The Role of Membrane Excitability in Insulin Regulation

Christopher Howard Emfinger

Washington University in St. Louis

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The Role of Membrane Excitability in Insulin Regulation
by
Christopher Emfinger

A dissertation presented to
The Graduate School
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2018
St. Louis, Missouri
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**List of abbreviations**

(GENE NAME/ Protein name)

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNCJ11</td>
<td>potassium voltage-gated channel subfamily J member 11</td>
</tr>
<tr>
<td>ABCC8</td>
<td>ATP Binding Cassette Subfamily C Member 8</td>
</tr>
<tr>
<td>Kir6.2</td>
<td>ATP-sensitive potassium channel pore subunit 6.2, inward-rectifying</td>
</tr>
<tr>
<td>SUR1</td>
<td>Sulfonylurea receptor 1</td>
</tr>
<tr>
<td>KNCJ8</td>
<td>potassium voltage-gated channel subfamily J member 8</td>
</tr>
<tr>
<td>ABCC9</td>
<td>ATP Binding Cassette Subfamily C Member 9</td>
</tr>
<tr>
<td>Kir6.1</td>
<td>ATP-sensitive potassium channel pore subunit 6.1, inward-rectifying</td>
</tr>
<tr>
<td>KNCJ11L</td>
<td>potassium voltage-gated channel subfamily J member 11-like</td>
</tr>
<tr>
<td>SUR2</td>
<td>Sulfonylurea receptor 2</td>
</tr>
<tr>
<td>Kir6.3</td>
<td>ATP-sensitive potassium channel pore subunit 6.3, inward-rectifying</td>
</tr>
<tr>
<td>VDCC</td>
<td>Voltage-dependent calcium channel</td>
</tr>
<tr>
<td>GJD2B</td>
<td>gap junction protein delta 2b</td>
</tr>
<tr>
<td>CX35b</td>
<td>Connexin 35b</td>
</tr>
<tr>
<td>GJD2</td>
<td>gap junction protein delta 2</td>
</tr>
<tr>
<td>CX36</td>
<td>Connexin 36</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon-like peptide 1</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to thank my mentors, Colin and Maria, for giving me the opportunity to work in their labs. They each have provided their own flavors of inspiration and insight, and without their continued support and guidance I would not have been able to do this. In particular, their willingness to give me the ability to explore the questions in this work from my own perspective and initiative has helped me develop a sense of independence and a love of discovery. They were always patient and willing to discuss any concerns, whether scientific or personal, and have provided invaluable personal, scientific, and career development advice. Maria and Colin have also always been willing to be in the lab, teaching me new techniques and helping me with experiments, which I find remarkable given the pressures of being a PI. They always made significant time for me, and I have always known they have been in my corner.

I would like to thank my thesis committee for their invaluable advice over the years of the project, and helping me develop my critical thinking as we worked through different aspects of the projects. In particular, I would also like to thank a former member of the committee, Kelly Monk, for her help in refining the zebrafish portion of the project.

In addition, there are individuals in other labs whose help has made this work possible. Reka, without your help none of the fish imaging would have been possible, not simply for the work itself but also because you helped me stay sane. Everyone in the Washington University fish community has been welcoming and helpful, providing both advice and technical help without which I, as a novice in working with fish, would have likely floundered. I must acknowledge Margot Williams, as without your advice and help with the molecular biology aspects of the project, I would not have had the animals which have formed half of this work.

I must also acknowledge the other members of the Nichols and Remedi labs. In addition to their invaluable roles in the projects forming this thesis, they have always supported for me, whether
with the technical aspects of the projects, with my writing and presentations, or with the various stressors and personal matters arising over the years. They are more than mere co-workers; they are dear friends without which I would not have been able to do this; you made the work enjoyable. Additionally, Yixi, you went above and beyond, and your help was invaluable.

There are also individuals whose names are not on the papers which make the body of this work. Theresa, you have been an invaluable friend and have done so much over the years without which this work would not have been possible.

I must also thank my friends outside the lab, particularly Zuzana, Kate, Sarah, and Angela (Bowie), who have helped me make St. Louis a second home. You have always had my back, and without you I do not think I would have the strength to do this; hopefully I can return the favor.

Finally, I do not think I would have been able to do any of this without the love and support of my family. You have celebrated with me when everything was working and have helped me recover at those inevitable times in any project when everything seems to be coming apart at the seams. Mom, Dad, and William, I love you all and would not be here without you.

This work was supported by grants from the National Institutes of Health to CGN (R01 DK069445), MSR (R01 DK 098584), and I received fellowship support from NIH T32DK108742–01. This work was additionally supported by institutional grants from the Washington University in St Louis Diabetes Research Center and the Washington University Center for Cellular Imaging.

Christopher Emfinger

Washington University in St. Louis

August 2018
Abstract

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In mammals, ATP-sensitive K⁺ (K\text{ATP}) channels are essential regulators of insulin secretion from pancreatic islet β-cells, illustrated by the finding that gain-of-function mutations in K\text{ATP} channels (K\text{ATP}-GOF) cause neonatal diabetes mellitus (NDM). However, variability in symptom severity and effectiveness of treatment is seen in NDM, even for those with the same mutation and in the same family. Short-term treatment of mice expressing K\text{ATP}-GOF mutations in β-cells (K\text{ATP}-GOF mice) with the K\text{ATP} blocker glibenclamide during disease onset results in two outcomes: one subset becomes severely diabetic (non-remitters), whereas the other subset remains below the glucose levels at which significant side effects occur (remitters). Remitters and non-remitters do not differ in insulin sensitivity early in the induction process, but remitter mice have lower levels of the inflammatory cytokines TNF-α and IL-6, as well as liver glucose production, suggesting roles for gluconeogenesis and inflammation in the pathogenesis of NDM.

Whether K\text{ATP}–dependent excitability is relevant to insulin secretory control in non-mammalian vertebrates is unclear. I have shown that zebrafish islet β-cells express functional K\text{ATP} channels, that these channels have similar properties to their mammalian orthologues, and that they regulate intracellular calcium and glucose homeostasis in zebrafish. Further, I have shown that zebrafish with inducible, β-cell specific K\text{ATP}-GOF mutations recapitulate loss of glucose-induced calcium response, severe hyperglycemia, and growth defects. These findings will inform studies of metabolism in zebrafish and enable the use of zebrafish for larger-scale studies of NDM to identify unknown modifiers regulating responses to β-cell membrane inexcitability.
Chapter 1: Introduction

1.1 Pancreatic insulin secretion is an essential regulator of blood glucose in mammals. In healthy individuals, blood glucose is tightly controlled, the balance of glucose production and disposal being regulated by two major hormones: insulin and glucagon, which are produced by endocrine cells in the islets of Langerhans in the pancreas (Campbell and Drucker, 2015; Haeusler et al., 2017; Saltiel and Kahn, 2001; Samuel and Shulman, 2016; Taylor, 2012).

Insulin acts on many tissues throughout the body. Insulin increases glucokinase and insulin transcription in β-cells (Haeusler et al., 2017; Rask-Madsen and Kahn, 2012; Zhang and Liu, 2014), blocks glucagon secretion from α-cells, and suppresses liver gluconeogenesis (Haeusler et al., 2017; Zhang and Liu, 2014). Moreover, it stimulates glucose uptake by regulating glucokinase expression, and stimulates storage of entering glucose by conversion into glycogen (Rask-Madsen and Kahn, 2012; Zhang and Liu, 2014). Additionally, insulin stimulates liver production of triglycerides from glucose, which are then exported in lipoproteins and then removed from the circulation into adipose cells for long term storage (Rask-Madsen and Kahn, 2012; Zhang and Liu, 2014). Insulin also acts on muscle tissue to stimulate translocation of glucose transporter 4 (Glut4) which allows rapid entry of glucose for oxidation and local storage as glycogen in muscle tissues (Rask-Madsen and Kahn, 2012; Zhang and Liu, 2014).

Insulin can also regulate neuronally driven behaviors by acting on subpopulations of cells in the central nervous system (Porte et al., 2005), particularly in hypothalamic nuclei. Insulin acts on neuropeptide Y (NPY) and pro-opiomelanocortin producing (POMC) neurons in the arcuate nucleus of the hypothalamus (ACNH)(Porte et al., 2005). These actions result in decreased food intake and increased energy expenditure (Porte et al., 2005). Central
administration of insulin limits adiposity and body weight gain in rats and mice, and antagonism of central insulin action with intracerebroventricular administration of insulin receptor antibodies increases food intake and weight gain (Porte et al., 2005).

1.2 Failure of insulin signaling causes diabetes. The importance of insulin signaling is highlighted by the disease consequences of defective signaling. Deficiency of insulin, or resistance to insulin signaling, causes multiple forms of diabetes, clinically defined by persistently elevated blood glucose (hyperglycemia; fasting blood glucose >126mg/dL (Menke et al., 2013) and post-prandial glucose >200mg/dL (Association, 2015)) and alterations in glycosylated hemoglobin (HbA1c >6.5%(Menke et al., 2013)).

Type 1 diabetes (T1D), which accounts for ~5-10% of known cases of diabetes (Maahs et al., 2010; Menke et al., 2013), is understood to result from auto-immune destruction of pancreatic β-cells, and potentially triggered by a combination of both genetic and environmental factors (Dean, 2004; Fourlanos et al., 2004; Maahs et al., 2010). HLA complex II gene mutations account for 30-50% of genetic risk in T1D (Dean, 2004; Steck and Rewers, 2011), but the onset trigger may involve other factors (Shojaeian, 2018). While there is no consensus incident giving rise to autoimmunity, as not all individuals with the genetic predisposition develop autoantibodies and subsequent diabetes, some incipient triggers the immune system to recognize islet components and begin destroying islet β-cells (Insel et al., 2015). Affected individuals become glucose intolerant (Insel et al., 2015; Sosenko et al., 2012a), and eventually, enough β-cells are destroyed that the body can no longer maintain normoglycemia and clinical manifestations of diabetes become apparent (Insel et al., 2015; Marro et al., 2017; Sosenko et al., 2012b).

Type 2 diabetes (T2D) typically occurs when insulin secretion is not sufficient to meet metabolic demands due to peripheral insulin resistance, and is the most common form of diabetes (Association, 2015). T2D is highly associated with obesity (Taylor, 2012), though there
are individuals with T2D that lack abnormalities in body composition (Matza et al., 2007). As with T1D, T2D is understood to result from a combination of genetic (Murea et al., 2012; Talmud et al., 2015) and environmental (Murea et al., 2012) factors. Causes of insulin resistance in T2D are not clear (Fung and Berger, 2016); some data implicate excess circulating triglycerides, lipotoxicity (Donath and Shoelson, 2011), and associated free fatty-acid-induced inflammation (DeFronzo, 2010; Donath and Shoelson, 2011; Giaccari et al., 2009), whereas other data suggest persistent activation of metabolism that triggers oxidative damage through reactive oxygen species (Giaccari et al., 2009).

In early stages, increasing pancreatic insulin secretion compensates for peripheral insulin resistance. However, persistently elevated insulin causes loss of insulin action in peripheral tissues beyond the liver (Harmancey et al., 2015; McGuinness et al., 1990) due to reduced expression of insulin receptor and serine phosphorylation of the remaining receptors (Kanety et al., 1994). Insulin resistance in α-cells, in part, results in dysregulation of glucagon secretion and hyperglucagonemia (Lee et al., 2014; Otero et al., 2014). Eventually, the metabolic demand exceeds the capacity of the β-cells to produce insulin and β-cell failure results, causing hyperglycemia (Weir and Bonner-Weir, 2004).

There are other forms of diabetes that do not fall into the T1D and T2D classifications (Aguilar-Bryan and Bryan, 2008; Naylor et al., 2011; Polak and Cave, 2007; Schwitzgebel, 2014). Genetic mutations that specifically disrupt insulin synthesis or secretion, such as mutations in the ATP-sensitive potassium channels (K_{ATP} channels), glucokinase (GK), the insulin gene itself, or transcription factors regulating β-cell maturation and identity, result in early-onset forms of diabetes including neonatal diabetes (NDM) and maturity-onset diabetes of the young (MODY), in the absence of insulin resistance. Reciprocally, mutations in the insulin receptor can also cause diabetes by blocking peripheral insulin action, independently of defects in insulin secretion (Boucher et al., 2014). Other, less severe mutations in the same genes that
cause NDM are also linked to risk of developing T2D (Nielsen et al., 2003). Given the complex underlying etiologies of T1D and T2D, the monogenic forms of diabetes may offer the opportunity to better differentiate the various mechanisms that underlie and link failure of insulin secretion and insulin action to systemic hyperglycemia. Many forms of monogenic diabetes result from mutations in genes linked to the control of insulin secretion from pancreatic β-cells.

### 1.3 Regulation of mammalian insulin secretion

Pancreatic β-cells store insulin in a large reserve of granules (Fu et al., 2013). Mammalian secretion of insulin in response to changes in nutrients is now well understood (Keane and Newsholme, 2014) (Figures 1.1 and 1.2). When plasma glucose is low, the β-cell metabolic rate is low, plasma membrane potential ($V_m$) is hyperpolarized (membrane potential ~-70mV, close to $E_K$), cytosolic calcium is low, and minimal insulin is secreted (Drews et al., 2010; Fu et al., 2013). As plasma glucose rises, it enters β-cells through glucose transporters (Donath and Shoelson, 2011; Fu et al., 2013): glucose transporter 1 and 3 (Glut1 and Glut3) (McCulloch et al., 2011) in humans and glucose transporter 2 (Glut2) in rodents (Drews et al., 2010; McCulloch et al., 2011).

As glucose enters β-cells, it is phosphorylated by glucokinase (GK) to glucose-6-phosphate (G6P) in the initial step of glycolysis. Two key features of this enzyme distinguish it from other hexokinase family members (Matschinsky, 2002): 1) it has a lower affinity for glucose than other hexokinase members; and 2) the enzyme is not inhibited by its product (G6P), making it a potentially rate-limiting component of glucose metabolism. The significance of this is illustrated by the ~150 identified mutations in GK (Gloyn, 2003; Matschinsky, 2002). Activating mutations cause GK-linked persistent hyperinsulinemic-hypoglycemia. Loss of function mutations result in diabetes; with more severely inactivating mutations or deletions resulting in GK-linked neonatal diabetes and less severely inactivating mutations causing MODY type 2.
Figure 1.1: Regulation of mammalian insulin secretion. (A, left) at low glucose, β-cell [ATP]/[ADP] is low, the cell’s K<sub>ATP</sub>-channels are open, membrane potential (V<sub>m</sub>) is hyperpolarized, voltage-dependent Ca<sup>2+</sup> channels (VDCCs) are closed, and minimal insulin is secreted. Rises in blood glucose (A, right) result in entry of glucose into the cell through glucose transporters (in rodents, Glut2 as seen in this diagram), conversion of glucose to G6P by glucokinase, and its subsequent breakdown in glycolysis. Entry of glycolytic products into mitochondria increases ATP by oxidative phosphorylation and the resulting rise in ATP and fall in ADP from glycolysis and mitochondrial respiration inhibits K<sub>ATP</sub> channels. The rise in glucose also triggers opening of the SWELL1 channel. Closure of K<sub>ATP</sub> triggers depolarization of the plasma membrane, opening of VDCCs and entry of calcium into the cell. The influx of calcium triggers insulin release. Oscillations in V<sub>m</sub>, intracellular calcium, and insulin release with increased glucose concentrations (illustrated in (B)), may result from the calcium-induced alterations in mitochondrial membrane potential (decreasing ATP production) as well as opening of the K<sub>V</sub> and K<sub>Ca</sub> channels which act to hyperpolarize the plasma membrane. As ATP production falls, K<sub>ATP</sub> channels open, the cell hyperpolarizes and the K<sub>V</sub> and VDCCs close. The fall in calcium restores mitochondrial membrane potential hyperpolarization, closes K<sub>Ca</sub> channels, glucose is metabolized, ATP increases, and the cycle begins again. This cycle repeats until peripheral actions of insulin lower blood glucose. At high glucose concentrations (25mM, B), the oscillations are so close that they essentially plateau.
Glucose is metabolized in subsequent steps of glycolysis (Keane and Newsholme, 2014; Newsholme et al., 2014) creating a net increase in ATP and fall in ADP. Subsequent metabolism of pyruvate in mitochondria generates further ATP via the electron transport chain (Hüttemann et al., 2007; Newsholme et al., 2014). Change in [ATP]/[ADP] ratio is sensed by K_{ATP} channels: they are activated by Mg-ADP and inactivated by ATP (see next section); thus, increased [ATP]/[ADP] ratio causes channel closure (Koster et al., 2006; Nichols, 2006) and consequent membrane depolarization.

β-cell membrane potential is coordinated by multiple ion channels (reviewed in Drews et al. (Drews et al., 2010)). A major hyperpolarizing K^+ current is generated by K_{ATP} channels (discussed below). Other significant K^+ currents that regulate subsequent action potential properties are provided by voltage-activated K^+ (K_V) channels which are inactive at resting membrane potential of -70mV); and calcium activated K^+ (K_{Ca}) channels which are closed at resting cytosolic calcium concentrations (Drews et al., 2010). SWELL 1 has recently been implicated as a significant Cl^- channel and is likely the previously suggested volume-sensitive anion channel (VSAC) identified in earlier studies in the 1990s (Kang et al., 2018). The transient-receptor potential channels (TRP channels) are important non-selective cation channels that contribute to incretin-induced depolarization and contribute to calcium oscillations (Drews et al., 2010). Multiple voltage-dependent Ca^{2+} channels (VDCCs) contribute to calcium entry into islet cells. L-type VDCCs (particularly Ca_{V}1.2) are clearly involved in insulin secretion, as evidenced by studies showing that inhibitors or genetically induced activity causing reductions in insulin secretion to basal levels(Drews et al., 2010).

In broad strokes, the closure of K_{ATP} channels induces membrane depolarization (Drews et al., 2010). The rise in V_{m} results in activation of VDCCs and Na_{V}, and V_{m} rises to a plateau potential, from which groups of action potential spikes occur, accompanied by intracellular Ca^{2+} elevation. Calcium entry through VDCCs triggers release of Ca^{2+} from the endoplasmic
reticulum, a process referred to as calcium-induced calcium release. The rise in intracellular calcium concentration triggers insulin vesicle fusion to the membrane and subsequent release. The VDCC-driven rise in calcium and the depolarized $V_m$ activate $K_{Ca}$ and $K_V$, respectively (Drews et al., 2010), which results in hyperpolarization of $V_m$ and consequent closure of VDCCs and lowering of intracellular calcium.

Oscillations in membrane potential, intracellular calcium, and insulin release have been observed in $\beta$-cells and islets incubated with stimulatory glucose concentrations (8-15mM) (Drews et al., 2010) (Figure 1.1). The frequency of oscillations, and the fraction of time spent in the active state, are dependent on the concentration of extracellular glucose, beginning at ~8mM and reaching a plateau of almost continuous electrical firing above 25mM glucose (Drews et al., 2010). Electrical activity across the $\beta$-cells in the islet is synchronized by the presence of gap junctions formed by connexin 36 (Perez-Armendariz, 2013).

1.4 Molecular basis of $K_{ATP}$ functions- subunit composition. $K_{ATP}$ channels are hetero-octomers composed of 4 pore-forming Kir6.x subunits, with each having an accessory Sulfonylurea receptor (SURx) subunit (Figure 1.2) (Nichols, 2006). There are two Kir6.x proteins (Kir6.1 and Kir6.2) and two SURx proteins (SUR1 and SUR2) (Nichols, 2006). Kir6.2 and SUR1 are encoded in the same locus of chromosome 11 by KCNJ11 and ABCC8, respectively (Nichols, 2006). Kir6.1 and SUR2 are encoded similarly on chromosome 12 by KCNJ8 and ABCC9, respectively (Nichols, 2006). Kir6.x and SURx subunits each have ER-retention signals (RKR sequences) that are masked by the association of Kir6.x and SURx, so that functional channels only assemble when the pore forming and accessory subunits are co-expressed (Crane and Aguilar-Bryan, 2004). While each pair of Kir6 and SUR subunits are typically co-expressed, the channels can be formed by any combination of the pore-forming and accessory subunits (Kir6.1/SUR2, Kir6.2/SUR2, Kir6.1/SUR1, Kir6.2/SUR2, or mixtures thereof). Each of the combinations has been detected in tissue-specific expression patterns. Kir6.2/SUR1
Figure 1.2: \(K_{ATP}\)-channel subunit composition and regulation. \(K_{ATP}\) channels (A), shown from the top (left) and membrane view (right), are hetero-octomers composed of 4 pore-forming Kir6.x subunits, each having its own accessory SURx subunits. (B) Kir6.2 and SUR1 are encoded on chromosome 11 by \(kcnj11\) and \(abcc8\), respectively, whereas Kir6.1 and SUR2 are similarly encoded on chromosome 12 by \(kcnj8\) and \(abcc9\), respectively. SUR2 is spliced into two primary variants (SUR2A and SUR2B) which differ in the incorporation of an alternative final exon 38 (B). A side-view (C) of the \(K_{ATP}\)-channel complex illustrates the binding site for ATP on Kir6 subunits (causing inhibition of the channel) and the hydrolytic cycle of magnesium adenonucleotides in the nucleotide binding domains of SUR subunits, which produce the magnesium ADP that activates the \(K_{ATP}\) channel via SURs. Activation by Mg-ADP and inhibition by ATP intrinsically link the channel activity to the metabolic state of the cell.
forms the primary $K_{ATP}$ channels in pancreatic endocrine cells (Koster et al., 2006), neurons (Shimomura et al., 2007), and cardiac atria (Flagg et al., 2008). Kir6.1/SUR2B forms the primary $K_{ATP}$ channels in the vascular and lymphatic smooth muscle tissue (Flagg et al., 2010; Garner et al., 2017). Kir6.2/SUR2 forms the primary channels in skeletal muscle, ventricular cardiomyocytes and sinoatrial node cells (Flagg et al., 2010). All four subunits have been detected in microglia under different circumstances (Rodríguez et al., 2013). The expression patterns of the $K_{ATP}$ subunits give rise to a spectrum of consequences of mutations in the channel: Kir6.2 and SUR1 are implicated in disorders of the pancreas and the central nervous system (Ashcroft, 2005; Koster et al., 2006; Koster et al., 2002); Kir6.1 and SUR2 subunits are implicated in diseases of the cardiovascular system (Nichols, 2016).

