New mutations in *KCNC3* and their functional effect in spinocerebellar ataxia type 13

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Abstract

Spinocerebellar ataxia (SCA) is an autosomal dominant disease characterized by degeneration of the spinocerebellum and impairment in locomotion. The disease is divided into many sub-types as the disease is caused by mutations in many different genes but has only small variations in the phenotype.

One of these sub-types is the rare SCA13 which is caused by missense mutations in the *KCNC3* gene. This gene encodes for the voltage-gated potassium channel Kv3.3 which is highly expressed in Purkinje cells. To date, 3 disease causing mutations are found in the *KCNC3* gene; R420H, R423H and F448L.

We screened 316 genetically undiagnosed Dutch SCA patients for the known SCA13 mutations and for possible new disease causing mutations in *KCNC3*, to indicate the frequency of SCA13 in our cohort. We found 3 patients with the R420H mutation and additionally 1 patient with a V535M mutation which is predicted to be (possibly) damaging and 1 patient with the A628A mutation which is predicted to be damaging or harmless depending on the used *in silico* prediction program.

After functional validation of Kv3.3 channel expression and localization, the 2 new mutations V535M and A628A are identified as probably not disease causing because of the similarity with the wild type. The R420H and R423H mutants are found to have less modificated monomeric forms and the R420H mutant is also more localized in the ER and perinuclear space than the wild type and other 4 mutants. The F448L mutant has a higher protein expression than the wild type Kv3.3. This indicates that the Kv3.3 mutations R420H, R423H and F448L induce SCA13, while the newly found mutations are probably not causative. This results into an incidence of SCA13 in our Dutch cohort of 0.63%.

List of abbreviations

β-ME	β-mercaptoethanol
BSA	Bovine Serum Albumine
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
ECL	Enhanced Chemiluminescence (horseradish peroxidase catalyses)
EGFP	Enhanced Green Fluorescent Protein
ER	Endoplasmatic Reticulum
FBS	Fetal Bovine Serum
HEK293T	Human Embryonic Kidney 293 cells
HeLa	Human cell line (cervical cancer cells) from Henrietta Lacks
KCNC3/Kv3.3	Gene/protein; homo sapiens potassium voltage-gated channel, Shaw-related subfamily, member 3
LSB	Laemmli Sample Buffer
PCR	Poly Chain Reaction
PEI	Polyethylenimine
Pen/Strep	Penicillin Streptomycin
PFA	Paraformalehyde
SCA	Spinocerebellar Ataxia
SDS	Sodium Dodecyl Sulfate
TTBS	Tween20 Tris-Buffered Saline
RIPA	Radioimmunoprecipitation assay buffer

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Introduction

The dysfunction of ion channels is the underlying cause of many different neurological disorders. [3] One of these diseases is spinocerebellar ataxia (SCA), an autosomal dominant disease characterized by impairment in locomotion and degeneration of the spinocerebellum which has an estimated prevalence of 1 to 4 in 100.000. SCA patients suffer from a progressive cerebellar ataxia characterized by loss of balance and coordination, slurred speech and abnormal eye movements, caused by selective degeneration of Purkinje cells, and subsequent loss of neurons in spinal cord and brainstem. [4; 5; 6] Although the clinical phenotype is very homogeneous, SCA is a genetically heterogeneous disorder, a disorder which can be caused by mutations in multiple different genes. [4; 7; 8] This variation in disease causing genes, divides SCA in multiple different sub-types. Until now, 31 SCA types have been described and 22 causative genes have been found (table 1). [2] All these genes cause similar phenotypes with slight variations in oculomotor deficits, dysarthria, dysmetria/kinetic tremor and gait. However the phenotypes also show overlap with some other neurological disorders, like episodic ataxias, hereditary spastic paraplegias, Huntington disease, essential tremor and hereditary sensory-motor neuropathies and can vary within a single SCA family. [4; 9] This clinical homogeneity complicates the diagnosis of a patient with a specific SCA type and makes genetic conformation necessary. The right diagnosis enlarges the possibility to treat a patient in a more effective way in the future and improves genetic counseling for family members. Until now, there are some therapies reported that reduce the symptoms in some SCA types, however there is not yet a complete treatment for any SCA type. [4; 10] Up till now, not all the disease causing genes are found and the complete molecular mechanism is unknown. Finding the disease causing genes and investigating their underlying

Table 1. A summary of the known spinocerebellar ataxia types. [1; 2]

SCA type	Location	Gene	Mutation type
SCA1	6p22.3	*ATXN1*	CAG expansion
SCA2	12q24.12	*ATXN2*	CAG expansion
SCA3	14q32.12	*ATXN3*	CAG expansion
SCA4	16q22.1	unknown	
SCA5	11p13.2	*SPTBN2*	In-frame deletion/ missense mutation
SCA6	19p13.2	*CACNA1A*	CAG expansion
SCA7	3p14.1	*ATXN7*	CAG expansion
SCA8	13q21	*ATXN8OS*	CTG expansion
SCA10	22q13.31	*ATXN10*	ATTCT expansion
SCA11	15q15.2	*TTBK2*	1bp indel/ stop mutation
SCA12	5q32	*PPP2R2B*	CAG expansion
SCA13	19q13.33	*KCNC3*	Missense mutation
SCA14	19q13.42	*PRKCG*	Missense mutation
SCA15	3p26.1	*ITPR1*	Deletion or missense mutation
SCA17	6q27	*TBP*	CAG expansion
SCA18	7q22-q32	Unknown	
SCA19	1p21-q21	*KCND3*	Missense mutation
SCA20	11q12		Chromosomal duplication
SCA21	7p21.3-15.1	Unknown	
SCA23	20p13	*PDYN*	Missense mutation
SCA25	2p21-p13	Unknown	
SCA26	19p13.3	Unknown	
SCA27	13q33.1	*FGF14*	Missense mutation
SCA28	18p11.21	*AFG3L2*	mutation
SCA30	4q34.3-q35.1	Unknown	
SCA31	16q21	*BEAN/ NEDD4*	TGGAA repeat
SCA32	7q32-q33	unknown	
SCA34	6p12.3-q16.2	unknown	
SCA35	20p13	*TGM6*	Missense mutation
SCA36	20p13	*NOP56*	GGCCTG expansion
SCA37	1p32	unknown	

pathway might result in a treatment or even a cure for SCA in the future. [11]

All known SCA types are caused by mutations in different genes. [6] Most of the SCAs such as the more common SCA1, SCA2, SCA3, SCA6 and SCA7s, are caused by a CAG repeat expansion, which encodes for an enlarged polyglutamine domain in the corresponding protein. [4; 6; 8] However, the disease can also be caused by non-coding repeat expansions, missense mutations, deletions or duplications. These mutations are characterized by severe maldevelopment or progressive degeneration of the cerebellum. [4; 6; 7]

