Clock genes, pancreatic function and diabetes

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ABSTRACT

Circadian physiology is responsible for the temporal regulation of metabolism to optimize energy homeostasis throughout the day. Disturbances in the light-dark cycle, sleep/wake schedule or feeding/activity behavior can affect the circadian function of the clocks located in the brain and peripheral tissues. These alterations have been associated with impaired glucose tolerance and type 2 diabetes. Animal models with molecular manipulation of clock genes and genetic studies in humans also support these links. It has been demonstrated that the endocrine pancreas has an intrinsic self-sustained clock, and recent studies have revealed an important role of clock genes in pancreatic beta-cells, glucose homeostasis and diabetes.

Keywords: Clock genes, diabetes, pancreas, beta-cell, insulin.

Type 2 diabetes and the circadian rhythm

Type 2 diabetes has become one of the most important health problems of modern society, affecting millions of people worldwide. Obesity and excessive weight gain play a major role in the onset of diabetes, along with other risk factors including genetics and lack of physical activity. In recent years, circadian disturbances have also been identified as contributors to this metabolic disease. Alterations in circadian rhythm derived from our current lifestyle, such as mistimed sleeping, shift-working, or eating at abnormal night time hours, have been related to type 2 diabetes, obesity and metabolic syndrome [1-3]. The discovery of these associations opens new possibilities for the design of novel treatments for these metabolic disorders based on the appropriate alignment or resetting of the circadian system [4-6]. In the case of type 2 diabetes, its association to circadian disruption was originally related to an altered function of the central clock located in the hypothalamus. However, recent findings show that this disease can also be attributed to an impaired function of the endocrine pancreatic clock [7-10]. Thus, the study of the pancreatic clock biology is important not only to understand circadian rhythms and their relationship with metabolism, but also to develop tools for the prevention and therapy of metabolic diseases such as type 2 diabetes.

Central and peripheral clocks

In mammals, the central pacemaker is located in the suprachiasmatic nuclei (SCN) in the hypothalamus. It is controlled by a transcriptional/translational autoregulatory feedback loop involving a set of clock genes (Figure 1). The core clock is composed of the transcription factors circadian locomotor output cycles kaput (CLOCK) and brain and muscle Arnt-like protein-1 (BMAL1), which bind to E-box

enhancers in the *period* (*Per*) and *cryptochrome* (*Cry*) promoters, producing PER and CRY expression [11,12]. PER and CRY proteins interact to form a complex that translocates to the nucleus and inhibits its own CLOCK:BMAL1-induced transcription. Phosphorylation of both proteins can also trigger their degradation by 26S proteasomes. The turnover of PER and CRY allows this cycle to continue. This clock network forms a self-sustained feed-back mechanism with an oscillatory pattern of approximately 24 hours (Figure 1). Several proteins encoded by clock genes regulate the expression of down-stream targets that further regulate the clock machinery, function as clock-controlled output genes relaying the clock information to down-stream proteins, or function in cellular processes such as metabolism. Another regulatory loop is composed by two nuclear receptor genes, which also possess an E-box enhancer activated by the CLOCK:BMAL1 heterodimer. REV-ERB ALPHA, so named because it is encoded by the reverse strand of the *c-erb-A* oncogene, can negatively regulate BMAL1 expression. In contrast, the retinoic-acid receptor-related orphan receptor alpha (ROR ALPHA) exerts a positive modulation of BMAL1 (Figure 1). Although the main input signal for this oscillator is the light/dark cycle, the SCN can receive additional information about nutrients and hormones as well [12]. It is also connected to a complex neural network with projections to different brain centers that regulate temperature, sleep, feeding, hormonal secretion and glucose homeostasis (Box1).

In addition to the central clock in the brain, peripheral clocks with similar molecular components exist in several organs, such as liver, kidney, heart, adipose tissue and pancreas [8,10,11,13-18]. The link between the central and peripheral clocks was shown in studies in which the ablation of the SCN eliminated the synchrony of peripheral tissues, suggesting that the SCN maintains the phase alignment of the peripheral clocks [19,20]. The temporal alignment of the peripheral clocks is governed

hierarchically by the central clock dependent upon the light-dark cycles, allowing to optimize metabolism and energy homeostasis throughout the day. The alteration of circadian rhythms and/or the misalignment of peripheral clocks with the SCN may be related to the onset of different metabolic diseases including type 2 diabetes [3,21].

Circadian rhythms, metabolism and health

Circadian variations in behavior and physiology are governed by internal biological clocks that are mainly regulated by light/dark inputs (sleep/activity cycles), but also by nutrient and hormonal signals [15,22,23] (Box 1). In addition to controlling the sleep/wake and feeding cycles, the circadian system regulates physiological processes such as lipid and glucose metabolism, body temperature, locomotor activity and hormone secretion [24], allowing the optimization of energy acquisition, use and storage throughout the day. Several parameters related to glucose metabolism such as glucose tolerance, insulin sensitivity as well as glucose, glucagon and insulin plasma levels are known to exhibit circadian variations along the day [25-30]. However, the regulation of this rhythmicity in glucose metabolism is still not well understood. Particularly, it is not totally clear how the interplay of all these factors that undergo 24 hour variations can modulate daily changes in plasma glucose levels [31]. For instance, plasma glucose concentrations and glucose uptake are maximal at the beginning of the activity period in animals or around awakening in humans and, almost simultaneously, glucose production is high [31]. At night, however, glucose tolerance in humans is decreased compared to the daytime, probably because of reduced insulin sensitivity [27,30,31].

