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The Circadian Clock Generates Anticipatory Insulin Secretory Cycles by Regulating Beta-cell Enhancers

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Mark Perelis

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Abstract

The circadian clock is a biological oscillator present in all terrestrial life forms that aligns organismal functions with the daily rising and setting of the sun. In mammals clocks are expressed both in pacemaker cells in the brain that are directly entrained by light as well as in all peripheral cells where they coordinate the timing of anabolic and catabolic processes in anticipation of daily changes in nutrient availability. It has long been recognized that humans exhibit a robust variation in blood glucose levels that are regulated in part by rhythmic secretion of the glucose-lowering hormone insulin across the sleep/wake cycle and there is now strong evidence that disruption of circadian cycles contribute to metabolic disorders including diabetes mellitus. Experimental genetic analyses in mice have recently revealed that circadian clock genes play an essential role in regulating insulin secretion in pancreatic β-cells. However, whether the circadian influence on β-cell insulin secretion is a consequence of cell-intrinsic or extrinsic timekeeping and the underlying transcriptional mechanisms were largely unknown. The studies presented in this dissertation demonstrate that the core circadian transcriptional activators CLOCK and BMAL1 act cell-autonomously to coordinate 24-hr rhythmic cycles in β-cell secretory capacity by regulating the transcription of genes that regulate the assembly, packaging, transport, and exocytosis of insulin containing vesicles. Examining the genome-wide binding patterns of CLOCK and BMAL1 in β-cells revealed that they regulate rhythmic genes by binding to cell-type specific transcriptional enhancers, providing a possible explanation for the marked differences in clock-controlled genes across mammalian organs. Finally, acute inhibition of circadian

transcription in β-cells of adult mice inhibits insulin secretion and causes severe glucose intolerance. Therefore, the developmentally established repertoire of transcriptional enhancers enables the circadian control of β-cells and regulates glucose metabolism throughout life.

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Chapter I

Introduction

Circadian clocks (from the latin circa diem, about a day) are found in all photosensitive organisms where they maintain temporal organization of cell and systems physiology in synchrony with the rotation of the Earth (Edgar et al. 2012, Milev and Reddy 2015, Bass 2012). In mammals, circadian clocks are organized hierarchically, with light-responsive 'master' pacemaker clocks located within the suprachiasmatic nucleus (SCN) of the hypothalamus, which in turn drive rhythmic cycles within extra-SCN neurons and peripheral organs. The SCN aligns peripheral tissue clocks with the environmental light cycle through a combination of direct autonomic nervous system efferent and neuroendocrine signals (such as through control of 24-hr cycles of the hypothalamic-pituitary-adrenal axis and rhythmic growth hormone production), and feeding-derived cues (la Fleur et al. 2000, Bartness et al. 2001, Buijs et al. 2001, Buijs et al. 2003, Dibner et al. 2010, Gerber et al. 2013).

The core circadian clock within both brain and peripheral cells is encoded by a negative transcription-translation feedback loop composed of transcription factors in the forward limb (CLOCK, NPAS2, and BMAL1 encoded by *Circadian locomotor output cycles kaput*, *Neuronal pas domain protein 2*, and *Bone and muscle Arnt-like 1* genes) that activate repressors within the negative limb (PER1/2/3 and CRY1/2 encoded by *Period1/2/3* and *Cryptochrome1/2* genes), which feedback to repress the activators (Partch et al. 2014). PER and CRY proteins are targeted for degradation by the ubiquitin-protesome by FBXL and SCL-betaTRCP ubiquitin ligases (Busino et al. 2007, Godinho et al. 2007, Reischl et al. 2007, Shirogane et al. 2005, Siepka et al. 2007), relieving the

inhibition of CLOCK/BMAL1 and enabling the re-initiation of the transcription cycle which lasts approximately 24-hrs (Partch et al. 2014).

A long-recognized aspect of circadian physiology in humans is the daily variation in blood glucose and insulin levels across the day (Polonsky et al. 1988; Gagliardino et al. 1984; Van Cauter et al. 1997). Clinical investigation in human subjects has shown that glucose clearance and insulin sensitivity in response to orally-administered isocaloric glucose challenge peak in the early morning and decline in the afternoon and evening (Aparicio et al. 1974, Bowen and Reeves 1967, Carroll and Nestel 1973, Jarrett and Keen 1969, Roberts 1964); moreover, both ultradian (<24-hr) and circadian (~24-hr) variation in glucose tolerance has been observed in response to intravenous glucose delivered in either continuous or oscillatory infusion (Shapiro et al. 1988; Sturis et al. 1995). The daily variation in response to isocaloric feeding arises due to both changes in insulin sensitivity and secretion of insulin from β-cells in the pancreas and has been demonstrated using isotopic labeling and modeling approaches (Saad et al. 2012). These observations suggest that intrinsic meal-independent processes give rise to daily cycles in glucose metabolism; however the underlying molecular basis for this observation has only recently emerged. Experimental genetic studies described here now demonstrate that cell-autonomous circadian transcription cycles within pancreatic β-cells produce rhythmic cycles in nutrient-dependent insulin secretion through interactions with cell-type specific regions of open chromatin. This dissertation focuses on the genomic and physiologic basis for the circadian control of glucose metabolism in pancreatic β-cells,

which may be applied towards understanding the pathophysiology of human diabetes mellitus by exploring 4 major questions:

1. Given it's functional redundancy for generating circadian rhythms in brain is pancreatic CLOCK expression necessary for maintaining glucose homeostasis?

2. Does the molecular clock exert cell-autonomous effects on the β-cell and what genes does it regulate?

3. How do circadian transcription factors regulate β-cell genes and are there differences in other tissues?

4. Is the expression of clock genes necessary for both the development of pancreatic β-cells and their function in adult-life?

Chapter II

Background

Circadian control of glucose metabolism

Insulin-producing β-cells exist in specialized organoids called islets of Langerhans within the pancreas, and their dysregulation and/or destruction is central to the pathogenesis of type 1 and type 2 diabetes (Muoio and Newgard 2008). Type 1 diabetes is an autoimmune disease involving the near complete destruction of pancreatic β-cell mass, whereas type 2 diabetes develops as a consequence of impaired β-cell compensation in the setting of impaired insulin action (insulin resistance) (Maclean and Ogilvie 1955, Matveyenko and Butler 2008). β-cells sense changes in blood glucose levels by its passive transport via glucose transporters (GLUT2 in mice but also GLUT1 and GLUT3 in humans) (Thorens 2014) its metabolism by glycolytic enzymes and ultimate oxidation in the mitochondria results in the generation of ATP. Rising ATP levels in the cytoplasm cause ATP sensitive potassium channels (K^+_{ATP}), which normally export K^+ ions to maintain plasma membrane polarization, resulting in membrane depolarization and the opening of voltage-gated L-type calcium channels. The rapid influx of Ca^{2+} ions triggers the immediate exocytosis of pre-docked insulin containing secretory vesicles and a variety of metabolites generated from mitochondrial TCA cycle flux such as glutamate and second messenger signaling molecules including 3',5'-cyclic adenosine monophosphate (cAMP) and Diacyl glycerol (DAG) cause the sustained trafficking and release of insulin vesicles (Seino 2012, Seino et al. 2011). The metabolic and electrochemical coupling of the β-cell to ambient glucose concentrations therefore enables the precise control of blood glucose levels.

Genetic analyses in mice first pointed to a key role for clock gene function in β-cells when it was discovered that multi-tissue *Clock*$^{\Delta 19/\Delta 19}$ mice, originally isolated from an ENU mutagenesis screen for circadian behavior phenotypes (Vitaterna et al. 1994), developed obesity and hyperglycemia by 6-8 weeks of age (Turek et al. 2005) in parallel with reduced circadian variation in blood glucose levels (Rudic et al. 2004). Paradoxically, *Clock*$^{\Delta 19/\Delta 19}$ mice did not display hyperinsulinemia, a hallmark of obesity-associated diabetes, providing the first clue that clock-deficiency leads to β-cell failure (Turek et al. 2005). Subsequent studies showed that multi-tissue circadian disruption in both *Clock*$^{\Delta 19/\Delta 19}$ and *Bmal1*$^{-/-}$ mutant mice caused reduced glucose tolerance and glucose-stimulated insulin secretion both *in vivo* and in isolated islets *in vitro* (Lamia et al. 2008, Sadacca et al. 2011, Marcheva et al. 2010, Lee et al. 2011), further implicating clock genes in the regulation of pancreatic insulin production. However, a conundrum in these early studies was that loss-of-function mutations in clock genes in liver also result in severe hypoglycemia during fasting (Lamia et al. 2008), which is likely due in part to mitochondrial dysfunction (Peek et al. 2013). Indeed, the idea that clock deficiency exerts distinct effects in different tissues emerged from the seemingly paradoxical observation that glucose levels vary according to nutrient state in multi-tissue mutants; thus hyperglycemia is found in the post-prandial condition whereas hypoglycemia occurs with prolonged fasting (Turek et al. 2005, Rudic et al. 2004).

The complex effects of multi-tissue circadian gene ablation on metabolism can mask an underlying functional defect, however, tissue specific and inducible gene ablation has been used effectively to delineate organ-specific clock gene functions. Tissue-specific

mutagenesis has confirmed the concept that clock function within endocrine β-cells plays a role in insulin secretion after feeding, whereas within liver it is critical in mitochondrial oxidative metabolism during fasting. Specifically, the use of tissue-specific gene targeting within pancreas (*PdxCre;Bmal1*$^{flx/flx}$) and β-cells (*RipCre;Bmal1*$^{flx/flx}$) showed that *Bmal1* deletion resulted in severe glucose intolerance and hypoinsulinemia, as well as impaired glucose-stimulated insulin secretion in islets isolated from these animals, confirming the idea that cell-autonomous expression of clock genes within the pancreas is essential for insulin secretion and glucose homeostasis (Marcheva et al. 2010, Sadacca et al. 2011, Lee et al. 2013). Notably, disruption of genes encoding repressors in the negative limb of the circadian clock in *Cry1/2*$^{-/-}$ mice results in hyperglycemia and impaired glucose tolerance due in part to altered regulation of glucagon and glucocorticoid signaling in the liver, and mice expressing a loss-of-function mutant allele of *Cry1* display reduced β-cell growth, reduced islet insulin content, and impaired insulin secretion (Lamia et al. 2011, Okano et al. 2013, Zhang et al. 2010). *Per2* mutant mice display reduced blood glucose levels and increased glucose-stimulated insulin secretion (Zhao et al. 2012), suggesting complex and distinct roles of individual clock components in regulating glucose homeostasis.

Insight into how disruption of the pancreatic clock might lead to impaired insulin secretion on a cellular level emerged from the initial finding that islets from both the multi-tissue and pancreas-specific *Bmal1* knockouts displayed normal insulin content in parallel with intact glucose-stimulated calcium influx, suggesting that the molecular pancreatic clock must control a late stage of insulin exocytosis. Exposure of

PdxCre;Bmal1$^{flx/flx}$ islets to KCl, an insulin secretagogue that bypasses glucose metabolism by directly depolarizing the plasma membrane, reduced insulin secretion in the *Bmal1* null islets, confirming an exocytotic defect in islets lacking an intact clock (Marcheva et al. 2010) (Fig. 1). However, studies using islets isolated from *RipCre;Bmal1*$^{flx/flx}$ have also found alterations in antioxidant gene expression and mitochondrial ATP production, indicating that multiple levels of clock regulation exist in the β-cell (Lee et al. 2013).

Further insight into the molecular defects underpinning the impaired exocytosis in circadian mutant islets arose from studies using signaling molecules generated from intermediates in glucose metabolism and second messenger signaling cascades that act on well-defined steps in insulin secretion, including 3',5'-cyclic adenosine monophosphate (cAMP), which is synthesized by adenyl cyclase downstream of G_s-coupled receptor activation following stimulation with circulating nutrient-sensitive incretin hormones such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP). cAMP enhances insulin secretion in β-cells by activating protein kinase A (PKA) and rap guanine nucleotide exchange factor 3 (RAPGEF3, also known as EPAC1/2) signaling (Seino et al. 2011). The observation that islets isolated from *Clock*$^{\Delta 19}$, *Bmal1*$^{-/-}$ and *PdxCre;Bmal1*$^{flx/flx}$ mice displayed reduced insulin secretion when stimulated with forskolin, an agonist of the cAMP biosynthetic enzyme adenylyl cyclase, 8-br-cAMP, an cAMP analog, and Exendin-4, an agonist of the upstream GLP-1 receptor revealed that the clock regulates this pathway in β-cells (Marcheva et al. 2010 (Fig. 1).

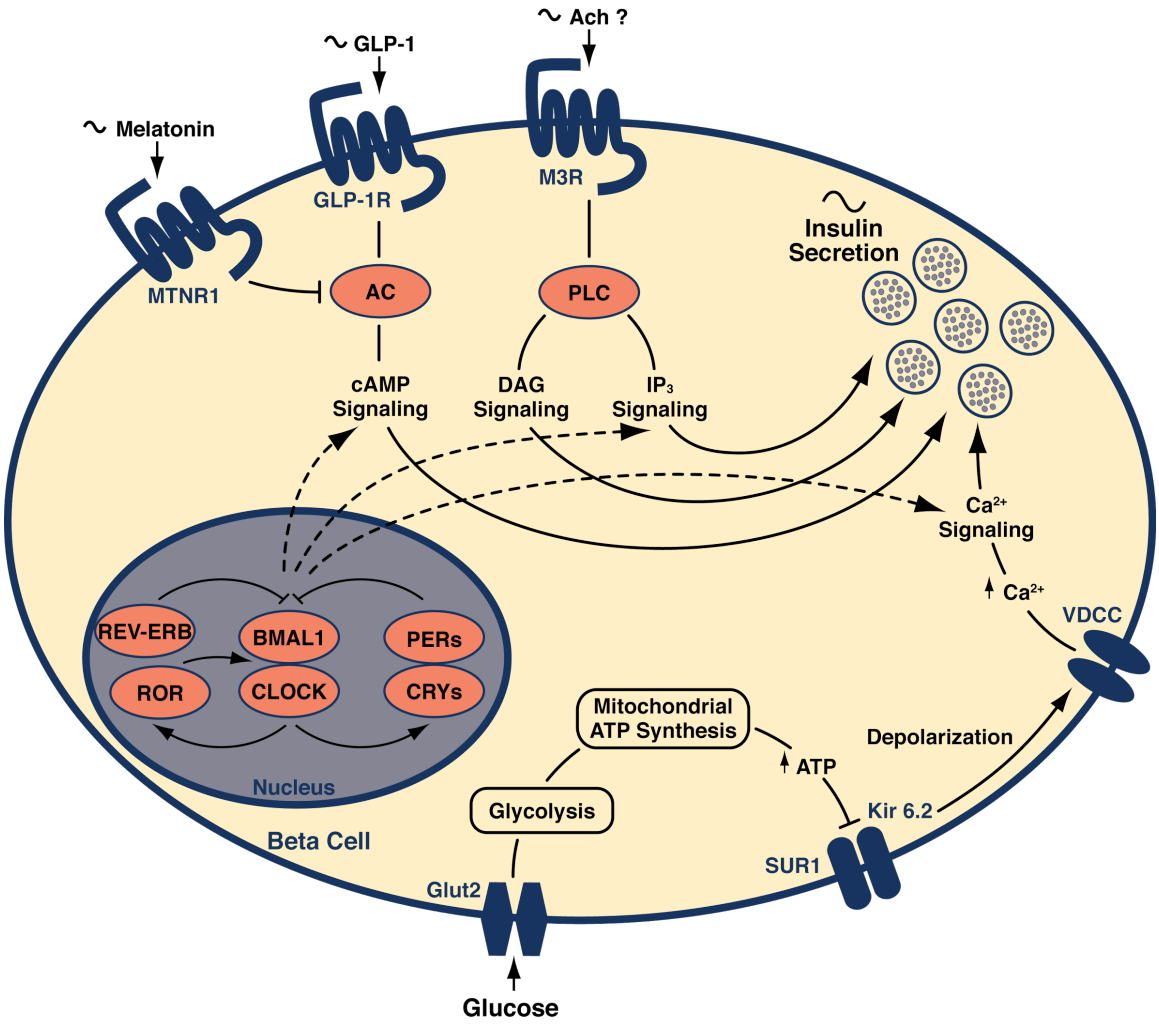

Fig. 1: Molecular clock in the β-cell regulates insulin secretion together with circulating systemic factors.

The circadian system regulates insulin secretion likely by promoting rhythmic transcription of gene networks involved in glucose-, cAMP-, and Ca^{2+}-stimulated exocytosis. Circulating levels of GLP-1 and melatonin modulate insulin secretion by augmenting or reducing intracellular cAMP levels, respectively. Acetylcholine-

stimulated DAG synthesis promotes insulin secretion, possibly linking brain-derived cholinergic signals to the temporal control of β-cell function.

Mechanisms of genome-wide circadian transcription

In the last 15 years, genome-wide transcription profiling studies using cDNA microarray and RNA sequencing (RNA-seq) technologies have been applied to define the repertoire of circadian-controlled genes in a variety of mouse tissues *in vivo,* enabling elucidation of the molecular underpinnings of tissue-specific circadian control of physiology (Panda et al. 2002, Storch et al. 2002, Koike et al. 2012, Menet et al. 2012, Vollmers et al. 2012, Zhang et al. 2014). These studies have revealed rhythmic oscillations of up to 43% of mRNAs in mice (Zhang et al. 2014), many of which encode key rate-limiting proteins important in organ-specific functions (Panda et al. 2002, Storch et al. 2002, Zhang et al. 2014). One approach to delineate the role of tissue-autonomous clock gene expression on cell physiology, versus systemically-driven oscillations of circulating serum factors or autonomic input (Gerber et al. 2013), has entailed application of tissue-specific genetic models of circadian disruption. Transgenic models for studies of inter-tissue circadian regulation of behavior and metabolism have included mice overexpressing the circadian repressor REV-ERBα within liver, which abolishes liver-specific core circadian gene activity (*LAP-tTA/TRE-Rev-erbα* mice) (Kornmann et al. 2007), or multi-tissue $Clock^{\Delta 19/\Delta 19}$ mice with genetic rescue of the wild-type CLOCK protein within the brain (*Scg2*::tTA;tetO::$Clock^{WT}$; $Clock^{\Delta 19/\Delta 19}$ mice), which restores circadian behavior (Hughes et al. 2012). Microarray analysis of mRNA expression in livers of these animals revealed that while oscillation of most genes depended on a functional cell-autonomous hepatic clock, a small subset of genes, including the core circadian repressor *Per2*, continued to oscillate robustly in clock-deficient hepatocytes in the presence of a functioning brain

clock, suggesting that *Per2* cycles in liver are likely generated by systemic signals rather than cell-autonomous clock gene expression within the intact animal. Together, these studies demonstrate that a combination of cell-autonomous and non-autonomous processes drive rhythmic transcription within liver; however, how distinct cell physiologic functions might be rhythmically controlled has remained less well understood.

Role of pancreatic clock in organismal glucose homeostasis *in vivo*

While *in vitro* studies have enabled the identification of molecular pathways regulating insulin secretion controlled by islet cell-autonomous clocks, as noted above, it has been challenging to tease apart the opposing effects of circadian disruption across distinct peripheral metabolic tissues in multi-tissue circadian mutants *in vivo*. For example, the diabetic phenotype in multi-tissue $Clock^{\Delta 19/\Delta 19}$ and *Bmal1* mutant mice is not as pronounced as in islet-specific circadian mutant mice. Despite significantly impaired glucose-stimulated insulin secretion observed in *isolated* pancreatic islets, early metabolic analyses of young multi-tissue $Clock^{\Delta 19/\Delta 19}$ mice did not detect hyperglycemia (Turek et al. 2005). This was later attributed to masking effects of clock disruption in the liver, which compensated for the reduced β-cell insulin secretion by impairing hepatic glucose production and increasing insulin sensitivity (Lamia et al. 2008, Zhang et al. 2010). Of note, when clock mutations were isolated to the liver, mice displayed hypoglycemia at times when the animals were inactive and fasting (Lamia et al. 2008, Peek et al. 2013), in contrast to the hyperglycemia and impaired insulin exocytosis

observed predominantly in the feeding period in pancreas-specific clock mutants (Marcheva et al. 2010). Thus, the composite effects of clock function in different tissues *in vivo* give rise to the overall 'net' glucose phenotype . Importantly, electrolytic ablation of the SCN pacemaker cells in mice also impaired hepatic insulin sensitivity and glucose production (Coomans et al. 2013), indicating that the brain clock also regulates hepatic glucose metabolism. Ultimately, pancreatic, hepatic, and pacemaker clocks appear to cooperate to maintain whole body glucose homeostasis throughout the day by promoting insulin-stimulated glucose clearance during the feeding periods while sustaining glucose levels by enhancing mitochondrial oxidative metabolism and glucose production in liver during periods of fasting (Fig. 2).

In addition to the cell-autonomous effects of the circadian clock within distinct metabolic tissues on glucose homeostasis, islet clocks *in vivo* are also exposed to a variety of systemic signals that in turn contribute to blood glucose homeostasis in the intact animal. For example, the β-cell clock is influenced by time-of-day-dependent variations in levels of the circulating glucagon-like peptide 1 (GLP-1) hormone. GLP-1 is secreted in response to nutrient ingestion by enteroendocrine L-cells in the intestine and potentiates glucose-stimulated insulin secretion in β-cells (Mojsov et al. 1987). Recent studies have demonstrated that GLP-1 secretion stimulated by administering an oral glucose load exhibits circadian rhythmicity in rats and that rhythmic expression of mRNAs encoding the circadian PAR-bZIP transcription factor thyrotroph embryonic factor (TEF) and extracellular signal-regulated kinase modulator pleiotropic regulatory locus-1 (PRL-1) contribute to circadian GLP-1 secretion in L-cells (Gil-Lozano et al. 2014). Interestingly,

maximal glucose-stimulated GLP-1 secretion and GLP-1-induced insulin secretion in rats occurred at ZT22, close to the transition from the dark-to-light period when β-cells are most sensitive to glucose in mice (Gil-Lozano et al. 2014, Marcheva et al. 2010) Rhythmic circulating GLP-1 levels are therefore an additional layer of systemic circadian control that acts synergistically with the pancreatic clock to regulate blood insulin and glucose levels throughout the day (Fig. 2).

Clocks throughout the body are also entrained and modulated by extracellular signals such as temperature fluctuations (Buhr et al. 2010, Saini et al. 2012, Morf et al. 2012), serum factors (Balsalobre et al. 1998, Balsalobre et al. 2000, Gerber et al. 2013), hormones (Le Minh et al. 2001, Chaves et al. 2014), and feeding (Damiola et al. 2000, Kohsaka et al. 2007, Hatori et al. 2012), and exposure to environmental conditions associated with disrupted circadian rhythms has also been shown to impact the pancreatic clock. For example, chronic exposure of mice to constant light (LL) for 10 weeks, which causes a total loss of SCN-driven behavioral rhythms, dampened the amplitude and altered the phase and synchrony of circadian *Per1-LUC* bioluminescence in isolated islets (Qian et al. 2013). Islets isolated from these LL-exposed rats displayed impaired insulin secretion in response to glucose, but not GLP-1 or tolbutamide, a secretagogue that acts by chemically closing K^{+}_{ATP} channels (Qian et al. 2013), suggesting shared regulation of some, but not all, pathways by the islet cell-autonomous clock and systemic circadian signals perturbed in LL. Similarly, a study testing the impact of simulated shift work on β-cell function found that a circadian disruption (CD) paradigm where mice were exposed to serial 6-hr advances in the light/dark cycle every 4 days for 8 weeks inhibited

glucose-stimulated insulin secretion and insulin-stimulated glucose clearance (Lee et al. 2013). β-cell-specific *Bmal1* knockout mice displayed more severely impaired insulin secretion when subjected to this CD protocol (Lee et al. 2013), suggesting that proper expression of clock genes in the β-cell might help to prevent hyperglycemia even in the setting of systemic circadian disruption in shift work.