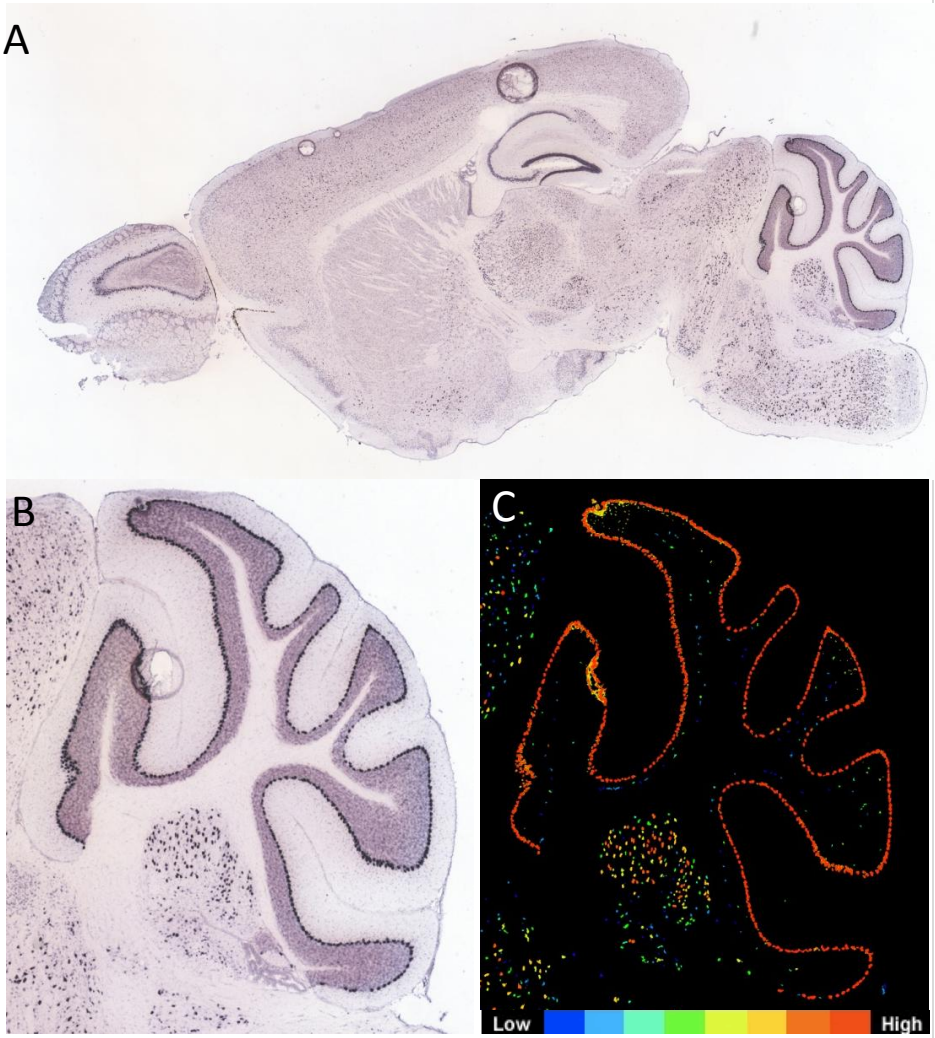

Figure 1. **A** sagittal section of a *Mus musculus* brain at an age of 56 days. Gene expression is localized using *In Situ* Hybridization. A. The whole mouse brain. A darker color indicates a higher expression of the *KCNC3* gene. **B** The cerebellum. **C** *KCNC3* expression localizes specifically to Purkinje cells in the cerebellum. Fluorescent *In Situ* Hybridization, red indicates a high expression of the *KCNC3* gene, blue indicates a low expression of the gene. [12]

One of these types is the SCA13 with a frequency varying between 0 to 2%. [3; 8; 13; 14] SCA13 is caused by missense mutations in the *KCNC3* gene, which encodes for the voltage-gated potassium channel Kv3.3. [3; 15] The Kv3.3 protein is highly expressed in the brain and has the highest expression in Purkinje cells (figure 1). The Kv3.3 channel facilitates the depolarization of dendritic calcium spikes and somatic sodium spikes in Purkinje cells, granule cells and deep cerebellar neurons. [5; 11]

Kv3.3 consists of 757 amino acids and is a protein with six transmembrane segments. [10; 11] The first four, S1-S4, are the voltage sensor domain and the last two, S5 and S6 together with a re-entrant loop form the ion-selective pore (figure 2). [11] Four of these Kv3.3 subunits together produce a functional channel complex. Kv3.3 channels are localized on axons and presynaptic terminals and facilitate the firing of hundreds of action potentials per second in neurons like Purkinje cells. [6] These action potentials are the only voltage change which activates and opens the Kv3.3 channel. [11] This opening and thereby the moving of potassium ions out of the neuron, contributes to a fast repolarization and helps recovering the sodium channels from inactivation. A proper functioning of the Kv3.3 channels gives a relatively short post-hyperpolarization and shortens the refractory period, whereby the neuron is

recovered fast and able to fire again. Defects in the voltage sensor or changes in the conformation of the channel can cause alterations in the potassium out flux. This results in a reduced firing rate of the neurons and therefore decreased signaling between these cells which causes problems in locomotion. A longer duration of the neuronal firing may also increase the flux of calcium into the cell, ultimately leading to neuronal death. [15]

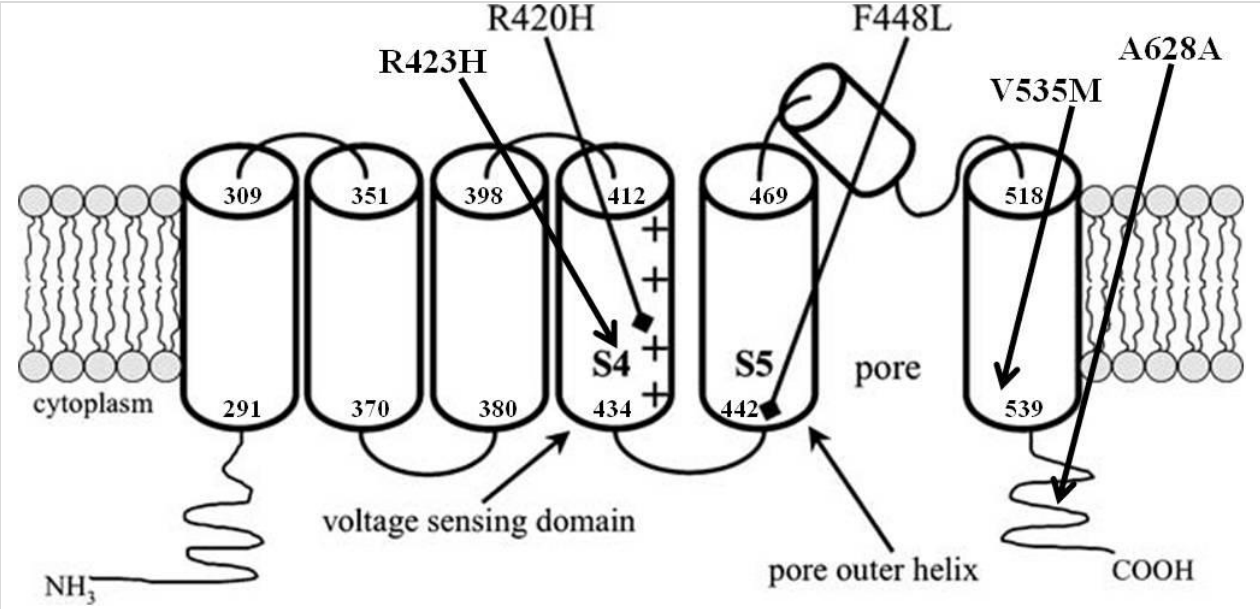

Figure 2. Schematic interpretation of the Kv3.3 channel. Showing the 6 transmembrane segments, the first and last amino acids of these segments are indicated with a number and the known and possibly new SCA13 causing mutations. [15]

