De novo *ITPR1* variants are a recurrent cause of early-onset ataxia, acting via loss of channel function

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Abstract

We explored the clinico-genetic basis of spinocerebellar ataxia 29 (SCA29) by determining the frequency, phenotype, and functional impact of *ITPR1* missense variants associated with early-onset ataxia (EOA). Three hundred thirty one patients from a European EOA target cohort ($n = 120$), US-American EOA validation cohort ($n = 72$), and early-onset epileptic encephalopathy (EOEE) control cohort ($n = 139$) were screened for de novo *ITPR1* variants. The target cohort was also screened for inherited *ITPR1* variants. The variants' functional impact was determined by IP3-induced Ca^{2+} release in HEK293 cells. 3/120 patients (2.5%) from the target cohort and 4/72 patients (5.5%) from the validation cohort, but none from the EOEE control cohort, carried de novo *ITPR1* variants. However, most *ITPR1* variants (7/10 = 70%) in the target cohort were inherited from a healthy parent, with 3/6 patients carrying disease-causing variants in other genes. This suggests limited or no phenotypic impact of many *ITPR1* missense variants, even if ultra-rare and well-conserved. While common bioinformatics tools did not discriminate de novo from other *ITPR1* variants, functional characterization demonstrated reduced IP3-induced Ca^{2+} release for all de novo variants, including the recurrent c.805C>T (p.(R269W)) variant. In sum, these findings show that de novo *ITPR1* missense variants are a recurrent cause of EOA (SCA29) across independent cohorts, acting via loss of IP3 channel function. Inherited *ITPR1* variants are also enriched in EOA, but often without strong impact, albeit rare and well-conserved. Functional studies allow identifying *ITPR1* variants with large impact, likely disease-causing. Such functional confirmation is warranted for inherited *ITPR1* variants before making a SCA29 diagnosis.

Introduction

Spinocerebellar ataxia type 29 (SCA29) is caused by missense variants in *ITPR1* [1]. However, *ITPR1* contains large numbers of missense variants of unknown significance (VUS), demonstrating the need to identify variants with high functional impact amongst the multitude of *ITPR1* VUS before making a diagnosis of SCA29.

Demonstration of de novo occurrence is a powerful tool to strongly support pathogenicity of missense variants

against a background of manifold rare VUS [2]. In fact, de novo variants in *ITPR1* might represent a particularly frequent cause of ataxia, especially in early-onset ataxia (EOA) [3]. Understanding the variant characteristics and mechanism of action of disease-associated de novo variants in *ITPR1* might guide interpretation of the functional impact of *ITPR1* variants and inform the genetic diagnosis of SCA29. So far, however, neither the exact frequency nor specificity of *ITPR1* de novo variants have been thoroughly determined in larger ataxia and control populations, and their functional mechanism of action in SCA29 remains unclear.

Here we aimed to systematically determine the frequency, phenotype, genetic characteristics, and functional mechanism of de novo *ITPR1* missense variants in EOA in two independent cohorts from different continents. Moreover, we also aimed to determine the overall mutational burden of *ITPR1* variants in EOA, including inherited *ITPR1* variants. We hypothesized that (i) de novo *ITPR1*

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missense variants are a recurrent and specific cause of EOA across independent cohorts; (ii) inherited *ITPR1* missense variants might also be frequent, yet with absent or small functional or phenotypic impact; (iii) functional characterization of *ITPR1* de novo variants might help to identify those *ITPR1* variants with large effect size, thus facilitating the diagnosis of SCA29.

Materials and methods

Patients

Three cohorts of patients were aggregated. *Cohort #1: Target cohort.* $N = 120$ consecutive index subjects with unexplained EOA (age of onset <30 years), “sporadic” family history (i.e., no ataxia in previous or same generation), and negative for Friedreich’s ataxia repeat expansions, were recruited at the University Hospital, Tübingen, Germany, from 2010–2016. All patients originated from European, Mediterranean or Middle Eastern countries. *Cohort #2: Validation cohort.* This cohort was used to confirm the frequency of *ITPR1* de novo variants observed in the target cohort in an independent mixed-population cohort resident in North America. $N = 72$ index subjects with unexplained EOA and “sporadic” family history (same criteria as above), and availability of parental DNA for trio sequencing (to confirm de novo inheritance), were compiled from consecutive referrals to Ambry Genetics, Aliso Viejo, USA, from 2012–2016. *Cohort #3: Disease control cohort.* This cohort served to rule out that the occurrence of *ITPR1* de novo variants—observed in the target and the validation cohort—was a nonspecific finding, unrelated to ataxia. $N = 139$ index subjects with unexplained early-onset epileptic encephalopathies (EOEE; age of onset <3 years) were recruited via the EuroEPINOMICS-RES network. Only subjects with “sporadic” family history (criteria see above), and availability of parental DNA for trio sequencing (to confirm de novo occurrence) were included.

