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# Compound heterozygous mutations in the SUR1 (*ABCC8*) subunit of pancreatic $K_{ATP}$ channels causes neonatal diabetes by perturbing the coupling between Kir6.2 and SUR1 subunits

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**Keywords:**  $K_{ATP}$  channel, *ABCC8*, SUR1, neonatal diabetes, compound heterozygous mutations, sulfonylureas, ATP

$K_{ATP}$  channels regulate insulin secretion by coupling  $\beta$ -cell metabolism to membrane excitability. These channels are comprised of a pore-forming Kir6.2 tetramer which is enveloped by four regulatory SUR1 subunits. ATP acts on Kir6.2 to stabilize the channel closed state while ADP (coordinated with  $Mg^{2+}$ ) activates channels via the SUR1 domains. Aberrations in nucleotide-binding or in coupling binding to gating can lead to hyperinsulinism or diabetes. Here, we report a case of diabetes in a 7-mo old child with compound heterozygous mutations in *ABCC8* (SUR1[A30V] and SUR1[G296R]). In unison, these mutations lead to a gain of  $K_{ATP}$  channel function, which will attenuate the  $\beta$ -cell response to increased metabolism and will thereby decrease insulin secretion.  $^{86}Rb^{+}$  flux assays on COSm6 cells coexpressing the mutant subunits (to recapitulate the compound heterozygous state) show a 2-fold increase in basal rate of  $^{86}Rb^{+}$  efflux relative to WT channels. Experiments on excised inside-out patches also reveal a slight increase in activity, manifested as an enhancement in stimulation by MgADP in channels expressing the compound heterozygous mutations or homozygous G296R mutation. In addition, the  $IC_{50}$  for ATP inhibition of homomeric A30V channels was increased ~6-fold, and was increased ~3-fold for both heteromeric A30V + WT channels or compound heterozygous (A30V + G296R) channels. Thus, each mutation makes a mechanistically distinct contribution to the channel gain-of-function that results in neonatal diabetes, and which we predict may contribute to diabetes in related carrier individuals.

## Introduction

ATP sensitive potassium ( $K_{ATP}$ ) channels are widely distributed in various tissues and cell types where they couple cell metabolism to cell excitability and therefore play important physiological roles.<sup>1</sup> The gating properties that are critical for the physiological function of  $K_{ATP}$  channels are their sensitivity to intracellular nucleotides ATP and ADP, whose concentrations fluctuate as glucose levels vary. Both Kir6.x and SUR subunits participate in nucleotide regulation of the channel; ATP inhibits channel activity by binding to the Kir6.x subunits, whereas  $Mg^{2+}$ -complexed ATP and ADP stimulate channel activity by interacting with SUR subunits. For pancreatic  $\beta$  cells, upon an increase in glucose concentration,  $K_{ATP}$  channels are driven to closure by the increase in ATP/ADP ratio, resulting in membrane depolarization. The depolarization activates voltage-gated calcium channels and causes insulin secretion.<sup>2-4</sup>

Over 65 gain-of-function mutations in  $K_{ATP}$  channels genes resulting in neonatal diabetes (NDM) have now been

