**RESEARCH ARTICLE**

**SYNE1 ataxia is a common recessive ataxia with major non-cerebellar features: findings from a large-scale multi-center screening**

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Abbreviations:

ARCA, autosomal recessive cerebellar ataxia; HSP, hereditary spastic paraplegia; pCA, pure cerebellar ataxia; CA plus, cerebellar ataxia plus; WES, whole exome sequencing, NGS, next generation sequencing; CNV, copy number variation.

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# Abstract (287 words)

Mutations in the synaptic nuclear envelope protein 1 (*SYNE1*) gene have been reported to cause a relatively pure, slowly progressive cerebellar recessive ataxia mostly identified in Quebec, Canada. Combining next-generation sequencing techniques and deep-phenotyping (clinics, MRI, PET, muscle histology), we here established the frequency, phenotypic spectrum and genetic spectrum of *SYNE1* in a screening of 434 non-Canadian index patients from 7 centers across Europe. Patients were screened by whole-exome sequencing or targeted panel sequencing, yielding 23 unrelated families with recessive truncating *SYNE1* mutations (23/434=5.3%). In these families, a total of 35 different mutations were identified, 34 of them not previously linked to human disease. While only 5/26 patients (19%) showed the classical *SYNE1* phenotype of mildly progressive pure cerebellar ataxia, 21/26 (81%) exhibited additional complicating features, including motor neuron features in 15/26 (58%). In three patients, respiratory dysfunction was part of an early-onset multisystemic neuromuscular phenotype with mental retardation, leading to premature death at age 36 years in one of them. PET imaging confirmed hypometabolism in extra-cerebellar regions like the brainstem. Muscle biopsy reliably showed severely reduced or absent SYNE1 staining, indicating its potential use as a non-genetic indicator for underlying *SYNE1* mutations.

Our findings, which present the largest systematic series of SYNE1 patients and mutations outside Canada, revise the view that SYNE1 ataxia causes mainly a relatively pure cerebellar recessive ataxia and that it is largely limited to Quebec. Instead, complex phenotypes with a wide range of extra-cerebellar neurological and non-neurological dysfunctions are frequent, including in particular motor neuron and brainstem dysfunction. The disease course in this multisystemic neurodegenerative disease can be fatal, including premature death due to respiratory dysfunction. With a relative frequency of ~5%, SYNE1 is one of the more common recessive ataxias worldwide.

# Introduction

*SYNE1* (OMIM 608441) is one of the largest genes in the human genome, with the longest isoform comprising 146 exons that encode the 8797 amino-acid *synaptic nuclear envelope protein 1* ([Gros-Louis et al., 2007](#_ENREF_9)). This protein, also known as Nesprin 1 (Nuclear envelope spectrin 1), is part of the spectrin family of structural proteins that share a common function of linking the plasma membrane to the actin cytoskeleton ([Gros-Louis *et al.*, 2007](#_ENREF_9)). Truncating recessive mutations in *SYNE1* have been reported to cause a slowly progressive, relatively pure cerebellar ataxia with only few extra-cerebellar symptoms (spinocerebellar ataxia, autosomal-recessive 8; SCAR8 / autosomal-recessive cerebellar ataxia type 1, ARCA1) ([Dupre *et al.*, 1993/2012](#_ENREF_5), [Dupre *et al.*, 2007](#_ENREF_6), [Gros-Louis *et al.*, 2007](#_ENREF_9), [Noreau *et al.*, 2013](#_ENREF_14), [Fogel *et al.*, 2014](#_ENREF_7)). So far, this ataxia has been mainly observed in Quebec, Canada: while it presents the third most common hereditary ataxia in Quebec (after ARSACS and Friedreich ataxia) ([Dupre *et al.*, 1993/2012](#_ENREF_5), [Gros-Louis *et al.*, 2007](#_ENREF_9)), only few families have been identified outside French Canadian populations so far ([Izumi *et al.*, 2013](#_ENREF_11), [Noreau *et al.*, 2013](#_ENREF_14), [Hamza *et al.*, 2015](#_ENREF_10)). This view on the phenotype and geographic distribution of *SYNE1* ataxia, however, might be rather preliminary, given that systematic screenings outside this founder population were confined to highly selected individual cases ([Izumi *et al.*, 2013](#_ENREF_11), [Noreau *et al.*, 2013](#_ENREF_14)). A systematic characterization of the clinico-genetic spectrum of a larger non-French Canadian subject group is missing. We here hypothesized that (i) *SYNE1* is a frequent cause of recessive ataxia also outside French Canadian populations, given the large size of the *SYNE1* gene, and that (ii) dysfunction in extra-cerebellar systems is not the exception but the rule. To test these hypotheses, we aggregated the genetic and phenotypic findings of a screening of 434 ataxia patients compiled by 7 different European centers, unravelling 23 novel index patients with truncating *SYNE1* mutations, including 34 mutations not previously linked to human disease. This large collection of *SYNE1* patients demonstrates that *SYNE1* ataxia is a common recessive ataxia also outside the French Canadian founder population, and that it commonly presents with multisystemic neurodegenerative disease. This includes in particular motor neuron and brainstem features and even complex neuromuscular syndromes, where respiratory dysfunction can lead to premature death.

# Methods

*Patients.*

N=434 index subjects with unexplained early-onset degenerative ataxia (age of onset <40 years) compatible with autosomal recessive inheritance (no ataxia in the parental generation) and negative for trinucleotide repeat expansions causing Friedreich’s ataxia (FRDA) were compiled from 7 different European ataxia centers (n=116 Tübingen, Germany; n=16 Essen, Germany; n=13 Munich, Germany; n=109 Milano, Italy; n=139 Strasbourg and Paris, France; n=41, Antwerp, Belgium). These patients originated from 36 different countries (Supplement 1).