Genetic screening by WES and high-throughput panel sequencing

Sequencing. Subjects from cohort #1 were screened for *ITPR1* mutations by WES and/or large next-generation sequencing panels (>120 ataxia related genes); subjects from cohort #2 and cohort #3 were screened by trio WES (for methodological details, see Supplement 1). *Filtering.* Variants from the target cohort (cohort #1) were filtered for (i) non-synonymous heterozygous variants in *ITPR1*, with (ii) absence or extremely low frequency (minor allele frequency <0.02%) in the public

databases EVS6500, 1000Genomes project, and ExAC as well as in GENESIS (<11 heterozygous or homozygous alleles in 5996 subjects in GENESIS [4], (iii) at least moderate conservation (GERP score >2 OR PhastCons score >0.5), and (iv) at least moderate genotype quality (GQ [GATK] quality index >35; read depth >8X). All *ITPR1* variants identified in the index patients from cohort #1 were subsequently tested in available family members by Sanger sequencing, revealing whether they occurred de novo or were parentally inherited. Variants from the EOA validation cohort (cohort #2) and the EOEE disease control cohort (cohort #3) were filtered only for *ITPR1* de novo variants, as these two WES trio cohorts only served to confirm and control the frequency of *ITPR1* de novo variants identified in the target cohort. Identified *ITPR1* variants and associated phenotypes were submitted to the public archive ClinVar (URL: <https://www.ncbi.nlm.nih.gov/clinvar/>; accession numbers SCV000700178 - SCV000700190).

Exploratory mutational burden analysis

To further explore the significance of *ITPR1* missense variants in EOA, we performed an exploratory analysis to determine whether *ITPR1* missense variants are enriched in EOA compared to the general population. The frequency of rare, conserved *ITPR1* missense variants in the EOA target cohort was compared to their frequency in ~60,606 control exomes (=121,612 alleles) from the Exome Aggregation Consortium (ExAC [5], accessed 07/2016). Identical filter settings were used to filter both datasets.

In silico characteristics of de novo vs. general ITPR1 missense variants

We next analyzed whether bioinformatic in silico characteristics of de novo *ITPR1* variants differed from the in silico characteristics of inherited *ITPR1* variants and of *ITPR1* variants in the general population. To this end, all de novo *ITPR1* variants identified in this study (cohort #1 and cohort #2) or reported previously in the literature were aggregated. They were then compared to the inherited *ITPR1* missense variants (identified from cohort #1) and *ITPR1* missense variants from general population databases ExAc, EVS6500, and GENESIS (see Supplement 4) for common genetic in silico characteristics.

Impact of ITPR1 missense variants on IP3-induced Ca^{2+} release

ITPR1 encodes an IP3-receptor acting as a Ca^{2+} release channel, localized predominantly in membranes of endoplasmic reticulum (ER) Ca^{2+} stores [6, 7]. Based on this

Table 1 Genetic characteristics of the *ITPR1* missense variants identified in this study and published autosomal-dominant families