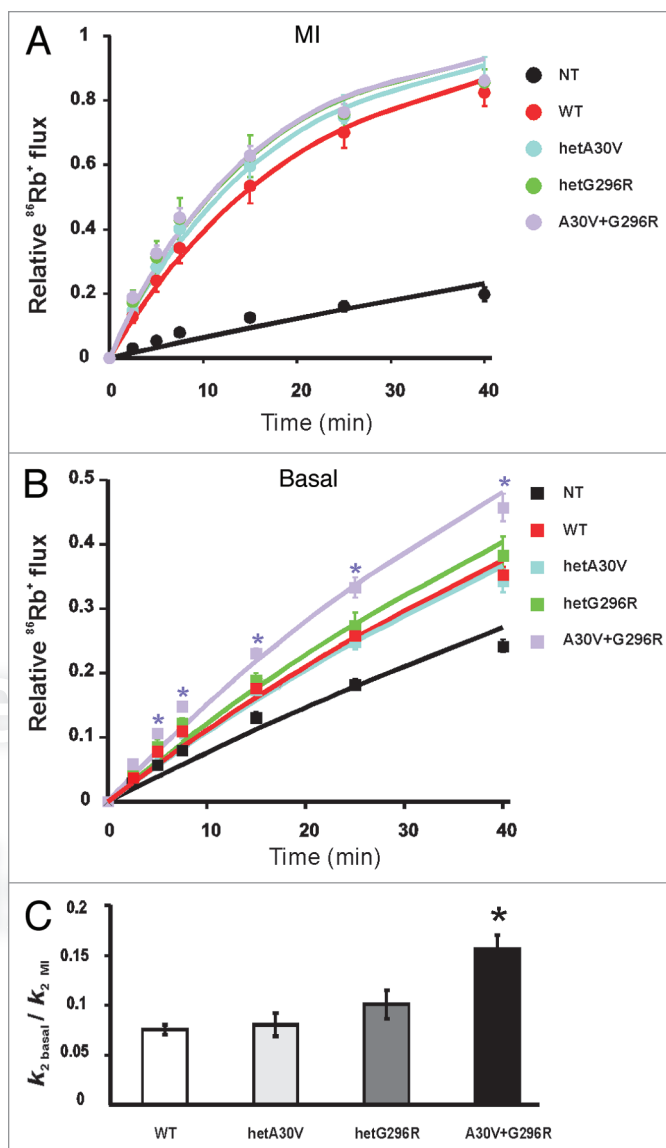
identified. These mutations result in overactive channels, leading to hyperpolarization of  $\beta$  cells and reduced insulin secretion. Sulfonylureas, which specifically inhibit the  $K_{ATP}$  channel by interacting with SUR subunits, can restore insulin secretion in these patients.<sup>5-8</sup> Such mutations in  $K_{ATP}$  channels account for approximately 50% of all cases of permanent neonatal diabetes and while mutations in *KCNJ11*, encoding Kir6.2, are more commonly inherited in an autosomal dominant pattern, in 43% of cases, *ABCC8*-related permanent neonatal diabetes is inherited in an autosomal recessive manner from unaffected parents with heterozygous mutations.<sup>9</sup> Increased activity of  $K_{ATP}$  channels resulting from mutations in *ABCC8* is caused by an increase in the magnesium-dependent stimulatory action of SUR1 on the pore<sup>8,10</sup> or by alteration in the inhibitory action of ATP on the Kir6.2 subunit.<sup>11</sup> Here we report the case of a child with permanent neonatal diabetes due to novel compound heterozygous mutations in *ABCC8* and the functional characterization of these mutant channels.

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## Results

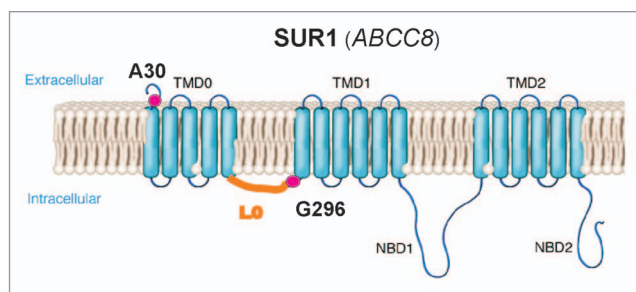
**Case report and identification of the compound heterozygous mutations in *ABCC8*.** A 7-mo-old Pakistani male presented with diabetic ketoacidosis. He was born via vaginal delivery, with birth weight of 3.416 kg and birth length was 48.9 cm (both appropriate for gestational age). At presentation he had fever, increased irritability and Kussmaul breathing. His weight was 9 kg (95<sup>th</sup> percentile), and he was severely dehydrated. Laboratory studies showed a pH of 7.06, bicarbonate of 5 mmol/L, glucose of 750 mg/dL (41.7 mmol/L), and HgA1c of >14%. He was fluid resuscitated and started on 0.1 unit/kg/hour of intravenous insulin. His acidosis resolved within 24 h and he was transitioned to a subcutaneous regimen of intermediate (NPH) and rapid-acting (aspart) insulin at a total daily dose of 0.6–0.8 unit/kg/day. Family history was remarkable for history of diabetes in maternal grandparents who were diagnosed in their 40s and treated with oral medications. The paternal grandfather was recently diagnosed with diabetes in his 50s and is also on oral medications. The mother had 2 h OGTT during pregnancy which was reported as normal and there is no history of diabetes in the father although he has not been tested. After starting subcutaneous insulin regimen, the proband HgA1c improved to 9.7%. A diabetes autoimmune panel, obtained after initiation of insulin, was weakly positive for insulin antibodies at 5.4 uU/mL (normal 0–5), and negative for GAD 65 and CA 512 antibodies (<0.5 U/mL and <0.8 U/mL, respectively). Direct sequencing of his DNA revealed two mutations in the *ABCC8* gene c.886 G > A (G296R) and c.89 C > T (A30V), the first of which is maternally derived and the second is paternally derived. Neither of these mutations has been previously described. At 12 mo of age, he was started on 0.2 mg/kg/day of glibenclamide (glyburide), increasing daily, until he was weaned off insulin, to a dose of 0.35 mg/kg/day. Baseline fasting C-peptide (prior to initiation of glibenclamide) was 0.119 ng/mL (blood glucose 118 mg/dL) and increased to 1.4 ng/mL (blood glucose 101 mg/dL) on glibenclamide. Four weeks after initiation of glibenclamide his HgA1c improved to 6.4%, random insulin level was 6.5  $\mu$ U/mL and C-peptide was 1.71 ng/mL (blood glucose 82 mg/dL). He has maintained good control with HgA1c in the range of 4.8–6.4% since initiation of therapy with glibenclamide.