Although feeding activity is one of the most important factors in the control of plasma insulin levels (Box 2), oscillations in plasma insulin are also reported during

day/night cycles in animals and humans independent of fasting/feeding behavior [32-34]. In humans, during the daytime there is a peak of insulin secretion to increase energy storage and utilization [24,32,33]. Reduced insulin secretion and increased glucose production are favored at night [24]. In rodents, this pattern is shifted 12 hours according to their nocturnal activity [25].

Circadian rhythms can be disrupted by impaired function of biological clocks as well as by desynchronization between the SCN and the external environment or the peripheral clocks. This may lead to unfavorable health effects and the development of diseases such as metabolic syndrome, obesity and type 2 diabetes [35]. Current lifestyle and social habits, such as eating or working at night, being exposed to artificial light at night and altered sleeping schedules are among the factors that can cause circadian disruption. Shift workers, who are a paradigmatic model of circadian misalignment, have alterations in the pancreatic beta-cell responses and in glucose and lipid metabolism [3,36,37], as well as an increased risk of developing metabolic syndrome, cardiovascular disease, cancer, obesity and type 2 diabetes [37-41]. Individuals with altered or restricted sleep exhibit an increased body mass index, impaired glucose tolerance and reduced insulin responsiveness [42-44]. In obese individuals or patients with type 1 or type 2 diabetes, the circadian rhythms and magnitude of insulin secretion and sensitivity as well as glucose tolerance are impaired [45]. Some of these changes have also been observed in diabetic patients subjected to sleep deprivation [42,46,47]. Thus, alterations in circadian rhythms may lead to metabolic disturbances that could predispose to diabetes.

Clock genes and metabolism: implications for glucose homeostasis and type 2 diabetes

Animal models with manipulated clock genes have revealed that circadian rhythms have a key regulatory function in metabolism and glucose homeostasis. These animal models have also shown that impaired function of clock genes may result in prediabetic conditions or overt diabetes. Some evidence of this was first found in *Clock* mutant mice studies [48]. These mice had disrupted rhythms in feeding and locomotor activity, hyperphagia, hyperlipidemia, hyperglycemia and hypoinsulinemia. Studies performed in mice deficient in *Bmal1* showed altered adipogenesis and hepatic carbohydrate metabolism [49-51]. While global inactivation of *Bmal1* suppressed the diurnal variation in glucose and triglycerides and led to impaired gluconeogenesis and recovery from insulin-induced hypoglycaemia [50], liver-specific deletion of this clock gene resulted in hepatic clock dysfunction, fasting hypoglycemia and altered glucose clearance throughout the day [49]. Clear diabetic phenotypes characterized by hyperglycemia and hypoinsulinemia were demonstrated in *Clock* and *Bmal1* mutants, as well as pancreas-specific *Bmal1* knockout (KO) mice [8]. In these animal models, circadian clock gene expression was impaired in the endocrine pancreas.

Several metabolic alterations have also been found in genetically manipulated animal models of CLOCK/BMAL1 downstream target genes. For instance, *Per2* KO mice displayed normal circadian clock function in white adipose tissue but altered lipid metabolism [52], while *Per3* single and *Per1/2/3* triple KO mice showed altered body mass regulation when fed a high-fat diet (HFD) [53]. When both *Cry1* and *Cry2* were deleted, behavioral and molecular circadian rhythms were impaired, lipid and glucose metabolism were modified and glucose intolerance emerged [54-56]. Conversely, hepatic *Cry1* over-expression in diabetic (db/db) mice led to increased insulin sensitivity and lower blood glucose levels [57]. It was also reported that cryptochrome proteins down-regulate liver gluconeogenesis during fasting by inhibition of glucagon-

induced cAMP/protein kinase A signaling and cAMP response element-binding protein phosphorylation [57]. The use of synthetic CRY1/2 agonists inhibited glucagon-induced gluconeogenesis in primary hepatocytes [58].

Studies on mice lacking the nuclear receptors REV-ERB ALPHA OR ROR ALPHA revealed alterations in bile and lipid metabolism as well as decreased adiposity and HDL levels [59,60]. In the case of *Rev-erb alpha* KO mice, although these animals displayed alterations in the molecular clock oscillatory properties, they did not show arrhythmic behavior in a constant environment [60]. The link with lipid metabolism was also found in the double KO mice for *Rev-erb alpha* and *beta* [61]. These animals presented altered circadian wheel running behavior and disrupted circadian clock gene expression in the liver. When diet-induced obese mice were treated with REV-ERB agonists, these animals decreased fat mass and weight gain as well as triglyceride and cholesterol levels [6]. Interestingly, REV-ERB ALPHA over-expression in the liver also altered energy, and carbohydrate and lipid metabolism [62]. Thus, several studies using animal models support the role of clock genes in lipid and glucose homeostasis, and also indicate that their impaired function may lead to metabolic pathologies.