ID	Genomic	cDNA	Protein	EVS6500 alleles	ExAC alleles	PhastCons	GERP	Provean	Sift	PolyPhen (HumDiv)	CADD	Solved by variants in other gene	Segregation
<i>Missense variants in the target cohort</i>													
P1	chr3:4706969A>G	c.1702A>G	p.(R568G)	A = 12264	Not in db	1	3.37	-6.53	0.002	0.986	19.09		De novo
P1	chr3:4725441A>G	c.3430A>G	p.(M1144V)	A = 12118	G = 58/A = 121376	0.997	4.21	-0.98	0.42	0.002	7.427		Not segregating
P1	chr3:4821291G>T	c.6205G>T	p.(A2069S)	T = 2/G = 12284	T = 7/G = 122299	0.217	4.39	-2.92	0.052	0.747	20.2		Not segregating
P2	chr3:4687362C>T	c.805C>T	p.(R269W)	C = 12376	Not in db	0.999	1.41	-7.47	0	1	21.4		De novo
P7	chr3:4853028T>C	c.7208T>C	p.(L2403P)	T = 12314	Not in db	1	5.48	-6.69	0.001	1	24.3		De novo
P8	chr3:4716885C>T	c.2732C>T	p.(A911V)	T = 1/C = 12167	T = 38/C = 122178	0.249	3.77	-1.51	0.44	0.688	12.8		Not segregating
P9	chr3:4741524C>T	c.4408C>T	p.(H1470Y)	C = 12186	Not in db	0.475	5.7	-3.89	0.841	0.997	12.53	GAN	Not segregating
P10	chr3:4753474A>C	c.4998A>C	p.(E1666D)	A = 11712	Not in db	0.71	-6.42	-2.05	0.195	0.09	14.33		Not segregating
P11	chr3:4706873C>T	c.1606C>T	p.(L536F)	C = 12274	Not in db	0.996	4.56	-3.46	0.01	0.959	19.01	SYNE1	Not segregating
P12	chr3:4725445A>G	c.3434A>G	p.(D1145G)	G = 11/A = 12127	G = 198/A = 120846	0.901	4.21	-1.39	0.113	0.485	10.29	ATM	Not segregating
<i>De novo variants in the validation cohort</i>													
P3	chr3:4687357C>T	c.800C>T	p.(T267M)	C = 12414	Not in db	0.703	4.97	-5.6	0	1	24.8		De novo
P4	chr3:4856866_4856868delAAG c.7687_7689delAAG	c.7687_7689delAAG	p.(K2563del)	A = 12872	Not in db	1	4.84	-12.02	N/A	0.999	26.1		De novo
P5	chr3:4687293G>A	c.736G>A	p.(E246K)	G = 12558	Not in db	0.96	4.97	-3.73	0.001	1	35		De novo
P6	chr3:4687362C>T	c.805C>T	p.(R269W)	C = 12376	Not in db	0.999	1.41	-7.47	0	1	21.4		De novo
<i>Published variants with autosomal dominant inheritance</i>													
	chr3:4709151A>G	c.1804A>G	p.(N602D)	A = 12200	Not in db	0.551	4.74	-4.61	0.002	0.979	18.65		Segregating (AD) [1], de novo [22]
	chr3:4725156C>T	c.3221C>T	p.(P1074L)	C = 12330	Not in db	0.994	4.5	-7.77	0.002	0.986	28.1		Segregating (AD) [23]
	chr3:4747877G>A	c.4657G>A	p.(V1553M)	G = 12302	Not in db	1	5.36	-1.23	0.306	1	20.5		Segregating (AD) [1]
	chr3:4821268A>G	c.6182A>G	p.(E2061G)	A = 12212	Not in db	1	5.28	-6.81	0	1	28.5		Segregating (AD) [8]

Variants were filtered from the Genesis database using the following filter criteria: MAF EVS < 0.02%, < 11 families in Genesis, GERP > 2 or PhastCons > 0.5, depth > 8, GQ (GATK) > 35. cDNA/protein positions refer to transcript ENST00000423119/NM_001099952.1 and Q14643-3 respectively. Annotation was obtained from SeattleSeq (<http://snp.gs.washington.edu/SeattleSeqAnnotation138/>). De novo variants are shown in bold. AD, autosomal-dominant.

Fig. 1 Pedigrees of families with de novo or inherited *ITPR1* missense variants from the target cohort, and cerebral MRI of *ITPR1* de novo patients.

A Pedigrees. Patient P1 carried 3 *ITPR1* variants, two of which were inherited paternally and arranged in cis, while the third variant (c.6205G>T) was de novo. Phasing between the de novo variant and the two paternally inherited variants cannot be determined from the available data. Three families (P9/11/12) carried *ITPR1* variants inherited by one parent in addition to biallelic variants in another ataxia gene (*GAN*, *SYNE1*, *ATM*) that fully explained the phenotype. Variants for which the phase cannot be determined are separated by a dash; dotted lines separate variants located on different chromosomes.

B Sagittal (i, iii, v) and parasagittal (ii, iv, vi) T1 cerebral magnetic resonance imaging (MRI) shows mild atrophy of the anterior cerebellar cortex with enlarged spaces between foliae (particular pronounced in patient P1, ii), and of the cerebellar vermis in patients P1 (i, ii) and P2 (iii, iv), but no cerebellar atrophy in patient P7 (v, vi).

well-established role of *ITPR1*, the functional effect of *ITPR1* variants was investigated by assessing their impact on IP3-induced Ca^{2+} -release in stable, inducible HEK293 cell lines that were constructed to express either one of these *ITPR1* variants or wild-type. IP3-induced Ca^{2+} release was determined by loading the HEK293 cells with an ER luminal Ca^{2+} dye (mag-fluo-4 AM), and measuring Ca^{2+} release upon increasing concentrations of IP3 (10–1000 nM) which were consecutively added to the cells (for methodological details, see Supplement 2). We established HEK293 cell lines for 4 exemplary paradigmatic *ITPR1* variants: one de novo variant from the target cohort (c.1702A>G (p.(R568G))), one de novo variant from the validation cohort (c.800C>T (p.(T267M))), one de novo variant present in both cohorts (c.805C>T (p.(R269W))), and one inherited variant with high in silico prediction scores (c.1606C>T (p.(L536F))).

