Circadian Rhythms and microRNAs in Energy Metabolism

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Abstract

Circadian clock and metabolic systems are intertwined at the molecular and genetic level to adapt energy utilization to geophysical time. Disruption of clockwork genes has been associated to metabolic and cellular malfunction; however, the exact molecular makeup of this relationship in glucose and lipid metabolism is still unclear. The potential relevance of microRNAs in this interplay is also largely unexplored.

Here, the role of the heterodimeric transactivator duo Clock-Bmal1 – a core constituent of the peripheral circadian machinery – was investigated in pancreatic beta cells and liver along with its relation to metabolic microRNAs based on three subprojects:

i) Firstly, mice deficient for either Clock or Bmal1 in pancreatic beta cells displayed glucose intolerance and diminished insulin release. The core circadian components were indispensable for appropriate vesicle exocytosis, but not for the early stages of glucose-stimulated insulin secretion. Clock-deficient beta cells displayed an abnormally elevated exocytotic rate, underscored by a regulation in numerous components of the exocytotic machinery. Collectively, the data suggested that Clock-Bmal1 promote insulin release through an action on membrane fusion dynamics and that disruption of the heterodimeric duo in beta cells represents a predisposing cause for type 2 diabetes.

ii) Secondly, it was found that *clock* is posttranscriptionally suppressed by the highest expressed microRNA in pancreatic beta cells, mir-375, which is a negative regulator of insulin secretion. mir-375 overexpression evoked downregulation of Clock *in vitro*, whereas loss of mir-375 induced Clock upregulation in pancreatic islets *in vivo*. Thus, *clock* was delineated as one target, mediating the negative impact of mir-375 on insulin secretion. Moreover, mir-375$^{-/-}$ islets displayed an enhanced intrinsic capacity to sustain circadian rhythms in the absence of external entrainment cues *ex vivo*. This suggested that mir-375 attenuated intercellular coupling in the functional syncytium of the islet pacemaker.

iii) Thirdly, in respect to liver function, Clock-deficiency resulted in impaired lipoprotein secretion. Clock-Bmal1 was demonstrated to physically interact with a conserved intronic E-box response element in *ppargc1b* driving expression of the transcriptional coactivator Pgc-1β and thereby promoting expression of downstream metabolic factors, involved in lipogenesis and lipoprotein assembly. Furthermore, a metabolic microRNA was found to be a Clock-Bmal1 target: Intronic mir-378 was co-transcribed with its host *ppargc1b* in an oscillating manner, yet, expression of its mature sequence remained constant over time.

Collectively, the results established a molecular nexus between Clock-Bmal1 function and hepatic lipoprotein metabolism.

In sum, the current work shed light on the molecular basis for Clock-Bmal1 action in two aspects of energy metabolism – insulin secretion and lipoprotein production. It further provided information on Clock-Bmal1 interconnection with mir-375 in beta cells and mir-378 in hepatocytes.

Zusammenfassung

Die circadiane Uhr und metabolische Systeme sind auf molekularer Ebene verbunden, um Energienutzung an die geophysikalische Zeit anzupassen. Fehlfunktion von Uhrwerkgenen ist mit metabolischen und zellulären Defekten assoziiert; jedoch sind die präzisen Mechanismen dieser Verbindung in Bezug auf Glukose- und Lipidmetabolismus noch unklar. Eine mögliche Relevanz von microRNAs in diesem Zusammenspiel ist auch weitgehend unerforscht.

Hier wurde die Rolle des heterodimeren Transaktivators Clock-Bmal1 – ein Kernelement des circadianen Uhrwerks – in pankreatischen Betazellen und Leber untersucht, sowie dessen potentielles Zusammenspiel mit microRNAs, anhand der folgenden drei Subprojekten:

i) Erstens, Mäuse defizient für Clock oder Bmal1 in pankreatischen Betazellen wiesen defekte Glukosetoleranz und Insulinsekretion auf. Die circadianen Kernkomponenten waren unabdingbar für den normalen Ablauf der Vesikel-Exozytose, jedoch nicht für die frühen Phasen des glukose-stimulierten Signaltransduktionswegs. Clock-defiziente Betazellen zeigten eine erhöhte Exozytoserate und veränderte Genexpression vieler Komponenten des Exozytose-Apparats. Insgesamt deuteten die Ergebnisse darauf hin, dass der circadiane Transaktivator die Membranfusion der Insulinvesikel kontrolliert und dass die gestörte Clock-Bmal1 Funktion in Betazellen ein begünstigender Faktor für die Entwicklung von Diabetes Typ 2 darstellt.

