Genome-edited zebrafish model of ABCC8 loss-of-function disease

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ABSTRACT
ATP-sensitive potassium channel (K\textsubscript{ATP}) gain-of-function (GOF) and loss-of-function (LOF) mutations underlie human neonatal diabetes mellitus (NDM) and hyperinsulinism (HI), respectively. While transgenic mice expressing incomplete K\textsubscript{ATP} LOF do reiterate mild hyperinsulinism, K\textsubscript{ATP} knockout animals do not exhibit persistent hyperinsulinism. We have shown that islet excitability and glucose homeostasis are regulated by identical K\textsubscript{ATP} channels in zebrafish. SUR1 truncation mutation (K499X) was introduced into the abcc8 gene to explore the possibility of using zebrafish for modeling human HI. Patch-clamp analysis confirmed the complete absence of channel activity in β-cells from K499X (SUR1\textsuperscript{−/−}) fish. No difference in random blood glucose was detected in heterozygous SUR1\textsuperscript{+/−} fish nor in homozygous SUR1\textsuperscript{−/−} fish, mimicking findings in SUR1 knockout mice. Mutant fish did, however, demonstrate impaired glucose tolerance, similar to partial LOF mouse models. In parallel, glucokinase and hyperinsulinism resulting from equivalent LOF mutations, these gene-edited animals provide valid zebrafish models of K\textsubscript{ATP}-dependent pancreatic diseases.

Introduction
Electrical activity is a key regulator of insulin secretion from β-cells and is critically regulated by ATP-sensitive potassium (K\textsubscript{ATP}) channels. In mammals, pancreatic K\textsubscript{ATP} channels are composed of four SUR1 subunits (encoded by ABCC8) and four Kir6.2 subunits (encoded by KCNJ11). At low plasma [glucose], K\textsubscript{ATP} channels are normally open, the cell membrane is hyperpolarized, and voltage-dependent calcium channels (VDCCs) are...
closed, thus inhibiting insulin secretion.\textsuperscript{4} Glucose metabolism increases the intracellular [ATP]/[ADP] ratio via enhanced β-cell glycolysis and oxidative phosphorylation. This causes closure of the K\textsubscript{ATP} channels, leading to membrane depolarization, calcium influx through VDCCs, and triggering of insulin release.\textsuperscript{5,6} Congenital hyperinsulinism (CHI) is the most common cause of hypoglycemia in neonates and infants\textsuperscript{7} and is often linked to loss-of-function (LOF) mutations in K\textsubscript{ATP}. LOF CHI mutations in either KCNJ11 or ABCC8\textsuperscript{8} result in reduced K\textsubscript{ATP} channel activity, β-cell hyperexcitability, and excessive insulin secretion.\textsuperscript{9} In direct contrast, gain-of-function (GOF) mutations in these same genes cause decreased membrane excitability and impaired insulin secretion, leading to neonatal diabetes mellitus (NDM).\textsuperscript{6,10,11} Mice with transgenic overexpression of GOF mutations first predicted a mechanism for human NDM and have provided valuable models for understanding disease progression.\textsuperscript{12–14} Similarly, mice with transgenic expression of K\textsubscript{ATP} LOF mutations, as well as mice with heterozygous KCNJ11 or ABCC8 gene knockout, reiterate persistent hyperinsulinism.\textsuperscript{15,16} However, homozygous K\textsubscript{ATP} knockout mice do not exhibit persistent hyperinsulinism; instead, they exhibit an unexplained loss of insulin secretion and glucose intolerance.\textsuperscript{17–20}

Current therapeutic approaches to both NDM and CHI will benefit from novel animal models and new insights into disease processes, leading the way to new opportunities for treatment. We have shown that K\textsubscript{ATP} channels are expressed in β-cells within the zebrafish (Danio rerio) islet, that they are functionally similar to their mammalian orthologues,\textsuperscript{21} and that they exert similar glucose-dependent control of intracellular [Ca\textsubscript{2+}] ([Ca\textsubscript{2+}]),\textsuperscript{22,23} Activation of these channels by the drug diazoxide\textsuperscript{21} or by overexpression of ATP-insensitive transgenes in β-cells\textsuperscript{23} can similarly alter the metabolic response to glucose. To provide a model of K\textsubscript{ATP} LOF, we have investigated a zebrafish model of the loss-of-function of K\textsubscript{ATP} in which an early nonsense mutation, predicted to lead to premature truncation of SUR1, was introduced into abcc8. This zebrafish mutant recapitulates key features of human K\textsubscript{ATP} LOF and provides a model for further analysis and testing of potential therapeutics, which may facilitate advances in clinical management and help identify new therapies by providing a high throughput platform for understanding mechanisms and testing potential therapeutic approaches.