*Genetic screening by next-generation sequencing methods.*

Subjects were screened for *SYNE1* mutations by one of the following five next-generation sequencing methods: 1. as part of a high coverage HaloPlex gene panel kit (Agilent, Santa Clara, CA, USA) which included >120 known ataxia genes (n=179) (Supplement 2A); 2. as part of a targeted exon-capture sequencing strategy (Agilent Sureselect kit) which included 57 known ataxia genes (n=139) (Supplement 2B); 3. as part of an amplicon-based customized panel (Illumina TrueSeq Custom Amplicon, TSCA) covering 76 known ataxia genes (n=21) (Supplement 2C); 4. as part of a targeted exon-capture sequencing strategy (Illumina Nextera Rapid Capture Custom kit) which included 107 known ataxia genes (n=54) (Supplement 2D). 5. as part of whole-exome sequencing using the SureSelect Human All Exon 50Mb kit (Agilent, Santa Clara, CA, USA) (n=41) (Supplement 2E). Variants were filtered for (i) non-synonymous homozygous or compound heterozygous truncating mutations in *SYNE1* (frameshift insertions or deletions, splice mutations, and stop-gain mutations) that were (ii) absent or extremely rare (minor allele frequency <0.5%) in the public databases GEM.app (5992 exomes from 4279 families), dbSNP137, NHLBI ESP6500, 1000Genomes project, and ExAc (60706 exomes; Exome Aggregation Consortium; Cambridge, MA <http://exac.broadinstitute.org>, accessed 06/2015) (for further details on the next-generation sequencing bioinformatics and filter criteria, see Supplement 2A-E). 90-95% of the coding exons of *SYNE1* have been covered with >20 reads by all five test formats.

In addition, to screen for *SYNE1* exon deletions/duplications, we performed a preliminary copy number variation (CNV) analysis on our panel-sequencing datasets from 229 patients of the ataxia screening cohort (for details see Supplement 3).

*Inclusion of SYNE1 missense variants.*

Due to the high number of private missense variants in the large *SYNE1* gene (often located in the numerous, modestly conserved spectrin repeats of the gene) and given the fact that only *truncating* *SYNE1* variants have been established as a cause of *SYNE1* ataxia, we did not include cases carrying only *SYNE1* missense variants. Following a conservative approach towards SYNE1 missense variants, subjects carrying a missense variant were only included if the respective missense variant (i) segregated *in trans* with a truncating *SYNE1* variant, (ii) was absent or rare in public exome databases (same criteria as above), (iii) located in highly conserved positions of the N-terminal actin-binding domain (codon 1 – 289), and (iv) predicted to be damaging by at least two out of three in silico algorithms (Mutation Taster ([Schwarz](#_ENREF_20" \o "Schwarz, 2010 #9535) *[et al.](#_ENREF_20" \o "Schwarz, 2010 #9535)*[, 2010](#_ENREF_20" \o "Schwarz, 2010 #9535), [Wang](#_ENREF_22" \o "Wang, 2010 #9537) *[et al.](#_ENREF_22" \o "Wang, 2010 #9537)*[, 2010](#_ENREF_22" \o "Wang, 2010 #9537)); SIFT ([Sim](#_ENREF_21" \o "Sim, 2012 #8764) *[et al.](#_ENREF_21" \o "Sim, 2012 #8764)*[, 2012](#_ENREF_21" \o "Sim, 2012 #8764)), and PolyPhen2 ([Adzhubei](#_ENREF_1" \o "Adzhubei, 2010 #9534) *[et al.](#_ENREF_1" \o "Adzhubei, 2010 #9534)*[, 2010](#_ENREF_1" \o "Adzhubei, 2010 #9534))). These criteria lead to the inclusion of 1 *SYNE1* missense mutation (p.F220S) in the final cohort. The mutation p.D5140G (family #7), which presented as a missense mutation on the genomic level, was shown to result in aberrant splicing of *SYNE1* transcript, thus representing *de facto* a null mutation.

*Burden analysis of SYNE1 missense variants*

To further explore the significance of SYNE1 missense variants in ataxia, we tested whether rare missense SYNE1 variants predicted to be damaging were more frequent in cases with ataxia than in controls. Cases comprised of a consecutive series of n=96 whole exome datasets from index patients with early-onset ataxia (same inclusion criteria as above). Controls comprised of a consecutive series of n=250 whole exome datasets from index subjects with early-onset Alzheimer’s Disease (EOAD), a condition which is not part even of the extended phenotypic spectrum of SYNE1 (for more details, see Supplement 4).

*mRNA analysis*

mRNA analysis was performed for selected mutations (mutations of families #6 and #7) to confirm the effect of the cryptic splice mutation identified in this study (family #7), and to exemplarily confirm the *loss-of-function* mechanism of truncating *SYNE1* mutations through nonsense-mediated decay of mutant mRNA (family #6). Total RNA was extracted from lymphocytes using a Maxwell® Extractor (Promega) with the Maxwell® 16 LEV simply RNA Cells kit. cDNA synthesis was carried out using Transcriptor FirstStrand cDNA synthesis kit (Roche) with random primers. PCR was carried out in 20 µL with an annealing temperature of 58°C and specific primer pairs designed to amplify cDNA fragments encompassing the different mutations of families #6 and #7. The amplified fragments were directly sequenced on an automated sequencer (ABI Prism 3100 Genetic Analyzer, Applied Biosystems) using the BigDyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer’s recommendations.

*Histological analysis and immunohistochemistry of muscle tissue*

Biopsies of the quadriceps muscle were available from three *SYNE1* subjects and compared to a healthy age-matched control. The samples were analysed by classical histological, histochemical, histoenzymatic and immunohistochemical techniques, as described previously ([Martin *et al.*, 1997](#_ENREF_13)). The antibodies used were mouse monoclonal anti-emerin (NCL-emerin, 1/100, acetone fixation - Novocastra, Newcastle-on-Tyne, UK); mouse monoclonal anti-lamin A/C (NCL-LAM-A/C, 1/1000, aceton/methanol fixation - Novocastra, Newcastle-on-Tyne, UK) and rabbit polyclonal anti-nesprin-1 (PRB-439P, 1/2000, unfixated – Covance, Princeton, New Jersey, USA). The avidin-biotin complex technique for the monoclonal antibodies and the peroxidase-antiperoxidase technique were used for the polyclonal antibody.

