Impaired cytoplasmic domain interactions cause co-assembly defect and loss of function in the p.Glu293Lys *KNCJ2* variant isolated from an Andersen-Tawil Syndrome patient

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Short title: Impaired CD-I interactions in Kir2.1 channels cause ATS

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Abstract (286 words)

Aims Subunit interactions at the cytoplasmic domain interface (CD-I) have recently been shown to control gating in inward rectifier potassium channels. Here we report the novel *KCNJ2* variant p.Glu293Lys that has been found in a patient with Andersen-Tawil Syndrome type 1 (ATS1), causing amino acid substitution at the CD-I of the inward rectifier potassium channel subunit Kir2.1. Neither has the role of Glu293 in gating control been investigated, nor has a pathogenic variant been described at this position. This study aimed to assess the involvement of Glu293 in CD-I subunit interactions and to establish the pathogenic role of the p.Glu293Lys variant in ATS1.

Methods and Results The p.Glu293Lys variant produced no current in homomeric form and showed dominant negative effect over wild type (WT) subunits. Immunocytochemical labelling showed the p.Glu293Lys subunits to distribute in the subsarcolemmal space. Salt bridge prediction indicated the presence of an intersubunit salt bridge network at the CD-I of Kir2.1, with the involvement of Glu293. Subunit interactions were studied by the NanoBiT split reporter assay. Reporter constructs carrying NanoBiT tags on the intracellular termini produced no bioluminescent signal above background with the p.Glu293Lys variant in homomeric configuration and significantly reduced signals in cells co-expressing WT and p.Glu293Lys subunits simultaneously. Extracellularly presented reporter tags, however, generated comparable bioluminescent signals with heteromeric WT and p.Glu293Lys subunits and with homomeric WT channels.

Conclusions Loss of function and dominant negative effect confirm the causative role of p.Glu293Lys in ATS1. Co-assembly of Kir2.1 subunits is impaired in homomeric channels consisting of p.Glu293Lys subunits and is partially rescued in heteromeric complexes of WT and p.Glu293Lys Kir2.1 variants. These data point to an important role of Glu293 in mediating subunit assembly, as well as in gating of Kir2.1 channels.

Translational perspective (94 words)

Andersen-Tawil Syndrome (ATS) is a rare genetic disorder characterized by the triad of periodic paralysis, dysmorphic features and ventricular arrhythmias. Symptoms can be mild and atypical, therefore, genetic screening of affected families is pivotal. This study describes the p.Glu293Lys variant of *KCNJ2* encoding the Kir2.1 ion channel subunit as pathogenic, thereby aiding genetic testing of ATS. The study also identifies disturbed interactions between the cytoplasmic domains of Kir2.1 subunits as the molecular mechanism of loss-of-function in the p.Glu293Lys variant. Targeting cytoplasmic domain interactions may represent a promising strategy for the development of Kir2.1 agonists.

1. Introduction

Andersen-Tawil Syndrome (ATS) is a rare genetic disorder characterized by the triad of periodic paralysis, dismorphic features and cardiac manifestations, such as frequent ventricular ectopic activity and the occurrence of sustained ventricular arrhythmias[1-5]. ATS type 1 (ATS1) is caused by mutations in the *KCNJ2* gene encoding for the inward rectifier potassium channel Kir2.1[6]. Kir2.1 is expressed in various tissues including the skeletal muscle and the heart. Kir2.1 can exists either in the form of homo- or hetero-tetrameric complexes that consist either four identical Kir2.1 subunits or various combinations of the structurally related members of the Kir2.x subfamily of inward rectifier potassium channels, respectively (reviewed in[7]). In the skeletal muscle, Kir2.1 combines with Kir2.2 and Kir2.6, whereas in the heart, Kir2.1, Kir2.2, Kir2.3 and Kir2.4 subunits co-assemble in a cell type-specific way to form ion channels generating the cardiac inwardly rectifying potassium current I_{K1}[8-10]. Kir2.1 dysfunction has been shown to underlie pathologies leading to periodic paralysis, craniofacial dismorphic features and cardiac symtpoms characteristic to ATS1[11-13].

Symptomes in ATS1 are often mild and long-term prognosis is thought to be relatively more favourable compared to other channelopathies. A recent multicenter study on the largest ATS1 patient cohort (n = 118) assembled to date, however, showed that adverse cardiac events are more frequent in ATS1 than previously anticipated[14]. Most importantly, pharmacological therapy including the use of β-

blockers alone or in combination with class Ic antiarrhythmic agents failed to reduce the occurrence of life-threathening arrhythmic events, despite suppressing arrhythmic symtpomes effectively[14]. These findings highlight the demand for evidence-based treatment strategies in ATS1, including the development of specific activators of I_{K1}.

Over 70 *KCNJ2* mutations causing ATS1 have been described, the majority being missense mutations and all but a few have a dominant negative effect over the wild type (WT) Kir2.1 variant (reviewed in[15]). Gating alterations[16, 17], defective protein trafficking[18] and structural changes in the conduction pathway[19, 20] have been identified as molecular mechanisms underlying loss-of-function of Kir2.1 channels.