Circadian control of pancreatic islet function in humans

Both epidemiologic and population genetics studies have shown strong associations between alterations in circadian clock function in shift workers and heritable genetic variants (single nucleotide polymorphisms) with the risk of hyperglycemia and diabetes in humans (Knutsson 2003, Dupuis et al. 2010). The effect of recurrent disruptions of the day/night cycle that occur in shift work can be simulated in the laboratory by exposing humans to 28-hr days, where the usual 12-hr:12-hr light:dark cycle is extended to 14-hr:14-hr, resulting in days that are 4-hrs longer than usual. In this paradigm, humans experience a complete 12-hr inversion of the external light/dark cycle after 3 successive 28-hr days, and circadian clock-controlled biomarkers such as blood cortisol levels display a pattern that is anti-phasic to its usual relationship with the light/dark cycle (Scheer et al. 2009). While misaligned, humans displayed significantly increased glucose and insulin levels after ingesting food at regularly scheduled mealtimes, which for some subjects reached levels seen in pre-diabetes (Scheer et al. 2009, Morris et al. 2015), indicating that circadian misalignment caused reductions in both insulin sensitivity

(increasing the demand for insulin secretion) and β-cell compensation for elevated glucose levels (insufficient insulin to lower blood glucose).

An additional link between circadian function and human glucose metabolism has come from genome-wide association studies (GWAS) testing for heritable genetic variation associated with fasting glucose levels and diabetes incidence in human populations. GWAS studies have identified associations between polymorphisms in close proximity to genes involved in circadian rhythm regulation to human β-cell function. Specifically, single nucleotide variants mapping to the circadian clock repressor *CRY2* (Dupuis et al. 2010) and a receptor for the circadian hormone melatonin *MTNR1B* (Bouatia-Naji et al. 2009, Dupuis et al. 2010) are associated with elevated fasting glucose levels in multiple human populations. Genetic variants in *BMAL1* and *CRY2* have also been linked with development of type 2 diabetes (Woon et al. 2007, Hu et al. 2010). In addition, allelic variants of *CRY2* have been associated with a decrease in two different oral glucose tolerance test-based disposition indices due to decreased insulin sensitivity (Boesgaard et al. 2010). While *CRY2* and *MTNR1B* are expressed in human pancreatic islets, they are also broadly expressed in other tissues involved in glycemic control (Dupuis et al. 2010). However humans carrying the *MTNR1B* variant associated with diabetes and hyperglycemia have been found to display reduced insulin secretion independently of their level of glycemic control (Fadista et al. 2014; Staiger et al. 2008), suggesting a role of *MTNR1B* in pancreatic β-cell function. Genomic analyses further substantiated a role of the *MTNR1B* variant in human islet cells, where *MTNR1B* mRNA expression was increased due to enhanced binding of key β-cell transcription factors *FOXA2* and

NEUROD1 to a transcriptional enhancer of the *MTNR1B* gene (Gaulton et al. 2015). Importantly, melatonin, the endogenous ligand for the *MTNR1B* receptor, is synthesized and released into circulation at night by endocrine cells in the pineal gland in response to rhythmic signals originating in the SCN (Mulder et al. 2009). Melatonin acutely inhibits glucose-, KCl- and cAMP-stimulated insulin secretion in rodent islets and β-cells when administered in conjunction with stimulating concentrations of glucose and the cAMP agonist forskolin (Peschke et al. 2002), most likely by coupling to inhibitory G_i proteins that limit the synthesis of the insulinotropic molecules cAMP and cGMP (von Gall et al. 2002). The inhibition of insulin secretion by melatonin is consistent with the decline in human insulin secretory capacity in the evening (Morris et al. 2015) and with impaired β-cell function in humans with increased sensitivity to melatonin signaling. However, the kinetics of melatonin are complex, and prolonged treatment of rodent β-cells with melatonin for 12 hrs, mimicking the endogenous exposure *in vivo*, also sensitizes cells to forskolin- and GLP-1-stimulated insulin secretion (Kemp et al. 2002), enhances transcription of cAMP response element binding protein (CREB) target genes (Nishiyama and Hirai 2014), and protects islets from stress-induced damage (Costes et al. 2015). Moreover, rare coding variants that inhibit MTNR1B function have also been found to associate with type 2 diabetes risk in human populations (Bonnefond et al. 2012) and reduced rates of nighttime melatonin secretion are associated with human diabetes (McMullan et al. 2013), suggesting that while melatonin appears to acutely suppress insulin secretion, it may also exert long-lasting effects that promote secretory function and β-cell survival.

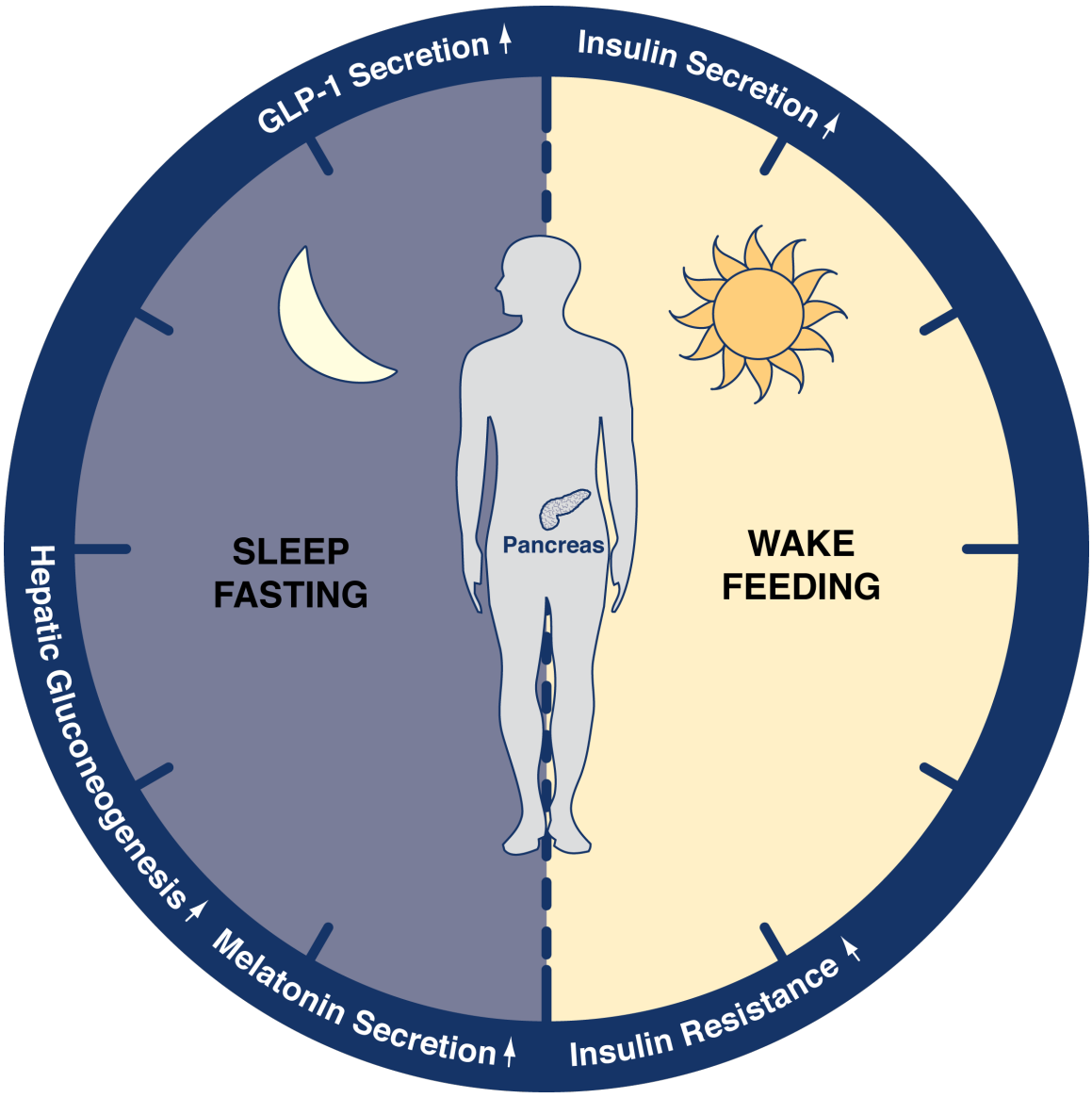

Fig. 2: Regulation of glucose homeostasis by the circadian clock.

Blood glucose levels are coordinately regulated across the 24 hr light-dark cycle by molecular clocks expressed throughout the body. The cell-autonomous clock within insulin-producing pancreatic beta cells anticipates the start of the active/feeding period by increasing glucose-stimulated insulin secretion which in turn stimulates the clearance of glucose from the blood. Both insulin secretion and peripheral insulin action decline as the

day progresses, while the liver clock increases glucose synthesis to sustain blood glucose levels in anticipation of sleep. Brain-derived melatonin increases in the evening in humans and may sensitize beta cells to secrete insulin in response to glucose in the morning. Molecular clocks distributed within beta cells, brain, and peripheral insulin sensitive tissues coordinate blood glucose levels across the day/night cycle.

Chapter III

Non-redundant function of pancreatic CLOCK in mammalian glucose homeostasis

Summary

The first mammalian gene discovered within the activator limb of the core clock is encoded by *Clock*, or *C*ircadian *l*ocomotor *o*utput *c*ycles *k*aput. *Clock* was identified by positional cloning following a chemical mutagenesis screen in which a single nucleotide transversion resulted in skipping of the DNA binding domain, generating a dimerization-competent protein that prevents CLOCK/BMAL1-mediated transcription and acts as a dominant-negative (Gekakis et al. 1998, King et al. 1997, Vitaterna et al. 1994). Although islets isolated from $Clock^{\Delta 19/\Delta 19}$ mice display primary defects in insulin secretion *ex vivo*, the mutant mice have moderate glucose intolerance *in vivo* because of the opposing, and insulin-sensitizing, effect of the $Clock^{\Delta 19}$ mutation within liver (Lamia et al. 2008, Peek et al. 2013). However, conditional ablation of *Bmal1*, a loss-of-function allele, has demonstrated that clock gene ablation within the pancreas is sufficient to induce hypoinsulinemic diabetes (Marcheva et al. 2010). A remaining question is whether expression of the *Clock* gene itself in pancreas is necessary for normal glucose homeostasis in the whole animal or whether NPAS2 functionally compensates for the loss of CLOCK within the pancreas as it does in the SCN (Debruyne et al. 2006, DeBruyne et al. 2007, DeBruyne et al. 2007).

Materials and methods

Animals. *Clock*$^{flx/flx}$ mice were crossed with *PdxCre* transgenic mice (kindly provided by Dr David R. Weaver, University of Massachusetts Medical School, and Dr Douglas Melton, Harvard University, respectively) to generate *PdxCre; Clock*$^{flx/flx}$ mice, as well as *Clock*$^{flx/flx}$ and *PdxCre* littermate controls (Debruyne et al. 2006, Gu et al. 2002). Offspring from this cross were on a mixed *B6x129xICR* background. Unless otherwise stated, mice were maintained on a 12:12 light:dark (LD) cycle and allowed free access to regular chow and water. All animal care and use procedures were conducted in accordance with regulations of the Institutional Animal Care and Use Committee at Northwestern University.

Glucose and insulin measurements. Blood glucose and plasma insulin levels in *ad libitum*-fed mice were assessed at ZT2 and ZT14 from tail vein bleeds. Glucose tolerance tests were performed in mice that were first fasted for 14 hours. Blood glucose and insulin levels were measured at the indicated times following intraperitoneal (IP) injection of glucose (2 or 3 g/kg body weight, as indicated). Plasma insulin levels were measured by ELISA (Crystal Chem Inc).

Pancreatic islet insulin secretion assays. Pancreatic islets were isolated via bile duct collagenase digestion (*Collagenase P*, Sigma) and Ficoll gradient separation and left to recover overnight at 37˚C in RPMI 1640 with 10% FBS, 1% L-glutamine, and 1%

penicillin/streptomycin. For insulin release assays, five equally-sized islets per mouse (in triplicate) were statically incubated in Krebs-Ringer Buffer and stimulated for 1 hour at 37˚C with glucose (various concentrations), 30mM KCl, 100nM exendin 4, 25μM forskolin, or 1mM 8-Br-cAMP. Supernatant was collected and assayed for insulin content by ELISA. Islets were then sonicated in acid-ethanol solution and solubilized overnight at 4˚C before assaying total insulin content by ELISA.

Immunohistochemistry. Mice were anesthetized with IP injection of phenobarbital (Nembutal, 50mg/ml) and perfused with heparinized saline, followed by 4% paraformaldehyde (Sigma) in PBS. Brain and pancreas were removed and post-fixed with 4% PFA overnight at 4°C. Pancreata were embedded in paraffin, and blocks of 6μm sections were mounted on slides. The following primary antibodies were used for staining: guinea pig anti-insulin (1:500, DAKO), mouse anti-glucagon (1:500, Sigma), and rabbit anti-CLOCK (1:500, Millipore). Secondary antibodies included: AMCA goat anti-guinea pig (1:400, Jackson ImmunoResearch), Alexa Fluor 488-conjugated goat anti-mouse (1:400, Invitrogen), and Alexa Flour 546-conjugated goat anti-rabbit (1:400, Life Technologies). Images were acquired with PictureFrame 1.0 using a Zeiss Axioskop 50.

Results

Local Ablation of the *Clock* Gene in Pancreatic Islets Impairs Glucose Homeostasis

To address whether the *Clock* gene *per se* is necessary for pancreatic islet cell function, we examined mice with conditional loss-of-function limited to pancreas. To achieve islet-specific ablation of *Clock,* we crossed $Clock^{flx/lfx}$ mice with *Pdx1-Cre* animals in order to excise exons 5 and 6 which encode the bHLH domain of CLOCK that is required for its nuclear translocation and interaction with BMAL1 (Huang et al. 2012, Kondratov et al. 2003, Marcheva et al. 2010, Rutter et al. 2001). We confirmed loss of CLOCK expression in the pancreatic islets, as expected since this mutation causes rapid degradation of the non-functional protein (Debruyne et al. 2006), by immunofluorescent staining in pancreatic sections from *PdxCre;*$Clock^{flx/flx}$ and their littermate *PdxCre* controls (Fig. 3a). In contrast to the control samples in which CLOCK was highly expressed throughout the entire pancreas, the CLOCK protein was nearly absent within the pancreatic islets of the conditional *Clock* knockout mice, without altering cellular architecture of the islet (Fig. 3a).

As noted, since mice with systemic ablation of *Clock* exhibit normal locomotor activity, we further sought to determine whether deletion of the *Clock* allele isolated to the pancreatic β-cell impacts activity or feeding behavior. To address this question, we first

monitored diurnal locomotor activity and feeding patterns in *PdxCre;Clock*$^{flx/flx}$ mice and their littermate controls. We found similar total levels of locomotor activity rhythms under regular l2:12 light:dark conditions among the genotypes (Fig. S1a-b), and under constant darkness, we observed normal period length (Fig. S1c). Total daily food consumption and feeding rhythms were also equivalent across genotypes, as were body composition and weight (Figs S1d-g). These observations indicate that glucose-regulatory effects of pancreas-specific *Clock* KO mice occur independently of effects on behavioral rhythms.

To determine whether *Clock* gene expression in the pancreas is important for glucose control across the 24 hour light/dark cycle, we examined *ad lib* glucose and insulin levels and glucose tolerance in 2-4 month old *PdxCre;Clock*$^{flx/flx}$ mice and their littermate controls at both ZT2 and ZT14. We observed a elevated glucose levels in *ad lib* fed pancreas-specific *Clock* knockout animals especially during the light phase, with a corresponding decrease in blood insulin levels in the mutant in both the light and dark periods, indicating insufficient islet insulin secretion in the *PdxCre;Clock*$^{flx/flx}$ animals (Figs S2a-b). Consistent with findings in the pancreas-specific *Bmal1* mutants (Marcheva et al. 2010), we further observed significant glucose intolerance and reduced insulin secretion following a glucose challenge in the pancreas-specific *Clock* KO mice compared to their littermate *PdxCre* and *Clock*$^{flx/flx}$ controls at both ZT2 and ZT14 (Figs 3b-c and S2c-d), indicating a requirement for both CLOCK and BMAL1 at the cellular level in the β-cell for maintenance of glucose homeostasis.

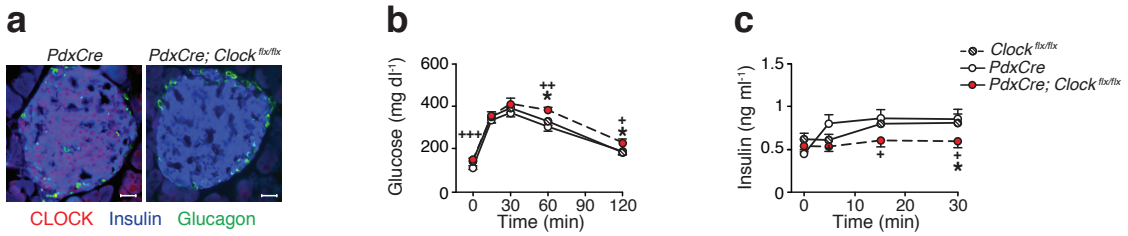

Fig. 3: Ablation of *Clock* gene in pancreatic islets impairs glucose homeostasis. (a) Immunofluorescent staining of CLOCK (red), insulin (blue), and glucagon (green) in *PdxCre* and *PdxCre;Clock*$^{flx/flx}$ control islets. Scale bars, 25μm. **(b)** Glucose and **(c)** insulin levels at the indicated time points following an intraperitoneal injection of glucose (2 or 3 g/kg body weight, respectively) in pancreas-specific *Clock* KO mice and littermate controls at ZT14 (n=7-12). Data were analyzed by Student's *t*-test. Asterisks denote significance between *Clock*$^{flx/flx}$ and *PdxCre;Clock*$^{flx/flx}$, while plus symbols denote significance between *PdxCre* and *PdxCre;Clock*$^{flx/flx}$. * and $^{+}p<0.05$, $^{++}p<0.01$, $^{+++}p<0.001$. All values represent mean ± SEM.

Loss of Pancreatic CLOCK Impairs Glucose-Stimulated Insulin Secretion in Isolated Islets

Next, to determine if altered glucose metabolism in the *PdxCre;Clock*$^{flx/flx}$ animals is due to a primary pancreatic islet defect, we examined glucose sensitivity and insulin secretion *ex vivo* in identically-sized islets isolated from pancreas-specific *Clock* KO mice and their littermate controls (Fig. 4). *Clock* KO islets displayed ~50% reduction in insulin secretion in response to 20mM glucose, consistent with a role of the CLOCK transcription factor in islet function (Fig. 4a). To test if impaired glucose-stimulated insulin secretion (GSIS) in *Clock* KO islets is caused by altered signaling downstream of glucose metabolism, we exposed islets to depolarizing concentrations of the insulin secretagogue KCl and observed significantly reduced insulin secretion in pancreas-specific *Clock* KO islets compared to controls (Fig. 4b). Pancreas-specific *Clock* KO islets also displayed impaired cAMP-mediated insulin secretion, as we found a reduction in insulin secretion in response to the cyclase activators forskolin and exendin-4 (a GLP1 mimetic) and 8-bromo-cAMP (a non-hydrolyzable analog of cAMP) in *Clock* KO islets compared to controls (Fig. 4b). The observation that pancreatic *Clock* ablation impairs insulin secretion induced by pharmacologic closure of the K_{ATP} channel is consistent with our previous findings in multi-tissue and pancreas-specific *Bmal1* KO mutant islets which likewise displayed defective β-cell function at the latest stage of stimulus-secretion coupling (Marcheva et al. 2010). These results provide additional evidence for a role of both transcription factors in the forward limb of the clock (CLOCK/BMAL1) in the cell-

autonomous regulation of local islet function.

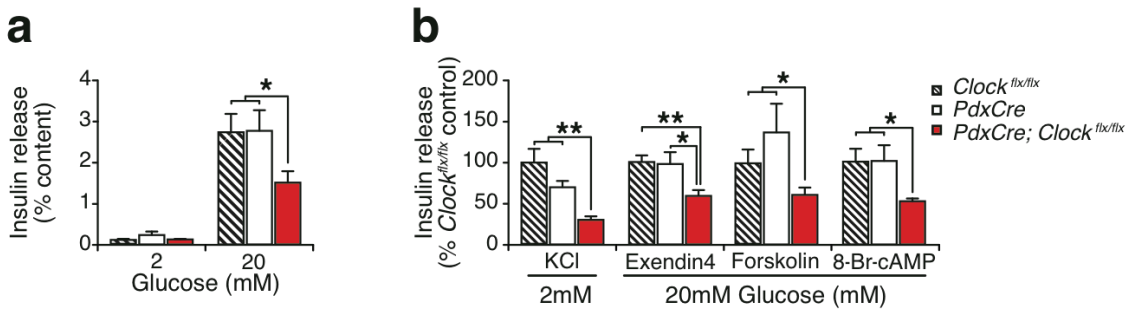

Fig. 4: Defective insulin secretion in pancreas-specific *Clock* KO mice. (a-b) Insulin release from islets isolated from pancreas-specific *Clock* KO and control mice in response to **(a)** glucose and **(b)** other secretagogues, including KCl, exendin-4, forskolin, and 8-Br-cAMP (n=5-7).

Discussion

While new genetic discoveries over the past several years have uncovered a central role for the biological clock network in the regulation of mammalian glucose homeostasis, the interplay between central and peripheral circadian signaling and the roles of individual circadian clock genes in the maintenance of blood glucose levels throughout the day is still incompletely understood. Our previous studies revealed that multi-tissue *Clock*$^{\Delta 19/\Delta 19}$ mutant mice develop obesity, hyperglycemia, hypoinsulinemia, and overt diabetes by ~6-8 months of age (Laposky et al. 2006). We demonstrate that impaired glucose tolerance and impaired insulin secretion in pancreas-specific *Clock* KO (*PdxCre;Clock*$^{flx/flx}$ mice) animals is evident much earlier in life by ~2-4 months of age, similar to our observations in pancreas-specific *Bmal1* KOs (*PdxCre;Bmal1*$^{flx/flx}$ mice) (Marcheva et al. 2010). Specifically, the *PdxCre;Bmal1*$^{flx/flx}$ loss-of-function mutants demonstrate a necessary role of the peripheral pancreatic clock in organismal homeostasis, and indicate the presence of opposing effects of clock disruption in distinct tissues of multi-tissue mutants. In further support of the idea that the clock exerts distinct physiological effects in different tissues, disruption of clock function in liver impairs hepatic gluconeogenesis and causes fasting-induced hypoglycemia, thus masking the effects of circadian disruption in the pancreas early in life (Lamia et al. 2008, Peek et al. 2013). We further note that comparison of the pancreas-specific *Clock* and *Bmal1* KOs reveals a similar phenotype (Marcheva et al. 2010), including reduced glucose tolerance and impaired

glucose-, KCl-, and cAMP-stimulated insulin secretion. Collectively, these observations reveal that CLOCK is essential for normal pancreatic β-cell function and regulates signaling pathways that overlap with those that are controlled by BMAL1.