*Clinical and electrophysiological assessments*

All index patients carrying two pathogenic *SYNE1* alleles (either in homozygous or compound heterozygous state) as well as their affected siblings received a systematic clinical assessment for disturbances in multiple neurological systems (Table 2) by a movement disorder specialist. Nerve conduction studies (NCS) and electromyography (EMG) investigations were performed in all patients available for these investigations. Patients were classified into two phenotypic categories: i) *pure cerebellar ataxia* (*pure CA*) = pure ataxia features without any evidence of non-ataxia features; ii) *cerebellar ataxia plus (CA plus)* = ataxia plus clinical evidence for damage of at least one additional neurological system, such as e.g. upper and/or lower motor neuron disease. Disease severity was rated by the Scale for the Assessment and Rating of Ataxia (SARA) ([Schmitz-Hubsch *et al.*, 2006](#_ENREF_19)).

*MRI and PET imaging*

Routine brain magnetic resonance imaging (MRI) including T1-, T2-, diffusion-weighted images (DWI) and fluid attenuated inversion recovery T2 (FLAIR) images were performed for at least one patient per family and were reviewed by both neuroradiologists and a neurologist. 18F- FDG positron emission tomography (PET) imaging was performed for two exemplary subjects with ataxia-motor neuron disease on a Siemens ECAT HR+ scanner (CTI, Knoxville, TN, USA) with a 128 × 128 voxel matrix (voxel size 2 mm), an axial field of view of 15.5 mm and a full-width-at-half-maximal resolution of 5 mm, according to previously published tracer-specific protocols ([Forster *et al.*, 2010](#_ENREF_8)). Resting-state PET recordings were obtained in the interval 30–50 min after iv application of 200 MBq 18-Fluoro-2-deoxyglucose (18F-FDG) in fasting state (>6 hrs), a brief attenuation scan was obtained with integrated 68Ge rod sources (for details of image analysis, see Supplement 5).

# Results

***A large cohort of novel SYNE1 index families and mutations from European and non-European populations***

We identified 22 index patients carrying two truncating *SYNE1* alleles and 1 index patient carrying one truncating plus one missense *SYNE1* allele, thus yielding a total of 23 index patients out of 434 early-onset ataxia patients (5.3%). Five index patients were from multiplex families, the remaining 18 index patients were simplex cases. Six index patients had consanguineous parents. In the 23 index patients, we observed a total of 35 different mutations, consisting of 7 frameshift, 20 nonsense mutations, 6 nucleotide changes affecting constitutive splice sites, 1 nucleotide change generating a cryptic exonic splice site, and 1 missense mutation in the actin binding domain (Table 1; for a discussion of the cryptic exonic splice site and the missense mutation, see below). The mutations were spread across the giant *SYNE1* gene, including the acting binding domain (also called “Calponin homology domain containing actin binding site”) and the spectrin repeat domains (Figure 1A). Five mutations (mutations 1-5) also affect the coding sequence of the SYNE1 isoform Nesprin 1α (nucleotide reference: <http://www.ncbi.nlm.nih.gov/nucleotide/119120815>), a region where no mutations linked to ataxia have been previously found ([Razafsky and Hodzic, 2015](#_ENREF_17)). 34 of the 35 mutations have not reported in association with human disease before, thus more than doubling the total amount of all *SYNE1* truncating mutations that have been published so far (for overview of all novel and published mutations, see Figure 1B). Only the variant c.3736G>T, p. E1246\* (mutation #29, table 1) has been reported before ([Hamza *et al.*, 2015](#_ENREF_10)). Two mutations were listed in dbSNP/1000 Genomes database, but also not yet linked with human disease. All 35 mutations were absent or had extremely low minor allele frequency (<0.001%) in GEM.app (5992 exomes from 4279 families), dbSNP137, 1000 Genomes database, NHLBI ESP (6500 exomes), ExAc (60706 exomes) (Table 1). Moreover, all 35 mutations yielded a scaled CADD score (Combined Annotation Dependent Depletion) of ≥19 (Table 1), ranking the predicted pathogenicity of each mutation among the top 1% of all 8.6 billion SNVs in the GRCh37/hg19 ([Kircher *et al.*, 2014](#_ENREF_12)). All mutations were confirmed by Sanger sequencing. In all families where DNA from the siblings was available (10/23), the variants cosegregated with disease in affected siblings, and unaffected siblings carried at most one pathogenic allele. For all families where DNA of at least one parent was available (12/23 families), we were able to show that the respective parent carried only one of the respective two *SYNE1* variants, indicating a biallelic localization of the variants in the index child. In 4 of the remaining 11 families, consanguinity was also suggestive of a biallelic location of the observed homozygous mutations. No other significant variants in known ataxia genes were identified in any of the 23 index subjects, as demonstrated by screening through whole exome sequencing and targeted panel sequencing, respectively.

No obvious *SYNE1* multi-exon deletions/duplications were observed by our preliminary NGS-based CNV analysis (supplement 3). This result, however, has to be interpreted with caution since no consensus currently exists on a method to perform CNV analysis on NGS data.

***Pathogenicity of selected base exchanges***

*Cryptic splice mutation*. While 6 out of 7 splice mutations affected constitutive splice sites (see Table1), the base exchange c.15419A>G (mutation #18, family #7) – which appeared as a p.D5140G missense change on the genomic level – was predicted by *in silico* analysis (NNSplice; ([Reese *et al.*, 1997](#_ENREF_18)) to act as a cryptic exonic splice mutation. Specifically, it was predicted to create a new donor site 26 bp upstream of the constitutive 5´ donor splice site of intron 80. To confirm this prediction, we performed reverse transcription and sequencing of *SYNE1* mRNA, revealing a heterozygous deletion of the last 26 nucleotides of exon 80 (r.15419\_15444del26) (Figure 2A) which would result in a protein truncated at residue 5140 (p.D5140Gfs1\*). This variant occurred in *trans* with the frameshift duplication/stop gain mutation p.C7895\*.

