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Graphical abstract

Human Cantu syndrome mutation SUR2[R1154Q] results in deletion of exon 28, with loss of mature protein and Kir6 channels in the heart and vasculature.

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Complex consequences of Cantu Syndrome SUR2 variant R1154Q in genetically modified mice

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The authors have declared that no conflict of interest exists.

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Abstract

Cantu Syndrome (CS) is caused by gain-of-function (GOF) mutations in pore-forming (Kir6.1, KCNJ8) and accessory (SUR2, ABCC9) K\textsubscript{ATP} channel subunits, the most common mutations being SUR2[R1154Q] and SUR2[R1154W], carried by ~30% of patients. We used CRISPR/Cas9 genome engineering to introduce the equivalent of human SUR2[R1154Q] mutation to the mouse ABCC9 gene. Along with minimal CS disease features, R1154Q cardiomyocytes and vascular smooth muscle showed much lower K\textsubscript{ATP} current density and pinacidil activation than WT cells. Almost complete loss of SUR2-dependent protein and K\textsubscript{ATP} in homozygous R1154Q ventricles revealed an underlying diazoxide-sensitive SUR1-dependent K\textsubscript{ATP} channel activity. Surprisingly, sequencing of SUR2 cDNA revealed divergent transcripts, one encoding full length SUR2 protein, and the other with in-frame deletion of 93 bases (corresponding to 31 amino acids encoded by exon 28) that was present in ~40% and ~90% of transcripts from hetero- and homozygous R1154Q tissues, respectively. Recombinant expression of SUR2A protein lacking exon 28 resulted in non-functional channels. SUR2[R1154Q] CS patient tissue and iPSC-derived cardiomyocytes showed only full length SUR2 transcripts, although further studies will be required to fully test whether SUR2[R1154Q] or other CS mutations might result in aberrant splicing and variable expressivity of disease features in human CS.

Brief Summary

The most common Cantu Syndrome (CS) mutation (SUR2[R1154Q]) causes aberrant mRNA splicing and loss of SUR2 protein, and hence minimal CS features, in mice.
Introduction

Cantu syndrome (CS), which has also been referred to as hypertrichosis-osteochondrodysplasia-cardiomegaly syndrome, is a rare multiorgan disease first recognized as such in 1982 (1). CS is characterized by congenital hypertrichosis, distinctive facial features, osteochondrodysplasia, and multiple cardiovascular abnormalities, including cardiomegaly, hypertrophy, pericardial effusion, pulmonary hypertension, and patent ductus arteriosus and cerebrovascular defects (2-5). Multiple reports have now confirmed that autosomal dominant gain-of-function (GoF) mutations in KCNJ8 and ABCC9, the genes encoding the Kir6.1 and SUR2 subunits of ATP-sensitive (K\textsubscript{ATP}) potassium channels, represent the genetic basis of CS (6-11). The severity of features varies widely between individuals, although genotype-phenotype correlations have been difficult to establish; interestingly, patients with the same mutation can span the clinical spectrum (6, 7, 12, 13).

Expressed in various tissues in the body, K\textsubscript{ATP} channels are nucleotide-gated, potassium selective channels that couple cellular metabolism to electrical excitability. KCNJ8 and ABCC9 are adjacent genes on human chromosome 12p12.1. A paralogous pair of genes (KCNJ11 [Kir6.2] and ABCC8 [SUR1]) is located on chromosome 11p15.1, with the result that multiple subunit combinations may exist in K\textsubscript{ATP} channels in different tissues. K\textsubscript{ATP} heterogeneity is further increased by the existence of variably spliced SUR isoforms (14-16); particularly prominent are 2 major splice isoforms of SUR2: SUR2A and SUR2B (17-19). K\textsubscript{ATP} channels in vascular smooth muscle are predominantly formed of Kir6.1 and SUR2B (20, 21), whereas SUR2A predominates in cardiac ventricular myocyte K\textsubscript{ATP} channels (22, 23).

To date, over 30 mis-sense mutations (24) have been identified in CS patients, and all mutations that have been functionally characterized result in gain-of-function (GoF) of K\textsubscript{ATP} channels (7-11). In a previous study, we generated ‘CS’ mice, in which the equivalent of human SUR2[A478V] and Kir6.1[V65M] mutations were introduced into the relevant mouse loci. In each case, key cardiovascular features of CS were replicated, and molecular, cellular and systemic consequences increased from heterozygous (Het) to homozygous (Hom) conditions. Importantly, as the number of genetically confirmed CS cases has risen, it has become clear that residue R1154 in SUR2 particularly susceptible to mutation, with 24/72 patients (20/57 unrelated families) carrying variants ABCC9[c.3461G>A], ABCC9[c.3460C>T], or ABCC9[c.3460C>G], which encode SUR2[p.R1154Q], SUR2[p.R1154W], and SUR2[p.R1154G], respectively (24). While this may reflect a susceptible genomic structure, at least the R1154Q mutation results in more severe GOF than many other CS mutations (7, 11), which raises the possibility that excessive occurrence of such mutations in patients reflects greater penetrance than other
mutations. To gain further insight to the disease consequences of the R1154Q mutation, we have used CRISPR/Cas9 genome engineering to generate CS mice carrying the SUR2[R1151Q] mutation, equivalent to human R1154Q. We show that the cardiovascular abnormalities in these CS mice are much less severe than in either of the earlier two animals, but this is accompanied by a marked decrease of SUR2-dependent $K_{\text{ATP}}$ density, in both cardiac and smooth muscle cells. Further experiments show that this is the result of the unanticipated appearance of ABCC9 mRNA splicing that results in in-frame exon deletion and loss of functional protein.
Results

The human R1154Q substitution causes $K_{\text{ATP}}$ GOF, but only a mild CS phenotype

CRISPR/Cas9 gene editing was used to introduce single nucleotide mutation
(ABCC9[c.3452G>A]; SUR2[p.R1151Q]) in the endogenous mouse ABCC9 locus, resulting in
protein substitution analogous to the most common human CS mutation SUR2[R1154Q]. Both
heterozygous (SUR2$^{\text{wt/RQ}}$) and homozygous (SUR2$^{RQ/RQ}$) mice were viable and fertile. We
subsequently analyzed cellular, organ, and whole animal phenotypes of these animals, which
we refer to as SUR2[R1154Q] mice to avoid confusion in reference to human Cantu Syndrome.