1.5 Regulation of $K_{ATP}$ channel activity in pancreatic $\beta$-cells. $K_{ATP}$ channels are differentially regulated by ATP and ADP in the presence or absence of magnesium. ATP, in the presence or absence of Mg, binds to the pore-forming Kir6.2 subunits with IC50 for ATP on Kir6.2 \( \sim 5-10 \mu M \) (Flagg et al., 2010) to inhibit the channel. Similar, but \(~10\)-fold less potent inhibition by ADP (in the absence of magnesium) and by non-hydrolyzable ATP analogues argue for direct electrostatic interactions of the phosphate moieties of ATP with the Kir6.2 subunit as the mechanism for inhibition (Flagg et al., 2010). The binding site for ATP, identified through mutagenesis, disease mutations, and now confirmed by cryo-EM structures (Lee et al., 2017; Martin et al., 2017b), is formed by several N and C terminal cytoplasmic residues (Flagg et al., 2010). Magnesium-bound nucleotides interact with nucleotide binding folds (NBFs) of the Sulfonylurea receptors to activate the channel (Flagg et al., 2010). Mg-dependent ATP hydrolysis occurs at the NBF2 domain (Vedovato et al., 2015). The open probability of the channel is thus reflective of the effects of both ATP and Mg-ADP on the channel: the ratio controls channel activity. $K_{ATP}$ channel activity is modulated by other factors, particularly phosphoinositides such as PIP2, which shift ATP sensitivity to higher concentrations, resulting
in increasing activation of the channel when added to excised patches (Shyng and Nichols, 1998).

\[K_{ATP}\] channels are the targets of many pharmacological modulators that bind to the SUR subunits (Martin et al., 2017a). Diazoxide activates SUR1-containing channels preferentially over SUR2-containing channels (Hambrock et al., 2004). Pinacidil acts preferentially on SUR2-containing \[K_{ATP}\]-channels (Russ et al., 2003). Given the predominance of SUR1 in the \(\beta\)-cells, diazoxide activation is sometimes beneficial in treatment of hyperinsulinemia resulting from loss-of-function \[K_{ATP}\] mutations (Sweet et al., 2013). \[K_{ATP}\] channels are inhibited by sulfonylurea drugs (glibenclamide, tolbutamide, glimepiride, and others)(Hambrock et al., 2002; Kharade et al., 2016; Koster et al., 1999b). Inhibition of \[K_{ATP}\] triggers insulin secretion, hence sulfonylureas have long been used in treatment of multiple types of diabetes.

### 1.6 \[K_{ATP}\] channel mutations result in metabolic disease

The importance of \[K_{ATP}\] channels to blood glucose regulation is highlighted by the diseases resulting from mutations in the channel subunits. Loss of function mutations in pancreatic \[K_{ATP}\] channel subunits result in congenital hyperinsulinism of infancy (CHI)(Loechner et al., 2011; Remedi and Nichols, 2009b; Saint-Martin et al., 2011). These mutations cause hypersecretion of insulin and, as a result, abnormally low glucose (hypoglycemia). The spectrum of symptom severity and the response to treatments such as diazoxide correlates with the degree of loss of function. Mutations which affect the sensitivity of the channels to ATP, but which do not block membrane trafficking (Koster et al., 2005; Shimomura et al., 2006; Snider et al., 2013), can respond to diazoxide treatment (Saint-Martin et al., 2011; Snider et al., 2013). Other mutations (frameshifts, premature stops, and mutations that alter membrane trafficking or protein folding) can result in hyperinsulinism which is refractory to diazoxide therapies, due to the absence of any channels at the membrane to be activated or loss of diazoxide sensitivity (Saint-Martin et al., 2011; Snider et al., 2013).
By contrast, gain-of-function mutations in pancreatic $K_{ATP}$ channels are the most common cause of neonatal diabetes (NDM) (Kataria et al., 2014; Polak and Cave, 2007; Remedi and Koster, 2010), characterized by early-onset (typically within the first few months of life) hyperglycemia due to insufficient insulin secretion. The significance of pancreatic gain-of-function mutations was predicted by early studies in mice with severe $K_{ATP}$ channel mutations (Koster et al., 2000), and confirmed later in patients in the early 2000s (Gloyn et al., 2004; Vaxillaire et al., 2004). NDM is a relatively rare disorder (1 in ~100,000-300,000 births) (Polak and Cave, 2007; Proks, 2013; Remedi and Koster, 2010); however, as $K_{ATP}$ mutations were identified only relatively recently, there may be many individuals previously diagnosed with type 1 diabetes who may in fact be suffering from NDM.

Some gain-of-function mutations result in decreased sensitivity to ATP in the pore-forming Kir6.2 subunits (Ashcroft, 2005); some SUR1 mutations can disrupt channel gating and decrease sensitivity to ATP whereas others can result in increased activation by ADP (Proks, 2013). Severity of disease frequently correlates with the severity of the mutation (Flanagan et al., 2006): the greater the reduction in ATP sensitivity or increase in ADP activation, the more severe the diabetes and the higher the dose of sulfonylurea that is required (Ashcroft, 2005; De Leon and Stanley, 2008; Patch et al., 2007). More severe mutations result in neurological side effects which include epilepsy and developmental delay in addition to neonatal diabetes, a syndrome called Developmental delay, Epilepsy, and Neonatal Diabetes (DEND) (Ashcroft, 2005; Proks, 2013; Shimomura et al., 2007). Less severe mutations generally do not present with secondary neurological effects, and in some cases, cause only transient diabetes that resolves in the first few months (Ashcroft, 2005; Marshall et al., 2015; Proks, 2013), with some affected individuals relapsing later in life (Vaxillaire et al., 2007; Yorifuji et al., 2005).

However, not all of the variation in symptom severity and treatment effectiveness is mediated by the severity of the mutation itself. There are several examples (Küçükemre Ay et
al., 2012; Marshall et al., 2015; Wambach et al., 2010) of individuals with identical mutations, and in some cases in the same family, who require much higher doses of sulfonylurea drugs than others to achieve glucose control. Finally, there are some mutations, particularly E23K in Kir6.2 (Nielsen et al., 2003; Schwanstecher et al., 2002; Villareal et al., 2009) and S1369A in SUR1 (Fatehi et al., 2012), which are not associated with neonatal diabetes, but are associated with increased risk of developing T2D later in life. These examples illustrate that despite the clear link between $K_{ATP}$ channel mutations and β-cell dysfunction, there must be additional factors influencing the outcome of insulin secretory defects and hyperglycemia that remain to be elucidated.

### 1.7 Secondary consequences of severe hyperglycemia: β-cells

Prolonged exposure to high glucose levels produces alterations in tissue function and cell viability, and poorly controlled diabetes therefore results in a number of secondary consequences (Aronson, 2008), including multiple direct effects on pancreatic islets. Several studies have indicated that prolonged exposure to high glucose, independent of food intake and other factors, results in β-cell dysfunction (Remedi and Emfinger, 2016). While initial loss of functional β-cell mass has frequently been attributed to β-cell death, more recent studies have suggested that loss of β-cell identity may play a more significant role (Bensellam et al., 2018; Brereton et al., 2014a; Cinti et al., 2016; Hunter and Stein, 2017; Remedi and Emfinger, 2016; Talchai et al., 2012; Wang et al., 2014).

β-cell function requires appropriate expression of relevant glycolytic enzymes, ion channels, insulin biosynthesis, gap junctions, as well as trafficking of insulin granules for normal secretory responses. β-cell identity is determined by expression of several key transcription factor pathways (Bensellam et al., 2018). Of particular importance to mature β-cells are the transcription factors MafA and MafB (MafA and MafB switch in mouse; they are co-expressed in
human β-cells), Pdx1, Nkx6.1, FoxO1, and Neurod1. Endocrine precursor cells, in contrast, express Neurogenin 3 (NGN3), Pax4, Pdx1, and FoxO1. Pancreatic α-cells express MafB, Arx, Irx1, Irx2, Neurod1, FoxO1, and glucagon. The expression of these different transcription factors and other proteins thereby functionally determines the identity of the pancreatic endocrine cells (Bensellam et al., 2018; Remedi and Emfinger, 2016). Transcription factor activity varies across cell populations and with age, stress and other factors (Talchai et al., 2012). Many of these genes are modulated by normal fluctuations in glucose levels (Bensellam et al., 2018). Specific deletion of these factors, for example FoxO1 (Talchai et al., 2012), can compromise insulin synthesis and alter transcription factor expression as well as β-cell function, without affecting cell survival.

In prolonged hyperglycemia, as results from KATP-gain-of-function diabetes (Brereton et al., 2014a; Remedi et al., 2009; Wang et al., 2014), expression of PDX1 and NIK6.1 is reduced, and the lineage precursor factor NGN3 is increased. The loss of identity, but in the absence of significant β-cell death, results in decrease of insulin synthesis, and eventual disappearance of insulin (Brereton et al., 2014a; Remedi and Emfinger, 2016; Talchai et al., 2012; Wang et al., 2014), with infiltration of glucagon-producing α-cells into the core of the islet (Brereton et al., 2014a; Wang et al., 2014). While some studies suggest the latter might occur as a consequence of trans-differentiation of β-cells into α-cells or bi-hormonal cells (Brereton et al., 2014a), others do not show significant numbers of bi-hormonal cells, nor overlap in the cell populations (Wang et al., 2014). Similarly, in db/db and GIRKO mice, which model insulin-resistant diabetes, diabetes is followed by loss of insulin staining, loss of FoxO1, and increased Oct4, L-Myc, and NGN3 (Talchai et al., 2012). Specific treatment of diabetic islets and isolated β-cells with inflammatory factors results in reduced FoxO1 expression, though this is only partly responsible for the dedifferentiation seen in hyperglycemia (Nordmann et al., 2017).
These consequences of persistent hyperglycemia can be prevented or reverted by normalization of glucose, either with insulin (Wang et al., 2014) or sulfonylurea therapy (Brereton et al., 2014a; Wang et al., 2014), or by syngenic wild-type islet transplantation under the kidney capsule (Remedi et al., 2009). Lineage tracing analysis demonstrated that restoration of β-cell mass is not a result of growth or proliferation of new β-cells from progenitors, but instead that the same dedifferentiated cells can re-differentiate to mature β-cells upon lowering of blood glucose (Brereton et al., 2014a; Wang et al., 2014). Interestingly, human T2D is associated with alterations in these same differentiation markers (Cinti et al., 2016; Talchai et al., 2012), suggesting this is not unique to rodents, though the degree and significance of dedifferentiation in human diabetes is still uncertain (Remedi and Emfinger, 2016).

1.8 Consequences of severe hyperglycemia in non-islet tissues. Severe hyperglycemia is known to have severe side effects in non-islet tissues, including reduced skeletal muscle function and muscle strength (Lime-Ma et al., 2017), adipose tissue inflammation (Chang and Yang, 2016; Esposito et al., 2002; Kanter et al., 2012; Lin et al., 2005; Pahwa and Jialal, 2016), as well as poor wound healing. This and the increase in inflammatory factors may be due to alterations in protein glycosylation and glucose action on macrophages, other cells in the immune system, and defective recruitment of endothelial cells to wound sites (Brem and Tomic-Canic, 2007; Terranova, 1991; Yang et al., 2016). Severe hyperglycemia is associated with altered neurological function (Malone, 2016). In severe hyperglycemia, excess urine is secreted and prolonged hyperglycemia can induce kidney damage and failure (Nasri and Rafieian-Kopaei, 2015; Reidy et al., 2014). Additionally, dysregulation of lipid handling due to insufficiency of insulin has been linked to dysregulation of cellular pH and a rise in ketone bodies, referred to as diabetic ketoacidosis (Westerberg, 2013). This is accompanied by polyurea, weight loss, fatigue, vomiting, abdominal pain, and, if improperly treated, death (Westerberg, 2013).
1.9 Major questions to be addressed in this thesis

The deleterious consequences of hyperglycemia in \( \beta \)-cells and other tissues illustrate the significance of normal glucose control. \( K_{ATP} \)-driven diabetes, as a monogenic disorder with a clear link between genetic mutations and insulin secretion defects, offers the possibility to identify modulatory pathways and factors independently of the complex etiologies of autoimmune-driven and insulin resistance-driven diabetes. Understanding whole-body responses to \( \beta \)-cell inexcitability driven by \( K_{ATP} \)-GOF mutations forms the scope of the work described in this thesis, organized around two major questions:

1.9.1 What are the mechanisms of remission in the context of \( K_{ATP} \)-driven diabetes (Chapter 2)?

As previously mentioned, the variation in symptom severity in individuals even with defined monogenic causes of diabetes nonetheless presents a challenge both to understanding both the complex evolution of the disease and developing treatments for it. That these variations can occur within families in individuals with the same mutation belies additional genetic and environmental factors which remain to be elucidated. A specific instance of variable outcome (either remission or permanent, progressive diabetes) has been identified in \( \beta \)-cell specific, inducible \( K_{ATP} \)-GOF mice (Remedi et al., 2011). When the \( K_{ATP} \)-GOF transgene is activated by Cre recombination induced by tamoxifen-binding to CreER\(^{T2} \) under the control of the PDX1 promoter, a rapid and profound hyperglycemia results (Remedi et al., 2011; Remedi et al., 2009). After early, short-term glibenclamide treatment, these animals exhibit two very different phenotypic outcomes (Remedi et al., 2011): most will become diabetic (non-remitter) following the end of glibenclamide treatment, but some do remit, and are able to regain significant control of glucose regulation (remitters) and remain below the blood glucose level at which severe secondary consequences (glycosuria, weight loss, ketoacidosis, changes in body
composition) occur. This is not due to differences in gene activation by tamoxifen or other factors, as islets from remitter animals show equivalent transgene marker expression and remain insensitive to glucose changes; this argues that an unknown pathway regulates responses to $K_{ATP}$-GOF in the remitting mice (Remedi et al., 2011). The study described in chapter two explores the pathways underlying the remission phenotype in the $K_{ATP}$-GOF mice.

1.9.2 What regulators of insulin secretion are conserved in non-mammalian vertebrates (Chapters 3 and 4)? The significance of insulin secretion in mammals and its regulation are well established. Whether control of insulin secretion, particularly excitability-driven regulation by $K_{ATP}$ channels and calcium oscillations, are conserved in and significant for non-mammalian vertebrates has not been well studied. Zebrafish are becoming more frequently used to study tissue regeneration (Moss et al., 2009; Moss et al., 2013) (including the pancreas), genetics and evolution (Postlethwait et al., 2000; Santoriello and Zon), metabolism (Fang et al., 2014; Kinkel and Prince, 2009; Li et al., 2014a), and organogenesis (Huang et al., 2001a; Lieschke and Currie, 2007). Given the significance of excitability-dependent control of insulin secretion in mammals, it is important to establish what role this pathway plays in zebrafish, both for understanding evolutionary conservation of these elements and extrapolating findings from zebrafish metabolic studies to mammals.

The variable outcomes in $K_{ATP}$-induced diabetes in mammals suggest that additional unknown factors or pathways may modulate consequences of $\beta$-cell membrane inexcitability. While specific instances of remission in mouse models of diabetes may inform mechanisms of remission of diabetes in humans, more comprehensive approaches to screening relevant mechanisms are a challenge. One approach to determining genetic modifiers is via forward genetic screens (Lieschke and Currie, 2007), for which zebrafish, due to their rapid and prolific reproduction, larval transparency, annotated genome, genetic similarity to vertebrates, and established mutagenic methods provide a uniquely suitable model organism (Lieschke and
Currie, 2007; Patton and Zon, 2001). Therefore, establishing the role of excitability in insulin secretion in zebrafish may be useful not only in understanding evolutionary conservation of these components but also in developing new approaches for identifying modifiers of excitability-driven and other forms of diabetes. *The studies discussed in chapters three and four explore the role of $K_{\text{ATP}}$ channels in the regulation of glucose homeostasis in zebrafish, regarding both native expression and function of the channels in zebrafish islet $\beta$-cells, as well as the consequences of $K_{\text{ATP}}$-GOF mutations in zebrafish $\beta$-cells.*
Chapter 2: Mechanisms underlying remission or persistent $K_{\text{ATP}}$-induced neonatal diabetes

Adapted from: Emfinger et al. 2018 “Mechanisms underlying remission or persistent $K_{\text{ATP}}$-induced neonatal diabetes” AJP Endo & Met - under review

Author Contributions:

I designed the studies here and performed the experiments. Colin Nichols and Maria Remedi helped design and perform the studies. Zihan Yan, Alecia Welscher, and Peter Hung also helped perform the studies. I wrote the manuscript. Maria Remedi and Colin Nichols reviewed and edited the manuscript. Paul Hruz reviewed the manuscript and provided comments.
2.1 Introduction

ATP-sensitive potassium (K\textsubscript{ATP}) channels play a critical role in many tissues by linking metabolism to electrical activity, and in pancreatic islet β-cells, thereby promoting insulin secretion (Nichols, 2006). As blood glucose rises, K\textsubscript{ATP} channels close and the β-cell plasma membrane depolarizes, with consequent opening of voltage-dependent calcium channels, calcium influx, which, in turn, triggers insulin secretion. Gain-of-function (GOF) mutations in the K\textsubscript{ATP} channel have been identified as the main cause of human neonatal diabetes (NDM) (Gloyn et al., 2004). In this case, K\textsubscript{ATP} channels remain open despite rising blood glucose and these patients develop diabetes. K\textsubscript{ATP} variants have been linked to development of type-2 diabetes (Riedel et al., 2005; Villareal et al., 2009). Patients with K\textsubscript{ATP}-GOF mutations are now treated with sulfonylurea (SU) drugs, widely used to treat type-2 diabetic patients, which pharmacologically close K\textsubscript{ATP} and stimulate insulin secretion (Marshall et al., 2015; Pearson et al., 2006).

One challenge to both understanding the underlying physiology of neonatal diabetes and increasing the effectiveness of available treatments is the significant variability of disease severity between patients (Ellard et al., 2007; Flanagan et al., 2006; Gloyn et al., 2005; Marshall et al., 2015; Pearson et al., 2006; Remedi and Koster, 2010; Shimomura et al., 2006; Vaxillaire et al., 2007; Wambach et al., 2010), which occurs even in individuals within the same family with the same mutation (Marshall et al., 2015; Wambach et al., 2010). Some individuals presenting with neonatal diabetes will enter remission within the first few months of life (transient NDM), whereas others require lifelong treatment (permanent NDM); however, underlying mechanisms remain elusive (Koster et al., 2005; Patch et al., 2007). We have previously generated a mouse with an inducible, β-cell specific K\textsubscript{ATP}-GOF mutation (K\textsubscript{ATP}-GOF mice) which rapidly develops severe diabetes upon tamoxifen induction (Remedi et al., 2009), reiterating many features of NDM. However, if these mice are treated for 6 days with the SU glibenclamide, a subset regain
control of blood glucose that lasts long-after the SU treatment ends (remitters), although the remainder become severely diabetic when treatment stops (non-remitters) (Remedi et al., 2011). With this differential outcome, these mice offer the exciting possibility to study underlying causes of transient vs. permanent NDM.

We previously demonstrated improved peripheral insulin sensitivity in remitter animals long after blood glucose separation (Remedi et al., 2011), but whether this was a cause or consequence of the differential outcome was not studied. To identify potential mechanisms underlying remission of diabetes, we have therefore studied K\textsubscript{ATP}-GOF mice before and after disease induction. We show here that large differences in insulin sensitivity between remitted and non-remitted animals are a consequence, rather than the cause, of the differences in blood glucose. Various potential candidate pathways that could be involved in remission do not change with diabetes progression, but alterations in inflammatory markers, particularly interleukin-6 (IL-6), correlate with the separation in glucose between the groups early in disease progression, and may represent the underlying mechanism of the remission phenotype.

2.2 Methods

2.2.1 Animal lines and maintenance Rosa26-Kir6.2[K185Q, ΔN30] mice were previously generated and crossed with Pdx1-Cre mice to generate β-cell specific K\textsubscript{ATP}-GOF mice (Remedi et al., 2009). These mice will not express the K\textsubscript{ATP}-GOF transgene until being treated with tamoxifen. All procedures were approved by the Washington University Institutional Animal Care. To generate pure remitter and non-remitting lines, remitted K\textsubscript{ATP}-GOF mice (blood glucose < 350 mg/dL following tamoxifen induction and SU treatment) were in-crossed with other remitted K\textsubscript{ATP}-GOF mice over 13 generations, and littermate single transgenic (Pdx1-Cre and Rosa26-Kir6.2[K185Q, ΔN30] mice from crosses generating non-remitting double transgenic animals were in-crossed for more than 13 generations, respectively.
2.2.2 **Gene induction** K$_{\text{ATP}}$-GOF mice were induced by 5 daily injections of 50 mg/kgBW tamoxifen (Sigma T5648) dissolved at 5 mg/mL in corn oil (Sigma C8267). Blood glucose measurements were made at the tail vein using OneTouch Ultra glucometers. Groups treated were injected with 50 mg/kgBW glibenclamide (Sigma, 20mg/mL stock in DMSO, final concentration 5mg/mL in PBS) for 14 days beginning at day 0. For the insulin-treated groups, the NovolinR dose was adjusted as indicated in Figure 2.1c. Initial injections of 1x/day (blue line) were insufficient to provide equivalent glucose control to the glibenclamide-treated groups and the dose was adjusted to 2x/day (red line) as indicated for all animals in the group until the final injection on day 14.

2.2.3 **Insulin tolerance tests (ITTs)** ITTs were performed as described previously (Remedi et al., 2011). Briefly, K$_{\text{ATP}}$-GOF and littermate control mice were fasted for 6 hours prior to the study. Mice were intraperitoneally injected with 0.5 mU/gBW insulin (NovolinR) diluted in saline, and blood glucose measurements by tail vein were performed at 0, 15, 30, 45, 60, 90, and 120 minutes post-injection using OneTouch Ultra glucometers.

2.2.4 **Glucose tolerance tests (GTTs)** GTTs were performed as previously described (Remedi et al., 2011). Briefly, K$_{\text{ATP}}$-GOF and littermate control mice were fasted overnight prior to the study. Mice were injected with 1.5 g/kgBW glucose dissolved in saline solution. Blood glucose measurements by tail-vein were performed at 0, 15, 30, 45, 60, 90, and 120 minutes post-injection using OneTouch Ultra glucometers.

2.2.5 **Fasting-refeeding study.** K$_{\text{ATP}}$-GOF mice were fasted overnight (16hrs) and blood glucose was measured before (time 0) and 1 hour after being provided ad lib access to chow food.

2.2.6 **Metabolic profiling.** Animals were induced as described above. Comprehensive metabolic, behavioral, and physiological variables were determined by TSE/PhenoMaster at
14 days after the first dose of tamoxifen, mice were placed into individual PhenoMaster cage units for measurement of oxygen (O$_2$) consumption, carbon dioxide (CO$_2$) generation, physical activity, and food intake. They were allowed to equilibrate for 2 days and then data were collected for 4 days and then analyzed in R. Food intake was considered as the amount of food consumed per gram of mouse lean body mass per 12 hours. The respiratory exchange ratio (RER) was calculated as the ratio between the amount of CO$_2$ produced in metabolism and the O$_2$ used. Metabolic efficiency was calculated as the ratio between body weight and food consumption.

2.2.7 Hyperinsulinemic-Euglycemic (HIEG) clamp HIEG clamp was performed as previously described (Remedi et al., 2011). Briefly, five days prior to the study, mice underwent surgeries to implant catheters into the jugular vein (for infusion) and femoral artery (for blood sampling). Surgeries were performed in the Hope Center Animal Surgery Core at the Washington University. Insulin (Novolin R) was dissolved in saline solution and administered at 4 mU/kgBW/min (2 µL/min). Glucose (50% dextrose) was administered at a variable rate to maintain blood glucose at 100 mg/dL, as measured using OneTouch Ultra glucometers from the femoral artery sampling line. Plasma samples were taken at 0, 120, and 145 minutes for hormone and metabolite analyses.