To date, three mutations in the *KCNC3* gene are found to be the cause of SCA13; F448L, R420H and R423H. [8; 11] The mutations G263D and R366H were also called as SCA13 causing mutations. However, these variations were identified as unlikely to be the disease causing mutation because one individual also carried a mutation in the SCA6 gene (G263D) or the mutation did not segregate in the family (R366H). [8; 13]

F448L

The first family clinically described with the SCA13 phenotype is of French origin. [9; 11] Clinical data was present for 8 affected individuals, 6 persons with unknown disease state and 4 spouses, in 4 generations. [16] In this family the age of disease onset is during childhood and clinical symptoms include a mild delay in motor development, increased reflexes, and slowly progressive mental retardation. [7; 9; 16]

This mutation changes the 448th amino acid from a phenylalanine into a leucine. This amino acid is not conserved across species. The mutation is located in one of the transmembrane domains forming the pore, as shown in figure 2, and causes a shift of the activation curve in the negative direction and slowed channel closing. This part of the pore converts the voltage change into the opening or closing of the channel. Therefore this mutation results in a faster activation and an increased stability of the open conformation of the channel. [8; 11; 15]

R420H

In a Filipino family with 11 affected and 6 unaffected individuals in 3 different generations, another SCA13 mutation was found. In this family the age of onset is between 22 and 60 years. [9] Further, a second family with three affected members, plus two German individuals and a French individual are found to carry this mutation. These individuals all had a slowly progressive ataxia and cerebellar atrophy starting at an age of between 25 and 51 years. [8; 15] Some of these individuals also developed epileptic seizures, which might be associated with SCA13. [17]
This mutation changed the conserved 420th amino acid from an arginine into a histidine. This mutation is, as shown in figure 2, located in the main voltage-sensing domain of the channel and results in a dominant negative effect on the channel activity. This is due to the absence of one of the positively charged arginines which senses and responds to changes in voltage. [8; 11; 15] The mutated subunit results in a reduced number of firings, longer duration of the action potential and reduced potassium flux out of the cell. In *Danio rerio* the R420H mutation is found to alter excitability which is supporting the idea that this mutation can cause neuronal dysfunction in humans and the pathogenesis of SCA13. [6]

It was hypothesized that alterations in the channel's conformational functioning (F448L) are more severe than reduced channel activity (R420H), which was thought to be the reason for the different age of onset between the families. [11; 15] However, when in two European families a third mutation was found, this hypothesis was proven to be wrong.

R423H

In three unrelated index cases of European and American Caucasian origin, and for each a second affected family member, a third mutation in the *KCNC3* gene was found. In all families the age of onset was during infancy and all showed a slowly progressive ataxia, and delay in motor development, but no signs of mental retardation. This mutation changes the conserved 423th amino acid from an arginine into a histidine. Just like the R420H mutation, this mutation is located in the main voltage-sensing domain of the channel and results into a reduced channel activity. However, the phenotype resembles more the French F448L mutation than the Filipino R420H mutation, with the exception of the mild mental retardation which is only seen in the French F448L family. It is still unknown how the R420H mutation and the R423H mutation are cellularly similar but can have such different phenotypes. [8; 13; 18]

In this study we screen a cohort of 316 genetically undiagnosed (no mutations in SCA1-3, 6, 7, 12, 17, 19 and 23 genes) SCA patients, for variations in *KCNC3*. This includes the known SCA13 mutations and possibly new disease causing variations. Furthermore, we will perform functional analysis on the known variants and on 2 possible disease causing mutations, V535M and A628A, which were found in diagnostic tests of 2 unrelated patients.

In this study we aim to find an answer on these questions: do we have genetically undiagnosed patients with the known SCA13 mutations in our cohort? Are there other possible mutations in *KCNC3* that can induce SCA13? And do these known and new mutations change the Kv3.3 expression (western blot) and the Kv3.3 localization (immunocytochemistry)? And what is the frequency of SCA13 in our cohort? These answers might lead to a better understanding of the molecular mechanism behind SCA13 and in the future this might even lead to a better screening, a treatment and/or cure.

Patients

DNA is collected from 316 Dutch patients with cerebelar ataxia who are recruited by the UMCG (University Medical Center Groningen) clinical genetics department and are screened negative for SCA 1, 2, 3, 6, 7, 12, 17, 19 and 23.

Sequence analysis

The PCR mixes are made with the conditions: 5% 25 µM forward primer, 5% 25 µM reverse primer, 40% AmpliTaq Gold Fast PCR Master Mix (Applied Biosystems), 25 ng DNA and is brought to a total volume of 10 µL with MilliQ. The mixture of exon 1 amplicon 4 also contained 1M betaine and exon 2 amplicon 1 also contained 10% glycerol, table 2. The DNA is amplified by a touchdown protocol: denaturation at 94 °C for 5 minutes, followed by 11 cycles of 94 °C for 30 seconds, 65-60 °C (every cycle lowering by 0.5 °C) for 30 seconds and 72 °C for 45 seconds, followed by 26 cycles of 94 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 45 seconds, and ends with 7 minutes at 72 °C in a SensoQuest Labcycler. DNA amplification is checked on 2% agarose gels (agarose, Eurogentec and 10x TBE buffer, Gibco) and thereafter the product is sequenced.

Sequencing reactions are made with the conditions: 1 µl PCR product, 2.2 µL 3.2 µM primer, 8 µL MilliQ and 6 µL of the 4x diluted Big Dye Terminator 3.1 cycle sequencing kit. The sequence reaction of exon 1 amplicon 4 also contains 1M betaine. The sequence reaction is amplified by the following protocol: 1 minute at 96 °C, followed by 25 cycles of 10 seconds at 96 °C, 5 seconds at 50 °C and 3 minutes at 60 °C. Next, the samples are purified with a purification liquid (3% 3M NaAc, 14.5% MilliQ, 80% EtOH), 70% ethanol and 10 µL HiDi Formamide (Applied Biosystems). The samples are heated for 2 minutes at 95 °C before sequence analysis in the 3730xl DNA analyzer, Applied Biosystems. The results are analyzed with Mutation Surveyor version 3.23, Soft Genetics.

Table 2. PCR Primers.

Exon	Amplicon	Forward primer	Reverse primer	Amplicon size [bp]	Protocol
1	4	CCATGAGACGTACCGCTCGA	GGAAGCAGAGGCGCTTGAG		TD + B
2	1	GATGCCTAGGTCACCTCT	GTCGATGATGTTGAGGCTG	332	TD + G
	2	CCCAGACAAGGTGGAGTTT	CGTAGTAAATCATGGTGGCGA	296	TD
	3	CTGCTGCTCATCATCTTCCT	TTGCAGTAGTTGGGCGAG	383	TD
	4	CAAGAAGAAGAACAAACACATCC	CAGCTACCTCCCCAGTC	376	TD
3		CTCTCTCCTTTGTCTCTCTGT	GGTCCCAGGGGATCAGTA	266	TD
4		TTCCTCACCACTGACCCTTC	GGTTAGTCAGGCAGGAGTGG	269	TD
5	1	CCCGTGACTCTGTGTATTTCT	AGGCTCTCACAGGCATC	334	TD

TD: Touchdown protocol, G: 10% glycerol, B: 1M betaine

Plasmids

Mayo Clinic Rochester provided a plasmid containing the human *KCNC3* and a CMV promoter. Because of the GC-rich exon 1, we only cut out the second part of the gene with EcoRV and XhoI (Pharmacia Biotech) and ligated it into pcDNA3.1(-). In this vector the mutations are applied by mutagenesis primers, Eurogentec, table 3 in combination with the QuikChange II XL Site-Directed Mutagenesis Kit, Agilent Technologies. Hereafter the sequence including the mutation

is ligated back into the original plasmid, resulting in a plasmid that contains the complete *KCNC3* gene with the possible disease-causing mutations.
Digestion is checked on a 1% agarose gel and extracted with the GeneJET Gel Extraction Kit, Thermo Scientific. Transformations are done in E.coli DH5α wherefrom the plasmid is extracted with the GeneJET Plasmid Miniprep Kit, Thermo Scientific. The correct *KCNC3* insertion and the presence of the desired mutations are checked by sequencing. Because of problematic mutagenesis of the A628A mutation in *KCNC3*, there are multiple primers designed resulting in 2 functioning primers, table 3.