ii) Zweitens, in pankreatischen Betazellen wurde nachgewiesen, dass *clock* durch die höchst exprimierte microRNA mir-375, die Insulinsekretion negativ reguliert, posttranskriptional supprimiert wird. mir-375 Überexpression bewirkte eine Herunterregulation von Clock *in vitro*, während mir-375 Deletion eine Hochregulation von Clock in Langerhans Inseln *in vivo* auslöste. Dementsprechend wurde *clock* als ein Zielgen von mir-375 ermittelt, welches zu der negativen Wirkung dieser microRNA auf Insulinsekretion beiträgt. Des Weiteren zeigten mir-375$^{-/-}$ Langerhans Inseln eine verbesserte Eigenschaft, autonome circadiane Rhythmen in der Abwesenheit von externen Zeitgebern *ex vivo* aufrechtzuerhalten. Dies deutete darauf hin, dass mir-375 die interzelluläre Kopplung des funktionellen Syncytiumm der Betazelluhr schwächt.

iii) Drittens, hinsichtlich Leberfunktion resultierte Clock-Defizienz in defekter Lipoproteinsekretion. Clock-Bmal1 bindete nachweislich an ein konserviertes E-box Element in *ppargc1b*, um die Expression des Transkriptionscoaktivators Pgc-1β anzutreiben, und somit die Expression von metabolischen Genen, involviert in Lipid- und Lipoproteinsynthese,

zu begünstigen. Weiterhin wurde gezeigt, dass eine metabolische microRNA durch Clock-Bmal1 aktiviert wird. Die intronische mir-378 wurde zusammen mit *ppargc1b* oszillierend transkribiert, wobei die Expression der reifen mir-378 konstant blieb. Insgesamt haben die Ergebnisse die Verbindung zwischen Clock-Bmal1 und Lipoproteinmetabolismus in der Leber aufgeklärt.

Zusammenfassend hat diese Arbeit die molekularen Mechanismen der Funktion von Clock-Bmal1 hinsichtlich zwei metabolische Aspekte – Insulinsekretion und Lipoproteinsynthese – erläutert. Des Weiteren hat sie zu der Aufklärung des Zusammenspiels zwischen Clock-Bmal1 und mir-375 in Betazellen sowie mir-378 in der Leber beigetragen.

I. Introduction

First, the physiological function of islet and liver in systemic glucose and lipid metabolism is introduced. Second, the genetic organization of the endogenous timing system and its crosstalk to metabolic networks is presented. Finally, the role of microRNAs in circadian and metabolic processes is laid out.

A. Systemic glucose and lipid metabolism

I.A.1 The Langerhans islet

The Langerhans islet is specialized in the maintenance of glucose homeostasis. Utilization of the universal energy source glucose is essential for vertebrate life. The carbohydrate monomer is directly and immediately absorbed into the circulation after food ingestion; however, excessive concentrations are pathological due to the protein glycosylation and the electrolyte imbalance it evokes (Vlassara et al. 1983; Tzamaloukas et al. 2008). Thus, maintaining blood glucose within the physiological range of ca. 4 – 6 mM is crucial (rev. in Bell and Polonsky 2001). This is achieved by the islet of Langerhans – a heterogeneous endocrine cell-population comprising only two percent of the whole pancreatic tissue and yet having a tremendous effect on physiology (Langerhans 1869; Orci 1982). This microorgan is composed predominantly of beta cells (60 - 90 %) and alpha cells (10 – 20%) (Elayat et al. 1995) **[Fig. I-1]**. The former secrete insulin, while the latter produce glucagon. Both hormones exert counteracting effects on glucose uptake and macromolecular turnover in peripheral tissues in order to maintain blood sugar concentrations and energy supply constant despite fluctuations in food availability (Pipeleers 1987) (see I.A.1.5).