2.2.8 Sequential plasma hormone and lipid measurements Blood samples were taken from the tail vein at 0, 5, and 14 days post tamoxifen induction for measurement of glucose, lipids, and plasma hormones. Blood was collected in heparinized tubes, with protease inhibitor cocktail containing 1µL each of benzamidine-hydrochloride (3.6mg/mL), aprotinin (Sigma, 1mg/mL), Sitagliptin (1mM in DMSO) and EDTA (25mM). Plasma lipids were measured at Diabetes Models Phenotyping Core, Washington University (http://diabetesresearchcenter.dom.wustl.edu/diabetes-models-phenotyping-core/). Plasma
hormones were measured using Luminex-Milliplex Mouse Metabolic Hormone panel at Vanderbilt Hormone Assay Core (http://hormone.mc.vanderbilt.edu/) and the Andrew and Jane Burskey Center for Human Immunology and Immunotherapy Program at Washington University (http://chiips.wustl.edu/iml-core-lab.html).

2.2.9 Anti-inflammatory treatment with Meloxicam Two independent cohorts of K\textsubscript{ATP}-GOF mice were treated with vehicle or meloxicam for 7 days beginning one day prior to the 6-day tamoxifen and glibenclamide injection. Twenty-four K\textsubscript{ATP}-GOF mice were treated with Meloxicam (20mg/gBW) and seventeen with vehicle (DMSO, 1ul/gBW). Blood glucose was followed over-time; and pyruvate tolerance test was performed at day 0 (before treatment) and at day 7 after meloxicam treatment.

2.2.10 Statistics Samples were analyzed by Tukey’s post-test following one-way ANOVA at the indicated time points, except where specifically indicated in the figure legends. Significant differences of *p < 0.05 are indicated in the figures, and non-significant differences are annotated with their specific values where noted. All data are represented as means ± standard error, except where specifically noted.

2.3 Results

2.3.1 Aggressive sulfonylurea therapy in K\textsubscript{ATP}-GOF mice can cause remission of permanent diabetes. Two-three month-old K\textsubscript{ATP}-GOF and littermate control mice were injected daily for 5 consecutive days with tamoxifen to induce transgene expression (Remedi et al., 2009). Within two weeks after induction, otherwise untreated K\textsubscript{ATP}-GOF mice develop severe diabetes, whereas littermate control mice are unaffected. We previously demonstrated that a short period of glibenclamide treatment (6 days) initiated at the same time as tamoxifen injection, results in two distinct outcomes: one group of K\textsubscript{ATP}-GOF became severely diabetic (blood glucose > 350mg/dL) after treatment ended (non-remitters), the others remained with blood glucose concentration of <350mg/dL (remitters), even long-after glibenclamide treatment
ended (Remedi et al., 2011). In initial breedings, $K_{\text{ATP}}$-GOF offspring of induced remitted mice showed a higher percentage of remitters when induced and treated with glibenclamide, whereas $K_{\text{ATP}}$-GOF offspring generated from single-transgenics from a non-remitter line produced non-remitting mice, which suggested a potential modifier gene as basis for the divergent outcomes. We therefore repeatedly in-crossed remitters, as well as littermates of non-remitting animals, in an attempt to generate lines with consistently remitting or non-remitting mice. Non-remitter lines did indeed breed true by this protocol (Fig. 2.1a, empty squares) and remitters generated a higher proportion of remitters in later (13+) generations (black circles) when compared to randomly generated $K_{\text{ATP}}$-GOF animals (Remedi et al., 2011), although 100% remittance was not achieved. Increasing the treatment with glibenclamide to 14 days did not increase remission rate, and instead resulted in the appearance of remitting animals in the ‘non-remitter’ line (Figure 2.1b). Failure of convergence of the phenotypes after many generations is inconsistent with a simple or purely genetic underlying basis. It is important to note that the remitter/non-remitter phenotype showed no sex differences (Figure 2.S3) and appeared even in older animals (Figure 2.1c, blue traces).

2.3.2 Remission is a consequence of lowering of blood glucose. Our interpretation of the remittance following glibenclamide treatment is that glibenclamide stimulated insulin secretion and slowed the rate of glucose elevation in remitted mice, but sulfonylurea drugs can have additional actions unrelated to inhibition of $K_{\text{ATP}}$ channels (Zhang et al., 2009). To test whether off-target effects of sulfonylureas underlay the remission phenotype, a cohort of $K_{\text{ATP}}$-GOF animals was treated with insulin injections (doses as indicated in Figure 2.1c, protocol upper panel) at the same time as initiation of tamoxifen injections, in a parallel protocol to the 14-day glibenclamide treatment. Insulin treatment, despite providing less precise control of blood
Figure 2.1 The remitter phenotype is not purely genetic. (A) Multiple generations of back-crossing tamoxifen induced remitting animals failed to consistently reach 100% remission by the original induction protocol (3-4 separate branches/generation; remitter = filled circles, non-remitter = empty squares). (b) Increasing period of glibenclamide injections to 14 days shows emergence of remission phenotype in line 4 (empty squares in panel a) which had not shown remission previously using the original 6 days of treatment protocol (8 remitters (blue), 4 controls (gray), and 13 non-remitters (red) compiled from several inductions). Animals were 9-12 weeks old at time of induction. (c) Short-term injections of insulin (varied in dose in an attempt to sustain lower blood glucose; dosage switched from 1x/day to 2x/day after the 2nd day), can result in remission (6 non-remitters (red), 3 remitters (blue), and 2 controls (gray) from two cohorts). Animals in the insulin experiment were 20-25 weeks old. Animals in the extended glibenclamide experiment (b) were 9-12
glucose, resulted in a similar proportion of remitting and non-remitting animals to glibenclamide treatment (Figure 2.1c), indicating that indeed the remission process was a result of blood glucose lowering, rather than any off-target effects of glibenclamide.

2.3.3 Changes in insulin sensitivity alone do not cause remission. Our earlier studies (Remedi et al., 2011) indicated lower insulin sensitivity in remitted animals compared to animals that remained diabetic: insulin tolerance and hyperinsulinemic-euglycemic clamp tests demonstrated that remitted animals were more insulin sensitive than control and non-remitter animals, and non-remitters were very insulin resistant. These findings raise the possibility that pre-existing differences in insulin sensitivity between individuals might underlie the remitter vs non-remitter outcomes, but since the insulin sensitivity tests were conducted long after blood glucose separation, the observed differences in insulin sensitivity might be a consequence of the prolonged differences in blood glucose, rather than the underlying cause of it. To test the possibility of pre-existing differences in insulin sensitivity, we have assessed insulin sensitivity before (day 0), near onset of (day 14), and late after (day 48) the blood glucose separation following glibenclamide-treatment in outbred cohorts (Figure 2.2a). It is extremely important to note that, prior to blood glucose separation, we could not predict which phenotype (remitter or non-remitter) K\textsubscript{ATP}-GOF mice would exhibit, therefore we conducted tests in cohorts of mice which were later re-grouped as remitters or non-remitters according to the subsequent phenotype. Because we cannot prospectively group the mice, all studies done prior to glucose separation are survivable procedures.

Glucose tolerance (Figure 2.2b) and insulin tolerance (Figure 2.2c) tests at baseline (day 0) demonstrated no significant differences between remitting, non-remitting and control mice. Blood glucose levels during insulin tolerance tests at day 14 demonstrate that remitters are more insulin sensitive than non-remitters (Figure 2.2d, left panel), but when normalized to the
Figure 2.2 Insulin sensitivity does not solely drive remission (a) Blood glucose curve for remitter (blue, N=9), non-remitter (red, N=15), and controls (gray, N=14) mice. Arrows indicate points at which GTTs, ITTs, or clamps were performed. All animals are 9-12 weeks old. Intra-peritoneal glucose tolerance test (IP-GTT) (b) and intra-peritoneal insulin tolerance test (IP-ITT) (c) performed on remitters (blue, N=8), non-remitters (red, N=10), and controls (gray, N=14) prior induction with tamoxifen. (d, left) Raw blood glucose curve for IP-ITT performed at day 14 post-induction. (d, right) ITT glucose curves normalized for basal glucose level at day 14 post-induction. (e) Hyper-insulinemic, euglycemic clamps at day 48 post-tamoxifen induction. Left panel shows blood glucose levels during the clamp and right panel shows glucose infusion rate (GIR). The clamps were performed on 3 remitters (blue) and 5 non-remitters (red) of the original cohort in (a).
very different baseline glucose between groups, these differences disappear (Figure 2.2d, right panel). At day 48, hyperinsulinemic-euglycemic clamp tests (Figure 2.2e) demonstrate that over the period when blood glucose levels were matched (shaded gray, Figure 2.2e, left panel), the glucose infusion rate (GIR) was much greater in remitters than in non-remitters (Figure 2.2e, right panel), confirming that remitters are significantly more sensitive to insulin than non-remitters, long after induction (Remedi et al., 2011).

Because early differences in basal glucose levels confound the interpretation of standard insulin tolerance tests, we sought an assay for insulin sensitivity that did not measure glucose. During fasting, adipose tissue triglycerides (TG) are hydrolyzed into free fatty (FFA) acids and glycerol, for oxidation and gluconeogenesis in the liver (Saltiel and Kahn, 2001; Savage et al., 2007). Plasma FFA and TG will increase in conditions of insulin resistance, therefore we measured plasma lipids at baseline (day 0), day 5 (1 day after tamoxifen ends) and day 14 (the day after glibenclamide treatment ends). While blood glucose already differs significantly between the groups at day 5 (Figure 2.3a), differences in FFA, TG, and cholesteryl esters (Figure 2.3b, c, and d) appear only at day 14. As expected, mice treated with vehicle alone show a more rapid rise in blood glucose upon tamoxifen induction (>600 mg/dL by day 5 after the study started, data not shown), accompanied by an increase in free fatty acids (>2.5mM, data not shown) compared with glibenclamide-treated mice. Together, these results suggest that the differences in insulin sensitivity between remitters and non-remitters are the consequence, rather than the cause, of the differential outcome in these animals.

2.3.4 Other glucoregulatory hormones do not differ between remitter and non-remitter animals. One possible explanation for the emergence of the remitter phenotype is the involvement of other hormones that control gluconeogenesis, glucose uptake, and baseline metabolism. Glucagon is a key regulator of gluconeogenesis, and lowering of glucagon or
Dysregulation of lipid metabolism follows changes in blood glucose (a) Blood glucose values taken from animals at day 0, 5, and 14 post tamoxifen induction in groups treated with glibenclamide or vehicle. Plasma free fatty acids (FFA) (b), triglycerides (c), and cholesteryl-esters (d) at day 14 post tamoxifen induction. Animals are 9-12 weeks old. Significant differences * p < 0.05, ** p < 0.01, **** p < 0.0001 are shown in the figures, comparing remitters and non-remitters via post-tests following ANOVA at the indicated time points. n=6 remitters (blue), n=20 non-remitters (red), and n=14 controls (gray triangles) treated with glibenclamide in each panel.
Figure 2.4 Changes in plasma metabolic hormones during induction process. Randomly sampled plasma at day 0, 5 and 14 post tamoxifen induction was analyzed to determine insulin (a), glucagon (b), leptin (c), active GLP-1 (d). All animals were 9-12 weeks old, n=3 remitters (blue), n=4 non-remitters (red) and n=4 controls (gray) for c and d. n=8 remitters (blue), n=9 non-remitters (red) and n=3 controls for a. n=21 remitters (blue), n=24 non-remitters (red), and 15 controls (gray) for b.
antagonism of its receptor is sufficient to prevent diabetes in many animal models of insulin insufficiency, showing promise as a potential therapy in early clinical trials (Bagger et al., 2011; Wang et al., 2015b). Additionally, leptin produced by adipose tissue can alter activity, feeding behavior, energy expenditure, and insulin action, thereby affecting glucose tolerance (Morton and Schwartz, 2011). The incretin glucagon-like peptide 1 (GLP-1) can potentiate insulin secretion and regulate metabolic state in many tissues, including the brain (MacDonald et al., 2002). We tested the levels of these hormones in remitter and non-remitter animals on samples taken at day 0, 5, and 14 post tamoxifen-induction. Insulin levels were not different between groups at early time points, but rapidly dropped below control levels as the disease progressed (Figure 2.4a), consistent with previous findings (Remedi et al., 2011). Glucagon, leptin and active GLP-1 plasma levels, were not different between groups (Figure 2.4b-d), indicating that differential counter-regulatory or modulatory hormone levels are not involved in the differential disease outcome. These data are consistent with an absence of change in body weight (Figure 2.S1) during the early phase (days 0-14) of any of the respective studies in Figures 2.1-2.7, suggesting that major fluctuations in body composition are unlikely to underlie the differential phenotype. Refeeding mice on day 6 after an overnight fast show no major differences in blood glucose between remitter and non-remitter animals an hour after the refeeding (Figure 2.S2), consistent with plasma GLP-1 concentration. Additionally, no differences are noted regarding food intake, movement, energy expenditure, respiratory exchange ratio, or oxygen consumption between groups at day 16 after initiation of the study (Figure 2.S4a-e, respectively).

### 2.3.5 Differential glucose production between remitters and non-remitters

As insulin-driven glucose disposal is not significantly different between the groups early in disease progression, another possible mechanism for divergence of blood glucose between the groups is basal glucose production by the liver. Animals with uncontrolled liver glucose production
Figure 2.5 Changes in inflammation correlate with separation in glucose. Randomly sampled plasma at day 0, 5 and 14 post tamoxifen induction was analyzed to determine TNF-α (A, B), and IL-6 (C, D) in remitters (n=15, blue), non-remitters (n=19, red) and controls (n=17, gray for a-d). Raw concentration traces are shown in a and c for IL-6 and TNF-α, respectively. AUC analyses for IL-6 and TNF-α are shown in b and d respectively. Specific p-values for comparisons between remitters and non-remitters are indicated at the time points on the curve. (*) indicates p < 0.05. All animals were 9-12 weeks old.
Figure 2.6 Basal glucose production before separation in glucose. Raw glucose traces (left) and change in blood glucose levels (right) following IP injection of sodium pyruvate into remitter (n=14, blue), non-remitter (n=70, red), and control (n=10, gray) animals prior to gene induction (a). For A, differences at indicated timepoints by Student's T between remitter and non-remitter animals are indicated. AUC analyses (b) of the change in blood glucose levels in the IP-PTT data in (a), significance evaluated by ANOVA with Tukey's post-tests. All animals were 9-12 weeks old.
become hyperglycemic even though basal and post-prandial insulin are significantly elevated (Michael et al., 2000). Therapies that target basal glucose production can significantly improve diabetic complications in humans and animals, and even eliminate diabetes in some animal models (Rines et al., 2016a; Wang et al., 2015a). To determine whether basal glucose production might differ between remitter and non-remitter animals, mice were given an IP injection of sodium pyruvate (IP-PTT), which is converted in the liver into glucose via gluconeogenesis. The change in glucose (Figure 2.6a, b) produced by the pyruvate injection was significantly reduced in the remitting animals, indicating an intrinsically lower basal rate of glucose production by the liver in remitting mice than in non-remitter and control mice.

2.3.6 Alterations in systemic inflammation predicts remission. Systemic inflammation (such as elevated tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6)) is known to affect islet function, insulin sensitivity and liver glucose production, and is associated with an increased risk of type 2 diabetes (Borst, 2004; Donath, 2014; Pitsavos et al., 2007; Shoelson et al., 2006). Notably, IL-6 was markedly lower at day 5 post tamoxifen-induction in remitter mice, compared with non-remitter and control mice (Figure 2.5a, b), a difference which persisted at day 14. The differences in IL-6 between the groups correlated temporally with the changes in plasma glucose, consistent with a potentially causal role in the separation of blood glucose between remitter and non-remitter animals. Non-remitter mice also showed higher levels of TNF-α at day 0, which remained higher at day 5, compared with remitter and control mice (Figure 2.5c, d). Importantly, KATP-GOF mice treated with vehicle instead of glibenclamide show rises in blood glucose and IL-6, and had elevated at baseline TNF-α, similar to non-remitting animals, again pointing to inflammation as a cause of blood glucose separation.
Figure 2.7 Meloxicam treatment increase the number of remitting animals (a) Blood glucose over-time on K$\text{ATP}$-GOF mice treated with Meloxicam or vehicle. Non-remitters (red) and Remitters (blue). Dashed lines are vehicle treated and solid lines show Meloxicam treated mice. (b) Pyruvate tolerance test (PTT) performed at day 0 and 7 days after meloxicam treatment. Blood glucose values and area under the curve during the PTT at day 0 (c) or at day 7 (d) after meloxicam or vehicle treatment. n=17 K$\text{ATP}$-GOF mice for vehicle and n=24 K$\text{ATP}$-GOF mice for Meloxicam (*) indicates p < 0.05.
2.3.7 Anti-inflammatory treatment with Meloxicam induce diabetes remission To further examine the role of inflammation on diabetes outcome, we treated mice with the anti-inflammatory agent Meloxicam, hypothesizing that lowering inflammation in vivo might induce more animals to remit. It is important to note that Meloxicam has toxic effects at high doses, independent of its anti-inflammatory action. At the dose used (20mg/gBW), the drug resulted in 9/24 and 4/17 deaths in the meloxicam and vehicle groups, respectively. From the surviving animals, the percentage of remitting animals almost doubled (from 23% in vehicle-treated to 40% in meloxicam treated, Figure 2.7a).

Finally, to examine potential interplay of hepatic glucose production and inflammation, we performed pyruvate tolerance test before (day 0) and at day 7 after Meloxicam- or vehicle-treatment. Although Meloxicam lowers fasting blood glucose, the area under the curve for the test was not significantly different between vehicle- and Meloxicam-treated animals (Figure 2.7b-d). This is consistent with Meloxicam keeping blood glucose low by reducing the glucose-mobilizing consequences of inflammation, whilst having no off target effects on liver glucose production directly.

2.4 Discussion

2.4.1 Progressive consequences of K$_{\text{ATP}}$-GOF-induced diabetes. Introduction of K$_{\text{ATP}}$ channel gain-of-function (GOF) in pancreatic β-cells leads to loss of glucose-dependent excitation and insulin secretion, and development of diabetes (Girard et al., 2009; Koster et al., 2000; Remedi et al., 2009). In addition to the immediate loss of glucose-dependent insulin secretion, we have shown loss of insulin content and β-cell mass as disease progresses (Remedi et al., 2009; Wang et al., 2014). Loss of β-cell mass was promoted by loss of β-cell identity and de-differentiation to islet-like progenitor cells (Wang et al., 2014), rather than β-cell death, as frequently assumed Similar outcomes have been reported as secondary consequences in other rodent mouse models of diabetes.
**Figure 2.8 Proposed model of remission in K\textsubscript{ATP}-GOF mice.** In un-induced K\textsubscript{ATP}-GOF mice (left panel), any differences at baseline regarding glucose production and inflammation between subsequent remitter (blue arrows) and non-remitter (red arrows) animals are countered by normal glucose-stimulated insulin secretion, and blood glucose levels are kept in the normal range. In the early phase of induction (middle panel), K\textsubscript{ATP}-GOF blocks insulin secretion in remitter and non-remitter mice and glucose begins to rise, but no differences exist in glucose disposal between the groups. Increased basal glucose production either at baseline or due to early rises in non-remitter inflammatory cytokines, or both, causes a more rapid increase in glucose in non-remitter mice (red arrows). This results in early glucotoxicity that then drives more inflammation and begins to inhibit glucose disposal in a feed-forward manner. In remitter mice basal glucose production and inflammation are low, and maintenance of low inflammatory responses allows for a slower rise in glucose, avoiding early glucotoxicity and enabling later alterations in sensitivity to basal insulin to compensate for gross insulin deficiency. In the late period (day 14+, right panel), glucotoxicity has resulted in diminished glucose disposal in non-remitters, and increased insulin sensitivity in remitters, resulting in persistent divergent outcomes. Known links are solid lines. Proposed links are dashed lines.
(Brereton et al., 2014b; Laybutt et al., 2003; Talchai et al., 2012). Strikingly, these secondary consequences can be prevented by lowering of blood glucose with sulfonylurea therapy, syngenic islet transplantation, or with caloric restriction by pair feeding (Ishida et al., 2017; Remedi et al., 2011; Remedi et al., 2009; Wang et al., 2014); and even reversed by intensive insulin therapy (Brereton et al., 2014a; Wang et al., 2014). Importantly, loss of islet β-cell identity has been demonstrated in pancreas from human diabetic organ donors (Cinti et al., 2016; Guo et al., 2013; Hunter and Stein, 2017) and even de-differentiation of β-cells has been observed in some cases (Cinti et al., 2016). The potential relevance of these findings for developing approaches for reversal is an exciting one, requiring a deeper understanding of underlying mechanisms.

2.4.2 Early treatment and precise glucose control can prevent progressive loss of islet function in neonatal diabetes. $\text{K}_{\text{ATP}}$-dependent NDM can present as permanent (PNDM), or as a transient form (TNDM) in which diabetes in infancy is followed by an unexplained remission, that may or may not revert to frank diabetes in maturity (Koster et al., 2005; Patch et al., 2007). Our previous studies highlight the significance of early intervention in both mice and human NDM: tight glucose control, provided by pharmacological inhibition of $\text{K}_{\text{ATP}}$, can lead to long-term remission (Marshall et al., 2015; Remedi et al., 2011; Wambach et al., 2010). We also demonstrated that maintenance of blood glucose below an apparent threshold around 300 mg/dl is sufficient to maintain insulin content and preserve markers of differentiated β-cell fate in $\text{K}_{\text{ATP}}$-GOF mouse islets (Remedi et al., 2009; Wang et al., 2014). While the islets expressing the $\text{K}_{\text{ATP}}$-GOF themselves remain glucose-unresponsive, basal levels of plasma insulin, as well as responsivity to sulfonylurea stimulation are maintained (Remedi et al., 2009; Wang et al., 2014). Our studies here show that remitting animals retain a high sensitivity to insulin, whereas non-remitting animals rapidly become very insulin resistant. Avoidance of severe hyperglycemia and loss of insulin content, and hence prevention of glucotoxic insulin resistance, in $\text{K}_{\text{ATP}}$-GOF mice
permits maintenance of islet responses to pharmacological secretagogues, consistent with our previous findings (Remedi et al., 2011; Remedi et al., 2009). Together, these findings suggest that interventions early in the disease progression will maintain sulfonylurea-responsive islets in NDM, and thereby lessen dose requirements for sulfonylurea therapies or insulin.

2.4.3 The role of inflammation in insulin resistance and disease progression. We show here that remitter animals have reduced circulating IL-6 and strong trends for reduced systemic TNF-α when compared to non-remitters. The differences in IL-6 between remitters and non-remitters appear at the onset of disease, suggesting that the level of inflammation may play a role in the divergence of blood glucose and supported by the increase in the percentage of remitting animals after treatment with the anti-inflammatory drug Meloxicam. Increased tissue inflammation is found in pre-diabetic obese and diabetic individuals, and elevated systemic inflammation is associated with increased risk of developing type-2 diabetes in a number of studies (Borst, 2004; Donath, 2014; Glund and Krook, 2008; Kim et al., 2009). Inflammation influences a number of different tissues, including fat, muscle, liver, brain, and islets (Donath, 2014; Kim et al., 2009). Elevated free fatty acids, endoplasmic reticulum stress, and increased reactive oxygen species are some of the factors present in hyperglycemic insulin-resistant or insulin-deficient states that can trigger activation of the inflammasome (via NOD-, LRR-, and pyrin domain-containing 3 (NLRP3) or caspase 1), eventually resulting in elevation of TNF-alpha, IL-6, and other cytokines (Donath, 2014). Elevated cytokines in turn can recruit additional immune cells to tissues and cause resident macrophages to adopt a more pro-inflammatory phenotype. TNF-α signaling has been suggested to cause insulin resistance that accelerates the development of type 2 diabetes (Borst, 2004; Lorenzo et al., 2008). Inhibition of TNF-α signaling can prevent or reverse insulin resistance in animals (Borst, 2004), and reduce risk of type-2 diabetes in humans (Borst, 2004; Donath, 2014). The reported relationship between IL-6 levels and glucose metabolism is conflicting. Acute elevations of IL-6 (such as in exercise) are
associated with increased glucose metabolism in muscle in some animal models (Glund and Krook, 2008). However, this seems dependent on increased energy expenditure (exercise) as IL-6 infusions in the absence of exercise are associated with increased muscle accumulation of fatty acyl-CoA, suggesting that chronic IL-6 may impair muscle function (Glund and Krook, 2008). Chronic elevations in IL-6 are associated with increased insulin resistance (Kim et al., 2009), and extreme elevations in IL-6 in some animal models can induce diabetic phenotypes; effects that can be reversed with anti-IL-6 antibodies. This mirrors findings in human patients taking IL-6 antagonists, who show improved metabolic parameters including significantly reduced HOMA-IR (Schultz et al., 2010).