Chapter IV

Widespread CLOCK/BMAL1-dependent circadian transcription of genes controlling insulin vesicle assembly, trafficking, and exocytosis

Parts of this chapter were adapted from:

Perelis M, et al (2015) Pancreatic beta cell enhancers regulate rhythmic transcription of genes controlling insulin secretion. *Science* 350: p. aac4250.

Summary

Previous studies using multitissue and tissue-specific circadian gene mutant mice have demonstrated that clock expression regulates multiple glucose-regulatory metabolic functions. For example, multitissue *Clock*$^{\Delta 19}$ and *Bmal1*$^{-/-}$ mice lose the diurnal variation of blood glucose levels (Rudic et al. 2004) and hyperglycemia due to reduced pancreatic insulin production in aging and when challenged with a high fat diet (Turek et al. 2005). Tissue-specific *Bmal1* mutant mice have helped to delineate discrete roles for peripheral tissue clocks in regulating insulin sensitivity in adipocytes (Paschos et al. 2012) and skeletal muscle (Dyar et al. 2014), mitochondrial oxidative function and gluconeogenesis in liver (Lamia et al. 2008, Peek et al. 2013), and glucose-stimulated insulin secretion in pancreatic β-cells (Lee et al. 2011, Lee et al. 2013, Marcheva et al. 2010, Rakshit et al. 2016, Sadacca et al. 2011).

While it is assumed that physiological functions altered in circadian gene mutants are coordinately regulated across the day/night cycle, there may be non-circadian effects of the core clock transcription factors. Indeed, a recent study using adult-life tamoxifen inducible *Bmal1* knockout mice found that many of the pathologies seen in constitutive *Bmal1*$^{-/-}$ mice are not found when the gene is deleted in all tissues postnatally, despite the complete loss of behavioral rhythms (Yang et al. 2016). The authors of that study argued that phenotypes such as accelerated aging and impaired glucose homeostasis observed only in constitutive but not postnatal *Bmal1*$^{-/-}$ mice likely reflect non-circadian functions of the *Bmal1* gene, whereas those phenotypes that persist reflect circadian-controlled physiological functions (Yang et al. 2016). The absence of alterations in glucose

homeostasis the multitissue postnatal *Bmal1* mutant mice may be due to the aforementioned distinct and opposing effects of clock mutation in liver (enhancing insulin sensitivity) and pancreas (inhibiting insulin secretion).

Circadian control of physiological processes at a cell- or tissue-autonomous level can also be defined by examining circadian gene transcription and cellular processes in organotypic tissue explants *ex vivo* (Peek et al. 2013, Peek et al. 2015). For example, one of the earliest studies examining circadian rhythms within isolated islet cells demonstrated that rat islets displayed a spontaneous circadian pattern of insulin release when continuously perifused with media containing a low concentration of glucose (Peschke and Peschke 1998), suggesting that islet cell-autonomous circadian transcription might regulate β-cell function across the 24-hr day/night cycle. Consistently, islets isolated from transgenic $Per2^{Luc}$ reporter mice, in which firefly luciferase is fused in frame with the endogenous *Per2* locus, displayed self-sustained bioluminescent rhythms *ex vivo* (Marcheva et al. 2010, Sadacca et al. 2011, Yoo et al. 2004), as did islets isolated from *Per1::LUC* transgenic rats (Qian et al. 2013). If insulin secretion is regulated cell-autonomously by circadian genes, rather than by a non-circadian function of CLOCK/BMAL1 in β-cells, it should display circadian variation in isolated islet cells cultured *ex vivo*. We therefore sought to test if isolated islets from wild type mice display self-sustained circadian patterns of glucose-stimulated insulin secretion. In addition, since the circadian clock is a transcriptional oscillator we used RNA sequencing (RNA-seq) to identify the repertoire of clock-controlled rhythmic genes underlying circadian control of islet function.

Materials and methods

Animals. Male wild-type C57BL/6J mice were purchased from The Jackson Laboratory. *Per2*^{Luc} (Yoo et al. 2004) and *PdxCre;Bmal1*$^{flx/flx}$ (Marcheva et al. 2010) mice were produced and maintained on a C57BL/6J background at the Northwestern University Center for Comparative Medicine. *Bmal1*$^{flx/flx}$ mice (Westgate et al. 2008) were crossed with *PdxCreER* transgenic mice (kindly provided by Dr Douglas Melton, Harvard University) (Gu et al. 2002) to generate *PdxCreER;Bmal1*$^{flx/flx}$ mice, as well as *Bmal1*$^{flx/flx}$ and *PdxCreER* littermate controls. Unless otherwise stated, mice were maintained on a 12:12 light:dark (LD) cycle with free access to regular chow and water. All animal care and use procedures were conducted in accordance with regulations of the Institutional Animal Care and Use Committee at Northwestern University.

Islet isolation, insulin secretion assays, and *in vitro* islet synchronization. Mouse pancreatic islets were isolated via bile duct collagenase digestion (*Collagenase P*, Sigma) and Ficoll gradient separation and left to recover overnight (16 hrs) at 37˚C in RPMI 1640 with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. For standard insulin release assays, 5 islets were statically incubated in Krebs-Ringer Buffer (KRB) and stimulated for 1 hr at 37˚C with various glucose concentrations or 30mM KCl. Supernatant was collected and assayed for insulin content by ELISA (Crystal Chem Inc). Islets were then sonicated in acid-ethanol solution and solubilized overnight at 4˚C before assaying total insulin content by ELISA. For rhythmic insulin release assays, islets were first synchronized with 10μM forskolin (Sigma) for 1 hr and allowed to recover for 16

hrs. Insulin secretion assays were then performed as above in individual groups of 5 islets every 4 hrs for 72 hrs (Fig. S1A). Human islets (obtained from IIDP) were cultured in RPMI 1640 with 10% human AB serum, 1% L-glutamine, and 1% penicillin/streptomycin (see table in Fig. S6A for details re: sex, age, BMI, and IIDP ID numbers of the three donors). For the rhythmic analysis of RNAs in murine and human islets, RNA was isolated (described below) in groups of 200 islets every 4 hrs for 48 or 24 hrs, respectively, starting 40 hrs following forskolin synchronization (Fig. S3A).

Lumicycle analysis. Approximately 2 hrs prior to the start of the dark period (i.e., lights off), ~100-150 pancreatic islets were isolated from *Per2*^{Luc} mice as described above. Islets were cultured on tissue culture membranes (Millipore) in 1.2ml DMEM media (Gibco) containing 352.5mg/ml sodium bicarbonate, 10mM HEPES (Gibco), 2mM L-glutamine, 2% B-27 serum-free supplement (Invitrogen), 25units/ml penicillin, 20mg/ml streptomycin (Gibco), and 0.1mM luciferin sodium salt (Biosynth AG). Sealed cultures were placed at 37˚C in a LumiCycle luminometer (Actimetrics) and bioluminescence from tissues was recorded continuously. After several days in culture, islets were synchronized by 10μM forskolin (Sigma) treatment for 1 hr followed by incubation in fresh media. Period was calculated via a modified best-fit sine wave analysis using Lumicycle Analysis software (Actimetrics).

Measurement of islet oxygen consumption. Following bile duct collagenase digestion, 40 purified pancreatic islets were plated in wells of a 96-well respirometry plate (Seahorse Bioscience) and cultured overnight in complete medium. The next day culture

medium was replaced with assay buffer containing 3mM glucose, 0.8mM Mg^{2+}, 1.8mM Ca^{2+}, 143mM NaCl, 5.4mM KCl, 0.91mM NaH2PO4, and Phenol red 15mg/ml (Seahorse Bioscience) and allowed to equilibrate at 37ºC in a CO_2-free incubator for 1-2 hrs. The plate was then loaded into a Seahorse XF96 instrument, and the oxygen consumption rate (OCR) was measured for 4 sequential 3-minute intervals at basal conditions and following injection of glucose (20mM final concentration), oligomycin (F1FOATPsynthase inhibitor) (5μM final concentration), and antimycin A (complex III inhibitor) (5μM final concentration). OCR values presented represent the average of 4 sequential measurements. Mitochondrial oxygen consumption was calculated by subtracting OCR values following antimycin A treatment (representing non-mitochondrial oxygen consumption).

Tamoxifen treatment. For *in vitro* administration of tamoxifen, isolated islets were incubated for 24 hrs with 1μM tamoxifen (dissolved in ethanol) prior to transfer to complete media for 24 hrs to recover. Islets were then synchronized with forskolin prior to insulin secretion assays as described above.

RNA isolation and qPCR mRNA quantification. Islets were added to microfuge tubes containing Tri Reagent (Molecular Research Center, Inc) and frozen at -80˚C. RNA was isolated according to the manufacturer's protocol and purified using RNeasy columns (Qiagen). cDNAs were then synthesized using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR analysis was performed with SYBR Green Master Mix (Applied Biosystems) and analyzed using an

Applied Biosystems 7900 Fast Real-Time PCR System. Relative expression levels were determined using the comparative C_T method to normalize target gene mRNA to *Gapdh*. *Exon-specific primer sequences for qPCR as follows*: *Bmal1* Exons 5-7 Forward: 5'-ATCGCAAGAGGAAAGGCAGT-3', Reverse: 5'- ATCCTTCCTTGGTGTTCTGCAT-3'; *Bmal1* Exons 7-9 Forward: 5'- AGGCCCACAGTCAGATTGAA-3' Reverse: 5'-TGGTACCAAAGAAGCCAATTCAT-3'; *Bmal1* Exon 8 Forward: 5'-GGCGTCGGGACAAAATGAAC-3' Reverse: 5'- TCTAACTTCCTGGACATTGCAT-3'; *Bmal1* Exons 8-9 Forward: 5'- TGCAATGTCCAGGAAGTTAGAT-3' Reverse: 5'-TGGTGGCACCTCTCAAAGTT-3'; *Bmal1* Exons 10-12 Forward: 5'-TAGGATGTGACCGAGGGAAG-3' Reverse: 5'- AGCTCTGGCCAATAAGGTCA-3'.

RNA-sequencing and analysis. Following RNA isolation (described above), RNA quality was assessed using a Bioanalyzer (Agilent), and sequencing libraries were constructed using an Illumina TruSeq Stranded mRNA sample prep kit LT (Illumina, RS-122-2101) according to the manufacturer's instructions. Libraries were quantified using both a Bioanalyzer (Agilent) and qPCR-based quantification (Kapa Biosystems) and sequenced on either an Illumina HiSeq 2000 or NextSeq 500 instrument to a depth of at least 30 million reads using 100bp or 75bp paired-end reads, respectively. *Differential Expression Analysis*: For differential expression comparison between *PdxCre;Bmal1*$^{flx/flx}$ and *Bmal1*$^{flx/flx}$ islets, RNA raw sequence reads were aligned to the reference genome (mm10) using STAR version 2.3.1s_r366 (Dobin et al. 2013). Differentially expressed RNAs were identified using DESeq2 version 1.6.3 (Love et al. 2014) (FDR-adjusted p-value <0.05). *Cycling RNA Analysis*: For cycling RNAs, raw sequence reads were

similarly aligned using STAR (mm10 index for mouse and hg19 for human) and uniquely mapped reads (tags) were normalized using the algorithm employed in DESeq2 (Love et al. 2014). The geometric mean of the raw read counts was calculated for each gene. A normalization factor was calculated for each sample using the median of the raw read counts of each gene divided by the geometric mean of the gene. The normalized read counts were computed by dividing the raw read counts by the normalization factor. The normalized tags for the mouse and human time series were separately concatenated and z-scored within each gene (Hutchison et al. 2015). Rhythm detection of the z-scored and normalized counts was performed with empirical JTK_CYCLE with asymmetry search, which increases sensitivity to detecting cycling transcripts by extending cosine wave-fiting to include asymmetric waveforms, which better represents expression patterns seen in biological data. Rhythmic time series were examined with reference waveforms with a period of 24 hr, a phase of either 0, 4, 8, 12, 16, or 20, and an asymmetry of either 4, 12, or 20. Due to the low number of waveforms compared, the Bonferroni correction was used instead of the empirical p-values. Genes with a Bonferroni-adjusted p-value below 0.05 were considered to be rhythmic. *Ontology Analysis*: For Kyoto Encyclopedia of Genes and Genomes (KEGG) ontology term enrichment (Kanehisa et al. 2014, Ogata et al. 1999), Ensembl gene IDs were supplied and analyzed using Homer (version 4.7.2) command "findGO" (Heinz et al. 2010). *Cycling Genes in Liver:* Genes exhibiting rhythmic mRNA accumulation *in vivo* in liver were derived from reported "exon cycling" transcripts (Koike et al. 2012).

Results

Isolated pancreatic islets exhibit rhythmic insulin secretion

To determine whether transcriptional oscillations in pancreatic islets give rise to rhythmic islet physiology, we examined the phase-dependence of pancreatic islet function by analyzing nutrient-induced insulin secretion in parallel with live-cell clock oscillation in islets from *Per2*^{Luc} reporter mice (Yoo et al. 2004). Following synchronization with forskolin (Marcheva et al. 2010, O'Neill et al. 2008), we assessed insulin secretion every 4 hrs in individual groups of 5 islets at each time point over the ensuing 72 hr window (see schematic in Fig. S3A and Materials and Methods) and observed a striking self-sustained, time-of-day-dependent variation in the magnitude of response to stimulatory concentrations of both glucose and KCl, an insulin secretagogue that triggers exocytosis through direct depolarization of the β-cell (Fig. 5A). Intracellular insulin content did not cycle (Fig. S3B) despite rhythmic glucose-stimulated insulin secretion (GSIS) (Fig. 3A), consistent with circadian regulation at a step following translation of insulin. We further confirmed that GSIS rhythms were autonomous by monitoring insulin secretion following forskolin synchronization at times corresponding to the nadir (36 hr post-forskolin shock) and zenith (48 hr post-forskolin shock) of the WT GSIS rhythm in islets isolated from *PdxCreER;Bmal1*$^{flx/flx}$ mice (see Fig. 5B and schematic in Fig. S3C), which when treated with tamoxifen *ex vivo* display >60% reduction in *Bmal1* expression (Fig. S3D). Vehicle-treated islets displayed significantly higher GSIS at the zenith than at the nadir, whereas tamoxifen-treated islets exhibited constitutively low levels of insulin

secretion (Fig. 5B). Together, these data suggest that the islet molecular clock gates the rhythmic secretory response downstream of membrane depolarization.

Isolated pancreatic islets exhibit rhythmic expression of secretory genes

We next sought to examine the genome-wide effect of rhythmic transcription on insulin secretory dynamics by performing RNA-sequencing (RNA-seq) over two circadian cycles in RNA isolated from wild-type islets synchronized *ex vivo* (see schematic in Fig. S3A and Materials and Methods). We analyzed polyadenylated RNAs using eJTK_CYCLE (Hutchison et al. 2015), a modified non-parametric algorithm with increased sensitivity for detecting cycling transcripts. We detected a total of 3,905 cycling transcripts (Bonferroni-corrected p-value <0.05) which accounted for ~27% of all expressed transcripts within the islet that met a minimum mean expression threshold of 10 normalized counts (Fig. 5C). As expected, we observed high amplitude rhythms for the core clock transcription factors, including *Bmal1*, *Clock, Npas2, Per2, Cry1*, *Rev-Erbα*, and *Rorα*, with *Bmal1* and its repressor *Rev-Erbα* displaying anti-phasic expression (Fig. 5C) (Ueda et al. 2005).

To determine the identity of functional circadian gene networks in the islet, we tested for overrepresentation of defined KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways amongst rhythmic RNAs. We observed enrichment of factors mediating vesicle

exocytosis, suggesting that daily variation in insulin secretory capacity arises from genomic regulation of the transport and release of peptidergic hormone (Fig. 5C and Table S1). Over-represented pathways in the circadian transcriptome included factors involved in *i)* vesicle budding, including genes encoding the COPII coat proteins (*Sec24a* and *Sec31a*), which mediate vesicle budding from the endoplasmic reticulum Chen et al. 2013, Noble et al. 2013; *ii)* cargo trafficking, specifically the motor proteins (*Kif1b*, *Myo9a*, and *Dync2h1*) that enable vesicle transport along cytoskeletal filaments Wang and Thurmond 2009; and *iii)* vesicle tethering and fusion to the plasma membrane, including v- and t-SNAREs such as *Vamp1*, *Vamp5, Vamp8, Stx1a*, *Stx4a,* and *Stx8* (Liu et al. 2011, Ohara-Imaizumi et al. 2004). In addition to the cycling of RNAs encoding factors involved in insulin exocytosis, we also identified rhythmic RNA expression of insulinotropic signals involved in vesicle movement and membrane fusion, including *i)* targets of cAMP/EPAC signaling (*Pclo*, *Rims2*, *Rab3b*, *Rap1a*, *Rap1b*, *Rapgef2*, *Rapgef6*), which mediate vesicle docking and fusion to the plasma membrane (Fujimoto et al. 2002, Takahashi et al. 2014); *ii)* Ca^{2+}-sensing synaptotagmins (*Syt11*, *Syt14, Syt16*), which stimulate membrane fusion of synaptic vesicles (Fukuda 2003, Milochau et al. 2014); and *iii)* calmodulin-dependent protein kinases (*Camk1*, *Camk4*, *Camkk2*, *Camk2g*), which regulate vesicle exocytosis and recycling (Easom 1999). Lastly, we detected significant oscillation in targets of phosphoinositide signaling, including the protein kinase C (*Prkca, Prkcb*) (Biden et al. 2008), exocyst actin interacting factors including *Exoc1*/*Sec3* (Zhang et al. 2008), and the cytoskeletal filament remodeling Rho GTPases *Rho*, *RhoA*, *RhoB*, *RhoC* (Wang and Thurmond 2009). Collectively, cycling of

RNAs encoding factors involved in insulin exocytosis and signaling components reveals a genomic basis for circadian variation in insulin secretion.

To further understand the physiologic function of tissue-specific rhythmic gene transcription, we next compared genome-wide rhythms of RNA expression in WT islets to those in pancreas-specific clock mutant mice (*PdxCre;Bmal1*$^{flx/flx}$) which exhibit severe hypoinsulinemic diabetes due to defects downstream of glucose metabolism and mitochondrial respiration (Fig. S4) (Marcheva et al. 2010). We performed RNA-seq using RNA immediately isolated from *PdxCre;Bmal1*$^{flx/flx}$ and control littermate islets at the start of the light phase (ZT2), the time of maximal GSIS impairment (Fig. S5A) (Marcheva et al. 2010). We identified changes in the expression of 1,757 genes in islets isolated from clock mutant animals relative to littermate controls (*Bmal1*$^{flx/flx}$), including transcripts that were both decreased (1,074) and increased (683) in expression, consistent with actions of the clock as both an activator and repressor of gene expression (FDR-adjusted p-value <0.05) (Fig. S3B-C). Importantly, many of the RNAs that were altered in islet-clock knockout mice were identified as cycling RNAs in WT islets - overall, a total of 720 oscillating genes exhibited altered expression in animals with disrupted pancreatic clock function (Fig. S5C), indicating an autonomous role of the islet clock in the rhythmic transcriptional regulation of insulin secretion. Among the most significantly changed RNAs were factors in the negative limb of the core clock containing the canonical E-box transcription motif, in addition to circadian PAR bZip transcription factors, including *Per2*, *Rev-Erbα (Nr1d1)*, *Tef,* and *E4bp4* (*Nfil3*) (Fig. 1D). We also found a broad range of alterations in cycling genes that are circadian outputs and grouped

into similar exocytosis networks by KEGG annotation as described for the WT islets, including genes encoding factors involved in trafficking, such as the vacuolar protein sorting factors *Vps13b* and *Vps13c*, the motor protein involved in vesicular transport *Myo9*, the kinesin transport factor *Kif21*, and the small GTPase *Rab11*, a factor in trans-golgi vesicular biogenesis (Jedd et al. 1997) (Figs. 1D, S3E-F, and Table S1). Ontology analysis also identified genes related to vesicle tethering and fusion as altered in clock deficient islets, including the conserved exocyst component *Exoc1*/*Sec3,* cAMP-EPAC controlled *Rims2* and *Pclo,* and the synaptogamnin *Syt14* (Fig. 5D), while canonical islet genes related to upstream steps in glucose sensing and in cell identity were unchanged (Table S2). Interestingly, whereas the complete set of cycling RNAs display broadly distributed peak phases (Fig. S5D), the majority of exocytosis-related RNAs that were differentially expressed in clock mutants exhibited peak expression at two distinct phases (48 and 60 hrs post forskolin shock) (Fig. 5D). Although this suggests that these genes may represent direct targets of CLOCK/BMAL1 and/or a clock repressor (REVERBα/β or E4BP4), nascent RNA-seq studies indicate that peak circadian mRNA phases are not directly correlated with nascent transcription (Menet et al. 2012). Collectively, sequencing results indicate that secretory pathway genes represent a major output of the islet clock.

Circadian transcription of islet genes is conserved in humans

To determine whether the rhythmic islet transcriptome is conserved from mouse to humans, we performed RNA-seq in RNA isolated from synchronized human islets (Fig.

S6A). Human islets displayed characteristic circadian patterns in the expression of core clock components *BMAL1* and *REV-ERBα* (Fig. S6B) (Pulimeno et al. 2013) and genome-wide rhythmic patterns in the transcriptome with 1,800 cycling RNAs (Bonferroni-corrected p-value <0.05) (Fig. S6B). While striking differences have been described between mouse and human islet cell composition and cytoarchitecture (Benner et al. 2014), the expression of key genes involved in insulin release are conserved between species (Benner et al. 2014). Remarkably, 481 of the rhythmic human islet genes were orthologous to those in mouse islets (Fig. S6C), including factors involved in exocytosis, trafficking, and fusion (Figs. 5E-F and S6C). Mapping cycling human islet RNAs onto KEGG-curated human insulin secretion pathways revealed regulation of GPCR, cAMP, Ca^{2+} and phosphoinositide-responsive signaling molecules important in nutrient responsive and hormone release (Fig. 5E-F). Specifically, these included the G_q protein *GNAQ*, insulin vesicle associated *RIMS2* and *PCLO*, calmodulin-activated protein kinase *CAMK2G*, and phospholipase C *PLCB4*, all of which were also rhythmic in mouse islets (Figs. 5E-F and S6D). Circadian gene regulation in the endocrine pancreas of both mice and humans thus converges on the late secretory pathway, demonstrating conservation of clock control of rhythmic tissue function across species.