In humans, genetic studies have also shown a link between clock genes and metabolism. For instance, genetic variants of *CLOCK* have been associated with increased susceptibility to total energy intake, obesity and metabolic syndrome [63-65]. *BMAL1* genetic variants have been correlated with hypertension, and gestational and type 2 diabetes [66,67], while *PER2* single-nucleotide polymorphisms have been associated with high fasting blood glucose levels and abdominal obesity [68,69]. *CRY2* variants have been related to type 2 diabetes and impaired fasting glucose [70,71]. A genetic polymorphism in the melatonin receptor, which plays a critical role in the modulation of the circadian rhythms, is also associated with increased susceptibility to

impaired insulin secretion and gestational and type 2 diabetes [72,73]. Thus, several genetic observations in humans point to the existence of a close link between clock gene dysfunction and pathological conditions such as metabolic syndrome, obesity and type 2 diabetes.

The role of clock genes in the pancreatic beta-cell function

The endocrine pancreas is a complex organ composed of different cell types that regulate glucose metabolism according to physiological demands. The main pancreatic cells involved in glucose homeostasis are the insulin-secreting beta-cells and the glucagon-secreting alpha-cells (Box 2). The secretion of these two hormones is pulsatile and occurs within minutes after a nutrient challenge [74]. However, plasma insulin and glucagon levels have also been shown to exhibit 24 hour oscillations independent of feeding, as mentioned above [25,26,28,32,33]. Moreover, the deregulation of circadian insulin secretion and action has been identified in first-degree relatives of patients with type 2 diabetes, who exhibited decreased insulin secretion and glucose uptake [45]. The exact mechanism behind these circadian oscillations is still unknown. Although this pattern was originally believed to be exclusively regulated by neuronal signaling and the SCN [75], an intrinsic circadian oscillator controlling insulin secretion was discovered in the rat pancreas [76]. Later, it was shown that the mRNA levels of Per1, Per2, Bmal1, Clock and Rev-erb alpha in the pancreas oscillated throughout a 24-hour period [11]. Additionally, a high expression of the transcription factors and clock-controlled output genes, d-binding protein (DBP) and thyrotroph embryonic factor (TEF), were shown to exist in human pancreatic islets and to have a circadian variation in insulin-secreting cells [77].

The islet clock appears to consist of the same components as those of other peripheral clocks, exhibiting oscillations in their gene expression throughout the day in different species. A circadian clock gene expression has been shown in rat pancreas [11], mouse pancreas [78], mouse islets [8,10], human islets and dispersed human islet-cells [79]. Additionally, it has also been observed in pancreatic cell lines such as MIN6 cells [10], INS-1 cells [80] and alphaTC1-9 cells [81]. DBP has also been found to oscillate in the pancreatic cell line MIN6 and in mouse islets [82]. All these findings support the existence of an intrinsic clock within the pancreatic islet.

Studies performed in whole-body, pancreas-specific and beta cell-specific KO mice have revealed the importance of islet clock genes in the control of insulin secretion and the development of diabetes. Additionally, these investigations have shown some of the potential mechanisms involved. *Clock*$^{\Delta 19/\Delta 19}$ mutant mice, as well as pancreas-specific *Bmal1* KO mice, exhibit hyperglycemia, hypoinsulinemia and glucose intolerance [8]. This diabetic phenotype has been associated with altered insulin secretory responses. Pancreatic islets isolated from *Clock* mutant mice had reduced size and impaired insulin release [8]. Consistent with these findings, these pancreatic islets presented decreased beta-cell proliferation as well as reduced expression or changes in the circadian pattern of different genes involved in glucose uptake and metabolism, insulin signaling, cell cycle and beta-cell growth [8] (Figure 2). In the same way, *Clock* antisense treatment during pancreatic embryogenesis resulted in changes of different cell cycle proteins via WNT and NOTCH signaling [83]. Analysis of insulin release in isolated islets from *Clock*$^{\Delta 19/\Delta 19}$ mice revealed impaired secretion in response to glucose but also to non-metabolic stimuli, indicating that defective exocytosis rather than altered glucose metabolism was likely involved [8]. The lack of changes in glucose-induced Ca^{2+} signaling (which precedes insulin secretion) as well as the decreased expression of

proteins involved in vesicle transport and docking, like VAMP3 and SYNTAXIN6, further supported this specific defect in beta-cell exocytosis.

Similar alterations in insulin secretion were observed in isolated islets of whole-body *Bmal1* KO and pancreas-specific *Bmal1* KO mice [8]. Another study reported hyperglycemia and hypoinsulinemia in global *Bmal1* KO mice and demonstrated that the impaired glucose tolerance in this animal model was due to defective first phase insulin secretion [84]. Both isolated islets from these KO mice and INS-1 cells with *Bmal1* knockdown exhibited a disrupted glucose-induced increase in the mitochondrial membrane potential gradient due to enhanced uncoupling protein 2 (*Ucp2*) expression, which led to decreased ATP levels and insulin secretion [84]. Inhibition of UCP2 partially reversed these defects. In contrast to the findings in $Clock^{\Delta 19/\Delta 19}$ mice [8], these latter results pointed to defects in glucose metabolism rather than alterations in exocytosis.