2.4.4 Glucose production and inflammation in neonatal diabetes outcomes. Retrospective analysis reveals that $K_{\text{ATP}}$-GOF mice which remit after early sulfonylurea treatment exhibit significantly lower basal glucose production rates prior to disease onset than those mice that do not remit. One possible explanation is that differences in basal glucose production reflect different levels of inflammation, since chronic elevations in cytokines can alter insulin signaling and increase liver glucose production (Hotamisligil, 2006b; Schmidt-Arras and Rose-John, 2016).

In Figure 2.8, we present a schematic model that we propose can explain the differential outcome in response to early sulfonylurea treatment: in the early period of the disease (middle panel), $K_{\text{ATP}}$-GOF mutations (black-arrows) block insulin secretion in both remitters (blue-arrows) and non-remitters (red-arrows), resulting in a rise in blood glucose. However, remitters exhibit reduced inflammation and lower basal glucose production. As a result, blood glucose rise less quickly in these animals, which thereby avoid early glucotoxicity, enabling a slow increase in sensitivity to remaining basal insulin to counter the loss of glucose-stimulated insulin secretion. By contrast, non-remitting animals (red-arrows) present with increased levels of inflammation and elevated basal glucose production. As a consequence, glucose rises more
quickly causing glucotoxicity, which may further increase inflammation (Montane et al., 2014; Shoelson et al., 2006), driving more glucose production (Okin and Medzhitov, 2016) in feedback cycle. Inflammation is also reported to increase de-differentiation and β-cell dysfunction (Nordmann et al., 2017). At later stages (right), reduced glucose disposal and elevated production is reflected in glucotoxic damage in non-remitters and increased sensitivity to remaining basal insulin in remitters. Meloxicam treatment, by reducing inflammation, prevents elevated glucose production, increasing the number of animals that remit, rather than progressing to the glucotoxic stage.

2.4.5 Conclusions. While the underlying basis of remission after early therapy in experimental NDM remains incompletely understood, our studies clearly demonstrate that early pharmacological intervention in combination with reduced inflammation can induce maintenance of low blood glucose in this animal model which can lead to long-term remission (Remedi et al., 2011). In a clear parallel, we have previously reported a human family, in which the mother and older sibling both carrying the same mutation suffered permanent non-remitting NDM that was, in each case, treated for many years with insulin, but then both achieved subsequent glucose control after switching to high dose sulfonylureas (Marshall et al., 2015; Wambach et al., 2010). In striking contrast, a younger sibling, carrying the same mutation, was treated with glibenclamide from the third-day of life and immediately achieved tight glucose control allowing the glibenclamide dose to be markedly decreased, and subsequently removed (N. White and B. Marshall, personal communication). A straightforward explanation is that the younger child has entered remission after tight glucose control. With similar observations in other reports, the differential outcomes we identify in mice are likely to inform the mechanistic basis of transient versus permanent outcomes in human NDM. Beyond NDM, the potential for inflammation and glucose production to exacerbate other forms of diabetes –or to ‘tip the balance’ from a compensated to a glucotoxic state- should be considered. Manipulation of the factors that affect
insulin sensitivity and insulin-independent pathways for glucose control may provide alternative means to lowering blood glucose in individuals with other forms of diabetes for whom secretagogues for insulin are not sufficiently effective. Understanding these pathways will therefore not only inform the basic mechanisms regulating metabolism but may also provide novel therapeutic targets for other metabolic disorders.

2.5 Supplemental figures
Figure 2.S1: Body mass curves for animals studied in figures 1-7. Body mass curves do not show significant differences between groups in the early induction phase (days 1-14) for any animal set. Body weights in these graphs correspond to groups in figure 2 (a), figures 3-5 (b), figure 6 (c), and figure 7 (d). Data are Mean ± SD.
**Figure 2.S2:** Refeeding responses in remitter and non-remitter mice. The change in blood glucose resulting from 1 hour of ad-lib re-feeding in K\(_{ATP}\)-GOF mice following an overnight fast at day 6 after the start of induction. Data are Mean ± SD. Animals are 9-12 weeks old (N=2 remitters, N=6 non-remitters).

**Figure 2.S3** No significant sex distribution of remission and non-remission. The proportion of remitting and non-remitting animals over the various cohorts studied in figures 1-7 is broken down in this graph by sex. Numbers of animals in each sex and group are expressed as a fraction of total K\(_{ATP}\)-GOF mice for their respective cohorts.
Figure 2.S4. Metabolic parameters in $K_{ATP}$-GOF mice. Energy expenditure (a), food intake (b), movement (c), respiratory exchange ratio (d), and oxygen consumption (e) of remitter (N=2) and non-remitter (N=6) $K_{ATP}$-GOF mice measured beginning at day 16 following the start of inductions.
Chapter 3: Expression and function of $K_{ATP}$ channels in zebrafish islet $\beta$-cells


Author Contributions:

I designed and performed the experiments (including tissue isolation, patch clamp, glucose injections, and PCR) and wrote the manuscript. Alecia Welscher, Zihan Yan, and Hannah Conway assisted with injection experiments and tissue isolation. Yixi Wang assisted with tissue isolation and PCR. Maria Remedi and Colin Nichols helped to design and perform the experiments, and also reviewed and edited the manuscript. Jennifer Moss and Larry Moss provided essential fish and technical instruction, and also reviewed and edited the manuscript.
3.1 Introduction:

In the pancreatic β-cell, ATP sensitive potassium (K\textsubscript{ATP}) channels link glucose metabolism and insulin secretion and are essential to the normal regulation of plasma glucose and other nutrients (Drews et al., 2010; Keane and Newsholme, 2014). At low glucose, intracellular [ATP]/[ADP] is low and K\textsubscript{ATP} channels are open, hyperpolarizing the cell membrane. As plasma glucose rises, it enters β-cells through glucose transporter 2 (GLUT2), increasing [ATP]/[ADP] which closes K\textsubscript{ATP} channels, inducing plasma membrane depolarization and opening voltage-dependent Ca\textsuperscript{2+} channels (VDCCs). Calcium influx through VDCCs subsequently triggers insulin secretion (Figure 3.S1A). The predominant role of K\textsubscript{ATP} channels is illustrated by the striking disease consequences of K\textsubscript{ATP} mutations. Loss-of-function mutations result in congenital hyperinsulinism (Remedi and Nichols, 2009a), whereas gain-of-function (GOF) mutations cause neonatal diabetes mellitus (NDM) (Gloyn et al., 2004; Koster et al., 2006), and polymorphisms are associated with the development of type-2 diabetes (Riedel et al., 2005).

K\textsubscript{ATP} channels have been well characterized in multiple mammalian tissues, and mechanisms coupling metabolism to insulin secretion have been well established in humans and other mammals. However, whether K\textsubscript{ATP} channel structure or function, as well as insulin secretion mechanisms, are conserved in and physiologically significant for lower vertebrates remains unclear. Studies in the zebrafish, Danio rerio, indicate that K\textsubscript{ATP} channels may be physiologically significant in fish: treatment of larvae with pharmacological activators of mammalian K\textsubscript{ATP} channels or transgenic expression of mammalian K\textsubscript{ATP} channels with GOF mutations is sufficient to raise larval whole-body glucose (Li et al., 2014a). Conversely, treatment of larvae with compounds that can close mammalian K\textsubscript{ATP} channels, or transgenic expression of mammalian K\textsubscript{ATP} channels with dominant-negative mutations, is sufficient to lower whole-larval glucose (Kimmel et al., 2015; Li et al., 2014a).
However, there have been very few mechanistic studies of insulin secretion in fish. Only a handful of papers even mention K\textsubscript{ATP} channels in zebrafish (Capiotti et al., 2014; Li et al., 2014a; Nam et al., 2015; Zhang et al., 2006), and direct analysis of expression and functional characterization are lacking. We have now developed approaches for efficiently identifying and isolating zebrafish islets, and for electrophysiological analysis of isolated \(\beta\)-cells. We show that zebrafish \(\beta\)-cells express functional K\textsubscript{ATP} channels with similar regulation, subunit composition, and pharmacology to their mammalian counterparts, and that pharmacologic K\textsubscript{ATP} channel openers can disrupt glucose tolerance in adult fish. Our results indicate that K\textsubscript{ATP} channels serve a highly conserved role in regulating metabolism in zebrafish, and that zebrafish may function as valuable models for metabolic studies.

### 3.2 Materials and Methods

#### 3.2.1 Nucleotide and amino acid alignments and identity determination

Comparisons of nucleotide and amino acid sequences of the orthologues of mammalian K\textsubscript{ATP} channel components were completed in DNASTAR Lasergene MegAlign using Clustal W alignment. Search query IDs giving the sequences analyzed for the components studied are indicated in supplemental table 1 for the nucleotide alignments and supplemental table 2 for amino acid alignments. Amino acid sequences were analyzed using InterPro 5 (Mitchell et al., 2015) ([https://www.ebi.ac.uk/interpro/](https://www.ebi.ac.uk/interpro/)) with Phobius (Kall et al., 2004) to determine predicted transmembrane, cytoplasmic, and extracellular residues.

#### 3.2.2 Animal lines and maintenance

Transparent Casper zebrafish (White et al., 2008) were used for injection experiments. Zebrafish expressing eGFP under the insulin promoter (Tg(\(-1.0\)ins:eGFP)sc1) were used for islet isolation studies (Moss et al., 2009). These fish were crossed into the Casper background for 3 generations (until external pigmentation was lost) and were maintained in the WU zebrafish facility. Details of standard operating procedures (SOPs)
for the facility can be found at http://zebrafishfacility.wustl.edu/documents.html. All procedures were approved by the Washington University in St. Louis IACUC.

3.2.3 Islet and β-cell isolation Zebrafish islets were isolated as described previously (Moss et al., 2009), with minor modifications. Briefly, fish were euthanized using cold-shock (8°C water immersion) followed by decapitation. Fish were rolled onto their right sides and the exterior skin and scales were removed using surgical forceps to expose the abdomen. Visceral organs were removed by gently applying pressure using forceps until fully separated. The islets were identified at the intersection of hepatic and bile ducts with the intestine (located using the gall bladder and spleen as regional indicators) and confirmed by eGFP fluorescence. Islets were removed by gently pinching ducts with forceps and separating the islets from the surrounding tissues.

Exocrine tissues surrounding islets were digested with collagenase (Sigma C9263, 0.4 mg/mL in Hank’s buffered salt solution, 0.5 mL/5-10 islets), during incubation at 29°C for 20 minutes, shaking gently every 5 minutes. Islets were then placed in RPMI (ThermoFisher 11875-093) supplemented with 1 mM HEPES, antibiotic solution (Sigma A5955, 10 mL/L solution), 10% fetal bovine serum, and diluted with glucose-free RPMI to 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 and clumps of cells were incubated a second time in the same conditions for 2 minutes. Dispersed cells were washed with media and re-suspended in ≤ 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% CO₂) on shards before being completely covered with media and incubated overnight in the same conditions.
3.2.4 Chemicals. Salts and glucose were purchased from Sigma Aldrich. Diazoxide (D9035), pinacidil (P154), tolbutamide (T0891), and glibenclamide (G0639) were purchased from Sigma Aldrich.

3.2.5 Whole-cell voltage-clamp and excised inside-out patch-clamp experiments. Whole-cell and inside-out excised-patch voltage-clamp experiments were performed as described for mammalian β-cells (Koster et al., 2002), with minor modifications. Isolated β-cells adhering to glass shards were transferred to bath solutions. Bath solution for whole-cell experiments was Tyrode’s solution containing 137 mM NaCl, 5.4 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 0.33 mM NaH₂PO₄, 5 mM HEPES, and 1 mM glucose. Bath solution for inside-out excised patch experiments (K-INT) contained 140 mM KCl, 10 mM HEPES, and 1 mM K-EGTA adjusted to pH 7.4 with KOH. In experiments testing ADP or drug action on K<sub>ATP</sub> channels, 0.5 mM free Mg<sup>2+</sup> was added to K-INT except for high [ATP] lanes. The amount of MgCl₂ used to reach this free Mg<sup>2+</sup> concentration was calculated using the CaBuf program (no longer accessible at webpages previously cited in other articles [ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip, as well as other sources]. We are happy to provide this to any requestors. For drug studies, drugs were kept as 100 mM stock solutions in DMSO (except diazoxide, which was kept at 300 mM in DMSO). Drug stocks were diluted to 100 µM in K-INT, with DMSO added to K-INT and ATP solutions to match the drug solution (0.1% DMSO).

Glass electrodes were pulled from Kimble-Chase 2502 micro-hematocrit capillary tubes using a P-97 (Sutter instruments) to yield 2-4 MΩ tips, when filled with K-INT. Recordings of currents were made using an Axopatch1B or Axopatch 200B amplifier and Axon pCLAMP software from Molecular Devices. For excised patches (seal >1 GΩ), membrane potential was kept constant at +50 mV. Once lifted, the pipet was moved through a mineral oil gate (Lederer and Nichols, 1989) to rip the cell free, leaving the patch in the micropipette. Patches were sequentially exposed to varying concentrations of ATP or ATP and activators, as noted. For
whole-cell recordings, membrane potential was held at -70 mV and repeatedly ramped between -120 to +40 mV.

3.2.6 RNA isolation, cDNA preparation, and channel subunit PCR. As young adult zebrafish pancreata typically contain only 1-3 large islets (~10,000 cells), biological replicates were designated as pools of islets from 8-15 fish each. RNA was isolated from pooled islets using the QIAGEN RNEasy mini-kit. cDNA was synthesized from isolated RNA with the ThermoFisher High-Capacity cDNA Reverse Transcription Kit. Genomic DNA was isolated from zebrafish hearts using the gMax mini-kit (IBI scientific). Primers for ion channel PCRs are listed in supplementary table 5. Primers were designed using the Primer-Blast NCBI online tool and checked for specificity for the selected genes ([http://www.ncbi.nlm.nih.gov/tools/primer-blast/](http://www.ncbi.nlm.nih.gov/tools/primer-blast/)) (Ye et al., 2012). Input parameters specified that primers not span exon-exon junctions. PCR reactions were run with Platinum Taq High-fidelity polymerase (ThermoFisher 11304-11) and products were separated on 1.5-2% agarose gels in TAE buffer and visualized with ChemiDoc. MP (Bio-Rad). PCRs on cDNAs from n > 5 separate pools of islets and n > 5 genomic DNAs were analyzed, with Figure 3.3 A being a representative sample.

For cell sorting, islets were dispersed as above and sorted using a BD FACSARia II (BD Biosciences) at the Washington University Flow Cytometry and Fluorescence Activated Cell Sorting Core ([http://pathology.wustl.edu/Research/cores/facs/index.php](http://pathology.wustl.edu/Research/cores/facs/index.php)). RNA was extracted from sorted cells as described (Gagnon et al., 2014) using TRIzol (ThermoFisher 15596026) and chloroform (Sigma C0549). DNA was removed from RNA samples using DNase I (ThermoFisher 18068015) for islet samples and TURBO DNA-free kit (ThermoFisher AM1907) for sorted cell samples prior to reverse transcription. The FACSARia II data file is included as online material.

3.2.7 Adult zebrafish injection studies Injections were performed as previously described (Kinkel et al., 2010), with modifications. Adult Casper zebrafish of both sexes, approximately 6-8
months of age, were anesthetized by cold water immersion. Animals were then transferred to pre-weighed cold water-soaked sponges in petri dishes with indentations cut to maintain hydration while holding fish immobilized. Fish were injected (10 µL/gBW) intraperitoneally (IP) using disposable 32 G needles (Acuderm) with luer-lock hubs on gas-tight 50 µL syringes (Hamilton 1705). For the IP glucose tolerance test, all solutions were prepared in 20% DMSO in 1 x PBS with 5 mg/mL phenol red. Following injection, animals were returned to warm water (28°C) for recovery. For plasma glucose measurements at indicated time points, individual fish were euthanized by immersion in cold water followed by decapitation across the gills. OneTouch Ultra glucometers were used to measure blood glucose by placing glucometer strip at sectioned heart at time of decapitation.

3.2.8 Data analyses

Initial experiments with ATP inhibition on excised patches from zebrafish established a variability similar to that seen for mammalian channels under similar conditions (Koster et al., 2000). [ATP]-response relationships were fitted with a modified Hill equation:

\[ I_{\text{rel}} = \frac{1}{1 + ([\text{ATP}] / IC_{50})^{n_H}} \]

where \( I_{\text{rel}} \) is the current relative to that in zero ATP; \( IC_{50} \) is the ATP concentration at which channels are half-maximally inhibited; [ATP] is the concentration of ligand; and \( n_H \) is the Hill coefficient. Fitting was done with GraphPad Prism software, using least-squares variable-slope log([inhibitor]) vs. normalized response function, with resulting R-squared value of 0.97.

Statistical comparisons between the various data sets were performed in GraphPad Prism, and the specific tests are indicated in the relevant figure legends. Data sets were tested for normality (Shapiro-Wilk) and whether variances were statistically different between groups (Bartlett’s test). Where normality and variance assumptions were met, ANOVA with Tukey’s multiple comparisons tests (3 or more groups) or Student’s T-test with Welch’s correction (2 groups) was used. As some data sets showed non-normal data or significantly different
variances within the groups, non-parametric tests were used (for tests of three or more groups, Kruskal-Wallis ranked test, with Dunn’s multiple comparisons; Mann-Whitney for two-group analyses). To improve interpretability of glucose tolerance tests, the values were log-transformed before statistical analysis.

3.2.9 Chapter supplemental material Included following the discussion section for the chapter are 3 supplemental tables and 3 supplemental figures. Supplemental table 1: references for nucleotide sequence alignments and identity determination. Supplemental table 2: references for amino acid sequence alignments and identity determination. Supplemental table 3: sequences and relevant data for the primers used in PCR reactions for the K<sub>ATP</sub> channel subunits in zebrafish. Figure 3.S1: amino acid and nucleotide identities for K<sub>ATP</sub> channel subunits in humans and zebrafish. Figure 3.S2: the alignment of zebrafish Kir6.x subunits relative to their mammalian orthologues highlighting residues of interest and functional domains. Figure 3.S3: alignment of zebrafish SURx subunit sequences relative to mammalian sequences, highlighting residues of putative functional significance in mammalian subunits that are or are not conserved in zebrafish orthologues.

3.3 Results

3.3.1 Orthologues of major genes involved in mammalian insulin secretion exist in zebrafish Key proteins involved in the electrical coupling of glucose metabolism to insulin secretion in mammals include glucose transporters (GLUT2), K<sub>ATP</sub> channels, and voltage-dependent Ca<sup>2+</sup> channels (VDCCs, Figure 3.S1A). Orthologues for each of these are present in the zebrafish genome, and predicted K<sub>ATP</sub> channel subunit sequences are highly conserved (Clustal W alignments, Figure 3.S1 B, C). In mammals, K<sub>ATP</sub> channels are generated as octameric complexes of 4 pore-forming Kir6.x subunits and 4 accessory sulfonylurea receptor (SURx) subunits (Nichols, 2006); Kir6.2 and SUR1 form K<sub>ATP</sub> channels in pancreatic islets and in the central nervous system, whereas Kir6.1 and SUR2 form K<sub>ATP</sub> channels in smooth and
striated muscles (Nichols, 2006). The genes for Kir6.2 (*KCNJ11*) and SUR1 (*ABCC8*) are immediately adjacent to one another in both zebrafish and human chromosomes (chromosome 25 in zebrafish and 11 in humans). The genes for Kir6.1 (*KCNJ8*) and SUR2 (*ABCC9*) are also adjacent to one another in both species (located on chromosome 4 in zebrafish and 12 in humans). Kir6.1, Kir6.2, SUR1 and SUR2 all show >70% amino acid identity between humans and zebrafish (Zhang et al., 2006) (Figure 3.S1B), with functional domains in SUR1 being highly conserved (Figure 3.S3). Kir6.3, a pore-forming subunit unique to zebrafish and likely to be derived from zebrafish Kir6.2 in a duplication event, is located on zebrafish chromosome 15 and has no SUR gene in its vicinity. Kir6.3 and SUR1 expression have previously been described in the zebrafish central nervous system, but expression of the different K<sub>ATP</sub> channel subunits has not been examined in other tissues (Zhang et al., 2006).

