase-conjugated anti-Flag antibody (Anti-Flag M2-HP-conjugate Sigma # A8592; 1 in 5000 in PBSTM), or an anti-a-actin antibody (clone C4 MilliporeSigma # MAB1501;1 in 3000 in PBSTM) for 1 h/RT. Anti-actin blots were washed (3x in PBSTM) and incubated with horseradish peroxidase-conjugated secondary antibody (Goat anti-mouse IgG antibody-HRP conjugate, Santa Cruz Biotechnology # sc-2005; 1 in 5000 in PBSTM) for 1 h at room temperature. A Pierce ECL Western Blotting Substrate kit was used for chemiluminescence detection; blots were analyzed and protein density quantified using ImageJ (NIH) and Excel (Microsoft). Actin and SUR2AA8 expression was normalized to values observed in WT SUR2A-Flag transfected cells in each independent experiment.
Symmetrical KINT solution which contained (in mM): 140 KCl, 10 HEPES, 1 EGTA (pH 7.4 with KOH) was used for pipette and bath solutions. ATP and pinacidil, when present, were at a concentration of 0.2 mM nucleotides and 1 mM MgCl₂, respectively. GTP was included at a concentration of 0.5 mM, yielding [MgGTP] = [MgATP].

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Generation of the SUR2 Lof mouse. All mouse studies were performed in compliance with the standards for the care and use of animal subjects as published in the NIH Guide for the Care and Use of Laboratory Animals, and were reviewed and approved by the Washington University Institutional Animal Care and Use Committee. SUR2-STOP mice were generated using CRISPR/Cas9-mediated genome engineering[9]. In mice in which an attempt was made to generate a Cantú Syndrome-associated missense mutation, an artefactual indel mutation in ABC9 (c.3446_3450delACTCTcinsGA) was identified in one founder individual (B6CBA F1) background, which results in the introduction of a premature stop codon (p. Y1148Stop). This founder (termed heterozygous SUR2-STOP) was viable and fertile and was crossed with C57BL/6 mice to generate F1 SUR2+/STOP mice. gDNA was isolated from one-cell stage zebrafish embryos. Each embryo was injected with 2 nl of solution containing ~12.5 ng of Cas9 mRNA and ~100 ng of single-stranded oligonucleotide. Injected embryos were grown to adulthood to generate SUR2+/STOP; SUR2+/STOP (heterozygous SUR2-STOP and wild-type and heterozygous littersmates, which were used for experiments.

Electrophysiological recordings of isolated myocytes. Ventricular myocytes were isolated from adult mice as previously described[24]. Inside-out patch clamp recordings were made in symmetrical pipette and bath KINT solutions which contained (in mM): 140 KCl, 10 HEPES, 1 EGTA (pH 7.4). Solution exchange was achieved using a Dynaflow Reverse perfusion chip (Flucell). MgATP and pinacidil (both Sigma Immunochemicals) were added to the perfusate as indicated. Prior to intracellular recordings, pipette electrodes were filled with KCl (140 mM) with the tip diameter being 1.1–1.2 μm. The resistance of the indel mutation in positive offspring. One positively identified SUR2+/STOP F1 mouse was selected, and further crossed against C57BL/6 to generate F2 SUR2+/STOP, SUR2+/STOP; SUR2+/STOP (hereafter referred to as SUR2-STOP) and wild-type and heterozygous littersmates, which were used for experiments.

STO mouse, heterozygote (Het) SUR2-STOP mouse, or a wild-type littermate control on the screen that was inclined to 60° with its head oriented down toward the base of the screen. After the mouse was stabilized on the screen, it was slowly inverted resulting in the mouse hanging upside down from the screen. Soft bedding was placed underneath the mouse to help protect it from injury. The time a mouse remained hanging upside down following inversion of the screen was recorded during an initial 3-min test trial. After the first trial, a mouse was placed into a holding cage for 5 min after which two more trials were conducted with 5 min being spent in the holding cage between trials. After completing the three trials, a mouse was placed back into the holding cage where it remained for 44 min, which was followed by the same 3 × 3-min test trial procedure described above. If a mouse remained hanging from the screen for the full 3 min duration it was removed from the screen and assigned a value of 180 s. The time a mouse remained hanging upside down from the screen (3-min maximum) was analyzed for each of the six trials as well as the total duration summed across all six trials.

Blood pressure recordings in SUR2-STOP mice. Arterial blood pressures were measured in 12-month-old SUR2-STOP mice and WT littermates anesthetized with sodium pentobarbital. The left common carotid artery was cannulated and a Millar pressure transducer advanced to the ascending aorta. Pressures were recorded using Powerlab data acquisition (ADInstruments), and mean arterial pressure (MAP) was calculated using LabChart 7 (ADInstruments).