We have identified the *KCNJ2* variant p.Glu293Lys in a patient with characteristic ATS1 phenotype. The amino acid Glu293 affected in this variant is located in cytoplasmic domain at the surface connecting adjacent subunits, the so-called cytoplasmic domain interface (CD-I)[21]. Subunit interactions between Kir subunits at the CD-I have recently been demonstrated to control gating of inward rectifier channels[22-24]. Considering the capacity of Glu293 to form salt bridges, a prominent role for Glu293 in mediating subunit CD-I interactions may be anticipated. To our knowledge, structure-function correlates of Kir2.1 at Glu293 and the surrounding region has not been investigated. In the light of the unmet need for a selective I_{K1} activator, insights into gating mechanisms and into structure-function relationships of Kir2.1-based ion channels might serve as the basis of rational drug design. Hence, this study aimed to investigate whether CD-I subunit interactions are affected in the p.Glu293Lys variant. Furthermore, since no mutation at codon 293 of *KCNJ2* has been associated to ATS1, we functionally characterized the p.Glu293Lys variant in order to establish the pathogenic role of p.Glu291Lys variant in ATS1, thereby aiding future genetic testing.

2. Methods

2.1 Patient's case history

The index patient was referred to the Cardiology Center, University of Szeged, Hungary (a European Reference Network GUARD-Heart Affiliated Partner) through a nation-wide collaboration aiming to establish centralized screening for patients with

ion channel diseases and was one of the six patients which were diagnosed with Andersen-Tawil syndrome in the frame of the project.

The female index patient was diagnosed with ATS1 at age of 7 years. She was born from an uneventful pregnancy at the end of 40 gestational weeks, with 2900 g birth weight. Dismorphic features were evident at the time of diagnosis and included hypertelorism, low-set ears, mandibular hypoplasia, broad nose, and broad forehead.

Symptomless premature ventricular beats (PVB), which were diagnosed as multifocal (with two different PVB morphology), sometimes bigeminal PVBs, with short runs of non-sustained ventricular tachycardia (NSVT) were detected at age of 4 years. On Holter examination, PVBs made up 38% of all recorded beats. Corrected QT interval (QTc) was increased and was in the range of 456-485 ms. Echocardiography did not reveal any significant structural heart disease. Due to regular NSVT, flecainide therapy was initiated at the age of 11 years. Ventricular PVBs ceased promptly after the initiation of iv. flecainide and remained so on po. therapy (2x100 mg). Beta blocker therapy (propranolol 3x20 mg) was continued. Control Holter revealed 0% PVB of all recorded beats.

Skeletal muscle symptoms, apart of general fatigue and occasional numbness of the feet, started to occur as periodical paralysis at age of 8 years. The latter was characterized by inability to raise and walk, lasting for 3 days, and was unrelated to serum potassium levels (3,86-4,6 mmol/l). Acetazolamide did not significantly improve symptoms. The episodes of periodical paralysis became more severe and the patient is wheelchair bound at age of 10 years. Flecainide treatment, which was shown to be particularly effective in reducing ventricular arrhythmic activity after one-year follow-up, seemed to have no effect on paralytic symptoms. Paralysis often affected all four extremities. The patient's mental development and ability to cooperate is according to age.

At the time of the last visit, the patient was 12 years old. She complains retrosternal chest pain, that does not occur at night. Shortness of breath also occurs. Respiratory function is in normal range in lying position, while a moderate restrictive respiratory dysfunction was demonstrated in upright position, characteristic for muscle weakness, possibly indicating intercostal muscle weakness. Rarely, abdominal pain develops. Abdominal ultrasound did not confirm any abnormality in the localization corresponding to the complaints.

2.2 Genetic analysis

Molecular genetic analysis was approved by the Institutional Ethics Committee of the University of Szeged, and conducted according to the Helsinki declaration after the patient's care-givers gave prior written informed consent. Coding sequences and exon-intron boundaries of major ion channel and ion-channel related genes, including the *KCNJ2* gene, were analyzed by next-generation sequencing using Agilent's SureSelect technology with custom-designed 120-mer RNA baits specific to target region (Agilent Technologies, Santa Clara, CA, United States). The *KCNJ2* gene sequence variant was validated by Sanger sequencing. Molecular genetic and bioinformatic analysis was carried out as described previously[25].

2.3 Plasmid constructs

The wild type (WT) mouse *KCNJ2* cDNA was available from a previous study[26], whose procedures conformed to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes, point mutations c.653G>A and c.877G>A encoding for the p.Arg218Gln and p.Glu293Lys variants were introduced using the overlap-extension PCR technique[27]. WT and mutant cDNAs were subcloned in the pcDNA3.1 expression vector and were used in the patch clamp and immunocytochemistry experiments.

