Decreasing Cx36 gap junction coupling compensates for overactive K\_ATP channels to restore insulin secretion and prevent hyperglycemia in a mouse model of neonatal diabetes.

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**Running title:** Modulating gap junctions to prevent diabetes.

Manuscript text word count: 4496.

Abstract word count: 199.

Number of figures: 7.
ABSTRACT

Mutations to the K<sub>ATP</sub> channel which reduce the sensitivity of ATP-inhibition cause neonatal diabetes mellitus, via suppression of β-cell glucose-stimulated free-calcium activity ([Ca<sup>2+</sup>]<sub>i</sub>) and insulin secretion. Connexin-36 (Cx36) gap junctions also regulates islet electrical activity: upon a knockout of Cx36 β-cells show [Ca<sup>2+</sup>]<sub>i</sub> elevations at basal glucose. We hypothesized that in the presence of overactive ATP-insensitive K<sub>ATP</sub> channels, a reduction in Cx36 would allow elevations in glucose-stimulated [Ca<sup>2+</sup>]<sub>i</sub> and insulin secretion to improve glucose homeostasis. To test this, we introduced a genetic knockout of Cx36 into mice that express ATP-insensitive K<sub>ATP</sub> channels and measured glucose homeostasis and islet metabolic, electrical and insulin secretion responses. In the normal presence of Cx36, following expression of ATP-insensitive K<sub>ATP</sub> channels, blood glucose levels rapidly rose to >500mg/dl. Islets from these mice showed reduced glucose-stimulated [Ca<sup>2+</sup>]<sub>i</sub> and no insulin secretion. In mice lacking Cx36 following expression of ATP-insensitive K<sub>ATP</sub> channels, normal glucose levels were maintained. Islets from these mice had near-normal glucose-stimulated [Ca<sup>2+</sup>]<sub>i</sub> and insulin secretion. We therefore demonstrate a novel mechanism by which islet function can be recovered in a monogenic model of diabetes. A reduction of gap junction coupling allows sufficient glucose-stimulated [Ca<sup>2+</sup>]<sub>i</sub> and insulin secretion to prevent the emergence of diabetes.
Glucose-stimulated insulin secretion from β-cells in the islet is regulated via a series of metabolic and electrical events. The ATP-sensitive K\(^+\) (K\(_{ATP}\)) channel provides a central role in coupling increases in ATP/ADP ratio following the metabolism of glucose, to membrane depolarization, elevated intracellular free-calcium activity ([Ca\(^{2+}\)]\(_i\)) and insulin granule exocytosis (1). The K\(_{ATP}\) channel is made up of inward-rectifying K\(^+\) channel Kir6.2 and sulfonylurea receptor 1 (Sur1). In humans, mutations in the genes encoding Kir6.2 (KCNJ11) and Sur1 (ABCC8) which reduce the sensitivity of ATP-inhibition and lead to overactive K\(_{ATP}\) channels (‘gain-of-function’ mutations) are the most common cause of neonatal diabetes mellitus (2). Transgenic mice which express ATP-insensitive K\(_{ATP}\) channels in β-cells can recapitulate the human disease (3-5). These mice exhibit marked hyperglycemia and reduced plasma insulin, and islets from these mice show a suppression of glucose-stimulated [Ca\(^{2+}\)]\(_i\) and insulin release (4,6). This demonstrates that a reduction of glucose-dependent excitability underlies the development of diabetes caused by K\(_{ATP}\) channel mutations. Inhibition of K\(_{ATP}\) channels with sulfonyleurases can recover elevations in [Ca\(^{2+}\)]\(_i\) and insulin secretion in islets of these mouse models (3,4,6). As such, sulfonyleurases can be applied to treat patients with NDM associated with K\(_{ATP}\) channel mutations (7,8). Nevertheless, some mutations render the K\(_{ATP}\) channel insensitive to sulfonyleurases (7,9,10).

Cellular interactions within the islet have long been known to be important for enhancing the regulation of insulin release (11), and are also important for regulating electrical activity (12). Connexin36 (Cx36) gap junction channels regulate islet electrical activity by coupling K\(_{ATP}\)-regulated membrane depolarization between β-cells of the islet. This synchronizes oscillations in membrane depolarization and [Ca\(^{2+}\)]\(_i\) at elevated glucose (13,14), leading to a coordination of first-phase insulin release and pulsatile second-phase insulin release (15). Under normal conditions Cx36 gap junction channels also enhance a suppression of spontaneous elevations in [Ca\(^{2+}\)]\(_i\) at basal
glucose (16,17): upon a knockout of Cx36, elevated \([Ca^{2+}]_i\) is observed at lower glucose levels (14,18). This suppression likely occurs as a result of the long-established presence of \(\beta\)-cell heterogeneity (19); where inexcitable cells in the islet suppress membrane depolarization and [Ca\(^{2+}\)]\_i\ in more excitable cells via gap junction coupling (16,18).

Given that Cx36 gap junction channels coordinate \(K_{ATP}\)-regulated membrane potential, we hypothesized that their action in suppressing [Ca\(^{2+}\)]\_i\ and insulin occurs more generally under conditions of \(K_{ATP}\) channel opening. Therefore, similar to their normal action at lower glucose levels, we hypothesized that Cx36 gap junctions will inappropriately enhance the suppression of [Ca\(^{2+}\)]\_i\ and insulin secretion at elevated glucose in the presence of overactive ATP-insensitive \(K_{ATP}\) channels in NDM. Therefore, we hypothesized that an absence of Cx36 gap junction coupling upon expression of ATP-insensitive \(K_{ATP}\) channels would reduce this suppression and lead to spontaneous elevations in [Ca\(^{2+}\)]\_i\ at elevated glucose. We anticipated that this [Ca\(^{2+}\)]\_i\ elevation would stimulate sufficient insulin release to prevent the severe hyperglycemia which emerges in these animals. However we anticipated that suppression of [Ca\(^{2+}\)]\_i\ and insulin release would be maintained at low glucose levels: in essence a decrease in Cx36 will left-shift the dose-response to compensate for a right-shift due to overactive \(K_{ATP}\) channels. Here, we tested this by introducing a knockout of Cx36 gap junction channels into a mouse model of NDM which expresses ATP-insensitive \(K_{ATP}\) channels in the \(\beta\)-cell under control of an inducible Cre\(^{ER}\)-recombinase (3). We tested whether mice with reduced or absent Cx36 gap junction coupling showed an improvement in glucose homeostasis, and whether islets from these mice showed a recovery of glucose-stimulated [Ca\(^{2+}\)]\_i\ and insulin secretion.
EXPERIMENTAL METHODS

Animal care – Experiments were performed in compliance with the relevant laws and institutional guidelines, and were approved by the University of Colorado Institutional Animal Care and Use Committee. Mice expressing Rosa26-Kir6.2^{∆N30,K185Q} (Kir6.2^{∆N30,K185Q} ‘gain-of-function’ mutant), Pdx-Cre^{ER} (pancreas-specific inducible Cre), and Cx36^{−/−} (global Cx36 knockout) were generated as described previously (3,20,21) and generously supplied by collaborating laboratories. β-cell conditional expression of Kir6.2^{K185Q,∆N30} is achieved through crossing Rosa26-Kir6.2^{AN30,K185Q} and Pdx-Cre^{ER} mice, to excise a loxP-flanked stop codon to drive Kir6.2^{K185Q,∆N30} expression. GFP is co-expressed via an IRES site. Rosa26-Kir6.2^{AN30,K185Q} and Pdx-Cre^{ER} mice had either normal gap junction coupling (to generate Cx36^{+/+};Kir6.2^{AN30,K185Q} mice); or were first separately crossed with Cx36^{+/−} mice to achieve a homozygous deletion and then bred together (to generate Cx36^{−/−};Kir6.2^{AN30,K185Q} mice). Cx36^{+/+} or Cx36^{−/−} littermate mice lacking Rosa26-Kir6.2^{AN30,K185Q} and/or Pdx-Cre^{ER} were used as controls. Mice were studied at generation F3-F5. To prevent genetic drift, new breeders were generated by crossing Cx36^{+/−} and Cx36^{−/−}-expressing breeders every 2-3 generations.