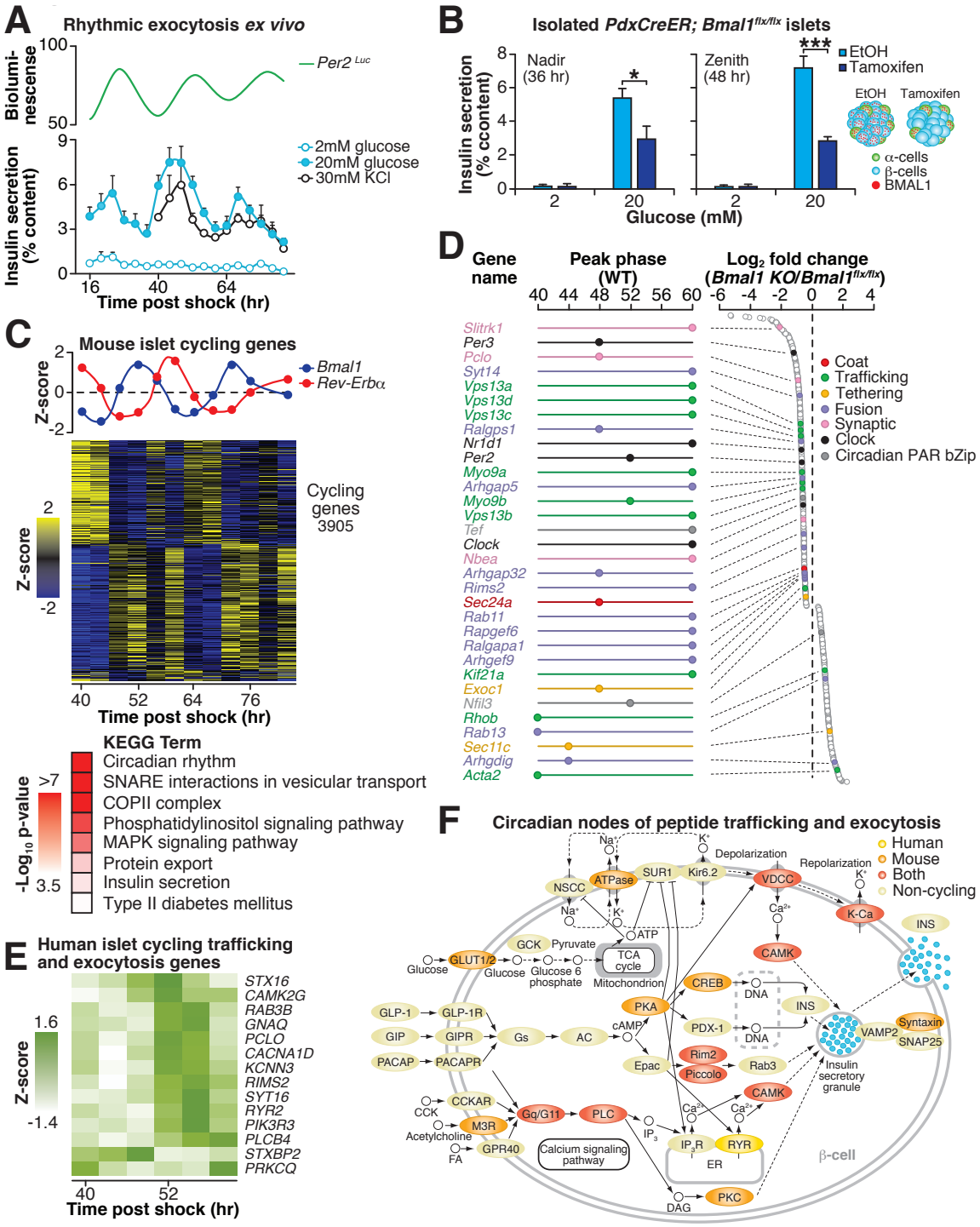

Fig. 5: Isolated pancreatic islets display rhythmic insulin secretion and transcription of secretory genes in mice and humans. **(A)** Glucose and KCl-stimulated insulin secretion in synchronized WT mouse islets across either 3 or 2 consecutive days,

respectively (n=3 replicate sets of islets pooled from 6-9 mice each) (bottom). Bioluminescence monitoring (counts/sec) in islets from *Per2*^{Luc} reporter mice was performed in parallel (top). **(B)** Glucose-stimulated insulin secretion in ethanol- or tamoxifen-treated islets from *PdxCreER;Bmal1*$^{flx/flx}$ mice at the nadir (36 hr post-forskolin shock) and zenith (48 hr post-forskolin shock) of cyclic insulin secretion in wild-type islets from Fig. 1A (n=4 islet pools per time point, 3 replicates per islet pool). Of note, ethanol-treated islets displayed significant difference in GSIS comparing 36- to 48-hrs (p=0.038), whereas tamoxifen-treated islets did not (p=0.974). **(C)** *Bmal1* and *Rev-erbα* RNA expression (top) and heatmap of all cycling genes identified by eJTK_CYCLE analysis (middle). Significantly enriched KEGG ontology pathways shown within the cycling gene set (bottom). **(D)** Peak phase expression (hrs post-forskolin shock) of cycling genes in synchronized WT islets (left) that were also altered in *PdxCre;Bmal1*$^{flx/flx}$ islets at ZT2. Log_2 fold change in expression in *PdxCre;Bmal1*$^{flx/flx}$ (KO) islets compared to *Bmal1*$^{flx/flx}$ (control) at ZT2 (right) for subset of genes relevant to insulin secretion. **(E)** Heatmap showing expression patterns of cycling trafficking and exocytosis genes in synchronized human islets. **(F)** Mapping of cycling RNAs in both human and mouse islets onto the "Insulin Secretion" KEGG pathway. All values represent mean ± SEM. *p<0.05, ***p<0.001.

Discussion

The observations that there is rhythmic variation in glucose- and KCl-stimulated insulin secretion are consistent with the impaired glucose- and KCl-stimulated insulin secretion in clock-deficient β-cells (Marcheva et al. 2010, Sadacca et al. 2011) and indicate that circadian regulation impacts the late stages of the insulin secretory pathway. One challenge of *ex vivo* studies of islet insulin secretion is extrapolation of the analyses to the intact animal. In this respect, *in vivo,* the acrophase of endogenous *Per2* mRNA peaks within the islets and liver during the transition from the dark to light period, the time of day when mice are most glucose-tolerant and display the greatest magnitude of glucose-stimulated insulin secretion (Marcheva et al. 2010). Interestingly, the timing of maximal glucose- and KCl-responsiveness at the light-dark transition was similar to the time of maximal $Per2^{Luc}$ bioluminescence in isolated islets, suggesting that the islet cell-autonomous clock anticipates the time of day when insulin demand is highest, thereby priming the β-cell for maximal responsiveness at the start of the active period.

The extensive circadian variation in gene expression in synchronized islets *ex vivo* indicates that the islet cell-autonomous molecular clock and downstream rhythmic transcription factors coordinate the timing of various β-cell functions across the day/night cycle. Previous circadian gene profiling studies using DNA microarray and RNA-seq technologies have similarly noted transcriptional regulation of key physiological processes such as mitochondrial oxidative metabolism in liver (Panda et al. 2002), neuropeptide synthesis and secretion in SCN (Panda et al. 2002), insulin-like growth

factor receptor signaling in kidneys (Zhang et al. 2014), and lipid and carbohydrate metabolism in skeletal muscle (Hodge et al. 2015). However, because these studies profiled RNA rhythms *in vivo* it is unclear if key circadian transcription pathways are regulated cell autonomously of by systemic circadian signals generated by the SCN pacemaker, such as glucocorticoids, body temperature rhythms, and feeding (Balsalobre et al. 2000, Balsalobre et al. 2000, Damiola et al. 2000, Schibler et al. 2015). The cell autonomous circadian regulation of genes encoding factors involved in insulin vesicle biogenesis, trafficking, and exocytosis indicates that circadian genes specifically in the β-cell contribute to the diurnal variation of insulin secretion. The observation that many genes in these pathways are dysregulated in islets from *PdxCre;Bmal1*$^{flx/flx}$ mice *in vivo* further suggests that their circadian regulation is critical for normal glucose homeostasis.

Importantly, human islets displayed extensive circadian gene expression revealing that the clock is similarly capable of cell-autonomously controlling secretory pathways in humans. Specifically, the circadian expression of genes that regulate stimulus-induced exocytosis, including effectors of muscarinic G_q coupled receptor signaling, cAMP signaling, insulin vesicle fusion, and glucose sensing reveal conservation of clock controlled pathways between mice and humans. A recent study comparing the transcriptomes of human islets transduced with either control or *Clock* siRNA lentiviruses found reduced expression of genes regulating glucose sensing and exocytosis, many of which were shared with cycling genes we identified including G_q receptor component *GNAQ* and cAMP sensitive *PCLO* (Saini et al. 2016). *siCLOCK* expressing islets also displayed a total loss of transcriptional rhythms and reduced insulin

secretion (Saini et al. 2016), supporting the idea that circadian transcription is critical for insulin secretion in humans. These observations support previous gene expression studies comparing the transcriptomes of human islets isolated from normoglycemic compared to type 2 diabetic donors have found significant reductions in the expression of several basic helix-loop-helix *Per-Arnt-Sim* (bHLH-PAS) transcription factors, including the core clock component *BMAL1* (Gunton et al. 2005), as well as *CRY2, PER2,* and *PER3* (Stamenkovic et al. 2012), in the diabetic state. Circadian gene expression therefore plays a cell-autonomous role in sustaining healthy levels of insulin secretion and its misregulation in human diabetes may contribute to disease by inhibiting glucose-stimulated insulin secretion.

Chapter V

CLOCK/BMAL1 regulate transcription of genes controlling insulin secretion by binding to β-cell-specific enhancers

Parts of this chapter were adapted from:

Perelis M, et al (2015) Pancreatic beta cell enhancers regulate rhythmic transcription of genes controlling insulin secretion. *Science* 350: p. aac4250.

Summary

The observation that cell-autonomous circadian regulation gives rise to widespread rhythmic oscillation of the transcriptome within various tissues, including the β-cell, raises the question as to how clock transcription factors mediate cell-type specific physiology (Perelis et al. 2015; Kornmann et al. 2007; Panda et al. 2002; Ueda et al. 2002; Zhang et al. 2014). Genetic studies originally demonstrated that the core clock transcription mechanism governs both behavior and physiology through the binding of the circadian transcriptional heterodimer CLOCK/BMAL1 to E-box elements in their own repressors, whereas more recent genomic studies in liver suggest that core clock factor binding within enhancer regions determines the phase of downstream oscillatory RNAs (Koike et al. 2012; Rey et al. 2011; Menet et al. 2012). Yet what has remained unclear is the mechanism through which the core clock cycle impacts differential physiologic function within distinct tissues. The best-characterized tissue for studies of clock transcription in mammals has been the liver, where rhythmic binding of both CLOCK/BMAL1 and the downstream circadian repressors REVERBα and E4BP4 to enhancers has been shown to regulate the transcription of enhancer-derived RNAs (eRNAs) and predicts phase-specific transcription of metabolic genes (Fang et al. 2014).

Cistrome studies have similarly identified β-cell transcriptional enhancer hubs as critical regulators of both development and function (Pasquali et al. 2014). Key transcription factors including the critical regulators of early pancreas organogenesis and adult-life β-cell function PDX1 and FOXA2 (Ahlgren et al. 1996, Ahlgren et al. 1998, Ang et al.

1993, Kaestner et al. 1993, Stoffers et al. 1997), and regulators of later β-cell development and function MAFB, NKX6-2, and NKX2-2 (Nishimura et al. 2006, Sussel et al. 1998, Taylor et al. 2013) co-localize within regions containing epigenetic signatures of accessible chromatin and active enhancers in human islets including deposition of the variant histone H2A.Z, monomethylated lysine 4 on histone 3 (H3K4Me1), and acetylated lysine 27 on histone 3 (H3K27Ac) (Creyghton et al. 2010, He et al. 2010, Pasquali et al. 2014).

Since our genome-wide RNA sequencing studies in islets indicate that genomic regulation by the clock gives rise to rhythmic insulin secretion, we sought to analyze how core circadian transcription factors (TFs) regulate this process by analyzing the extent of binding by BMAL1 and CLOCK to rhythmically expressed genes. Transcriptional enhancers play key roles in both the regulation of rhythmic genes in liver and in the activation of essential β-cell genes so we combined analysis of CLOCK/BMAL1 with epigenetic histone posttranslational modifications to distinguish genomic regions corresponding to accessible chromatin (H2A.Z), accessible promoter or enhancer regulatory sites (dimethylated lysine 4 on histone 3: H3K4Me2) and transcriptional activity (H3K27Ac) to test the hypothesis that enhancers play a role in regulating rhythmic islet genes.

Materials and methods

β-cell culture. Beta-TC6 cells were purchased from ATCC (CRL-11506) and cultured in DMEM supplemented with 15% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. All cells used in experiments were at fewer than 15 passages.

Mouse BMAL1 and CLOCK polyclonal antibody generation. Guinea pig anti-mouse BMAL1 and CLOCK polyclonal antibodies were generated using a 37- and 39- amino acid peptide fragment of the mouse BMAL1 and CLOCK proteins, respectively (RS synthesis). Guinea pigs were immunized with KLH-conjugated peptides (Pocono Farms), and BMAL1- and CLOCK-specific antibodies were affinity-purified from whole serum using resin cross-linked with antigen peptides (Pierce).

Chromatin immunoprecipitation (ChIP). Beta-TC6 cells (~40-160 million) were fixed for 30 minutes in 2mM DSG and for 10 minutes in 1% formaldehyde and then either frozen at -80˚C or processed immediately. Nuclei were isolated in buffer containing 1% SDS, 10mM EDTA, 50mM Tris-HCl pH 8.0, and protease inhibitors and sonicated using a Diagenode Bioruptor to shear chromatin to 200-1000bp fragments. Protein-DNA complexes were incubated with antibodies against BMAL1 and CLOCK (affinity-purified guinea pig IgGs as described above), H3K4Me2 (Abcam), H3K27Ac (Active Motif), H2AZ (Active Motif), or PDX1 (Novus Biologicals) and immunoprecipitated with IgG paramagnetic beads (Invitrogen). Eluted chromatin was isolated using MinElute PCR purification columns (Qiagen).

ChIP-sequencing and analysis. Sequencing libraries were generated using KAPA DNA Library Preparation kits (Kapa Biosystems, KK8504) according to manufacturer's instructions. Library concentrations were assessed by both a Bioanalyzer (Agilent) and qPCR-based quantification (Kapa Biosystems). Libraries were sequenced using 75bp single-end reads on an Illumina Next-Seq 500 instrument to a depth of > 10 million mapped reads. *Alignment and Peak Finding*: Raw sequence reads were aligned to the mm10 reference genome and displayed using UCSC annotated genes using bowtie version 1.1.1 (Langmead et al. 2009) with parameters "--best" and "-m 1" to ensure reporting of uniquely mapped reads (tags). ChIP-Seq peaks were designated as regions with 4-fold enrichment over both the input sample and the local background and were normalized to 10 million reads using default parameters for the Homer "findPeaks" command (Heinz et al. 2010) and specifying "-style factor" for BMAL1, CLOCK, and PDX1 and "-style histone" for H2A.Z, H3K4Me2, and H3K27Ac. For BMAL1 and CLOCK peaks, promoter binding was defined as peaks occurring within 2kb of the nearest gene TSS, and distal binding was defined as those occurring greater than 2kb from a nearest TSS. *Motif Analysis*: To identify consensus motifs for BMAL1 and CLOCK, we scanned 50bp windows surrounding TF peaks using "findMotifsGenome.pl" with standard background (random genomic sequences sampled according to GC content of peak sequences). We determined the occurrence of tandem E-boxes with variable length spacing by generating synthetic canonical E-box motifs separated by the indicated number of random spacers (i.e. CACGTGNNNCACGTG = 3 spacers) using "seq2profile.pl" allowing for 2 mismatches and testing for their occurrence at BMAL1

and CLOCK peaks using “annotatePeaks.pl”. *Analysis of Liver ChIP-Seq Data*: Fastq files for all BMAL1 and H3K27Ac ChIP-Seq were downloaded from the ENA server (Study Accession Number: SRP014752) and raw sequence reads for 12 sequential time points were concatenated into a single file. Alignments and peak calling were performed using bowtie and Homer as described above. Shared BMAL1 binding sites were identified by comparing binding locations between β-cells and liver using the Homer command “mergePeaks” and specifying “-d 200” which identified peaks occurring within 200bp as shared across tissues.

Results

BMAL1 and CLOCK bind proximate to active cell-type specific enhancers in pancreatic islets

To determine the intersection between circadian TF regulation and genomic binding at regulatory loci, we performed chromatin immunoprecipitation sequencing (ChIP-seq) in the mouse β-cell line Beta-TC6 (Fig. 6A). As expected, we found that both BMAL1 and CLOCK physically bound to sites at core clock and other gene targets in β-cells that were enriched for the canonical CACGTG E-box motif, often occurring in tandem, as previously reported at BMAL1 binding sites in liver (Fig. S7A) ($p=10^{-38}$ and $p=10^{-91}$, respectively) (Koike et al. 2012, Rey et al. 2011). Moreover, we also observed significant overlap in the genome-wide binding of BMAL1 and CLOCK (Fig. S7B). A representative UCSC genome browser track at the *Rev-erbα* (*Nr1d1*) locus is shown in Fig. 6A, revealing co-localization of BMAL1 and CLOCK binding sites at three distinct regulatory regions at the *Nr1d1* locus, including within both the promoter region (shaded light orange and defined as within 2kb of the transcription start site (TSS)) and within intragenic and intergenic distal enhancer regions (shaded light green and defined as binding regions greater than 2kb from the TSS) (Fig. 6A). Of importance, histone markers representing active and accessible chromatin (H3K27Ac and H2A.Z, respectively) localize to the same promoter and enhancer regions within the *Nr1d1* locus, indicating active transcriptional regulation by BMAL1 and CLOCK (Fig. 6A).

To determine whether BMAL1 and CLOCK directly regulate the oscillating transcripts identified in the synchronized WT islets (Fig. 5C), we evaluated the overlap between the BMAL1 and CLOCK cistromes with genes oscillating in the WT islets. Among binding sites localized to expressed RNAs, 30% (862 binding sites) and 29% (330 binding sites) of the BMAL1- and CLOCK-targets, respectively, exhibited rhythmic transcription in synchronized WT islets (Fig. 6B), which collectively accounted for 742 cycling direct target genes of which 165 were differentially expressed in *Bmal1* knockouts (Fig. S7C), suggesting direct BMAL1 and CLOCK regulation. Moreover, KEGG analysis of the direct gene targets in mouse islets that were present in BMAL1 and CLOCK cistromes revealed enrichment in pathways related to protein export, COPII-mediated vesicle budding from the endoplasmic reticulum, and SNARE vesicular transport and membrane fusion, in the cycling compared to non-cycling set of BMAL1 and CLOCK-controlled transcripts (KEGG pathways listed in order of descending $-\log_{10}$ p-values, Fig. 6C, Table S1). Together, these findings identify direct transcriptional targets of CLOCK/BMAL1 that mediate rhythmic islet physiology.

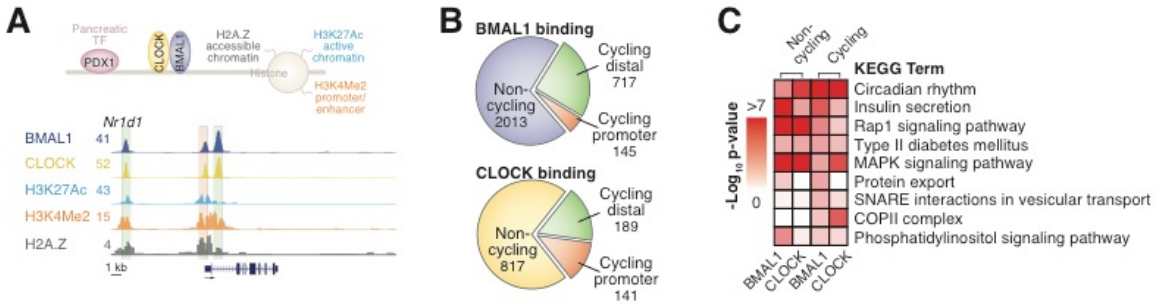

Fig. 6: BMAL1 and CLOCK bind to cycling genes at distal regulatory sites. (A) Model of transcriptional targets and chromatin modifications for ChIP-seq experiments (top). UCSC genome browser tracks at *Nr1d1* (*Rev-erbα*) locus in β-cells. Maximum track heights within viewable window are indicated to the right of each factor (bottom). **(B)** Distribution of BMAL1 and CLOCK peaks at cycling and non-cycling gene targets. Binding sites at cycling genes are separated into promoter proximal and distal sites (< and >2kb from TSS of nearest gene, respectively). **(C)** KEGG ontology terms enriched in cycling and non-cycling BMAL1 and CLOCK target genes.

Given the evidence for tissue-specific regulation at enhancers as a predominant mode of circadian regulation in liver (Fang et al. 2014), we next analyzed the binding position of core clock TFs BMAL1 and CLOCK in relation to the transcription start site (TSS) of rhythmic genes in β-cells. We classified binding events occurring within 2kb of the nearest annotated gene TSS as promoter-proximal since genome-wide promoter activity studies and epigenetic characterization of mammalian regulatory regions have indicated that the majority of core promoter activity is localized within 2kb of the TSS (Carninci et al. 2006, Cooper et al. 2006, Heintzman et al. 2007). Surprisingly, we found that BMAL1 and CLOCK bind predominantly at distal sites (defined as greater than 2kb from the TSS) rather than at proximal promoter sites (defined as less than 2kb from the TSS) of rhythmically regulated genes (Fig. 6B and Fig. S7D), suggesting that the islet clock TFs impact rhythmic physiology through binding to distal regulatory sites, an observation concordant with the general finding that TFs exert physiologic effects through regulation within tissue-specific enhancers (Pasquali et al. 2014).

While clock factors have been shown to exert distinct physiologic functions across tissues, a major gap remains in understanding the underlying genomic mechanisms accounting for these tissue-specific functions. To determine whether BMAL1 regulates rhythmic genes through unique sites in the β-cell compared to liver, the tissue in which the circadian cistrome has been best characterized (Fang et al. 2014, Gaulton et al. 2015, Koike et al. 2012, Menet et al. 2012, Vollmers et al. 2012), we compared sites of BMAL1 occupancy in the β-cells to a published set of liver BMAL1 peaks (Koike et al. 2012).

Unexpectedly, although there was a significant overlap of genes identified as direct BMAL1 binding targets in β-cells and liver (40%, 1063 genes out of 2660 total β-cell target genes) (Fig. 7A), BMAL1 binding at the regulatory regions of those shared gene sets localizes to distinct sites (Fig. 7A). Remarkably, in comparing genome-wide binding patterns, we only observed common locations of binding in 4% of these instances; thus BMAL1 binding at all β-cell and liver-defined sites are uncorrelated (Fig. 7A, Fig. S7E) (R^2=0.01874 and 0.03286 for BMAL1 binding at β-cell and liver sites, respectively), while binding at canonical E-box sites in *Per2*, *Cry1,* and *Dbp* was similar between tissues (Fig. S7F). Furthermore, when we compared the shared set of BMAL1 target genes that were rhythmic in islets and also reported to be rhythmic at the mRNA level in liver, BMAL1 likewise bound to unique sites (Koike et al. 2012) (Fig. 7B). These data suggest convergent regulation of BMAL1-targets in β-cell and liver through divergent regulatory elements.