Plasmid constructs for the intracellular protein:protein interaction assays were generated by following the instructions for the NanoBiT Protein:Protein Interaction System (Promega, Madison WI, USA). For the extracellular display of the NanoBiT tags, reporter constructs were equipped with an additional transmembrane domain, the so-called 'Snorkel' tag, following standard laboratory procedures[28]. Each resulting plasmid construct was sequence verified. A detailed description of the molecular cloning procedures is provided in the **Supplementary material online.**

2.4 Cell culture and transient transfections

Human embryonic kidney cells (HEK, ATCC CRL-11268, Manassas, VA, USA) were cultured in DMEM : F12 medium (Lonza, Verviers, Belgium) supplemented with 10% FBS (EuroClone, Pero (MI) Italy) at 37 °C in a humidified atmosphere containing 5% CO_2, Chinese hamster ovary (CHO, ATCC CCL-61, Manassas, VA, USA) cells were cultured as described previously[26]. 25 KDa linear poliethyleneimine (PEI, Polysciences Inc., Warrington PA, USA) was used as a transfection reagent, the

transfection mixture contained a total amount of 4 µg plasmid DNA and 16 µg PEI in 1.5 ml reduced serum medium (UltraMEM; Lonza, Verviers, Belgium). In the patch clamp expeirments, transfection mixtures contained 1 µg wild type Kir2.1 (WT-Kir2.1) or 1 µg mutant cDNA constructs. Co-transfections with WT- and mutant Kir2.1 variants were carried out with 1 µg WT- and 1 µg mutant cDNA plasmid. A GFP-encoding plasmid was used to keep DNA content constant in each experimental group and as a transfection marker. Transfection mixtures for the NanoBiT experiments contained 2 µg of each NanoBiT reporter construct with no GFP-expressing plasmid added. All subsequent experiments were carried out 48 hours post-transfection.

2.5 Electrophysiology

Whole cell voltage-clamp experiments were carried out as previously described[26]. Briefly, normal Tyrode solution was used externally, containing (in mmol/L): NaCl 142, NaH2PO4 0.4, KCl 4, MgSO4 0.53, CaCl2 1.8, glucose 5.5, and HEPES 5; pH was adjusted to 7.4 with NaOH. Electrodes were filled with a solution containing (in mmol/L): KOH 110, KCl 40, K2ATP 10, HEPES 5, EGTA 5, and MgCl2 0.1; pH was adjusted to 7.2 with aspartic acid. Currents were recorded at 37 °C from the GFP-positive cells. Cell capacitance was estimated by the integral of the capacitive current elicited by a -10 mV hyperpolarizing pulse. Cell capacitance was canceled and series resistance was compensated. Voltage error after compensation was below 0.5 mV at -60 mV in each experiment. Kir2.1 current amplitudes were recorded as Ba^{2+}-sensitive (30 µM) current at the end of a 300 ms long pulses to potentials in the voltage range of -120 mV to 40 mV with 10 mV increments, with the interpulse holding potential set at -80 mV. Current densities were calculated by the current amplitude and cell capacitance quotient.

2.6 Immunocytochemistry

CHO cells were cultured and transfected according to the protocol described for the patch clamp experiments. The cell membrane was labelled with Wheat Germ Agglutinin Texas Red-X Conjugate (WGA-TxRed, 1:400, Life Technologies, Carlsbad, CA USA), Kir2.1 was labelled by the Anti-Kir2.1 primary antibody (APC-026, 1:100, Alomone Labs, Jerusalem, Israel) recognizing an epitope on the cytoplasmic C-terminus of Kir2.1 and by the subsequently applied FITC-conjugated

Anti-Rabbit IgG secondary antibody (1:450, DakoCytomation, Santa Clara CA, USA). Fluorescent images were captured by a LSM 880 (Zeiss) laser scanning confocal microscope using the Plan-Apochromat 40x/1.4 Oil DIC M27 objective. FITC and WGA-TxRed were detected in the 493 – 574 nm and 574 – 689 nm wavelength range, respectively. The resulting pixel size was in the range of 0.03 – 0.1 µm (meidan 0.04 µm, 25% percentile 0.04 µm, 75% percentile 0.05 µm). Images were quantitatively analyzed by the the ImageJ software (1.52p) as the following. A 1 px wide line was placed crossing the cell and avoiding the nucleus. The red channel showing WGA-TxRed fluorescence was used to identify the membrane (M) and cytoplasmic (C) regions independently of Kir2.1 fluorescence. M areas were defined by the maximal intensity value and equal number of pixels on both sides of it, all together covering a 280 – 320 nm wide area on each edge of the cell. The C region was defined as a 490 – 510 nm wide area overlapping with the geometric axis between the two membrane regions. Identical pixels corresponding to the M and C regions were selected in the green channel showing Kir2.1 immunofluorescence and average intensity values were calculated for M and C regions of each cell. Perasons`s correlation coefficients for the WGA-TxRed and Kir2.1 immunofluorescent signals were calculated by the Coloc2 plugin of the ImageJ software (version 1.52p) with no filtering or background subtraction applied.

2.7 Prediction of salt bridges

Salt bridges were predicted by using the ESBRI software[29] in the 1U4F crystal structure of the cytoplasmic domain of the WT Kir2.1[21] and in the 2GIX crystal structure of the cytoplasmic domain of mutant Kir2.1 carrying the pathogenic ATS1 mutation p.Arg218Gln and a rescue mutation p.Thr309Lys[20]. The 3SPI model of Kir2.2 is used for illustration purposes only (**Figure 2A**)[30]. The models were obtained from the RCSB Protein Data Bank (https://www.rcsb.org/) and were visualized by using the RasWin Molecular Graphics software (2.7.5.2.).