In-vivo measurements – Each experimental group (age-matched Cx36^{+/+};Kir6.2^{AN30,K185Q} and Cx36^{+/−};Kir6.2^{AN30,K185Q} with respective littermate Cx36^{+/+} and Cx36^{−/−} controls) received five daily doses of tamoxifen (50mg/g body-weight) at experimental days 1-5. Blood glucose was measured daily as previously performed (3) with a glucometer (Ascensia Contour, Bayer). Plasma insulin was measured at day 29 from blood samples centrifuged at 14krev/min for 10 minutes and assayed using mouse ultrasensitive insulin ELISA (Alpco). Mice were sacrificed at day 30-32 for islet isolation. Glucose tolerance tests (GTT) were performed at day 30-32. Littermate or age-matched mice were
fasted overnight for 16h, received intra-peritoneal (IP) injection of 2g/kg body-weight of glucose, and blood glucose was measured on tail vein blood samples pre-injection (0 minutes) and 15,30,60,90,120 minutes post-glucose delivery. Insulin tolerance tests (ITT) were performed at day 30-32. Littermate mice were fasted for 6 hours, received IP injection of 0.75U/kg body-weight of human recombinant insulin (Novolin, Novo-Nordisk), and blood glucose was measured on tail vein blood samples pre-injection (0 minutes) and 15,30,45,60,90 minutes post-injection.

*Islet isolation and insulin secretion* – At day 30-36 islets were isolated from pancreata of each experimental mouse and maintained in islet medium (RPMI medium, 10% fetal bovine serum, 11mM glucose, 100U/ml penicillin, 100µg/ml streptomycin) at 37°C under humidified 5% CO₂ for 24 hours. For static insulin secretion measurements, islets (5/column, in duplicate) were pre-incubated for 60 minutes at 37°C in Krebs-Ringer buffer (128.8mM NaCl, 5mM NaHCO₃, 5.8mM KCl, 1.2mM KH₂PO₄, 2.5CaCl₂, 1.2mM MgSO₄, 10mM HEPES, 0.1% BSA, pH=7.4) plus 2mM glucose; then incubated for 60 min at 37°C in Krebs-Ringer buffer plus different glucose concentrations and/or reagents as indicated. After the incubation period, the medium was removed and insulin concentration assayed using mouse ultrasensitive insulin ELISA. To estimate insulin content, islets were lysed in 1% TritonX-100 and frozen at -20°C overnight.

*Microscopy* – All isolated islets were imaged using established methods (13), in polydimethylsiloxane microfluidic devices (16), maintained at 37°C, with imaging medium (125mM NaCl, 5.7mM KCl, 2.5mM CaCl₂, 1.2mM MgCl₂, 10mM HEPES, 2mM glucose, 0.1% BSA, pH=7.4).
To measure $[\text{Ca}^{2+}]_i$ response and dynamics, islets were loaded with 4µM FuraRed-AM (Invitrogen) for 2 hours at room-temperature, and imaged on a Marianas spinning-disk microscope (31) with a 40x 1.3NA Plan-NEOFluar oil-immersion objective (Zeiss). Images were acquired 10 minutes after glucose stimulation. FuraRed was imaged using a 488nm diode laser for excitation and a 580-655nm band-pass emission filter (Semrock). GFP was imaged using a 488nm diode laser for excitation and a 500-550nm band-pass emission filter. There was no detectable cross-talk between FuraRed and GFP.

To measure $[\text{Ca}^{2+}]_i$ concentration, islets were loaded with 2µM Fura2-AM for 30 minutes at room-temperature, and imaged on an Eclipse-Ti widefield microscope (Nikon) with a 20x 0.75NA PlanApo objective. Images were acquired 10 minutes after glucose stimulation or immediately after KCl stimulation. Fura2 was imaged sequentially, using 340nm and 380nm (±5nm) Arc-lamp excitation, and a 470-550nm band-pass emission filter (Chroma).

To measure NAD(P)H, islets were imaged on a LSM510 microscope (Zeiss), with a 40x 1.2NA C-Apochromatic water-immersion objective. NAD(P)H autofluorescence was imaged with 2-photon excitation using a 710nm mode-locked Ti:sapphire laser (Coherent), and a 400-500nm band-pass emission filter (Chroma) and non-descanned detector. GFP fluorescence was imaged using a 488nm Ar$^+$ laser line for excitation and a 500-550nm band-pass emission filter. Z-stacks of 6 images were acquired at 2µm spacing. No GFP fluorescence was detected in the NAD(P)H channel.

To measure mitochondrial membrane potential changes, islets were loaded with 50nM Rhodamine123 for 20 minutes at 37°C, and imaged on a LSM510 microscope with a 40x 1.2NA C-Apochromatic water-immersion objective. Images were acquired 10 minutes after glucose stimulation. Rhodamine123 was imaged using a 488nm Ar$^+$ laser line for excitation and a 500-550nm band-pass emission filter. Z-stacks of 6 images were acquired at 2µm spacing.
Image Analysis – Images collected on the different microscope systems (3I, Zeiss; Nikon) were analyzed offline in Matlab (Mathworks) using established methods (6,18,22). For Fura2, 340nm and 380nm intensities were averaged across each islet, and the ‘background’ intensity averaged over an unstained islet subtracted. Time-averaged \([\text{Ca}^{2+}]_i\) concentration were calibrated from the background-subtracted time-averaged 340nm/380nm intensity ratio using the Fura2 calibration kit (Invitrogen).

NAD(P)H and Rhodamine123 fluorescence were averaged across each islet, with Z-positions corrected for focal drift. The mean NAD(P)H autofluorescence for each experimental group was normalized to the average autofluorescence in Cx36\(^{+/+}\) islets at 2mM. The mean Rhodamine123 fluorescence of each islet was normalized to the fluorescence at 2mM glucose.

To estimate the proportion of the islet showing elevations in \([\text{Ca}^{2+}]_i\), FuraRed images were first smoothed using a 3x3 average filter. The variance (intensity fluctuation) was calculated over the time-course of each pixel. A threshold variance was calculated for a silent cell, identified as quiescent at 2mM glucose, which represents image noise. An active area showing \([\text{Ca}^{2+}]_i\) fluctuations was defined as having a variance significantly greater (95% confidence) than the variance of the silent cell.