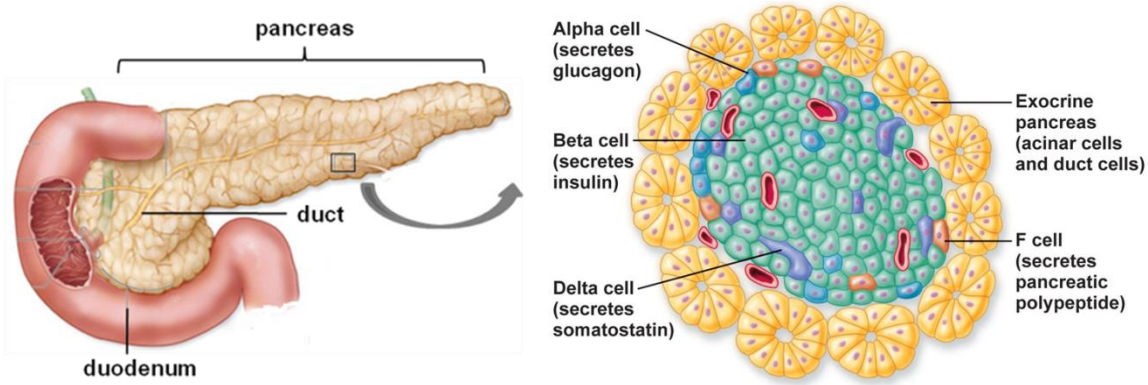


Fig. I-1: Structure of the pancreatic islet
The majority of the pancreatic tissue represent exocrine acinar and duct cells, which secrete digestive enzymes (amylases, peptidases etc.) via the pancreatic duct into the duodenum. Only 2-3% of the pancreatic tissue is concentrated in the Langerhans islets and fulfill endocrine functions (right). The predominant cell population in the endocrine pancreas are beta cells; Langerhans islets also contain glucagon-secreting alpha cells, somatostatin-producting delta cells and pancreatic polypeptide-secreting F sells.

I.A.1.1 The insulin secretion pathway

Granulogenesis

The beta cell-produced hormone is constitutively synthesized at the endoplasmic reticulum as a 110 amino acid-long preproinsulin (Steiner et al. 1967). After signal peptide cleavage, proinsulin transits the Golgi Network and is packaged into immature secretory granules (rev. in Kim et al. 2006). While their lumen is acidified and sorted out from constitutive secretory proteins not intended for regulated exocytosis, vesicle outer is released from its clathrin coat. Over the course of this maturation process, proinsulin is proteolytically processed by prohormone convertase 2/3 and carboxypeptidase H to finally yield the crystalline insulin content, formed of hexamer polypeptide aggregates associated with zinc cations (Hutton 1994) **[Fig.I-2]**. Newly formed dense-core insulin vesicles translocate to the plasma membrane in an ATP-dependent manner to await extracellular release upon glucose stimulation. They can be stored in the cytoplasm for several days before extracellular release (Schnell et al. 1988). Vesicles, which do not undergo secretion are subjected to crinophagy i.e. intracellular destruction by lysosomes (Schnell et al. 1988).