One of the most consistent features of CS patients is pronounced cardiomegaly (24, 25).
Consistent with this, hearts were larger than WT in heterozygous SUR2$^{\text{wt/RQ}}$ but not obviously
more so in homozygous SUR2$^{RQ/RQ}$ mice (Fig. 1A,B). Therefore, R1154Q hearts display
chamber dilation and cardiac enlargement similar to, although much less dramatic, than that
seen previously in A478V or V65M CS mouse hearts (26). Isolated aortic diameter was higher in
SUR2$^{\text{wt/RQ}}$ than WT at all pressures (Fig. 1C) although, again, there was no further increase in
SUR2$^{RQ/RQ}$, and carotid artery dimensions were not different between WT and R1154Q animals
(Fig. 1D). Slope compliance (reflecting non-contractile biomechanical properties) was not
obviously different between genotypes (Fig. 1C). As shown in Fig. 2A, both SUR2$^{\text{wt/RQ}}$ and
SUR2$^{RQ/RQ}$ mice maintain diurnal fluctuation in blood pressure but, unlike SUR2[A478V]-
expressing mice (26), blood pressures are not significantly lower than control in either SUR2$^{\text{wt/RQ}}$
or SUR2$^{RQ/RQ}$ mice (Fig. 2A). Moreover, while pinacidil had similar BP lowering effect in control
and Het SUR2$^{\text{wt/RQ}}$ mice, it had almost no effect on BP in SUR2$^{RQ/RQ}$ mice (Fig. 2C). In contrast,
pinacidil raised heart rates similarly in all genotypes (Fig. 2D).

Unexpected $K_{\text{ATP}}$ channel properties in R1154Q cardiac and vascular smooth muscle
cells

Mice expressing introduced SUR2[A478V] and Kir6.1[V65M] CS GOF mutations exhibit marked
lowering of blood pressure and cardiac enlargement (26). Since previous studies show that
recombinant SUR2[R1154Q] causes a significant GOF (in both human SUR2 (7) and in rat
SUR2 with the identical DNA mutation (27)), the above results (i.e. limited or no increase in
vessel diameters and compliance, lack of effect on BP, and lack of pinacidil action in
SUR2$^{RQ/RQ}$) are unexpected, and raise questions regarding the level and nature of $K_{\text{ATP}}$
channels in these mutant tissues. We therefore examined the density of $K_{\text{ATP}}$ channels, and
sensitivity to $K_{\text{ATP}}$ channel openers, pinacidil (acting primarily on SUR2) and diazoxide (acting
primarily on SUR1), in excised membrane patches from ventricular myocytes (Fig. 3A). Overall
$K_{\text{ATP}}$ channel density was much lower than WT in SUR2$^{\text{wt/RQ}}$ myocytes, and dramatically so in homozygous SUR2$^{\text{RQ/RQ}}$ (Fig. 3B). Moreover, in striking contrast to the findings in recombinant R1154Q channels, pinacidil-activation was essentially absent in homozygous SUR2$^{\text{RQ/RQ}}$ cardiomyocytes, while relative diazoxide-activation was markedly enhanced (Fig. 3C).

This unexpected lowering of channel density and apparent switch in pharmacological sensitivity from pinacidil to diazoxide suggests that levels of SUR2-dependent channel complexes are reduced in R1154Q hearts, almost completely in homozygous SUR2$^{\text{RQ/RQ}}$, and that the remaining functional sarcolemmal channels are predominantly SUR1-dependent. To test the latter suggestion directly, we additionally generated SUR2$^{\text{RQ/RQ}}$ on a SUR1$^{-/-}$ background (28); in this case, no $K_{\text{ATP}}$ was detected (Fig. 3B), indicating that channels in SUR2$^{\text{RQ/RQ}}$ animals are essentially exclusively SUR1-dependent channels. We further examined underlying $K_{\text{ATP}}$ subunit levels in isolated ventricular tissue, by Western blot (Fig. 3D). This revealed a marked decrease of core- and complex-glycosylated SUR2A proteins in homozygous SUR2$^{\text{RQ/RQ}}$ hearts compared to littermate control hearts (Fig. 3D). In contrast, levels of core-glycosylated SUR1 protein are increased in SUR2$^{\text{RQ/RQ}}$ hearts (Fig. 3D). Both SUR1 and SUR2A in WT hearts are normally associated with Kir6.2 (29), but only core-glycosylated ~140 kDa forms of both SUR1 and SUR2A are present in Kir6.2$^{-/-}$ hearts (Fig. 4A), demonstrating that they both require association specifically with Kir6.2 to mature. As shown in Fig. 4B, treatment with Peptide:N-glycosidase F (PNGase F) resulted in complete deglycosylation of both SUR1 and SUR2A, demonstrating that total SUR1 is indeed increased in SUR2$^{\text{RQ/RQ}}$ hearts, although the excess is incompletely glycosylated. The results confirm that, consistent with loss of SUR2A-dependent $K_{\text{ATP}}$ current and relative increase in SUR1-dependent channels, mature SUR2A protein levels are reduced, while total SUR1 protein is increased, in SUR2$^{\text{RQ/RQ}}$ hearts.

The effects of the introduced mutation on $K_{\text{ATP}}$ channel function were also examined in vascular smooth muscle cells (VSMCs). In contrast to the findings in A478V and V65M animals (26), whole-cell patch clamp recordings, using an intracellular pipette solution containing no ATP (see Methods), revealed no elevation of basal $K_{\text{ATP}}$ conductance in acutely isolated aortic smooth muscle cells from SUR2$^{\text{wt/RQ}}$ compared to WT, and significantly lower conductance in SUR2$^{\text{RQ/RQ}}$ compared to WT (Fig. 5A,B). Application of pinacidil provoked a significant increase in conductance in WT VSMC, but there was less of an effect in SUR2$^{\text{RQ/RQ}}$ and very little effect in VSMC from SUR2$^{\text{RQ/RQ}}$ mice (Fig. 5A,B). These results indicate that $K_{\text{ATP}}$ density was also markedly reduced in R1154Q smooth muscle, although SUR2$^{\text{RQ/RQ}}$ VSMC were hyperpolarized relative to WT VSMC following break-in in current clamp mode (Fig. 5C,D).
indicating at least some net K\(_{\text{ATP}}\) gain-of-function under intact cell physiological conditions in SUR2\(^{\text{RQ/RQ}}\) VSMC.

Taken together, the data indicate that, while the expected molecular consequences of the SUR2[R1154Q] substitution is a significant gain-of-function of SUR2-dependent K\(_{\text{ATP}}\) channels in blood vessels and in the heart, there are actually minimal cardiovascular CS features, with an unexpected down-regulation of SUR2-dependent K\(_{\text{ATP}}\) channel density in heterozygous SUR2\(^{\text{wt/RQ}}\) cardiac and vascular smooth muscle myocytes, dramatically so in homozygous SUR2\(^{\text{RQ/RQ}}\) tissues, accompanied by an increase of SUR1 levels in the heart.