Table 3. Mutagenesis primers.

Mutation	Forward primer	Reverse primer
R420H	GTCCGCTTCGTCCACATCCTGCGCATC	GATGCGCAGGATGTGGACGAAGCGGAC
R423H	GTCCGCATCCTGCACATCTTCAAGCTG	CAGCTTGAAGATGTGCAGGATGCGGAC
F448L	CAGCACCAACGAGTTACTGCTGCTCATCATC	GATGATGAGCAGCAGTAACTCGTTGGTGCTG
V535M	CATCGCCATGCCTATGCCCGTCATT	CAATGACGGGCATAGGCATGGCGATG
A628A (1)	CTCAGGGGGGAGCAGGTGGGCT	AGCCCACCTGCTCCCCCCCTGAG
A628A (2)	GGGGAGCAGGTGGGCTGGGGATCAT	ATGATCCCCAGCCCACCTGCTCCCC

Cell culture & transfection

HeLa cells and HEK293T cells are cultured in DMEM, Gibco with 10% FBS, Invitrogen and 1% Pen/Strep, Gibco at 37 °C and 5% CO_2. After 24 hours the cells are transfected with PEI, Polysciences Inc. The mixtures shown in table 4 are incubated for 15 minutes at room temperature before being added to the cells. The cells are incubated for 48 hours at 37 °C and 5% CO_2. The EGFP-N1 and GFP-Kv3.3 plasmids are included as negative and positive control and to indicate transfection efficiency.

Table 4. PEI mixtures.

	Western blot		Immunocytochemistry	
	HEK	HeLa	HEK	HeLa
DMEM [µl]	150	150	100	100
PEI [µl]	5 (1 µg/µl)	7,5 (2 µg/µl)	3,75 (1 µg/µl)	7,5 (2 µg/µl)
DNA [ng]	1	1	0,75	0,75

Protein extraction for western blot

After transfection the medium is removed and the cells are washed with DPBS, Lonza. Next, the cells are lysed with SDS lysis buffer or RIPA lysis buffer. SDS (2% SDS, Invitrogen in PBS and 25x proteases inhibitor, Roche Diagnostics or RIPA) and RIPA treated cells are scraped out of the wells and sonificated. The amount of protein is quantified with the Pierce BCA Protein Assay kit, Thermo Scientific. Further, LSB 4x (125 mM Tris-HCL, ph 6.8, 8% SDS, 40% glycerol, 0.008% bromophenol blue) with 10% β-ME, Sigma) is added and the samples are incubated at 65 °C for 5 minutes.

Western blot

A Tris-glycine SDS-polyacrylamide electrophoresis gel is made from 10% resolving gel and 5% stacking gel. 50 µg protein sample and 4 µl PageRuler Plus Prestained Protein ladder (Thermo Scientific) are loaded and at 130 V. The samples are transferred from the gel to the nitrocellulose membrane, Bio-Rad for 90 minutes at 250 mA. Protein transference is checked with Ponceau S Solution, Serva. Next, the membrane is blocked with 5% milk in 1x TTBS (Tween 20, Sigma-Aldrich and 10x TBS, Bio-Rad) for 1 hour at room temperature and incubated with the primary antibody overnight at 4 °C, table 5. The membrane is washed with TTBS and incubated

with the HRP-conjugated secondary antibody for 1 hour at room temperature, table 5. Then the membrane is washed with TTBS and incubated for 1 minute with ECL (homemade) to detect the proteins.

Table 5. Western blot antibodies diluted in 5% milk in TTBS.

Antibody	Type	Source	Dilution	Company
α-Kv3.3	Primary	Rabbit	1:500	Alomone Labs
α-actin	Primary	Mouse	1:5000	MP Biochemicals
α-rabbit	Secondary	Goat	1:10.000	Bio-Rad
α-mouse	Secondary	Goat	1:10.000	Bio-Rad

Immunocytochemistry

After transfection the medium is removed and the cells are washed with DPBS. The cells are fixed on the cover slip with 4% PFA for 15 minutes at room temperature and are washed with DPBS. The cells are incubated with 0.1% triton in PBS for 10 minutes at room temperature for permeabilization and incubated with 5% BSA, Sigma in PBS for 30 minutes at room temperature for blocking. Next, the cells are incubated with the primary antibody for 1 hour at room temperature in a dark humid chamber, table 6. The cells are washed with PBS and incubated with the fluorescent secondary antibody for 1 hour at room temperature in the dark, table 6. Finally, the cover slips are washed with PBS and mounted on slides with Vectashield mounting medium containing DAPI, Vector Laboratories.

Table 6. Immunocytochemistry antibodies diluted in 5% BSA in PBS.

Antibody	Type	Source	Dilution	Company
α-Kv3.3	Primary	Rabbit	1:200	Alomone Labs
Cy3	Secondary	Donkey α-rabbit	1:250	Jackson Laboratories

Results

In order to try to find more individuals with SCA13 including heterozygous carriers of the known mutations R420H, R423H and F448L in *KCNC3,* but also patients with possible new disease causing mutations, we screened a Dutch ataxia cohort (316 ataxia patients) for rare variations in the coding regions of *KCNC3.* The exons of the gene were amplified by PCR, where after this PCR product was Sanger sequenced to determine the base pair order.

We found 2 seemingly independently referred genetically undiagnosed Dutch SCA patients who carried the known heterozygous SCA13 mutation c.1259, p.R420H located in exon 2 (figure 3A-C). The family history was investigated for both patients to see if the R420H mutation occurred twice in different families or just once in one family with 2 known affecteds. These individuals, 15988 and 04919, appeared to be cousins who came out of a large family with more affected family members (figure 4). DNA was available of a third family member, the daughter of the first cousin 19962.This woman was also screened and was found to be carrier of the R420H mutation too.