### 3.3.2 K<sub>ATP</sub> channels regulate glucose homeostasis in adult zebrafish

We initially probed the glucose metabolism, and the role of K<sub>ATP</sub> in glucose control, in adult zebrafish using a glucose tolerance test. Intraperitoneal (IP) injection of glucose elevates blood glucose in adult zebrafish beyond vehicle alone, and glucose gradually normalizes as insulin is secreted and peripheral glucose uptake is activated (Figure 3.1A, blue dotted line). As shown in Figure 3.1 A, co-injection of diazoxide along with glucose significantly slows the return of glucose to baseline. These results are consistent with the effect of diazoxide on IP glucose tolerance in mammals, and with previous experiments on larval zebrafish (Hodson et al., 2014).
Figure 3.1: Whole-cell voltage-clamp of zebrafish β-cells reveals functional $K_{ATP}$ channels. (A) Glucose tolerance in adult zebrafish. Blood glucose at each time point in (A) was compared between groups by ANOVA with Tukey’s multiple comparisons on log-transformed data sets. N = 7-13 at each time point except for the baseline values which were 24. (***) denotes $p < 0.001$, group vs. vehicle; (****) denotes $p < 0.0001$, group vs. vehicle; p-values are from Tukey’s multiple comparisons test following ANOVA of log-transformed values. (†††) denotes $p < 0.001$, group vs. glucose, also from Tukey’s multiple comparisons test. Data in this panel are compiled from multiple injection experiments performed over several days. (B) Expression of eGFP in the fish pancreas allows visualization of β-cells in live adults (left image), isolated islets (middle image), and dispersed β-cells (right image). Scale is indicated for the live adult image. The middle and right images are at 20x and 40x, respectively. For image panels, bright-field and fluorescence images were superimposed for adult fish and whole islets. Adult fish brightfield image was contrast-enhanced prior to superimposing it with fluorescence image to enhance visibility in final combined image. (C) Whole-cell voltage-clamp detection of $K_{ATP}$ in zebrafish β-cells. Voltage ramps (lower) were applied from -120mV to +40 mV over 4 seconds. Following break-in, the initial ramp (blue) elicits large voltage-dependent K currents above -30mV. These currents gradually rundown in successive voltage ramps, and a weakly inwardly rectifying $K_{ATP}$ conductance gradually increases to maximal (green) and then in turn runs down to baseline (red). Right panel shows currents between -120 and 0 mV for more clear visualization of $K_{ATP}$ currents.
Figure 3.2: Excised-patch clamp reveals functional properties of $K_{ATP}$ channels in zebrafish $\beta$-cells. (A) Individual $K_{ATP}$ channels (4.35 pA at -50mV) are detected in three representative membrane patches excised from zebrafish $\beta$-cells. (B) These $K^+$ currents are inhibited by ATP, with (C) $IC_{50} = 22.6$ mM, $n_H = 1.01$. (D) These channels are also activated by increasing [Mg-ADP], quantified in (E). (****) indicates $p < 0.0001$ (Mann-Whitney test). The dose-response curve was generated from 16 cells derived from 6 pools of zebrafish islet (biological replicates). The ADP response graph comprises 9 cells derived from 4 biological replicates. (A) (B) and (D) show representative traces.
3.3.3 Zebrafish β-cells express functional $K_{\text{ATP}}$ channels

To examine $K_{\text{ATP}}$ channel expression and function in zebrafish β-cells, we have developed approaches to isolate islets and individual β-cells for gene expression as well as whole-cell and excised-patch voltage-clamp techniques (Fig. 3.1B). Fish that express eGFP using the zebrafish insulin promoter allowed isolation and dispersion of pancreatic islets to yield individual β-cells as described in methods (Moss et al., 2009) (Figure 3.1 B). Whole-cell patch clamp of these isolated zebrafish β-cells (Figure 3.1 C), demonstrated activation of large, almost linear potassium conductances that are maximal within a few minutes after initial dialysis of the cell with zero ATP solution. The weak inward rectification (evident above ~0mV, Fig. 3.1 C, left), and reversal potential very close to $E_K$ (-80 mV, Fig. 3.1 C, right), as well as the amplitude of this conductance, are indistinguishable from typical $K_{\text{ATP}}$ currents activated in mammalian β-cells (Misler et al., 1989). Inside-out excised-patch voltage-clamp experiments on zebrafish β-cells reveal potassium channels with single channel conductance of ~87 pS (4.35 pA at -50 mV driving force; Figure 3.2 A). Again, this property is indistinguishable that of from mammalian $K_{\text{ATP}}$ channels formed from Kir6.2+SUR1 subunits (Ashcroft, 1988; Tinker et al., 2014). These channels are inhibited by increasing concentrations of ATP at the intracellular surface (Figure 3.2 B, C), with $IC_{50}$ of 22.6 µM ($n_H = 1.01$), very similar to reported values for mammalian β-cell $K_{\text{ATP}}$ channels in the same conditions ($IC_{50} \sim 10-20$ µM (Ashcroft, 1988; Koster et al., 2000; Mannikko et al., 2011; Miki et al., 1999)). Furthermore, these channels are activated by addition of Mg-ADP to the cytoplasmic face (Figure 3.2 D, E), again similar to properties of mammalian $K_{\text{ATP}}$ channels (Dunne and Petersen, 1986). Taken together, these data show that zebrafish β-cells express functional $K_{\text{ATP}}$ channels with activation and inhibition properties that are essentially the same as those expressed in mammalian β-cell $K_{\text{ATP}}$ channels.
Figure 3.3: Zebrafish β-cell K\textsubscript{ATP} channels are similar in composition to mammalian β-cell K\textsubscript{ATP} channels. (left) PCR of RNA-derived cDNA from different pools of islets shows bands for orthologues of KCNJ11 (Kir6.2), KCNJ8 (Kir6.1), KCNJ11L (Kir6.3), and ABCC8 (SUR1), but no bands for ABCC9 (SUR2). Plasmid DNA is a negative control for non-specific replication by primer mix; genomic DNA (gDNA) is a positive control for presence of target genes. These reactions were repeated over N ≥ 5 separate pools of cDNA and gDNA for validation. Primers for KCNJ11L, KCNJ8, and ABCC8 span exons to distinguish gDNA from cDNA. There are no introns in KCNJ11. (right) PCR of RNA-derived cDNA from eGFP-sorted β-cells shows bands for orthologues of KCNJ11 and ABCC8, but no bands for the other subunits. Images were cropped and resized, and in some cases contrast-enhanced to improve clarity. Original images are in Figures 3.S4 and S5. Orange and red boxes highlight transcript presence in islet and β-cell cDNAs, respectively.
3.3.4 Zebrafish β-cell $K_{ATP}$ channels show similar subunit composition and pharmacology to mammalian β-cell $K_{ATP}$ channels

We performed PCR on cDNA generated from RNA isolated from zebrafish islets, to characterize Kir6 and SUR subunit expression in zebrafish. Genes for Kir6.1, Kir6.2, Kir6.3, SUR1, and SUR2 were all detected in zebrafish genomic DNA (gDNA) but only Kir6.2, Kir6.1, Kir6.3 and SUR1 were consistently detected in islet cDNA (Figure 3.3 A). The similarity of zebrafish $K_{ATP}$ currents to those expressed in mammalian β-cells is consistent with both being formed of Kir6.2 and SUR1 subunits, raising the question of the relevance of Kir6.1 and Kir6.3 expression, Kir6.3 having been detected in fish neurons by RNA in situ hybridization studies (Zhang et al., 2006). cDNA derived from eGFP-sorted β-cells indicates transcription of only $KCNJ11$ (Kir6.2) and $ABCC8$ (SUR1) (Figure 3.3). While β-cells form the majority of cells in the islet, islets are innervated and permeated by capillaries (Moss et al., 2013). The presence of $KCNJ8$ (Kir6.1) and $KCNJ11L$ (Kir6.3) transcripts in whole islets may reflect the presence of these other cell types.

Mammalian SUR subunits respond differentially to activator and inhibitor compounds: the potassium channel opener (KCO) diazoxide is a more effective activator of SUR1-containing $K_{ATP}$ channels and pinacidil is a more effective activator of SUR2-containing channels (Inagaki et al., 1996; Shyng et al., 1997). Sulfonylureas, furthermore, typically close SUR1-containing $K_{ATP}$ channels ~100 to 1000-fold more effectively than SUR2-containing $K_{ATP}$ channels in mammals (Vila-Carriles et al., 2007). Residues involved in drug sensitivity are conserved between zebrafish and mammalian SUR subunits (Figures 3.S2 and 3.S3). In excised zebrafish β-cell membranes, addition of Mg$^{2+}$ and diazoxide is sufficient to activate $K_{ATP}$ channels (Figure 3.4 A, E), whereas pinacidil is ineffective (Figure 3.4 B, E) at the same concentration. Two sulfonylurea drugs, tolbutamide (Figure 3.4 C, F) and glibenclamide (Figure 3.4 D, F), both inhibit the zebrafish β-cell $K_{ATP}$ at relatively low concentrations in excised
Figure 3.4: Zebrafish β-cell K<sub>ATP</sub> channels are similar in pharmacology to mammalian β-cell K<sub>ATP</sub> channels. Zebrafish β-cell K<sub>ATP</sub> channels show activation by diazoxide (A) but not by pinacidil (B) in excised patches. These channels also show inhibition by both tolbutamide (C) and glibenclamide (D). (E) and (F) show quantification of activation (E) and inhibition (F) of the K<sub>ATP</sub> channels. (*) indicates p < 0.05 by the Mann-Whitney test. In (E), the right panel indicates the increase in I<sub>rel</sub> produced by each drug, whereas the left panel indicates the fraction of overall maximum current in each condition. These are quantified from recordings of 6 cells for diazoxide, 4 cells for pinacidil and glibenclamide, and 5 cells for tolbutamide.
patches, similar to the level of inhibition seen for mammalian Kir6.2+SUR1 channels at the same drug concentrations in similar conditions (Koster et al., 1999a). Potent response to sulfonylureas and diazoxide, but not to pinacidil, is consistent with the expression data showing that SUR1 is the only SUR detected in islet cDNA. Taken together, these data indicate that the subunits comprising zebrafish β-cell K_ATP channels and the drug responsivity of these channels are essentially the same as their mammalian counterparts.

3.4 Discussion

3.4.1 Structure and functional properties of β-cell K_ATP channels are conserved between zebrafish and mammals Mammalian K_ATP represents a family of potassium channels generated by various combinations of Kir6.1/2 and SUR1/2 subunits (Flagg and Nichols, 2011; Wheeler et al., 2008). Expression patterns and functional properties have been extensively characterized, and shown to be generally well conserved between mammalian species (Brereton and Ashcroft, 2013; Dean and Annilo, 2005; Koster et al., 2000; Koster et al., 2006; Nichols, 2006; Seino et al., 2000). K_ATP channel subunit orthologues are clearly present in all sequenced vertebrate genomes, but there have been surprisingly few studies of structural or functional properties of K_ATP channels in islets from non-mammalian vertebrate classes. Studies of K_ATP structure and function in fish have been very limited, in part due to technical difficulties of identification and isolation of specific cell types. Zhang et al. identified a third, unique, pore-forming subunit, Kir6.3 (Zhang et al., 2006), in zebrafish, and showed that this subunit is expressed in the central nervous system via RNA-in situ hybridization), but expression and potential roles in other tissues was not explored. Here, using fluorescently tagged β-cells in transparent Casper fish, we have succeeded in efficiently identifying, isolating and dissociating zebrafish islets. We show that zebrafish β-cells express functional K_ATP channels that exhibit very similar composition (Kir6.2 and SUR1) and pharmacology (activation by diazoxide, but not
pinacidil) to those in mammalian β-cells, and that modulation of these channels affects adult fish glucose homeostasis similarly to the effects in mammals.

### 3.4.2 Conservation of $K_{ATP}$ channel-dependent insulin secretion mechanisms between teleost fish and mammals

Rapid responses to metabolic changes are challenges faced by all organisms, and the potential importance of insulin signaling in such responses is highlighted by the high conservation of insulin structure and insulin signaling pathways across vertebrates and invertebrates, with evolutionary lineages that diverged long ago (Das and Dobens, 2015; Ellsworth et al., 1994). However, the last common ancestor between teleost fish and humans is estimated to have lived ~450 million years ago (Taylor et al., 2001), and while insulin and other hormones are structurally conserved across the vertebrates, whether secretory regulation and functional consequences are as conserved is less clear.

The finely tuned properties and regulatory features of β-cell $K_{ATP}$ channels are absolutely key to the regulation of mammalian insulin secretion (Drews et al., 2010; Koster et al., 2006). A role for $K_{ATP}$ channels in modulating glucose metabolism in zebrafish has been implied by the demonstration that treatment of fish larvae with the $K_{ATP}$ opener diazoxide increases whole-larval glucose and that the inhibitor glibenclamide lowers whole-larval glucose (Li et al., 2014a). Transgenic over-expression of mammalian $K_{ATP}$ channels with gain-of-function mutations was also sufficient to increase larval glucose in these studies, whereas a dominant-negative mammalian $K_{ATP}$ channel lowered larval glucose (Li et al., 2014a). It has also been suggested that diazoxide interferes with regeneration of pancreatic islets in fish after alloxan treatment, while glimepiride, a sulfonylurea, enhances recovery (Nam et al., 2015). However, despite implicating $K_{ATP}$ in metabolic control, none of these earlier studies characterized the properties of native zebrafish islet $K_{ATP}$ channels or directly assessed their role in insulin secretion. We are unaware of ex vivo analyses of insulin secretion in zebrafish islets, but our findings suggest that $K_{ATP}$ channels are conserved in both functional expression and properties between zebrafish
and mammalian β-cells (Figures 3.2, 3, and 4). Manipulation of $K_{\text{ATP}}$ channels in vivo with the pharmacological activator diazoxide suggests that $K_{\text{ATP}}$ channels are also key to normal glucose tolerance in adult fish (Figure 3.1 A).

### 3.4.3 Zebrafish as model organisms for studying metabolic diseases

In addition to their use in tracking temporal expression patterns of transcription factors in endocrine development, zebrafish have been used to model atherosclerosis, the consequences of high fat diet feeding, hyperglycemia, and other metabolic interventions, as well as the regeneration of key endocrine organs, including the pancreas (Seth et al., 2013a). Our finding that zebrafish β-cells express functional $K_{\text{ATP}}$ channels with very similar biophysical properties and pharmacology to mammalian channels, and that channel activation significantly impairs whole-body glucose clearance in adult fish, will lend further support to the use of these animals to model metabolic diseases.

Forward genetic screens can be powerful tools for unmasking subtle modifiers of disease phenotypes, but infrequent reproduction, low litter numbers, long maturation time, and high cost limit the utility of mammalian species for such studies. Zebrafish reproduce frequently and with large clutches (potentially hundreds of embryos per clutch), allowing analysis of thousands of individuals in short periods. The genome is fully sequenced, and techniques for introducing mutations in zebrafish have been well streamlined (Lieschke and Currie, 2007; Maddison et al., 2014b). Larvae are transparent, allowing easy visualization of genetic markers or fluorescent dyes (Patton and Zon, 2001; White et al., 2008). Zebrafish develop metabolic abnormalities when fed high-fat diets (Fang et al., 2014; Oka et al., 2010), show similar complications of persistently high glucose, and have many of the same transcription factor pathways involved in development of endocrine, liver, and other organs important in controlling metabolism (Lieschke and Currie, 2007; Tehrani and Lin, 2011). While zebrafish may thus offer
major advantages for screening diabetes modifiers, details of comparative organ biology must first be evaluated and further studies like those we describe here are required.

3.5 Supplemental Results

3.5.1 Significant residue conservation in SUR1 and Kir6.2 orthologues in zebrafish Many residues which, when mutated, cause loss of function in mammalian $K_{\text{ATP}}$ (Babenko et al., 2006; de Lonlay-Debeney et al., 1999; Fernández–Marmiesse et al., 2006; Henwood et al., 2005; Huopio et al., 2002; Magge et al., 2004; Nestorowicz et al., 1998; Nestorowicz et al., 1996; Nichols et al., 1996; Ohkubo et al., 2005; Otonkoski et al., 2006; Saint-Martin et al., 2015; Shepherd et al., 2000; Shyng et al., 1998; Suchi et al., 2005; Tanizawa et al., 2000; Taschenberger et al., 2002; Thornton et al., 2003; Tornovsky et al., 2004; Yan et al., 2007), are conserved in zebrafish SUR1 (highlighted green in Figure 3.S3). Additional residues which, when mutated, have been shown to cause $K_{\text{ATP}}$ gain-of-function (Ellard et al., 2007; Küçükemre Ay et al., 2012; Vaxillaire et al., 2007) (and consequently diabetes) in mammals, are also conserved in zebrafish SUR1 (also highlighted green in Figure 3.S3). Several residues with mutations associated with loss of function (Aguilar-Bryan and Bryan, 1999; Henwood et al., 2005; Lin et al., 2006; Mannikko et al., 2011; Marthinet et al., 2005; Ohkubo et al., 2005; Pinney et al., 2008; Suchi et al., 2005; Taneja et al., 2009; Tornovsky et al., 2004) and hyperinsulinemia in mammalian Kir6.2, (Figure 3.S2, highlighted green), as well as many residues which, when mutated, cause gain of function (Bonnefond et al., 2012; Flanagan et al., 2006; Florez et al., 2007; Gloyn et al., 2005; Männikkö et al., 2010; Shimomura et al., 2006; Shimomura et al., 2007; Tammaro and Ashcroft, 2007; Vaxillaire et al., 2004; Yorifuji et al., 2005) and diabetes are fully conserved (Figure 3.S2, also highlighted green).

3.5.2 Not all residues suggested to be functionally significant for mammalian Kir6.2 are conserved in zebrafish Kir6.2 (rel. to Supplemental Fig. 2, highlighted in red) The D101 residue in zebrafish Kir6.2 replaces the A101 in human Kir6.2, and the A101D mutation in
humans causes congenital hyperinsulinism (Suchi et al., 2005). L147, found mutated to proline in congenital hyperinsulinism with loss of $K_{ATP}$ channel expression (Aguilar-Bryan and Bryan, 1999), is a valine residue in zebrafish Kir6.2. However, this substitution may be tolerated as both are nonpolar and as valine substitutions for alanine can still form essential tertiary structures in many proteins (Gregoret and Sauer, 1998). S372 and T224, which are phosphorylated by PKA (Aziz et al., 2011; Lin et al., 2000), are likewise not conserved. However, it should be noted that it is difficult to assign functional effects to S372 and other nearby residues, as large portions of the C terminal region of zebrafish Kir6.2 differ from those in mammalian Kir6.2.

3.5.3 Not all residues suggested to be functionally significant for mammalian SUR1 are conserved in zebrafish SUR1 (rel. to Supplemental Fig. 3, highlighted in red)

Glycine 111 in human SUR1 (which, when mutated to arginine in humans causes congenital hyperinsulinism (Tornovsky et al., 2004)) is a cysteine in zebrafish SUR1. Valine 187, mutated to aspartic acid in human congenital hyperinsulinism (Huopio et al., 2002), is an isoleucine in zebrafish SUR1. K1336, also linked to hyperinsulinism (Snider et al., 2013), is a glutamic acid residue in zebrafish SUR1. L1551 in mammalian SUR1 (Campbell et al., 2003) is a methionine residue in zebrafish SUR1. Several other residues near the first nucleotide binding fold, mutations at which trigger hyperinsulinism or diabetes in mammals (Fernández–Marmiesse et al., 2006; Saint-Martin et al., 2015; Snider et al., 2013; Vaxillaire et al., 2007), are shifted from those exact positions in zebrafish SUR1 (as is the second walker motif in the first nucleotide binding fold).
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**Table 3.1:** References for nucleotide sequence alignments and identity determination. Databases queried and query IDs for the orthologues of SURx and Kir6.x subunits in humans and zebrafish are indicated.
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**Table 3.2:** References for amino acid sequence alignments and identity determination. Databases queried and query IDs for the orthologues of SURx and Kir6.x subunits in humans and zebrafish are indicated.
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<td>507 (i), 423 (e)</td>
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**Table 3.3:** Sequences and relevant data for the primers used in PCR reactions for the K<sub>ATP</sub> channel subunits in zebrafish. For primers with products differing by whether the sequence contains an intron, the product length for samples containing the target (i) precedes the length of the target which does not contain introns (e). For figure 3.3 A, ABCC9 primer set from the NCBI reference was used. For Figure 3.3 B, the ABCC9 primer set for the Ensembl sequence reference was used. References for Ensembl are Chromosome #: nucleotide position. The reference genome for Ensembl queries was GRCz10.
Figure 3.S1: Zebrafish possess orthologues for $K_{\text{ATP}}$ channels with high amino acid conservation. At low plasma glucose (A, left), mammalian β-cell $K_{\text{ATP}}$ channels are open due to low intracellular [ATP]/[ADP], hyperpolarizing the cell. As plasma glucose rises (A, right), glucose enters the cell through Glut2 and is metabolized to ATP, increasing [ATP]/[ADP]. This inhibits $K_{\text{ATP}}$ channels, and $V_m$ increases, opening VDCCs. Influx of calcium triggers insulin secretion. Orthologues of many components of mammalian metabolism-secretion coupling are present in the zebrafish, including $K_{\text{ATP}}$ channels (single channel diagram in B, top view). Identity alignments for amino acid sequences for Kir6.x and SURx subunits (B) are scaled, with darker grey indicating more identity between the proteins compared. The query IDs for amino acid alignments are indicated in Supplemental table 2. There is also high identity between the nucleotide sequences for these genes (C). Query IDs for nucleotide alignments are in supplemental table 1.
Figure 3.S2: Residues suggested to be functionally significant for mammalian Kir6.2 are conserved in zebrafish Kir6.2 Alignments for the Kir6.x subunits from human and zebrafish are shown highlighting residues suggested to be significant for gain-of-function (neonatal diabetes) or loss-of-function (congenital hyperinsulinism). Green highlighting indicates conservation of the residue; red highlighting indicates the residue is not conserved. Red box outlines indicate regulatory residues post-translationally modified in mammalian Kir6.2 which are not conserved in zebrafish Kir6.2, while green outline boxes show residues which are post-translationally modified in mammalian Kir6.2 that are conserved in zebrafish Kir6.2.
Figure 3.S3: Many residues with functional significance in mammalian SUR1 are conserved in zebrafish SUR1. The sequences for zebrafish SUR1, zebrafish SUR2, and human SUR2A and SUR2B are shown aligned to human SUR1. Residues identical to those in human SUR1 are highlighted in grey. Specific residues of functional significance to human SUR1 which are conserved in zebrafish SUR1 are highlighted green. Green outlines highlight conserved residues which are important for subunit regulation in mammals. The predicted nucleotide binding folds are indicated by blue outlines. Within these, red outlines highlight the Walker motifs. Residues highlighted red are significant for SUR1 function in mammals which are not identical in zebrafish SUR1. Above the residue ruler, two bars indicate the topological domains predicted by Interpro 5 software for zebrafish (upper bar) and human (lower bar) SUR1. Pink color (light for zebrafish, dark for human) indicates predicted cytoplasmic domains. Blue color (light for zebrafish, dark for human) indicates predicted extracellular domains. Grey color (zebrafish) and black color (human) indicate predicted transmembrane domains. Query IDs for these sequences are shown in Table 3.2.
Chapter 4: Excitability-driven diabetes in the zebrafish

Adapted from work which is currently drafted into the following paper: Emfinger, C.H. and Lőrincz, R.*, Huang, Y., Wang, Y., Hyrc, K., Reissaus, C., Piston, D., Meyer, D., Remedi, M.S., and Nichols, C.G. “Excitability-driven diabetes in the zebrafish” (* co-first-author) *This paper is being submitted in August 2018*

Author contributions:

I designed and conducted *ex vivo* islet imaging experiments, as well as tissue, digest, fixation, and culture. Reka Lorincz, Chris Reissaus, Krzysztof Hyrc, and Yixi Wang participated in *ex vivo* islet imaging experiments. Reka Lorincz and Yixi Wang also performed tissue dissection, digest, fixation, and culture. I designed and performed molecular biology experiments to generate constructs for transgenic fish lines. Yixi Wang also performed molecular biology experiments to generate constructs for transgenic fish lines. Reka Lorincz did the live larval injection and imaging experiments. Yan Huang performed the whole-cell patch clamp experiments. I wrote the initial draft of the paper, which was further edited by Reka Lorincz, Colin Nichols, and Maria Remedi. Colin Nichols and Maria Remedi participated in molecular lab work, design of experiments, and drafting the manuscript. David Piston and Dirk Meyer participated in the design of experiments.
4.1 Introduction:

Zebrafish (*Danio rerio*) possess orthologues of many genes essential to metabolic control in mammals (Lieschke and Currie, 2007; Postlethwait et al., 2000; Sanhueza et al., 2009; Tehrani and Lin, 2011), have similar organ structures to mammals (Howe et al., 2013b; Kimmel and Meyer, 2016; Tehrani and Lin, 2011), and develop abnormalities when overfed (Seth et al., 2013a). Zebrafish are increasingly being used as a model organism in which to study metabolic diseases (Kamel and Ninov, 2017; Kimmel and Meyer, 2016; Kinkel and Prince, 2009) and, owing to their frequent and prolific reproduction and larval transparency, they offer the potential to screen for modifiers of metabolism that may be relevant to mammalian disease (Maddison et al., 2014a; Obholzer et al., 2012; Patton and Zon, 2001). However, the details of metabolic regulation and to what degree it may mirror metabolic regulation in mammals remains insufficiently explored.