*Missense mutation*. The missense variant c.659T>C, p.F220S (observed in family #20) (i) segregated in *trans* with the nonsense variant c.2776A>T, p.K926\*, (ii) was absent in all public databases described above, (iii) predicted to be damaging by three out of three in silico algorithms (Mutation Taster ([Schwarz *et al.*, 2010](#_ENREF_20), [Wang *et al.*, 2010](#_ENREF_22)); SIFT ([Sim *et al.*, 2012](#_ENREF_21)), and PolyPhen2 ([Adzhubei *et al.*, 2010](#_ENREF_1))), (iv) ranked among the top 1% of all 8.6 billion SNVs in the GRCh37/hg19 (CADD score: 28.8) ([Kircher *et al.*, 2014](#_ENREF_12)), and (v) located at a highly conserved position in the actin-binding domain of SYNE1 (Supplement 6). This domain is conserved even in non-metazoan species (paralogues in ichthyospora, fungi and slime mold, E-values in the range of 5x10-40 to 10-50), whereas the spectrin domains of SYNE1 provide no significant alignment with paralogues of the same non-metazoan species (E-values above 10). The p.F220S mutation affects one of the 6 invariant amino acid positions of the second calponin-homology domain in nesprin, spectrin and alpha-actinin homologues (for figure, see Supplement 6). The biallelic truncating and the missense *SYNE1* variants were also found in the affected sibling of the index patient in family #20, indicating segregation with disease.

*Burden analysis for missense variants in SYNE1*. Rare missense *SYNE1* variants were observed in 14/192 alleles (=7.3%) from ataxia cases and in 28/500 alleles (=5.6%) from disease controls (early-onset Alzheimer dementia) (Supplement 4). Also missense variants in the N-terminal actin-binding domain of *SYNE1* (codon 1 – 289) were not more frequent in ataxia cases than in controls (Supplement 4). This finding suggests that rare missense *SYNE1* variants seem to present a ubiquitous finding unrelated to specific disease conditions or phenotypes.

In general, these results from the cryptic splice and the missense mutation as well as from the missense variant burden analysis indicate that careful interpretation of single *SYNE1* nucleotide variants is necessary to evaluate possible effects detrimental to SYNE1 function. Such a critical one-by-one debate of *SYNE1* single nucleotide variants is of particular relevance in the future, given the large size of *SYNE1* with its substantial variability.

***Absence of SYNE1 transcript adds support for a loss-of-function mechanism***

Truncation of proteins can lead to either toxic gain of function (exerted by the residual protein) or to loss-of-function. To exemplarily investigate the mechanism of action for truncating *SYNE1* mutations, we investigated the pathomechanism of the changes p.L132\* and p.G4752Efs\*10 (observed in family #6). No *SYNE1* transcript was amplified by reverse transcriptase-PCR in patient’s cells (Figure 2B), suggesting that both the early nonsense mutation p.L132\* and the later frameshift mutation p.G4752Efs\*10 cause nonsense-mediated decay of mutant mRNA. The absence of the transcript adds support for a loss-of-function mechanism underlying the detrimental effect of truncating SYNE1 mutations in human SYNE1 disease, as hypothesized earlier ([Gros-Louis *et al.*, 2007](#_ENREF_9), [Attali *et al.*, 2009](#_ENREF_3)).

***Severely reduced to absent SYNE1 staining in muscle tissue***

Muscle biopsy (available for 3 different SYNE1 index patients [patient 13-1, 10-1, and 6-1] originating from 3 different countries) showed neurogenic changes in all patients, thus corresponding to the clinical/EMG finding of frequent lower motor damage in SYNE1 disease (see below). Upon routine light microscopy and also immunohistochemistry with relevant antibodies, the nuclei had a normal peripheral distribution in the three patients comparable to the control individual (Figure 3). More precisely, no myopathic and in particular no dystrophic muscle features were seen in any of the three patients, thus contrasting previous findings on dystrophic changes in patients with Emery-Dreifuss muscular dystrophy Type 4 that were reported in association with heterozygous “dominant” *SYNE1* (and *SYNE2*) mutations ([Zhang *et al.*, 2007](#_ENREF_24)).

In line with the suggested loss of protein in SYNE1 patients with truncating mutations, staining of SYNE1 was severely reduced in two patients and absent in one patient (Figure 3). To control for the possibility that this lack of SYNE1 staining might be an unspecific result or artefact in the SYNE1 samples, control stainings were performed. Control immunolabelling showed normal results for emerin at the inner nuclear membrane and of lamin A/C also in the SYNE1 muscle samples (Figure 3). No mislocalization of emerin or lamin A/C was seen, thus contrasting previous findings of a mislocalization of these proteins reported in patients with heterozygous *SYNE1* mutations ([Zhang *et al.*, 2007](#_ENREF_24)).

***A broad range of additional non-cerebellar features, including severe and complex early-onset syndromes***

We aggregated clinical data from 26 affected subjects belonging to 23 families (median age of disease onset: 22 years, range: 6-40 years; last examination in each individual performed after a median of 25 years of disease duration). Disease started with gait coordination disturbances in 24/26 subjects; in two subjects it began with focal upper limb dystonia (writer’s cramp) (patient 20-1) and lower limb weakness (patient 23-1), respectively. Five out of 26 patients (19%) showed the classical *SYNE1* phenotype of pure cerebellar ataxia that was mildly progressive and started in adulthood (range: 19-36 years) (*pure cerebellar ataxia, pCA*). However, the large majority of patients (21/26 =81%) exhibited at least one additional complicating feature from a large range of non-cerebellar features, both in neurological and non-neurological domains (*cerebellar ataxia plus, CA plus*) (see Table 2 and Figures 4A and 4B).