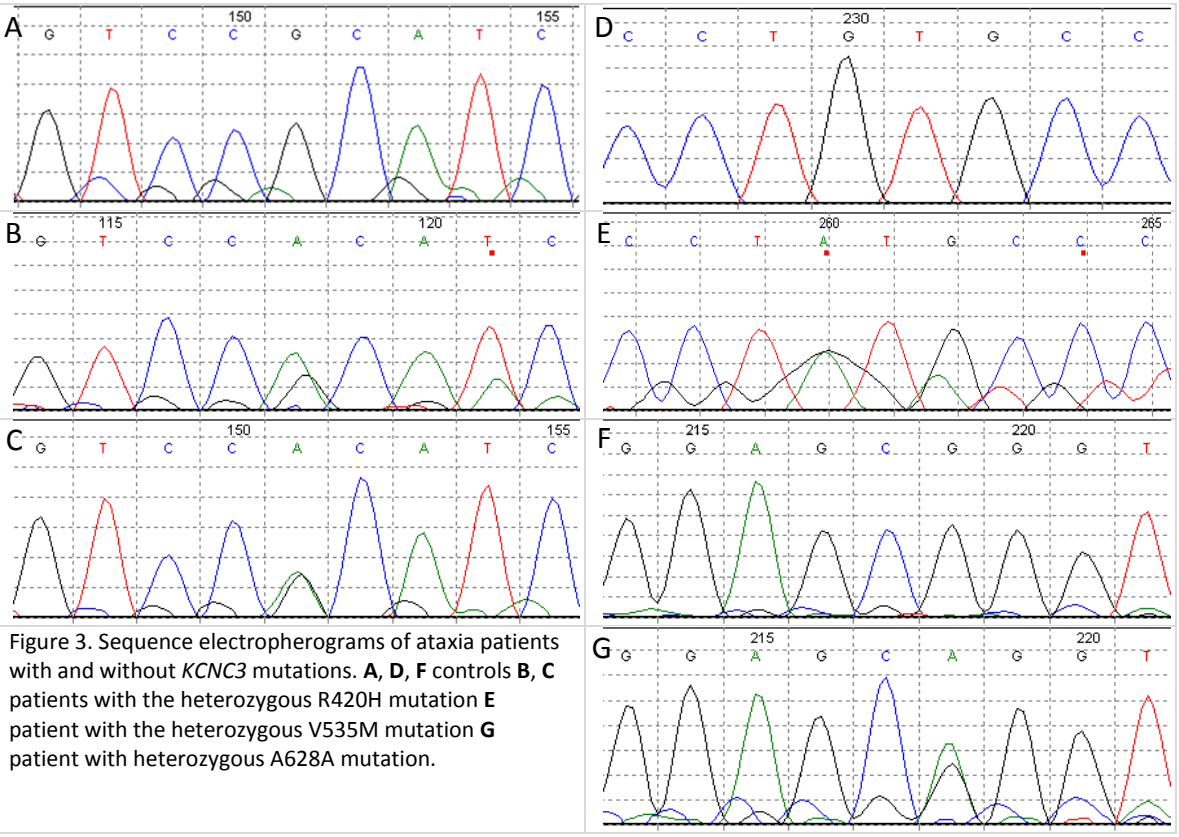

Figure 3. Sequence electropherograms of ataxia patients with and without *KCNC3* mutations. **A**, **D**, **F** controls **B**, **C** patients with the heterozygous R420H mutation **E** patient with the heterozygous V535M mutation **G** patient with heterozygous A628A mutation.

Additionally we found 2 other cases with rare variations in *KCNC3*. These patients were heterozygous carriers of the c. 1603, p.V535M and c.1884, p.A628A, variations located in exon 2 (figure 3D-G). These variations were not observed in healthy Dutch controls (GoNL database; 500 exomes) or healthy European-Americans (EVS; 4300 exomes) (table 7).

To investigate whether the novel variations will have damaging effects to the protein function or structure, we used the *in silico* prediction programs PROVEAN, SIFT, PolyPhen2, mutation taster

and mutation assessor to indicate if they are harmless or disease causing (see table 7). The first variant, V535M, affects the semi-conserved 535th amino acid and is found across species as valine or isoleucine. These 2 amino acids only differ in the addition of 1 methyl group. This variant is predicted very likely to be disease causing. The second variant, A628A is a synonymous variant, but is still predicted as possibly disease causing as the program mutation taster predicts that this mutation affect a splice site leading to a novel donor site in the intron between exon 2 and 3.

Additionally our screen yielded 3 patients with the mutation c.1641 p.S547S which is indicated as a SNP in EVS and GoNL, rs2301357.

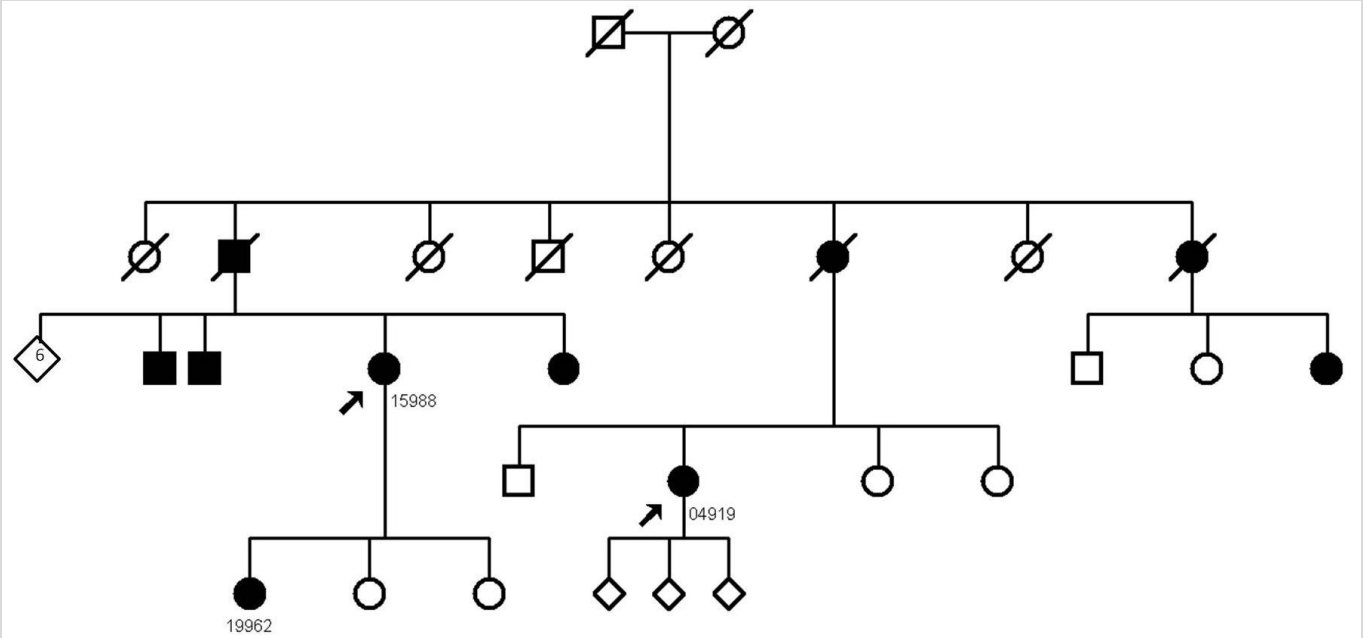

Figure 4. Pedigree of the Dutch R420H family. The persons indicated with arrows were found in our cohort screen to carry the R420H mutation.

Table 7. *KCNC3* variations found in undiagnosed SCA patients and *in silico* damage prediction.

Mutation	SNP	Genomic position	Type	Affecteds	GoNL	EVS	PROVEAN	SIFT	Poly-Phen2	Mutation-taster	Mutation-assessor
R420H	rs104.894.699	50.826.951	Missense	3	-	A=0 G=8600	+	+	+/-	+	+/-
R423H	-	50.826.942	Missense	0	-	-	+	+	+/-	+	+/-
F448L	rs104.894.700	50.826.868	Missense	0	-	-	+	-	+/-	+	-
V535M	-	50.826.607	Missense	1	-	-	+	+	+/-	+	+/-
A628A	-	50.826.325	Synonymous	1	-	-	-	-		+	

+ disease causing or damaging +/- probably damaging – not damaging

To further prove that these two variants were really damaging we searched for changes in Kv3.3 protein localization, expression and activity of the channel compared to the 3 known *KCNC3* mutations and the wild type Kv3.3 channel.