2.8 The NanoBiT structural complementation reporter assay

3.2 x 10^6 HEK cells were plated in 6 cm culture dishes on the day before the transfection. 48 hours post-transfection, 1.6 x 10^5 cells were loaded per well in 100 µl UltraMEM in a 96-well plate. A five-fold working solution of the cell-permeable NanoGlo Live Cell Reagent (Promega, Madison WI, USA) was prepared in a reduced

light environment and 25 µl of it was added to the cells per well. The plate was then quickly moved into a FLUOstar Optima Microplate Reader (time = 0 min) and was read in luminescence mode. Luminescence values were collected every minute for 1 hour. Luminescence values collected between 16–17 min and between 0-1 min were used for statistical analysis with the cytoplasmic and extracellular NanoBiT reporters, respectively. Transfections were carried out on separate days containing all experimental groups and three replicates for each group in parallel. One data point reported corresponds to the numerical average of three parallel replicates.

2.9 Statistical analysis

Numerical data in the text are presented as the arithmetic mean of data points and the 95% confidence interval of the mean (95% CI [lower limit, upper limit]), in order to indicate the range of values which contains the mean of the measured parameter of the population of experimental subjects with 95% probability. For the same reason, numerical data in the figures are presented as individual values and the arithmetic mean ± the lower and upper limits of 95% CI. Luminescence values presented in **Figure5** and **6** were log-transformed to achieve homogeneous variance. Group means were compaired either by Student's t-test or by one-way ANOVA followed by post hoc tests with Bonferroni's correction for multiple comparisons by using the Graphpad Prism software (version 8.1.1).

3. Results

3.1 Genetic analysis

The NM_000891.2: c.877G>A (p.Glu293Lys) heterozygous genetic variant in the *KCNJ2* gene has been detected using targeted resequencing and was validated by standard capillary sequencing. This variant has not been reported previously, nor was it present in 374 control chromosomes representing subjects from the same geographical area. The mutation was not detected in the clinically unaffected parents and brother of the proband (data not shown), indicating that the mutation may have arisen ‘de novo’ (paternity proven). No mutations were found in the proband in any other member of the *KCNJ* gene family. The p.Glu293Lys variant was regarded as ‘pathogenic’ according to the joint consensus recommendation of the American

College of Medical Genetics and Genomics and the Association for Molecular Pathology[31].

3.2 Electrophysiological characterization of the p.Glu293Lys Kir2.1 variant

WT and mutant Kir2.1 subunits were heterologously over-expressed in CHO cells. Cells expressing the WT Kir2.1 alone or in combination with p.Glu293Lys exhibited robust inward current with strong inward rectification (**Figure 1A**). Current amplitudes were markedly reduced when cells were transfected with the combination of cDNAs encoding the WT and p.Glu293Lys variants (**Figure 1A**). No current was detected in cells expressing the p.Glu293Lys variant alone (n = 10, **Figure 1A**). The outward component of the current-voltage relationships measured at the physiologically relevant membrane potential range between -80 to 0 mV was prominent (**Figure 1B**).

To assess conductivity of Kir2.1-based ion channels in the presence and in the absence of the p.Glu293Lys variant, current densities that were recorded at the test potential -60 mV were compared. The *KCNJ2* allele p.Arg218Gln with loss of function and dominant negative effects and with well documented pathogenic role in ATS1 has been included as positive control (**Figure 1B**)[16, 32]. Average current densities were the highest in cells expressing WT Kir2.1 alone (9.6 pA / pF, 95% CI [7.2, 12.0], n = 15, **Figure 1B**). Current densitites with p.Glu293Lys or with the p.Arg218Lys variants were practically non-detectable (-0.2 pA / pF, 95% CI [-0.8, 0.3], n = 7 and 0 pA / pF, 95% CI [-0.7, 0.6], n = 9, respectively) and were significantly lower compater to WT ($p < 0.0001$ in both cases, **Figure 1B**). Modelling the heterozygous patient genotypes by co-expression of WT and mutant *KCNJ2* variants resulted in significantly decreased average current densities both with the p.Glu293Lys (3.6 pA / pF, 95% CI [1.7, 5.6], n = 10, $p = 0.0006$) and the p.Arg218Gln variant (4.5 pA / pF, 95% CI [1.6, 7.5], n = 10, $p = 0.0045$) compared to homomeric WT channels (**Figure 1B**). In summary, these data indicate a prominent dominant negative effect of the p.Glu293Lys variant over WT channels, resulting in loss of function on the heterozygous genetic background.

3.3 Intersubunit salt bridges predicted in the vicinity of Glu293

Tetrameric complexes of ion channels based on Kir2.x subunits consist of two distinct structural domains, the membrane-spanning transmembrane domain (TD) and the cytoplasmic domain (CD, **Figure 2A**). The glutamic acid at position 293, that