**Compound heterozygous mutant channel are more active in whole cells.** To test the hypothesis that the compound heterozygous channels have an overall gain of channel function in intact cells, we examined  $^{86}\text{Rb}^+$  efflux across the plasma membrane in COSm6 cells cotransfected with Kir6.2 and WT or mutant SUR1. Efflux curves were fit with a two-pathway model (see materials and methods); untransfected cells providing the efflux rate ( $k_1$ ) for the non  $K_{\text{ATP}}$  pathway and the indication of channel activity,  $k_2$ , is calculated from transfected cells. As shown in **Figure 1A**, neither heteromeric single mutant channels ([A30V + WT], referred to as hetA30V and [G296R + WT], referred to as hetG296R) nor compound heterozygous channels ([A30V + G296R]) exhibited significantly different maximum  $^{86}\text{Rb}^+$  efflux rates in the presence of metabolic inhibitors. However, in basal conditions without metabolic inhibitors, compound heterozygous but not single mutant



**Figure 1.** Increased basal  $K_{\text{ATP}}$  activity in compound heterozygous channels compared with WT channels.  $^{86}\text{Rb}^+$  efflux of heterozygous mutant channels coexpressed with each other or WT in 1:1 DNA ratio. NT: Non Transfected cells. The NT curve is used to calculate the non  $K_{\text{ATP}}$  channel pathway rate  $k_1$  (see Materials and Methods). (A) In the presence of metabolic inhibition, compound heterozygous and hetA30V, hetG296R channels exhibit similar maximal Rb efflux rates. (B) Compound heterozygous channels display a 2-fold increase in the basal  $^{86}\text{Rb}^+$  efflux without metabolic inhibitors. Data points indicate means  $\pm$  SEM of  $n = 4$ –9 experiments. Fitted lines correspond to least squares fits of a double-exponential equation. \* $p < 0.05$  compared with WT by One-Way ANOVA analysis. (C) Rate constant for  $K_{\text{ATP}}$ -dependent  $^{86}\text{Rb}^+$  efflux ( $k_2$ ) in basal conditions relative to metabolic inhibition (MI).  $k_{2,\text{basal}}/k_{2,\text{MI}}$  calculated from WT, het A30V, het G296R and SUR1[A30V + G296R] are  $0.07 \pm 0.005$ ,  $0.08 \pm 0.01$ ,  $0.1 \pm 0.01$  and  $0.16 \pm 0.01$  ( $n = 4$ –9). \* $p < 0.05$  compared with WT by One-Way ANOVA analysis.