First, we aimed to detect possible changes between wild type and mutant (V535M, A628A, R420H, R423H, and F448L) Kv3.3 channel expression in HEK cell extracts using western blot analysis. As we know that Kv channels form tight tetramers intracellular, we first wanted to test which type of lysis buffer (2% SDS or RIPA) would be able to disrupt these complexes yielding only monomeric Kv3.3.

Therefore, all HEK cells expressing wild type and all mutant Kv3.3 channels were lysed with 1) 2% SDS lysis and 2) RIPA. These extracts were run on different SDS page gels for comparison. After western blotting, we noticed that in the SDS extracts less high molecular weight complexes were seen than in RIPA (see figure 5A, indicated by the square) and more monomeric Kv3.3 (~110kDa, indicated by asterix). Next, in the SDS extracts we noticed clearer extra bands just above the monomeric Kv3.3, (indicated by double or triple asterixes). The high molecular weight complexes seen in RIPA treated cells, figure 5B, make it harder to quantify the expressed amount of Kv3.3. Thus 2% SDS lysis buffer works more efficient than RIPA, and therefore all further experiments are performed using SDS lysis buffer.

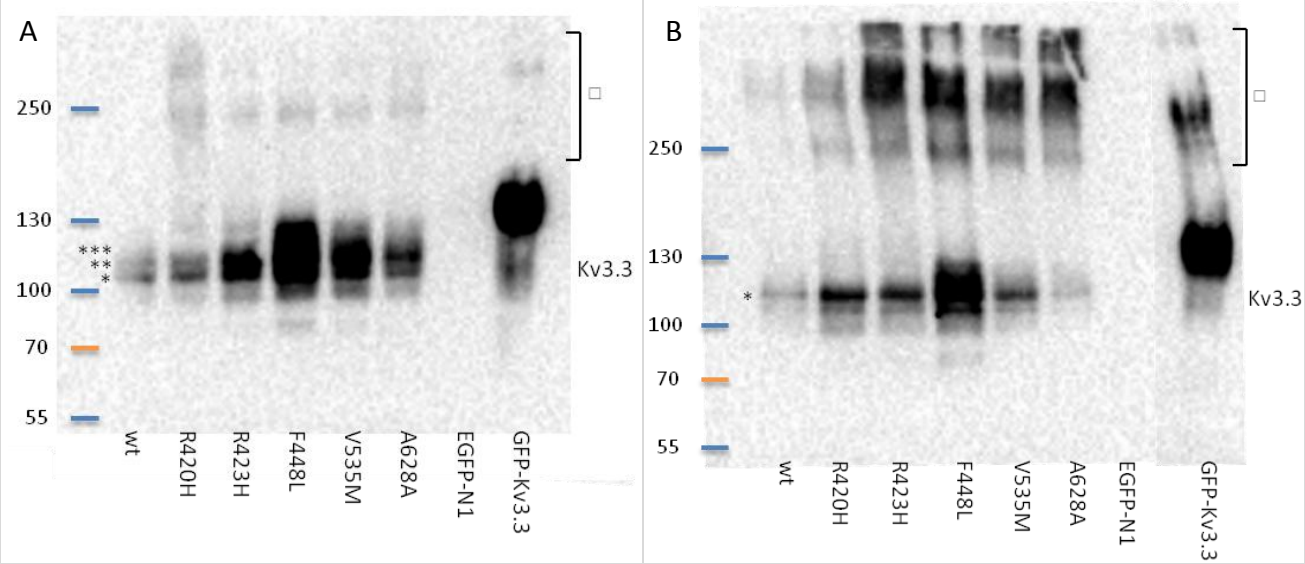

Figure 5. Western blots of HEK293T cells extracted with **A** 2% SDS and **B** RIPA run on a 6% SDS page gel. Protein expression of wild type and mutant Kv3.3, EGFP-N1 as negative control and Kv3.3-GFP as positive control.

To finally compare the expression levels of the various Kv3.3 proteins, an independent experiment was performed in which the HEK cells were lysed with 2% SDS and equal amounts of protein was loaded on 10% SDS page gels (figure 6). Although not very clear in figure 6, we noticed that the Kv3.3 protein with the F448L mutation has an overall higher protein expression than wild type Kv3.3.

Furthermore, Kv3.3 with the F448L, V535M, and A628A mutations seem to have the same protein modifications as wild type (indicated by the asterixes in figure 6), even though they have a higher expression in figure 5. However, Kv3.3 carrying the mutations R420H and R423H are not or less modified as these extra bands are not detected. Finally, the R423H mutation led to increased Kv3.3 protein expression compared to wild type Kv3.3.

Note: because of the high expression Kv3.3-GFP only 5 µg was loaded on the SDS page gel instead of 50 µg. This results in a lower actin signal on the blot.