**Unanticipated alternate splicing of SUR2 exon 28 in R1154Q tissues**
The above results lead us to conclude that, although the R1154Q mutation indeed causes a GOF in K\(_{\text{ATP}}\) channel properties (since smooth muscle is still relatively hyperpolarized), the expressivity of CS features is severely blunted in these animals by the unexpected reduction in SUR2-dependent K\(_{\text{ATP}}\) density that is not seen in other (A478V, V65M) CS mice (26). In homozygous SUR2\(^{\text{RQ/RQ}}\) mice there is almost complete disappearance of SUR2-dependent K\(_{\text{ATP}}\) channels from both heart and blood vessels, and a consequent reduction in disease severity, as reflected in lack of obvious effects on BP, and reduced effects on heart size (Fig. 1,2). We considered the possibility that CRISPR-generated mistakes may have led to additional mutations that resulted in defective protein, but sequencing of genomic DNA >5000bp either side of the introduced mutation failed to detect any additional mutations (not shown). It has long been recognized that there are multiply spliced forms of the SUR2 protein(15, 30-32), the best characterized being the SUR2A and SUR2B isoforms that result from alternate splicing of the terminal exon 38A/B. The R1154Q and R1154W mutations are in exon 27 and, while there is no evidence in the literature for alternate splicing of this region of the gene, the specific location of the underlying mutations (13 and 14 bases, respectively, before the end of exon 27, Fig. 6A-C), places them in a potential exon splicing enhancer (ESE) region that may influence exon splicing(33). We isolated mRNA from WT and R1154Q mouse hearts, generated cDNA corresponding to SUR2A and SUR2B, and sequenced the entire coding region. The introduced c.3452G>A mutation was present in <50% of heterozygous SUR2\(^{\text{wt/RQ}}\) and close to 100% of homozygous SUR2\(^{\text{RQ/RQ}}\) but, strikingly, heterozygous cDNA reads became doubled sequences immediately following the last nucleotide of exon 27 (Fig. 6B). Close inspection reveals that this corresponds to ~half of the reads in heterozygous SUR2\(^{\text{wt/RQ}}\), and essentially all reads in homozygous SUR2\(^{\text{RQ/RQ}}\), reflecting an exact in-frame deletion of the 93 bases in the following exon 28 (Fig. 6B).
It might be hypothesized that this exon 28 splicing of SUR2 mRNA could be a cellular regulatory mechanism to moderate $K_{ATP}$ channel functional activity as a response to abnormally increased $K_{ATP}$ activity. However, this does not seem likely, since no alternative splicing of exon 28 was detected in heterozygous or homozygous SUR2[A478V] or Kir6.1[V65M] hearts (not shown), nor in WT hearts (Fig. 6B). Instead, the tight dependence of splicing on the presence of the c.3452G>A mutation indicates that the nucleotide change itself is directly responsible for the splicing event.

To assess the effect of deleting exon 28 on $K_{ATP}$ channel activity, we engineered SUR2A cDNA with exon 28 deleted. When co-expressed with WT Kir6.2, SUR2A[R1154Q,Δexon28] failed to generate significant $K_{ATP}$ channel activity in heterologous expression (Fig. 7A,B). In subunit mixing experiments, with equal transfection of WT SUR2A and SUR2A[R1154Q,Δexon28] cDNA, there was no evidence for dominant-negative suppression of heterologously expressed $K_{ATP}$ channels by exon-deleted subunits (Fig. 7C). The data were best fit by assuming that even one full length WT subunit was sufficient to rescue function (Fig. 7C), consistent with truncated subunits being rapidly degraded and not incorporated into $K_{ATP}$ complexes.

**Lack of splicing in human R1154Q mutant tissues or iPSC-derived cells**

The introduced mutation thus results in alternate splicing and consequent loss of SUR2 protein in CS mice. In turn, this leads to significantly blunted phenotype severity, despite the R1154Q mutation showing a marked molecular GOF (27). If the same splicing is similarly present in humans, it would tend to mitigate the effects of the mutation. In addition, variable SUR2 splicing between individuals could potentially account for the quite variable expressivity in CS individuals with the R1154Q mutation (24). We further examined the tissue-dependence of exon 28 skipping in cDNAs generated from mRNA isolated from multiple R1154Q mouse tissues. As shown in Fig. 7D, the apparent fraction of spliced transcripts was similar in skeletal, smooth and cardiac muscle, being ~25-35% in heterozygous SUR2$^{wtRQ}$ and ~75-100% in homozygous SUR2$^{RQ/RQ}$ animals. This further indicates that alternate SUR2 splicing is driven by the nucleotide change via a cell-autonomous mechanism, independent of tissue type. The less than stoichiometric ratio of spliced to unspliced transcript in the heterozygous case further suggests slower transcription or reduced stability of the mutant mRNA. We obtained a skin and skeletal muscle biopsy sample from a single R1154Q patient and successfully isolated ABCC9 mRNA. However, PCR from both samples revealed only single product bands corresponding to full length SUR2A cDNA and no band corresponding to exon 28-deleted cDNA (Fig. 7E).
We also obtained peripheral blood mononuclear cells (PBMCs) from a patient with the R1154Q mutation, and renal epithelial cells (RECs) were obtained from a patient with the R1154W mutation. Induced pluripotent stem cells (hiPSCs) were generated from these primary cells using Sendai Virus-based reprogramming vectors. Two subclonal hiPSC lines were produced for each mutation, and DNA sequencing analysis confirmed the expected mutation in each CS hiPSC line. A GCaMP6-expressing hiPSC line from an unaffected individual was used as a control. Expression of human pluripotency-associated genes and a normal karyotype were confirmed for all hiPSCs prior to subsequent experiments. WT and CS hiPSCs were differentiated into cardiomyocytes as previously described (34). Using this approach, hiPSC-CMs exhibiting robust rhythmic contractile behavior were present by Day 7-9. Subsequently, a 10-day lactate purification step was used to metabolically select for cells (Fig. 7F) with cardiomyocyte-specific biochemical properties enabling survival exclusively via lactate metabolism, as previously described (35). For unknown reasons, we were unable to detect K$_{ATP}$ channels in these myocytes (not shown). However, RT-PCR analysis of RNA isolated from WT, RQ, and RW hiPSC-CMs at Day 45 revealed full-length SUR2A transcripts in each genotype, with no evidence of detectable alternate splicing (Fig. 7F). Although the amino acid sequence in this region of SUR2 is identical between mouse and human (Fig. 6C), there are slight variations in codon usage between the two species (Fig. 6A) that could affect ABCC9 mRNA splicing. Nevertheless, the lack of detectable alternate splicing in human R1154Q and R1154W iPSC-derived cardiomyocytes suggests that the disease mutation may not lead to alternate splicing in native human tissues.
Discussion

The cellular pathology of $K_{ATP}$ GoF in Cantu Syndrome: additional outcome twists with R1154Q

The present study demonstrates that, when introduced into the mouse genome, the most common CS-associated ABCC9 mutation (encoding human SUR2[R1154Q]) results in qualitatively the same cardiovascular features as the SUR2[A478V] and Kir6.1[V65M] mutations (26), providing further confirmation of the common cardiovascular outcome of vascular dilation and cardiac enlargement resulting from SUR2- or Kir6.1-dependent $K_{ATP}$ GOF in CS. However, in SUR2[R1154Q] animals the disease severity was quantitatively much less than naively predicted, based on the molecular severity of the mutation. The reason for the reduced severity of outcome is shown to be a reduction of overall $K_{ATP}$ density in both vascular smooth muscle and in the heart, particularly in the homozygous SUR2RQ/RQ case. This in turn is shown to be a result of the genomic c.3452G>A mutation causing altered pre-mRNA splicing, with deletion of the following exon 28 and generation of non-functional SUR2 proteins, and downregulation of overall $K_{ATP}$ density. SUR2A levels are very strongly reduced in homozygous SUR2RQ/RQ, with the SUR2A protein that is present showing lower complex glycosylation, indicative of ER localization, as seen in Kir6.2 knockout animals (29).