**Materials and methods**

**Ethical approval**

All animal procedures were approved by the Washington University in St. Louis Institutional Animal Care and Use Committee.

**SUR1 ENU generated nonsense mutation**

ENU-mutagenesis was performed at the Sanger Institute, as part of the Zebrafish Mutation Project, using N-ethyl-N-nitrosourea (ENU) mutagenesis to attempt to identify knockout alleles for all protein-coding regions in the zebrafish genome (https://www.sanger.ac.uk/resources/zebrafish/zmp/). This project outcrosses ENU-mutagenized F\textsubscript{0} males to create a population of F\textsubscript{1} fish heterozygous for ENU-induced mutations, which were then obtained through the Zebrafish International Research Consortium (ZIRC). The abcc8(sa15863) nonsense mutant allele (K499-STOP, TTCGCGCTC C GATGCACAG[A > T]AAAGCACATTG GTG AGCTATCTTATTTGGTAAATGTCCCTAATGAGGCCA) was obtained from the Zebrafish Mutation Project,\textsuperscript{24} through ZIRC. Homozygous K499-STOP mutants were generated by in-crossing heterozygous carriers, and the progeny was genotyped by Transnetyx using restriction digest with the inserted digestion site for HpyCHRIII, which is inserted into the mutant allele (Forward primer: TTGTTGTTGCTGCTT TGGG; Reverse primer: TTTACAAGCACAGCGCTCAC) to identify homozygotes.

**Animal lines and maintenance**

In addition to the mutant lines above, we used AB wild-type fish as well as the previously described β-cell-specific GCaMP6s-expressing transgenic fish Tg(−1.0ins:gCaMP6s)\textsuperscript{25} and insulin reporter line Tg(−1.0ins:eGFP)\textsuperscript{25}c1. Wild-type controls
were on the AB background. All fish lines were housed in the Washington University Zebrafish Facility under standard conditions, the details of which can be found at: http://zebrafishfacility.wustl.edu/documents.html. Briefly, tanks and feeding are managed on the Tritone robotic system. Beginning day 4–6 post fertilization, larvae are housed at a density of 8–10/L and fed a combination of microalgae and/or rotifers. As the fish progress in their growth and development, larger food items will be provided, until they are moved to the adult fish-holding areas. If live foods are not available for first-feeding larvae, dry diets may be used. Adult fish are moved from the Nursery to the Adult Fish Holding rooms at ~42 days, or once 50% of the tank has reached sexual maturity, and are housed at a density of <1–12 fish/L. Adult fish held on the recirculating system are fed a minimum of once per day and may be fed up to five times per day, using prepared dry food and/or rotifers. Unless otherwise stated, all experiments were performed on adult zebrafish of reproductive age (10 weeks to 9 months of age) and on roughly equal proportions of males and females.

**Electrophysiological analyses**

Islets were isolated from zebrafish, and single β-cells were dissociated, as previously described, and recordings were performed on GFP-positive cells.21 Excised patch recordings were performed using pipettes with a resistance of 1–2 MΩ when filled with pipette solution. Bath and pipette solution (K-INT) contained (in mM): 140 KCl, 10 HEPES, 1 EGTA (pH 7.4 with KOH). All recordings were performed at ~50 mV holding potential, and the absence or presence of nucleotides was adjusted in bath solution as indicated. $K_{ATP}$ currents were normalized to the basal current in the absence of nucleotides. The data were tested for statistical significance using Welch’s $t$-test. A $p$ value of <0.05 was considered significant.

**Isolation of islets and β-cells**

Islets and β-cells were obtained as described.21 Briefly, fish were euthanized using cold-shock (2–4°C water immersion) followed by decapitation. Under a fluorescent dissecting microscope, fish were placed onto their right sides, and the exterior skin and scales were removed using surgical forceps to expose the abdomen. Visceral organs were gently dissected away with forceps. The primary islets were identified at the intersection of hepatic and bile ducts with the intestine and confirmed by eGFP fluorescence. Islets were removed by gently pinching the ducts with forceps and separating the islets from the surrounding tissues and stored in islet media (see below) until all dissections were complete.