To determine high-GFP (GFP+) cells, GFP images were first smoothed using a 3x3 average filter. A threshold intensity was calculated from the autofluorescence in the GFP channel from control (GFP negative) Cx36\(^{+/+}\) or Cx36\(^{-/-}\) islets. A GFP+ region was identified as having GFP fluorescence greater than the threshold intensity.
RESULTS

Inducible β-cell expression of mutant ATP-insensitive K\textsubscript{ATP} channel subunit (Kir6.2\textsuperscript{[AN30,K185Q]}) forms K\textsubscript{ATP} channels with mixed mutant and wild-type Kir6.2 (3), which reduces the sensitivity of ATP-inhibition. To test whether reduced Cx36 gap junction coupling could recover sufficient elevation in [Ca\textsuperscript{2+}]; and insulin secretion in the presence of ATP-insensitive K\textsubscript{ATP} channels, age-matched Rosa-Kir6.2\textsuperscript{[AN30,K185Q]};Pdx-Cre\textsuperscript{ER} expressing mice with normal Cx36 gap junction coupling (Cx36\textsuperscript{+/+};Kir6.2\textsuperscript{[AN30,K185Q]}) and Rosa-Kir6.2\textsuperscript{[AN30,K185Q]};Pdx-Cre\textsuperscript{ER} expressing mice with a lack of Cx36 gap junction coupling (Cx36\textsuperscript{-/-};Kir6.2\textsuperscript{[AN30,K185Q]}) were selected along with littermate ‘control’ mice (Cx36\textsuperscript{+/+}, Cx36\textsuperscript{-/-} respectively). Each group of these 4 mice underwent tamoxifen injections to induce Kir6.2\textsuperscript{[AN30,K185Q]} expression and were studied in parallel.

Cx36 knockout prevents hyperglycemia upon mutant K\textsubscript{ATP} channel expression –

In Cx36\textsuperscript{+/+};Kir6.2\textsuperscript{[AN30,K185Q]} mice, following the induction of Kir6.2\textsuperscript{[AN30,K185Q]} expression ad-lib fed blood glucose levels rapidly rose to >500mg/dl compared to littermate Cx36\textsuperscript{+/+} control animals (figure 1A). Plasma insulin levels were substantially and significantly diminished in Cx36\textsuperscript{+/+};Kir6.2\textsuperscript{[AN30,K185Q]} mice compared to littermate Cx36\textsuperscript{+/+} controls (figure 1B). In Cx36\textsuperscript{-/-};Kir6.2\textsuperscript{[AN30,K185Q]} mice which lack β-cell gap junction coupling, following the induction of Kir6.2\textsuperscript{[AN30,K185Q]} expression ad-lib fed blood glucose levels remained unchanged compared to Cx36\textsuperscript{-/-} littermate control animals (figure 1C). Insulin levels were not significantly reduced in Cx36\textsuperscript{-/-};Kir6.2\textsuperscript{[AN30,K185Q]} mice compared to littermate Cx36\textsuperscript{-/-} controls (figure 1D). At day 30 post induction, Cx36\textsuperscript{+/+};Kir6.2\textsuperscript{[AN30,K185Q]} mice showed substantially greater blood glucose compared to Cx36\textsuperscript{+/+} controls, whereas Cx36\textsuperscript{-/-};Kir6.2\textsuperscript{[AN30,K185Q]} showed no significant increase compared to littermate Cx36\textsuperscript{-/-} controls, and similar to Cx36\textsuperscript{+/+} controls (figure 1E). Over the 30 days post-
induction, Cx36<sup>+/+</sup>;Kir6.2<sup>[ΔN30,K185Q]</sup> mice showed significantly less weight gain compared to Cx36<sup>+/+</sup> littermate controls, whereas Cx36<sup>−;/−</sup>;Kir6.2<sup>[ΔN30,K185Q]</sup> showed no significant difference compared to Cx36<sup>−;/−</sup> littermate controls (figure 1F).

In Cx36<sup>+/−</sup>;Kir6.2<sup>[ΔN30,K185Q]</sup> mice with ~50% gap junction coupling, following the induction of Kir6.2<sup>[ΔN30,K185Q]</sup> expression ad-lib fed blood glucose levels also rose compared to littermate control animals (supplemental figure S1). This initial elevation was slightly reduced compared to that in Cx36<sup>+/−</sup>;Kir6.2<sup>[ΔN30,K185Q]</sup> mice studied in parallel. However, mice ultimately progressed to similar high blood glucose levels >500mg/dl. Plasma insulin levels were also significantly diminished in Cx36<sup>+/−</sup>;Kir6.2<sup>[ΔN30,K185Q]</sup> mice compared to control mice.

Cx36<sup>+/−</sup>;Kir6.2<sup>[ΔN30,K185Q]</sup> mice also showed substantially greater fasting blood glucose compared to Cx36<sup>+/−</sup> controls and poor glucose tolerance during an IP glucose tolerance test (IPGTT), as expected given the marked elevation in fed glucose levels (figure 2A). While Cx36<sup>−;/−</sup>;Kir6.2<sup>[ΔN30,K185Q]</sup> mice showed similar fasting blood glucose compared to Cx36<sup>−;/−</sup> controls; they showed significantly elevated glucose levels during an IPGTT, indicating a reduced glucose tolerance (figure 2B). During an insulin tolerance test (ITT) Cx36<sup>−;/−</sup>;Kir6.2<sup>[ΔN30,K185Q]</sup> mice showed only a small elevation in blood glucose levels compared to Cx36<sup>−;/−</sup> controls; indicating a small reduction in insulin sensitivity (figure 2C). Therefore in the presence of Cx36 gap junction coupling, expression of ATP-insensitive K<sub>ATP</sub> channels results in severe diabetes, whereas in the absence of Cx36 gap junction coupling only glucose intolerance occurs.

**Cx36 knockout improves [Ca<sup>2+</sup>]<sub>i</sub> insulin secretion upon mutant K<sub>ATP</sub> channel expression**

To discover whether the improved glycemic control originated from improvements in stimulus-secretion coupling within the islet, we isolated islets from Cx36<sup>+/−</sup>;Kir6.2<sup>[ΔN30,K185Q]</sup> and Cx36<sup>−;/−</sup>
Kir6.2\(^{\Delta N30,K185Q}\) mice with their respective littermate controls at 30 days post induction. Islets isolated from Cx36\(^{+/+}\);Kir6.2\(^{\Delta N30,K185Q}\) and Cx36\(^{-/-}\);Kir6.2\(^{\Delta N30,K185Q}\) mice showed similar levels of GFP co-expression above the level of autofluorescence (figure 3A), with on average 50% (each ranging from 30-65%) of the islet GFP+ (figure 3B). This indicates similar levels of high mutant-Kir6.2\(^{\Delta N30,K185Q}\) expression in each case.

Significantly elevated insulin secretion was observed at elevated glucose levels (20mM) in islets from Cx36\(^{-/-}\);Kir6.2\(^{\Delta N30,K185Q}\) mice compared to low glucose levels (2mM), whereas no significant elevation in insulin secretion was observed in islets from Cx36\(^{+/+}\);Kir6.2\(^{\Delta N30,K185Q}\) mice (figure 3C). At elevated glucose levels (20mM), insulin secretion from Cx36\(^{+/+}\);Kir6.2\(^{\Delta N30,K185Q}\) islets was significantly lower compared to Cx36\(^{-/-}\);Kir6.2\(^{\Delta N30,K185Q}\) islets, as well as compared to Cx36\(^{+/+}\) and Cx36\(^{-/-}\) control islets. While mean glucose-stimulated insulin secretion from Cx36\(^{-/-}\);Kir6.2\(^{\Delta N30,K185Q}\) islets was significantly greater than Cx36\(^{+/+}\);Kir6.2\(^{\Delta N30,K185Q}\) islets, it was ~50% less than less than Cx36\(^{+/+}\) and Cx36\(^{-/-}\)control islets. Under elevated KCl stimulation, similar levels of insulin secretion were observed in all cases (figure 3C). Insulin content was also slightly reduced in Cx36\(^{+/+}\);Kir6.2\(^{\Delta N30,K185Q}\) islets compared to Cx36\(^{+/+}\) controls, whereas similar insulin content was observed in both Cx36\(^{-/-}\);Kir6.2\(^{\Delta N30,K185Q}\) and Cx36\(^{-/-}\) islets (figure 3D). Therefore in the absence of Cx36 gap junction coupling, ATP-insensitive K\(_{ATP}\) channels have a reduced effect in suppressing glucose-stimulated insulin secretion.