heterozygous channels exhibit significantly increased  $k_2$  compared with WT channels (**Fig. 1B and C**). The increased  $^{86}\text{Rb}^+$  efflux rate in the compound heterozygous channels indicates that they have higher channel activity when both mutants are present from

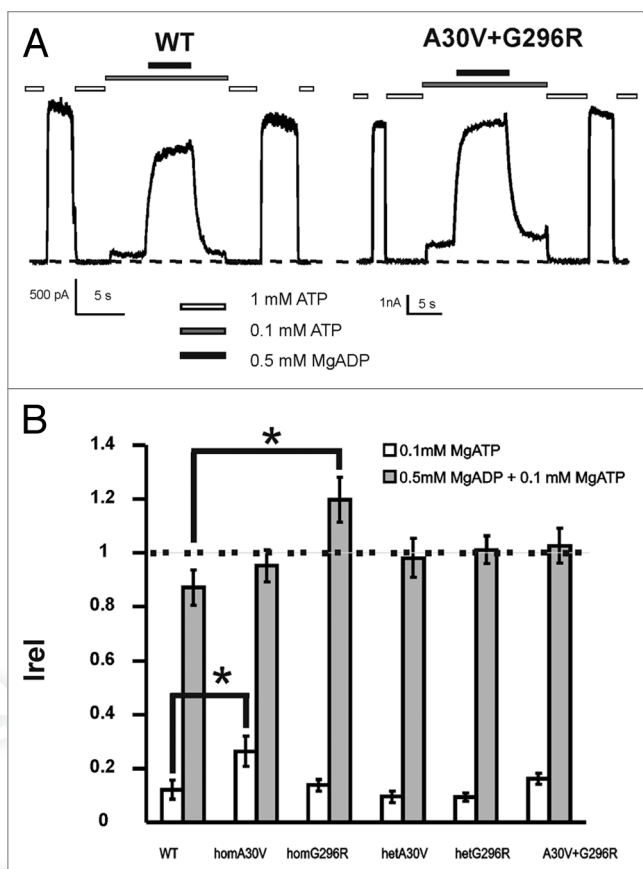


**Figure 2.** Membrane topology of SUR1 and the predicted locations of A30V and 296R. The red filled circles indicate the predicted positions for A30V at the N terminus of its 1st transmembrane domain (TMD0) and G296R at the L0 linker region before TMD1 domain. L0 is colored in orange.

each allele and reflects a recessive effect of the two mutations that will manifest as significantly increased  $K_{ATP}$  conductance only in the compound heterozygous case, as in the proband.

**HetG296R channels exhibit increased sensitivity to MgADP stimulation while HetA30V channels exhibit slightly decreased ATP sensitivity.** A30V and G296R mutants are located at the TMD0 and the cytosolic linker region (L0) of SUR1<sup>3</sup> (Fig. 2). The TMD0 and L0 domains at the beginning of the SUR1 protein have been proposed to interact with the Kir6.2 subunit and to functionally couple the core nucleotide binding domains (NBD1 and NBD2) to the channel pore.<sup>8,12-17</sup> Because of the functional/physical coupling between SUR1 and Kir6.2, any mutant in SUR1 could affect the intrinsic channel gating of Kir6.2 or simply affect the transduction of Mg-nucleotide stimulation. Here we characterized the sensitivity to MgADP activation, as well as inhibitory ATP sensitivity (without  $Mg^{2+}$ ), of homomeric A30V channels (labeled as homA30V), homomeric G296R channels (labeled as homG296R), hetA30V, hetG296R and compound heterozygous channels [A30V + G296R].<sup>8,18,19</sup> Representative recordings of channel response to MgADP from WT and compound heterozygous mutants are shown in Figure 3A and summary results are shown in Figure 3B. Homomeric G296R channels exhibit dramatically increased MgADP activity, while homomeric A30V channels show no significant enhancement (Fig. 3B). However, hetG296R and compound heterozygous channels show a small but not significant increase in MgADP stimulation. On the other hand, it is noticeable that A30V homomeric channels show significant increased current in 0.1 mM MgATP, reflecting an additive consequence of the two distinct mutant effects (Fig. 3B).