The most frequent complicating finding was motor neuron dysfunction, observed in 15/26 (58%) of the total cohort (Figure 4A). This comprised a combination of upper motor neuron dysfunction (bilateral positive extensor plantar reflex and/or spasticity) and lower motor neuron dysfunction (muscle atrophy combined with reduced reflexes; fasciculations clinically or on EMG; or chronic and/or acute neurogenic changes on EMG) in 5/26 patients (19%), only upper motor dysfunction in 8/26 patients (31%), and only lower mutation in 2/26 (8%) patients. Other frequent complicating features across the cohort included: scoliosis (5/26; 19%), sometimes combined with kyphosis, indicative of skeletal abnormalities in SYNE1; slowing of saccades (4/26; 15%), indicative of brainstem dysfunction in SYNE1; and reduced vibration sense of the lower limbs (3/26; 12%), indicative of peripheral nerve and/or dorsal column dysfunction in SYNE1 (for a relative frequency of all main features, see Figure 4A). A broad range of non-cerebellar oculomotor deficits was seen in single patients across the cohort: square wave jerks, ophthalmoparesis, and strabism including esotropia (see Table 2 and Figure 4A). One patient exhibited mild macroglossia in addition to esotropia, that could not be related to any other disease other than SYNE1 (for photo, see Supplement 7).

Three *CA plus* patients (3/26= 12%; patients 13-1, 13-2, 23-1; no consanguinity in either of the two families) showed a particularly severe and complex multisystemic phenotype, comprising of very early onset (6-10 years of age) ataxia, spasticity, weakness and muscle wasting of all four limbs, mental retardation (IQ 49-60), dysphagia, pes cavus, and a broad range of variable skeletal and soft-tissue abnormalities such as sacral cysts, pseudarthrosis clavicula, hyperlaxity of joints, achilles tendon contractures, kyphosis, scoliosis, cataract, hypertelorism. All three developed respiratory dysfunction in late adolescence, necessitating BiPAP (bi-level positive airway pressure) ventilation in 2/3 subjects and leading to premature death at age 36 years in one of them. No obvious second hit in any other disease gene was detected in these patients (for details, see supplement 8). Thus, the complex phenotype in these three subjects does not seem to be explained by a second hit in another gene or to present a qualitatively different condition; rather it seems to illustrate one end on the continuous spectrum of SYNE1-disease, which literally encompasses *all* of the aforementioned SYNE1-associated domains and includes also mental retardation and respiratory distress.

One of the three subjects also showed developmental abnormalities of the visceral organs, such as malrotation of the colon and unilateral positioning of both kidneys at right pelvis. However, future studies in SYNE1 patients are warranted to confirm whether such visceral features are indeed part of its phenotypic spectrum.

***Electrophysiological findings***

Motor evoked potentials, available for 8 patients, were prolonged or not evoked in 6/8 patients (75%), all of them showing also clinical signs of upper motor neuron damage (Table 2). Nerve conduction studies, available for 19 patients, were normal in 17/19 patients (89%), while 1 patient showed a peripheral motor neuropathy and 1 patient a mild sensory axonal neuropathy, demonstrating that peripheral neuropathy is a rather infrequent feature in SYNE1 disease.

***Imaging findings***

Cerebral MRI, available from 24 patients, consistently showed marked cerebellar atrophy in all 24/24 (100%) patients (for exemplary illustration, see Figure 5). 18F- FDG positron emission tomography (PET) imaging performed in two exemplary subjects with ataxia-motor neuron disease revealed a marked homogenous 18F-FDG decrease in both cerebellar hemispheres in both subjects and, on visual inspection, also of the pontine brainstem, which reached statistical significance in subject 10-1 with a Z-score of 3 and a trend in subject 9-1 with a Z-score of 1.5 (semiquantitative PET, normalization to global mean activity and comparison to an age-adjusted database) (Figure 5). These imaging findings provide proof-of-concept functional imaging evidence that dysfunctions in SYNE1 patients extend beyond the cerebellar domain to include in particular the pons, thus complementing our clinical findings of extra-cerebellar features in SYNE1.

# Discussion

***SYNE1: a recurrent recessive ataxia worldwide, with mutations spanning across the entire gigantic gene***

So far, autosomal-recessive cerebellar ataxia (ARCA) due to *SYNE1* mutations has been described mainly in Quebec, Canada, with only very few families having been identified outside French-Canadian populations ([Izumi *et al.*, 2013](#_ENREF_11), [Noreau *et al.*, 2013](#_ENREF_14)). Reporting the by far largest series of *SYNE1* patients outside Quebec, we here show that SYNE1 deficiency is in fact a relatively common cause of recessive ataxia worldwide, yielding an estimated frequency of at least 5.3% after exclusion of Friedreich’s ataxia and the most common repeat expansion SCAs. This is probably a rather conservative estimate, given the fact that our approach used only a very preliminary NGS-based approach to screen for CNVs and that it was very restrictive on including missense variants. Future studies might be able to identify damaging CNVs in the large *SYNE1* gene, which would further increase the relative frequency of subjects with truncating *SYNE1* mutations.

Our genetic findings more than double the number of truncating *SYNE1* mutations identified so far: while <15 different truncating *SYNE1* mutations have been described, we here identified 35 novel truncating *SYNE1* mutations, which are scattered throughout all parts of the gigantic gene. Almost all mutations are private, identified in single families without obvious mutational hot spot regions. Five mutations affected the coding sequence of the SYNE1 isoform Nesprin 1α. As no mutations linked to ataxia had been previously found in this domain ([Razafsky and Hodzic, 2015](#_ENREF_17)), it was suggested that a protein encoded by these exons may underlie a distinct pathology in humans ([Razafsky and Hodzic, 2015](#_ENREF_17)). However, here we show that mutations in this sequence can lead to the same phenotypic features as mutations in other parts of *SYNE1* (see mutations 1-5, table 1).

Taken together, these findings have also major implications for future clinic-genetic testing strategies in unresolved ARCA patients around the world: they demonstrate that also in non-Canadian populations current genetic testing approaches have to include the large *SYNE1* gene by using approaches that tightly capture all of its exons.