Islets were digested with collagenase (Sigma C9263, 0.4 mg ml$^{-1}$ in Hank’s buffered salt solution, 0.5 ml/5–10 islets) to remove surrounding exocrine and connective tissues by incubation at 29°C for 20 min, shaking gently every 5 min. Islets were then placed in Islet Media made up of RPMI (ThermoFisher 11875–093) supplemented with 1 mM HEPES, antibiotic solution (Sigma A5955, 10 ml l$^{-1}$ solution), 10% fetal bovine serum and diluted with glucose-free RPMI to a final glucose concentration of 6.67 mM.

For experiments involving individual β-cells, islets were dispersed with StemPro Accutase (ThermoFisher A11105) for 10 min at 37°C. Any remaining clumps of cells were incubated a second time in the same conditions for 2 min. Dispersed cells were washed with islet media and re-suspended in less than or equal to 100 μl of media, then transferred to glass shards cut from coverslips. Cells were allowed to adhere for 30 min in incubator (28°C, 0% CO$_2$) on shards before being completely covered with media and incubated overnight in the same conditions.

**Ex-vivo microscopy of adult zebrafish islet calcium**

Islets were isolated and perfusion imaging experiments performed as described.21 Briefly, glass-bottomed 35 mm dishes (MatTeK) were coated with 1% agarose. A well was created in the center of the plate using a plastic pipette tip to remove a section of agarose. Islets were individually transferred to wells and immersed in pH 7.4 Krebs Ringer’s solution buffered with HEPES (KRKH) containing 2 mmol/L glucose. The KRKH base solution consisted of (in mmol/L): NaCl 114, KCl 4.7, MgSO$_4$ 1.16, KH$_2$PO$_4$ 1.2, CaCl$_2$ 2.5, NaHCO$_3$ 5, and HEPES 20, with 0.1% BSA. KRKH solutions
of indicated glucose concentrations were flowed into the plate chamber through lines running into and out of the chamber.

High-resolution images were captured using a Nikon Spinning Disk confocal microscope (a motorized Nikon Ti-E scope equipped with PerfectFocus, a Yokogawa CSU-X1 variable speed Nipkow spinning disk scan head, and Andor Zyla 4.2 Megapixel sCMOS camera) at the Washington University Center for Cellular Imaging (http://wucci.wustl.edu/). Time-lapse images used 100 msec exposure at 1 sec intervals. All images were analyzed in FIJI. To correct for movement in x- and y-planes, images were stack registered (using StackReg, rigid body) in Fiji before analysis. A single z-stack for each time-lapse was analyzed, with a region of interest (ROI) drawn to surround the border of the islet. Because the baseline electrical activity of an islet, and thus the intensity of islet fluorescence, can vary in our abcc8 mutants, fluorescent response to glucose is shown normalized using min-max normalization.

Quantification of islet β-cell density

To calculate β-cell density, whole islets were isolated as above and imaged in low glucose conditions at a single time point. A single representative z-stack was analyzed for each islet using FIJI software to calculate the islet area. Cells were counted using the FIJI plug-in StarDist.

Blood glucose measurements and glucose tolerance test

Blood glucose was measured as described in random (fed) adult zebrafish. Zebrafish were fasted for 18–20 hours prior to glucose tolerance tests. Intraperitoneal glucose tolerance test was performed as described on similarly fasted zebrafish.

Growth measurements

To obtain growth data, fish were briefly anesthetized in tricaine, and excess water removed with Kimwipe and then weighed on a digital scale with a precision of 1 µg, before returning the fish to reverse osmosis water to recover from anesthesia.

Chemicals

All salts, amino acids, and other compounds were purchased from Sigma, except where indicated above.

Statistics

Statistical analyses were performed in GraphPad Prism. Data on blood glucose over time and glucose tolerance test were tested for statistical significance using a one- or two-way ANOVA with Tukey test. Data on animal weight and β-cell density used Welch’s t-test and measurements of relative fluorescence used multiple t-tests. A p value of <0.05 was considered significant.