In general, genetic or pharmacological manipulations that alter the levels of any $K_{ATP}$ subunits, even complete knockout of any given subunit, have not been shown to result in marked compensatory changes in other subunits, in any tissues (36-38). In the present case, there is a surprising increase in relative diazoxide sensitivity of cardiac $K_{ATP}$, accompanied by a small increase in absolute levels of diazoxide-sensitive current, and of mature, glycosylated SUR1 protein, in the heart. Previous studies have suggested that SUR1, while present in the heart, loses out to SUR2A in competition for association with Kir6 subunits, resulting in low levels of fully mature, glycosylated, SUR1 being present in $K_{ATP}$ channels at the membrane surface (29). We speculate this may be because SUR2A-containing channels normally leave the secretory pathway more efficiently, so that when mutant SUR2A protein is depleted, SUR1 accesses Kir6.2, explaining higher levels of core-glycosylated SUR1 and more diazoxide-activatable current, in R1154Q hearts.

Cardiac hypertrophy and enhanced cardiac output is a consistent finding in CS patients (25) and in both Kir6.1[V65M] and SUR2[A478V] mutant mice (26). As we have shown in these mice, cardiac hypertrophy arises independently of ventricular $K_{ATP}$ activity, as a secondary consequence of enhanced vascular $K_{ATP}$ activity, resulting in vasodilation, reduced vascular resistance and, in response, enhanced renin-angiotensin signaling, adrenergic signaling or
other vasoresponsive pathways (26, 39). We also see cardiac enlargement in SUR2[R1154Q] mutant mice, but this is less marked than in V65M or A478V mice, consistent with reduced overall expression of SUR2-dependent \( K_{ATP} \) channel levels in the vasculature (as well as the heart). Cardiac \( \beta \)-AR activation promotes cardiac hypertrophy (40) and, as we have also shown, can enhance trafficking of SUR1-dependent \( K_{ATP} \) channels to the myocardial surface membrane (29). This in turn could then contribute to increased SUR1-containing channels at the cell surface in R1154Q hearts.

**Variable disease causing/modifying consequences of alternate splicing in ABCC genes**

Strikingly, the SUR2[R1154Q] (\( ABCC9 \) c.3461G>A) mutation induces alternate splicing of SUR2 mRNA, generating a truncated SUR2 protein that lacks the 30 amino acids of exon 28. When expressed together with Kir6.2 in recombinant cells, the SUR2[R1154Q,\( \Delta \)exon28] construct failed to generate active \( K_{ATP} \) channels. In mixed expression with full length SUR2A cDNA, there was no evidence for a dominant-negative effect of the SUR2[R1154Q,\( \Delta \)exon28] construct; the data were best fit by assuming that even one full length subunit was sufficient to rescue function. This can explain the observed reduction of overall channel density, yet persistence of vascular hyperpolarization and cardiac enlargement in R1154Q animals; in the heterozygous case, the disease features were less marked than seen in heterozygous SUR2[A478V] animals (26, 39), even though the molecular consequence of the mutation itself is more severe (7, 11). In contrast to SUR2[A478V] animals, the disease features of homozygous SUR2[R1154Q] animals were no more dramatic than the heterozygous case, and \( K_{ATP} \) channel activities were more markedly decreased, particularly in smooth muscle, explained by the enhanced degree of splicing.

In many genes, exon inclusion/skipping is becoming recognized as a more common consequence of disease mutations than previously assumed (41-43). Multiple intra-intronic and intra-exonic mutations in CFTR (\( ABCC7 \)), a very closely related gene to \( ABCC8 \), have been associated with non-functional protein and cystic fibrosis (CF) disease (44). In one systematic study involving both *in silico* predictions and analysis of exon skipping in recombinant minigenes (45), 9/19 disease-associated CFTR mutations induced exon skipping in a fraction of transcripts, but did not abolish wild type expression completely, potentially underlying variably milder phenotypes. Mutations occurring at conserved intron–exon boundaries (i.e. splicing junctions at \(-1\), \(-2\), \(-3\), and \(+1\), \(+2\), \(+3\) positions) are expected to affect splicing of the immediately adjacent exons. The consequence of such mutations, for example, c.1117-1G>A and c.1209+1G>A in \( ABCC7 \), are generally considered to be severe, whereas mutations...
occurring at more distant positions, for example, +5, +6, or −5 and −6, are mild, typically associated with only mild CF disease (46).

Alternate splicing is well recognized as a component of ABCC9 regulation; the canonical finding is that cardiac myocytes express SUR2A, a variant containing exon 38A, whereas smooth muscle cells typically express SUR2B, containing the alternate C-terminal exon 38B. Previous studies in mouse have also identified multiple additional potential spliced SUR2 variants (14, 15, 19), including short forms of only 28 and 68 kDa (30), in addition to the full length (~150-kDa) form in the WT cardiac sarcolemmal membrane. Some small exon deletions modulate channel ATP sensitivity (15), whereas co-immunoprecipitation of short forms lacking NBD1 but containing NBD2 with Kir6.1 or Kir6.2 suggests that abnormal channel properties could be generated (30). Other studies identified an additional 55-kDa form of the protein, lacking exons 5-28 in mitochondria (termed mitoSUR2) generated by a nonconventional intraexonic splicing (IES) event within the 4th and 29th exons of SUR2 mRNA (47). Specific deletion of exon 5 of ABCC9, to ablate expression of both plasma membrane and the mitoSUR2 short form, resulted in neonatal cardiomyopathy, potentially due to failure of the heart to transition normally from fetal to mature myocardial metabolism (48). Conversely, mice overexpressing the 55kDa short form protein had improved recovery from ischemia-reperfusion injury relative to WT hearts (49).