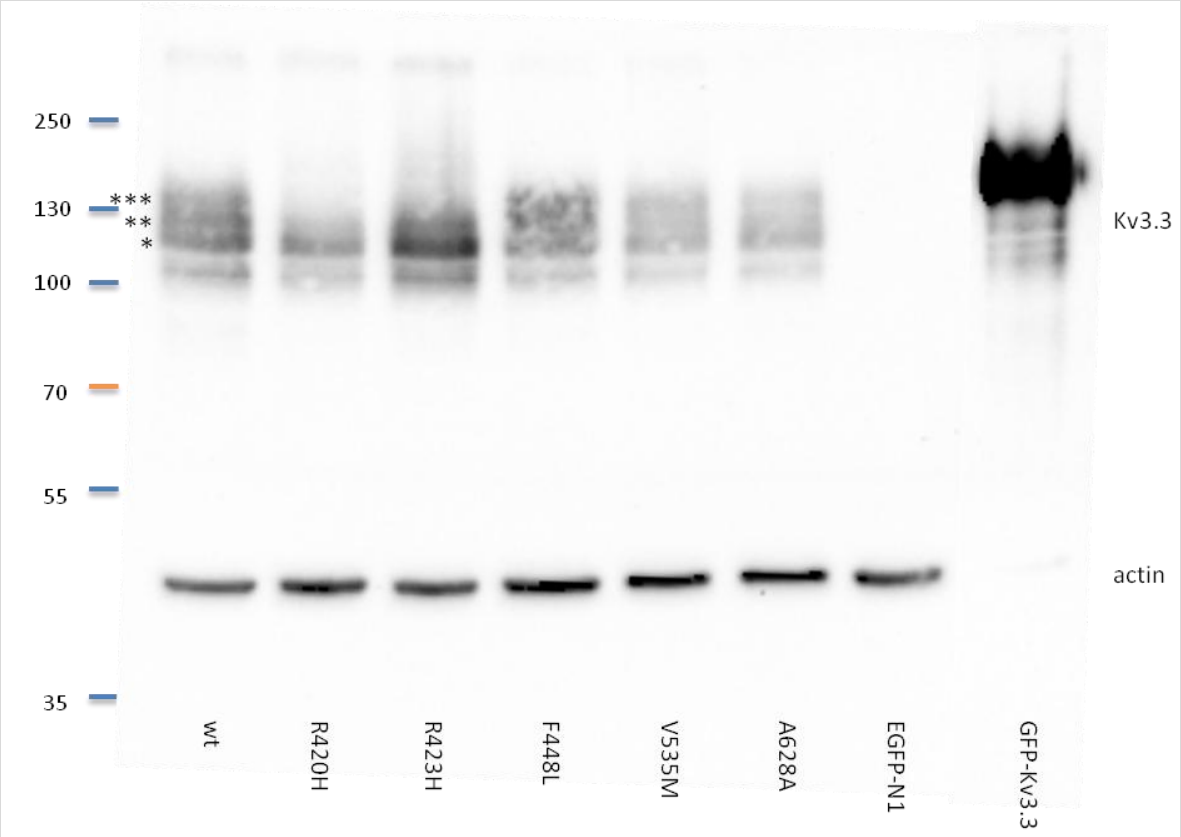

Figure 6. Western blot analysis of HEK293T cells extracted with 2% SDS run on a 10% SDS-page gel. Protein expression of wild type and mutant Kv3.3, EGFP-N1 as negative control and Kv3.3-GFP as positive control.

To determine the effect of the mutations on the cellular localization of Kv3.3, we performed immunocytochemistry on HEK cells expressing wild type Kv3.3 and the various mutants. We hypothesized that mutant Kv3.3 shows altered cellular localization compared to wild type Kv3.3 as R420H, R423H, F448L and V535M are all located in transmembrane regions. After 48 hours the HEK cells were fixed and stained with anti-Kv3.3 antibody (red) (figure 7). In contrast to what we expected, wild type Kv3.3 was mostly detected in dots-like structures (vesicles) that were located all over the cell and concentrated near the nucleus, and only rarely cells were found with clearer staining on the edges representing the plasma membrane. Cells with ER like staining were also observed (indicated by a green arrow in figure 7A). No striking differences were seen between the R423H, F448L, V535M and A628A mutant Kv3.3 and wild type Kv3.3 as these mutant proteins seem to localize to the same organelles (Figure 7 D-G). In the case of R420H mutant Kv3.3 a change in cellular localization might be there, as strong dot-like Kv3.3 staining was observed and seems to accumulate near the nucleus (indicated by blue arrows in figure 7B), but again also here some cells showed suggestive ER localization (indicated by a green arrow in figure 7C). Overall a mixed population of cells was seen with various Kv3.3 patterns ranging from vesicles throughout the cell, vesicles perinuclear and ER staining.

Note: we did not yet stain with organelle markers to perform co-localization studies.

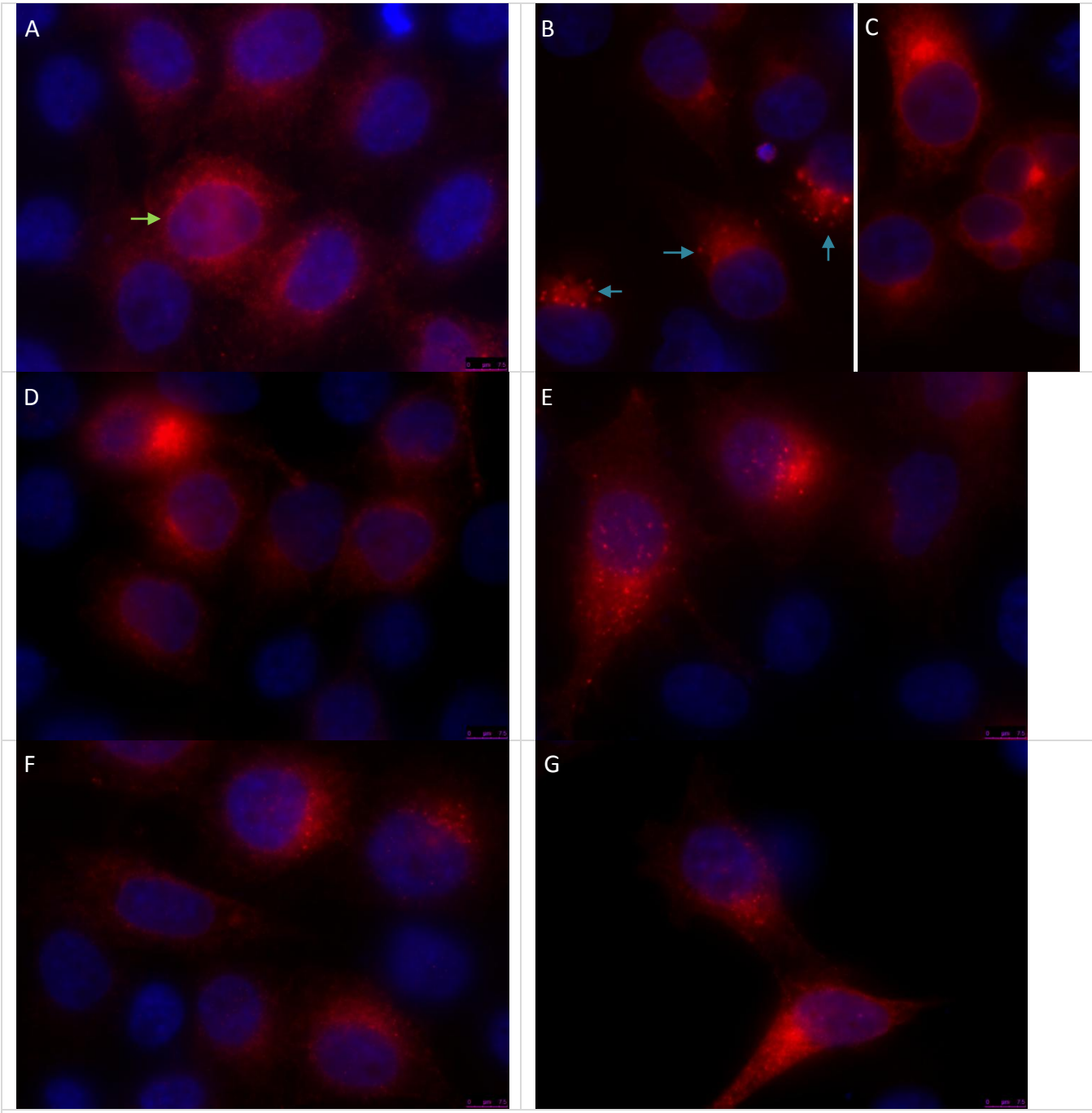

Figure 7. HeLa cells transfected with wild type and the various mutant Kv3.3 plasmids. 100x objective Blue: DAPI, Red: Kv3.3., Green arrow: ER, Blue arrow: perinuclear space **A** wild type **B & C** R420H **D** R423H **E** F448L **F** V535M **G** A628A.

Conclusion and Discussion

In our 316 genetically undiagnosed SCA patients cohort we found 2 patients with the known SCA13 mutation R420H. These patients appeared to be related and had 1 additional family member who also carried the R420H mutation. The known *KCNC3* mutations R423H and F448L were not found in our Dutch cohort. However, there were 2 patients found with a V535M and an A628A mutation. *In silico* prediction programs determined V535M as (possible) disease causing and A628A was predicted as damaging but also as not damaging, depending on the used program.