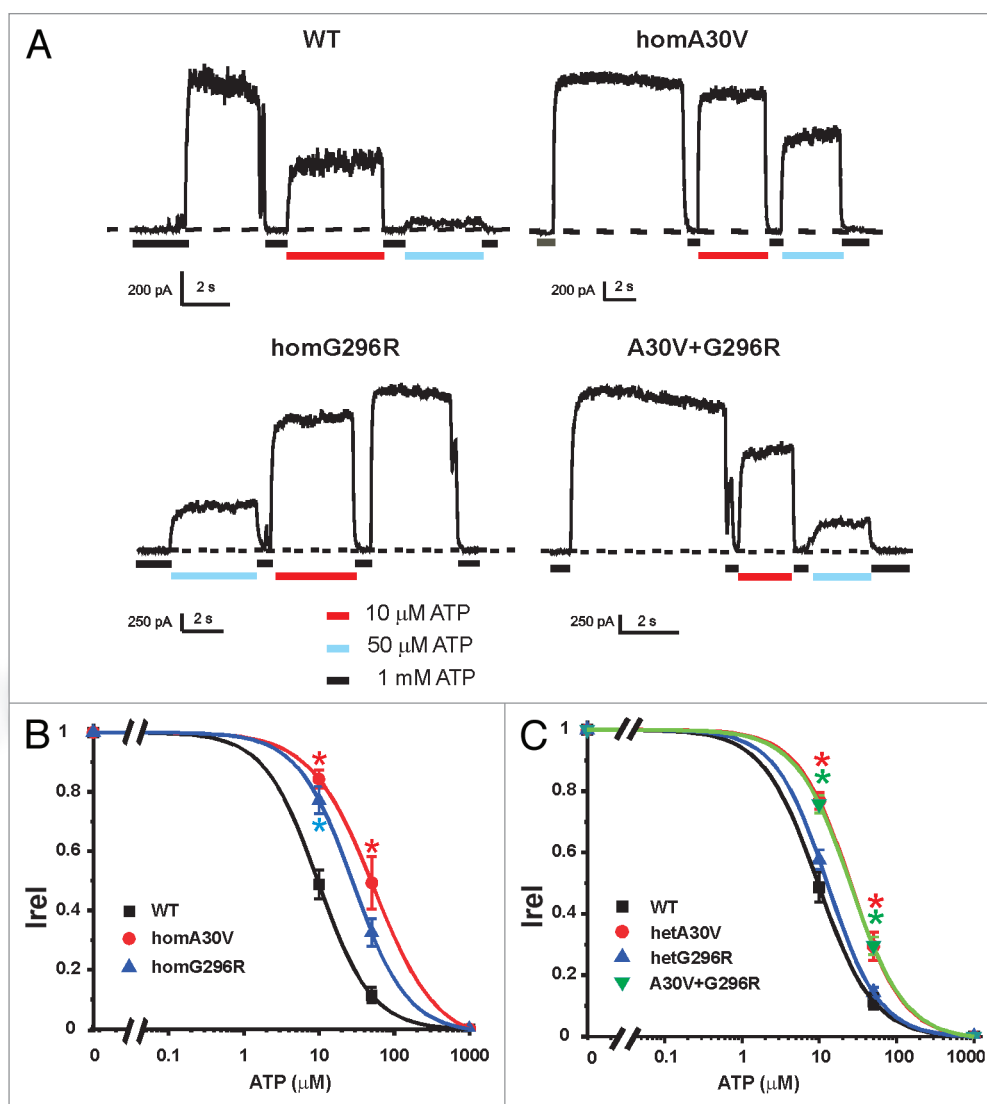
**ATP response of WT and mutant channels.** Given that homomeric A30V channels showed greatly reduced ATP sensitivity in 0.1 mM ATP (with 0.5 mM free  $Mg^{2+}$ ), we characterized the sensitivity to inhibitory ATP, without any confounding effects of Mg-nucleotides. Representative recordings of channel response to different concentrations of ATP from WT, homA30V, homG296R and compound heterozygous mutants are shown in Figure 4A. Correspondingly, homA30V channels exhibit significantly reduced ATP sensitivity compared with WT channels. A summary of the ATP dose response-curves for WT and mutant