Such studies indicate that exon splicing could result in distinct forms of the protein that are expressed in different cellular compartments, with profoundly different effects on cell function. The present data raise the possibility that R1154Q (or R1154W, and perhaps other) CS mutations might not only result in a functional K_{ATP} GOF but, by causing exon skipping, might also result in a truncated protein and hence an effective mixed loss/gain of function phenotype, potentially explaining variable expressivity of disease features in human CS (24). In cardiac myocytes derived from R1154Q CS patient iPSCs, we failed to detect any exon 28 skipping. The amino acid sequence in the region of R1154 is identical between mouse and human, but there is some variation in codon usage between the two species and, although splicing prediction algorithms suggest that both the human mutation and the CRISPR introduced mouse mutation should both alter exon splicing similarly, it is possible that the mouse sequence is more susceptible. It is also possible, given the unnatural differentiation process for iPSC-derived cells in vitro, that different outcomes might be obtained in native tissues, and additional studies will be necessary to confirm whether or not such splicing occurs in R1154 mutant human CS. Nevertheless, the finding that R1154Q induces such splicing in any genome illustrates the principle that SUR2 GOF mutations can also be associated with additional LOF.
resulting from variable splicing that results in reduced protein levels, such that the net effect could be either GOF or LOF in different tissues. We have demonstrated that isolated SUR2 LOF results in a very distinct constellation of features in ABCC9-related Intellectual Myopathy Syndrome (AIMS) (50). Hence, dual GOF/LOF consequences of CS mutations could result in not just quantitatively, but qualitatively variable outcomes, and marked variability of CS pathologies, and hence might underlie the marked variation in severity of CS consequences that is actually seen in patients (24).

Conclusions
First recognized as a distinct syndrome 30 years ago, recent studies have defined the genetic basis of Cantu syndrome (CS) gain-of-function (GOF) in $K_{ATP}$ channel genes, and have further defined the consequent mechanistic basis of multiple CS features. The most common human CS mutations, SUR2[R1154Q] and [R1154W], are present in ~30% of CS patients. In the present study, we have shown that, when introduced into the mouse locus, the SUR2[R1154Q] equivalent mutation causes canonical features of CS, but also demonstrates the unanticipated consequence of alternate mRNA splicing, which results in decrease in functional SUR2 protein levels. This is effectively a LOF that counteracts the mutational GOF action, and leads to a lower CS phenotypic severity. While studies in cells from human SUR2 R1154Q and R1154W patient cells failed to reveal similar outcome, the possibility remains that these or other GOF CS mutations might result in a counteracting loss of functional protein levels by a similar mechanism, which could then help explain, and have significant implications for, the wide variability of CS disease expressivity.
Methods

**CRISPR/Cas9 genome editing**

Using CRISPR/Cas9-mediated genome engineering technology (51) we generated knock-in mice carrying human gain-of-function mutation in the *ABCC9* gene, which encodes the accessory SUR2 subunit of the $K_{ATP}$ channel, respectively. Guide RNA (gRNA) target sequences predicted using the MIT CRISPR design tool ([http://crispr.mit.edu](http://crispr.mit.edu)) were cloned into plasmid pX330 (Addgene # 42230). sgRNA activity was validated in vitro by transfection of N2A cells using ROCHE Xtremegene HP, followed by T7E1 assay (NEB). T7 sgRNA template and T7 Cas9 template were prepared by PCR amplification and gel purification, followed by RNA in vitro transcription with the MEGAshortscript T7 kit (gRNA) or the T7 mMessage mMachine Ultra kit (Cas9). After transcription, RNA was purified with the Megaclear kit (Life Technologies). 200nt ssODN donor DNAs with the appropriate mutation centered within the oligo were synthesized from IDT as ultramer oligos.

B6CBA F1/J female mice (3–4 wk old; Jackson Laboratory) were superovulated and mated overnight with B6CBA F1/J male mice (>7 wk old). Zygotes were harvested from the ampullae of superovulated females and placed in potassium-supplemented simplex optimized medium (KSOM; MR106D) before microinjection. Microinjection of the Cas9, sgRNA, and ssDNA template (at a final concentration of 50 ng/μL Cas9 WT RNA, 25 ng/μL gRNA, and 20 ng/μL ssODN DNA) was performed in flushing holding medium (FHM; MR-024-D; EmbryoMax; Millipore). After injection, zygotes were incubated at 5.5% CO$_2$ at 37 °C for 2 h, and surviving embryos were transferred to ICR recipient mice by oviduct transfer. Founders were identified using Qiagen pyrosequencer and Pyromark Q96 2.5.7 software. We identified multiple viable and fertile positive founder animals carrying CS mutation mouse SUR2[R1151Q] (equivalent to human SUR2[R1154Q]), and we refer to these as SUR2[R1154Q] mice for direct comparison to the human Cantu syndrome equivalent. Successful mutation was verified in founder (F0) mice by Sanger sequencing of genomic DNA. Mutant mice were subsequently crossed with C57BL/6J mice to generate heterozygous F1 SUR2$^\text{Het}$ lines. PCR was used to generate amplicons of *ABCC9* spanning >5 kb either side of the introduced mutation, from gDNA isolated from mouse tails, and resultant PCR products were sequenced to confirm absence of unintended additional mutations. After verification, one F1 animal from one line of each genotype was selected and subsequently bred with C57BL/6J for multiple (>6) generations to generate Het and Homo R1154Q as well as wild type littermates that were used in experiments.

**Generation of human iPSCs and analysis of derived cardiac myocytes**
All studies were approved by the Washington University Human Studies Committee and carried out with the full consent of participating patients. Human R1154W patient renal epithelial cells (RECs) were reprogrammed to hiPSCs by the WUSM Genome Engineering and iPSC Core (GEiC) using Sendai Virus-based reprogramming vectors. After four unsuccessful attempts to reprogram human R1154Q patient RECs, peripheral blood mononuclear cells were provided by the patient, and were successfully reprogrammed by the GEiC using the Sendai Virus-based reprogramming cocktail. hiPSCs were maintained on a 4-day passaging cycle. Differentiation to cardiomyocytes was carried out in entirely chemically defined conditions via temporal modulation of canonical Wnt signaling(34).

**RNA extraction and analysis** RNA was isolated from freshly dissecting cardiac apices or from iPSC-derived cardiomyocyte cultures using TRlzol (Thermo Fisher) and first strand cDNA was synthesized using SuperScript™ III First-Strand Synthesis System (Thermo Fisher).

**Protein analysis**

*Protein extraction from heart tissue* Snap frozen tissue was thawed on ice and equilibrated with ice-cold homogenization buffer (protease inhibitors, 50 mM NaCl, 0.32 M sucrose, 2mM EDTA, 20 mM HEPES pH 7.4). Atria were dissected from ventricles. The ventricular tissue was diced, resuspended in homogenization buffer and homogenized via a Miccra D-1 homogenizer and subsequent strokes by a manual glass-teflon dounce homogenizer. The suspension was then centrifuged at 100,000 g. The obtained membrane pellet was resuspended in homogenization buffer, aliquoted and snap frozen with liquid nitrogen. Membranes were resuspended in solubilization buffer (1.5% Triton X-100, 0.75% sodium deoxycholate, 0.1% SDS, protease inhibitors in 10 mM NaCl, 5mM EDTA, 2.5 mM EGTA, 50 mM Tris-HCl pH 7.35) and centrifuged at 50,000 g at 4°C. Supernatant was subjected to TCA to a final concentration of 12.5 % and incubated for 30 minutes on ice. The pellet was acetone washed twice and air-dried at 37°C, supplemented with 1x SDS sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 0.1% Bromophenol blue, 10% glycerol) containing 100 mM DTT and resuspended for subsequent analysis by SDS-PAGE.