We next examined potential alterations in [Ca\(^{2+}\)]\(_{i}\) in islets from these mice. At elevated (11mM) glucose levels, transient elevations (oscillations) in [Ca\(^{2+}\)]\(_{i}\) were observed in Cx36\(^{+/+}\) control islets (figure 4A, top). These oscillations were rarely observed in Cx36\(^{+/+}\);Kir6.2\(^{\Delta N30,K185Q}\) islets at elevated glucose levels (figure 4A, middle), but observed frequently in many cells of Cx36\(^{-/-}\);Kir6.2\(^{\Delta N30,K185Q}\) islets at elevated (20mM) glucose (figure 4A, lower). The proportion of cells
showing transient elevations in $[\text{Ca}^{2+}]_i$ was significantly greater in Cx36$^{-/-}$;Kir6.2$^{[\text{AN30,K185Q}]}$ islets compared to Cx36$^{+/+}$;Kir6.2$^{[\text{AN30,K185Q}]}$ islets at elevated glucose (figure 4B). $[\text{Ca}^{2+}]_i$ oscillations were observed at a greater extent in low-GFP- cells in Cx36$^{-/-}$;Kir6.2$^{[\text{AN30,K185Q}]}$ islets compared to high-GFP+ cells (figure 4B and supplemental figure S2), and to a similar extent as Cx36$^{+/+}$ control islets. Interestingly, a large proportion of high-GFP+ cells still showed $[\text{Ca}^{2+}]_i$ oscillations in Cx36$^{-/-}$;Kir6.2$^{[\text{AN30,K185Q}]}$ islets, albeit with a plateau fraction of $<10\%$, compared to between 30-90% in low-GFP- cells (figure 4A). Upon elevated glucose levels, the time-averaged free-calcium concentration was also significantly reduced in Cx36$^{+/+}$;Kir6.2$^{[\text{AN30,K185Q}]}$ islets compared to Cx36$^{+/+}$ islets (figure 4C). At high glucose levels, Cx36$^{-/-}$ islets normally show a reduced time-average calcium concentration compared to wild-type islets (18), and no significant difference was observed between Cx36$^{-/-}$;Kir6.2$^{[\text{AN30,K185Q}]}$ islets and Cx36$^{-/-}$ islets. Again similar elevations in $[\text{Ca}^{2+}]_i$ concentration were observed in all groups upon elevated KCl. Therefore in the absence of Cx36 gap junction coupling, ATP-insensitive $K_{\text{ATP}}$ channels have a reduced effect in suppressing glucose-stimulated $[\text{Ca}^{2+}]_i$; consistent with the observed recovery of glucose-stimulated insulin secretion.

**Cx36 knockout improves $[\text{Ca}^{2+}]_i$, insulin secretion upon diazoxide-induced $K_{\text{ATP}}$ opening** –

To further test whether modulating gap junction coupling can enhance $[\text{Ca}^{2+}]_i$ and insulin secretion upon $K_{\text{ATP}}$ channel opening, this time in the presence of normal β-cell heterogeneity, we applied varying concentrations of the $K_{\text{ATP}}$-activator diazoxide. At 11mM glucose in Cx36$^{+/+}$ islets, 100µM diazoxide suppressed $[\text{Ca}^{2+}]_i$ elevations across most of the islet (Figure 5A, top), whereas in Cx36$^{-/-}$ islets many cells still showed $[\text{Ca}^{2+}]_i$ elevations (figure 5A, bottom). $[\text{Ca}^{2+}]_i$ elevations were significantly greater in Cx36$^{-/-}$ islets upon 100µM diazoxide but not upon 250µM diazoxide (Figure 5B). Similarly upon 100µM diazoxide at 11mM glucose insulin secretion from Cx36$^{-/-}$ islets was
significantly greater than Cx36\textsuperscript{+/+} islets. Therefore in a more general case of K\textsubscript{ATP} channel opening, a reduction in gap junction coupling elevates [Ca\textsuperscript{2+}]; and insulin secretion.

\textit{Cx36 knockout reduces metabolic dysfunction following mutant K\textsubscript{ATP} channel expression –}

A secondary consequence of the chronic hyperglycemia and hypoinsulinemia which occurs in Kir6.2\textsuperscript{[ΔN30,K185Q]} expressing mice is marked mitochondrial dysfunction (6), characterized by reduced mitochondrial NAD(P)H accumulation and reduced mitochondrial membrane depolarization at elevated glucose. We quantified these parameters in mice with reduced gap junction coupling that are protected from diabetes. Cx36\textsuperscript{+/+};Kir6.2\textsuperscript{[ΔN30,K185Q]} islets showed elevated NAD(P)H at low levels of glucose compared to control Cx36\textsuperscript{+/+} islets, but similar low levels of NAD(P)H were observed in Cx36\textsuperscript{−/−};Kir6.2\textsuperscript{[ΔN30,K185Q]} islets and control Cx36\textsuperscript{−/−} islets (figure 6A,B). Similar levels of NAD(P)H were observed at elevated glucose in all sets of islets (figure 6A). As a result, Cx36\textsuperscript{+/+};Kir6.2\textsuperscript{[ΔN30,K185Q]} islets showed significantly reduced glucose-stimulated accumulation of NAD(P)H compared to other experimental groups (figure 6C). Cx36\textsuperscript{+/+};Kir6.2\textsuperscript{[ΔN30,K185Q]} islets also showed significantly reduced mitochondrial membrane depolarization compared to Cx36\textsuperscript{−/−};Kir6.2\textsuperscript{[ΔN30,K185Q]} islets at elevated glucose, as indicated by Rhodamine123 fluorescence (figure 6D-F). Therefore reducing gap junction coupling in Kir6.2\textsuperscript{[ΔN30,K185Q]} expressing mice leads to an absence of secondary mitochondrial dysfunction.
DISCUSSION

In this study we tested whether a reduction in islet Cx36 gap junction coupling could compensate for overactive ATP-insensitive $K_{ATP}$ channels in a model of NDM, and therefore prevent the emergence of diabetes. Upon a knockout of Cx36, the elevation in glucose-stimulated $[Ca^{2+}]_i$ and insulin secretion which we measured explains the normalization in blood glucose levels. Based on results presented here and prior studies, we propose the following mechanisms of action, schematically represented in figure 7A. A reduction in Cx36 gap junctions effectively left-shifts the dose-response of $[Ca^{2+}]_i$ (although insulin remains suppressed at low glucose due to $[Ca^{2+}]_i$-independent mechanisms of suppression (18)); whereas expression of overactive $K_{ATP}$ channels effectively right-shifts the dose-response of $[Ca^{2+}]_i$ and insulin secretion (with a largely suppressed response over the physiological glucose range in the presence of Cx36). Therefore in the presence of overactive $K_{ATP}$ channels, a reduction in Cx36 partially normalized the dose-response, albeit with disrupted insulin-secretion dynamics associated with a loss of Cx36. This is further detailed in figure 7B, where in the normal presence of Cx36 inexcitable cells that are present due to heterogeneity prevent membrane depolarization and $[Ca^{2+}]_i$ elevations in normally excitable cells at elevated glucose, via a gap junction-mediated current. This suppresses $[Ca^{2+}]_i$ elevations and insulin release across the islet. In the absence of Cx36 gap junction coupling, this current is absent (17) and therefore normally excitable cells are free to depolarize, elevate $[Ca^{2+}]_i$ and release insulin at elevated glucose levels.