**Figure 3.** Analysis of response to MgADP. Representative inside-out patch clamp recordings of WT and A30V + G296R in different MgATP and MgADP concentrations (A).  $Mg^{2+}$  is included in all ATP-containing solutions (free  $Mg^{2+}$  = 0.5 mM). (B) Relative channel currents in 0.1 mM MgATP and plus 0.5 mM MgADP for WT, homA30V, homG296R, hetA30V, hetG296R and compound heterozygous [A30V + G296R] channels as indicated. Each bar is the mean  $\pm$  SEM (n = 4–9). \*p < 0.05 compared with WT by One-Way ANOVA analysis.

channels is shown in Figure 4B. Homozygous G296R channels exhibit a slightly right-shifted dose-response, but homozygous A30V channels exhibit a marked and significantly right-shifted ATP sensitivity (Fig. 4B). In the heteromeric case, mimicking the disease condition, hetG296R channels are not markedly different from WT, but hetA30V and compound heterozygous A30V + G296R channels both exhibit a significant rightward shift when compared with WT channels (Fig. 4C).

**The compound heterozygous mutation does not alter sulfonylurea sensitivity of the channel.** Because some Kir6.2 mutations can significantly change sulfonylurea (SU) sensitivity,<sup>22,25</sup> we examined the SU sensitivity of these mutations. Representative recordings of channel response to different concentrations of glibenclamide are shown in Figure 5A. A summary of the relative current in different concentrations of glibenclamide are shown in Figure 5B. Glibenclamide sensitivity was not significantly altered in the compound heterozygous channel. Consistent with this, the patient responded to oral sulfonylurea glibenclamide and he was weaned off insulin, at a dose of 0.35 mg/kg/day.



**Figure 4.** ATP-response curves of WT and mutant channels. (A) Representative currents recorded by inside-out excised patch-clamp technique from COSm6 cells expressing WT, homA30V, homG296R and [A30V + G296R] at +50 mV pipette potential. Patches were exposed to different concentrations of Mg-free ATP as indicated. (B) ATP dose-response relationships, fit by Hill equation as described in methods. Data points indicate means  $\pm$  SEM of  $n = 6-9$  patches. \* $p < 0.05$  compared with WT by One-Way ANOVA analysis. Fitted  $K_{1/2}$  for WT, homA30V and homG296R channels are 9.65, 53.31 and 28.11 (in  $\mu$ M) and the Hill coefficients are 1.22, 1.04 and 1.19, respectively. (C) The fitted  $K_{1/2}$  for WT, hetA30V, hetG296R and [A30V + G296R] are 9.65, 25.75, 12.75 and 25.45 (in  $\mu$ M) and the Hill coefficients are 1.22, 1.28, 1.29 and 1.23, respectively.