***SYNE1 spectrum: from late-onset pure cerebellar ataxia to complex neuromuscular disease with mental retardation***

Recessive ataxia due to *SYNE1* mutations is commonly perceived to present as a slowly progressive, relatively pure cerebellar ataxia with only mild and infrequent extra-cerebellar symptoms (e.g. brisk reflexes in 33%; ([Dupre *et al.*, 2007](#_ENREF_6))), starting in adult age ([Dupre *et al.*, 1993/2012](#_ENREF_5), [Gros-Louis *et al.*, 2007](#_ENREF_9), [Noreau *et al.*, 2013](#_ENREF_14), [Fogel *et al.*, 2014](#_ENREF_7)). However, here we show that pure cerebellar ataxia represents rather a relatively infrequent phenotypic cluster along a broad multidimensional SYNE1-associated spectrum of neurodegenerative disorders. With 81% of all patients exhibiting variable additional non-cerebellar features both in neurological and non-neurological domains, a multisystemic phenotype seems to be the rule rather than the exception in SYNE1 disease. Apart from cerebellar ataxia, this spectrum includes upper motor neuron disease, lower motor neuron disease, brainstem dysfunction (e.g. saccadic slowing), and a variable range of musculoskeletal abnormalities (e.g. kyphosis, scoliosis, pes cavus, contractures). Variable combinations of these features can be found in different SYNE1 patients along a continuous spectrum of disease. This spectrum ranges from (i) pure cerebellar ataxia via (ii) cerebellar ataxia plus damage of one or two additional systems (such as motor neuron disease, thus giving rise to spastic ataxia and complicated hereditary spastic paraplegia phenotypes) to (iii) cerebellar ataxia as part of severe multisystemic neurodegenerative syndromes. The latter encompass literally *all* of the aforementioned SYNE1-associated domains and include also mental retardation, respiratory distress and possibly visceral organ involvement (Table 2; for graphic illustration of the SYNE1 disease continuum, see Figure 4B and 4C).

The complex early-onset neurodegenerative syndrome does not seem to present a mere coincidental finding (that might be unrelated to the *SYNE1* mutations), but rather reflects a phenotypic cluster systematically related to *SYNE1* mutations. This is indicated by the fact that we identified 3 patients from two different families with this severe phenotypic combination. In fact, a fourth patient with early-onset complex *SYNE1* ataxia-motor neuron disease and respiratory dysfunction has recently been described as a single case in a Japanese family ([Izumi *et al.*, 2013](#_ENREF_11)), thus increasing the evidence to three different families. Here we now show that this phenotypic cluster is not a qualitatively distinct syndrome, but rather a *combination* of *SYNE1*-assosciated features that can each be found in isolation or as part of less complex phenotypic clusters in other *SYNE1* patients along a continuous spectrum of disease. Although we did not find obvious mutation-phenotype correlations, different *SYNE1* mutations might differentially affect the multiple SYNE1 splicing isoforms which vary greatly in size and tissue-specific expression patterns ([Zhang *et al.*, 2010](#_ENREF_23), [Razafsky and Hodzic, 2015](#_ENREF_17)), thus possibly explaining parts of the phenotypic variability observed here. In fact, *SYNE1* mutant mice show variable phenotypes depending on the splicing isoform that was targeted (for overview see ([Zhang *et al.*, 2010](#_ENREF_23)).

Our findings have important implications for clinico-genetic counselling. They revise current clinical notions about the benign disease course of *SYNE1*-disease, including the notion that life expectancy was normal ([Dupre *et al.*, 1993/2012](#_ENREF_5), [Gros-Louis *et al.*, 2007](#_ENREF_9)). They show that, at least in some patients, *SYNE1*-disease can start already as a developmental disease with mental retardation and early-onset ataxia in the first decade of life, and that it can lead to premature death due to respiratory dysfunction in the mid-adult age (age 30- 40 years).

***Bridging the gap between SYNE1 ataxia and arthrogryposis syndromes***

Truncating recessive *SYNE1* mutations have been described as a cause of a arthrogryposis multiplex congenita syndrome in a single Palestinian family presenting with infantile-onset hypotonia, bilateral club foot, progressive motor decline in the first decade, scoliosis and restrictive lung disease, yet without ataxia or motor neuron disease ([Attali *et al.*, 2009](#_ENREF_3)) (for location of these mutations, see Figure 1B). This musculoskeletal infantile-onset phenotypic cluster was proposed as a “distinct human disease phenotype” of SYNE1, separate from the neurological phenotype ([Attali *et al.*, 2009](#_ENREF_3)). As several of our ataxic SYNE1 patients, however, (i) also show scoliosis/kyphosis, restrictive lung disease, foot deformities, and other neuromuscular abnormalities as part of their early-onset multisystemic disease, and (ii) also carry truncating mutations in the same or neighboring gene region (Figure 1B), we here suggest that such arthrogryposis syndromes do not represent qualitatively distinct phenotypes, but rather clusters of variably combined neurologic and non-neurologic features along the continuum of *SYNE1* disease.

It remains more questionable whether this SYNE1 disease continuum also includes the dominant form of Emery-Dreifuss muscular dystrophy type 4 (EDMD4; MIM 612998) ([Zhang *et al.*, 2007](#_ENREF_24)). Although a phenotypic overlap might be theoretically conceivable, as indicated by our clinical findings, this syndrome has been linked only to *heterozygous missense* mutations in *SYNE1 (*[*Zhang et al., 2007*](#_ENREF_24)*)* (see Figure 1B), but not to *biallelic truncating* mutations. As advanced genomic techniques now allow us to appreciate the immense variability of the *SYNE1* gene and to determine the frequency of rare *SYNE1* missense variants in controls (e.g.28/500 [=5.6%] rare missense alleles in our control dataset with early-onset Alzheimer dementia; Supplement 4), the pathogenicity of such variants previously related to EDMD4 needs to be critically evaluated against current standards. Furthermore, also findings on muscle biopsies between EDMD4 subjects and the here reported cases with biallelic truncating mutations differ substantially: while EDMD4 subjects showed dystrophic myopathic changes and in particular mislocalization of emerin or lamin A/C ([Zhang *et al.*, 2007](#_ENREF_24)), none of these features was seen in the muscle cells of any of our subjects (Figure 3). Although this might be explained by a different *SYNE1*-related pathomechanism, this patho-morphological discrepancy in addition to the genetic concerns mentioned above indicates a possible over-interpretation of heterozygous *SYNE1* variants in the context of EDMD4 and warrants a critical re-evaluation of these historic findings.