These dissimilar effects might be due to different CLOCK and BMAL1 pleiotropic actions in addition to their circadian role. A similar diabetic phenotype and secretion defects were reported in beta-cell specific *Bmal1* KO mice [85]. This latter study indicated that BMAL1 is important for the prevention of oxidative stress. Pancreatic islets isolated from these mice displayed augmented reactive oxygen species, increased *Ucp2* expression and impaired glucose-induced insulin release [85] (Figure 2). Oxidative stress prevention or UCP2 suppression were able to reverse these defects. Additionally, it was shown that the key antioxidant regulatory factor *Nrf2* was a direct target of BMAL1. In agreement with these reports, selective pancreas deletion of *Bmal1* also produced glucose intolerance and defective insulin secretion in mice and isolated islets [86].

In addition to the core clock components *Clock* and *Bmal1*, other clock genes such as *Rev-erb alpha* are also important for the pancreatic beta-cell function. *Rev-erb alpha* is expressed in pancreatic islets and exhibits circadian oscillations in MIN6 cells [10]. When this gene was silenced in mouse pancreatic islets, glucose-induced insulin secretion and beta-cell proliferation decreased [10]. Similarly, *Rev-erb alpha* exhibited circadian changes in alphaTC1-9 cells and, when it was down-regulated, glucose-modulated glucagon secretion became impaired [81]. The effect of *Rev-erb alpha* on both beta and alpha-cell secretion may be related to the exocytotic process, as expression of exocytotic genes like *Vamp3*, *Syntaxin1*, *Munc18* and *Snap25* were decreased when *Rev-erb alpha* was silenced [81], in agreement with previous results in $Clock^{\Delta 19/\Delta 19}$ mice [8]. Thus, although further work is necessary to understand the regulatory networks, it seems that clock genes may influence beta-cell mass and function by modulation of different processes (Figure 2).

Interestingly, in addition to clock genes, the expression of several other genes exhibits a circadian rhythm in the pancreas, isolated islets and beta-cell lines. These genes include *Glut2, Glucokinase, Syntaxin1A, Insulin, Ucp2, Nrf2, CyclinD1, Pdx1* and other genes involved in beta-cell metabolism, secretion, growth and insulin signaling [77,87]. It has been reported that *Nrf2* is a transcriptional target of BMAL1 [85], TEF transactivates *Glut2* by direct interaction with the promoter [77] and DBP directly increases the transcription of *Arnt/Hif1beta* [82], a key gene in beta-cell metabolism and secretion (Figure 2). It would be interesting to know whether the circadian expression of all these above-mentioned genes is coupled to an oscillatory functional output in different beta-cell processes. In this regard, there is a high correlation between the diurnal changes in insulin secretion and the glucose-induced reduction in beta-cell K^+ conductance, most likely via ATP-dependent K^+ channel inhibition, which was

264 measured at the same time of the day [88]. Likewise, a circadian expression of
265 functional K^+ channels has been reported in rat pancreatic islets [89].

266 The information about clock genes in human islets is scarce. Human islets also
267 express *BMAL1*, *CLOCK*, *PER1*, *PER2*, *PER3*, *CRY1* and *CRY2*, and have been found
268 to exhibit self-sustained circadian oscillations of *Bmal1*-luciferase expression [79].
269 Islets from type 2 diabetes patients have decreased *CRY2*, *PER2* and *PER3* expression,
270 which positively correlated with insulin content [90]. Additionally, the exposure of
271 human pancreatic islets to high glucose and palmitate levels decreased *PER3* expression
272 [90]. Although various animal studies support that clock genes have an essential
273 function in the endocrine pancreas, further research will be required to unravel their role
274 in the human pancreas.

275

276 **Environmental conditions that alter circadian rhythms and the pancreatic clock**

277 Current lifestyle frequently involves alterations in the natural rhythms governed
278 by the circadian system and the daily light/dark cycle. Diet composition as well as
279 eating and activity at inappropriate times, which can disrupt circadian clocks, have been
280 related to type 2 diabetes [40,41,91]. Although sleep loss has also been associated with
281 this disease [43,44,47], recent studies indicate that circadian misalignment induced by
282 changing the sleeping schedules can also increase diabetes risk independent of sleep
283 loss [21]. SCN ablation affects the circadian pattern of glucose tolerance, as well as
284 glucose, insulin and glucagon plasma levels [26-28], suggesting that this central clock
285 plays an important role in glucose homeostasis. However, several environmental factors
286 can also affect the function of these circadian oscillators not only in the SCN, but also in
287 peripheral locations (Box1).

In recent mouse studies, constant exposure to light or a HFD or combination of both altered the SCN function, affecting the circadian patterns of feeding, energy expenditure and insulin sensitivity [92]. Experiments with mice fed a HFD have shown changes in the circadian expression of clock genes and clock-regulated metabolic genes in the hypothalamus, liver, and adipose tissue [16]. Additionally, mice fed a HFD during the light phase gained more weight than those fed during the dark phase [93]. Temporal feeding restriction in mice produced a misalignment between the SCN and the peripheral clocks in liver, kidney, heart, and pancreas, but did not affect the circadian behavior of the central clock [94]. In contrast, caloric restriction can affect both central and peripheral clocks [95]. Simulated shift work in rats led to abdominal obesity and dampened glucose rhythms. Both alterations were reversed when the feeding time was shifted back to the activity phase [96]. The majority of these results show the importance of appropriate feeding timing for the maintenance of circadian function. Thus, feeding schedules uncoupled from natural day/night cycles can lead to altered circadian rhythms and lack of synchrony between central and peripheral clocks that may favor metabolic diseases.