Zebrafish maintenance. All zebrafish experiments were conducted under the guidelines of the animal welfare committee of the Royal Netherlands Academy of Arts and Sciences (KNAW) and were approved by the local ethics committee at the Royal Dutch Academy of Sciences (KNAW). Adult zebrafish (Danio rerio) were maintained at 28 °C and subjected to the 14-h light/10-h dark cycle. Embryos were produced by natural mating and kept in a humidified incubator at 28 °C[36]. All Crisp/Cas9 injections were performed in the wild-type strain Tubingen longfin.

CrisPR/Cas9 design and embryo injections. A target site within the abcc9 open-reading frame was chosen using CHOPCHOP (http://chopchop.cbu.ualberta.ca/). sgRNA template was purchased at Integrated DNA Technologies (IDT) as standard desalted same-day oligos and synthesis was carried out using the Ambion MEGAscript T7 or SP6 kits (Ambion). Purification of the in vitro synthesized mRNA was achieved with the RNasey Mini Kit (Qiagen)[42]. gRNA and Cas9-encoding mRNA were co-injected into one-cell stage zebrafish embryos. Each embryo was injected with 2 nl of solution containing ~12.5 ng of gRNA and ~100 ng of Cas9 mRNA. Newly hatched embryos were placed in 1× E3 solution to generate “founder” fish (F0), and screened for germline transmission of CrisPR-induced indel mutations using an adapted method for detecting simple sequence-length polymorphisms (SSLPs). Each putative founder adult fish was crossed with a wild-type adult fish (F1). Sequencing analysis confirmed successful introduction of 13n deletion and segregation in a Mendelian fashion. Homozygous fish (F2) were generated by inbreeding heterozygous mutant carriers.

Quantitative RT-PCR. Zebrafish larvae at 5 dpf were pooled in groups of 60 (three different groups for WT and four groups for homozygous mutants) and homogenized. RNA was purified using Trizol following standard procedures and cDNA synthesized using High-Capacity cDNA Reverse Transcription Kit. qPCR was performed using a StepOne Real Time PCR System (Applied Bioscience) and SYBR green primers and methods (Integrated DNA Technologies). Relative gene expression (2-ΔΔCt) was calculated by normalizing abcc9 expression to the mean expression observed in WT pools using Gapdh as a reference gene and the Pfaffl Method[45].

Isolation of adult zebrafish ventricular myocytes. For isolation of cardiomyocytes, 1-year-old animals were anesthetized by transfer into ice water before they were decapitated. After removal of the skin and opening of the pericardial sac, the hearts were harvested and both bulbus arteriosus and atrium were removed. Ventricles from three to four fish were pooled together and placed in an epi containing perfusion buffer: (mM) 10 HEPES, 30 Taurine, 5.5 Glucose, 10 BDM in PBS. Ventricular myocytes were obtained by enzymatic dissociation (perfusion buffer supplemented with 12.5 μM CaCl₂, 5 mg/ml collagenase II and IV) at 32 °C and 800 rpm for 40 min. Dissociation was ended by transfer to stopping buffer (perfusion buffer supplemented with 10% (vol/vol) FBS, 12.5 μM CaCl₂, 5 mg/ml BSA). Cells were dispersed by gentle trituration using a Pasteur pipette.
Electrophysiological recordings of isolated myocytes. Pipettes were made from soda lime glass microhemocytocap tubes (Kimble) and had a resistance of 1–2 MΩ when filled with intracellular solution. All recordings were made in an intracellular K+NT solution (mM) 140 KCl, 10 HEPES, 0.5 EGTA (pH 7.4 with KOH). ATP (Sigma) was applied as indicated. Free Mg2+ was maintained at 0.5 mM by supplementing ATP-containing solutions with MgCl2 where necessary. Currents were recorded at −50 mV holding and filtered at 1 kHz using an Axopatch 1D (Molecular Devices). KATP current was calculated as the difference between the current in the absence of nucleotides and in the presence of a fully inhibiting concentration of 5 mM ATP. The data were analyzed using Clampfit (Molecular Devices) and Excel (Microsoft), and are presented as individual replicates in scatter plots, alongside mean ± SEM.