is substituted to lysine in the p.Glu293Lys variant, is located in the cytoplasmic domain at the surface connecting two adjacent subunits, also known as the CD-I region (**Figure 2B**). Glutamic acid has the capacity to form salt bridges with positively charged resdues, when in the proximity to each other. Since salt bridges can mediate subunit interactions and such interactions have been shown to have profound effects on Kir2.1 gating, we asked whether or not Glu293 can be involved in the formation of salt bridges. To this end, salt bridges were predicted in the 3D crystallography models of the Kir2.1 cytoplasmic domain (1U4F and 2GIX) by using the ESBRI software[29]. According to these predictions, Glu293 participates in a network of salt bridges. There are four amino acids involved. Glu319 is located in the same subunit proximal to Glu293, while Arg46 and Lys50 are situated in the opposite subunit. One salt bridge was predicted between Glu293 and Arg46, two between Arg46 and Glu319 and an additional one between Glu319 and Lys50 (**Figure 2C**). Each of these four salt bridges are formed between amino acids that reside in different subunits and therefore connect two subunits to each other. Because of the four-fold symmetry of the Kir2.x ion channel complex, anaolgous CD-I regions are present between each adjacent subunit pair. The same set of salt bridges were predicted at all four locations in both protein structure models. A complete list of the predicted salt bridges in the region is provided in **Supplementary material Table S1.** The charge reversal at position 293 in the p.Glu293Lys variant, therefore, may be expected to impair this intersubunit salt bridge network and to prevent the formation of 4 salt bridges between cytoplasmic domains of adjacent Kir2.1 subunits. Moreover, alignment of amino acid sequences revealed that Arg46, Lys50, Glu293 and Glu319 are highly conserved in Kir2.1 proteins from vertebrate ~~species~~ and in the human inward rectifier potassium channel protein family, suggesting a crucial role for these amino acids and the predicted intersubunit salt bridge network in structure-function relationships of inward rectifier potassium channels (**Figure 2D**).

3.4 Assessment of subcellular localization

Intersubunit salt bridges can stabilize the binding of monomers in protein complexes and mediate subunit assembly. We hypothesized, therefore, that the disruption of the predicted salt bridge network at the CD-I in the p.Glu293Lys variant might affect co-assembly of Kir2.1 subunits, which in turn might result in the cytoplasmic accumulation of misfolded protein complexes. To assess this possibility,

the subcellular localization of the heterologously over-expressed WT, p.Glu293Lys and p.Arg218Gln Kir2.1 variants was assessed and compared by immunofluorescent labelling and confocal microscopy (**Figure 3**). Visual observation of confocal images showed prominent labelling of the cell membrane (**Figure 3A**), line profiles consisting of pixel intensity values along a 1 pixel wide line placed crossing the cytoplasm but not the nucleus showed intensity peaks near the edges of the cell (**Figure 3B**). Membrane (M) and cytoplasmic (C) regions were then defined for each cell, based on the fluorescent signal of the aspecific membrane dye WGA-TxRed. Average Kir2.1 immunofluorescense signal intensities were significantly higher in the M compared to the C area with all three variants ($p < 0.0001$, **Figure 3C**). Fold difference of average M versus C Kir2.1 expression were similar in all experimental groups, with a statistically non-significant decrease with the p.Glu293Lys variant ($p=0.12$, **Figure 3D**). Colocalization of the Kir2.1 immunofluorescence with WGA-TxRed fluorescence was then assessed by calculating Person`s correlation coefficients (PCC). While average PCC values were similar with the WT and p.Arg218Gln variants, significantly lower PCC values were found in cells expressing p.Glu293Lys subunits (0.68, 95% CI [0.63, 0.73], n=11) both compared to WT (0.81, 95% CI [0.77, 0.85], $p = 0.0002$, n=13) and p.Arg218Gln (0.82, 95% CI [0.78, 0.86], $p < 0.0001$, n=12) groups (**Figure 3E**). Therefore, we found that while an obvious cytoplasmic accumulation is not evident, the p.Glu293Lys variant tends to distribute less in the cell membrane. These data suggest that oligomerization of the p.Glu293Lys variant into a homo-tetrameric complex is deficient and that misfolded p.Glu293Lys subunits distributre preferentially in the submembrane area.

3.5 Assessing physical interaction between Kir2.1 subunits

To confirm and further explore deficient oligomerization of the p.Glu293Lys Kir2.1 variant, two distinct variations of the NanoBiT assay was developed (**Figure 4A, 6A**)[33]. The NanoBiT assay is based on the complementation of the large (LgBiT) by the small (SmBiT) fragments of the split NanoLuc luciferase enzyme. The LgBiT and SmBiT fragments complement each other and form a functional enzyme when in the proximity of each other[33]. Therefore, LgBiT and SmBiT fragments can be used as reporter tags to investigate portein:protein interactions, including intersubunit interactions within the ion channel complex. First, the LgBiT and SmBiT tags were fused to the cytoplasmic C-termini of Kir2.1 subunit variants (**Figure 4A**). To assess