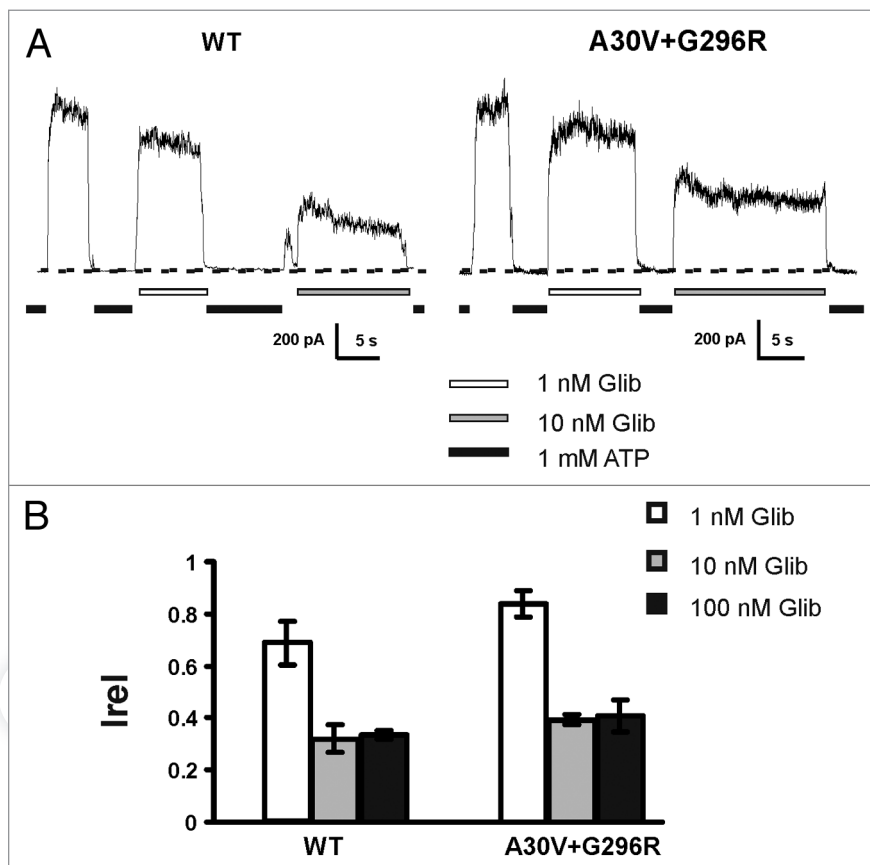
## Discussion

**Clinical case.** We have described the case of a child presenting with diabetic ketoacidosis at 7 mo of age. Although the standard definition of neonatal diabetes refers to diabetes presenting in the first 6 mo of life,<sup>20</sup> not uncommonly, like in our case, presentation is beyond this age, prompting some to propose to replace the term “neonatal diabetes mellitus” with “diabetes mellitus of infancy”.<sup>21</sup> Each *ABCC8* mutation carried by this child makes a unique contribution to enhancement of channel activity, resulting in marked stimulation of basal Rb efflux only in the compound heterozygous case (Fig. 1B and C), and explaining the presentation of the disease in the proband, as well as the responsiveness to sulfonylureas.<sup>22,23</sup> The G296R mutation is inherited from the

patient’s mother who is normal in her OGTT, but it is noticeable that the both maternal grandparents and the paternal grandfather reportedly have Type-2 Diabetes, which might come from a high risk of predisposition conferred by the heterozygous mutation in *ABCC8*, even though previous studies have indicated that carrier parents of similarly inherited compound heterozygous mutations are non-diabetic.<sup>24</sup> Thus we may speculate that similar carriers of individual variants such as this may be predisposed to diabetes. Interestingly, the G296R variant was identified as a rare variant in whole exome sequencing of over 1,000 individuals (<http://evs.gs.washington.edu/EVS/>).

**Importance of TMD0 and L0 domains of SUR1 in regulating Kir6.2 subunits.** A30 and G296 are located in the TMD0 and L0 domain, which has been proposed to confer SUR1





**Figure 5.** Glibenclamide sensitivity of WT and mutant channels. (A) Representative currents recorded by inside-out excised patch-clamp technique from COSm6 cells expressing WT and [A30V + G296R] at +50 mV pipette potential. Patches were exposed to different concentrations of glibenclamide as indicated (free Mg-nucleotide). (B) Current in different concentrations of glibenclamide relative to control solution (zero glibenclamide). Data points indicate means  $\pm$  SEM of  $n = 4-6$  patches.

ligand-independent channel gating of Kir6.2.<sup>10,14,17</sup> More specifically, SUR1 fragments lacking the TMD0 domain failed to modulate Kir6.2 activity, which suggests a potential physical interaction between TMD0 of SUR1 and the N terminus of Kir6.2. The cytosolic linker L0 domain has also been proposed to directly transduce the Mg-nucleotide stimulatory action from the SUR1 core to the Kir6.2 pore<sup>14</sup> and a previous study demonstrated that another NDM mutation (L213R) in the L0 region also shifts intrinsic ATP sensitivity.<sup>13</sup> The present study thus provides further evidence that mutations in the TMD0-L0 region alter the  $K_{ATP}$  channel activity and further supports the hypothesis that TMD0-L0 is closely associated with Kir6.2.

## Materials and Methods

**Clinical data.** Clinical information was obtained by chart review. The study was approved by The Children's Hospital of Philadelphia Institutional Review Board. Informed consent was obtained from the parents of the child.