*Gap junction suppression of $[Ca^{2+}]_i$ is physiologically important –*

Previous work has shown that membrane depolarization and $[Ca^{2+}]_i$ elevations in excitable cells of an islet can be suppressed by gap junction coupling to inexcitable cells. This was demonstrated in
islets expressing a $K_{\text{ATP}}$ ‘loss-of-function’ mutation (closes in the absence of ATP) at low (~2mM) glucose (16); where a reduction of gap junction coupling elevated $[\text{Ca}^{2+}]_i$. Similar observations have been made in normal islets at basal (~5mM) glucose (17,18). Here, in the presence overactive ATP-insensitive $K_{\text{ATP}}$ channels (figure 4), or diazoxide-induced $K_{\text{ATP}}$ channel opening (figure 5), a reduction of gap junction coupling also elevated $[\text{Ca}^{2+}]_i$. This shows that a general principle exists whereby gap junction channel closure/deletion can (at least partially) counteract the effect of $K_{\text{ATP}}$ channel opening to elevate $[\text{Ca}^{2+}]_i$.

Importantly in the presence a ‘gain-of-function’ mutation to the $K_{\text{ATP}}$ channels, there was no elevation in $[\text{Ca}^{2+}]_i$ and insulin release at lower glucose levels. As described above, likely a reduction in Cx36 imparts a ‘left-shift’ to the glucose-stimulated $[\text{Ca}^{2+}]_i$ response rather than a constitutive elevation, counteracting the ‘right-shift’ following ATP-insensitive mutant $K_{\text{ATP}}$ channel expression. Thus a reduction in Cx36 cannot counteract the very strong $K_{\text{ATP}}$ opening that results from low glucose and ATP-insensitive $K_{\text{ATP}}$ channels. This suggests that a Cx36 gap junction reduction would be unlikely to counteract very strong mutations to the $K_{\text{ATP}}$ channel, as is also suggested by very high diazoxide treatment (figure 5).

Interestingly, in islets from Cx36$^{-/-}$;Kir6.2$^{[\Delta N30,K185Q]}$ mice even those high-GFP+ cells that were inexcitable in the presence of gap junctions showed some $[\text{Ca}^{2+}]_i$ elevations. These were less frequent than in the more excitable low-GFP- cells, but significantly more than in islets of Cx36$^{+/+}$;Kir6.2$^{[\Delta N30,K185Q]}$ mice (figure 4). Electrically uncoupled cells will likely show some stochastic ‘channel noise’, as suggested by modeling studies (23,24), which may be sufficient to transiently depolarize and elevate $[\text{Ca}^{2+}]_i$. This suggests that electrically uncoupled cells may inherently behave different to β-cells in normal islets; warranting further study.
Importantly, the elevated \([\text{Ca}^{2+}]_i\) at elevated glucose levels that follows a reduction of gap junction coupling also led to elevated insulin secretion (figures 3,5). This contrast with the behavior at lower glucose levels in normal islets (18), where this effect is normally masked due to other mechanisms of cell-cell communication that suppress insulin release independent of \([\text{Ca}^{2+}]_i\). However, it was suggested that these other communication mechanism(s) that suppresses insulin are inactive at elevated glucose (18,25), as we have demonstrated.

Of further importance, the gap junction mediated suppression of islet \([\text{Ca}^{2+}]_i\) and insulin release is physiologically important. By eliminating gap junction coupling in mice expressing ATP-insensitive \(K_{\text{ATP}}\) channels we eliminated hyperglycemia (figure 1). Gap junction coupling in the islet has been shown to play an important physiological role, to coordinate and enhance the first-phase of insulin secretion and coordinate the pulsatile second-phase of insulin secretion (15). In its absence glucose intolerance occurs, and we observed glucose intolerance in Cx36\(^{-/-}\);Kir6.2\(^{\text{ΔN30,K185Q}}\) mice, albeit slightly less than previously observed (15) likely due to the younger age of the mice. This physiological role gives a new fundamental understanding of the multicellular properties of the islet, specifically how the electrically coupling of \(\beta\)-cells in via gap junction channels can be critically important to coordinate \(K_{\text{ATP}}\) channel-regulated electrical activity to regulate insulin secretion and glucose homeostasis.

Relevance to human NDM –

The majority of cases of neonatal diabetes mellitus (~60%) occur as a result of ‘gain-of-function’ mutations to the Kir6.2 or Sur1 subunits of the \(K_{\text{ATP}}\) channel (26). In the normal presence of Cx36, we observed similar results to a prior mouse model that expresses a Kir6.2 mutation associated with NDM (4), including: marked hyperglycemia; reduced weight gain; reduced islet insulin content;
suppression of glucose-stimulated $[\text{Ca}^{2+}]_i$ and insulin. Despite the reduced insulin content, normal depolarization induced $[\text{Ca}^{2+}]_i$ and insulin release was observed, also as previously observed (4). Given that a reduction of Cx36 gap junction coupling so dramatically normalized glycemic control, we anticipate an improvement in islet function would occur in humans with NDM. However some subtle differences must be discussed.

The distribution of Kir6.2$^{[\Delta N30, K185Q]}$ expression is likely more heterogeneous in islets from this mouse model compared to islets of humans with the disease, due to the stochastic and variable nature of Pdx-Cre$^{\text{ER}}$ mediated recombination (21). Therefore the heterogeneity in β-cell function, from which the gap junction-mediated suppression of $[\text{Ca}^{2+}]_i$ and insulin partly occurs, will not be exactly the same. However, it is well established that human and mouse β-cells lacking cellular proximity are very heterogeneous in their glucose-response (27,28). This suggests a Cx36 gap junction reduction will elevate glucose-stimulated $[\text{Ca}^{2+}]_i$ and insulin even in the presence of uniform Kir6.2$^{[\Delta N30, K185Q]}$ expression. Results from diazoxide treated islets (figure 5) support this, as diazoxide treatment likely provides uniform $K_{\text{ATP}}$ opening across the islet. In this case the endogenous β-cell heterogeneity would be closer to that of human islets with NDM mutations. Further, gap junction coupling suppresses excitability in other conditions of $K_{\text{ATP}}$-channel opening at lower glucose levels. In Cx36$^{-/-}$ islets, elevated $[\text{Ca}^{2+}]_i$ is observed in ~50% of β-cells at 5mM glucose (18), and in ~40% of β-cells upon 100µM diazoxide (figure 5), each similar to that following expression of Kir6.2$^{[\Delta N30, K185Q]}$ (figure 4). The precise quantitative balance between β-cell heterogeneity and gap junction coupling in determining islet function remains to be determined. Nevertheless, the presence of any heterogeneity will lead to gap junction coupling suppressing $[\text{Ca}^{2+}]_i$ over a certain range of glucose levels. Likely the broader the heterogeneity the greater the
difference in the presence and absence of gap junction coupling (18), and the more dramatic the
effect in modulating gap junction coupling.