**Glycosidase treatment** 40 µl of glycoprotein denaturing buffer (reconstituted with 1x glycoprotein denaturing buffer, 2,5% NP-40, 1x G7 in ddH$_2$O) was added to TCA-precipitated air-dried pellets from ~100 µg of total protein and agitated at room temperature for 30 minutes. Subsequently,
1.5 µl of the glycosidase (PNGaseF (750 U); New England Biolabs Inc.) was added to the mixture. After incubation at 37°C for 1 hour at 1000 RPM, the mixture was supplemented with 5x SDS sample buffer and 100 mM DTT, agitated for 30 minutes and analyzed by SDS-PAGE.

**Protein analysis by western-blotting** For separating proteins via SDS-PAGE, 6% polyacrylamide gels were used for proteins greater than 100 kDa and 12% for other proteins. Electrophoresis was performed at constant current, limited to 15 mA per gel. Gels with separated proteins were put onto a nitrocellulose membrane and placed between two blotting papers and electroblotted for 90 minutes in transfer buffer (25mM Tris, 192mM glycine, pH 8.3) at 4°C with a constant voltage of 60 V and the current limited to 1A. Membranes were washed and blocked with blocking buffer (5% w/v milk powder, 25 mM Tris/HCl pH 7.4, 135 mM NaCl, 3 mM KCl, 0.02% IGEPAL).

As previously described, the anti-Kir6.2 antibody (raised in guinea pig and yielded as serum of the third bleeding (52)) recognizes the last 36 amino acids of the protein, and was characterized on native tissue against Kir6.2-knockout controls (29). Information about antibodies against proteins other than Kir6.2 are given in Table 1. Primary antibodies were diluted in blocking buffer as given in the table below and incubated over night at 4°C. For antibodies against SUR-proteins a differing blocking buffer (“SUR-blocking buffer”: 4% w/v milk powder, 25 mM Tris/HCl pH 7.4, 135 mM NaCl, 3 mM KCl, 0.1% Tween-20) was used. Subsequently, membranes were washed three times with their respective blocking buffer and incubated with IRDye LiCOR- secondary antibodies (800CW) diluted in blocking buffer at 1:4000. Blots were incubated for 90 minutes at room temperature, washed with washing buffer (25 mM Tris/HCl pH 7.4, 135 mM NaCl, 3 mM KCl, 0.1% Tween-20 for SUR-proteins, 5% w/v milk powder, 25 mM Tris/HCl pH 7.4, 135 mM NaCl, 3 mM KCl, 0.02% IGEPAL for others) and antibody-signals were subsequently visualized using an Odyssey® Sa Infrared imaging system.

**Patch clamp electrophysiology**

*Isolated vascular smooth muscle cells*: Mice were anesthetized with 2.5% avertin (10ml/kg, intraperitoneal Sigma-Aldrich) and the ascending aorta was rapidly dissected and placed in ice-cold Physiological Saline Solution (PSS) containing (in mM): NaCl 134, KCl 6, CaCl2 2, MgCl2 1, HEPES 10, and glucose 10, with pH adjusted to 7.4 with NaOH. Smooth muscle cells were enzymatically dissociated in dissociation solution containing (in mM): NaCl 55, sodium glutamate 80, KCl 5.6, MgCl2 2, HEPES 10, and glucose 10, pH 7.3 with NaOH, then placed into dissociation solution containing papain 12.5 µg/mL, dithioerythreitol 1 mg/mL, and BSA 1
mg/mL for 25 minutes (at 37°C), before immediate transferal to dissociation solution containing collagenase (type H:F=1:2) 1 mg/mL, and BSA 1 mg/mL for 5 minutes (at 37°C). Cells were dispersed by gentle trituration using a Pasteur pipette, plated onto glass coverslips on ice and allowed to adhere for >1 h before transferal to the recording chamber.

Whole-cell $K_{ATP}$ currents were recorded using an Axopatch 200B amplifier and Digidata 1200 (Molecular Devices). Recordings were sampled at 3 kHz and filtered at 1 KHz. Currents were initially measured at a holding potential of -70mV in High Na’ bath solution containing (in mM): NaCl 136, KCl 6, CaCl$_2$ 2, MgCl$_2$ 1, HEPES 10, and glucose 10, with pH adjusted to 7.4 with NaOH before switching to a High K’ bath solution [KCl 140, CaCl$_2$ 2, MgCl$_2$ 1, HEPES 10, and glucose 10, with pH adjusted to 7.4 with KOH] in the absence and presence of pinacidil and glibenclamide as indicated. The pipette solution contained (in mM) potassium aspartate 110, KCl 30, NaCl 10, MgCl$_2$ 1, HEPES 10, CaCl$_2$ 0.5, $K_2$HPO$_4$ 4, and EGTA 5, with pH adjusted to 7.2 with KOH.

**Isolated ventricular myocytes:** Ventricular myoctes were isolated from adult mice, anesthetized using 2.5% Avertin (10 ml/kg), and the heart and ascending aorta were removed and immersed in ice-cold calcium free Wittenberg Isolation Medium (WIM; in mM): 116 NaCl, 5.4 KCl, 8 MgCl$_2$, 1 NaH$_2$PO$_4$, 1.5 KH$_2$PO$_4$, 4 NaHCO$_3$, 12 Glucose, 21 HEPES, 2 Glutamine plus essential vitamins (GIBCO) and essential amino acids (GIBCO) (pH 7.40). The heart was cannulated via the aorta and Langendorff perfused with WIM for 5 min at 37°C, followed by 20 min perfusion of WIM supplemented with 270 units/ml of collagenase type 2 (Worthington Biochemical) and 10 µM CaCl$_2$ at 37°C. The heart was then transferred to WIM containing 50 mg/ml BSA, 12.5 mg/ml taurine and 150 µM CaCl$_2$, and ventricular tissue was manually dissociated using forceps before single cell dissociation by trituration with a fire-polished Pasteur pipette.