***A widespread functional role of SYNE1 in various tissues***

Our findings on widespread extra-cerebellar dysfunctions in human SYNE1 disease complement and recapitulate some of the key features of SYNE1/Nesprin 1 knock-out mouse models. Key features in mice include kyphoscoliosis, respiratory failure, and shortened survival ([Zhang *et al.*, 2007](#_ENREF_25), [Puckelwartz *et al.*, 2009](#_ENREF_16), [Zhang *et al.*, 2010](#_ENREF_23)). We now show systematically that these features are also part of the human disease spectrum, thus bridging the cross-species gap.

Moreover, our findings on extra-cerebellar dysfunctions might provide new insights into the pathophysiology underlying SYNE1-associated neurodegeneration. Recent work has shown that a specific SYNE1 isoform devoid of the KASH domain (KLNes1g) is specifically abundant in the cerebellum (in particular in the granule cell layer), where it might be involved in vesicular trafficking and/or in dendritic membranes’ structural organization ([Razafsky and Hodzic, 2015](#_ENREF_17)). Our clinical, electrophysiological and imaging observations of multisystemic damage in *SYNE1* patients, however, indicate that intact SYNE1 transcripts seem to be functionally important not only for cerebellar, but also for motor and brainstem neurons. They thus stimulate future molecular research aiming to identify the specific functional role of SYNE1 in these neuron types. For example, the proposed interaction of SYNE1 transcripts with vesicular trafficking proteins such as Kif5c, suggested for cerebellar neurons ([Razafsky and Hodzic, 2015](#_ENREF_17)), might also apply to motor neurons. Aberrant vesicular trafficking dynamics is a common process underlying motor neuron degeneration (as e.g. in HSPs ([Crosby and Proukakis, 2002](#_ENREF_4))), and mutations in KIF5C have been shown to include upper motor neuron damage ([Poirier *et al.*, 2013](#_ENREF_15)).

In sum, our clinical, electrophysiological and imaging findings suggest a widespread functional role of SYNE1 in many different tissues in humans, which is in line with its ubiquitous expression ([Apel *et al.*, 2000](#_ENREF_2), [Gros-Louis *et al.*, 2007](#_ENREF_9), [Zhang *et al.*, 2010](#_ENREF_23)). This ubiquitous expression of SYNE1 – and, in turn, its absence in case of protein truncation - might help to find diagnostic biomarkers for the disease. Our results suggest that severely reduced to absent SYNE1 staining in muscle tissue – which was observed in all 3/3 available samples – may represent a diagnostic marker that indicates underlying truncating *SYNE1* mutations. If confirmed in larger sample cohorts, SYNE1 staining of muscle tissue might thus become a helpful auxiliary tool in the histochemical work-up of muscle biopsies of future patients with unresolved neurodegenerative and neuromuscular diseases. It is likely that many more SYNE1 patients with both relatively pure ataxia as well as complex neuromuscular phenotypes will be identified worldwide in the very next years.

# Figure Legends

**Figure 1: *SYNE1* mutations**. **(A) Graphical overview of the mutations found in this study in relation to the SYNE1 domains**. Numbers indicate the mutation IDs of the mutations identified in this study (see table 1). Their position indicates the position of the respective mutations in the *SYNE1* gene. Blue color coding= N-terminal Actin binding domain (= Calponin homology domains containing actin binding sites [IPR001715]) and mutations affecting this domain; orange color coding = Spectrin/alpha-actinin repeat domains (IPR018159) and mutations affecting these domains; turquoise color coding= KASH domain (=C-terminal klarsicht domain) (IPR012315); black color coding= mutations not affecting any of these domains. Mutation #33 (black circle) is the only missense mutation in the present study. **(B) Overview of the variant types and their location of all published and novel *SYNE1* mutations.** The presentation of the giant SYNE1 gene is split in a first part (chr6:152.44.819-152.644.000; upper panel) and a second part (chr6:152,644,000-152,958,534; bottom panel). It presents the variant types of all SYNE1 mutations found in the present study (bottom row of each panel) and other studies (Human Gene Mutation Database) (top row of each panel), their location and their annotation with the associated clinical phenotypes (ATX, ataxia, AMC, arthrogryposis multiplex congenita; ED, Emery-Dreifuss muscular dystrophy; CM, cardiomyopathy; MR, mental retardation; HSP, hereditary spastic paraplegia). Note that, except ATX and AMC, all other phenotypes have been associated only with missense mutations, not truncating mutations in SYNE1. Green colored arrows and boxes= indel mutations; blue colored arrows and boxes= stop mutations; red colored arrows and boxes = splice site mutations; purple colored arrow and box = missense mutation. HG19 genome build. Transcript: NM\_033071 > NP\_149062.

**Figure 2. Exemplary mechanisms of *SYNE1* mutations. (A) An exonic base exchange leads to activation of a cryptic splice site.** (A) *SYNE1* transcript analysis reveals that the c.15419A>G (p.D5140G) mutation (identified in patient 7-1) creates a new splice donor site within exon 80 (GT, underlined in bottom panel) resulting in a 26bp deletion (p.D5140Gfs1\*). Upper left panel: sequence analysis of PCR products of amplified genomic DNA showing the A-to-G variant (minus strand) that changes codon 5140 from GAT (Asp) to GGT (Gly). Upper right panel: sequence analysis of amplification products following reverse transcriptase-PCR of *SYNE1* transcript (exons 78-82) from patient’s cells showing the presence of the 26-nt deletion (r.15419\_15444del26). Bottom panel: schematic diagram showing the aberrant splicing mechanism which leads to the premature truncation of the protein through the deletion of 26 nucleotides in exon 80 (in red). Premature TAA termination codon in exon 81 is shown in bold-red italics. **(B) Truncating *SYNE1* mutations can lead to nonsense-mediated decay of *SYNE1* mRNA**. *SYNE1* mRNA carrying the p.L132\* and the p.G4752Efs\*10 null mutations undergoes nonsense-mediated decay. PCR amplification from retro-transcribed mRNA was carried out with primers specific for *SYNE1* (exons 4-9) and *SPG7* (exons 6-10) transcripts. Note the complete absence of the *SYNE1* mutant transcript in patient 6-1 as compared to the control.