Results

Frequency and specificity of de novo *ITPR1* missense variants

We identified 10 *ITPR1* missense variants in a total of 8 of 120 index patients from the EOA target cohort (cohort #1), yielding an *ITPR1* missense carrier frequency of 6.6% (8/120) (Table 1). One patient (P1) carried 3 different *ITPR1* variants (Fig. 1a). While 7/10 *ITPR1* variants (70%) were parentally inherited, the remaining 3/10 variants (30%) were de novo, yielding a de novo carrier frequency of 2.5% (3/120) in our EOA target cohort. Out of the 72 index patients from the EOA validation cohort (cohort #2), four subjects carried a de novo *ITPR1* variant (4/72 = 5.5%) (Table 1), thus confirming the carrier frequency observed in the target cohort. No de novo *ITPR1* variant was found in

Table 2 Clinical, imaging, and electrophysiological features of de novo *ITPR1* ataxia

	P1	P2	P3	P4	P5	P6	P7
cDNA	c.1702A>G (de novo) c.3430A>G c.6205G>T (not de novo)	c.805C>T	c.800C>T	c.7640_7642delAGA	c.736G>A	c.805C>T	c.7208T>C
Protein	p.(R568G) (de novo) p.(M1144V) p.(A2069S) (not de novo)	p.(R269W)	p.(T267M)	p.(K2563del)	p.(E246K)	p.(R269W)	p.(L2403P)
Origin/gender	Germany/f	Germany/f	USA/m	USA/m	Malaysia/f	USA/m	Germany/m
SARA score	13	12 at age 18 (14.5 at age 10 years)	N/A	N/A	N/A	36 (not able to walk)	9 at age 12 (11 at age 10 years)
Progressive/non-progressive	np	np	N/A	N/A	np	np	np
Age of onset (years)	1	1	<2	<2	<1	1	1
First clinical symptom	Delayed motor development	Delayed motor development	N/A	N/A	Hypotonia	Delayed motor development, nystagmus and hypotonia	Delayed motor and mental development
Age at last examination (years)	10	18	3.5	2	6	3	12
Disease duration (years)	9	16	2	1	6	2	11
Phenotype category	Infantile onset ataxia	Infantile onset ataxia	Infantile onset ataxia	Infantile onset ataxia	Infantile onset ataxia (hypotonia, dystonia)	Infantile onset ataxia (nystagmus, hypotonia)	Infantile onset ataxia
Cerebellar ataxia; age of onset (years)	+, 1	+, 1	+, <2	+, <2	+, <1	+, 1	+, 1
Cognitive impairment (formal testing)	—, IQ 97	Isolated dyscalculia	N/A	N/A	N/A	+, Single words of extremely limited repertoire	+, SON R test score 81; single words of limited repertoire
Pyramidal features	—	—	N/A	N/A	N/A	—	—
Muscle tendon reflexes	Normal	Normal	N/A	N/A	N/A	Normal	Normal
Epilepsy	—	—	N/A	N/A	—	—	—
Oculomotor apraxia	—	Slowing of horizontal saccades (COMA)	N/A	N/A	—	—	—
Myoclonus	—	Yes, extremities and trunk	N/A	N/A	N/A	—	—
Iris hypoplasia	—	—	—	+, Total aniridia	—	—	—
Additional clinical features	Divergent strabismus	Convergent strabismus	—	—	Strabismus	Strabismus	—

Table 2 (continued)

	P1	P2	P3	P4	P5	P6	P7
MRI brain	Mild cerebellar atrophy (vermis)	Mild cerebellar atrophy (vermis)	N/A	N/A	No cerebellar or cortical atrophy	Mild cerebellar atrophy	No cerebellar or cortical atrophy
NCS (sural/tibial)	Normal	Normal	N/A	N/A	N/A	Normal	Normal
Motor evoked potentials	N/A	Normal	N/A	N/A	N/A	N/A	Normal

Oculomotor apraxia was defined as impairment in initiating voluntary saccades, often accompanied by compensatory head thrusting. It was in this case congenital (congenital oculomotor apraxia = COMA [9]). SON-R 2½-7test = standardized non-verbal intelligence test, developed by P.J. Tellegen, J.A. Laros and F. Pete
m male, *f* female, + present, – absent, *SARA* scale for the assessment and rating of ataxia, *MRI* magnetic resonance imaging, *NCS* nerve conduction studies, *N/A* information not available, *np* nonprogressive

the 139 patients from the EOEE disease control cohort (cohort #3) (0/139 = 0%). This demonstrates that the frequency of *ITPR1* de novo variants observed in the two EOA cohorts cannot be attributed to a general high baseline prevalence of *ITPR1* de novo variants, unrelated to ataxia ($p = 0.046$, Fisher's exact test, 2-sided).