subunit interactions in homomeric Kir2.1 channels, HEK cells were co-transfected with LgBiT- and SmBiT-tagged, but identical *KCNJ2* variants. When identical Kir2.1 variants tagged with either NanoBiT fragment were co-expressed, average $\log_e$ luminescence (ln(RLU)) values were similar in cells transfected with WT-LgBiT and WT-SmBiT (6 ln(RLU), 95% CI [5.2, 6.9], n = 4) and in cell co-expressing the p.Arg218Gln reporter pair (6 ln(RLU), 95% CI [5.4, 6.6], n = 4, p > 0.9999). In contrast, ln(RLU) values were significantly lower in cells expressing the p.Glu293Lys reporter constructs (4.3 ln(RLU), 95% CI [3.3, 5.3], n = 4), both compared to homomeric combinations of the WT (p = 0.0033) or the p.Arg218Lys variant (p = 0.0045, **Figure 4B**). Furthermore, these latter values were statistically indistinguishable from cells expressing WT-SmBiT and the LgBiT-tagged, structurally unrelated, six-transmembrane-domain voltage-gated potassium channel subunit KvLQT1 used as a negative control (4 ln RLU, 95% CI [3, 4.9], n = 4, p > 0.9999, **Figure 4B**). To model the heterozygous genotype, experiments were carried out with the intracellular NanoBiT constructs in cells co-expressing the appropriate reporter pair of the WT and each mutant *KCNJ2* variant (**Figure 4C**). Mean ln(RLU) values were not different in cells expressing WT-LgBiT and p.Arg218Gln-SmBiT (6.6 ln(RLU), 95% CI [6, 7.1], n = 5) compared to the positive control (WT-LgBiT + WT-SmBiT: 6.4 ln(RLU), 95% CI [5.9, 7.2], n = 5, p > 0.9999, **Figure 4C**). Interestingly, co-transfection of WT-LgBiT with p.Glu293Lys-SmBiT produced luminsecence values (5.6 ln(RLU), 95% CI [5.1, 6.1], n = 5) that were significantly lower compared to the positive control (p = 0.0266), but significantly higher than the negative control (WT-SmBiT + KvLQT1-LgBiT: 4.4 ln(RLU), 95% CI [3.9, 4.9], n = 5, p = 0.0042, **Figure 4C**). These data indicate that the oligomerization of Kir2.1 subunits is impaired in the presence of p.Glu293Lys, but not the p.Arg218Gln mutation. Importantly, p.Glu293Lys subunits are still able to form heteromers with WT subunits, although to a lower extent compared to WT or to p.Arg218Gln variants.

If p.Glu293Lys and WT subunits are able to co-assemble to a certain extent, they might be able to form properly folded heteromeric complexes that are transported to and are present in the cell membrane. To test this possibility, the extracellular variant of the NanoBiT assay was applied (**Figure 5**). In these reporter constructs, an extra transmembrane domain, the so-called Snorkel tag (-Sn-) is fused to the C-termini of Kir2.1 subunits, which are further extended by the NanoBiT fragments, so that the LgBiT and SmBiT reporter tags are displayed extracellularly (**Figure 5A**). The

extracellular presentation of the Snorkel-NanoBiT fragments could be verified by observing the time course of the development of the bioluminescent signal (**Supplementary Figure S1**). When the cytoplasmic NanoBiT reporters were used, maximal bioluminescent signal intensities were reached at 15.6 minutes on average. This relatively slow signal development can be attributed to the time needed for the cell-permeable substrate of NanoLuc to perfuse the cell interior. In sharp contrast to this, the Snorkel-NanoBiT reporters generated the maximal bioluminescent intensities typically at the earliest time point (**Supplementary Figure S1**), indicating the presence of the reconstituted NanoLuc on the extracellular side of the membrane. Hence, to gain information on the membrane-resident fraction of the Snorkel-NanoBiT reporter constructs, in these experiments, bioluminescent intensities collected at 0 min were compared.

Average ln(RLU) values were not different in the positive control (WT-Sn-LgBiT + WT-SN-SmBiT: 6.8 ln(RLU), 95% CI [6.1, 7.4], n = 6), in cells expressing p.Arg218Gln-Sn-LgBiT and WT-Sn-SmBiT (7.4 ln(RLU), 95% CI [6.6, 8.1], n = 6, p = 0.5406) or in cells that were co-transfected with p.Glu293-Sn-LgBiT and WT-Sn-SmBiT reporters (6.8 ln(RLU), 95% CI [6.1, 7.5], n = 6, p > 0.9999, **Figure 5B**). On the other hand, each of these group means were significantly higher compared to that of the negative control group (4.5 ln RLU, 95% CI [4, 5], n = 6, p < 0.0001 for both comparisons), in which KvLQT1, tagged with LgBiT was co-expressed with WT-Sn-SmBiT (**Figure 5B**). These data with the extracellular Snorkel-NanoBiT reporters indicate that both the p.Arg218Gln and the p.Glu293Lys variants co-assemble with WT subunits and heteromeric complexes composed of WT and either of the mutant variants exist in the cell membrane.

Based on these evidence we conclude that the p.Glu293Lys, but not the p.Arg218Gln variant affects heteromerization of Kir2.1 subunits, as demonstrated by immunocytochemistry and by the experiments with the cytoplasmic NanoBiT reporter constructs. On the other hand, the p.Glu293Lys variant is still able to co-assemble with WT subunits, although to a lower degree, and these heteromeric ion channels are present in the cell membrane. Furthermore, the amount of the membrane-resident heteromeric channels containing p.Glu293Lys subunits is similar to homomeric WT channels, but current amplitudes are reduced in the presence of the p.Glu293Lys variant. Therefore, we conclude that in addition to subunit heteromerization, Glu293 has an important role in maintaining Kir2.1 channel

conductivity, possibly mediated by an intersubunit salt bridge network at the CD-I of Kir2.1 channels.