**Figure 3. Severely reduced to absent SYNE1 staining in muscle tissue of *SYNE1* patients**. Immunohistological findings in control tissue (A, E, I), as well as in the quadriceps muscle biopsies of three different *SYNE1* patients from three different countries: patient 13-1, Belgian (B, F, J); patient 10-1, German (C, G, K); and patient 6-1, Italian (D, H, L). All three patients show a severely reduced to absent staining of the nuclear envelope after immunolabelling of nesprin-1 (B, C, D), whereas staining was normal in the control (A) (Peroxidase-antiperoxidase technique). To control for an unspecific lack of staining in the SYNE1 patients, further control stainings were performed. Immunolabelling of Emerin and Lamin A/C at the inner nuclear membrane was normal in all three patients as well as the control (E-H and I-L respectively) (Avidin-biotin complex technique).

**Figure 4: The phenotypic spectrum of SYNE1 disease. (A) Frequency of non-cerebellar features in SYNE1.** Relative frequencies of non-cerebellar features observed in the n=26 SYNE1 patients investigated in this study. Note that several patients showed ≥2 non-cerebellar disease features. **(B)** **The relative share of main SYNE1 phenotypes.** This figure illustrates that only a minor share of SYNE1 patients shows pure cerebellar ataxia (blue), whereas the large majority shows cerebellar ataxia plus phenotypes (yellow), in particular cerebellar ataxia plus motor neuron disease (MND). The latter combination is thereby not a distinct ataxia plus-phenotype, but is commonly associated also with other non-MND signs (see overlap group comprising of n=10 subjects). **(C) The continuous spectrum of SYNE1 disease**. The clinical spectrum of neurological and non-neurological *SYNE1* features presented in figure A unfolds along a continuous spectrum of disease. Variable combinations of these features can be found in different SYNE1 patients reaching from pure cerebellar ataxia (left side) via cerebellar ataxia plus damage of one or two additional systems (like e.g. motor neuron disease [MND]) to cerebellar ataxia as part of severe multisystemic neurodegenerative syndrome (right side). In addition to ataxia and MND, the latter can include also mental retardation, respiratory distress and visceral organs. The phenotype of mild, slowly progressive pure cerebellar ataxia, which has been considered the most prominent phenotype of SYNE1 so far ([Gros-Louis *et al.*, 2007](#_ENREF_9), [Noreau *et al.*, 2013](#_ENREF_14)), thus represents only one far edge of this broad disease spectrum.

**Figure 5: Cerebral 18F- FDG PET and MRI in exemplary SYNE1 patients.** **(A, C)** Surface projections of the 18F- FDG PET scans of subject 10-1 at age 37 years (A) and subject 9-1 at age 45 years (C). The upper rows in A and C show the surface projection map of cerebral glucose metabolism normalized to the maximum of the acquisition. The lower rows show the deviation of an age adjusted normal database after normalization to a global mean calculation (GLB) of metabolic activity. On both projections, a marked homogenous bilateral reduction of cerebellar FDG metabolism can be seen in both subjects. **(B, D)** Tomographic projections after automated fitting. For both subject 10-1 (B) and subject 9-1 (D) a reduction of FDG-metabolism is also clearly visible in the brain stem (white arrows), which reached statistical significance in subject #10 with a Z-score of 3 and a trend in subject 9-1 with a Z-score of 1.5 (semiquantitative PET, deviation after normalization to the global mean). **(E, F)** T2 weighted MRI scans in subject 18-1 at age 21 years shows marked vermian atrophy (F) and cortical hemisphere atrophy (E) of the cerebellum (red arrows). **(G)** Fusion image of 18F- FDG PET scan of subject 10-1 onto a central paramedian sagittal T2 weighted MRI scan shows considerable hypometabolism in the cerebellum and in the pons, whereas FDG metabolism is normal in the cortical cerebral regions.

# Tables

**Table 1: SYNE1 mutations identified in this study**. Genomic positions of the variants according to genome build hg19. DNA changes according to NM\_033071.3. Variant type and protein changes according to GVS function based on NP\_149062. pCA= pure cerebellar ataxia; CA plus, cerebellar ataxia plus clinical evidence for damage of at least one additional neurological system. PhyloP = PhyloP conservation score based on base-wise conservation across 100 vertebrates. CADD score = scaled Combined Annotation Dependent Depletion score, integrating many diverse annotations into a single measure (C score) for each variant. The predicted pathogenicity of each variant is scored and ranked relative to all ~8.6 billion SNVs of the GRCh37/hg19 reference. A scaled CADD score of 20 indicates variants at the top 1%, a CADD score of 30 indicates variants at the top 0.1%, etc. ([Kircher *et al.*, 2014](#_ENREF_12)). MAF = minor allele frequency. ExAC= Exome Aggregation Consortium. EVS = Exome Variant Server 6500 exomes all from the NHLBI GO Exome Sequencing Project. HGMD = Human Gene Mutation Database.

**Table 2: Clinical, imaging and electrophysiological features of SYNE1 patients.** Patient ID = family number\_individual number; m =male; f = female; pure CA= pure cerebellar ataxia; CA plus = cerebellar ataxia plus at least one additional system damage; + = present; - = absent; SARA = scale for the assessment and rating of ataxia; upper motor neuron signs = extensor plantar response positive and/or hyperreflexia of muscle tendon reflexes; UL = upper limb, LL = lower limb; MRI= magnetic resonance imaging; NCS= nerve conduction studies; EMG= electromyography; n.d. = not done.

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