Phenotype of patients with *ITPR1* de novo variants

All 7 EOA patients with *ITPR1* de novo variants (3 from cohort #1; 4 from cohort #2) presented with infantile onset cerebellar ataxia starting before the age of 2 years, including delayed motor milestones (Table 2). Cognitive deficits of variable degree were observed in 3 out of 4 patients where this information was available, reaching from only mild dyscalculia (P2) to severe intellectual disability with a speech vocabulary of only a few words (P7 at age 12 years). In contrast, patient P1 showed normal intelligence with an IQ of 97. Aniridia was noted only in 1/7 patients (P4), demonstrating that the “Gillespie syndrome” [7, 8] is a relatively infrequent presentation of *ITPR1* variants. In contrast, 4/7 patients showed strabismus (Table 2), suggesting that it is a frequent feature of de novo *ITPR1* ataxia. One patient (P2) also revealed congenital horizontal oculomotor apraxia (COMA [9]), i.e., slowed initiation and performance of horizontal saccades. The oculomotor apraxia slowly ameliorated throughout the following disease course, but still precludes her from obtaining driving license at her current age of 18 years. P2 also showed limb action myoclonus throughout all prospective longitudinal assessments, thus extending the movement disorder spectrum associated with *ITPR1* ataxia. In contrast, none of the patients identified here showed evidence for other non-ataxia signs that are frequently observed in other EOAs [10, 11] such as epilepsy, pyramidal tract affection or peripheral neuropathy (Table 2). Cerebellar atrophy was seen only in 2 out of 5 patients where MRI was available (Fig. 1b, Table 2), suggesting that it might not be an obligate feature of *de novo ITPR1* ataxia, at least not early in the disease course. One patient (P6) showed no cerebellar, but cerebral atrophy, suggesting that not only cerebellar, but also cerebral cortical neurons are susceptible to *ITPR1* dysfunction (for further discussion on the MRI findings and the clinically non-progressive disease course, see Supplement 3).

An enrichment of *ITPR1* variants in EOA

Our exploratory mutational burden analysis showed an enrichment of *ITPR1* missense variants in the EOA target cohort compared to the general population (odds ratio 4.27, $p = 0.0002$, 95% confidence interval 2.013–8.014), which prevailed even after removing the *ITPR1* de novo variants (odds ratio 2.95, $p = 0.01181$, 95% confidence interval

1.170–6.188). At least some of the inherited *ITPR1* variants might therefore possibly contribute to the EOA phenotype, acting e.g., as risk factors or low-penetrance alleles (for a detailed presentation of the results and further discussion see Supplement 5).

Characteristics of inherited *ITPR1* missense variants and of *ITPR1* missense variants in the general population

We next explored the 7 inherited *ITPR1* variants from cohort #1 in more detail. In line with the inclusion criteria of only *sporadic* index patients, all parents of the patients with inherited *ITPR1* variants were healthy, without even mild cerebellar signs, demonstrating lack of a clinical phenotype for these 7 inherited variants in the parental generation. 3/6 patients with an inherited *ITPR1* variant carried damaging biallelic variants in other well-established EOA genes (*SYNE1*, *GAN1*, *ATM*; Table 1 and Fig. 1a) which fully explained the phenotypic presentation. The subject carrying two inherited *ITPR1* variants in addition to one de novo variant (patient P1) did not show a more severe disease phenotype or progression than the subjects carrying only one de novo variant. Rather, her phenotype was similarly mild as the phenotype seen in patient P2 and she even improved over time. Compared to the severely affected patient P7 her phenotype was much less severe, indicating that the two inherited *ITPR1* variants additionally identified in P1 have no major effect on the phenotype. Taken together, these findings suggest that the majority of the inherited *ITPR1* missense variants likely have minimal or no impact on the clinical phenotype.

Screening of >70,000 WES from the three general population databases identified 550 rare, well conserved *ITPR1* missense variants (see Supplement 4), supporting the notion that many of these variants might indeed be associated with a minimal or no direct effect on a particular clinical phenotype. Common bioinformatics tools did not allow to distinguish the inherited *ITPR1* variants from cohort #1 and the *ITPR1* variants observed in the general population (many of them likely with limited or no direct effect) from *ITPR1* de novo variants (most of them likely with a strong effect). All *de novo* variants, but also many of these other variants were: (i) very rare or even private according to ExAC, EVS, and Genesis; (ii) highly conserved according to PhastCons or GERP scores (Fig. 2c); (iii) predicted to be “deleterious” by commonly used in silico prediction tools like PolyPhen2, PROVEAN, SIFT [12] and CADD scores (Combined Annotation Dependent Depletion) [13] (Fig. 2b); and (iv) located in functional domains like e.g., the MIR, RIH or Ion-TM domains (Fig. 2a). This suggests that while bioinformatics tools might be helpful to discard those *ITPR1* missense variants

which likely have no effect (namely variants which are frequent, not conserved, and predicted to be tolerated), they do *not suffice in themselves* to demonstrate whether a certain variant has a sufficiently deleterious effect to cause a phenotype or not.