Results

Genome-modified zebrafish model of SUR1 LOF

A number of K<sub>ATP</sub> mutations have been described to cause CHI, with the causal mutation being in the SUR1 subunit more often than in Kir6.2. Although gating mutations are a common underlying cause, mutations that result in loss of functional protein or failure to traffic to the cell membrane are also prominent. To model loss of functional protein, we obtained an abcc8 mutant line that was originally generated by ENU mutagenesis, from the Zebrafish Mutation Project. These fish contain an early stop codon (X) mutation in exon 10 of SUR1 (K499X), which results in disruption of transmembrane domain 1 (Figure 1a), and is expected to result in complete loss of functional SUR1 protein. Complete knockout of channel activity was confirmed in isolated membrane patches of β-cells from homozygous mutant fish (see below), which we thus term SUR1<sup>−/−</sup>. Infants with CHI often have macrosomia, but, as with mouse SUR1 knockouts, there was no significant difference in growth between SUR1<sup>−/−</sup> mutants and wild type (Figure 1b). Islet development, as indicated by β-cell density, also showed no difference in the mutants (Figure 1b). In mice with such a marked loss of K<sub>ATP</sub> channels, a common theme of early transient hyperinsulinemic hypoglycemia followed by normoglycemia or impaired glucose tolerance as adults has been described.
A similar progression has also been seen in some humans with CHI due to K<sub>ATP</sub> LOF. Preliminary measurements suggest that SUR1<sup>−/−</sup> larvae also have lower whole-body glucose compared to wildtype (not shown), but random fed blood glucose in both homo- and heterozygous fish was not different from WT (Figure 1c).

Intraperitoneal glucose tolerance test (IPGTT) was performed on adult zebrafish. We have previously shown that IPGTT in wild-type zebrafish has a peak in blood glucose around 30 minutes with a slow return to normal over four to six hours. Here, we found higher baseline fasting and higher peak glucose in SUR1<sup>−/−</sup> compared to wild type, but similar glucose values by 4 hours post injection (Figure 1d). This parallels the findings in mouse SUR1 and Kir6.2 knockout models of complete loss of K<sub>ATP</sub>, which lack persistent hypoglycemia and instead exhibit glucose intolerance and loss of insulin secretion as adults.

**Molecular consequences of introduced gene modifications**

We crossed SUR1 mutant fish to ins:GFP expressing fish to assess morphology and insulin gene promoter activity. GFP fluorescence was used to identify β-cells in isolated islets via confocal microscopy. There was no obvious deficit of β-cell density or GFP density in SUR1 mutants compared to WT (not shown). Inside-out patch clamp recordings from β-cells isolated from primary islets of SUR1 homozygous mutant and wild-type fish, both containing ins:GFP transgene, were also identified by the presence of green fluorescence. Excised inside-out patch-clamp experiments (Figure 2a,b) confirmed the effective knockout, with complete absence of K<sub>ATP</sub> channels in SUR1 homozygous mutant β-cells. These recordings additionally confirmed no responsivity to the channel opener diazoxide, consistent with a severe loss of function.

**Excitability consequences of introduced gene modifications**

K<sub>ATP</sub> channel LOF is predicted to increase islet excitability and increase Ca<sup>2+</sup> entry into β-cells. Mutant fish were crossed with transgenic GCaMP6s fish carrying Tg(ins:GCaMP6s), and time-lapse confocal fluorescent microscopy was carried out on ex vivo perifused whole adult islets. Consistent with prior findings, controls showed...
a significant increase in relative fluorescence when transitioned from low (2 mM) to high (20 mM) glucose (Figure 3a, b). In SUR1 mutant zebrafish, carrying the same Tg(ins:GCaMP6s), [Ca2+] imaging revealed elevated basal fluorescence at low (2 mM) glucose concentration, reflective of basal depolarization in mutant β-cells, but a smaller increase from baseline in high (20 mM) glucose, when compared to the increase seen in controls (Figure 3a, b). These results are very similar to previous findings in SUR1 knockout mice.34

**Discussion**

In the current study, we have validated the first K_{ATP}-knockout zebrafish and demonstrate a recapitulation of the essential consequences seen in mammalian K_{ATP} knockout animals. The premature stop mutation at position 499 is expected to result in a severely truncated protein, containing only the TMD0 region and half of the second TMD1 region and lacking both nucleotide binding folds (NBFs). Previous studies have shown that essentially no functional channels are formed when NBF1 is absent and, accordingly, we first show that homozygous ENU-generated SUR1 truncation mutant fish generate a functional K_{ATP} channel knockout, with no measurable K_{ATP} channels in isolated pancreatic β-cells. Secondly, this knockout results in elevated intracellular [Ca2+] at physiologically basal glucose levels (2 mM), even though [Ca2+]i is elevated at higher (20 mM) glucose, as is also seen in rodent SUR1 knockout islets.34