Electrical activity couples metabolism to insulin secretion in mammals (Drews et al., 2010; Koster et al., 2006). At low plasma glucose, ATP-sensitive potassium (K\textsubscript{ATP}) channels are open. The cell membrane is consequently hyperpolarized, voltage-dependent calcium channels (VDCCs) are closed, and minimal insulin is secreted. As plasma glucose increases, it enters β-cells via glucose transporter 2 (GLUT2), and metabolism of glucose increases the [ATP]/[ADP] ratio, resulting in closure of K\textsubscript{ATP} channels and depolarization of the plasma membrane. Consequent calcium influx through VDCCs then triggers insulin release. That gain-of function mutations in K\textsubscript{ATP} channels cause diabetes, whereas loss of function in these channels reciprocally causes hyperinsulinism and hypoglycemia, illustrates the significance of excitability in regulating insulin secretion in mammals (De Leon and Stanley, 2008; Drews et al., 2010; Remedi and Koster, 2010). Some calcium channel (Ca\textsubscript{v}1.2) mutations cause hypersecretion of insulin (Yang and Berggren, 2006), and mutations in Ca\textsubscript{v}1.3 and Ca\textsubscript{v}2.1 have been associated
with a subgroup of diabetic patients, further highlighting the relevance of excitability in regulating insulin secretion (Morotti et al., 2017; Reinbothe et al., 2013).

While excitability-dependence of insulin secretion has been well elucidated in mammalian islets, its presence in and significance for insulin secretion in lower organisms remains incompletely assessed. Previous work in zebrafish identified a role for K\textsubscript{ATP} channels in early islet responses to overnutrition; activation of K\textsubscript{ATP} either pharmacologically or with inducible transgenes generated increased \(\beta\)-cell growth in response to excess nutrients (Li et al., 2014a). We have recently shown that K\textsubscript{ATP} channels are expressed in zebrafish \(\beta\)-cells, that they are functionally similar to their mammalian orthologues, and that pharmacologic manipulation of these channels via diazoxide can similarly alter glucose tolerance (Emfinger et al., 2017). While this argues that excitability is important for insulin secretory control in zebrafish, whether the downstream processes that are present in mammals have parallels in zebrafish, and whether alterations in excitability can drive persistent changes in glucose control in zebrafish, remain unknown. In this study, we show that intracellular \([Ca^{2+}]\) in zebrafish islets and individual \(\beta\)-cells is glucose-sensitive, similar to mammals. We further show that transgenic expression of K\textsubscript{ATP}-GOF mutations blocks glucose-dependent \([Ca^{2+}]\) elevations, resulting in severe hyperglycemia, paralleling the consequences of \(\beta\)-cell inexcitability in mammals.

4.2 Materials and Methods:

4.2.1 Generation of constructs for islet-specific cytosolic gCAMP6s-expressing fish.

Cytosolic gCAMP6s expression in zebrafish islet \(\beta\)-cells was achieved using constructs optimized for tol2-transposase insertion (Kwan et al., 2007). The construct was generated using gateway recombination of plasmids containing the promoter, gCAMP6s protein, and poly-A stop sequence (sequence for the Tg(-1.0ins:gCAMP6s)\textsuperscript{std41} transgenic fish (cgCAMP6s fish), Figure 4.1C). All plasmids were purified using the Qiagen Plasmid Mini-prep kit (Qiagen catalog # 27104). The sequence containing the zebrafish insulin promoter (zINSp) was amplified from an
existing plasmid (Addgene # 53787) by PCR using a Phusion high-fidelity polymerase (NEB Catalog # M0530S) and modified to overlap sequences from a tol2kit 5’ entry vector (gift of Dr. Lila Solnica-Krezel). The PCR product was run on a 1.5% agarose gel and the target band excised. The fragment was extracted from the gel using the QIEX II gel extraction kit (Qiagen Catalog # 20021). The 5’ entry vector was digested using BamHI (NEB Catalog# R0136S) and XhoI (NEB Catalog # R0146S) according to manufacturer instructions, and the digested plasmid was run on a 1.5% agarose gel to separate the larger fragment containing the ATT sites. The ATT-containing fragment band was excised and the fragment extracted using the QIEX II gel extraction kit. The zINSp was inserted into the fragment using Gibson assembly (Gibson et al., 2009). Precise conditions and reagent mixes are provided in the supplementary data. DH5-α E. coli (ThermoFisher # 18265017) were transformed with the resulting vector, and the plasmid was amplified and selected using antibacterial resistance on agar plates made with Luria Broth (LB) overnight. Subsequent colonies were expanded in LB containing relevant antibiotics.

For the middle entry vector (MEV) containing the calcium sensor gCAMP6s, the gCAMP6s-containing fragment was PCR amplified from a plasmid provided by Dr. Solnica-Krezel using Phusion HF polymerase and modified to add CACC on the 5’ end. The plasmid was inserted into the PENTR/D Topo vector (ThermoFisher # K240020) according to manufacturer instructions and amplified using One Shot® Top 10 chemically competent E. coli provided in the kit with antibacterial-resistance selection.

The 3’ entry vector (p3E-polyA, plasmid #302 of the tol2kit) and the destination vector (pDestTol2CG2, plasmid #395 of the tol2kit, gifts of Dr. Solnica-Krezel). 5’ entry vector, MEV, 3’ entry vector, and destination vector were combined using LR Clonase II (ThermoFisher catalog # 11791100) according to manufacturer instructions. DH5-α E. coli were transformed with the resulting vector, the plasmid was amplified and selected using antibacterial resistance on agar plates made with Luria Broth, and resulting colonies were expanded in LB containing relevant
antibiotics. Sequences for all plasmids, PCR primers for the relevant modifications of selected fragments, and reactions mixes and cycling conditions are provided in the online supplementary data.

4.2.2 Generation of constructs for fish conditionally expressing Kir6.2 with gain-of-function mutations. Constructs for generating fish which conditionally express gain-of-function \( K_{\text{ATP}} \) channels only in islets (Tg(-1.0ins:LoxP_mCherry_polyA_LoxP,Kir6.2(K185Q,\Delta N30)-GFP)\textsuperscript{stl443}, Figure 4.3A) were created as per the insulin-gCAMP6s vector, with modifications. The 5’ entry vector was the same as the cgCAMP6s fish construct. For the MEV, the sequence with mCherry and SV40-polyA stop sequence was cloned from vector Addgene 24334 and inserted into the PENTR/D Topo vector according to manufacturer’s instructions. For the 3’ entry vector, the mutant Kir6.2 subunit gene was cloned from an existing vector containing a gain-of-function mutation in the subunit (Kir6.2(K185Q,\Delta N30)-GFP), which has been previously described. Overlaps were introduced on this sequence to match it to the p3E-polyA vector after linearization with BamHI. The backbone and gene fragment were combined using Gibson assembly. 5’ entry, middle, and 3’ entry vectors were combined into the pDestTol2CG2 destination vector using LR Clonase II.

4.2.3 Generation of transgenic fish. Transgenic fish were created as follows. For each of the previously described constructs, 2nL of injection solution containing 25ng/\( \mu \)L of construct and 25ng/\( \mu \)L of Tol2 transposase RNA were injected into AB zebrafish embryos at the single-cell stage. The pDestTol2CG2 vector contains eGFP expressed under the cardiac myosin light-chain promoter as a transgenesis marker, permitting detection of subsequent founders by visible green fluorescence in the heart.

4.2.4 Animal lines and maintenance. In addition to the generated lines, we used AB wild-type fish as well as previously described \( \beta \)-cell-specific eGFP expressing fish (Tg(-1.0ins:eGFP)\textsuperscript{sc1})
(Moss et al., 2009), membrane-tethered insulin promoter-driven gCAMP6s fish (Tg(ins:lynGCaMP6s,ins:H2B:RFP) zebrafish (Kimmel and Meyer, 2016), and ubiquitin-gCAMP6s fish (Chen et al., 2017). 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. All animal procedures were approved by the Washington University in St. Louis IACUC.

4.2.5 Heat shock of zebrafish. For heat shock induction in larvae, larvae were placed in 20mL glass scintillation vials at 40-70 larvae/vial and heated at 37°C from day 1-5pf in a water bath for 3 hours/day. For adult induction, fish were transferred to glass beakers (7 fish/500mL) with air stones and placed in a 37°C water bath for 3hr/day for 2-10 days (as indicated in the figures).

4.2.6 Tissue isolation, dispersion, and culture. Islets were isolated, cultured, and dispersed as previously described (Emfinger et al., 2017), with minor modifications. For imaging, extracted islets were used on the day of isolation following 1-hour recovery.

4.2.7 PCR for genomic DNA analysis.

For genomic DNA analysis, zebrafish hearts were collected with forceps at the time of islet isolation from individual fish, digesting and gDNA was extracted using the PureLink Genomic Mini-kit (ThermoFisher).

4.2.8 PCR for gene expression analysis.

For islet gene expression studies, islet cDNAs were collected and prepared as previously described (Emfinger et al., 2017)

Zebrafish brains were collected by taking the head following decapitation across the gills, and isolating the brain using forceps as previously described (Lopez-Ramirez et al., 2016). Isolated brains were digested and RNA purified using the Qiagen RNeasy mini kit. cDNAs from brains
were prepared from extracted RNA using the ThermoFisher High-Capacity cDNA reverse transcription kit. Forward (5′-GAA TGG ACA ATT CTC GAG CGT CTC-3′) and reverse (5′-GCA AAT GAA TCG GAA GTT CCA AAA C-3′) primers for the connexin 35b have been previously described (Carlisle and Ribera, 2014).

4.2.9 Ex-vivo microscopy of adult zebrafish islet calcium. Islets were isolated as described (Emfinger et al., 2017). Glass-bottomed 35mM dishes (MatTeK) were coated with 1% agarose, and glass pipet tips were used to remove a section of agarose at the plate center, creating a well. Individual islets were transferred to wells and immersed in pH 7.4 Kreb’s Ringer’s solution buffered with HEPES (KRBH) containing 2mM glucose. The KRBH base solution consisted of (in mM): 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. Solutions of varying glucose concentrations were flowed into the plate chamber through lines running into and out of the chamber lid (Figure 4.1C). Bulk islet data were captured using a Zeiss Axiovert 200M microscope equipped with a Lambda DG-4 illumination system and EM-CCD camera and a Till photonics microscope with PolyChrome V monochromator and cooled CCD camera in the CIMED Live Cell Imaging Core (https://research.wustl.edu/core-facilities/cmed-live-cell-imaging-core/). Time lapse images used 100msec exposure at an interval of 500msec. For single-cell comparisons and determination of cell coupling, hi-res images were captured using the Nikon Spinning Disk confocal microscope (a motorized Nikon Ti-E scope equipped with PerfectFocus, a Yokagawa 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/). Images of ubiquitinating-gCAMP6s fish islets were collected on a Zeiss LSM 880 Airyscan confocal microscope equipped with two non-descanned detectors for two-photon imaging, also at the Washington University Center for Cellular Imaging. Time lapses images used 100msec exposure at 1 sec intervals. All images were analyzed in Fiji (Schindelin et al., 2012). To correct for movement in
x- and y-planes, images were stack registered (using StackReg, rigid body) in Fiji before analysis. All calcium image data are presented as change in fluorescence intensity vs baseline fluorescence intensity. Because the maximum excitability of an islet or β cell can vary, and the intensity of islet fluorescence can vary, glucose responses are shown normalized to the change in fluorescence in response to KCl (showing maximum islet depolarization) for the summary curve (Figure 4.1E). For determining trace cross-correlation and synchronicity, ROI measurements were analyzed using PeakCaller in MATLAB (Artimovich et al., 2017). The KCl response was excluded from segments in which cross-correlation analysis was performed, to capture the responses to glucose only.

4.2.10 Whole-cell voltage-clamp of zebrafish islet β-cells. Isolated β-cells were transferred into a recording chamber and Ca\(^{2+}\) currents were recorded in the whole-cell patch. The extracellular solution contained (in mM): NaCl 137, CsCl 5.4, CaCl\(_2\) 1.8, MgCl\(_2\) 0.5, Glucose 10, HEPES 5, NaHCO\(_3\) 3, NaH\(_2\)PO\(_4\) 0.16 (pH 7.4). The patch pipette was filled with a solution containing (in mM) CsCl 130, TEA-Cl 20, MgCl\(_2\) 1, CaCl\(_2\) 0.5, K\(_2\)ATP 3, EGTA 5, HEPES 10 (pH 7.4). The resistance of the pipettes was 2–3 MΩ. Currents were recorded from holding potentials of −70 mV during linear voltage ramps from −70 mV to -45 mV, followed by 10 voltage step starting from −45 to 65 mV. Data were filtered at 1 kHz and recorded at 3 kHz. Current records were analyzed using pClamp.

4.2.11 Live larval yolk glucose injections. Larvae (Tg(ins:lynGCaMP6s,ins:H2B:RFP) were cold-anesthetized (by setting the embryos in egg water-containing dishes on ice) and injected into the yolk with 10nL of 1mg/mL α-bungarotoxin (BTX, B137, Sigma). After 10-15 minutes embryos were embedded in low-melt agarose (1.2%) and imaged on Leica DM6000B microscope with SPOT-RT3 digital camera (Diagnostic Instruments, Inc.). Time-lapse images were captured at 500msec-1sec intervals with 100msec exposure. Larvae were injected in the yolk with a GB120F-10 borosilicate glass capillaries with filament and cut ends (Science
Products, GmBH) with glucose (1nL at 0.5M glucose, estimated final concentration of 10-20mM) in combination with Rhodamine-B isothiocyanate-Dextran (Sigma, R9379) as an indicator of injection success. Larvae were then imaged after the time lapses indicated. All images were analyzed in Fiji.

4.2.12 Analysis of mouse islet imaging in supplemental figures. Images from Johnston et al. (Movie S5, (Johnston et al., 2016)) and Kenty and Melton (Movie S2, (Kenty and Melton, 2015)) were downloaded from and [https://www.cell.com/action/showImagesData?pii=S1550-4131%2816%2930306-0](https://www.cell.com/action/showImagesData?pii=S1550-4131%2816%2930306-0) and [https://doi.org/10.1371/journal.pone.0122044.s006](https://doi.org/10.1371/journal.pone.0122044.s006), respectively. Images were analyzed using ImageJ and correlation analysis was performed using PeakCaller in MATLAB, as described above.

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

4.2.14 Statistics. Statistical analysis was done in GraphPad prism, except as noted. Each data set was tested for deviation from normal distribution (D’Agostino-Pearson). For multiple group column data comparisons, the indicated data were analyzed by ANOVA, followed by Tukey’s post-tests where normality assumptions were met. In comparisons of 2 groups, Student’s T test with Welch’s correction was used. In cases of non-normal distributions, the Kruskal-Wallis (more than 2 groups) or Mann-Whitney (2 groups) tests were used. All values are indicated as mean ± SEM, except where noted. For the Ca²⁺ response curve (Figure 4.1E), the points were fitted using nonlinear regression (log(agonist) vs response- 4 parameter variable slope).

4.3 Results:

4.3.1 Zebrafish islets express L-type calcium channels. The fish genome contains orthologues of the calcium channels found in mammalian islets, and RNA encoding these channels is present in islets (Sanhueza et al., 2009; Sidi et al., 2004; Tarifeño-Saldivia et al.,
2017; Zhou et al., 2008), but functional demonstration of Ca\(^{2+}\) channels in fish islet is lacking. Whole-cell voltage-clamp recordings from isolated zebrafish β-cells reveal nifedipine-sensitive calcium currents (Figure 4.1A), with current/voltage profiles (Figure 4.1B) and pharmacology very similar to those of mammalian L-type calcium currents (Kuryshev et al., 2014; Lipscombe, 2002; Mangoni et al., 2006; Striessnig et al., 2015), the observed major VDCCs in mammalian islets (Drews et al., 2010)). Similar currents were identified in both primary islet and secondary islet cells (Figure 4.1B).

4.3.2 Zebrafish adult islet calcium is glucose responsive. In order to image whole-islet calcium responses, we generated fish (Tg(-1.0ins:gCAMP6s)\(^{stl441}\), referred to as cGCAMP6s fish) expressing cytosolic gCAMP6s driven by the zebrafish insulin promoter. Ex vivo imaging (Figure 4.1C, schematic of the process) of isolated islets from these fish shows rapid and dramatic increases in fluorescence in response to changes in glucose, a change which correlated with the concentration of glucose (Figure 4.1D, E). The overall dependence of calcium on glucose concentration (Figure 4.1E) was similar to that seen in mammalian islets, with a sigmoidal response that plateaus above ~20mM glucose and EC50 of 10.17 mM glucose, slightly higher than the typically reported 5-9mM for mouse(Antunes et al., 2000), rat(Antunes et al., 2000), and human(Henquin et al., 2006) islets. Adult fish islets were relatively unresponsive at concentrations of glucose that evoke significant activity (8mM, for example) in mammalian islets(Henquin et al., 2006; Liu et al., 2008), the amino acids glutamine, alanine, and leucine caused no shift in responsivity of the islets when combined with 8mM glucose (Figure 4.1F) or in 2mM glucose (data not shown). Consistent with the nifedipine sensitivity of calcium currents in Figure 4.1A, the glucose-induced calcium responses are abolished by the addition of 50µM nifedipine (Figure 4.S1). Finally, islets did not show any response to sucrose (Figure 4.1G, representative trace), which indicates that these responses are not due to osmotic shock or other stressors.
Figure 4.1: Zebrafish adult β-cell calcium currents are glucose responsive. (A) Representative trace of isolated β-cell whole-cell patch-clamp revealing Ca2+ currents (left) which were inhibited by the nifedipine (right). Successive voltage steps from -45 to +65mV (lower panels) match corresponding current traces (upper panels). (B) Summary current-voltage relationship for calcium fluxes in isolated zebrafish β-cells (14 total primary and 2 secondary islet cells). (C-D) Imaging of islet calcium fluxes use isolated islets use the method diagramed in (C, left). Isolated β-cell-cGCAMP6s islets are placed in wells of agarose-coated glass-bottomed plates and solutions of variable glucose concentrations are flowed over the islet during continuous imaging. The right panel of C shows frames of islets at low glucose (left), high glucose (middle), and KCl (right). Representative fluorescence traces of whole islets (D), normalized to initial fluorescence, during transitions to low glucose (2mM, left), intermediate glucose (10mM, middle), and high glucose (20mM, right), followed by maximum excitation in KCl. (E) Summary curve for glucose-responsiveness of islet calcium, with change in fluorescence at the indicated concentrations normalized to the total change in fluorescence elicited by KCl depolarization (N=8-17 islets/concentration). (F) Calcium responses to amino acids when compared to 8mM glucose. The indicated amino acid concentrations were added to solutions of 8mM glucose in KRBH. The change in fluorescence is normalized to the maximum excitation in KCl. (G) Representative trace for islet calcium responses to sucrose (20mM).
Figure 4.2: Zebrafish islets are electrically uncoupled.

(A) Single-cell relative fluorescence traces for islets reveal lack of synchronicity and diverse glucose sensitivity. The far-left panel is the summary of the 87 tracked cells from this islet. Several cells (blue, left middle panel) were active at basal (2mM) glucose. Others (green, middle right panel) activated beginning at 12mM glucose or (orange, far right panel) or 20mM glucose. (B) Individual traces from the early 12mM transition (upper panel) and early 20mM glucose transition (lower panel) from islet in (A). The traces are from given clusters of adjacent cells in the larger islet, given in the inset of each panel and pseudo-colored in ImageJ. (C) Cross-correlation matrix (determined by PeakCaller) of the cells from the islet in (A). Color map key is given to the right of the panel. (D, left panel) Cell traces from an islet explant, color-keyed for the cells beginning oscillations at 12mM (green) and 20mM (orange) glucose. (D, right panel) Correlation matrix for the explant imaged in the left panel, with color key to the right of the matrix graph. (E) Cell traces from live imaging of islets following glucose injections into immobilized larvae. The schematic for the process (upper panel) shows the injection into the yolk. The islet image in green (membrane-gCAMP6s) and red (H2B-RFP) show the islet cells and the ROIs for the cell traces in the middle panel. Arrows in the lower panel indicate peaks for individual cells or groups. (F) Representative PCR of cDNAs from islets, brains, and hearts of zebrafish. Sets A and B are different pools of islets (biological replicates). Below the original panel is one contrast-enhanced to show the absence of any band in the islet cDNA lanes. The gDNA is isolated from hearts, and the plasmid (Addgene 24334) is a control for nonspecific replication.
4.3.3 Zebrafish islets are electrically uncoupled. Synchronous electrical and calcium oscillations across groups of β-cells, due to gap-junction coupling, is characteristic of mammalian islets (Farnsworth and Benninger, 2014). In contrast, adult zebrafish islets did not show synchronous calcium oscillations; instead gCAMP6s fluorescence oscillations were observed in individual β-cells. High-resolution images of adult islets confirmed that β-cell Ca\(^{2+}\) oscillations are asynchronous (Figure 4.2). In mammals, individual β-cells vary in their expression of metabolite transporters, metabolic enzymes, and ion channels involved in the insulin secretion response. Thus isolated mammalian β-cells exhibit variable sensitivities to glucose (Benninger et al., 2014; Silva et al., 2014), and gap-junction coupling is required to ensure uniform electrical and secretory responses across the intact islet. Without this coupling, cells across the islet would show independent glucose sensitivities, as is seen in the fish islets (Figure 4.2A), in which individual cells become active at very different glucose levels (i.e. low (2mM glucose, blue traces), intermediate (12mM, green), or high (20mM, orange traces)). Even cells that are physically close together lack synchronicity in their calcium spikes and glucose sensitivity (Figure 4.2B, traces from the islet in Figure 4.2A are highlighted for specific regions of adjacent cells and show transitions between low and intermediate (upper panel) and intermediate and high glucose (lower panel)). This lack of synchrony is evident in correlation coefficients for the cell traces from the islet (Figure 4.2C; the cross-correlation matrix corresponds to the cell traces for the islet in Figure 4.2A). These behaviors are present even in undigested explants (Figure 4.2D; lack of synchrony is shown in the raw traces (left) and correlation coefficient matrix (right)), illustrating that this is not an artifact of exocrine tissue digestion with collagenase. Additionally, explants and islets isolated from zebrafish expressing gCAMP6s under the ubiquitin promoter also show these asynchronous behaviors (Figure 4.52). Low correlation-coefficients for the cell traces in zebrafish islets is in stark contrast to mouse
islets; analysis of published islet images (Johnston et al., 2016; Kenty and Melton, 2015) shows high correlation (Figure 4.S3A, S6, and S7) in mouse islets at high glucose, whereas zebrafish islets (Figure 4.S3B, Figure 4.2A-C) do not show such strong correlations.

In mammalian islets, coupling of β-cells is created by the gap junction protein connexin 36 (Farnsworth and Benninger, 2014), and these junctions can be disrupted by 18-α-glycyrrhetinic acid (Davidson and Baumgarten, 1988). Calcium responses from islets that were pre-incubated in media with 18-α-glycyrrhetinic acid and then assessed in the presence of 18-α-glycyrrhetinic acid were not obviously different from those of untreated islets (Figure 4.S4).