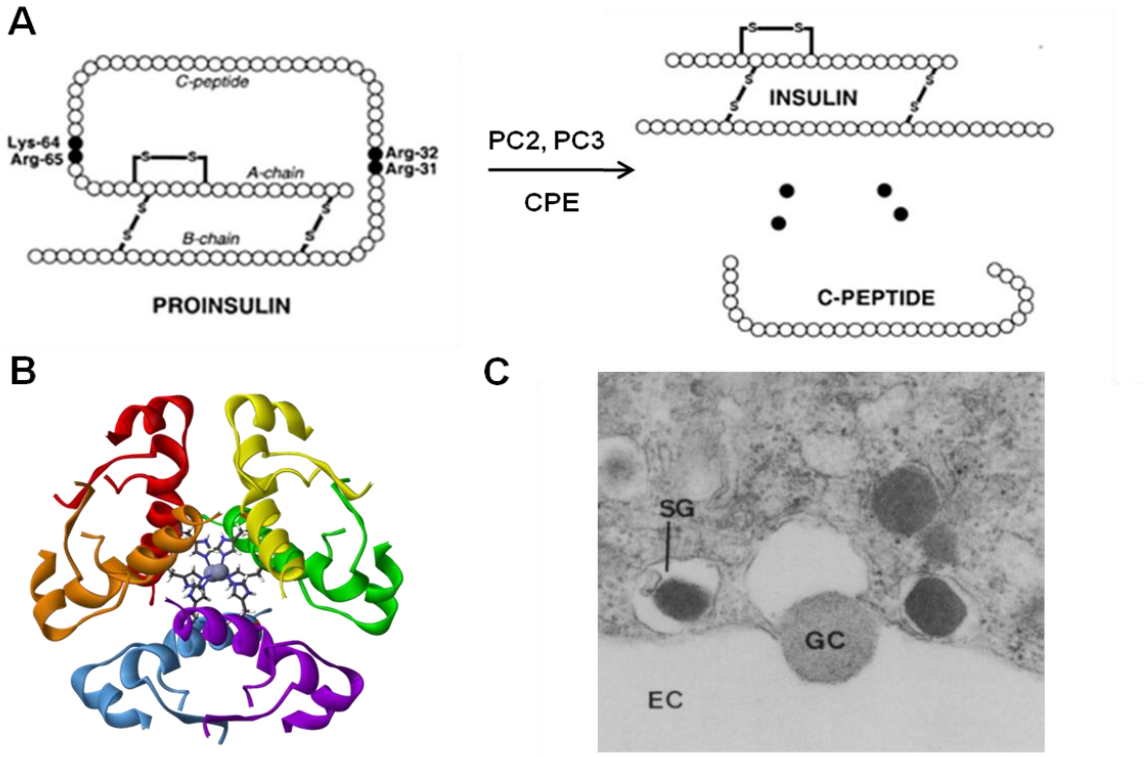


Fig.I-2: Insulin synthesis and structure
(A) The product of the insulin gene enters the secretory pathway as a biologically inactive proinsulin, which undergoes proteolytical processing over the course of vesicle maturation in Golgi, mediated by different endo- (prohormone convertase PC2, PC3) and exopeptidases (carboxypeptidase H or CPE) (modified from LeRoith et al. 2004). (B) Structure of insulin hexamers in the presence of Zn-cations, represented by a Richardson diagram (source: Wikimedia Commons). (C) Docked insulin vesicles, which are secreted. They have a diameter of ca. 350 nm and contain electron-dense Zn_2-$insulin_6$ crystals. A single granule contains ca. 10^6 molecules or 8 fg insulin (Orci 1982; Rorsman and Renström 2003). SG – secretory granule; GC – granule core: EC – extracellular space

Glucose-stimulated secretion

Secretion of insulin granules is induced upon supraphysiological circulating glucose concentration above 7 mM, in linear proportion to the hexose amount and a saturation reached above 20 mM (rev. in Bell and Polonsky 2001; Rorsman 1997). Stimulatory signals are derived from the intracellular metabolism of glucose rather than a receptor-ligand interaction **[Fig.I-3]**. After glucose internalization by the specialized transporter Glut2, the hexose is metabolized to pyruvate and oxidized in the Krebs cycle. The resulting rise in cellular ATP/ADP ratio induces closure of ATP-dependent K^+-channels and, subsequently, membrane depolarization. The latter activates voltage-gated Ca^{2+}-channels leading to elevation in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) and, ultimately, to exocytosis of insulin-containing secretory granules.

Insulin secretion in the Langerhans islets is further characterized through a biphasic time course (Curry et al. 1968). Immediately after glucose stimulation, beta cells respond with release of vesicles in direct proximity to the plasma membrane, i.e. the readily releasable vesicle pool [**Fig. I-4]**. Particularly, this involves granules, situated in the vicinity of Ca^{2+}-channels and released immediately after elevation of local [Ca^{2+}]. This pool comprises ca. 5-7% of all vesicles and accounts for the rapid and strong hormone peak during the acute phase of secretion (Bokvist et al. 1995; Barg et al. 2002).

Once the readily releasable vesicle pool is exhausted, a replenishment and mobilization of more distant granules to the cell membrane is required (Ohara-Imaizumi et al. 2004). This corresponds to a lower second secretion phase with a slower release rate (ca. 0.05% per minute), which is sustained until euglycemia is achieved (Anello et al. 1999). Along their journey to the plasmalemma, insulin granules interact with cytosolic compounds, such as adaptor and motor proteins, and guanosine triphosphatases. They are loaded onto microtubules and mobilized to the cell periphery by means of kinesin-based transport, whereas their precise allocation at the site of secretion occurs through actin microfilaments (Parsons et al. 1995; Gromada et al. 1999; Varadi et al. 2002).

Glucose represents the most potent and physiologically relevant stimulator of insulin secretion since it is a common component of dietary carbohydrates. Other secretagogues, which together with glucose act in a synergistic manner, include amino acids, fatty acids, and gastrointestinal hormones (Nuttall and Gannon 1991). They act through amplification of the glucose signaling cascade, e.g. through mobilization of Ca^{2+} from intracellular stores (Rorsman and Renström 2003).