The effect of some of these environmental factors has also been analyzed in the endocrine pancreas. The effect of light exposure at night has been investigated in the rat pancreas using *Per1*-driven luciferase [97]. While changes in the light/dark cycle *in vivo* entrained the phase of islet clock transcriptional oscillations, ten weeks of continued exposure to light at night (constant light regime over 24 hours) impaired the amplitude, phase, and inter-islet synchrony of clock transcriptional oscillations. In these circumstances, glucose-stimulated insulin secretion was impaired due to a decrease in the insulin secretory pulse mass [97]. Interestingly, it was also observed that glucose regulates the amplitude and period of *Per1* circadian oscillations, indicating a nutrient-

sensing mechanism in the islet clock. It has also been reported that constant light regime or 6-h advance of the light cycle every 3 days accelerates the development of diabetes in Sprague Dawley rats transgenic for the human islet amyloid polypeptide [7]. Exposure to HFD has been shown to change the circadian expression pattern of *Rev-erb alpha*, *Clock*, *Per1* and *Per2* in mouse pancreatic islets as well as the circadian insulin secretion [10]. Circadian misalignment by simulation of shift work induced oxidative stress and altered insulin release in pancreatic islets similar to that observed in beta-cell specific *Bmal1* KO mice [85]. Thus, several studies have demonstrated that environmental conditions that affect circadian rhythms can also impair the pancreatic clock and the function of this endocrine organ.

Conclusions and future perspectives

Numerous studies have revealed an association between alterations in human circadian rhythms and metabolic diseases such as type 2 diabetes. Moreover, animal models with genetically manipulated clock genes, as well as genetic studies in humans, have further demonstrated a link between clock genes and glucose homeostasis. Although glucose metabolism and its circadian variations may depend on several tissues and factors, which also present daily activity oscillations, the demonstration of an existing self-sustained circadian clock in the endocrine pancreas is of critical importance. Indeed, several animal models with an ablated function of different clock genes, either in the whole body, the pancreas or beta-cells, exhibit diabetic phenotype features including glucose intolerance, hyperglycemia and/or hypoinsulinemia. Additionally, the pancreatic islets from these animal models show impaired exocytosis and insulin release, altered mitochondrial function and alterations in cell proliferation or cell size. Several of these observations have also been reported in human islets. It has

been recently demonstrated that the pancreatic clock and islet function are also affected by some environmental factors known to disrupt circadian rhythms. It could therefore be concluded that the pancreatic clock plays an important role in the endocrine pancreas function and in glucose homeostasis, and that its malfunction may lead to glucose intolerance and type 2 diabetes. Since the research focused on the pancreatic clock is relatively new, there are yet important questions to be answered (Box 3).

Understanding the role of the pancreatic clock may help in the design of therapeutic interventions for metabolic diseases such as type 2 diabetes, when they are a consequence of circadian alterations. For instance, since Cry1 over-expression in diabetic mice increased insulin sensitivity and lowered blood glucose levels [57], it would be interesting to explore the role of CRY1/2 agonists in the treatment for diabetes. In this regard, the agonist KL001 was proved to be useful in preventing the glucagon-mediated activation of hepatic glucose production, which could be beneficial in the control of fasting glucose levels [58]. The use of REV-ERB agonists may also be valuable for the treatment of diabetes and obesity, since these molecules enhance energy expenditure, reduce fat mass and improve dyslipidaemia and hyperglycemia in diet-induced obese mice [6]. ROR GAMMA antagonists have been recently suggested to be a therapeutic strategy in type 2 diabetes by modulation of insulin resistance and glucose tolerance [98]. Other potential drugs to be explored in metabolic diseases could include ligands for ROR ALPHA [99-101] as well as for other components of the central clock [91]. Although several of these ligands, agonists and antagonists modulate metabolic processes in peripheral clocks located in the liver, adipose tissue and muscle, their actions in the pancreatic clock remain to be explored.

Taking into consideration the pancreas chronobiology also implies evaluating the appropriate daily times for medical treatments in diabetic patients or for the

evaluation of glycemic parameters. In this regard, recent clinical studies have shown that there exists a circadian variation in the response to the glucose challenge test in pregnancy, which may influence the diagnosis of gestational diabetes [102]. In addition, it would be important to analyze whether alterations of circadian rhythms (i.e. shift work) affect the human pancreatic clock and function. If so, it would be also interesting to evaluate whether life style changes adapted to the natural light/dark cycle and to appropriate feeding and activity times have a positive impact on the pancreas clock in terms of preventing and treating metabolic diseases such as diabetes.

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Box 1. The central and peripheral clocks.