The discovery that NDM can be caused by ‘gain-of-function’ mutations to the Kir6.2 and
SUR1 subunits of the $K_{\text{ATP}}$ channel has meant that patients can switch from insulin therapy to oral
sulfonylurea treatment with improved glycemic control (7). While successful for treating NDM,
hypoglycemic episodes have been reported to occur during sulfonylurea treatment (29), which may
occur due to constitutive glucose-independent $K_{\text{ATP}}$ channel closure which reduces the low glucose-
regulation of insulin secretion. Chronic sulfonylureas therapy can also cause glucose intolerance
(30), possibly resulting from over-stimulation of $[\text{Ca}^{2+}]_i$. As discussed above, a modulation of gap
junction coupling retains a suppression of $[\text{Ca}^{2+}]_i$ and insulin secretion at low glucose (figure 3,4,7)
and thus would be anticipated to lessen potential hypoglycemia. More importantly, some Kir6.2 and
Sur1 mutations reduce the sensitivity of sulfonylurea inhibition (7,9,10), reducing sulfonylurea
effectiveness. We speculate that modulating Cx36 gap junction coupling may provide an alternative
route to elevate glucose-stimulated $[\text{Ca}^{2+}]_i$ and insulin secretion, particularly in the presence of
sulfonylurea-insensitive mutations.

Here, a near complete reduction in gap junction coupling (>95%, Cx36$^{-/-}$) normalized blood
glucose levels, but a partial reduction (~50%, Cx36$^{+/+}$) had a minimal effect (figure S1). This Cx36
dose-response is similar to how reducing Cx36 gap junction coupling disrupts glucose tolerance via
first-phase and second-phase insulin dynamics (15). As a potential therapeutic target, we therefore
anticipate a gap junction inhibition of >50% would be required. Current inhibitors are weak with
non-specific effects, although a recent study developed a novel screen for gap junction modulators
(31) which may yield more potent and specific inhibitors. A partial disruption to gap junction
coupling can also result from hyperglycemia (32). However in Cx36$^{+/+}$;Kir6.2$^{[\Delta N30,K185Q]}$ islets
significant coupling is still present, as shown by the coordinated residual \( [\text{Ca}^{2+}] \). Therefore any Cx36 decrease resulting from hyperglycemia would likely be insufficient to significantly alter insulin release in this model.

In principle, we anticipate reducing gap junction coupling could similarly correct defects in insulin release caused by mutations proximal to \([\text{Ca}^{2+}]\) influx, including Glucokinase (33,34) and genes that regulate mitochondrial function (35,36). Defects in proximal steps could affect the ‘amplifying’ pathways that regulates insulin release (37), and reduce the effect of gap junction modulation. However, altering the \( \text{K}_\text{ATP} \)-regulation of membrane potential can compensate for an absence of Glucokinase (38). Therefore modulating Cx36 gap junction coupling may be more broadly applicable to normalize islet function.

To summarize, we have shown that by reducing gap junction coupling between \( \beta \)-cells in a model of NDM caused by expression of ATP-insensitive \( \text{K}_\text{ATP} \) channels, we can eliminate severe hyperglycemia and islet dysfunction. This is achieved through a novel pathway where a reduction in Cx36 can partially compensate for overactive \( \text{K}_\text{ATP} \) channels and prevent suppression of electrical activity across the islet. This restores glucose-stimulated \([\text{Ca}^{2+}]\), insulin secretion and glucose homeostasis. This yields a better understanding for how the islet functions as a coupled unit of \( \beta \)-cells, and may ultimately provide a potential therapeutic target for treating NDM and other monogenic forms of diabetes.
ACKNOWLEDGEMENTS

Author Contributions- L.M.N researched data, M.P. researched data; T.H.H. researched data; R.K.P.B. designed experiments, researched data and wrote manuscript.

Acknowledgements- We thank Colin G Nichols (Washington University St Louis) and Maria S Remedi (Washington University St Louis) for helpful comments and suggestions in performing this study and for reviewing this manuscript. Richard KP Benninger (University of Colorado) is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors acknowledge that no conflict of interest exists. This study was primarily supported by NIH grants R00 DK085145 (to RKPB), NSF grant DGE0742434 (THH), and CU internal funds. Experiments on the 3I Marianas spinning disk microscope and the Zeiss LSM510 2-photon microscope were performed through the use of the CU AMC advanced light microscopy core (P30 NS048154, UL1 RR025780).
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synchronization of glucose-induced Ca2+ and insulin oscillations, and basal insulin release. Diabetes 2005;54:1798-1807


FIGURE CAPTIONS

Figure 1: Prevention of Kir6.2\[^{\Delta N30,K185Q}\]-induced hyperglycemia. A) Mean time-course of *ad-lib* fed blood glucose levels following tamoxifen injection in Cx36\(^{+/+}\);Kir6.2\[^{\Delta N30,K185Q}\] mice (empty diamond) and control Cx36\(^{+/+}\) mice (solid square). Duration of daily tamoxifen injections indicated by solid line. B) Mean plasma insulin levels at day 29 following tamoxifen injection in Cx36\(^{+/+}\);Kir6.2\[^{\Delta N30,K185Q}\] mice (hashed bar) and control Cx36\(^{+/+}\) mice (solid bar). C) As in A for Cx36\(^{-/-}\);Kir6.2\[^{\Delta N30,K185Q}\] mice (empty diamond) and control Cx36\(^{-/-}\) mice (solid square). D) As in B for Cx36\(^{-/-}\);Kir6.2\[^{\Delta N30,K185Q}\] mice (hashed bar) and control Cx36\(^{-/-}\) mice (solid bar). E) Mean *ad-lib* blood glucose levels for all groups of mice, averaged over day 27-29 post-induction. F) Mean weight change for all groups of mice, between day 1 and day 29 post-induction. Data in A,C,E,F averaged over n=22 (Cx36\(^{+/+}\);Kir6.2\[^{\Delta N30,K185Q}\]) and n=21 (Cx36\(^{+/+}\)) littermates. Data in B,D,E,F averaged over n=21 (Cx36\(^{-/-}\);Kir6.2\[^{\Delta N30,K185Q}\]) and n=24 (Cx36\(^{-/-}\)) littermates. * indicates significant difference (p<0.05, 2-tailed student’s t-test), ‘ns’ indicates no significant difference (p>0.05) between each experimental group as indicated. Error bars represent mean±s.e.m.