Functional effect of *ITPR1* missense variants on IP₃-induced Ca²⁺ release

We investigated the functional effect of selected *ITPR1* variants from both the EOA target cohort (cohort#1) and EOA validation cohort (cohort#2) on IP₃-induced Ca²⁺ release in HEK293 cells. Compared to wildtype, all three HEK293 cell lines expressing a *de novo* *ITPR1* variant (c.800C>T (p.(T267M)); c.805C>T; (p.(R269W)) or c.1702A>G (p.(R568G))) showed a markedly reduced fractional Ca²⁺ release (i.e. smaller drop in the steady-state ER Ca²⁺ level) upon induction by IP₃ (Fig. 3a, b) ($p < 0.001$). This demonstrates a (strong) loss-of-function effect as the mechanism of action of these de novo *ITPR1* variants. In contrast, the IP₃ response of the HEK293 line expressing the inherited *ITPR1* variant (c.1606C>T (p.(L536F))) did not differ from wildtype ($p = \text{n.s.}$), yet differed significantly from the 3 de novo *ITPR1* variants ($p < 0.001$) (Fig. 3a, b). Thus, functional analyses can help to disentangle those *ITPR1* missense variants with a large effect size from those *ITPR1* variants with no or only minimal effect on Ca²⁺ release.

Discussion

De novo variants in *ITPR1*/SCA29 are a recurrent cause of EOA, not observed in other early-onset neurological disease

Here we report the first systematic and largest screening series of *ITPR1* patients in several independent cohorts. We show that de novo missense variants in *ITPR1*/SCA29 account for a substantial share of patients with so far unexplained sporadic ataxia, yielding an estimated frequency of 2.5% to 5.5% of sporadic EOA in Europe and North America. The high number of de novo variants identified in our EOA cohorts (seven de novo mutations, four of them reported here the first time) demonstrates that not only autosomal-recessive variants, but also de novo variants in SCA genes need to be appreciated as a recurrent cause of EOA. Our finding receives particular support from its validation in a second, independent large screening cohort, and from the use of a specific neurologic control cohort (EOEE) where a high share of de novo variants can generally be expected [14]. Exploiting this disease control cohort, we show that the increased frequency of *ITPR1* de

novo variants is not due to a ubiquitous high frequency of de novo variants in *ITPR1* in neurologic populations, unrelated to ataxia. At the same time, this finding shows that—unlike many other de novo variants in ataxia genes, which might also present with prominent epileptic encephalopathy phenotypes (e.g., *KCNA2* [15, 16] or *KCNB3* [17])—de novo variants in *ITPR1* do not seem to present a common cause of epileptic encephalopathy. This is also of

particular interest as epileptic seizures are a major phenotypic feature in the *mouse* model of *ITPR1* [18].

Loss of channel function: a common mechanism of *ITPR1* de novo missense variants causing SCA29

The mechanism of action of *ITPR1* variants causing SCA29 has not yet been systematically studied in a larger series.