Numerous biological processes such as body temperature, feeding/fasting and sleep/wake behavior, as well as hormonal release, display circadian patterns which are mainly driven by the central clock located in the suprachiasmatic nuclei (SCN) of the hypothalamus. These circadian rhythms are responsible for the optimization of energy homeostasis throughout the light/dark cycle of the day. The neuronal population that forms this master clock works as a self-sustained circadian oscillator that is controlled by autoregulated transcriptional feedback loops. The SCN also sends neural projections to other brain areas that are important for the regulation of sleep, feeding, energy homeostasis and temperature [22]. Several external cues (also called zeitgebers) synchronize the internal clocks to 24 hour cycles. The change of environmental light throughout the day/night cycle is the main entraining signal for the SCN, which in turn also synchronizes other clocks located in extra-SCN brain areas and peripheral tissues to the photic signals [103]. Additionally, other external cues like the time of feeding/fasting or food restriction can also affect the circadian rhythm of internal clocks. Different metabolic sensors can integrate information from the nutritional state and transfer it to the clock machinery. In this way, changes in NAD/NADH or AMP/ATP during exercise, fasting or caloric restriction can be sensed by the clocks [22].

Peripheral tissues are also equipped with autonomous clock oscillators with similar molecular components as those of the SCN. The circadian activity of these peripheral clocks is temporally aligned to the central SCN clock mainly by autonomic innervations, hormonal signals (i.e. glucocorticoids) and feeding-related cues [103]. Just as the central pacemaker, peripheral clocks contribute to global energy metabolism but also participate in local metabolic functions such as those involved in glucose and lipid

406 homeostasis. Although light-dependent time alignment with the SCN is important, the
407 fasting/feeding cycles and changes in several nutritional factors also play an important
408 role in the entrainment of peripheral clocks [22]. For instance, a high-fat diet and caloric
409 restriction affect both peripheral and central clocks, while temporal feeding restriction
410 only entrains peripheral clocks [95].
411
412

Box 2. Endocrine pancreas function and type 2 diabetes.

The endocrine pancreas is composed of a heterogeneous collection of cell types that form the islets of Langerhans, which are dispersed throughout the whole pancreatic tissue. The main cell types are the insulin-secreting beta-cells and the glucagon-releasing alpha-cells. These cell populations respond reciprocally to plasma glucose levels [104]. While beta-cells secrete insulin at increasing glucose concentrations, alpha-cells release glucagon at lower glucose levels. Insulin mainly acts through the PI3K (phosphatidylinositol-4,5-bisphosphate 3-kinase) pathway in muscle and adipose tissue to induce glucose uptake, and in the liver to reduce hepatic glucose production. These processes result in decreased plasma glucose levels. In contrast, glucagon mainly binds to liver receptors, activating gluconeogenesis and glycogenolysis via the protein kinase A pathway, which allows for an increase in plasma glucose levels. Thus, glucose is maintained within a physiological range by the fine regulation of islet secretion [104].

Type 2 diabetes results from insulin resistance along with impaired beta-cell function. In this condition, insulin action is attenuated, leading to decreased insulin-induced glucose uptake in the adipose tissue and muscle as well as enhanced hepatic glucose production [105]. In the majority of individuals, insulin resistance induces a compensatory adaptation in the pancreas, resulting in an augmented beta-cell mass and function to increase insulin release, which ensures the maintenance of normoglycemia. However, when this beta-cell compensation is insufficient, hyperglycemia may progressively develop. This deficient adaptive response can be also accompanied by beta-cell failure and loss. It has been suggested that hyperglucagonemia and alpha-cell dysfunction are also involved in the pathophysiology of type 2 diabetes, and may exacerbate the high plasma glucose levels.

Box 3. Outstanding questions.

- What is the exact contribution of the pancreatic clock to glucose homeostasis and the daily variations in plasma glucose and insulin?

- Which mechanisms allow for the communication between the SCN and the pancreatic clock?

- Is the misalignment of the pancreatic clock with the SCN sufficient to induce alterations in glucose homeostasis and diabetes?

- Will pharmacological modulation of the clock proteins lead to useful therapeutics to treat metabolic diseases?

- Which environmental factors known to produce circadian disruption are able to affect the pancreatic clock and the function of this organ? What are the mechanisms?

- Could the reversal of or the intervention in these environmental factors restore the normal circadian pattern in the pancreas?