Because BMAL1 predominantly bound at distal regulatory regions in islets that were divergent from liver, we next sought to examine the chromatin regulatory context at all cycling genes in β-cells. To do so, we defined all regulatory regions at cycling loci using H3K4Me2 peaks within 2kb of the TSS (promoter) and >2kb from the TSS (enhancer) (Fig. 7C). The binding patterns of the histone marks H3K4Me2, H2A.Z, and H3K27Ac (which represent promoter/enhancer regulatory regions, chromatin accessibility, and enhancer activity, respectively), as well as binding of the lineage-determining TF for β-cells PDX1 (Stoffers et al. 1997) at promoters and enhancer regions are displayed in heatmaps in Fig. 7C. By performing hierarchical clustering, we found that all epigenetic

and PDX1 signals at promoter and distal enhancer regions at cycling genes more frequently displayed correlated binding than did H3K27Ac at these loci in liver, as indicated by the clustering dendogram (Fig. 7C). Accordingly, the genomic coordinates in liver corresponding to enhancers defined in β-cells displayed markedly reduced H3K27Ac, indicating that these enhancers defined specific loci of β-cell regulation (Fig. 7C). Frequent binding of PDX1 at distal enhancer loci suggested that tissue-specificity arose from early events in islet cell development (Fig. 7C) (Hoffman et al. 2010). Consistent with tissue-specific clock TF regulation at β-cell regulatory regions, BMAL1 displayed a greater degree of binding to promoter and enhancer regions at cycling genes in β-cells than in liver, particularly at active enhancers containing both H3K4Me2 and H3K27Ac (Fig. 7D). These results indicate that clock TFs generate unique patterns of rhythmic RNA expression across tissues according to the pattern of cell-specific enhancer repertoires and provide a molecular basis for the distinct and opposing effects of the clock in pancreas and liver, which primarily affect post-prandial and fasting glucose metabolism, respectively (Lamia et al. 2008, Marcheva et al. 2010).

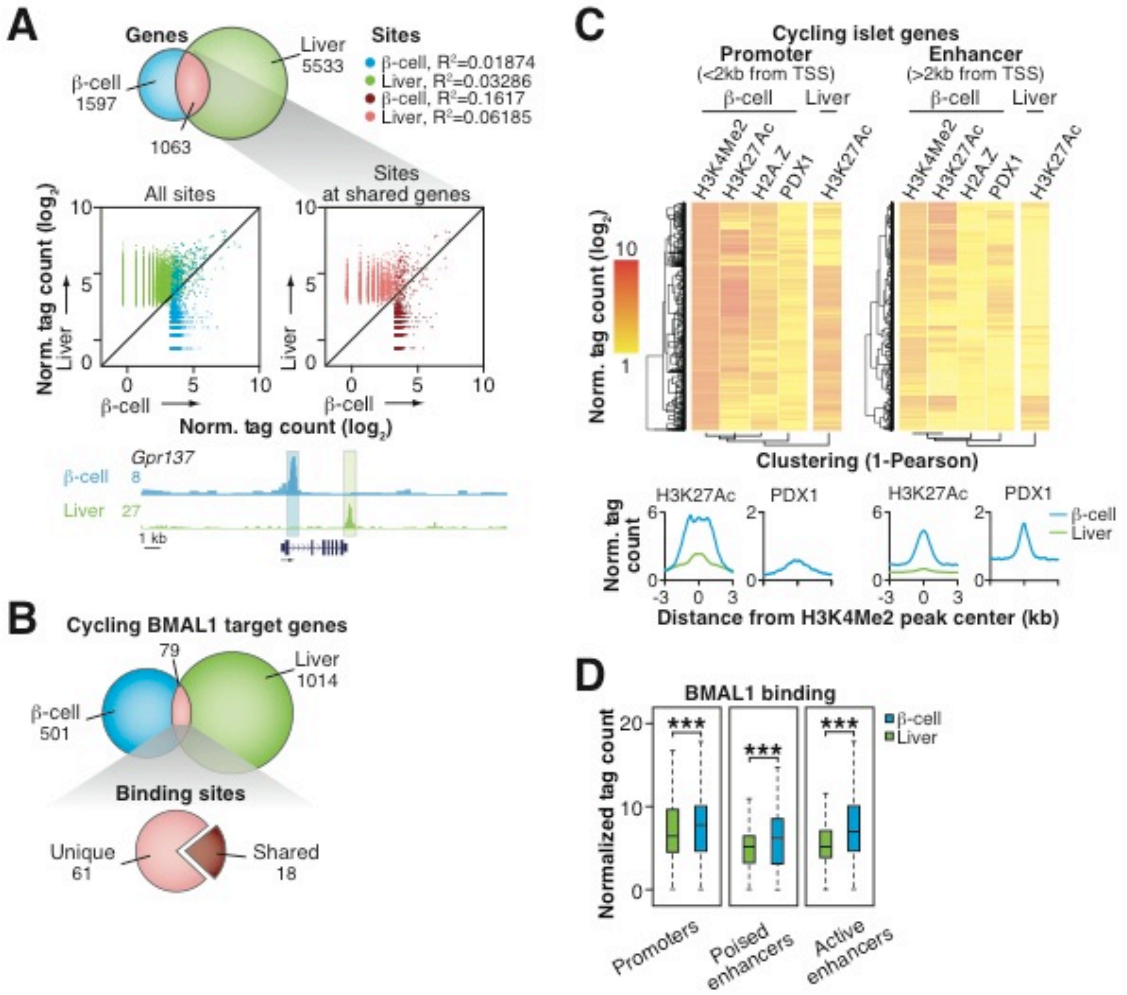

Fig. 7: β-cell circadian cistrome is determined by tissue-specific enhancer repertoire. **(A)** Overlap of genes identified at BMAL1 binding sites in β-cells and liver (top). Scatter plots show BMAL1 binding in liver (y-axis) versus β-cells (x-axis) within 500bp windows surrounding peaks identified in each tissue (middle). Browser track view of BMAL1 binding in β-cells and liver at the *Gpr137* locus (bottom). **(B)** Overlap of cycling and direct BMAL1 target genes in β-cells that have been reported to cycle in liver (top). Cycling BMAL1 direct target genes containing shared or unique binding sites in β-

cells and liver (bottom). **(C)** Heatmaps comparing binding of indicated factors within 1kb windows surrounding promoter (3,492) and enhancer (5,771) localized H3K4Me2 peaks annotating to genes containing cycling RNAs in WT islets. Histograms summarizing normalized tag counts for H3K27Ac (in β-cells and liver) and PDX1 (in β-cells) across 6kb span centered at all β-cell H3K4Me2 peaks (bottom). **(D)** Box and whiskers plots (whiskers represent IQR 1.5) comparing BMAL1 binding in β-cells and liver at loci corresponding to H3K4Me2 peaks defined in heatmaps. Poised enhancers refer to H3K4Me2 sites that do not co-localize with H3K27Ac, whereas active enhancers are defined as H3K4Me2 sites co-localized with H3K27Ac. ***$p<0.0001$ by Mann-Whitney non-parametric, unpaired *t*-test. All reported ChIP-seq tag counts normalized per 10^7 reads.

Discussion

Cistrome profiling in β-cells revealed that CLOCK/BMAL1 regulate cycling genes involved in insulin secretion by interacting with cell type-specific enhancer elements. This observation is in agreement with the prominent roles of enhancers in controlling the expression of cycling genes in liver and essential β-cell genes in development and adulthood. A likely explanation for the divergence between BMAL1 binding in liver and β-cells is that the repertoire of available E-box elements differs due to tissue-specific activities of transcription factors that interact with chromatin to expose these binding sites during organ development. Consistent with this idea, enhancers proximate to cycling islet genes were enriched for the PDX1 transcription factor, which cooperates with a "pioneer" transcription factor FOXA2 that is capable of binding to and displacing nucleosomes to facilitate chromatin opening and establish enhancer sites (Hoffman et al. 2010, Sekiya et al. 2009).

CLOCK/BMAL1 have also been proposed to exert pioneer-like functions by coordinating a daily rhythm in nucleosome occupancy in mouse liver (Menet et al. 2014) and interestingly ectopic expression of *Clock* outside of pacemaker neurons in *Drosophila* induces the *de novo* formation of circadian oscillators suggesting a fundamental role in establishing new transcription programs (Zhao et al. 2003). Besides potential pioneer-like functions CLOCK/BMAL1 are known to influence transcriptional activity at their binding sites by recruiting histone acetyltransferase complexes including cAMP response element binding protein binding partner (CBP), p300, and p300/CBP-associated factor

(PCAF) (Curtis et al. 2004, Etchegaray et al. 2003, Lee et al. 2010, Takahata et al. 2000). The core circadian repressor PER2 recruits the SIN3B-HDAC1 histone deacetylase and the downstream rhythmic repressor *Rev-erbα* recruits the nuclear corepressor NCoR and histone deacetylase 3 (HDAC3) to rhythmically inhibit transcription (Duong et al. 2011, Feng et al. 2011, Naruse et al. 2004, Sun et al. 2011). CLOCK/BMAL1 can also influence the epigenetic state of gene promoters and transcriptional enhancers by recruiting the histone methyltransferase Mixed lineage leukaemia 1 (MLL1) to promote trimethylation of lysine 4 on histone 3 (H3K4Me3) at gene promoters (Katada and Sassone-Corsi 2010), which contributes to transcriptional activation. The degree of H3K4Me1 deposition within enhancers similarly displays circadian variation (Koike et al. 2012) due to rhythmic occupancy of the Mixed lineage leukaemia 3 (MLL3) methyltransferase in mouse liver (Valekunja et al. 2013). While these studies point to possible roles for CLOCK/BMAL1 in both the formation of enhancers and their acute epigenetic modification to influence transcriptional activity, the disparity between BMAL1 binding in β-cells and liver suggests CLOCK/BMAL1 most likely influence rhythmic gene transcription by binding to and modifying the activity of developmentally established binding sites rather than forming new enhancers.

The surprising finding that BMAL1 binds to distinct sites even within genes that cycle in both islets and liver raises the possibility that CLOCK/BMAL1 may interact with unique co-regulatory transcription factors to influence tissue-specific physiological functions. Whereas CLOCK/BMAL1 have been shown to regulate hepatic gene transcription predominantly by recruiting transcriptional coactivators and RNA polymerase II (Koike

et al. 2012, Menet et al. 2012, Vollmers et al. 2012), BMAL1 represses transcription of inflammatory genes in myeloid cells by recruiting the polycomb repressor 2 (PRC2) complex proteins Enhancer of zeste 2 (EZH2), Extra-sex comb (EED) and Suppressor of zeste 12 (SUZ12) to gene promoters (Nguyen et al. 2013). It is possible that this discrepancy in BMAL1 function may be related to its co-recruitment to target sites by distinct sets of activating or repressive transcription factors. A comparative cistrome analysis of the circadian repressor REVERBα in liver, brown adipocytes, and brain revealed that the rhythmic expression of a small number of shared target genes across the three tissues are regulated primarily by competition between repressive REVERB and activating ROR transcription factors, while REVERBα was recruited to liver-specific targets by the hepatic transcription factor HNF6 (Zhang et al. 2015). In the future it will be important to determine if CLOCK/BMAL1 and downstream rhythmic transcription factors cooperate or compete with islet-specific transcription factors other transcriptional coregulators to influence rhythmic gene transcription.

Chapter VI

Acute silencing of the β-cell clock in adulthood impairs second messenger-induced insulin secretion and causes hypoinsulinemic diabetes

Parts of this chapter were adapted from:

Perelis M, et al (2015) Pancreatic beta cell enhancers regulate rhythmic transcription of genes controlling insulin secretion. *Science* 350: p. aac4250.

Summary

Genetic analyses in mice have demonstrated that the circadian clock is a critical regulator of mammalian glucose homeostasis, with established roles in promoting glucose synthesis during fasting in the liver (Lamia et al. 2008;Peek et al. 2013) and insulin secretion in pancreatic β-cells in the fed state (Turek et al. 2005; Marcheva et al. 2010). Studies in mice and humans have revealed that the ability of β-cells to secrete insulin in sufficient quantities to maintain normal blood glucose levels depends on the formation of adequate β-cell mass during postnatal development and in some disease states, in addition to the intrinsic secretory capacity of individual β-cells (Sachdeva and Stoffers 2009). While the observation that size-matched islets from $Clock^{\Delta 19}$, $Bmal1^{-/-}$, or $PdxCre;Bmal1^{flx/flx}$ mice secrete significantly less insulin than littermate controls (Lee et al. 2011, Marcheva et al. 2010, Sadacca et al. 2011) clearly indicates that the clock regulates secretory capacity, $Clock^{\Delta 19}$ and $Bmal1^{-/-}$ mice displayed reduced islet size and proliferation suggesting that the clock might also regulate islet growth during development (Marcheva et al. 2010). To differentiate the effects of circadian gene deletion on adult-life function and development in β-cells we generated tamoxifen-inducible $PdxCreER;Bmal1^{flx/flx}$ mice, which enables the acute deletion of the *Bmal1* gene following injection of tamoxifen (Gu et al. 2010, Gu et al. 2002).

Materials and methods

Animals. All mice were produced and maintained on a C57BL/6J background at the Northwestern University Center for Comparative Medicine. *Bmal1*$^{flx/flx}$ mice (Westgate et al. 2008) were crossed with *PdxCreER* transgenic mice (kindly provided by Dr. Douglas Melton, Harvard University) (Gu et al. 2002) to generate *PdxCreER;Bmal1*$^{flx/flx}$ mice, as well as *Bmal1*$^{flx/flx}$ and *PdxCreER* littermate controls. Unless otherwise stated, mice were maintained on a 12:12 light:dark (LD) cycle with free access to regular chow and water. All animal care and use procedures were conducted in accordance with regulations of the Institutional Animal Care and Use Committee at Northwestern University.

Tamoxifen treatment. For *in vivo* delivery of tamoxifen (Sigma, dissolved in corn oil), mice received three IP injections of 200μg tamoxifen/g body weight, administered every other day. Subsequent experiments were conducted 10-14 days following tamoxifen treatment.

Immunohistochemical analysis. Mice were anesthetized with intraperitoneal injection of phenobarbital (Nembutal, 50mg/ml) and perfused with heparinized saline, followed by 4% paraformaldehyde (PFA) (Sigma) in PBS. Brain and pancreas were removed and post-fixed with 4% PFA overnight at 4°C. Brain tissues were then cryoprotected in 30% sucrose (Sigma), frozen in O.T.C. (Tissue Tek), and 30μm brain sections collected for antibody staining. Pancreata were embedded in paraffin, and blocks of 6μm sections were mounted on slides. The following primary antibodies were used for staining: guinea pig

anti-insulin (1:500, DAKO), mouse anti-glucagon (1:500, Sigma), and rabbit anti-BMAL1 (1:500, Novus Biological). Triple staining was visualized with the following secondary antibodies: AMCA goat anti-guinea pig (1:400, Jackson ImmunoResearch), Alexa Fluor 488-conjugated goat anti-mouse (1:400, Invitrogen), and Alexa Flour 546-conjugated goat anti-rabbit (1:400, Life Technologies). Nuclei were counterstained with DAPI as indicated. Images were acquired with PictureFrame 1.0 using a Zeiss Axioskop 50. β–cell mass was assessed by morphometric analysis of insulin immunostained pancreatic sections (DAKO; Histomouse^{TM}*Plus* kit, Life Technologies). Four pancreatic sections, spaced 50μm apart, were stained for each animal, and endocrine versus total pancreas area was calculated using Image-Pro Premier software (Media Cybernetics) using the smart segmentation feature.

Glucose and insulin measurements and glucose tolerance tests. Blood glucose and plasma insulin levels in *ad libitum* fed mice were assessed at ZT2 and ZT14 from tail vein bleeds. Glucose tolerance tests were performed in mice following a 14 hr fast, and blood glucose and plasma insulin levels were measured at the indicated times following intraperitoneal (IP) glucose injection of either 2 or 3g/kg body weight, respectively. Plasma insulin levels were measured by ELISA.

Behavioral analysis. Locomotor activity was analyzed in 2-4 month old pancreas-specific *Bmal1* knockout mice and their respective littermate controls following tamoxifen treatment. All animals were individually housed in standard mouse cages equipped with running wheels and allowed free access to food and water. Mice were

placed in a 12:12 LD cycle for 14 days, followed by 14 days in constant darkness (DD). Total activity data was recorded and analyzed in 6-minute bouts using ClockLab software (Actimetrics). The free-running period was determined as the duration of time between the major activity periods on consecutive days in DD. Period was calculated using a Chi-square periodogram for days 7-14 in DD. Food consumption was analyzed in pancreas-specific *Bmal1* knockout mice and their littermate controls prior to and following tamoxifen treatment. All animals were individually housed with free access to water and regular chow. Day- and night-time food consumption was determined by manual measurement of food at both ZT0 and ZT12 for 3 consecutive days.

Islet isolation and insulin secretion assays. Mouse pancreatic islets were isolated via bile duct collagenase digestion (*Collagenase P*, Sigma) and Ficoll gradient separation and left to recover overnight (16 hrs) at 37˚C in RPMI 1640 with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. For standard insulin release assays, 5 islets were statically incubated in Krebs-Ringer Buffer (KRB) and stimulated for 1 hr at 37˚C with various glucose concentrations, 30mM KCl, 2.5μM forskolin, 1mM 8-Br-cAMP, 10mM L-leucine + 2mM L-glutamine, 1mM carbachol, 10μM PMA, or 10μM ionomycin. Supernatant was collected and assayed for insulin content by ELISA (Crystal Chem Inc). Islets were then sonicated in acid-ethanol solution and solubilized overnight at 4˚C before assaying total insulin content by ELISA.

Intracellular calcium determination. BetaTC-6 cells were plated at a density of 100,000 cells per well in black 96-well plates with clear bottoms and cultured overnight

at 37°C and 5% CO_2. Islets were dispersed to single cells by incubating in 0.05% Trypsin-EDTA at 37°C for 3 minutes and plated at a density of 100 islets per well in laminin-treated black 96-well plates with clear bottoms and cultured in complete media for 48-hours at 37°C and 5% CO_2. Cells were then washed with BSA-free KRB buffer with no glucose and loaded with 5mm Fura-2 (Invitrogen) and 0.04% Pluronic F-127 (Invitrogen) for 30 min at 37°C. Following a wash with BSA-free KRB, Fura-2 intensity was measured following injection of either glucose or ionomycin (Sigma) to final concentrations of 20mM or 10μM, respectively. Cells were alternately excited with 340 nm and 380 nm wavelength light, and the emitted light was detected at 510 nm using a Cytation 3 Cell Imaging Multi-Mode Reader (Bio Tek) at sequential 30-second intervals. Raw fluorescence data were exported to Microsoft Excel and expressed as the 340/380 ratio for each well.

Results

Clock disruption during adulthood causes β-cell failure due to blockade of second messenger signaling

To test the hypothesis that clock genes modulate genome-wide transcription on a daily basis throughout adult life, we examined the impact of acute clock inhibition on glucose metabolism in *PdxCreER;Bmal1*$^{flx/flx}$ mice at 2-3 months of age following administration of tamoxifen, which abrogates BMAL1 expression exclusively within the β-cell (Fig. S8) (Gu et al. 2010, Taylor et al. 2013). While these mice displayed normal wheel running rhythms, period length, food intake, and body weight (Fig. S9) compared to littermate tamoxifen-treated *PdxCreER* and *Bmal1*$^{flx/flx}$ animals, they developed significant hyperglycemia, impaired glucose tolerance, and hypoinsulinemia within 10-14 days following tamoxifen administration during both the day (ZT2) and night time (ZT14) (Figs. 8A-B and S11), despite no differences in islet mass (Fig. S11A). These results establish that circadian disruption in fully differentiated cells is sufficient to induce metabolic disease independent of effects on early development.

We further found that islets isolated from tamoxifen-treated *PdxCreER;Bmal1*$^{flx/flx}$ mice secreted significantly less insulin compared to littermate controls when exposed to (i) 20mM glucose, (ii) 10mM leucine combined with 2mM glutamine, which bypasses glycolysis to trigger mitochondrial ATP production, or (iii) 30mM KCl, which chemically closes the K_{ATP} channel, thus inducing membrane depolarization distal to

glucose metabolism and an increase in cytosolic calcium (Fig. 8C-D), while glucose-stimulated calcium influx was unchanged (Fig. S11B). Remarkably, this data is consistent with our observation that circadian oscillation in insulin secretory capacity is regulated downstream of K_{ATP} channel closure. Consistent with impaired heterotrimeric G protein-coupled receptor (GPCR) signaling, *PdxCreER;Bmal1*$^{flx/flx}$ islets also secreted significantly less insulin than controls in response to glucose together with the cyclase agonist forskolin and the non-hydrolyzable cAMP analogue 8-br-cAMP (Fig. 8C, E).

Finally, we tested the response to G_q-type GPCR signaling by stimulating islets with the muscarinic agonist carbachol, the DAG mimetic PMA, and Ca^{2+} ionophore ionomycin. Surprisingly, carbachol and PMA restored insulin secretion in *PdxCreER;Bmal1*$^{flx/flx}$ islets (Fig. 8C, F), while the response to ionomycin, which raised intracellular Ca^{2+} in β-cells (Fig. 8F), was significantly reduced in mutants, indicating that the DAG-arm of the G_q pathway restored second messenger signaling. DAG regulates exocytosis in β-cells and other neurosecretory cells by acting as a ligand for the vesicle priming protein Munc13-1 (Kang et al. 2006) and PKC, which phosphorylates and activates SNAP25 and MUNC18-1 to initiate vesicle fusion (Genc et al. 2014). We observed rhythmic RNA expression of the PKC activating Rho and Rap GTPases *Rho*, *Rhoa*, *Rhob*, and *Rap1a* in WT islets, raising the intriguing possibility that elevated DAG concentrations in carbachol or PMA treated islets pharmacologically bypassed a deficiency in Rho- and Rap-mediated signaling. Together, these results demonstrate that pharmacologic Gq agonism reverses the insulin secretory blockade induced by clock disruption, indicating convergence of cholinergic and phosphoinositol signaling within the β-cell in temporal homeostasis.