Larval locomotor assay. The Viewpoint Zebrabox system (Viewpoint Behaviour Technology, Lyon, France) was used to evaluate larval locomotor activity in 5 dpf zebrafish. Larvae were transferred to a 48-well plate with 500 μl of embryo medium placed into the Zebrabox and subjected to a 30 min acclimatization period to the plate and Zebrabox environment. A total of 24 replicates (one embryo placed individually in each plate well per replicate) were used per genotype. Behavior was monitored at 28.5°C using a Zebrabox system (Viewpoint Behaviour Technology) consisting of a soundproof chamber with an infrared camera capable of recording 60 frames per second; analyses were performed using Zebralan locomotion tracking software (ViewPoint Behavior Technology). The integration period was set to 30 s for the 10-min duration of the experiment. A nontransparent background mode with a detection threshold of 20 was used and a minimum locomotion tracking software (ViewPoint Behavior Technology). The integration period was set to 30 s for the 10-min duration of the experiment. A nontransparent background mode with a detection threshold of 20 was used and a minimum

Heart extraction and hematoxylin and eosin (H&E) staining. Adult zebrafish hearts were dissected and fixed in 4% paraformaldehyde (in phosphate buffer with 4% sucrose). All recordings and subjected to paraffin embedding and sectioning at 10 μm intervals. Heart sections were stained with H&E following standard procedures. Six fishes were used for each genotype. Image acquisition was conducted using a Leica DM4000 B LED upright automated microscope.

TUNEL assay. TUNEL apoptosis staining was performed on 10 μm cryosections and detected using the in situ cell death detection kit from Roche (Mannheim, Germany) according to the manufacturer's instructions. Nuclei are shown by DAPI (4',6-diamidino-2-phenylindole) staining. Confocal images were acquired using a Leica SP8 confocal microscope.

Statistical analysis. Rubidium flux, patch clamp electrophysiology and echocardiography data were analyzed using two-tailed t-test or Mann–Whitney U test as described. For the inverted screen test of mice, data were analyzed using a repeated measures (rm) ANOVA model that contained one between-subjects variable (genotype) and two within-subjects variables (trials and sessions), followed by pairwise comparison by two-tailed t test with Bonferroni correction. Behavioral tests in mice were analyzed using a repeated measures (rm) ANOVA model that contained two between-subjects variable (genotype and sex) and one within-subjects variable (trial block). The data are presented as mean ± SEM.

For zebrafish studies sample size was not predetermined by statistical analysis. In all experiments involving zebrafish embryos, selection was random for scoring. Exact numbers of analyzed embryos are reported at relevant locations in the main text or figure legends. Statistical analysis was carried out with Prism (GraphPad). Distribution of the data sets was tested by D'Agostino–Pearson omnibus normality test. Depending on the outcome of this test, comparison of two conditions with each other was carried out using a Student’s two-tailed t test or Mann–Whitney two-tailed U test throughout the manuscript. All values are expressed as mean ± SEM.

Web resources. For CHOPCHOP, see http://chopchop.cbu.uib.no/; for Ensemble, see http://www.ensembl.org/; for ExAC Browser, see http://exac.broadinstitute.org/; for gnomAD, see https://gnomad.broadinstitute.org/; for OMIM, see https://www.omim.org/; for UCSC, see https://genome.ucsc.edu/.

Cardiac dimensions and function in SUR2-STOP zebrafish. The time interval between three heat beats was measured, and the heart rate (bpm) was calculated in triplicates. Sequential still frames from high-speed movies were used to outline the perimeter of the ventricle. Measurement analysis was carried out by “fit-to-ellipse” algorithm, which calculates the center of mass and subsequently the best fitting ellipse. The long axis length (a) and short axis length (b) at diastole and systole were determined and used to calculate ventricular end-systolic (ESV) and diastolic volumes (EDV) in triplicates applying the formula: V = 4/3πab2. The stroke volume was calculated as the difference between three ventricular EDVs and ESVs. Cardiac output was obtained by multiplying the heart rate with stroke volume. The percent shortening fraction (SF) was calculated using the formula: SF = (length at diastole – length at systole)/length at diastole) x 100. Fractional area change (FAC) was calculated as follows: FAC = (area at diastole – area at systole)/area at diastole) x 100. Measurements were performed blindly.

Cardinal vein blood flow velocity. Zebrafish larvae were mounted in lateral position at 5 dpf and the cardinal vein region behind the cloaca was imaged for 5 s at 28 ± 0.3°C. Vein blood flow velocity was calculated by assessing frame-by-frame motion of three single erythrocytes per fish determined from high-speed images to assess mean erythrocyte cell velocity (mm/sec) using ImageJ. Cardinal blood flow velocity was measured over ten frames at a video frame rate of 150 fps as non-pulsatile venous flow allows frame-by-frame analysis.

References


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Author contributions

Competing interests
The authors declare no competing interests.

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