Zebrafish larvae are sufficiently transparent to allow for visualization of the pancreas (Kimmel and Meyer, 2016). It is therefore possible to image islet glucose responses in vivo. Injecting larval yolk with glucose results in glucose diffusion into the larval circulation and a gradual change of glucose across the islet. Fish larvae expressing membrane-tethered gCAMP6s were injected with glucose and individual β-cells in islets of these fish still show asynchronous responses (Figure 4.2E), also apparent in previous in vivo imaged islets (Figure 4.S5, data from Kimmel and Meyer (Kimmel and Meyer, 2016)). These findings suggest that the lack of electrical coupling is not an artifact of ex vivo analysis.

Connexin 36 is the primary gap junction coupling protein in mammals (Farnsworth and Benninger, 2014). As zebrafish islets showed uncoupled behavior, we next asked whether the major orthologue of mouse connexin 36 in fish, connexin 35b (Carlisle and Ribera, 2014; Jabeen and Thirumalai, 2013; Watanabe, 2017), was expressed in zebrafish islets. Connexin 35b protein expression is well characterized in zebrafish brain (Carlisle and Ribera, 2014; Jabeen and Thirumalai, 2013)) and cDNA was only detected in brain and not in islets or heart by PCR (Figure 4.2F).
Figure 4.3: Islet inexcitability results in diabetes in zebrafish. (A) Structure of construct for conditionally expressing KATP-GOF mutations in the zebrafish islet, before (upper panel) and after (lower left panel) Cre recombination. A F2 larva is shown in the right lower panel, with the islet highlighted in the yellow circle. (B) GFP (left column) and bright-field (right column) images from dissected islets from control (upper), uninduced KATP-GOF fish (middle), and induced KATP-GOF (lower) fish. These images are taken at 12x. (C) Random blood glucose levels in WT fish (N=25) and KATP-GOF fish with (N=15) and without (N=4) 5 days of heat-shock. The blood glucose was measured 2 days after the last heat shock. (D) Random glucose levels in KATP-GOF fish heat shocked for 2 (dashed line) or 5 (solid line) days. For 2 days HS, N = 3-13 fish. For 5 days HS, N = 4-20 fish. Data are mean ± SEM. (E) Representative trace for calcium responses in KATP-GOF islets. WT (gCAMP6s only, N=8) and islets from two lineages of KATP-GOF/gCAMP6s fish (M111 (N=7), and M203 (N=8) are compared at 2mM and 20mM glucose, as well as KCl. Because the exact time of switching to the relative solutions differs slightly between islets, the time in each solution for each group is aligned to the others for showing relative change in the solution and for quantitative comparison of solution effects. Colors for each trace are indicated in the insert, and error bars are indicated above each curve. (F) Area under the curve for the 20mM portion of the curves in (E), in absolute time in solution x relative change in FIU from baseline. (G) Glucose levels from KATP-GOF fish sacrificed as adults which were either uninduced (N=4), induced as larvae (N=4), or induced as adults (N=15). (H) Mass (left panel, N=10-14) and body length (right panel, N=18) in KATP-GOF and control fish heat shocked as larvae. (C), (F), and (G) are analyzed by 1-way ANOVA followed by Tukey’s post-tests. *p <0.05, **p <0.01, ***p <0.001, and ****p<0001. Day 7 in (D) as well as the columns in (H) are analyzed with the Mann-Whitney test, with corresponding p-values displayed on the graphs.
4.3.4 Zebrafish with inexcitable islets become profoundly diabetic. The above studies demonstrate that \( K_{\text{ATP}} \)-dependent excitability is important in control of insulin secretion in the zebrafish. To examine whether \( \beta \)-cell membrane excitability controls whole animal glucose levels as in mammals, we generated additional transgenic fish (Tg(-1.0ins:LoxP_mCherry_polyA_LoxP,Kir6.2(K185Q,\Delta N30)-GFP),\( K_{\text{ATP}} \)- fish) which express a GFP-tagged gain-of-function Kir6.2 subunit \( \beta \)-cells after Cre recombination (schematic in Figure 4.3A). We crossed these \( K_{\text{ATP}} \)-fish to fish expressing HSP-16 inducible Cre, to generate \( K_{\text{ATP}} \)-GOF zebrafish. These fish show expression of the transgene (determined by visualizing tagged GFP) in \( \beta \)-cells after heat-shock (Figure 4.3B) and develop severe hyperglycemia (blood glucose >400mg/dL, Figure 4.3C). The persistence of the hyperglycemia varies with the degree of heat shock (Figure 4.3D), with 5 days of heat-shock producing a more severe and more persistent hyperglycemia.

4.3.5 Zebrafish \( K_{\text{ATP}} \)-GOF islets lose calcium responses. We crossed \( K_{\text{ATP}} \)-GOF fish to cGCAMP6s fish, and isolated islets after heat-shock induction of the GOF transgene, in order to assess glucose-responsivity of intracellular calcium. \( K_{\text{ATP}} \)-GOF fish show significantly diminished glucose-induced calcium responses, even at high (20mM) glucose levels (Figure 4.3E, F), but show normal KCl responses. These results are consistent with \( K_{\text{ATP}} \)-GOF inhibiting electrical activity at all [glucose] by hyperpolarizing cells, an effect that is overcome by direct KCl-induced depolarization, which in turn suggests that \( \text{Ca}^{2+} \) channels are unaffected.

4.3.6 \( K_{\text{ATP}} \)-GOF zebrafish develop secondary consequences of insulin insufficiency. Mammals with untreated neonatal diabetes develop significant secondary consequences including growth limitations (Girard et al., 2009; Remedi et al., 2009). \( K_{\text{ATP}} \)-GOF zebrafish induced as larvae showed increased blood glucose levels (similar to those in adult-induced fish, Figure 4.3G) as well as significantly reduced body length and weight at 10 weeks of age, when compared to tankmates from the same clutches that were also heat-shocked but which lacked
the Cre gene (Figure 4.3H). Many $K_{\text{ATP}}$-GOF fish were too small to obtain sufficient blood to measure glucose (reflected in lower number of glucose readings from the analysis in Figure 4.3G than numbers in the length and mass measurements in Figure 4.3H). These data suggest that $\beta$-cell $K_{\text{ATP}}$-GOF induces similar secondary effects in zebrafish as it does in mammals.

4.4 Discussion:

4.4.1 Conservation of metabolic regulation mechanisms in the zebrafish. We previously reported the expression and function of $K_{\text{ATP}}$ channels in zebrafish islet $\beta$-cells (Emfinger et al., 2017) and showed that pharmacological activation of $K_{\text{ATP}}$ channels worsened glucose tolerance in adult fish, indicating a role for native $K_{\text{ATP}}$ in zebrafish metabolism. Li et al. showed that conditional larval activation of a distinct $K_{\text{ATP}}$-GOF transgene, or treatment of larvae with diazoxide resulted in increased larval glucose levels and block of over-nutrition induced $\beta$-cell expansion (Li et al., 2014a). In the present study we have shown that intracellular calcium is glucose sensitive, and that the glucose dependence of calcium oscillations is similar, but not identical, to mammalian islets. We further show that islet calcium responses to glucose can be blocked by the $\beta$-cell specific induction of GOF mutations in $K_{\text{ATP}}$, resulting in profound diabetes. This suggests that the key components of excitability governing insulin secretion and nutrient responses in mammals are fully conserved in zebrafish.

However, several important differences should be noted between our results and findings from mammalian islets. Firstly, amplification of glucose signals by amino acids, which is observed in mammalian islets (Henquin et al., 2006; Liu et al., 2008), was not seen in adult fish islets (Figure 4.1F). The absence of an amplifying effects of amino acids suggests either that additional signaling intermediates present in vivo may be lost ex vivo, or that the links between the initiating and amplifying pathways in insulin that are present seen in mammals may be absent in fish. More detailed experiments will be required to determine what components of
known amplifying pathways (Anders and Erik, 2017; Henquin et al., 2006; Henquin, 2000; Kalwat and Cobb, 2017) in mammals may be conserved in zebrafish.

Secondly, the adult zebrafish islet is electrically uncoupled. Mammalian islet electrical coupling is critical for normal glucose tolerance (Farnsworth and Benninger, 2014; Klee et al., 2008). In mice lacking connexin 36, which forms the primary gap junctions in islet β-cells, basal insulin is elevated, and glucose responses of the overall islet are slowed, which worsens relative glucose tolerance in otherwise healthy animals (Head et al., 2012). Disruption of rapid, pulsatile insulin secretion is seen in many forms of diabetes. The absence of coupling in zebrafish β-cells may explain, in part, the relatively decreased sensitivity to glucose in zebrafish islets. Slowed release of insulin and reduced sensitivity to glucose relative to mammals may be why glucose tolerance of wild-type fish, both from the studies of others (Eames et al., 2010) and ourselves (Emfinger et al., 2017), is relatively reduced when compared to mammalian glucose tolerance (Andrikopoulos et al., 2008) (with a higher peak, and a longer, more gradual return to baseline glucose after a glucose injection). Zebrafish diets do not typically contain high sugars (wild diets consist of algae, insects, and zooplankton (Spence et al., 2007; Watts et al., 2012)) and conceivably, the evolutionary pressure of plant-based diets in land animals may have led to the development of gap junction coupling to ensure more robust glucose-responses.

Because of the lack of coupling, we noted very variable glucose-sensitivities in individual cells within the intact islet. Prior reports from early zebrafish development also showed differential glucose-sensitivities of β-cells ex vivo in larval and early juvenile zebrafish (Singh et al., 2017); ex vivo recordings of these younger zebrafish islets clearly show cells activating independently at thresholds from 5-20mM glucose.

Lack of coupling may also help to explain variability in glucose levels in K<sub>ATP</sub>-GOF fish following transgene induction. Shorter periods of induction produce more transient rises in
glucose levels (Figure 4.3D) and response variability increases with time. Li et al. showed that, when functional β-cells are present, overnutrition can induce β-cell mass expansion (Li et al., 2014a). One possible explanation for variability in K\textsubscript{ATP}-GOF fish long after induction is that recombination induced by heat-shock is uneven across the islet, such that production of new β-cells from unrecombined precursor cells may occur and, consequently, blood glucose responses may gradually normalize.

4.4.2 Zebrafish recapitulate major features of mammalian neonatal diabetes. Mice expressing K\textsubscript{ATP}-GOF at birth have reduced growth rates (Girard et al., 2009; Remedi et al., 2009), and in the case of severe mutations, die early (Koster et al., 2000). Growth retardation has also been observed in some neonatal diabetics (Polak and Cave, 2007). K\textsubscript{ATP}-GOF zebrafish are profoundly hyperglycemic, and show similar growth limitation when induced as larvae, though they can survive and reproduce (data not shown). Other secondary consequences of hyperglycemia can occur directly in the islet, with loss of β-cell identity and insulin content (Brereton et al., 2014b; Wang et al., 2014). Whether this occurs in zebrafish islets exposed to high glucose for long periods is unknown. Future lineage tracing studies using models such as ours may clarify these questions. Finally, what consequences of prolonged hyperglycemia may be preventable or reversible by pharmacological lowering of glucose (via insulin treatment, sulfonylurea treatment, or treatment with SGLT2 inhibitors, which have been reported to reduce glucose levels in zebrafish larvae (Dalgin and Prince, 2015; Li et al., 2014a), and, in some cases, adults (Capiotti et al., 2014)) remains to be determined.

4.4.3 Unique advantages of the zebrafish as a model organism for metabolic disease?

We have shown that zebrafish exhibit many similarities to mammals in their β-cells glucose responsivity, and in the hyperglycemia that results from electrical glucose-unresponsivity. While lack of electrical coupling distinguishes zebrafish islets from mammalian islets, and may affect quantitative relationships, it also offers the potential to functionally correlate individual cell
behavior with gene and protein expression. In wild-type mammalian islets, synchrony of
responsivity in islet β-cells masks variation in glucose sensitivities and calcium responses in
individual cells within the islet. Some studies in mice suggest that subpopulations of β-cells
within the islet may regulate overall islet behavior, and may be more vulnerable to stressors
known to induce β-cell dysfunction (Johnston et al., 2016; Weir and Bonner-Weir, 2017).
Zebrafish may therefore provide advantages for determining which β-cells may be most
vulnerable to stressors, and what changes in gene expression correlate with loss of function
during exposure to those stressors. Additionally, transparency of larval zebrafish allows this to
be done directly in vivo via injection of immobilized larvae (our study here, as well as Kimmel
and Meyer (Kimmel and Meyer, 2016)), which is not possible in mice. Recent findings from
larval zebrafish (Singh et al., 2017) illustrate the ex vivo advantages of this approach as applied
to studying different populations of islet cells and their relative functional vs. proliferative
capacities. Applying such approaches to disease states may be informative regarding identifying
mechanisms of disease development in ways not possible in mammalian systems.

4.4.4 Can we identify novel regulators of neonatal diabetes in zebrafish? Zebrafish are
transparent as larvae, reproduce frequently and in large clutches, have a fully sequenced
genome, and have many methods of generating mutant lines, making them well-suited to large-
scale studies like drug and genetic screens (Barros et al., 2008; Henke et al., 2013; Howe et al.,
2013a; Howe et al., 2013b; Huang et al., 2001b; Kamel and Ninov, 2017; Lieschke and Currie,
2007). We now provide a potentially useful disease model for screening to identify novel
regulators of diabetes that avoids disruption of pancreatic development via transcription factor
mutations (Dalgin and Prince, 2015; Huang et al., 2001b; Kimmel et al., 2015; Kimmel and
Meyer, 2016), pharmacological issues with hyperglycemic induction (potential off-target effects
of drugs), or regeneration of the pancreas following cell ablation (Moss et al., 2009). These
features, as well as persistent hyperglycemia (Figure 4.3D,G) and development of secondary
consequences (Figure 4.3H) suggest this model will be useful as a background for drug and genetic screens. It is known that significant variation in disease severity and treatment responses occurs even in patients and animals with single mutation-induced diabetes, such as those with $K_{\text{ATP}}$-GOF mutations (Marshall et al., 2015; Philla et al., 2013; Polak and Cave, 2007; Remedi et al., 2011; Thurber et al., 2015; Vaxillaire et al., 2007). Our findings here, and the techniques adapted by our group (Emfinger et al., 2017; Kimmel and Meyer, 2016) and others (Capiotti et al., 2014; Fang et al., 2014; Heckler and Kroll, 2017; Kimmel and Meyer, 2016; Kinkel and Prince, 2009; Li et al., 2014a; Moss et al., 2009; Singh et al., 2017) to studying zebrafish metabolism, suggest that screens using $K_{\text{ATP}}$-GOF zebrafish might be possible to identify such hidden modulators. However, given that not all mammalian islet responses to glucose are conserved in zebrafish, and given that some properties of islets (such as pancreatic regeneration) distinguish zebrafish from mammals, careful phenotyping and characterization of metabolism is required before such studies can begin.

4.5 Supplemental figures
Figure 4.S1: Nifedipine blocks glucose-induced calcium oscillations. (A) Representative trace showing islet treated with 20mM glucose and 10μM nifedipine. (B) Summary of nifedipine block of calcium responses to high glucose. 20mM glucose, N= 8; 20mM glucose + nifedipine, N=10. (*) p<0.05.
Figure 4.S2: Glucose-responsive calcium oscillations in ubiquitin-gCAMP6s zebrafish islets. (A) Cell traces from pancreatic explants reveal asynchronous firing at high (16.7mM) glucose (left panel). Cells inactivate in low (1mM) glucose (middle panel) and some reactivate at low glucose with K<sub>ATP</sub> inhibitor tolbutamide (right panel). (B) Cross-correlation matrices for the cells tracked in (A) at high and low glucose. (C) Cell traces from an islet from ubiquitin-gCAMP6s fish show similar asynchronous firing at high (left panel) and low (right panel) glucose levels.
Figure 4 S3: Islet synchrony comparison between mouse and zebrafish. (A) ROI (upper left panel) on Johnston et al. movie S5 (Johnston et al., 2016), which shows a mouse islet stained with Fluo2 and incubated in 11mM glucose. Traces from the ROIs (lower left panel; individual cells are grey and average is in red) were analyzed with PeakCaller and the cross-correlation matrix (right panel) shows high correlation between the cells in the islet. (B) ROIs from cGCAMP6s zebrafish islet (upper panel). Traces from the ROIs are in the lower panel (average of the islet is in red). (C) A magnification of some of the islet traces in (B) show variation in β-cell Ca²⁺ amplitude, frequency, and timing. (D) Cross-correlation matrix of traces in (B).
Figure 4.S4: Gap junction inhibitor does not alter zebrafish β-cell Ca\textsuperscript{2+} synchrony. (A, left) Traces from islet treated with 20\textmu M 18-α-glycyrrhetinic acid (18AGA). Islets were incubated in media with the 18AGA for 1hr prior to imaging, and all imaging buffers in this trace contained the same concentration of 18AGA. Cells active beginning at 2mM glucose, 12mM glucose, and 20mM glucose are indicated in blue, green, and orange, respectively. (A, right panel) A magnification of the trace data in the left panel, with a subset of cells individually colored to highlight variation in islet β-cell Ca\textsuperscript{2+} amplitude, frequency, and timing of activation. (B) Cross-correlation matrix of the cell traces in (A).
Figure 5.S5: In-vivo asynchronous islet cell responses following glucose injection. Previously imaged islet (Kimmel and Meyer, 2016) from Kimmel and Meyer 2016 shows the same asynchronous behavior as seen in islet from figure 2D. (Left) Regions of interest in the islet image showing individual cells. (Right) Arrows show cells firing out of sync with the majority of the islet (average is in red).
Figure 4.S6: Cross-correlation analysis of mouse islets (set 1: islets 1, 1b, 2 and 3) from Movie S2 (Kenty and Melton, 2015). (A), (B), (C), and (D) represent individual islets from Movie S2 previously published by Kenty & Melton. The traces (grey, individual cells; red is the islet average) from each islet are in the left panel. The middle panel indicates the ROIs for the islet traces. The right panel shows the cross-correlation matrix for the islet.
Figure 4.S7: Cross-correlation analysis of mouse islets (set 2: islets 4, 5, 6 and 7) from Movie S2 (Kenty and Melton, 2015). (A), (B), (C), and (D) represent individual islets from Movie S2 previously published by Kenty & Melton. The traces (grey, individual cells; red is the islet average) from each islet are in the left panel. The middle panel indicates the ROIs for the islet traces. The right panel shows the cross-correlation matrix for the islet.
Chapter 5: Discussion

This thesis focuses on understanding mechanisms linking membrane excitability, diabetes, and the consequences of these phenomena for whole-body glucose homeostasis. The work I have carried out informs both basic physiology and the etiology of diabetes. My studies provide new insights into the secondary consequences of hyperglycemia in an established mouse model ($K_{\text{ATP}}$-GOF mice) and further elucidation of the role of excitability in function of the β-cells of a non-mammalian vertebrate, the zebrafish, both through exploring native properties of zebrafish $K_{\text{ATP}}$ and the consequences of $K_{\text{ATP}}$-GOF mutations in zebrafish β-cells.

5.1: Novel insights to and implications for excitability-driven diabetes

5.1.1 Secondary consequences of hyperglycemia and diabetes-a role for inflammation?

The clinical definition of diabetes (elevated blood glucose) belies the complex underlying physiology of the different forms of the disease. Even with presumably clear links between mutation and pathway defect, monogenic forms of diabetes (Marshall et al., 2015; Pearson et al., 2006; Philla et al., 2013; Polak and Cave, 2007; Vaxillaire et al., 2007; Wambach et al., 2010) and hyperinsulinism (Martínez et al., 2016; Mazor-Aronovitch et al., 2007) can present with significant variation in disease severity and treatment response, illustrating that additional uncontrolled factors are involved. Several factors, such as age, time on insulin therapy, and degree of glucose control, have been suggested to play a role in patients with neonatal diabetes (NDM) regarding the success of transitioning to sulfonylurea therapies and dose of sulfonylurea required to regulate glucose levels (Marshall et al., 2015; Thurber et al., 2015). However, these factors do not explain certain observations regarding variability of incidence and disease severity in NDM (Vaxillaire et al., 2007); therefore both identifying underlying mechanisms involved in disease severity and identifying potential biomarkers for predicting relapse in
transient NDM will be of potential interest for developing new therapies to achieve remission in otherwise permanent NDM.

It has previously been observed that correction of hyperglycemia prevents or reverses some of the secondary consequences of severe diabetes in NDM animal models (Brereton et al., 2014b; Remedi et al., 2011; Remedi et al., 2009; Wang et al., 2014). There may be also be reduction of β-cell mass in humans with K_{ATP} channel mutations (Busiah et al., 2014; Greeley et al., 2017), though underlying reasons for it remain unclear. Following an early, short period of glibenclamide treatment, diabetes dramatically remits in a subset of β-cell K_{ATP}-GOF mice. These remitting animals maintain glucose levels below the threshold at which severe effects of hyperglycemia induces loss of β-cell identity and dedifferentiation, as is otherwise seen in untreated or non-remitting K_{ATP}-GOF mice (Remedi et al., 2011; Wang et al., 2014). These observations make this mouse model unique for studying mechanisms regulating control of glycemia in the context of transient vs. permanent NDM.

My results in these K_{ATP}-GOF mice suggest a previously unconsidered role of inflammation and hepatic glucose production in the early progression of the disease, leading to changes in glucose levels that can have lasting influences on disease outcome. Prior to disease induction, animals that subsequently remit exhibit decreased glucose production, and lower circulating TNF-α than those animals that which develop extreme hyperglycemia following the end of treatment (Figure 2.8). Additionally, IL-6 levels of the remitters do not rise during the induction process. These results suggest the following model: low glucose production and inflammation in remitter mice results in glucose rising only slowly during the induction process, thereby limiting glucotoxic damage such that an increase in sensitivity to basal insulin then enables remitters to maintain non-toxic glucose levels even in the absence of glucose-stimulated insulin secretion (GSIS). By contrast, non-remitter mice begin with higher glucose production and TNF-α, as well as demonstrating increases in IL-6 which are concurrent with the
separation in glucose. As GSIS is lost, glucose rises rapidly in non-remitters due to higher glucose production, and subsequent glucotoxic damage induces increases in systemic inflammation, driving up glucose production and more rapid increases in glucose levels in a feed-forward cycle (Figure 2.8). This prevents the non-remitters from increasing sensitivity to basal insulin, and non-remitters consequently remain severely diabetic.