Inside-out patch clamp recordings were made in symmetrical KINT solution which contained (in mM): 140 KCl, 10 HEPES, 1 EGTA (pH 7.4 with KOH). Varying MgATP concentrations were applied using a Dynaflow Resolve perfusion chip (Cellectricon). MgCl$_2$ was added to each solution to achieve a free [Mg$^{2+}$] 0.5 mM according to calculations using CaBuf (Katholieke Universiteit Leuven). Membrane currents were sampled at 3 KHz, filtered at 1 KHz, at a holding potential of -50 mV using an Axopatch 700B amplifier and Digidata 1200 (Molecular Devices). $K_{ATP}$ channel currents in solutions of varying nucleotide concentrations were normalized to the basal current in the absence of nucleotides and dose-response data were fit with a four-parameter Hill fit according to equation (1):

$$Normalized\ current = I_{min} + (I_{max} - I_{min})/(1 + ([X]/IC_{50})^h)$$

1
where the current in $K_{\text{ext}} = I_{\text{max}} = 1$, $I_{\text{min}}$ is the normalized minimum current observed in MgATP, $[X]$ refers to the concentration of MgATP, $IC_{50}$ is the concentration of half-maximal inhibition and $H$ denotes the Hill coefficient.

Whole-cell patch clamp recordings of voltage-gated calcium channel activity were made in a bath solution which contained (in mM): 116 NaCl, 5.4 CsCl, 0.16 NaH$_2$PO$_4$, 10 glucose, 1.8 CaCl$_2$, 0.5 MgCl$_2$, 5 HEPES, 3 NaHCO$_3$, 0.01 tetrodotoxin (pH 7.4 with NaOH), using a pipette solution which contained (in mM): 120 CsCl, 20 TEA-Cl, 5 K$_2$ATP, 10 HEPES (pH 7.3 with KOH). Cell capacitance and series resistance were determined from 5 mV square pulses from a holding potential of -70 mV following establishment of the whole-cell configuration. All recordings were performed at 20-22 °C.

**Arterial compliance**

After mice were euthanized under isoflurane anesthesia, the ascending aorta and left common carotid artery of 3 week-old mice were excised and placed in a physiologic saline solution (PSS) containing 130 mM NaCl, 4.7 mM KCl, 1.18 mM MgSO$_4$-7H$_2$O, 1.17 mM KH$_2$PO$_4$, 14.8 mM NaHCO$_3$, 5.5 mM dextrose, and 0.026 mM EDTA (pH 7.4). The vessels were then cleaned from surrounding fat, mounted on a pressure arteriograph (Danish Myo Technology) and maintained in PSS at 37°C. Vessels were visualized with an inverted microscope connected to a charge-coupled device camera and a computerized system, which allows continuous recording of vessel diameter. Intravascular pressure was increased from 0 to 175 mmHg by 25-mmHg increments and the vessel outer diameter was recorded at each step (12 seconds per step). The average of three measurements at each pressure was reported.

**Blood pressure measurement**

*In anesthetized mice* Mice were anesthetized with 1.5% inhaled isoflurane and restrained on a heating pad to maintain body temperature. A 2- to 3-mm incision was made in the midline of the neck; the thymus and muscle were separated to expose the right carotid artery. A Millar pressure transducer (model SPR-671) was carefully inserted into the right carotid artery and moved to the ascending aorta. Systolic BP, diastolic BP and heart rate were recorded using the PowerLab data acquisition system (ADInstruments), and data were analyzed using LabChart 7 (ADInstruments). For blood pressure measurements in conscious mice, a radio-telemetry pressure transmitter (Data Science International, DSI) was surgically inserted into the left
carotid artery and moved to the ascending aorta, where blood pressures in day and night were recorded by DSI data acquisition system after mice recovered from surgery.

**Telemetry probe implantation and telemetry recording** Mice (6–8 month old) were implanted with TA11PA-C10 (Data sciences Inc., USA) telemetric implants under anesthesia with a gas concentration of 1.5-2.5% isoflurane. The catheter was advanced into the ascending aorta via the left carotid artery and the body of transmitter was slipped into the pocket subcutaneously in the right flank. Animals were housed in an isolated recording room and allowed for at least 1 week recovery before recordings. Systolic (SBP), diastolic (DBP), mean arterial pressure (MBP = DBP+1/3(SBP-DBP)), and heart rate (HR), were collected using the Dataquest ART system. Data were sampled by averaging 10 seconds of each 1 minute period. Values were presented as means of per 10 min in the traces. Values of day and night were averages of day time (6 am – 6 pm) or night time (6 pm – 6 am). After 3 days of baseline recording, the mice were injected with pinacidil (ip. 0.01, 0.1, 1 mg) daily.

**Heart weight measurement and histology** Mice were anesthetized with 2.5 % Avertin and hearts were excised, rinsed with PBS which contained (in mM): 137 NaCl, 2.7 KCl, 10 Na₂HPO₄, KH₂PO₄ (pH 7.4 with NaOH). The hearts were arrested in diastole with 10% KCl, and blotted to remove excess liquid. Hearts were then weighed and weight was normalized to tibia length. After weighing, the hearts were fixed in 10% buffered formalin for 24 h, and embedded in paraffin. Sections (3 μm) were cut and stained with hematoxylin and eosin (H&E) for the morphometric analysis.

**Echocardiography** Short-axis left ventricular scans were obtained via M-mode echocardiography using an ATL 5000cv instrument (Phillips, Bothell, WA) with a 15-MHz compact linear array. The operator was blinded to genotype. Left ventricular end-diastolic dimension (LVEDD), left end-systolic dimension (LVESD), end diastolic anterior wall thickness (AWT), end diastolic posterior wall thickness (PWT), R-R interval, ejection time (ET) were recorded from three separate cardiac cycles for each mouse. Wall thickness divided by chamber radius was calculated at diastole. Left ventricular mass (LVM) were calculated using the Devereux equation (). Fractional shortening (FS%) refers to (LVEDD-LVESD) / LVEDD as a percentage. Stroke volume (SV) refers to the amount of blood ejected by the left ventricle in one contraction, determined by subtracting left end-systolic volume (LVESV) from left ventricular end-diastolic volume (LVEDV)
(LVEDV – LVESV), assuming LVEDV and LVESV are simply cubed. The ejection fraction (EF%, SV/LVEDV) refers to the percentage of blood that is pumped out of the ventricles with each contraction.

**Statistics** Unless otherwise noted, all data are presented as mean ± S.E.M., and were tested for statistical significance using one-way ANOVA, with post hoc Tukey test, or 2-tailed Student’s t-Test as indicated. P values < 0.05 were considered statistically significant.

**Study Approval**
Studies were performed in compliance with the standards for the care and use of animal subjects defined in the NIH Guide for the Care and Use of Laboratory Animals (53) and were reviewed and written approval was provided by the Washington University Institutional Animal Care and Use Committee.

**Author contributions:** All authors made equally significant contributions to this study: HZ, MSR, CGN originally conceived the study; MSR, CGN oversaw the generation of the mutant mice; HZ, AH, TSdA, CMcC, CE, ECA, TH, ZH, PC, SB, AK, carried out the experiments; RPM, GKS, DKG, BS contributed key technical help or clinical background; HZ, AH, CMcC, BS, MSR, CGN wrote the paper, which was edited by the other authors.