Fig. 2 **A** Schematic representation of the *ITPR1* structure and location of *ITPR1* variants. *ITPR1* transcript variant NM_001099952/ENST00000423119 encodes a protein of 2710 amino acid residues length (Q14643-3). The *ITPR1* protein contains several conserved protein domains (NCBI conserved domains database): an inositol 1,4,5-trisphosphate/ryanodine receptor domain (Ins145, aa4-225) for inositol 1,4,5-trisphosphate binding, a MIR domain (aa 232-433), that conveys ligand transferase function, two RIH domains (aa 472-677, aa1176-1355), a ryanodine receptor homology domain (RIH_assoc, aa1923-2035) and a transmembrane ion transport domain (Ion-TM, aa2235-2550) containing six putative transmembrane motifs. Both de novo (red circles and stars) and autosomal dominant (AD, yellow circles and stars) variants associated with ataxia are spread over most part of the protein; the variant associated with the unique phenotype (infantile onset non-progressive ataxia, pontocerebellar hypoplasia) described by van Dijk et al. [21] is marked by a blue border. Missense variants associated with infantile onset ataxia without iris hypoplasia (marked by circles) tend to cluster in the N-terminal part of the protein, preferentially the Ins145 domain, the MIR domain, and the N-terminal RIH-domain. In contrast, variants leading to ataxia complicated by iris hypoplasia (Gillespie syndrome, marked by stars) exclusively locate to the C-terminus. However, as also shown here, not *all* variants at this C-terminal end include iris hypoplasia (see red circle in Ion-TM domain, indicating patient P7). All *ITPR1* variants reported to cause autosomal recessive Gillespie syndrome are truncating in nature (orange stars or circles). All but one variant are associated with infantile onset ataxia; the autosomal-dominant variant p. (P1074L) that reportedly causes adult onset ataxia is circled by a gray border. Rare conserved variants reported in public databases (grey dots; EVS, ExAC, Genesis) are equally spread over the whole protein and don't appear to spare certain functional domains. **B, C** Rare conserved variants were extracted from public databases (EVS, ExAC, Genesis) and annotated with four established scores predicting the deleteriousness of variants (PROVEAN, SIFT, PolyPhen-2, CADD) **B** and two scores evaluating the conservation (GERP, PhastCons). The 'deleterious' range of each in silico prediction score is marked by an orange shaded region (cutoff PROVEAN < -2.5, SIFT < 0.05, PolyPhen-2 > 0.85, CADD > 15). Although most de novo (red circles) and AD (yellow circles) variants cluster in the 'deleterious' range for each score, none of the scores discriminates well between ataxia-associated variants and variants retrieved from public databases (gray dots). **C** The same holds true for the conservation scores (GERP, PhastCons). Although most de novo (red circles) and AD (yellow circles) variants cluster in the highly conserved range for each score, neither one of the scores per se nor the combination thereof discriminates well between ataxia-associated variants and variants retrieved from public databases (gray dots). Please note that no variants were shown in the low range of the conservation scores (lower left quarter) as we only filtered for variants with a score PhastCons > 0.5 OR GERP > 2. **D** De novo and AD variants associated with ataxia tend to cluster at the extreme end of both the SIFT and PolyPhen-2 score. We therefore evaluated whether a combination of both scores can be used to predict deleteriousness of *ITPR1* variants. While a 'tolerated' prediction in SIFT and polyphen-2 are a strong indicator against pathogenicity of an *ITPR1* missense variant, 'deleterious' predictions in these two scores do not reliably discriminate de novo *ITPR1* variants from *ITPR1* missense variants in public databases

Our findings demonstrate that loss of the IP₃ channel function, demonstrated by impairment of the IP₃-induced endoplasmic reticulum Ca²⁺ release, is a common mechanism associated with *ITPR1* variants causing SCA29, here observed in all 3 of 3 de novo variants that were analyzed, including the recurrent R269W variant which we

and others [19, 20] have identified in different cohorts. This loss of IP₃ channel function could very well be a result of a dominant-negative mode of action of mutant IP₃ subunits on the tetrameric IP₃ channel, as speculated by McEntagart et al. [8]. This does, of course, not exclude the possibility that also gain of IP₃ channel function might lead to SCA29 in some cases with other *ITPR1* variants.

Our findings might help to guide future pharmacological therapies for SCA29, prioritizing those compounds that enhance the sensitivity of endoplasmic reticulum Ca²⁺ release and thus compensate for the loss of channel function effect of SCA29-associated *ITPR1* variants.

Extending and specifying the phenotype of de novo *ITPR1* ataxia

Our phenotypic findings show that the onset of de novo *ITPR1* ataxia is limited to the *infantile* end of the EOA disease spectrum (i.e., it does not start at >2 years of age) and is frequently complicated by cognitive deficits. This phenotypic combination makes it less likely that affected subjects will reproduce, which might explain why the majority of the published cases with *ITPR1*-associated ataxia are de novo, while descriptions of autosomal dominant pedigrees are relatively sparse.

Moreover, our phenotypic results show that the oculomotor presentation of *ITPR1* can also encompass congenital oculomotor apraxia, elaborating on a previous report of de novo *ITPR1* patients [3]. Our findings thus extend the genetic basis of the well-recognized syndrome of "congenital oculomotor apraxia" (COMA [9]; OMIM #257550), where the genetic background beyond Joubert syndrome had still remained largely elusive [9].

Phenotype-genotype relations in *ITPR1* de novo ataxia

Our findings confirm the classic Gillespie cluster (=EOA plus aniridia; OMIM #206700) as a striking (albeit overall relatively infrequent; 1/7 = 14% of the cases) phenotype of *ITPR1* variants (patient P4) [7, 8]. In fact, P4 carried the same variant as observed previously in four independent subjects with this particular phenotypic cluster (c.7687_7689delAAG (p. (K2563del)), referred to as NM_001168272.1:c.7786_7788delAAG, p.Lys2596del in [8]) [7, 8], thus indicating a relatively high degree of genotype-phenotype correlation of this particular variant with the classic Gillespie cluster. Variants associated with the Gillespie syndrome cluster at the C-terminus at protein positions >2000, with predilection for the transmembrane ion transport domain (see Fig. 2a). However, as also shown here, not *all* variants at this C-terminal end are associated with iris hypoplasia (see patient P7, Fig. 2a). In contrast to