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FIGURE LEGENDS

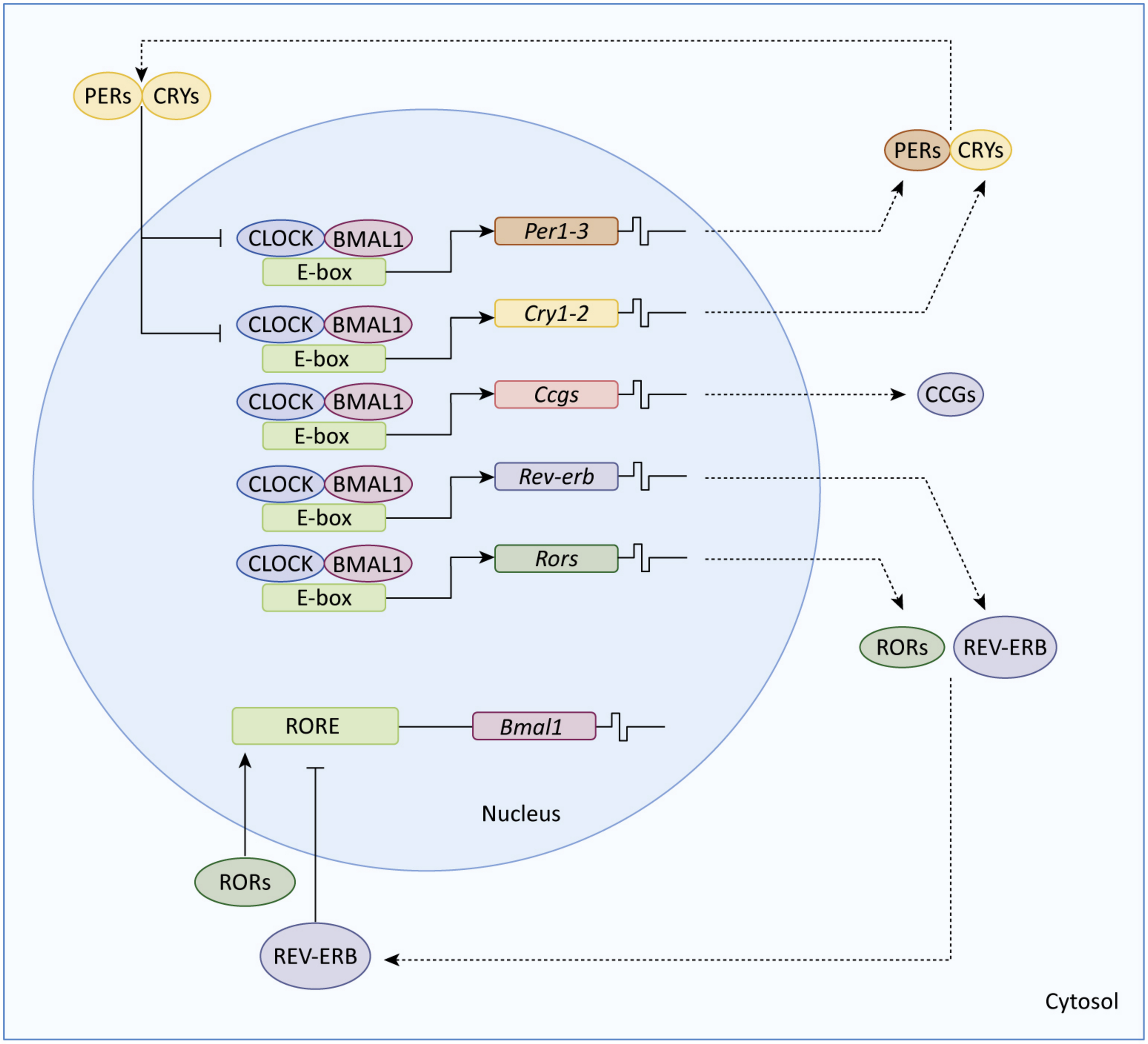
Figure 1. Feedback loop in the core circadian clocks. The clock machinery is composed of different feedback loops. In the primary loop, BMAL1 and CLOCK drive the transcriptional expression of *Per* and *Cry* through the activation of E-box enhancers. PER and CRY proteins form heterodimers that translocate to the nucleus, repressing their own transcription by interaction with CLOCK:BMAL1 complexes. The turnover of PER and CRY is also regulated by protein casein kinase 1 and AMP kinase, which phosphorylate these proteins, inducing their degradation in the cytosol by the 26S proteasome complex. This feedback in the primary loop occurs during 24 hours and then, a new cycle starts. In another loop, CLOCK:BMAL1 heterodimers drive the transcriptional expression of *Rev-erb* and *Ror*, which inhibit and activate *Bmal1* expression, respectively. Some clock-controlled genes (*Ccgs*), which are involved in metabolic pathways, are also under the modulation of the clock machinery.

Abbreviations: BMAL1, brain and muscle ARNT-like 1; CLOCK, circadian locomotor output cycles protein kaput; PER, period homolog drosophila; CRY, cryptochrome; REV-ERB, reverse-eritroblastosis virus; RORS, retinoic acid receptor-related orphan receptor; RORE, ROR response element.

Figure 2. Clock genes in the regulation of pancreatic beta-cell function. Glucose enters the pancreatic beta-cell via glucose transporter 2 (GLUT2) and is metabolized by mitochondria, resulting in the rapid generation of ATP and the subsequent closure of ATP-sensitive K^+ (K_{ATP}) channels. The closure of these channels leads to plasma membrane depolarization (ΔVm), opening voltage-gated Ca^{2+} channels (VOCC). The consequent Ca^{2+} influx triggers insulin release through exocytosis of insulin secretory granules. Pancreatic beta-cell regulation by clock genes may involve several processes

and mechanisms. Impaired glucose-induced insulin secretion (GSIS) has been shown in islets deficient in CLOCK, BMAL1 or REV-ERB. CLOCK and REV-ERB may regulate insulin secretion by modulating the expression of genes involved in exocytosis (*Vamp3*, *Syntaxin1*, *Munc18*, *Snap25*). Additionally, CLOCK and REV-ERB regulate beta-cell proliferation, probably through the modulation of different genes involved in beta-cell survival and growth (*CyclinD1*, *Hnf4α*, *InsR*, *Pdx1*). BMAL1 is also required for normal beta-cell GSIS by reducing UCP2-mediated mitochondrial uncoupling and oxidative stress and by increasing antioxidant defenses. This latter action is mediated by direct transcriptional modulation of *Nrf2* by BMAL1. The clock-controlled output proteins TEF and DBP have also been shown to directly modulate the transcriptional activity of the glucose transporter 2 (*Glut2*) and *Arnt*, respectively. This latter gene plays a key role in beta-cell metabolism and GSIS.