This model makes several predictions: i) If inflammation plays a causal role in the separation of glucose levels, altering inflammation during induction will change the potential outcome in excitability-driven hyperglycemia, and perhaps in other forms of diabetes. Reducing inflammation will limit the rise of glucose and will result in more remittance, whereas increasing inflammation during disease onset will reduce remittance. Consistent with this hypothesis, preliminary studies in which I treated $K_{\text{ATP}}$-GOF mice with Meloxicam, a broad-spectrum non-steroidal anti-inflammatory agent, did increase the proportion of animals that remitted in several cohorts over the fraction of remitting mice seen in the vehicle co-treatment (Figure 2.7). ii) As with prior observations of late intervention using sulfonylureas (Remedi et al., 2009), anti-inflammatory treatments will fail to rescue established diabetes in these $K_{\text{ATP}}$-GOF mice, presumably due to lack of basal insulin production in the already severely diabetic mice at the time of treatment. Indeed, co-treatment with Meloxicam did not rescue $K_{\text{ATP}}$-GOF mice following the development of severe diabetes (Figure 5.1). Which specific inflammatory factors are involved in this process, where they are produced, and which tissues they act upon remain to be determined. iii) These results may have important implications for disease outcome. Thus, if treatment of the mice with pro-inflammatory cytokines prevents or reverses remission could be illuminating, as stressors which alter insulin sensitivity, such as puberty, are suspected to be why some individuals relapse during adolescence in NDM (Akihiko et al., 2018; Polak and Cave, 2007; Vanelli et al., 1994).
Figure 5.1: Early but not late intervention with anti-inflammatory agents rescues diabetes in K<sub>ATP</sub>-GOF mice. K<sub>ATP</sub>-GOF mice treated with tamoxifen and sulfonylurea vehicle (10% DMSO in 1x PBS) rapidly become severely diabetic. Late intervention (6 day treatment with daily injections of the Cox inhibitors Sulindac and Meloxicam, dose indicated in the figure, or vehicle) fails to persistently reduce blood glucose in the diabetic mice. Sulindac is known to have off-target effects on blood coagulation, which is one possible explanation for the deaths in some of the mice treated with that agent. (B) By contrast, co-treatment of K<sub>ATP</sub>-GOF mice with meloxicam during induction (upper panel) increases the fraction of remitting animals over treatment with vehicle (1uL/gBW DMSO) alone (lower panel). (B) is reproduced from Figure 2.7 for comparison.
Disrupting TNF-α levels or downstream signaling prevents or reverses insulin resistance in some mouse models (Borst, 2004). Hyperglycemia and insulin deficiency, as well as increased free fatty acids, endoplasmic reticulum stress, and reactive oxygen species production, have been shown to activate pro-inflammatory responses (Donath, 2014; Gonzalez et al., 2012), and elevated cytokines like TNF-α can reduce insulin action in the periphery (Borst, 2004; Lorenzo et al., 2008). Some studies also suggest that prolonged elevation of inflammatory cytokines can increase hepatic glucose production (Chen et al., 2012; Hotamisligil, 2006a; Sheng et al., 2012). Recent data also suggest a role for inflammation in regulating β-cell maturity and functionality (Nordmann et al., 2017). In these studies, treatment of islets with cytokines, particularly IL-1β and TNF-α, caused a reduction in markers of β-cell function and maturity. Diabetic mice in this study also showed elevations in cytokines and reduction in mature β-cell markers that were reversed by anti-inflammatory treatments (Nordmann et al., 2017). The feed-forward cycle of increased inflammation, dysregulated glucose control, and glucotoxicity in the model I present here, as well as the inflammatory changes seen in the non-remitting mice, are consistent with the above studies of inflammation and insulin resistance. Taken together, these findings point to a key causal role for inflammation in the pathogenesis of multiple forms of diabetes and suggest that treatments targeting inflammation may prove beneficial beyond the context of neonatal diabetes.

5.1.2 Are findings regarding inflammation in mouse models relevant to human NDM? Pro-inflammatory cytokines are elevated in type 2 diabetes (T2D) and chronic elevation of cytokines is associated with increased risk of developing T2D in obese non-diabetic individuals (Borst, 2004; Donath, 2014; Duncan et al., 2003; Glund and Krook, 2008; Jahromi et al., 2011; Kim et al., 2009). Reciprocally, treatment of patients with chronic inflammatory disorders with antibodies to IL-6, TNF-α, or the receptors of these cytokines, resulted in improvements in insulin sensitivity and metabolic function (Borst, 2004; Burska et al., 2015; Donath, 2014; Gupta-
Ganguli et al., 2011; Schultz et al., 2010), suggesting therapeutic potential in modulating inflammation and glycemic control. Determining whether variation in pro-inflammatory markers correlates with disease phenotypes in human NDM, and whether modulating them has therapeutic value, are potentially important areas for further investigation following from the results I present here (Chapter 2).

5.2.3 A role for hepatic glucose production in NDM? $K_{ATP}$-GOF mice that subsequently remit have reduced hepatic glucose production prior to disease onset when compared to non-remitters which eventually develop severe diabetes (Figure 2.6). If increased liver glucose production does play a causal role in subsequent development of severe diabetes in $K_{ATP}$-GOF mice, and if lower hepatic glucose production is one key reason that some $K_{ATP}$-GOF mice remit, one major prediction is that altering liver glucose production would affect the outcome of $K_{ATP}$-GOF: increasing liver glucose production will reduce remittance, whereas decreasing glucose production will increase the fraction of remitters, i.e. potentially convert otherwise permanent NDM to transient disease. This prediction is consistent with data from other studies: animals with uncontrolled liver glucose production become hyperglycemic even with significant hyperinsulinemia (Michael et al., 2000), while blocking glucose production can reverse or prevent diabetes in animals lacking sufficient insulin secretion (Gomez-Valades et al., 2006; Rines et al., 2016b), and anti-glucagon therapies show improvements in glucose control in some human studies (Kazda et al., 2016; Rines et al., 2016b).

This may be of particular significance for early neonates since variation in birth weight and timing of birth can significantly alter glucose production. Low body weight infants and preterm infants have greatly increased liver glucose production (Hemachandra and Cowett, 1999; Keshen et al., 1997; Mitanchez, 2007). While direct study of gluconeogenesis in NDM patients is lacking, lower-than-normal birth weight for gestational age is frequently seen in NDM (Aguilar-Bryan and Bryan, 2008; von Mühlendah and Herkenhoff, 1995). Whether
pharmacological modulation of glucose production will be consequential in our mouse model, and whether alterations in glucose production play a role in human NDM, require further study.

5.2: Novel insights to and implications of excitability-dependent insulin secretion in zebrafish

5.2.1 Conservation of insulin secretion mechanisms in non-mammalian vertebrates. The importance of insulin signaling is illustrated by conservation of insulin protein and many components of the insulin signaling pathway between mammals, other vertebrates, and some invertebrates, despite evolutionary lineages diverging long ago (Das and Dobens, 2015; Ellsworth et al., 1994; Schlegel and Stainier, 2007). Zebrafish, for example, possess orthologues of many key genes involved in development of insulin-secreting β-cells (Dalgin and Prince, 2015; González-Alvarez et al., 2009; Huang et al., 2001a; Huang et al., 2001b; Tehrani and Lin, 2011) and in insulin responses of many tissues which are targets of insulin signaling in mammals (Maddison et al., 2015; Schlegel and Stainier, 2007; Seth et al., 2013a). As zebrafish become more widely used to model metabolic disease processes, it will be important to establish what similarities and differences exist between zebrafish and mammalian metabolic regulation.

I have shown in this work (Chapter 3) that zebrafish, like mammals, possess K_{ATP} channels in pancreatic β-cells, that these channels have similar properties to mammalian K_{ATP}, and that pharmacologically over-activating these channels reduces glucose tolerance in adult zebrafish. I have also shown that intracellular Ca^{2+} responds similarly to glucose in zebrafish β-cells. These findings demonstrate conservation of key elements of insulin control pathway and lend support for zebrafish as a model in which to probe insulin secretion and disease.
One interesting finding of these studies is that zebrafish islets are not electrically coupled (Figure 4.2). In mammals, electrical coupling of islet $\beta$-cells to one another is integral to normal glucose tolerance and can dramatically influence glucose tolerance when disrupted (Head et al., 2012; Nguyen et al., 2014; Perez-Armendariz, 2013). In mammals, inhibition of gap junction coupling causes glucose intolerance and increases basal insulin secretion (Head et al., 2012). In mouse islets lacking gap junctions, the calcium responses of $\beta$-cells are slowed (Benninger et al., 2008; Benninger et al., 2014). These results may explain, in part, why fish require longer time to return to basal glucose levels following glucose injections when compared to mammals given the same injection (3-4 hours in fish vs 2 hours in mice, both in our hands (Figure 3.1A, for example) and those of others). Lack of gap junction coupling and hence lower glucose sensitivity may reflect a dietary difference: zebrafish native diets consist of zooplankton and insects (Spence et al., 2007; Watts et al., 2016; Watts et al., 2012) and being less carbohydrate rich, such diets may not need rapid insulin secretion to control glucose levels. In mammals, gap junction coupling may have resulted from evolutionary pressures as diets shifted to plants in land-based mammals. What consequences the uncoupling has for nutrient responses in zebrafish is an area of further interest.

One approach to studying this is to artificially couple the islet by introducing gap junctions into zebrafish $\beta$-cells. In an attempt to do so, I have generated zebrafish which express inducible, $\beta$-cell specific mouse connexin 36 (Figure 5.2). Preliminary results in these fish have indicated that transgene induction decreases calcium responses to glucose, although the precise mechanisms for and consequences of this are unclear, and will require further characterization. What the consequences of artificially coupling the islet will be for the zebrafish may provide insight into the evolution of that trait in the different lineages of vertebrates, as well as helping to clarify the mechanisms regulating metabolism and islet function in zebrafish.
Figure 5.2: Transgenic expression of gap junction proteins in zebrafish β-cells alters islet calcium responses. (A, left) Schematic for the transgene recombination in zebrafish expressing inducible, β-cell specific mouse connexin 36 (MsCx36) (MsCx36-Tg fish). Zebrafish with this gene but lacking Cre express mCherry in islet β-cells. When crossed to Cre-expressing fish, the activation of Cre results in loss of mCherry expression and activation of mouse Cx36 only in β-cells. (A, right) A 5dpf F2 larva of the MsCx36-Tg fish, showing the mCherry expression in b-cells (highlighted by the yellow circle). (B) Normalized relative fluorescence intensity unit (RFIU) graphs of islets from zebrafish possessing the MsCx36, cGCAMP6s, and HSP-16 Cre genes, after 10 days of heat-shock to activate the Cre (blue line, N=3), as well as islets expressing only β-cell cGCAMP6s (WT, black line, N=4). These islets are sequentially incubated (for procedures, see Chapter 4, schematic in Figure 4.1C) in the concentrations of glucose indicated in gray, with final maximum excitation elicited using 20mM glucose and KCl. Because the exact transition times for individual traces do not perfectly align, segments of each curve corresponding to the solutions in question are adjusted to match alignment and normalized to response to KCl.
5.2.2 What are the consequences of severe hyperglycemia in non-mammalian vertebrates? Zebrafish metabolism differs from that of mammals in several key respects. For example, zebrafish lack Glut4 (Tseng et al., 2009), a key insulin-responsive transporter in mammals which mediates a significant portion of insulin-stimulated glucose uptake in the periphery. Additionally, some metabolic regulating hormones such as leptin differ in function between mammals and zebrafish: leptin, which is poorly conserved between mammals and zebrafish, is an important regulator of lipid homeostasis in the former and a regulator of glucose and tissue regrowth in the latter (Michel et al., 2016). Leptin is expressed in fish liver rather than in adipose tissue, and increases in multiple different types of fish during fasting (Gorissen et al., 2009), in contrast to the fall of leptin during fasting in mammals (Ahima et al., 1996). Leptin also has strong effects on liver glucose production pathways in fish (Michel et al., 2016), and deficiency in leptin signaling in zebrafish fails to cause infertility, weight gain, or increases in overall adiposity as it does in mammals (Michel et al., 2016). Findings from mutations in insulin receptor are unclear: partially abrogating insulin action results in weight gain in fish (Riddle et al., 2018), rather than growth defects as seen in some mammalian models and in knock-down experiments in fish (Toyoshima et al., 2008; Yang et al., 2017). Additionally, many fish organs, including the pancreas, can regenerate (Moss et al., 2009), in contrast to mammals. What consequences result from dysregulation of insulin production and signaling as well as hyperglycemia will therefore be important to determine when examining metabolism in zebrafish.

As previously discussed (chapter 1, and above), severe hyperglycemia and insulin deficiency in mammals can result in growth defects, β-cell dedifferentiation, and dysfunction in many organ systems. In this work, I show that β-cell-specific K<sub>ATP</sub>-GOF mutations can cause severe hyperglycemia in zebrafish (Figure 4.3). Induction of the GOF mutation beginning at the larval stage results in defective growth, mirroring both other genetic mutations which disrupt
zebrafish β-cell development and cause hyperglycemia, as well as paralleling consequences of severe K\textsubscript{ATP}-GOF in mammals. Whether other consequences of K\textsubscript{ATP}-induced diabetes, such as dedifferentiation, ketoacidosis, and organ failure that are observed in mammals also occur in zebrafish remains to be determined. Other models of severe hyperglycemia in zebrafish suggest these consequent effects may occur (Heckler and Kroll, 2017; Olsen et al., 2010). Sequential streptozotocin injections in adult zebrafish produce steady-state glucose levels of 300mg/dL and result in kidney glomerular basement membrane thickening, increased glycated protein levels, retinal thinning, and defects in wound repair, comparable to effects of similar hyperglycemia in mammals (Olsen et al., 2010). Genetic mutations that disrupt β-cell differentiation in embryogenesis similarly cause wound repair defects and smaller body weight (Kimmel et al., 2015). Few of these studies (Capiotti et al., 2014; Li et al., 2014a; Zang et al., 2017) have shown that correction of glucose levels can ameliorate the effects attributed to the high glucose. Systemic administration of hypoglycemic agents such as sulfonylureas or phlorizin, which are tolerated by adult zebrafish ((Capiotti et al., 2014), and discussion, figure 3A) and which lower glucose levels in larvae (Dalgin and Prince, 2015; Li et al., 2014a), may allow us to resolve these questions.

One interesting feature to consider in exploring these questions is the lack of cell-cell electrical coupling in islets. In larvae exposed to high glucose and lipids, β-cell expansion can occur. I have observed (Figure 4.3D) that K\textsubscript{ATP}-GOF fish show increasing variation in glucose levels with time after transgene induction, and that fewer rounds of heat-shock gene induction produce a less severe, transient hyperglycemia. One possible explanation for this (Figure 5.3B) is that there may be incomplete Cre-recombination across the islet, and that in such cases where sufficient numbers of unrecombined β-cells remain, the growth of new β-cells from unrecombined progenitors can restore insulin secretion over time, since the functions of the new cells would not be influenced by the cells arising from those that had undergone recombination.
An alternative possibility is that unknown pathways may cause variation in the response to $K_{\text{ATP}}$-GOF, as is seen in mouse models and humans (discussed above).

That $\beta$-cells in zebrafish islets are electrically and chemically uncoupled from one another may offer the possibility of correlating changes in function within individual $\beta$-cells to gene expression and pathway signaling within those same cells (as, for example, was done recently in examining early pancreatic development (Singh et al., 2017)), something difficult to do in mice because the synchrony of $\beta$-cells can mask variation in cell behavior. There is some indication in mice that subpopulations of cells within the islet may regulate overall islet behavior, and may be more vulnerable to stressors known to induce $\beta$-cell dysfunction (Johnston et al., 2016; Weir and Bonner-Weir, 2017). Zebrafish may therefore prove advantageous for determining which sub-populations of $\beta$-cells may be most vulnerable to stressors, and what changes in gene expression correlate with loss of function during exposure to those stressors.

5.2.3 Can we identify unknown modulators of metabolism in zebrafish? Zebrafish possess many traits that make them well-suited for high-throughput, systematic studies of metabolism. Transparency of larvae makes possible both tracking of specific proteins and organ development by in vivo imaging, as well as making it possible to track in vivo responses of cell populations within the islets or other tissues to injections of glucose (Figure 4.2D and Kimmel and Meyer (Kimmel and Meyer, 2016)) or other metabolites. Zebrafish reproduce frequently and in large clutches, making them well-suited to large-scale studies like drug and genetic screens.
Figure 5.3: KATP-GOF zebrafish as a model for diabetes. (A) Adult zebrafish survive chronic treatment with the sodium-glucose co-transporter inhibitor phlorizin, without significant alterations in blood glucose levels in the absence of diabetes, consistent with prior reports in larvae (Dalgin and Prince, 2015). (B) Model for transience of glucose phenotypes in heat shock of HSP-Cre-inducible KATP-GOF zebrafish. Glucose levels (Figure 4.3D) vary in shorter periods of heat-shock and return to normal after a week with 2 days of heat shock. The variability in glucose levels seen in the fish may partly be explained by β-cell expansion. In this model, zebrafish islets with incomplete recombination in both β-cells and progenitors may undergo expansion in unrecombined β-cells and progenitors, giving rise to additional functional β-cells, as seen in larvae treated with overnutrition (Li et al., 2014b). Eventually, functional β-cell mass restores GSIS and glucose levels normalize. By contrast, when the islets and progenitors undergo complete recombination, newer β-cell mass, being unexcitable, may not be generated (Li et al., 2014b), and any new β-cell mass would lack GSIS, resulting in persistently elevated glucose levels. (C) Model for F3 genetic modifier screen in KATP-GOF zebrafish. In the breeding scheme (left panel), randomly mutagenized fish crossed with homozygous KATP-GOF fish produce heterozygous mutants containing both genetic elements. Breeding these produces fish containing the randomly mutated locus, the KATP-GOF, both mutations, or neither. Comparison of secreted glucose levels (right panel) would identify mutagenized fish in the GOF background which would be grown for further phenotyping and breeding. Genetic comparison (lower right) would identify the mutated loci of interest. (D) Proof of principle for non-destructive measurement of glucose in zebrafish larvae. 16-hour treatment of larvae with phlorizin in egg medium (E3) results in secretion of glucose into medium by larvae with hyperglycemia, which can be measured using plate assays. Diabetes in this cohort is simulated by co-treatment with the KATP opener diazoxide.
One current limitation in zebrafish is measurement of \textit{in vivo} glucose levels. Larval glucose measurements frequently require the destruction of the larvae and measurement of whole-larval glucose (Kamel and Ninov, 2017; Seth et al., 2013b) and many studies of hyperglycemia have used this method (Dalgin and Prince, 2015; Jurczyk et al., 2011; Kimmel et al., 2015; Li et al., 2014a). There are some methods of indirect measurement of glucose levels, such as sensors of glucose uptake using fluorescent compounds (Park et al., 2014) and readouts of glucose production (Gut et al., 2013). However, glucose production or uptake may not reflect glucose levels, and studies are lacking on the effects of the fluorescent compounds used on viability, reproduction, and other measures of zebrafish health. While some FRET-based techniques for glucose measurement are in development ((Maioli et al., 2016; Veetil et al., 2010) for example), they have yet to be validated in zebrafish. Finally, as diabetes and high glucose levels have been shown to have significant secondary effects, mutations that affect glucose levels may also affect growth and survival in ways that limit some phenotyping measurements more easily done in adults, such as insulin and glucose injections (Kinkel et al., 2010) and tissue regeneration (Kimmel et al., 2015).

It has been demonstrated that the sodium-glucose co-transporter 2 inhibitor phlorizin, used to lower glucose in diabetic patients by causing secretion of glucose in urine (Gerasimova et al., 2014), can lower glucose in zebrafish larvae (Dalgin and Prince, 2015). Adult zebrafish tolerate the drug without major ill effects, and it does not cause hypoglycemia in wild-type adult zebrafish (Figure 5.3A). To overcome limitations of current larval glucose assays, I have developed a prototype, non-destructive assay for larval glucose (Figure 5.3D) by incubating larvae with phlorizin to stimulate secretion of glucose into egg medium, and then measuring that glucose. This assay has the potential to measure glucose secretions from individual larvae (data points in figure 5.3D represent individual larvae). The method is non-toxic and adult fish arising from larvae so-treated show no secondary effects. The assay demonstrates that secretion of
glucose is increased in hyperglycemic larvae: co-treatment of phlorizin-incubated larvae with diazoxide to block insulin release resulted in increased secretion of glucose into egg medium above treatment with phlorizin alone (Figure 5.3D). Together with other techniques that I have adapted in this work to study zebrafish pancreatic function and ion channels (Chapters 3 and 4), this assay may enable phenotyping of fish at different developmental stages to dissect mechanisms that may worsen or ameliorate hyperglycemia. The inducible and β-cell specific $K_{\text{ATP}}$-GOF model provides a consistent background on which to screen for unknown genetic modifiers of glucose homeostasis (Figure 5.3C), independently of other dietary and environmental factors and without the need for exogenous compound administration to generate the hyperglycemia.
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**Academic positions/Employment**

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<td>Washington University in St. Louis</td>
<td>Bruce Carlson, PhD and Erik Herzog, PhD</td>
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<td>05/2016</td>
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<td>Colin Nichols, PhD, and Bhavna Murali</td>
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Professional society memberships

Society of General Physiologists (15 June 2016-present)

Research Fellowship Awards

NIH T32DK108742-01

Academic and Professional Honors

- First prize for poster presentation, Midwest Islet Club 2018, St. Louis, MO, United States
- First prize for poster presentation, CIMED Research Day 2017, St. Louis, MO, United States
- Second prize for poster presentation, basic science category; Diabetes Research Center Diabetes Research Day Symposium, 2016, St. Louis, MO, United States
- National Merit Scholarship 2003-2008
- Naval Reserve Officer Training Corps Scholarship 2003-2005 (Discharged for Medical Reasons)
- Dean’s list, Georgia Institute of Technology, 2005

Students mentored in lab

- Yixi Wang, November 2015-May 2018
- Felesha Clarke, May 2016-August 2016
- Hannah Steber, September 2015-December 2015
- Peter Hung, June 2015-August 2015

Published papers:


**Papers submitted:**


**Papers in preparation:**


**Conference poster presentations (presenter in bold):**


**Talks given:**


**Other work presented (presenter in bold):**


**Relevant skills:**

1) **Computational:**
   a. *Writing/graphics:* Microsoft Office, GraphPad PRISM, Corel Suite (Photopaint & Draw)
   b. *Statistics:* GraphPad PRISM, R
   c. *Sequence design:* LaserGene Suite (MegAlign, SeqBuilder), ApE, SerialCloner
   d. *Image analysis:* Fiji (ImageJ), MATLAB

2) **Laboratory:**
   a. *Protein analysis:* Tissue protein isolation, western blot, IF, ELISA
   b. *Tissue isolation, lipid, and protein/RNA prep:* (mouse/zebrafish), particularly of metabolic tissues (liver, fats, brain, muscle tissue, and pancreas), chloroform/methanol extraction of tissue lipids, glycogen determination, and TLC separation of various lipids
   c. *Molecular biology:* PCR; cDNA prep for gene expression; plasmid design, growth, and processing for Tol2 vectors (for making transgenic zebrafish)
   d. *Primary and cell line tissue culture:* particularly of whole islets and islet β-cells (from both mouse and zebrafish), INS-1 and Min-6 cell lines; GSIS
   e. *Electrophysiology:* whole cell & excised-patch experiments, particularly of islet β-cells (mouse/zebrafish) and cardiomyocytes.
   f. *Live animal studies:* tolerance tests (pyruvate, insulin, glucose injections; in both mice and zebrafish), clamp experiments (hyperinsulinemic-euglycemic, hyperglycemic, hyperinsulinemic-hypoglycemic; these include administering radiotracers for glucose uptake, lipid processing, and glucose production), fasting/re-feeding studies, diet studies, compound incubation (zebrafish, both adult and larvae)
   g. *Microscopy:* calcium imaging using dyes & genetic calcium indicators, both epifluorescence and confocal (Spinning Disk)
   h. *Plasma analysis:* lipoprotein analysis (density-gradient and HPLC lipoprotein isolation; peptide analysis (Skyline)), lipid analysis (TG/CE assays and TLC separation)

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