Thirdly, these fish reiterate the counterintuitive elevation of basal [glucose] and relative glucose intolerance that is seen in mammalian K_{ATP} knockout animals.36 It is likely that many human HI mutations will cause only incomplete loss of K_{ATP} channel activity,37,38 and both active K_{ATP} channels and sensitivity to the K_{ATP} channel opener diazoxide have been detected in some HI patients with K_{ATP}channel mutations.28,39 Mice with partial loss of K_{ATP} activity mimic this hyperinsulinemic phenotype and secrete insulin at lower [glucose] than controls.16 However, while mice with complete loss of K_{ATP} exhibit elevated serum insulin and hypoglycemia in the neonatal period,16,18,19,31 they then rapidly develop hyperglycemia with reduced insulin secretion, a phenomenon that persists through adulthood. This cross-over to loss of secretion, in the face of continual excitation, reflects a marked downregulation of the secretory process itself. The underlying cause remains elusive, and it is unknown whether this is a mouse-specific progression or reflects processes that may also be involved in human HI, although the correlative data is substantial.36 In demonstrating a similar glucose-intolerant and non-hypoglycemic

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**Figure 2.** SUR1 LOF (499X) abolishes K_{ATP} channel activity (a) Representative K_{ATP} channel activity in inside-out patch clamp recordings from inside-out patch clamp recordings of WT (above) and homozygous SUR1(499X) (SUR1−/−) mutant β-cells (below) in the presence of ATP (μM), or addition of diazoxide (mM), as indicated. Voltage was clamped at −50 mV. (b) K_{ATP} channel density in WT and SUR1−/− patches (n = 17,10).
phenotype in zebrafish that completely lacks $K_{ATP}$ channels, these SUR1$^{-/-}$ fish thus confirm a common finding from fish to mouse and may provide a useful model for further exploring the unexplained phenomenon of glucose intolerance and even diabetes in $K_{ATP}$-dependent HI patients.

Conclusions
In paralleling features of mammalian hyperinsulinism resulting from equivalent loss-of-function mutation, these gene-edited animals provide a valid zebrafish model of $K_{ATP}$ LOF dependent pancreatic diseases.

Abbreviations

KCNJ11 potassium voltage-gated channel subfamily J member 11

ABCC8 ATP Binding Cassette Subfamily C Member 8

Kir6.2 ATP-sensitive potassium channel pore subunit, inward-rectifying

SUR1 Sulfonylurea receptor 1

Figure 3. SUR1$^{-/-}$ islets exhibit elevated basal $[Ca^{2+}]$ and reduced responsivity to glucose (a) Representative recordings of intracellular calcium in the presence of 2 mM glucose (2 G) and following switch to 20 mM glucose (20 G) and then 30 mM KCl (30 K), for WT (above, n = 6) and SUR1$^{-/-}$ (below, n = 12). Fluorescence is normalized to maximum fluorescence in 30 K ($f = 1$), and minimum fluorescence ($f = 0$) anywhere within the record. Values for each islet are also shown. (b) Average calcium in each condition for WT (N = 8) and SUR1$^{-/-}$ (n = 14). Data in B are analyzed by 1-way ANOVA followed by multiple unpaired t-tests. (*) $p < .05$, (**) $p < .01$. 
Key points

- Gain and loss-of-function in the Kir6.2 (KCNN11) and SUR1 (ABCC8)-encoded pancreatic islet β-cell K<sub>ATP</sub> channel underlies neonatal diabetes mellitus (NDM) and congenital hyperinsulinism, respectively. Mouse models reiterate key features, but zebrafish models could provide a powerful model for further analysis and therapy testing.
- An early nonsense mutation in exon 10 of SUR1 was generated by ENU mutagenesis.
- Homozygous SUR1 truncation mutants had normal random glucose but impaired fasting glucose and glucose tolerance as adults, mimicking findings in mouse SUR1 knockouts.
- Patch-clamp analysis revealed an absence of K<sub>ATP</sub> channels in the SUR1 truncation mutants.
- Ca<sup>2+</sup> imaging demonstrated elevated basal [Ca<sup>2+</sup>]<sub>i</sub> in β-cells with SUR1 truncation.
- In paral-lelling features of mammalian diabetes and hyperinsulinism resulting from equivalent loss-of-function mutations, this gene-edited animal provides a valid zebrafish model of K<sub>ATP</sub>-LOF dependent pancreatic diseases.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Ethics statement

All procedures were approved by the Washington University Institutional Animal Care and Use Committee.

Author contributions

CGN and JMI conceived the study; JMI, RCT, SSS, NWY, LY carried out the experiments; JMI, RCT, SSS, NWY analyzed the data; CGN, MSR, JMI, RCT, SSS, NWY interpreted the results; MSR and JMI participated in the design of experiments. JMI and CGN wrote the paper, which was edited by other authors. All authors gave final approval for the manuscript.

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