Fig. 3 IP₃-induced Ca²⁺ release in HEK293 cells expressing *ITPR1* wildtype versus *ITPR1* variants. **A** Stable, inducible HEK293 cells expressing the *ITPR1* wildtype (WT) or *ITPR1* variants were loaded with an ER luminal Ca²⁺ dye (mag-fluo-4). Fluorescence intensity of mag-fluo-4 loaded cells expressing ITPR1-WT (black trace), c.800C>T (p.(T267M)) (red); c.805C>T (p.(R269W)) (pink), c.1606C>T (p.(L536F)) (orange); or c.1702A>G (p.(R568G)) (green) was measured continuously before and after repeated additions of various concentrations of IP₃ (10, 30, 100, 300, and 1000 nM), followed by addition of 1000 nM IP₃ plus 10 μ M tBHQ. The steady-state mag fluo-4 signal before the first addition of IP₃ was taken as the maximum ER Ca²⁺ level (F_{max} , 100%), whereas, the steady-state mag fluo-4 signal after the addition of IP₃ (1000 nM) plus tBHQ (10 μ M)

was taken as the minimum ER Ca²⁺ level (F_{min} , 0%). Compared to WT, all three HEK293 cell lines expressing a *de novo* *ITPR1* variant (c.800C>T (p.(T267M)); c.805C>T; (p.(R269W)); or c.1702A>G (p.(R568G))) showed a reduced fractional Ca²⁺ release (i.e., smaller drop in the steady-state ER Ca²⁺ level) upon induction by IP₃. In contrast, the IP₃ response of the HEK293 cells expressing the inherited *ITPR1* variant (c.1606C>T (p.(L536F))) did not differ from wildtype ($p = n.s.$), yet differed significantly from the three *de novo* *ITPR1* variants ($p < 0.001$). **B** The steady-state ER Ca²⁺ levels (%) at different cumulative concentrations of IP₃ in HEK293 cells expressing *ITPR1* WT or variants were determined and normalized to the maximum ER Ca²⁺ content ($F_{max} - F_{min}$). Data shown are mean $\pm$ SEM ($n = 4-6$) (* $P < 0.001$ vs WT or c.1606C>T (p.(L536F)))

the variants associated with the Gillespie cluster, variants associated with congenital ataxia *without* iris hypoplasia tend to cluster in the N-terminal part of the protein (preferentially the Ins145 domain, the MIR domain, and the N-terminal RIH-domain, see Fig. 2a).

Apart from this partial genotype–phenotype association, phenotypic variability in de novo *ITPR1* ataxia can be substantial. Two subjects carried an identical variant (c.805C>T; p.(R269W)), but showed very different phenotypes and disease severity (P2: mild ataxia, isolated mild cognitive deficits, mild vermian cerebellar atrophy; P6: severe ataxia, severe mental retardation; no cerebellar atrophy).

Functional confirmation is warranted to disentangle disease-causing *ITPR1* missense variants

Although rare and well conserved *ITPR1* missense variants are enriched in EOA, we here show that they also frequently occur in the general population (~1%). Caution therefore should be used in claiming pathogenicity of *ITPR1* missense variants and, consequently, in diagnosing SCA29. It demonstrates the need to disentangle those *ITPR1* missense variants with a large effect on channel function (and thus high likelihood to produce a phenotype) from those *ITPR1* missense variants with no or only minimal effect-size (and thus likely without direct contribution to the phenotype).

As shown here, commonly used *in silico* criteria (e.g., variant frequency, evolutionary conservation, domain location, or combinations thereof) alone do not suffice to distinguish between these types of *ITPR1* missense variants. Correspondingly, these bioinformatic criteria did also not allow to distinguish the three (de novo) *ITPR1* missense variants functionally shown to have a large effect-size from the *ITPR1* missense variant (c.1606C>T (p.(L563F))) shown to have no effect. In contrast, this effect difference could be well demonstrated by a functional analysis of the IP₃-induced Ca²⁺ release.

In conclusion, we suggest that, at least for inherited *ITPR1* variants, functional confirmation of the variant effect on IP₃ channel function, e.g., demonstrated by alterations of IP₃-induced Ca²⁺-release from the ER, should be obtained before making a SCA29 diagnosis and should thus become state-of-the-art. The functional impact observed in *ITPR1* de novo variants (strong loss of channel function) might thereby serve as a paradigmatic functional pattern to identify those *ITPR1* variants with large effect sizes.

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Compliance with ethical standards

Conflict of interest MS received consulting fees from Actelion Pharmaceuticals Ltd. KLH, EP and ST are employed by Ambry Genetics. IK-M received consulting fees from Actelion Pharmaceuticals Ltd. PB is Chief Operating Officer at Centogene AG, Rostock, since January 2016. This company has no direct market-related interests in this study and was not involved in any parts of this study. The other authors declare that they have no conflict of interest.

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