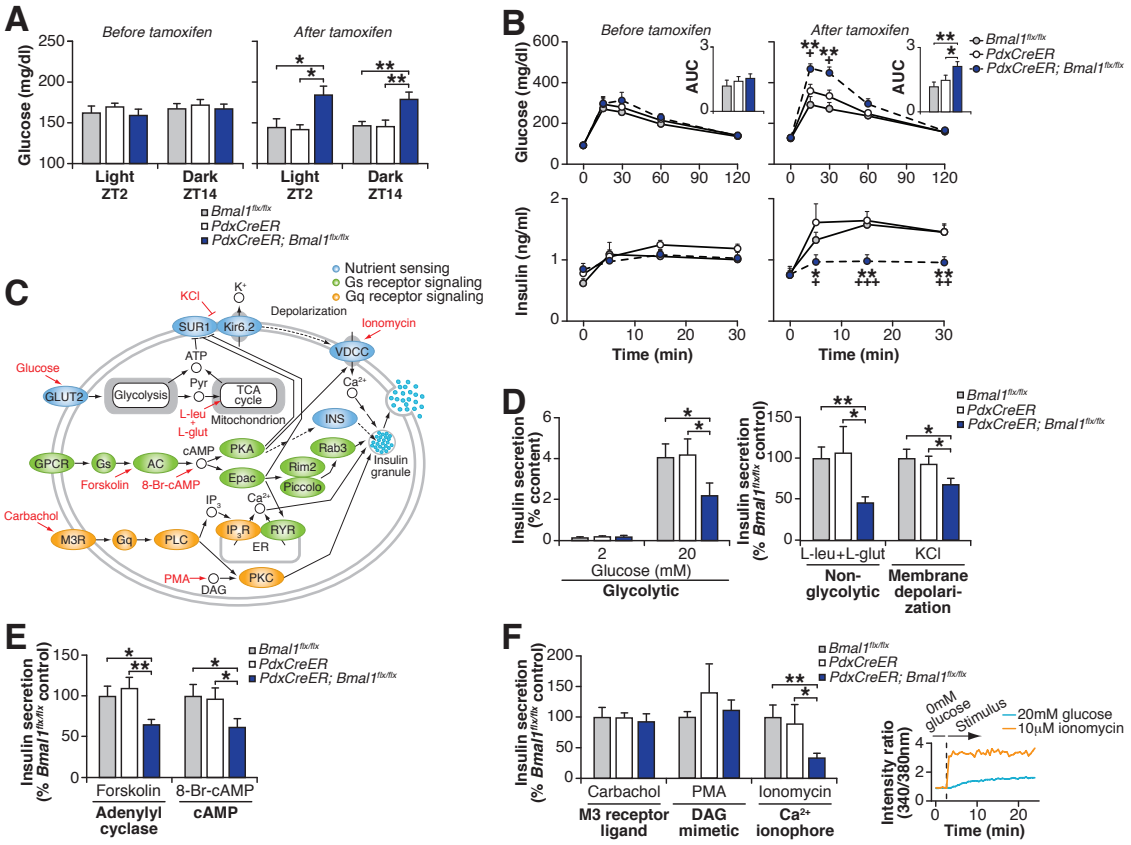

Fig. 8: Clock disruption in β-cells during adulthood causes acute hypoinsulinemic diabetes in mice. (A) Blood glucose levels in *ad libitum* fed mice before and after tamoxifen administration (n=6-12 mice per genotype). **(B)** Glucose tolerance and insulin secretion at ZT2 following intraperitoneal glucose administration in *PdxCreER;Bmal1*$^{flx/flx}$ mice and littermate controls before and after tamoxifen treatment (n=4-11 mice per genotype). Inset represents area under the curve (AUC) for glucose (10^4 mg/dl/120min). **(C)** Model of intersecting pathways driving insulin exocytosis highlighting nutrient, Gs, and Gq receptor signaling that are used to stimulate insulin secretion in (D-F). **(D)** Glucose and nutrient-stimulated, **(E)** cyclase-pathway, and **(F)** catecholamine-stimulated insulin secretion in islets isolated from tamoxifen-treated *PdxCreER;Bmal1*$^{flx/flx}$ and

control mice (n=3-8 mice per genotype, 3 repeats per mouse). Inset is ratiometric determination of intracellular Ca^{2+} using Fura2-AM dye in Beta-TC6 cells in response to insulin secretagogues (n=3 replicates per condition). All values represent mean ± SEM. For (B), asterisks denote significance between *Bmal1*$^{flx/flx}$ and *PdxCreER;Bmal1*$^{flx/flx}$, while plus symbols denote significance between *PdxCreER* and *PdxCreER;Bmal1*$^{flx/flx}$. *p<0.05, **p<0.01, ***p<0.001

Discussion

The observation that acute deletion of *Bmal1* in β-cells of adult mice rapidly precipitates hyperglycemia and impaired glucose tolerance demonstrates that a functional β-cell clock is required to maintain blood glucose levels in adulthood independently of potential effects on islet development. Furthermore these results are consistent with impaired insulin secretion in *PdxCreER;Bmal1*$^{flx/flx}$ islets following acute *Bmal1* deletion *in vitro* and with the pronounced effect of acute changes in BMAL1 activity across the 24-hr circadian cycle in isolated islets.

Recently, a similar effect of acute β-cell *Bmal1* deletion in adult mice using *RipCreER;Bmal1*$^{flx/flx}$ mice where a tamoxifen sensitive Cre recombinase is expressed under the control of the rat *Ins2* promoter was reported (Rakshit et al. 2016), confirming these results. Interestingly, that study also found reduced β-cell proliferation in *RipCreER;Bmal1*$^{flx/flx}$ mice following 10 weeks of high fat feeding due in part to impaired expression of proliferative genes downstream of GLP-1 signaling (Rakshit et al. 2016) suggesting that the proliferative response to diet- or obesity-induced signals is compromised in clock mutant β-cells. High fat diet causes insulin resistance resulting in increased circulating glucose and insulin levels which contribute to β-cell mass expansion in mice (Kulkarni et al. 1999, Martinez et al. 2006, Okada et al. 2007). The primary effectors of glucose and insulin required for β-cell proliferation include insulin and IGF1 (insulin-like growth factor 1) receptors and downstream signaling via the insulin receptor substrate IRS2 and the serine/threonine kinase AKT (Bernal-Mizrachi et al. 2001,

Terauchi et al. 2007, Tuttle et al. 2001, Withers et al. 1998). Several downstream effectors of Insulin-IRS2-AKT signaling have been found to influence β-cell proliferation during high fat feeding including inhibition of the transcription factor FoxO1 (Kitamura et al. 2002, Okamoto et al. 2006) and the serine/threonine kinase GSK3β (Tanabe et al. 2008), and activation of the mammalian target of rapamycin (mTOR) kinase and its target p70/ribosomal S6 kinase (Pende et al. 2000, Rachdi et al. 2008). Interestingly, GLP-1 signaling similarly induces β-cell growth by activating the cAMP-sensitive protein kinase PKA (protein kinase A) leading to phosphorylation of the cAMP-response element binding protein (CREB) which binds to and activates transcription and and activity of IRS2 (Jhala et al. 2003, Van de Velde et al. 2011). The finding that islets isolated from *PdxCreER;Bmal1*$^{flx/flx}$ mice displayed refractory secretory responses to adenylyl cyclase agonists and cAMP agonists downstream of the GLP-1 receptor reveal that the clock acutely regulates the potentiation of insulin secretion and suggests it may also influence the inducibility of β-cell growth by this pathway.

Muscarinic receptor (m3AchR) signaling, which surprisingly rescues insulin secretion in *PdxCreER;Bmal1*$^{flx/flx}$ islets, similarly affects both insulin release and β-cell proliferation (Thorens 2014). Muscarinic receptor activation by acetylcholine is stimulated by autonomic signals that respond to glucose sensing in the brain. Specifically, glucose sensing by taste buds and glucose sensing neurons in the hepatoportal vein stimulate activity of the vagus nerve and promote insulin secretion (Berthoud et al. 1981, Berthoud and Jeanrenaud 1982, Berthoud and Powley 1990, Berthoud et al. 1980, Niijima 1982, Niijima 1984). A population of these glucose sensing neurons have cell bodies in dorsal

motor nucleus of the vagus (DMNX) in the brain stem in close proximity to parasympathetic neurons in the nucleus of the solitary tract (NTS) which project onto pancreatic ganglions (Ionescu et al. 1983, Jansen et al. 1995). Contact between glucose sensitive DNMX and NTS neurons integrates central glucose sensing to regulate acetylcholine-stimulated insulin secretion (Thorens 2014). Deletion of glucose transporter 2 (GLUT2) in central and peripheral neurons in mice disrupts this axis and results in decreased insulin secretion, reduced postnatal β-cell proliferation, and diabetes by approximately 6 months of age (Tarussio et al. 2014).

The restoration of insulin secretion by m3AchR signaling raises the intriguing possibility that brain-derived autonomic signals might override the β-cell autonomous clock under certain conditions. While autonomic control of the pancreas has not been examined in clock mutant mice, it is possible that altered autonomic function may explain reduced β-cell mass in multitissue mutants that was not observed after acute deletion of *Bmal1* in adulthood. Finally, studies are warranted to determine if this pathway represents a novel target for therapeutic intervention in diabetic subjects with a circadian component to their disease.

Chapter VII

Conclusions

Collectively, the *in vivo* and *in vitro* studies presented here reveal that multiple circadian control mechanisms modulate the secretion of insulin across the day/night cycle. Studies using pancreas-specific *Clock* knockout mice reveal that both major components of the forward limb of the clock (CLOCK/BMAL1) are required to sustain β-cell function and normal glycemic control and that loss of *Clock* is not functionally compensated for by its paralog *Npas2*. Isolated islet studies demonstrate that cell-autonomous expression of CLOCK/BMAL1 in β-cells drives genome-wide cycles in the transcription of mRNAs regulating the formation, trafficking, and exocytosis of insulin-containing vesicles that prime cells to maximally secrete insulin at specific windows throughout the day. These cycles are coordinated by the binding of CLOCK and BMAL1 proteins to distal regulatory enhancers that are unique to the β-cell, which helps to explain how the core oscillator expressed in all mammalian tissues can exert tissue-specific functions. The rhythmic regulation of secretory genes by CLOCK/BMAL1 is critical for maintaining normal blood glucose levels since even acute inhibition of the oscillator rapidly precipitates glucose intolerance and hyperglycemia.

Additional layers of regulation establish the rhythmicity of glucose homeostasis in the intact animal, including variation in hepatic transcription cycles and in the gastrointestinal tract where incretin hormones are produced (such as GLP-1), as well as the brain-driven melatonin, neuroendocrine, and behavioral cycles. While still incompletely known, white adipose, brown adipose, and skeletal muscle clocks also contribute to energy balance and in turn likely impact glucose homeostasis (Paschos et al.

2012; Gerhart-Hines et al. 2013; Schroder et al. 2015; Hodge et al. 2015). In fact, receptors for the adipose-derived hormone leptin, which is elevated in obesity, are present in β-cells although their effect on β-cell health is likely context dependent since pancreas-specific deletion of the receptor impairs glucose tolerance, insulin secretion and compensatory cell growth in high fat feeding (Morioka et al. 2007) while exogenous addition of leptin to cultured rodent and human islet cells impairs insulin secretion and causes apoptosis (Kulkarni et al. 1997, Maedler et al. 2008, Seufert et al. 1999). Additionally, muscle-specific deletion of the PPARγ coactivator PGC-1α causes impaired islet insulin secretion in mice that is attributed to decreased levels of muscle-derived IL-6, which has a potentiating effect on GLP-1 stimulation of β-cells (Handschin et al. 2007). Studies in *Bmal1*$^{-/-}$ islets demonstrate that the circadian clock regulates GLP-1-stimulated insulin secretion possibly by controlling the expression of cAMP-sensitive genes, however future studies are warranted to better understand how the β-cell clock might respond to or integrate cues from other glucose regulatory tissues such as adipocytes.

Disruption of external circadian entrainment by light cycle manipulation or simulated shift work has been shown to inhibit clock function and insulin secretion in the β-cell (Lee et al. 2013; Qian et al. 2013), and may account in part for sub-types of diabetes in humans. Additional mechanisms of circadian disruption in shift-workers may involve alterations in autonomic nervous system signals or feeding-derived factors that impact islet function. However, the relative contribution of the brain and peripheral clocks, as well as the repertoire of secreted factors that confer timing cues to the β-cell, remain

largely unknown. For example, *Per2* transcriptional oscillations in isolated islets can be entrained by mimetics of circulating and physiologic conditions, including the cAMP agonist forskolin which mimics downstream GLP-1 signaling, the glucocorticoid agonist dexamethasone, and simulated body temperature cycles (Marcheva et al. 2010; Perelis et al. 2015; Pulimeno et al. 2013; Saini et al. 2016), and therefore alterations in the circadian pattern of circulating GLP-1, glucocorticoid hormones, and body temperature rhythms (Balsalobre et al. 2000; Balsalobre et al. 2000; Schibler et al. 2015) may represent mechanisms that contribute to the dysregulation of the islet clock during shift work by producing misalignment between the oscillator and entraining signals. Further, the observation that mimetics of cholinergic signaling bypass the insulin secretory defects in *PdxCreER;Bmal1*$^{-/-}$ islets presents the intriguing possibility that, under some circumstances, the autonomic nervous system may override impairment of the cell-autonomous clock to promote insulin secretion. Indeed, studies have long noted the existence of a pre-absorptive "cephalic phase" of insulin secretion that is highly conserved across animal species and regulated by brain-derived signals transmitted by cholinergic vagal innervation (Power and Schulkin 2008; Powley and Berthoud 1985). In addition to providing insight into new potential mechanisms for circadian regulation of the β-cell, the observation that muscarinic acetylcholine receptor activity and downstream DAG signaling restores insulin secretion suggests that this pathway may be a therapeutic target to enhance insulin secretion in diabetic humans with a circadian component to their disease.

Conversely, there is extensive evidence that circadian clocks are sensitive to nutrient excess and are dysregulated in obesity and diabetes. For example, genetic rodent models for obesity and metabolic dysregulation, such as the leptin-deficient *ob/ob* mice (*Lep*$^{ob/ob}$), the leptin receptor-deficient *db/db* mice (*Lepr*$^{db/db}$) and the Zucker obese rats, all exhibit disruptions in circadian behavior, including feeding rhythms, diurnal locomotor activity rhythms and sleep (Laposky et al. 2006; Laposky et al. 2008; Megirian et al. 1998; Mistlberger et al. 1998). Interestingly, changes in peripheral clock gene expression in *Lep*$^{ob/ob}$ mice precede metabolic abnormalities (Ando et al. 2011), suggesting that altered clock function may contribute to metabolic decline. A recent study found mitigating effects of the small molecule Nobiletin on metabolic disorders, including impaired insulin secretion, in *Lepr*$^{db/db}$ mice by enhancing the amplitude of circadian rhythms (He et al. 2016), suggesting that enhanced circadian amplitude in β-cells is protective in diabetes. Studies using high fat diet feeding, which causes obesity and diabetes as well as dysregulation of behavioral, molecular and metabolic circadian rhythms in mice (Kohsaka et al. 2007; Eckel-Mahan et al. 2013), have shown that restoring the feeding rhythm by restricting feeding time to the "correct" time of day protects mice from developing pathologies associated with diabetes (Arble et al. 2009; Hatori et al. 2012). Moreover, clock expression specifically in the β-cell may exert a protective role during high fat feeding, since β-cell-specific *RipCreER;Bmal1*$^{flx/flx}$ mice display decreased compensatory β-cell proliferation and increased glucose levels (Rakshit et al. 2016; Kohsaka et al. 2007). It has become clear that the circadian clock is sensitive to metabolic and hormonal changes that occur as consequences of nutrient excess and

future studies are needed to determine if clock-controlled signaling in the β-cell might be a therapeutic target to improve glucose control in diabetes.

Recent advances in genome-wide transcriptional and cis-regulatory mapping of circadian regulation has not only identified the repertoire of clock-controlled genes within each tissue and the studies presented here have revealed tissue-specific differences in clock control of enhancer landscapes in distinct tissues. However, many outstanding questions remain regarding the mechanisms and consequences of circadian transcription factor regulation in β-cells. First, the observation that CLOCK/BMAL1 regulate circadian islet genes by binding to enhancers co-occupied by developmental TFs such as PDX1 suggests that there may be reciprocal regulation of cycling genes by other pancreatic TFs in the β-cell. For example, physiological inhibition of PDX1 or other TFs that occurs following high fat feeding (Reimer and Ahren 2002) in wild type mice may impair β-cell function by inhibiting CLOCK/BMAL1 activity. Second, since circadian gene transcription cycles are enabled by the activity of not only the core circadian activators and repressors, but also downstream CLOCK/BMAL1-controlled circadian PAR-bZIP TFs (DBP, HLF, TEF, and E4BP4) (Fang et al. 2014, Gachon et al. 2004, Gachon et al. 2006) and nuclear receptors (RORα/β/γ and REV-ERBα/β) (Fang and Lazar 2015, Zhang et al. 2015), it is therefore possible that alterations in the expression or genomic localization of these TFs may direct and modulate circadian gene transcription in the β-cell. For example, *Nrf2* is a downstream target of CLOCK/BMAL1 and encodes a transcription factor that regulates the circadian pattern of antioxidant gene expression in β-cells (Lee et al. 2013). A key related outstanding question is how do circadian transcription factors regulate

transcriptional responses mediated by extracellular cues such as GLP-1 and acetylcholine, which signal via CREB, Hypoxia inducible factor 1α (HIF1α) and other transcription factors (Jhala et al. 2003, Van de Velde et al. 2011). Interestingly, chromatin-immunoprecipitation coupled to DNA promoter array analysis, an early and more limited approach to sequencing, revealed that CREB occupies a similar set of promoters in human islet and liver cells, however upon stimulation with cAMP agonists islets and hepatocytes display highly divergent patterns of active, phosphorylated CREB occupancy (Zhang et al. 2005). The mechanisms underlying differential occupancy of active but not inactive CREB across tissues, however are unknown. An intriguing possibility is that transcription factors such as CLOCK/BMAL1, which are recruited to tissue-specific sites and known to interact with CREB-related coactivators p300/CBP and P-CAF, may establish a permissive environment for CREB-induced transcription. Lastly, information gained by genomic mapping can be used to interrogate how circadian dysregulation and heritable genetic sequence variants associated with human diabetes contribute to disease risk. The discovery of variants mapping to *CRY2* and *MTNR1B* raises the possibility that there may be subpopulations of individuals with increased metabolic vulnerability to circadian disruption. In the future it will be important to identify the genome-wide sites of circadian transcription factor binding in human islets to determine if the clock regulates enhancers containing disease-associated variants and if the sequence variants impact circadian transcription factor binding and rhythmic gene transcription. Such studies would help to explain how alterations in the level or timing of circadian transcription factor activity might adversely affect β-cell health and predispose individuals to diabetes.

Collectively, genetic analyses and physiologic studies indicate that the cell-autonomous circadian clock within pancreatic β-cells is a key regulator of mammalian glucose homeostasis, and its dysregulation is strongly associated with human diabetes. Recent drug screening studies have identified small molecule drugs that enhance circadian clock function and protect mice from metabolic syndrome (He et al. 2016; Bass 2016), raising the possibility that the circadian system may ultimately represent a therapeutic target to enhance β-cell health and function.

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Appendix

Supplementary figures: S1-11

Supplementary tables: S1-2

Supplementary figures

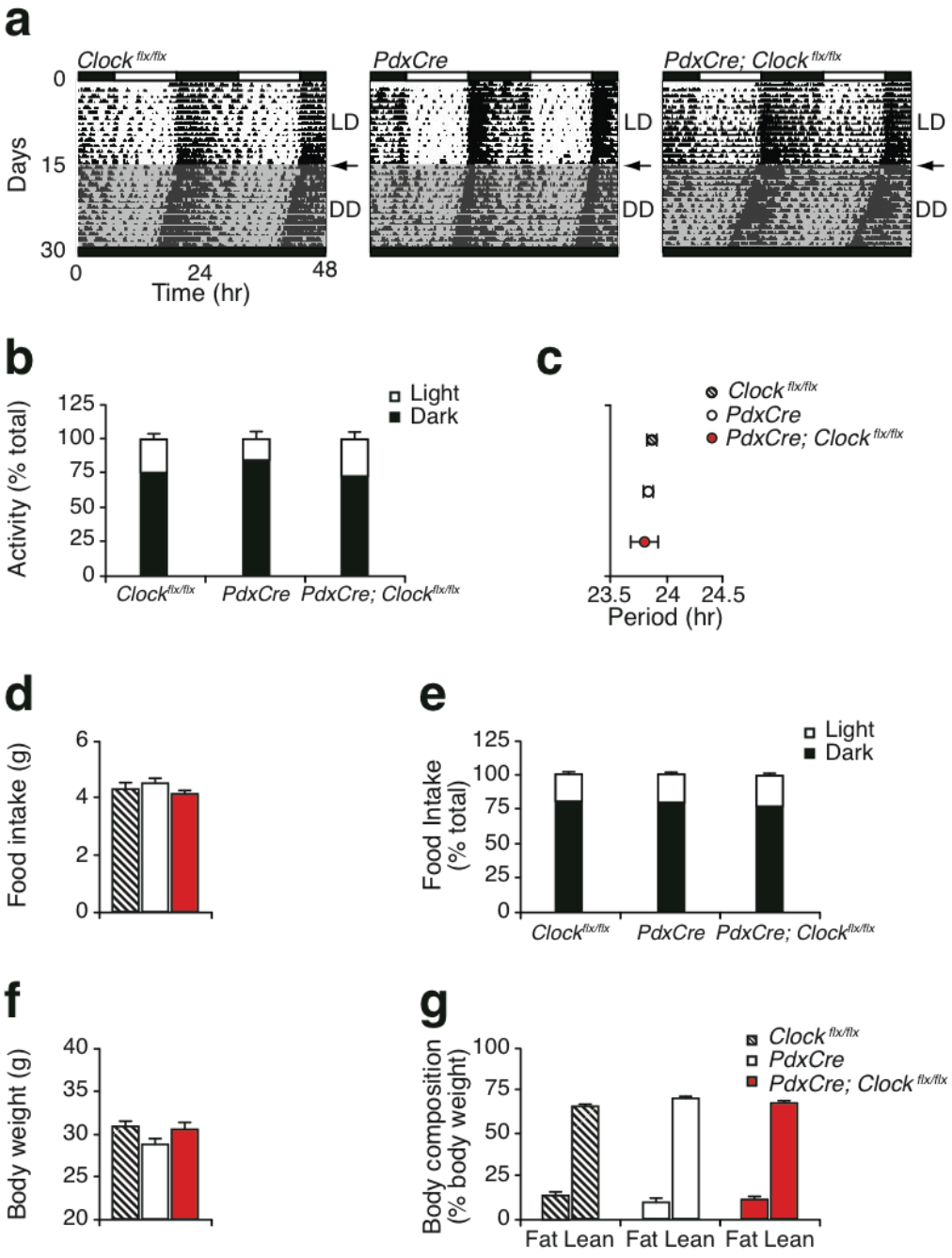

Fig. S1: Disruption of pancreatic *Clock* gene does not influence activity and feeding rhythms. (a) Actograms showing locomotor activity over a 30 day period in

representative *Clock*$^{flx/flx}$, *PdxCre*, and *PdxCre;Clock*$^{flx/flx}$ mice. Arrow denotes switch from 12:12 LD to constant darkness (DD). **(b)** Diurnal rhythm of locomotor activity and **(c)** period of activity in DD, calculated using Chi-square periodogram for days 18-23 (n=3). **(d)** Total daily food intake and **(e)** percentage of feeding during either the light or dark period (n=8). **(f)** Body weight and **(g)** body composition in pancreas-specific *Clock* KO mice and littermate controls (n=15-17). Data were analyzed by Student's *t*-test. All values represent mean ± SEM.