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References


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Figure 1: Cardiovascular phenotype of R1154Q mice. (A,B) Cardiomegaly in heterozygous SUR2<sup>wt/RQ</sup> (wt/RQ) and homozygous SUR2<sup>RQ/RQ</sup> (RQ/RQ) hearts. (C,D) (Left) Isolated ascending aorta of wt/RQ and RQ/RQ shows similar increase in diameter at all pressures, relative to WT (C), but carotid artery mechanical properties are not different from WT (D). (n = 5 for WT, 7 for wt/RQ, 6 for RQ/RQ). Statistical significance was determined by two-way ANOVA followed by post-hoc Tukey test correction for multiple comparisons, asterisks indicate significantly different than WT (* p<0.05; ** p<0.01).
Figure 2: Cardiovascular function of R1154Q mice. (A) Mean arterial pressures (MAP) and (B) mean heart rates in conscious wild type (WT), heterozygous SUR2<sup>wt/RQ</sup> and SUR2<sup>RQ/RQ</sup> mice in day and night. (C) Mean arterial pressure (MAP) and (D) heart rate (HR) in anesthetized mice showing blunted response to the K<sub>ATP</sub> channel activator pinacidil in SUR2<sup>RQ/RQ</sup>. Statistical significance was determined by one-way ANOVA followed by Tukey test for pairwise comparison, asterisks indicates significant difference (p<0.05) within genotypes.
Fig. 3: Decreased $K_{\text{ATP}}$ channel density and switch to SUR1-dependence in SUR2A[R1154Q] hearts. (A) Representative inside-out patch-clamp recordings of $K_{\text{ATP}}$ channel activity from acutely dissociated ventricular myocytes from WT (above) and SUR2$^{RQ/RQ}$ (below) mice showing inhibition by 10 or 0.1 μM MgATP and the response to K channel openers Pinacidil (Pin) and Diazoxide (Diaz) at 100 μM, in the presence of MgATP (recording at -50 mV membrane potential). (B) Absolute $K_{\text{ATP}}$ current level in zero ATP, from experiments as in A. (C) $K_{\text{ATP}}$ current, as a fraction of current in zero ATP, from experiments as in A. (D) Western-blot analysis of the membrane fraction from ventricular heart tissue of WT and SUR2$^{RQ/RQ}$ mice (four biological replicates each) showing protein steady-state levels of $K_{\text{ATP}}$ channel subunits and Na/K-ATPase α subunits. Since both SUR subunits are only core-glycosylated when the Kir6.2 subunit is missing (29) tissue from a single Kir6.2$^{-/-}$ mouse is also shown for reference. Statistical significance was determined by one-way ANOVA followed by Tukey test for pairwise comparison, * p<0.05, ** p < 0.01, *** p < 0.001.
Fig. 4. SUR1 and SUR2A maturation in SUR2A[R1154Q] hearts. (A) Western blot analysis of the membrane fraction from ventricular heart tissue of WT and Kir6.2−/− mice. Representative result from 3 biological replicates. (B) PNGase F treatment (18.75 U/µL) of ventricular heart membrane lysates of WT, SUR2^WT/RQ, SUR2^RQ/RQ and Kir6.2−/− mice. Representative Western-blot of 2 biological replicates.
Figure 5 Loss of pinacidil-sensitive SUR2-dependent $K_{\text{ATP}}$ channels in SUR2A[R1154Q] VSM. (A) Representative whole-cell voltage-clamp recordings from acutely isolated aortic smooth muscle cells from WT (top) and SUR2$^{RQ/RQ}$ (bottom) mice. Cells were voltage-clamped at -70 mV. (B) Summary of whole-cell current densities from voltage clamp recordings as IUN, showing significantly reduced pinacidil-activated $K_{\text{ATP}}$ conductances SUR2$^{RQ/RQ}$. Statistical significance was determined by multiway ANOVA followed by 2-tailed t-test pairwise comparison with Bonferroni correction for multiple comparisons (adjusted α = 0.008), * denotes p < 0.008. (C) Representative whole cell current-clamp recordings from acutely isolated aortic smooth muscle cells from WT (left) and homozygous SUR2$^{RQ/RQ}$ (right) using an intracellular pipette solution absent of nucleotides. (D) Summary of initial (init) and stable (stab) membrane potentials from experiments as in C. Statistical significance was determined by one-way ANOVA followed by Tukey tests, * denotes p < 0.05, ** p < 0.01.
Figure 6: Abnormal splicing in SUR2A[R1154Q] mRNA. (A) Canonical cDNA sequence for human and mouse SUR2 over exons 27-29 region (non-identities indicated by grey). Human nucleotide c.3461(mouse c.3452) G>A mutation generating p.R1154Q is indicated by red box. (B) cDNA PCR product analyzed by gel electrophoresis (above), and by direct sequencing of the selected bands from (below) reveals an exact deletion corresponding to the 93 nucleotides of exon 28 in ~half of heterozygous wt/RQ- and almost all homozygous RQ/RQ mouse transcripts (red arrowheads). (C) (above) Amino acid sequence of residues 1149-1194 (human) is identical in human and mouse SUR2. (below) Model of the Kir6/SUR complex (pdb ID: 5WUA) indicates the predicted location of the R1154Q mutation and amino acids encoded by exon 28.
Figure 7: Functional consequence of SUR2[R1154Q] mRNA splicing. (A) Representative Rb efflux experiments from untransfected COSm6 cells, and from untransfected cells (Unt) and cells transfected with WT, R1154Q (RQ) and R1154Q[delta exon 28] SUR2A, plus Kir6.2. (B) Fractional efflux at 40 minutes, from experiments as in A. Statistical significance was determined by one-way ANOVA followed by Tukey tests, *** denotes p < 0.001. (C) Fractional efflux at 40 minutes, from similar experiments to A, in cells transfected with SUR2[R1154Q,delta exon 28] subunits in addition to SUR2[R1154Q] (plus Kir6.2) subunits. Dashed lines are predicted levels of efflux assuming that 1, 2, 3 or 4 WT subunits in a randomly assembling complex are necessary to restore function. (D) cDNA PCR product analyzed by gel electrophoresis reveals similar levels of splicing in R1154Q ventricle, smooth muscle and skeletal muscles, suggesting that SUR2 function will be significantly reduced in all tissues. (E) cDNA PCR product from human R1154Q patient skeletal muscle and skin analyzed by gel electrophoresis reveals only a single band corresponding to 642 bp fragment from full length SUR2A cDNA and no band corresponding to predicted 549 bp from exon 28-deleted cDNA (red arrowheads). (F) Image of human R1154Q patient iPSC-derived cardiac myocytes (above, scale bar 50 μm), and cDNA PCR product from human R1154Q or R1154W patient iPSC-derived cardiac myocytes analyzed by gel electrophoresis reveals only a single band
corresponding to 325 bp fragment from full length SUR2A cDNA and no band corresponding to predicted 232 bp fragment from exon 28-deleted cDNA (red arrowheads) (representative result from n= 3 repeats).