Abbreviations: BMAL1, brain and muscle ARNT-like 1; CLOCK, circadian locomotor output cycles protein kaput; CRY, cryptochrome; DBP, d-binding protein; PER, period homolog drosophila; REV-ERB ALPHA, reverse-eritroblastosis virus alpha; TEF, thyrotroph embryonic factor; UCP2, uncoupling protein 2.


PERs
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REV-ERB
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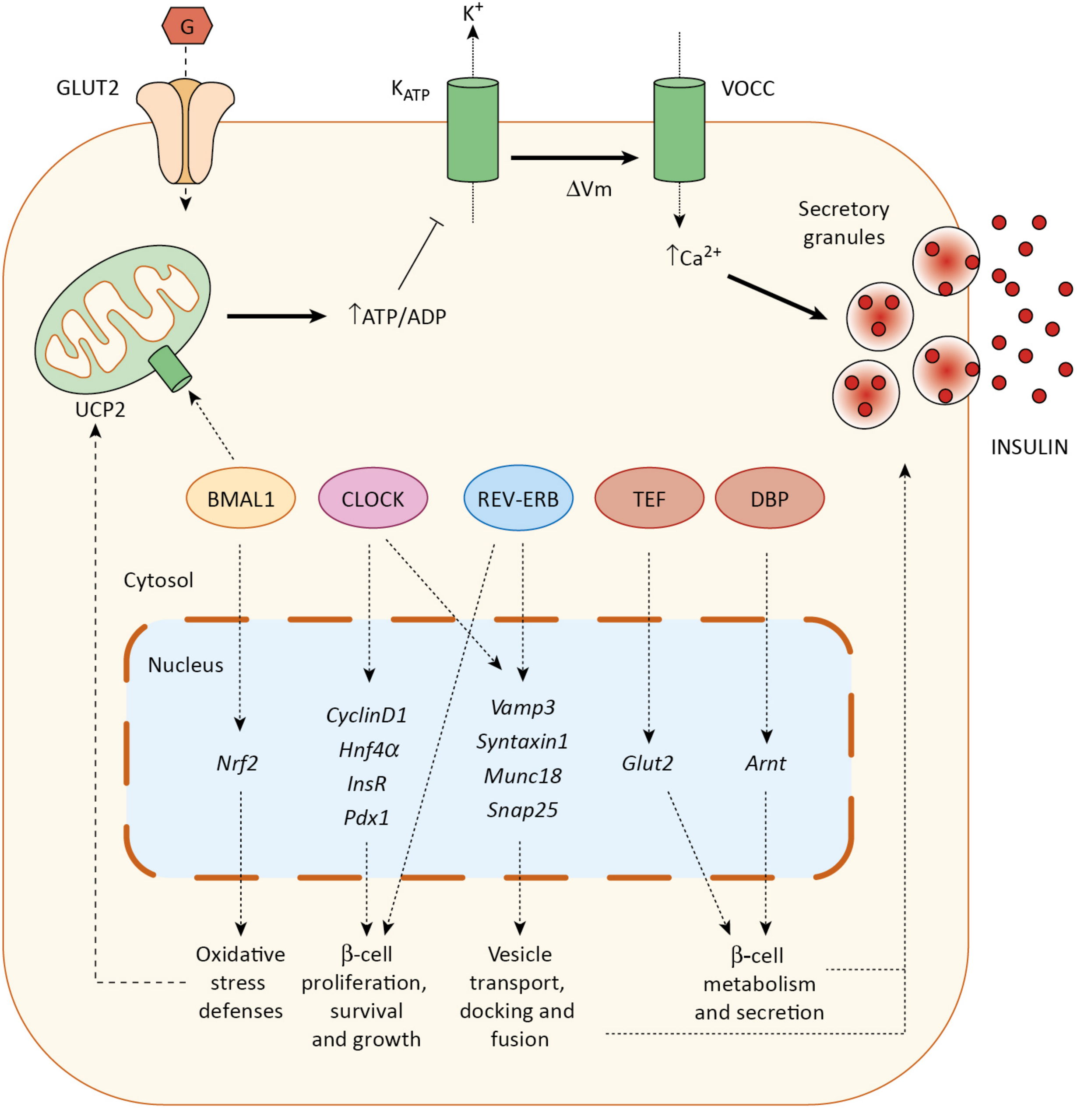
G
GLUT2
K^+
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VOCC
ΔVm
Secretory granules
↑Ca^{2+}
↑ATP/ADP
INSULIN
UCP2
BMAL1
CLOCK
REV-ERB
TEF
DBP
Cytosol
Nucleus
Nrf2
CyclinD1
Hnf4α
InsR
Pdx1
Vamp3
Syntaxin1
Munc18
Snap25
Glut2
Arnt
Oxidative stress defenses
β-cell proliferation, survival and growth
Vesicle transport, docking and fusion
β-cell metabolism and secretion