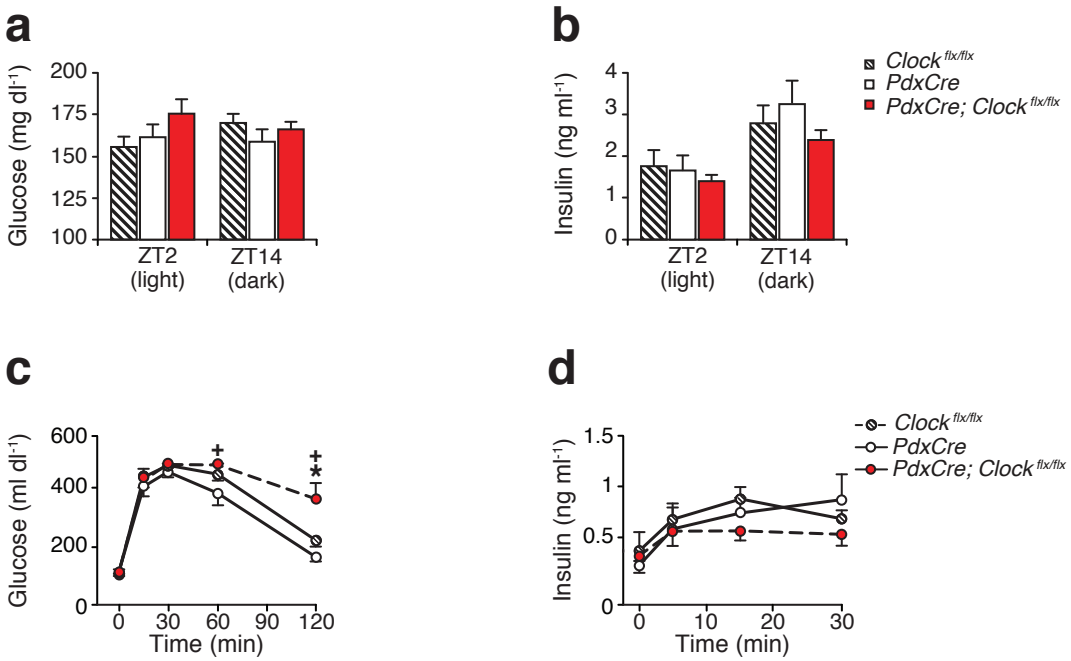

Fig. S2: Pancreatic *Clock* gene required for glucose homeostasis. (a) Blood glucose and **(b)** insulin levels in *ad libitum* fed mice at ZT2 and ZT14 (n=10-13). **(c)** Glucose and **(d)** insulin levels at the indicated time points following an intraperitoneal injection of glucose (2 or 3 g/kg body weight, respectively) in pancreas-specific *Clock* KO mice and littermate controls at ZT2 (n=4).

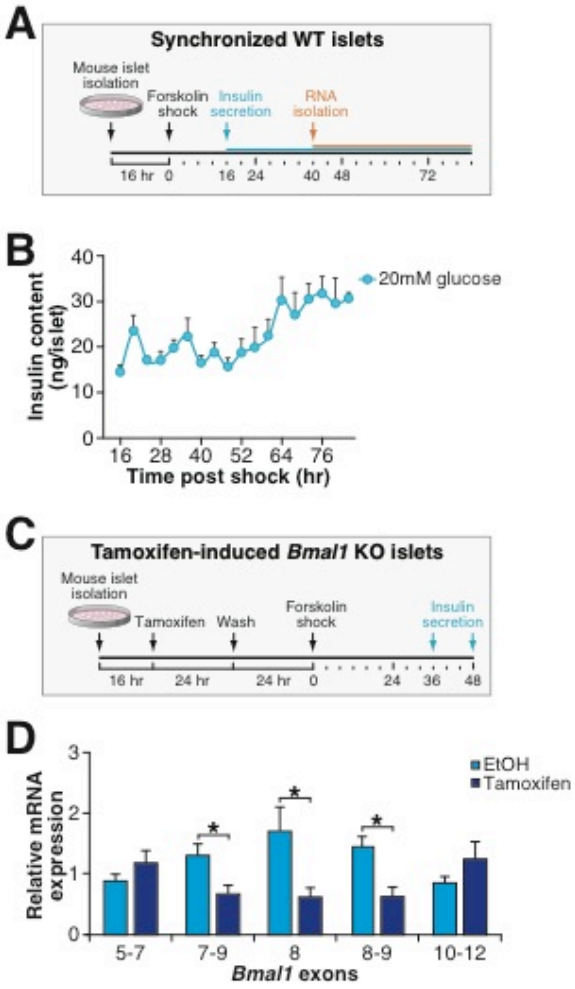

Fig. S3. Islet cell autonomous circadian clock controls rhythmic insulin secretion. **(A)** Schematic of *ex vivo* experimental design for insulin secretion assays in forskolin-synchronized mouse islets. **(B)** Average intracellular insulin content in WT islets stimulated with glucose at indicated time points following forskolin treatment (n=4 islet pools per time point, 3 replicates per islet pool). **(C)** Schematic of *ex vivo* tamoxifen-

induced *Bmal1* ablation and insulin secretion in islets isolated from *PdxCreER;Bmal1*$^{flx/flx}$ mice. **(D)** Excision of exon 8 of the *Bmal1* gene in islets from *PdxCreER;Bmal1*$^{flx/flx}$ mice after *in vitro* tamoxifen treatment as assessed by real time PCR using primers specific to indicated exons (n=4 samples per condition). All values represent mean ± SEM. *$p<0.05$.

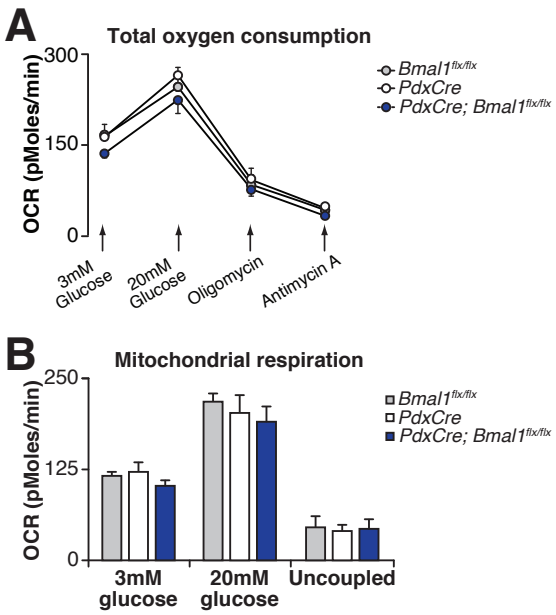

Fig. S4. Impaired glucose-stimulated insulin secretion in circadian mutant islets is independent of mitochondrial respiration. (A) Total oxygen consumption rates (OCR) and **(B)** mitochondrial respiration in islets in the presence of glucose, oligomycin (an ATP synthase inhibitor which inhibits mitochondrial respiration, enabling measurement of uncoupled respiration), and antimycin A (a mitochondrial toxin which enables measurement of non-mitochondrial respiration). Mitochondrial respiration is calculated by subtracting OCR value in the antimycin A condition from basal, glucose- and oligomycin-stimulated islets (n=3-4 mice per genotype, 4 replicates per mouse). All values represent mean ± SEM.

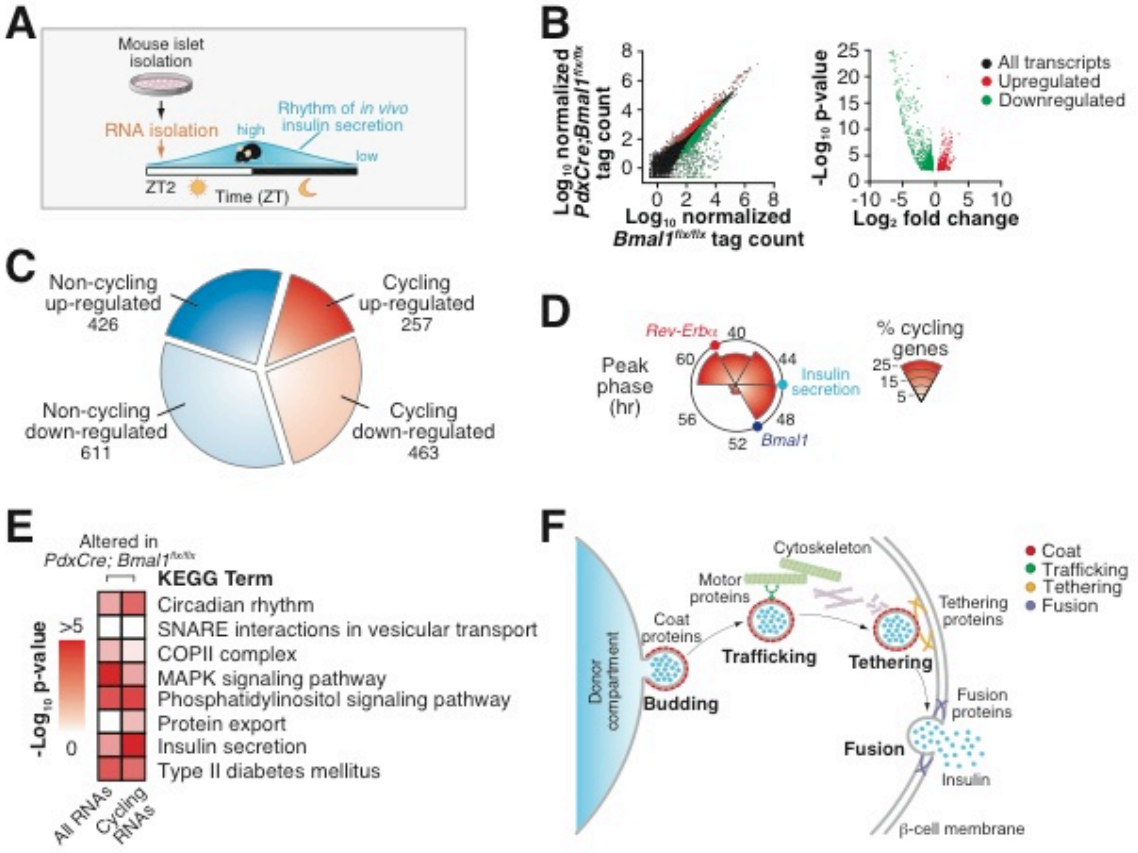

Fig. S5. Circadian control of secretory gene expression is dependent on the pancreatic clock. (A) Schematic showing timing of *PdxCre;Bmal1*$^{flx/flx}$ islet isolation (ZT2) for RNA-seq in relation to endogenous diurnal patterns of *in vivo* insulin secretion. **(B)** Scatterplot showing RNA expression levels in *PdxCre;Bmal1*$^{flx/flx}$ and control *Bmal1*$^{flx/flx}$ islets and volcano plot comparing FDR-adjusted p-values and fold-change among significantly differentially expressed genes (FDR-adjusted p<0.05). Up-regulated genes are shown in red and down-regulated genes are in green. **(C)** Breakdown of up- and down-regulated genes in *PdxCre;Bmal1*$^{flx/flx}$ islets and overlap with cycling genes

identified in synchronized WT islets. **(D)** Peak phase distribution of all cycling genes with reference to timing of maximal glucose-stimulated insulin secretion and *Bmal1* and *Rev-erbα* expression. **(E)** Enrichment of KEGG terms among all RNA and cycling genes differentially expressed in *PdxCre;Bmal1*$^{flx/flx}$ islets. **(F)** Model of basic vesicular transport pathway depicting proteins involved in i) vesicle budding from the donor membrane, ii) trafficking along cytoskeletal filaments, iii) tethering to the target membrane, and iv) fusion with the target membrane.

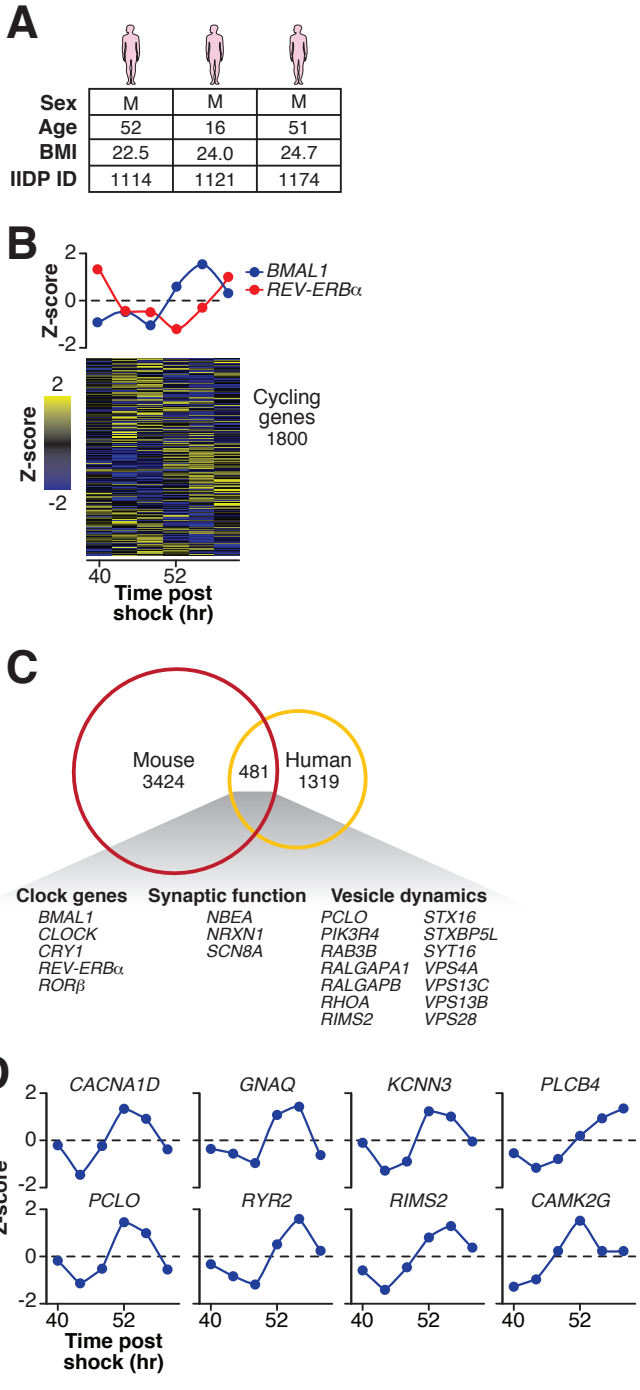
A
Sex M M M
Age 52 16 51
BMI 22.5 24.0 24.7
IIDP ID 1114 1121 1174
B
Z-score
2
0
-2
BMAL1
REV-ERBα
Cycling genes 1800
40 52
Time post shock (hr)
C
Mouse 3424
481
Human 1319
Clock genes
BMAL1
CLOCK
CRY1
REV-ERBα
RORβ
Synaptic function
NBEA
NRXN1
SCN8A
Vesicle dynamics
PCLO
PIK3R4
RAB3B
RALGAPA1
RALGAPB
RHOA
RIMS2
STX16
STXBP5L
SYT16
VPS4A
VPS13C
VPS13B
VPS28
D
CACNA1D
GNAQ
KCNN3
PLCB4
PCLO
RYR2
RIMS2
CAMK2G
Z-score
Time post shock (hr)

Fig. S6. The circadian transcriptome is conserved in human islets. **(A)** Summary of human islet donor information. **(B)** RNA expression of *BMAL1* and *REV-ERBα* (top) and heatmap showing expression patterns of all cycling RNAs in human islets identified by eJTK_CYCLE analysis (bottom) (Bonferroni corrected p <0.05). **(C)** Overlap between cycling RNAs identified in mouse and human islets, highlighting the significant enrichment in shared genes involved in synaptic and vesicle signaling. **(D)** Expression profiles for cycling genes mapping to the "Insulin Secretion" KEGG pathway in Fig 1F. Average z-score values from the 3 donors are shown.

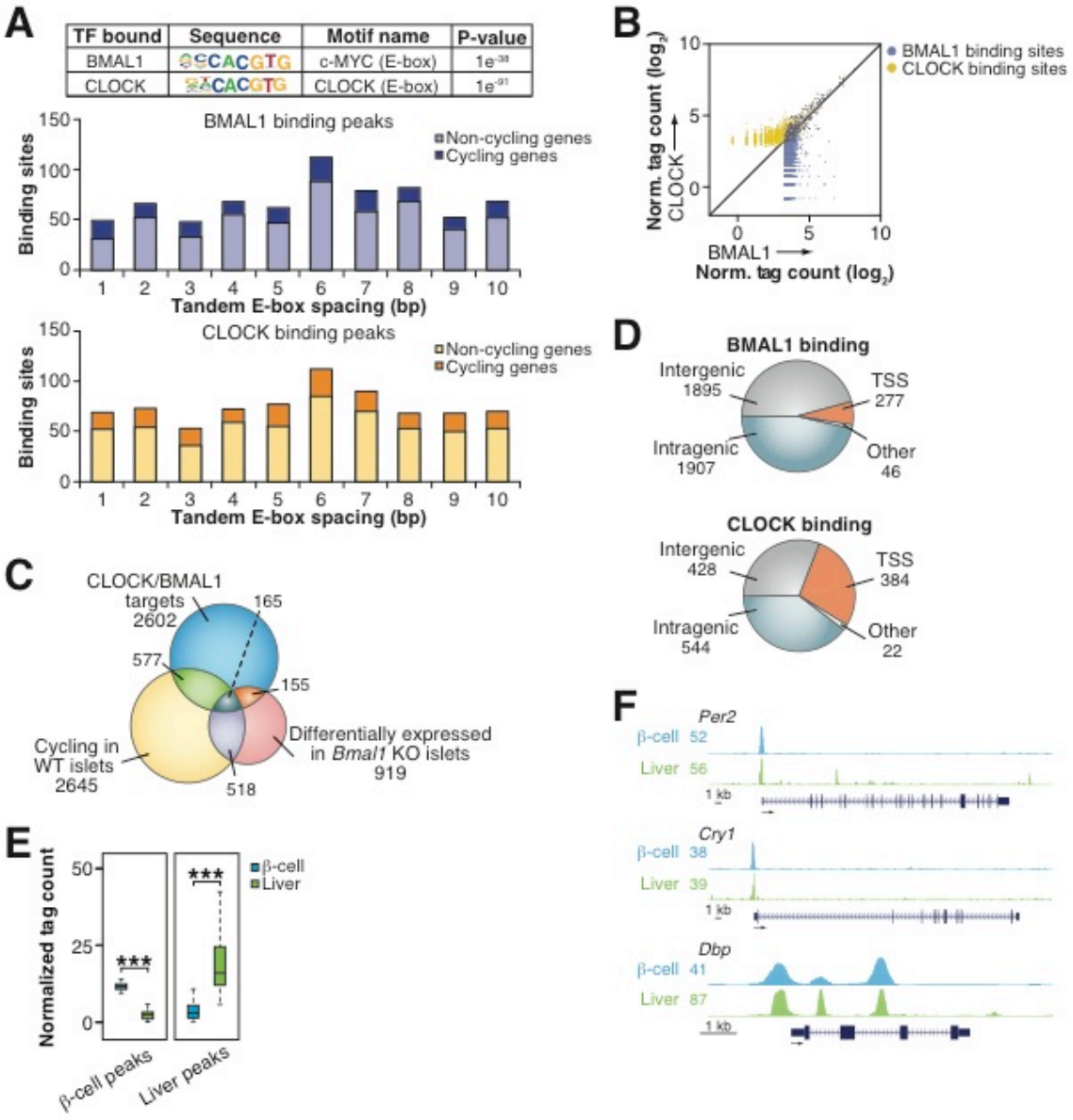

Fig. S7. BMAL1 and CLOCK bind distinct enhancer regulatory regions genome-wide in β-cells compared to liver. (A) Top known HOMER motifs enriched at BMAL1

and CLOCK binding sites from Chip-seq analysis in β-cells (top panel). Number of tandem CACGTG E-boxes allowing for 2 nucleotide mismatches within BMAL1 and CLOCK peaks at non-cycling and cycling genes. Column numbering corresponds with number of nucleotides separating sequential E-box motifs (bottom panels). **(B)** Scatter plot showing BMAL1 (x-axis) and CLOCK (y-axis) binding as $\log_2$ normalized tag count within 500bp windows surrounding BMAL1 peaks (blue) and CLOCK peaks (yellow) normalized per 10 million tags. **(C)** Venn diagram showing overlap of CLOCK/BMAL1 targets in BetaTC6 cells, cycling RNAs in wild type islets, and genes that are differentially expressed genes in *PdxCre;Bmal1*$^{flx/flx}$ compared to *Bmal1*$^{flx/flx}$ controls. **(D)** Distribution of genomic annotations of BMAL1 and CLOCK peaks from Chip-seq in β-cells. **(E)** Box and whiskers plots (whiskers represent IQR 1.5) comparing BMAL1 ChIP-seq tags normalized per 10 million tags in β-cells and liver at loci corresponding to 500bp windows surrounding BMAL1 peaks identified in either β-cells or in liver. **(F)** UCSC genome browser tracks at *Per2*, *Cry1*, and *Dbp* loci in β-cells and liver show comparable tag density in both liver and β-cells at core clock loci. Maximum BMAL1 track heights within viewable window are indicated to the right of tissue. ***p <0.0001 by Mann-Whitney non-parametric, unpaired *t*-test.

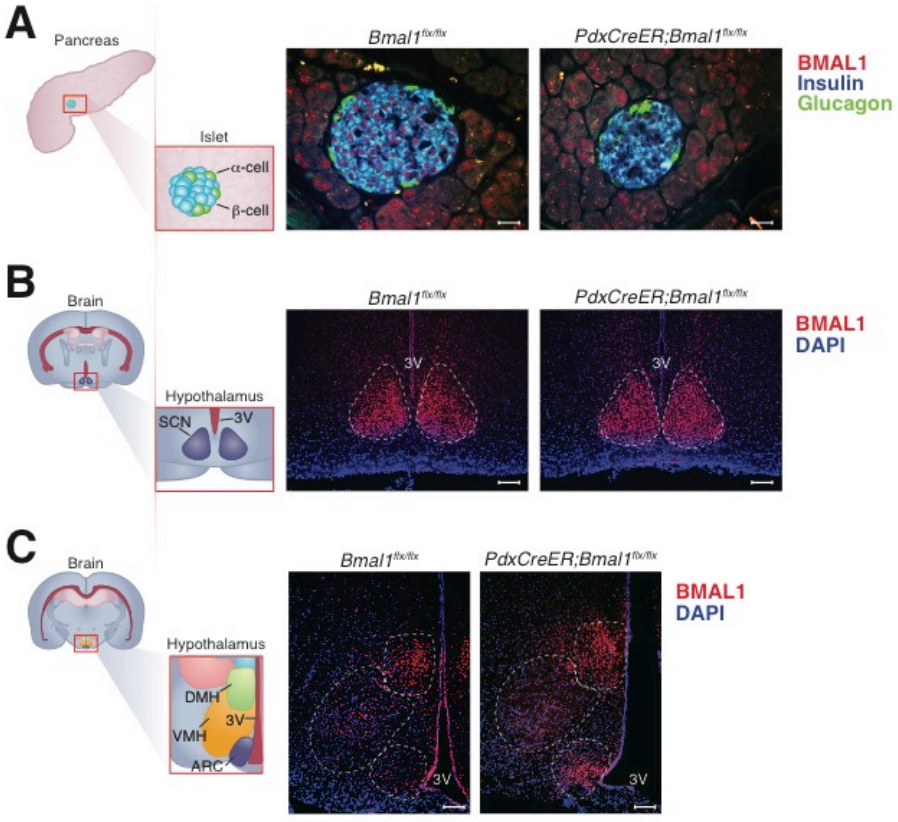

Fig. S8. Tamoxifen-induced adult-life *Bmal1* deletion is limited to pancreatic β-cells. **(A)** Immunofluorescent staining of BMAL1 (red), insulin (blue), and glucagon (green) in *PdxCreER;Bmal1*$^{flx/flx}$ and control islets. Scale bars, 25μm. Immunofluorescent staining of BMAL1 (red) and DAPI (blue) in **(B)** suprachiasmatic nucleus and **(C)** feeding centers in the hypothalamus of *PdxCreER;Bmal1*$^{flx/flx}$ and *Bmal1*$^{flx/flx}$ mice. SCN, suprachiasmatic

nucleus. ARC, arcuate nucleus. DMH, dorsomedial hypothalamic nucleus. VMH, ventromedial hypothalamic nucleus. V3, third ventricle. Scale bars, 50μm.