Protein expression and localization studies revealed that, contrary to the prediction programs, V535M and A628A might be harmless variations in the DNA. In contrast, the Kv3.3 R420H mutant seems to have no modificated monomeric forms and is also more expressed in the ER and accumulates more in the perinuclear space than the other 4 mutants and the wild type. However, some cells also express Kv3.3 in vesicles in the entire cell just like wild type Kv3.3. These observations suggest that the R420H mutation is a damaging variation. The Kv3.3 R423H mutant also seems to have no modificated monomeric forms but is expressed in vesicles all over the cell. This might even be a more damaging variation than the R420H mutation. The Kv3.3 F448L mutant has a higher protein expression than wild type Kv3.3, indicating that also this mutation might be disease causing.

The V535M and A628A mutation seemed to have increased the incidence of SCA13 in our Dutch genetically undiagnosed SCA cohort to 1.27%. However, the functional studies indicated both mutations are probably harmless and changed the incidence of SCA13 in our cohort into 0.63%.

The first exon of *KCNC3* is a very GC-rich area. These kind of areas are often hard to amplify because of the 3 hydrogen bonds between G and C nucleotides in comparison with 2 hydrogen bonds between A and T nucleotides. Therefore, more energy is needed to break these bonds and separate the strands. Also, the G and C nucleotides make it harder for primers and polymerases to bind. The usage of a touchdown PCR protocol, primers with low GC content or the addition of enhancers like glycerol, DMSO, betaine or GC-enhancer provided by companies often clear this problem. [19] However, after these changes we were still unable to amplify and sequence the first exon of *KCNC3*. Recently, we did manage to amplify a part of exon 1 between amino acids 120-246. In this part 1 additional patient was found that carried the c.385, p.D129N mutation where the 1st nucleotide of the codon was changed from G into an A. Except from PolyPhen2, all the prediction programs used predicted that this mutation is damaging. However, co-segregation in the family and functional analysis are not tested yet. This proves that all the exons of a gene are important for its function and indicates that even the N-terminus of *KCNC3* might contain new SCA13 causing mutations.

Therefore, it is impossible to assume that R420H, V535M and A628A are the only possible SCA13 causing mutations in our cohort. Notably, all the disease causing mutations, R420H, R423H, F448L, V535M, and A628A, appear in the second exon encoding for the 6 transmembrane segments. Supporting this idea, the A628A is a mutation located in the C-terminus of the protein and not in the transmembrane segments and therefore might not be damaging. This was also supported by our functional assays as is therefore assumed to be a polymorphism.

Further, we cannot be 100% on the outcome of the prediction programs. Since no *in silico* program makes a prediction based on the molecular mechanisms in a cell, especially because all the mechanisms are not known yet. Most of the prediction programs only look at a few mutation causing aspects and base their prediction on those data. The programs predict how damaging a mutation is on different criteria, for example the change of amino acid, amino acid conservation across species, splice site changes and conservation of the domains. These different criteria cause the *in silico* prediction programs to have different outcomes as was the case for the V535M and A628A mutations.

Only for three mutations we can conclude that the expression and/or modification of the Kv3.3 protein is altered including R420H, R423H and F448L. The F448L mutation led to increased protein stability whereas the R420H and R423H Kv3.3 proteins showed altered protein modifications. Examples of post-translational modifications are glycosylation and phosphorylation. These changes occur in the Golgi apparatus and prepare proteins for their final destination like transport to the membrane. The absences of the extra bands on the western blots for the R420H and R423H mutants might indicate that these proteins are not modificated in the Golgi apparatus or do not reach the Golgi apparatus. Therefore, we expect that these 2 mutations are more localized in the ER than the wild type and the other mutants (F448L, V535M and A628A) which have multiple modificated forms.

The F448L mutant has a higher protein expression than the wild type and the other mutants. This might be the result of a more stable protein and a longer half life that is induced by this mutation. This might lead to a higher amount of tetrameric channels in the plasma membrane and increased potassium out flux. An elevated extracellular potassium concentration makes it harder for intracellular potassium ions to flow out during repolarization. Just like all other ions, potassium prefers to diffuse down its concentration gradient and not in the other direction. This decreased potassium out flow causes an extension of the repolarization phase or even an incomplete repolarization. This is leading to a slower recovery of the neuron and therefore a slower firing. An elevated extracellular potassium concentration also results in an increased membrane potential, hereby the activation threshold of action potentials is reached earlier and activation of the cell occurs earlier than in the wild type. This corresponds with the findings of waters et al. [(11)]

The 2 possible new mutants V535M and A628A do not alter Kv3.3 expression and have similar modified forms as wild type Kv3.3. This may suggest that the Kv3.3 mutants V535M and A628A are not disease causing.

Kv3.3 is thought to be a transmembrane protein in the plasma membrane. However, after our localization studies it seems that wild type protein expressed in HeLa cells, is rarely expressed in the plasma membrane. The wild type Kv3.3 is mainly expressed in vesicles in the cytosol or in ER. This indicates that the production of Kv3.3 might be inefficient and that most of the encoded protein is not transported to the plasma membrane due to absence of its regulatory subunits in HEK or HeLa cells. This is seen for Kv4.3, where the regulator subunit KChIP improves the transportation from ER to Golgi apparatus to plasma membrane. However, this regulatory subunit is not present in HeLa cells. [(20)]

The accumulation of Kv3.3 in the ER of the R420H and R423H mutant might indicate that these mutations cause misfolding of the Kv3.3 protein and get ER retained and therefore the protein cannot be modified in the Golgi apparatus. This might explain the absence of the modificated

monomeric proteins in the protein expression study. Thus even though the *in silico* prediction programs determined the V535M and A628A mutation as damaging or probably damaging. The protein expression study and the localization study do not see changes compared to the wild type. Based on these experiments the V535M and the A628A mutation in *KCNC3* might be harmless SNPs.

The best functional test to determine if a variant is harmless or disease causing is to determine the channel activity of Kv3.3. Unfortunately, due to time we were not able to perform this test but will be in the near future.

Minassian et al. found that R420H subunits incorporated into active wild type Kv3.3 channel have little effect on the functional properties of the channel. [18] This finding gives the idea that most of the proteins with the R420H mutation stay in the ER and are degradated, but when they leave for the membrane they only suppress the Kv3.3 current amplitude. In humans this heterozygous mutation could lead to a lower amount of functional channels which leads slowly to damage. This corresponds with the phenotype of slowly progressive ataxia and cerebellar atrophy in these patients.

This group also found that multiple R423H subunits can be incorporated into active wild type Kv3.3 channels, unlike R420H were only 1 subunit can incorporate. When the tetramer is completely made of R423H subunits or in combination with wild type Kv3.3 subunits, the gating properties of the channel are affected. [18] Tetrameric Kv3.3 channels consisting of multiple mutated subunits might be more harmful, leading to a more degenerative phenotype and earlier onset corresponding to the R423H phenotype.

The accomplishment of functional studies in other groups might be due to their choice of cells. Most of the SCA13 data results from two-electrode voltage clamp experiments in *Xenopus* oocytes. [8; 11; 13; 18] With exception of the study of Issa et al. who patch-clamped CaP neurons in zebrafish. [6] However, mammalian cells are more stable to be transfected and can be more easily generated in a higher number than *Xenopus* oocytes. [21] Mammalian cells also resemble the intracellular composition of human diseases more than non-mammalian cells. This makes it more likely that experiments done in mammalian cells are a better representation for the real Kv3.3 functioning in human cells.

In the end more research has to be done to unravel the whole molecular mechanism behind SCA13. More knowledge might lead to a better screening, a treatment and/or cure for SCA13 affected patients.

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