**Genetics and molecular biology.** Genomic DNA was prepared from peripheral blood white cells or oral mucosa cells

from the child and both parents. The 39 exons of *ABCC8* were directly sequenced. Two mutations were found in *ABCC8* in the proband: c.886 G > A, predicted to cause a G296R amino acid change, was maternally transmitted, and c.89 C > T, predicted to cause A30V amino acid change, was paternally transmitted. DNA from 54 healthy control subjects was screened for these mutations and they were negative. Hamster SUR1 was cloned into the pECE vector and the parental plasmid DNA was used to generate SUR1 mutations using the QuickChange Site Directed Mutagenesis Kit (Stratagene). The mutation was confirmed by further sequencing. The mouse Kir6.2 was cloned into the pCDNA3.1 vector (Invitrogen).

**Expression of  $K_{ATP}$  channels in COSm6 cells.** COSm6 cells were cultured and transfected with cDNA using FuGENE 6 Transfection Reagent (Roche Diagnostics) as previously described in reference 10. Green fluorescent protein (as a marker for positive transfection) was mixed with FuGENE 6 and preincubated for 1 h. Cells were incubated in the presence of the transfection mixture for 18–30 h, and plated on sterile glass coverslips overnight before patch-clamp experiments.

**$^{86}\text{Rb}^+$  efflux assay.** COSm6 cells transfected with GFP, SUR1 and Kir6.2 cDNA were incubated for 5–12 h in culture medium containing  $^{86}\text{RbCl}$  (1  $\mu\text{Ci/ml}$ ) 24 h post-transfection. Before measurement of  $^{86}\text{Rb}^+$

efflux, cells were incubated for 5 min at room temperature in Krebs-Ringer solution with metabolic inhibitors (2.5  $\mu\text{g/ml}$  oligomycin and 1 mM 2-deoxy-D-glucose). At selected time points the solution was aspirated from the cells and replaced with fresh solution. At the end of a 40 min period, the cells were lysed with 2% SDS. The  $^{86}\text{Rb}^+$  in the aspirated solution and the cell lysate was counted. The percentage efflux at each time point was calculated as the cumulative counts in the aspirated solution divided by the total counts from the solutions and the cell lysates.  $^{86}\text{Rb}^+$  efflux curves were fitted by assuming that a single non- $K_{ATP}$  conductance was active in the untransfected cells, using the equation,  $\text{Eq.1 Rb efflux} = 1 - \text{Exp}(-k_1 \cdot t)$  where  $\text{exp}$  is an exponential function,  $t$  is time, and  $k_1$  is the rate constant for efflux through the endogenous, non- $K_{ATP}$  pathway. In transfected cells, efflux curves were fitted by assuming that both a  $K_{ATP}$  and a non- $K_{ATP}$  conductance pathway were active in parallel and were determined as  $\text{Eq.2 Rb efflux} = 1 - \text{Exp}[-(k_1 + k_2) \cdot t]$  where  $k_1$  is the rate constant for efflux through the endogenous, non- $K_{ATP}$  pathway (obtained from untransfected cells by Eq. 1) and  $k_2$  is the rate constant for efflux through the  $K_{ATP}$  pathway.  $k_2$  is then directly proportional to the  $K_{ATP}$  conductance.

**Electrophysiological methods.** Membrane patches were voltage-clamped and currents were measured at a membrane potential of -50 mV (pipette voltage, +50 mV), with inward currents shown as upward deflections. Data were collected using the pClamp 8.2 software suite (Axon Instruments) and Microsoft Excel (Microsoft). The bath (intracellular) and pipette (extracellular) solution (K-INT) had the following composition: 140 mM KCl, 10 mM Hepes, 1 mM EGTA, pH 7.4. ATP was added as the dipotassium salt.

**Quantitative analysis of ATP inhibition.** Channels in inside-out patches were exposed to varying concentrations of ATP in K-INT solution. Channel activity was normalized to that seen in the absence of ATP. The curves were fit by the Hill equation

( $I_{rel} = 1/(1 + \{[ATP]/K_{1/2,ATP}\}^H)$ );  $I_{rel}$  = current in [ATP]/current in zero ATP; H = Hill coefficient;  $K_{1/2,ATP}$  = [ATP] causing half-maximal inhibition to averaged data.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

#### Acknowledgements

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