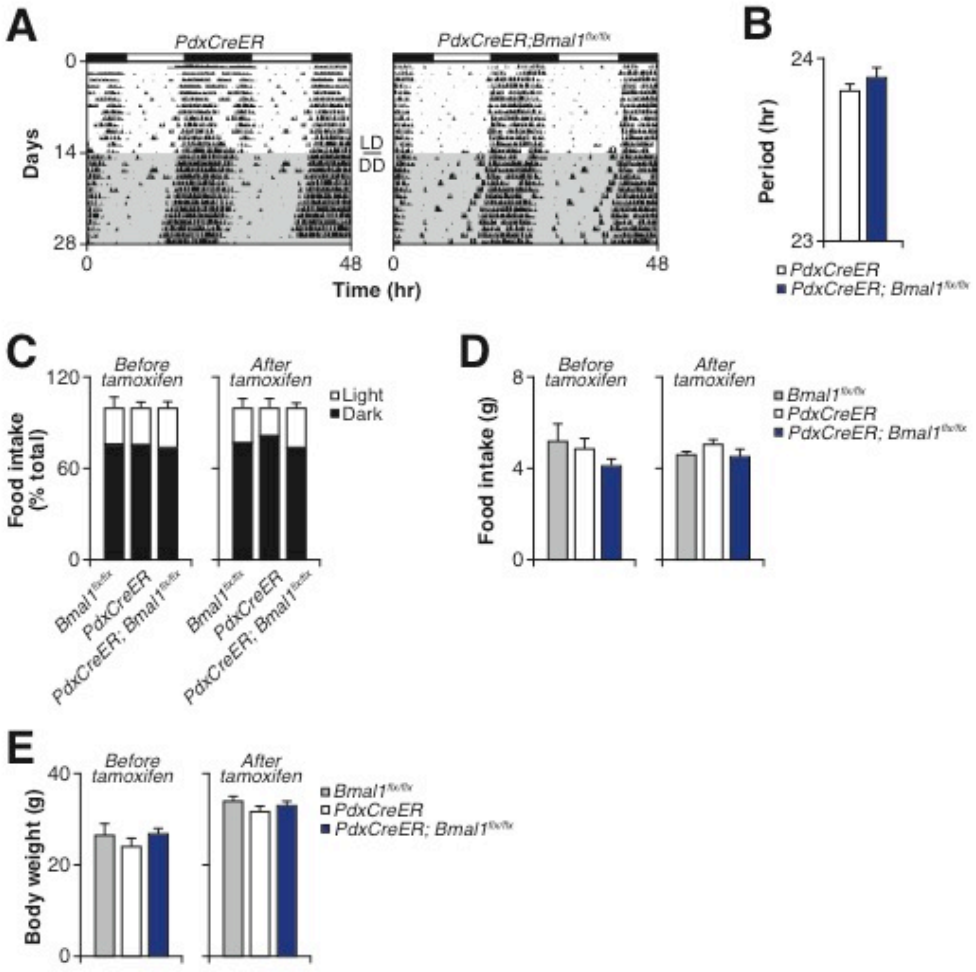

Fig. S9. Adult-life pancreatic β-cell-specific loss of BMAL1 does not impact behavior, feeding, or body weight. **(A)** Representative actograms showing locomotor activity over a 28 day period in individually-housed *PdxCreER* and *PdxCreER;Bmal1*$^{flx/flx}$ mice post-tamoxifen treatment. **(B)** Period of activity in total darkness (DD), calculated using Chi-

square periodogram for days 7-14 in DD (n=4-5 mice per genotype). **(C)** Food intake (% total) during either the light or dark periods and **(D)** total food intake (g) in *PdxCreER;Bmal1*$^{flx/flx}$ and littermate control mice before and after tamoxifen treatment (n=3-5 mice per genotype). **(E)** Body weight in *PdxCreER;Bmal1*$^{flx/flx}$ and littermate control mice before and after tamoxifen treatment (n=7-10 mice per genotype). All values represent mean ± SEM.

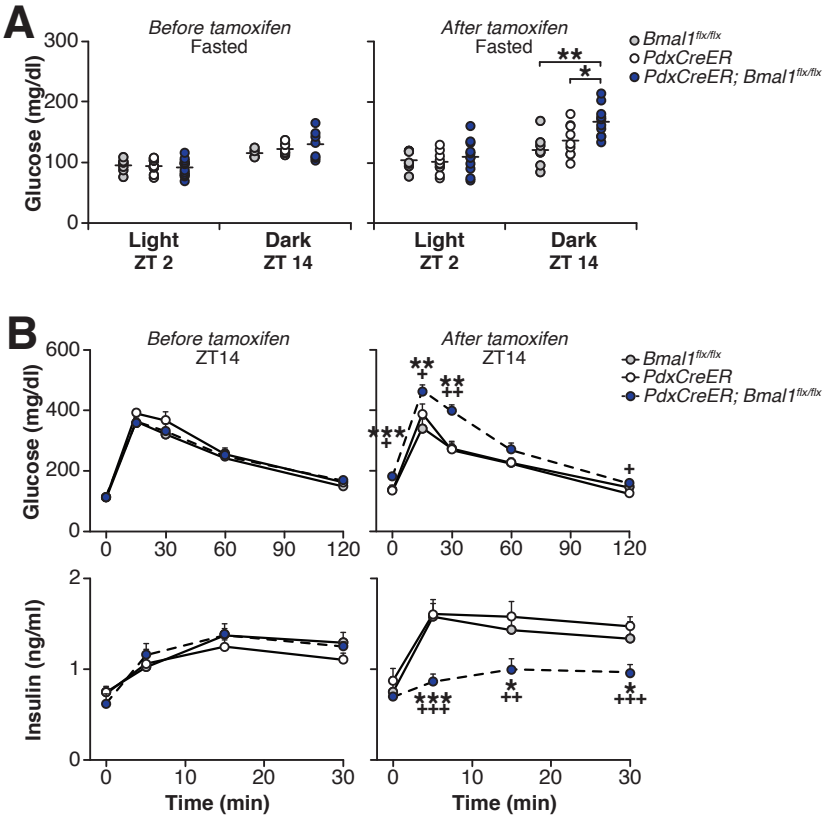

Fig. S10. Acute *Bmal1* deletion in adult β-cells impairs glucose homeostasis. (A) Fasting glucose in *PdxCreER;Bmal1*$^{flx/flx}$ and littermate control mice before and after tamoxifen treatment (n=7-11 mice per genotype). **(B)** Glucose tolerance and insulin secretion at ZT14 following intraperitoneal glucose administration of 2 and 3g/kg body weight, respectively, in *PdxCreER;Bmal1*$^{flx/flx}$ mice and littermate controls before and after tamoxifen treatment (n=4-10 mice per genotype). *p<0.05, **p<0.01, ***p<0.001.

For B asterisks denote significance between *Bmal1*$^{flx/flx}$ and *PdxCreER;Bmal1*$^{flx/flx}$, and plus symbols denote significance between *PdxCreER* and *PdxCreER;Bmal1*$^{flx/flx}$. All values represent mean ± SEM.

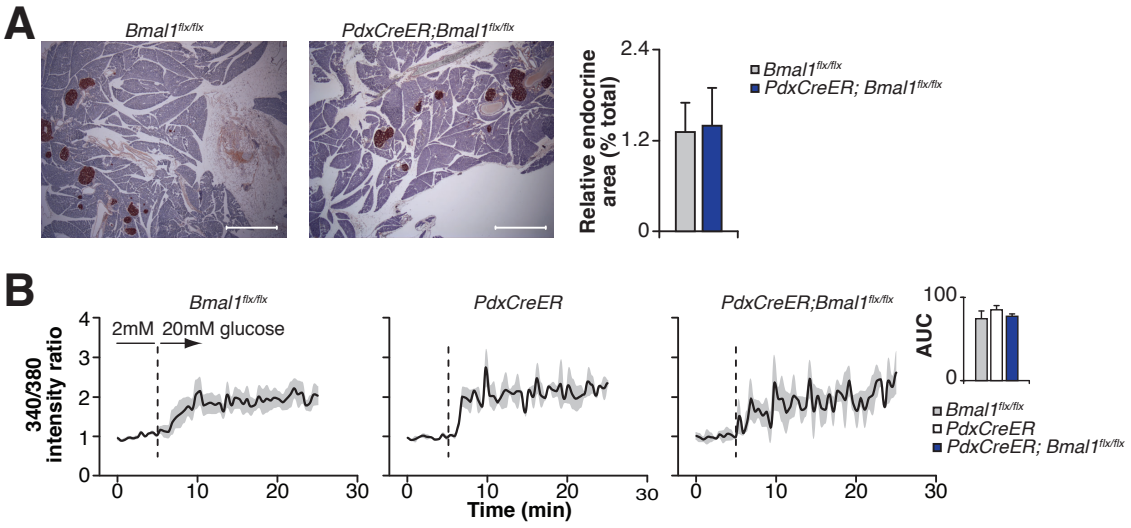

Fig S11. Islet mass and glucose-stimulated calcium influx are normal in adult-life *Bmal1* knockout islet cells. **(A)** Morphometric analysis of insulin-positive area in the pancreas of *PdxCreER;Bmal1*$^{flx/flx}$ and *Bmal1*$^{flx/flx}$ control mice (n=3 mice per genotype). Scale bars, 1000μm. **(B)** Ratiometric determination of intracellular Ca^{2+} using Fura2-AM dye in islets isolated from *PdxCreER*, *Bmal1*$^{flx/flx}$, and *PdxCreER;Bmal1*$^{flx/flx}$ mice following *ex vivo* challenge with 20mM glucose, where the dashed line indicates the time when glucose was injected (left) and area under the curve (right) (n=2-4 mice per genotype). All values represent mean ± SEM.

Table S1: KEGG pathways

Cycling in WT Islets

KEGG Term	-log$_{10}$ P-value	Genes in Term	Target Genes in Term	Fraction of Targets	Gene Symbol
Circadian rhythm	14.2992954	30	17	0.013832384	*Nr1d1, Prkaa2, Clock, Rorc, Rbx1, Npas2, Creb1, Rorb, Fbxw11, Per2, Rora, Per3, Prkag2, Arntl, Btrc, Prkaa1, Cry1*
Insulin secretion	10.86222887	86	22	0.017900732	*Cacna1c, Atf2, Chrm3, Prkacb, Slc2a2, Atf4, Camk2g, Prkcb, Prkx, Plcb1, Prkca, Cacna1d, Kcnn4, Gnaq, Creb1, Creb3, Plcb4, Pclo, Kcnn3, Atp1b2, Rims2, Stx1a*
SNARE interactions in vesicular transport	10.86222887	33	16	0.013018714	*Sec22b, Gosr1, Vti1b, Vamp8, Vamp5, Stx8, Ykt6, Vamp1, Bnip1, Vti1a, Vamp4, Stx17, Use1, Stx16, Stx4a, Stx1a*
COPII complex	7.449579942	11	7	0.005695688	*Sar1b, Sar1a, Sec31b, Sec24b, Sec31a, Sec24a, Sec13*
Phosphatidylinositol signaling system	6.130040566	81	24	0.019528072	*Calm1, Synj2, Plcd1, Dgke, Pikfyve, Itpr2, Inpp5k, Inpp5e, Prkcb, Plcb1, Dgkh, Plcg1, Prkca, Calm2, Pi4ka, Impa2, Ocrl, Inpp4a, Pip5k1b, Itpr1, Plcb4, Plcd3, Pik3r3, Synj1*
MAPK signaling pathway	5.435454827	253	58	0.04719284	*Rps6ka4, Il1r1, Atf4, Map2k5, Pdgfa, Prkx, Prkacb, Taok1, Cacna1a, Rapgef2, Ikbkb, Cacna1d, Nr4a1, Mapk8ip3, Map3k5, Nlk, Nfkb2, Dusp10, Rap1a, Gadd45b, Mapk10, Map2k3, Stmn1, Rap1b, Dusp4, Traf6, Sos1, Mapkapk2, Taok3, Rps6ka5, Hspa8, Map2k1, Cacna1c, Atf2, Elk4, Braf, Ppm1b, Pla2g4b, Prkcb, Mapk11, Prkca, Hspb1, Mapkapk5, Elk1, Ngf, Map3k3, Cacna2d1, Tnfrsf1a, Raf1, Cacna1b, Crkl, Rps6ka3, Sos2, Mknk1, Dusp3, Mapk9, Nf1, Nfatc3*
Protein export	4.23117336	25	9	0.007323027	*Sec11c, Spcs2, Hspa5, Immp2l, Sec61b, Srp9, Spcs1, Srp14, Sec11a*
Type II diabetes mellitus	3.580764239	50	14	0.011391375	*Cacna1c, Mapk10, Cacna1b, Slc2a2, Mtor, Insr, Cacna1a, Ikbkb, Cacna1d, Pik3r3, Irs1, Mapk9, Socs4, Prkce*

Table S1 continued

Differentially Expressed in *PdxCre;Bmal1*$^{flx/flx}$ Islets

KEGG Term	-$\log_{10}$ P-value	Genes in Term	Target Genes in Term	Fraction of Targets	Gene Symbol
MAPK signaling pathway	4.105230153	253	26	0.052631579	*Dusp6, Cacna1c, Elk4, Cd14, Braf, Atf4, Myc, Prkcb, Taok1, Cacna1a, Gadd45g, Chuk, Hspb1, Rasa2, Cacna2d3, Tgfbr1, Map3k2, Cacna2d1, Cacna1h, Cacna2d2, Cacna1b, Akt3, Rras2, Pdgfra, Nf1, Jund*
Type II diabetes mellitus	3.580764239	50	7	0.01417004	*Cacna1a, Cacna1c, Hkdc1, Socs2, Pklr, Cacna1b, Socs4*
Phosphatidylinositol signaling system	3.205302246	81	10	0.020242915	*Pikfyve, Prkcb, Plcb4, Itpr1, Plcb3, Itpk1, Pik3c2a, Synj11a, Dgkh, Dgkb*
Insulin secretion	2.164694745	86	9	0.018218623	*Cacna1c, Atf4, Prkcb, Plcb4, Plcb3, Pclo, Kcnn4, Atp1b3, Rims2c13*
Circadian rhythm	2.009822141	30	4	0.008097166	*Prkab2, Clock, Per2, Per3*
COPII complex	1.817835746	11	2	0.004048583	*Sec23a, Sec24a*
Protein export	0.691657973	25	2	0.004048583	*Sec11c, Srp9*
SNARE interactions in vesicular transport	4.31E-12	33	0	0	0

Table S1 continued

BMAL1 Targets Non-Cycling

KEGG Term	-log$_{10}$ P-value	Genes in Term	Target Genes in Term	Fraction of Targets	Gene Symbol
Rap1 signaling pathway	12.44593154	214	34	0.065637066	*Pdgfc, Sipa1l1, Adcy2, Plce1, Pard6b, Magi2, Bcar1, Rapgef1, Igf1r, Efna5, Akt3, P2ry1, Adcy8, Kit, Gnai1, Fgfr2, Kitl, Src, Pard6a, Vegfc, Pard6g, Itgb1, Hgf, Sipa1l2, Fgf14, Kdr, Ins1, Prkd1, Tek, Magi3, Rac1, Gnao1, Mapk1, Nras*
MAPK signaling pathway	8.881669516	253	34	0.065637066	*Cd14, Gadd45g, Ecsit, Map3k7, Mapk8, Rps6ka2, Cacng4, Dusp1, Cacna2d3, Rps6ka1, Map3k14, Jun, Akt3, Ppm1a, Rasgrf2, Tgfb2, Flnb, Fgfr2, Nfatc1, Dusp6, Ppp3ca, Casp3, Myc, Mef2c, Fgf14, Nfkb1, Rac1, Tgfbr1, Mapk1, Pla2g4a, Nras, Dusp5, Grb2, Cacnb3*
Insulin secretion	7.184122363	86	15	0.028957529	*Gcg, Atp1b1, Rab3a, Cckar, Camk2d, Kcnma1, Kcnn2, Creb3l2, Ins1, Abcc8, Adcy2, Adcy8, Pdx1, Atp1a1, Atp1a3*
Phosphatidylinositol signaling system	2.936660459	81	10	0.019305019	*Cds1, Dgkb, Dgkg, Inpp4b, Pi4kb, Pi4k2a, Plce1, Itpk1, Pten, Inpp5j*
Circadian rhythm	2.927736192	30	5	0.00965251	*Cry2, Cul1, Bhlhe40, Bhlhe41, Per1*
Type II diabetes mellitus	2.031842078	50	6	0.011583012	*Mapk1, Mapk8, Hk3, Ins1, Abcc8, Pdx1*
Protein export	1.386777127	25	3	0.005791506	*Sec63, Spcs3, Sec62*
SNARE interactions in vesicular transport	0.387042009	33	2	0.003861004	*Vamp3, Stx18*
COPII complex	1.38097E-11	11	0	0	0

Table S1 continued

CLOCK Targets Non-Cycling

KEGG Term	-log$_{10}$ P-value	Genes in Term	Target Genes in Term	Fraction of Targets	Gene Symbol
Rap1 signaling pathway	12.87814504	214	23	0.085185185	*Pard6a, Vegfc, Pard6g, Itgb1, Cdc42, Hgf, Sipa1l2, Dock4, Prkd1, Adcy2, Magi2, Mapk1, Bcar1, Pard6b, Rapgef1, Efna5, Akt2, Gnai3, Ngfr, F2rl3, Vegfb, Kit, Gnai1*
MAPK signaling pathway	7.105451517	253	20	0.074074074	*Dusp7, Dusp6, Cdc42, Map3k4, Map3k7, Tab2, Max, Mapk8, Dusp1, Mecom, Akt2, Gng12, Mapk1, Rps6ka1, Jun, Map4k2, Dusp5, Mknk2, Tgfb2, Ppp3ca*
Circadian rhythm	5.503601854	30	5	0.018518519	*Cry2, Fbxl3, Bhlhe40, Bhlhe41, Per1*
Insulin secretion	2.396637964	86	6	0.022222222	*Abcc8, Adcy2, Kcnj11, Camk2d, Kcnma1, Atp1b3*
Type II diabetes mellitus	2.241662226	50	4	0.014814815	*Mapk1, Mapk8, Abcc8, Kcnj11*
Phosphatidylinositol signaling system	0.567315545	81	3	0.011111111	*Dgkg, Inpp4b, Pten*
SNARE interactions in vesicular transport	0.34973543	33	1	0.003703704	*Gosr2*
COPII complex	1.40878E-11	11	0	0	0
Protein export	1.1483E-11	25	0	0	0

Table S1 continued

BMAL1 Targets Cycling

KEGG Term	-log$_{10}$ P-value	Genes in Term	Target Genes in Term	Fraction of Targets	Gene Symbol
Circadian rhythm	10.2035023	30	6	0.037037037	*Nr1d1, Prkag2, Prkaa2, Rbx1, Arntl, Clock*
Insulin secretion	4.527557	86	6	0.037037037	*Pclo, Chrm3, Slc2a2, Camk2g, Rims2, Gnaq*
Rap1 signaling pathway	3.138964233	214	9	0.055555556	*Rapgef6, Ngf, Magi1, Skap1, Insr, Pard3, Cnr1, Adora2b, Gnaq*
Type II diabetes mellitus	2.361287911	5	3	0.018518519	*Irs1, Slc2a2, Insr*
MAPK signaling pathway	2.306955066	253	9	0.055555556	*Ngf, Il1r1, Dusp4, Ppm1b, Mknk1, Taok1, Rps6ka3, Rps6ka5, Nfkb2*
Protein export	2.285014334	25	2	0.012345679	*Sec61b, Immp2l*
SNARE interactions in vesicular transport	1.829545138	33	2	0.012345679	*Stx17, Stx16*
COPII complex	1.537746457	11	1	0.00617284	*Sec31a*
Phosphatidylinositol signaling system	1.355737038	81	3	0.018518519	*Pikfyve, Pip5k1b, Itpr2*

Table S1 continued

CLOCK Targets Cycling

KEGG Term	-log$_{10}$ P-value	Genes in Term	Target Genes in Term	Fraction of Targets	Gene Symbol
Circadian rhythm	11.92035309	30	6	0.037037037	*Nr1d1, Rorc, Arntl, Per2, Cry1, Per3*
MAPK signaling pathway	4.899304791	253	10	0.055555556	*Cacna1c, Cacna2d1, Stmn1, Rps6ka4, Dusp4, Mknk1, Cacna1a, Prkca, Hspb1, Nfatc3*
COPII complex	4.353710244	11	2	0.00617284	*Sec31a, Sec24a*
Insulin secretion	1.828132526	86	3	0.037037037	*Cacna1c, Prkca, Chrm3*
Type II diabetes mellitus	1.649314642	50	2	0.018518519	*Cacna1a, Cacna1c*
Rap1 signaling pathway	1.339863213	214	5	0.055555556	*Prkca, Cnr1, Vegfa, Calm2, Pfn2*
Phosphatidylinositol signaling system	0.977936239	81	2	0.018518519	*Prkca, Calm2*
SNARE interactions in vesicular transport	0.878529011	33	1	0.012345679	*Stx16*
Protein export	1.66591E-11	25	0	0	0

Table S2: Expression of metabolic and developmental β-cell genes

Signature β-Cell Genes	Cycling	Differentially Expressed in *Bmal1* KO at ZT2	BMAL1 Target
Glut2	Yes	No	Yes
Gck	No	No	No
Kcnj11	No	No	No
Abcc8	No	No	Yes
Pcsk1	No	No	No
Glp1r	No	No	No
Ins1	No	No	Yes
Ins2	No	No	No

Key Transcription Factors	Cycling	Differentially Expressed in *Bmal1* KO at ZT2	BMAL1 Target
Pdx1	No	No	Yes
Nkx2.2	No	No	No
Pax6	No	No	No
NeuroD1	No	No	No
MafA	No	No	No
Ngn3	No	No	No
Pax4	No	No	No

"Disallowed" Genes	Cycling	Differentially Expressed in *Bmal1* KO at ZT2	BMAL1 Target
Ldha	Yes	No	No
Slc61a1	No	No	No
Pdgfra	No	2.3 fold increase	No
Cxcl12	No	No	Yes
Maf	No	0.3 fold increase	Yes
Lmo4	No	1.8 fold increase	No
Hk1	No	No	No