Figure 2: Improved glucose tolerance. A) Intra-peritoneal glucose tolerance test on Cx36\(^{+/+}\);Kir6.2\[^{\Delta N30,K185Q}\] mice (empty diamond) and control Cx36\(^{+/+}\) mice (solid square), following 2g/kg b.w. IP glucose injection. Left displays area under the curve (AUC) of the glucose excursion. n=6 littermate mice in each group. B) As in A for Cx36\(^{-/-}\);Kir6.2\[^{\Delta N30,K185Q}\] mice and control Cx36\(^{-/-}\) mice. n=11 littermate mice in each group. C) Insulin tolerance test for Cx36\(^{-/-}\);Kir6.2\[^{\Delta N30,K185Q}\] mice and control Cx36\(^{-/-}\) mice following 0.075U/kg IP insulin injection after 0 time point. n=13 littermate mice in each group. * indicates significant difference (p<0.05, 2-tailed paired student’s t-test) between each experimental group as indicated. Error bars represent mean±s.e.m.
Figure 3: Recovery of glucose-stimulated insulin secretion. A) Representative images of GFP (green) in islets from Cx36⁺/⁺;Kir6.2[ΔN30,K185Q], Cx36⁻/⁻;Kir6.2[ΔN30,K185Q], and Cx36⁺/+ mice. B) Mean % coverage of GFP in islets from Cx36⁺/⁺;Kir6.2[ΔN30,K185Q] and Cx36⁻/-;Kir6.2[ΔN30,K185Q] mice. n=9 age-matched mice in each group, 2-6 islets per mouse. C) Insulin secretion under low glucose (2mM), high glucose (20mM) and high glucose plus KCl (+20mM) in islets isolated from Cx36⁺/⁺;Kir6.2[ΔN30,K185Q] and Cx36⁺/+ littermate control mice and Cx36⁻/-;Kir6.2[ΔN30,K185Q] and Cx36⁻/- littermate control mice. D) Insulin content in islets isolated from Cx36⁺/⁺;Kir6.2[ΔN30,K185Q] and Cx36⁺/+ littermate control mice and Cx36⁻/-;Kir6.2[ΔN30,K185Q] and Cx36⁻/- littermate control mice. Data in C,D averaged over n=6 age matched mice. * indicates significant difference (p<0.05, 2-tailed student’s t-test), ‘ns’ indicates no significant difference (p>0.05) between each experimental group as indicated. Error bars represent mean±s.e.m.

Figure 4: Recovery of glucose-stimulated [Ca²⁺]. A) Representative time-courses of [Ca²⁺], as measured from FuraRed fluorescence, in islets isolated from Cx36⁺/+ mice (top), Cx36⁺/⁺;Kir6.2[ΔN30,K185Q] mice (middle) and Cx36⁻/-;Kir6.2[ΔN30,K185Q] mice (bottom). Displayed is the inverted, normalized FuraRed fluorescence from a number of non-adjacent cells with low GFP expression (black) or high GFP expression (green) at 2mM glucose (left) and 10 minutes after elevation to 20mM glucose (right), or at 2mM and 11mM glucose in Cx36⁺/+ islets. Time courses are offset for clarity and vertical scale bar indicates 50% change in FuraRed fluorescence. B) Mean % of cells displaying dynamic changes in [Ca²⁺] at 20mM glucose, in Cx36⁺/+;Kir6.2[ΔN30,K185Q] islets, Cx36⁻/-;Kir6.2[ΔN30,K185Q] islets and at 11mM glucose in Cx36⁺/+ islets; averaged over high GFP expression only or low GFP expression only. Data averaged over n=3 age-matched mice in
each group, 3-7 islets per mouse. C) Mean [Ca$^{2+}$], concentration measured from Fura2 fluorescence, under low glucose (2mM), high glucose (20mM) and high glucose plus KCl (+20mM) in islets isolated from Cx36$^{+/+};$Kir6.2$[^{AN30,K185Q}]$ and Cx36$^{+/+}$ littermate control mice and Cx36$^{-/-};$Kir6.2$[^{AN30,K185Q}]$ and Cx36$^{-/-}$ littermate control mice. Data averaged over n=6 age-matched mice, 2-8 islets per mouse. * indicates significant difference (p<0.05, 2-tailed student’s t-test), † indicates significant difference (p<0.05, 2-tailed paired student’s t-test), ‘ns’ indicates no significant difference (p>0.05) between each experimental group as indicated. Error bars represent mean±s.e.m.

Figure 5: Recovery of [Ca$^{2+}$]$_i$ in diazoxide treated islets. A) Representative time-courses of [Ca$^{2+}$]$_i$, as measured from Fluo4 fluorescence, in islets isolated from Cx36$^{+/+}$ mice (top), and Cx36$^{-/-}$ mice (bottom) at 11mM glucose before and after Diazoxide treatment. Displayed is the normalized Fluo4 fluorescence from a number of non-adjacent cells within an islet at 11mM glucose alone (left), and 11mM glucose 10 minutes after treatment with 100µM Diazoxide. Time courses are offset for clarity and vertical scale bar indicates 50% change in Fluo4 fluorescence. B) Mean % of cells displaying dynamic changes in [Ca$^{2+}$]$_i$ at 11mM glucose, in Cx36$^{+/+}$, Cx36$^{-/-}$ islets at several diazoxide concentrations. Data averaged over n=3 age-matched mice in each group, 2-5 islets per mouse. C) Insulin secretion under high glucose (11mM) in Cx36$^{+/+}$, Cx36$^{-/-}$ islets at several diazoxide concentrations. Data averaged over n=6 age-matched mice in each group. * indicates significant difference (p<0.05, 2-tailed student’s t-test), † indicates significant difference (p<0.05, 1-tailed paired student’s t-test). Error bars represent mean±s.e.m.
Figure 6: Prevention of secondary mitochondrial defects. A) Representative NAD(P)H autofluorescence image at 2mM glucose (top) and 20mM glucose (bottom) in a Cx36+/+ islet. B) Mean NAD(P)H autofluorescence in islets isolated from Cx36+/+;Kir6.2[ΔN30,K185Q] and Cx36+/+ littermate control mice and Cx36−/−;Kir6.2[ΔN30,K185Q] and Cx36−/− littermate control mice. Data is normalized to mean NAD(P)H fluorescence measured in Cx36+/+ islets at 2mM glucose. C) Change in NAD(P)H from 2mM to 20mM glucose in islets from each experimental group in B. Data in A, B averaged over n=5 age-matched mice in each group, 2-4 islets per mouse. D) Representative Rhodamine123 fluorescence image at 2mM glucose (top) and 20mM glucose (bottom) in a Cx36+/+ islet. E) Mean Rhodamine123 fluorescence in islets isolated from Cx36+/+;Kir6.2[ΔN30,K185Q] and Cx36+/+ littermate control mice and Cx36−/−;Kir6.2[ΔN30,K185Q] and Cx36−/− littermate control mice. Data is normalized to fluorescence at 2mM glucose in each experimental group. F) Change in Rhodamine123 fluorescence from 2mM to 20mM glucose in islets from each experimental group in E Data in E,F averaged over n=5 age-matched mice in each group, 2-6 islets per mouse. * indicates significant difference (p<0.05, 2-tailed student’s t-test) between each experimental group as indicated. Error bars represent mean±s.e.m.