4. Discussion

Despite the efforts taken towards the refinement of the ATS1 phenotype, phenotypic variability and the incidence of atypical cases seem to be common in ATS1[34, 35]. Efficient genetic screening of affected kindreds supports early diagnosis and establishment of adequate management. Therefore, the identification and functional characterization of pathogenic *KCNJ2* variants with the aim to define genotype-phenotype correlations is paramount.

In the present study, the novel causative ATS1 mutation p.Glu293Lys is reported. We demonstrate that p.Glu293Lys causes loss of function and exerts a dominant negative effect on Kir2.1 currents, as indicated by the patch clamp experiments. Importantly, the dominant negative effect was detected over a physiologically relevant membrane potential range between -70 mV to -10 mV and it was comparable to that of a well-known ATS1 variant p.Arg218Gln[32]. These findings demonstrate the causative role of the p.Glu293Lys mutation in ATS1.

Salt bridges, electrostatic interactions between an Aspartic or Glutamic acid and an Arginine, Lysine or Histidine stabilize folded protein structures and mediate subunit interactions. Gating of inward rectifier potassium channels has recently been shown to be dependent on intersubunit salt bridges linking Kir subunits within the ion channel complex [22, 23]. It has been demonstrated that destabilizing the intersubunit salt bridges at the CD-I of inward rectifiers results in a substantial dilation (10-20 Ä) of the cytoplasmic domain and favors an inactivated state with lower open probability of the channel[23]. Therefore, it seems that the strength of CD-I interactions within the cytoplasmic domain has a pivotal role in gating of inward rectifier potassium channels. Our study corroborates the above findings and exemplifies the functional consequense of impaired CD-I interactions by clinical observations.

In this study, we investigate intersubunit interactions within the Kir2.1 ion channel complex in the presence of the clinically relevant Kir2.1 variants p.Glu293Lys and p.Arg218Gln, by using two distinct versions of the NanoBiT split reporter assay. The NanoBiT split reporter assay is based on the structural complementation between two fragments of the NanoLuc luciferase, the 1.3 kDa SmBiT peptide and the 18 kDa

LgBiT polypeptide[33]. The LgBiT and SmBiT fragments have been selected to have the lowest binding affinity to each other, which ensures that a functional, bioluminescent enzyme is formed only when there is association between the target proteins. In addition, we employed the Snorkel tag schema to display the reporter tag pair on the extracellular side of the cell membrane[28]. The distinctly different time course of signal development with the Snorkel- compared to cytoplasmic NanoBiT reporter constructs allowed us to gain qualitative and quantitavie information on the subunit composition of the membrane-resident fraction of heteromeric Kir2.1 ion channels. The NanoBiT assay has recently been used in studies on subunit interactions of the A_3 adenosine receptor[36], on the subunit assembly of the NADH oxidase 4[37] and for the real time monitoring of cullin1 neddylation[38]. Heteromeric assembly of the pore-forming subunits of potassium channels is a general phenomenon underlying an enormous functional diversity, however, the full spectrum of possible subunit interactions has not been explored yet (reviewed in[39]). To our knowledge, the present study is the first to demonstrate the applicability of the NanoBiT assay to investigate subunit interactions of potassium channels, thus representing a novel experimental approach in this area.

Allosteric modulation of protein oligomerization including stabilization of protei:protein interactions are emerging concepts of drug discovery (reviewed in[40, 41]). Flecainide, propafenone, nadolol and zacopride have been shown to increase I_{K1}, but no specific activators of I_{K1} have been identified to date[42-44]. Therefore, it is tempting to speculate whether the CD-I of Kir2.1 could represent a promising target for the development of I_{K1} agonists that modulate I_{K1} via strengthening intersubunit interactions at the CD-I.

It is important to note that the amino acid Arg218 that is substituted by Gln in the p.Arg218Gln variant is located at the surface of the cytoplasmic domain of Kir2.1, distant from the CD-I[20, 21]. Arg218 resides in the amino acid cluster mediating the binding of PIP_2, a major activator of Kir2.1 channels[16, 21]. Furthermore, the p.Arg218Gln variant has been shown to cause displacement of the cytoplasmic G-loop, a key structural element in the K^+ permeation pathway[20]. Therefore, the molecular mechanisms leading to loss of function in the p.Glu293Lys and the p.Arg218Gln variants are distinctly different. Indeed, the behavior of the p.Arg218Gln was qualitatively and quantitatively similar compared to WT subunits in both variations of the NanoBit assays.

Our data indicate, that p.Glu293Lys is a novel causative *KNCJ2* variant in ATS1, exerting dominant negative effect on the WT allele on a heterozygous genetic background. Furthermore, Glu293 plays an important role in mediating subunit interactions within the Kir2.1 ion channel complex, possibly maintaining a salt bridge network at the CD-I. The charge reversal in the p.Glu293Lys variant leads to impaired subunit co-assembly in homomeric channels consisting of p.Glu293Lys subunits and to gating abnormalities in heteromeric complexes of WT and p.Glu293Lys subunits.

5. Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

6. Funding

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7. Authors` contribution

Sz. Déri, T. Hartai, Á. Visnyovszki, B. Ördög: molecular cloning, heterologous expression system, immunostaining, patch clamp experiments, NanoBiT assays; R. Arora and A. J. Labro advised designing and evaluate the NanoBiT experiments; R. Sepp, L. Hategan, B. Csányi, J. Borbás, I. Nagy, Z. Hegedűs: genetic and bioinformatic analysis; T. Madácsy, J. Maléth: confocal microscopy; R. Sepp, L. Környei, J. Borbás:

clinical diagnosis and case history; Sz. Déri, B. Ördög,: manuscript writing; B. Ördög, R. Sepp, A. Varró, A. J. Labro: revising the manuscript.

8. Conflict of interest

None to declare.

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10. Figure legends

Figure 1. Electrophysiological characterization of the p.Glu293Lys Kir2.1 variant by whole-cell voltage clamp experiments in transiently transfected CHO cells. **A:** Representative current recordings obtained from cells expressing the wild type (WT) or mutant (p.Glu293Lys) variants alone or simultaneously (WT + p.Glu293Lys). **B:** Current-voltage relationships. The outward component of Kir2.1 currents detected between -80–0 mV are shown in the bottom panel. **C:** Current densities elicited by a -60 mV test pulse. Data are presented as mean ± 95% CI. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$ *vs* WT by one-way ANOVA with Bonferroni's correction for multiple comparisons.

Figure 2. **A**: The 3D crystallography modell of the Kir2.2 ion channel complex (3SPI). TD: transmembrane domain, CD: cytoplasmic domain. **B**: The cytoplasmic domain of Kir2.1 in the 1U4F model (upper view). **C:** Up-close view of the region harboring the amino acids involved in the formation of the predicted salt bridge network in the 1U4F model. Atoms are designated according to the PDB nomenclature, salt bridges are indicated by the dashed yellow lines. **D**: Alignment of amino acid sequences of human (h), mouse (m), rat (r), guinea pig (gp), dog (d), Xenopus tropicalis (x) and zebra fish (zf) Kir2.1 (upper panel) and alignment of amino acid sequences of human inward rectifier potassium channel subunits (lower panel). Positions indicated are relative to the amino acid sequence of human Kir2.1, conserved amino acids are shown in blue shading. Glu293 and its interacting partners predicted to participate in the salt bridge network (Arg46, Lys50, Glu319) are highlighted in red.

Figure 3. **A**: Representative photomicrographs show preferential labelling of the plasma membrane both with WGA Texas red and anti-Kir2.1 staining. The scale bar represents 10 µm. **B**: Line profiles of cells with anti-Kir2.1 staining from panel A. Pixel intensities were normalized to the maximal intensity value for each cell. **C**: Statistical analysis of average pixel intensities in the membrane (M) and in the cytoplasmic (C) region. **D:** Relative pixel intensities in the M vs C regions. **E:** Fluorescence colocalization analysis. (n=13 in WT, n=12 in p.Arg218Gln and n=11 in the p.Glu293Lys group). Data are presented as mean ± 95% CI. ***: $p < 0.001$ by

Student's t-test *vs* the M region (C) and one-way ANOVA followed by post-hoc tests with Bonferroni's correction for multiple comparisons (D and E).

Figure 4. Subunit interactions within the Kir2.1 complex as assessed by intracellular NanoBiT reporter constructs. **A**: Schematic structure of the intracellular NanoBiT reporters showing two opposite Kir2.1 subunits carrying the intracellular NanoBiT reporter tag pair. **B**: Relative $\log_e$ luminescence (ln(RLU)) readings from cells expressing combinations of LgBiT- and SmBiT-tagged Kir2.1 variants in homomeric (i.e. WT + WT or mutant + mutant) configuration. **C**: Ln(RLU) from HEK cells co-expressing wild type (WT) and mutant (p.Arg218Gln or p.Glu293Lys) Kir2.1 subunits tagged with intracellular NanoBiT reporter constructs. Data are presented as mean ± 95% CI of log-transformed values (n=4 in panel B and n=5 in panel C). *: $p < 0.05$, **: $p < 0.01$, n.s: non-significant by one-way ANOVA followed by post-hoc tests with Bonferroni's correction for multiple comparisons.

Figure 5. Subunit interactions within the Kir2.1 complex as assessed by extracellular NanoBiT reporter constructs. **A**: Schematic strucutre of the extracellular Snorkel-NanoBiT reporters, showing two opposite subunits assembled in the tetrameric Kir2.1 complex. A transmembrane domain (Snorkel TMD) was fused to the cytpolasmic C-terminus of Kir2.1 so that the NanoBiT reporter tag pair (LgBiT and SmBiT) is presented extracellularly. TD: transmembrane domain, CD: cytoplasmic domain. **B**: Relative $\log_e$ luminescence (ln(RLU)) from HEK cells co-expressing the Snorkel-NanoBiT reporter constructs (Sn-LgBiT or Sn-SmBiT). WT-Sn-LgBiT + WT-Sn-SmBiT co-expression was used as positive control, the structurally unrelated six-transmembrane-domain potassium channel subunit KvLQT1 tagged with LgBiT co-expressed with WT-Sn-SmBiT was used as a negative control. Data are presented as mean ± 95% CI of log-transformed values (n=6). ***: $p < 0.001$, n.s: non-significant by one-way ANOVA followed by post-hoc tests with Bonferroni's correction for multiple comparisons.