Figure 7: Schematic describing recovery of insulin secretion. A) Schematic representation for glucose-stimulated [Ca²⁺]ᵢ as a result of changes to Cx36 and KᵦTP activity. Top: Reduction in Cx36 activity due to genetic knockout leads to a left-shift and more gradual dose-response of [Ca²⁺]ᵢ (solid red). Disrupted dynamics not shown. Note insulin at low glucose is still suppressed due to action of other communication mechanism (dotted red). Middle: Increase in KᵦTP activity due to mutations that reduce the sensitivity of ATP-inhibition leads to a right shift in dose-response in [Ca²⁺]ᵢ (dashed black), such that it is largely suppressed within experimental glucose ranges. Insulin
follows this dose response. Bottom: In the presence of increased $K_{\text{ATP}}$ activity a reduction in Cx36 activity leads to a left-shift in the dose-response of $[Ca^{2+}]_i$ back to within a physiological range (dashed red), albeit with glucose-stimulated activity slightly blunted compared to a reduction in Cx36 activity alone. Insulin follows this dose response, although at low glucose it is still suppressed due to action of other communication mechanism (dotted red). B) Two representative, neighboring cells in an islet, with one inexcitable cell (intrinsically inexcitable or expressing high levels of ATP-insensitive $K_{\text{ATP}}$ channels: lower, green), and one excitable cell (with near-normal $K_{\text{ATP}}$ channels: upper, grey). At elevated glucose, the inexcitable cell remains hyperpolarized ($V_m$ low). In isolation the excitable cell would depolarize ($V_m$ increase), elevate $[Ca^{2+}]_i$, and insulin release. However Cx36 gap junction coupling mediates a hyperpolarizing current ($I_{\text{h-pol}}$) from the inexcitable cell to the excitable cell, preventing depolarization and $[Ca^{2+}]_i$ elevations, thereby suppressing $Ca^{2+}$-triggering of insulin secretion across the islet. B) In the absence of Cx36 gap junction coupling, the hyperpolarizing current is abolished and the more excitable cell depolarizes and elevates $[Ca^{2+}]_i$, which triggers insulin secretion.
Figure 1

A

Blood Glucose (mg/dl) vs. Day

B

Plasma Insulin (ng/ml) vs. Day

C

Blood Glucose (mg/dl) vs. Day

D

Plasma Insulin (ng/ml) vs. Day

E

Blood Glucose (mg/dl) vs. Cx36 and Kir6.2

F

Weight change (g) vs. Cx36 and Kir6.2

* ns

Diabetes
Figure 2

A

Blood Glucose (mg/dl)

Time (min.)

Cx36+/+

Cx36+/+;Kir6.2[ΔN30,K185Q]

AUC (mg/dl)

B

Blood Glucose (mg/dl)

Time (min.)

Cx36−/−

Cx36−/−;Kir6.2[ΔN30,K185Q]

AUC (mg/dl)

C

Blood Glucose (norm.)

Time (min.)

Cx36+/+

Cx36+/+;Kir6.2[ΔN30,K185Q]

AUC (mg/dl)
Figure 3

**A**

Diabetes

**B**

**C**

**D**

Insulin secretion (ng/hour/5 islets) vs. % area GFP+ for different Cx36 and Kir6.2 genotypes.

* indicates statistical significance, **ns** indicates no significant difference.
Figure 4

A

2mM gluc.

20mM gluc.

Cx36^{+/+} (11mM)  

Cx36^{+/+}; Kir6.2_{[\Delta N30,K185Q]}  

Cx36^{-/-}; Kir6.2_{[\Delta N30,K185Q]}

FuraRed (normalized, inverted)

Time (sec.)

B

% cells active

GFP-  

GFP+

Cx36^{+/+}  

Cx36^{-/-}; Kir6.2_{[\Delta N30,K185Q]}  

Cx36^{+/+}; Kir6.2_{[\Delta N30,K185Q]}

0%  

25%  

50%  

75%  

100%

0%  

25%  

50%  

75%  

100%

C

[Ca^{2+}]_i (µM)

2mM gluc.  

20mM gluc.  

20mM gluc. + KCl

Cx36^{+/+}  

Cx36^{-/-}; Kir6.2_{[\Delta N30,K185Q]}  

Cx36^{+/+}; Kir6.2_{[\Delta N30,K185Q]}

0.00  

0.05  

0.10  

0.15  

0.20  

0.25  

0.30  

0.35

*  

ns  

†
**Figure 5**

**A**

11mM glucose + Diazoxide

<table>
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<th>Diazoide Concentration</th>
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<th>Cx36-/-</th>
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<tr>
<td>100μM</td>
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**B**

[Ca$^{2+}$]$_i$ (% cells active)

![Bar chart](#)

**C**

Insulin secretion (ng/ml)

![Bar chart](#)
Figure 7

A

Wild-Type

Cx36^{-/-}

[Ca^{2+}] (~ insulin)

Wild-Type

Kir6.2^{[K185Q,ΔN30]}

[Ca^{2+}] (~ insulin)

Kir6.2^{[K185Q,ΔN30]};Cx36^{-/-}

[Ca^{2+}] (~ insulin)

B

High glucose

glycolysis

mitochondria

KATP

KATP

CaV

CaV

ATP

Vm

[Ca^{2+}]_i

glycolysis

mitochondria

ADP

Vm

Insulin

Insulin

I_h-pol

Cx36

[Ca^{2+}]_i

High glucose
Figure S1

(A) Blood glucose (mg/dl) over time for Cx36+/+;Kir6.2[ΔN30,K185Q] and Cx36+/−;Kir6.2[ΔN30,K185Q].

(B) Plasma insulin (ng/ml) on Day 29 for Cx36+/+;Kir6.2[ΔN30,K185Q] and Cx36+/−;Kir6.2[ΔN30,K185Q]. No significant difference (ns) was observed.
Figure S2

(A) Time series of FuraRed normalized and inverted signal intensity over time for different regions. 

(B) Representative images showing the locations of regions analyzed for FuraRed signal intensity.
Supplemental information

"Modulation of Cx36 gap junction coupling increases insulin secretion and prevents hyperglycemia in a mouse model of $K_{ATP}$-induced neonatal diabetes"

Figure S1. Minor change in hyperglycemia with 50% gap junction coupling. A) Mean time-course of ad-lib fed blood glucose levels following tamoxifen injection in Cx36$^{+/+}$;Kir6.2$^{[\Delta N30, K185Q]}$ mice (empty diamond) and Cx36$^{+/+}$;Kir6.2$^{[\Delta N30, K185Q]}$ mice (solid diamond). B) Mean plasma insulin levels at day 29 following tamoxifen injection in Cx36$^{+/+}$;Kir6.2$^{[\Delta N30, K185Q]}$ mice (solid bar) and Cx36$^{+/+}$;Kir6.2$^{[\Delta N30, K185Q]}$ mice (hashed bar). Data in A,B averaged over n=9 (Cx36$^{+/+}$;Kir6.2$^{[\Delta N30, K185Q]}$) and n=10 (Cx36$^{+/+}$;Kir6.2$^{[\Delta N30, K185Q]}$) age-matched mice. * indicates significant difference (p<0.05, 2-tailed paired student’s t-test) between each experimental group as indicated.
Figure S2. Time-course of [Ca^{2+}]_{i} in Kir6.2^{[AN30, K185Q]} expressing islets with and without gap junction coupling. 

A). Representative time-courses of [Ca^{2+}]_{i}, as measured from FuraRed fluorescence, in islets isolated from Cx36\textsuperscript{+/+};Kir6.2^{[AN30, K185Q]} mice (top) and Cx36\textsuperscript{−/−}; Kir6.2\textsuperscript{[AN30, K185Q]} mice (bottom). Displayed is normalized FuraRed fluorescence from cells with low GFP expression (black) or high GFP expression (green) at 20mM glucose (Time courses are offset for clarity and vertical scale bar indicates 50% change in FuraRed fluorescence. 

B). Images of Cx36\textsuperscript{+/+};Kir6.2^{[AN30, K185Q]} islet (top) and Cx36\textsuperscript{−/−}; Kir6.2\textsuperscript{[AN30, K185Q]} islet (bottom), with GFP in green and FuraRed in red; and those cells indicated, from which time courses are displayed in A.