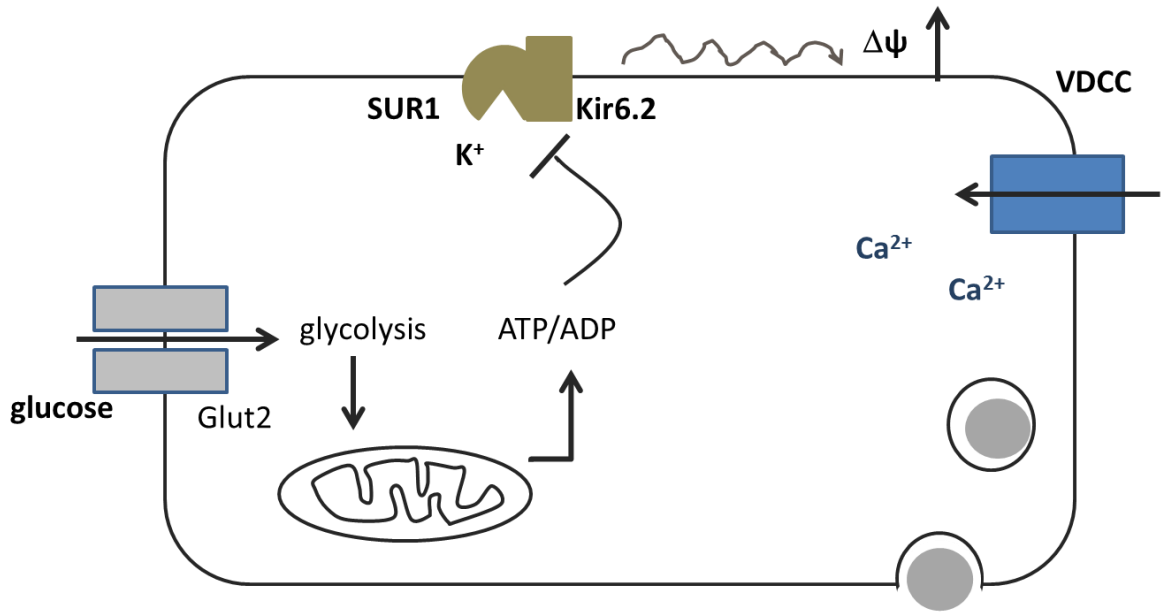


Fig.I-3: Mechanism of glucose-stimulated insulin secretion
Glucose enters the glycolytic pathway by facilitated diffusion and is oxidized in the mitochondria. The rise in generated ATP closes ATP-sensitive K^+ channel, inducing a shift in the membrane potential. The resulting depolarization opens voltage-gated Ca^{2+} channels and leads to Ca^{2+}-influx, which eventually triggers vesicle fusion. Note that regulated exocytosis affects only the release of granules, while, in contrast, their *de novo* synthesis occurrs constitutively (adapted from De León and Stanley 2007).

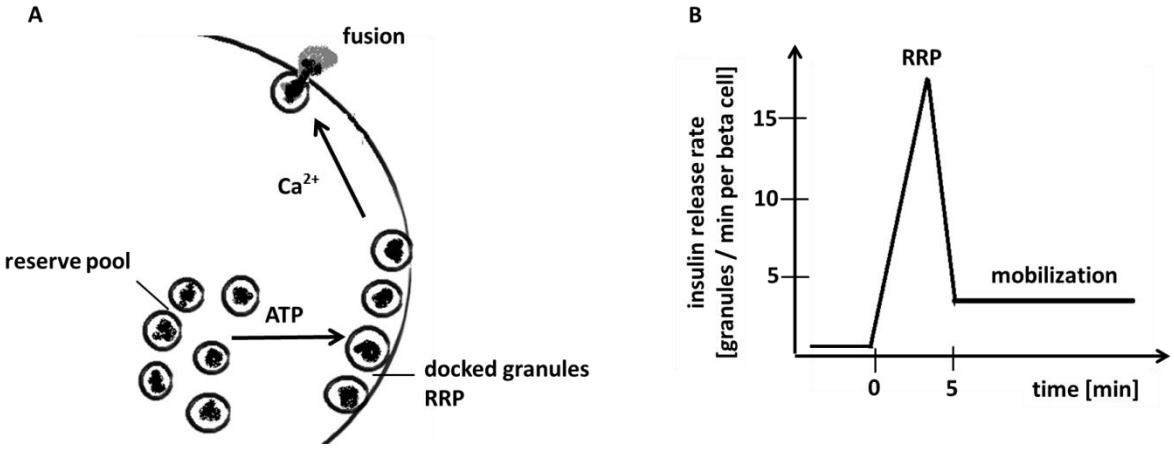


Fig. I-4: Biphasic glucose-stimulated insulin secretion results from release of distinct vesicle pools
(A) Beta cells are equipped with distinct populations of insulin-containing vesicles: The readily releasable pool (RRP) is docked at the cell membrane and is immediately released (within 5 min) after glucose stimulation. After that, a more distant vesicle pool is mobilized in an ATP-dependent manner. (B) Insulin release rate during the first and the second phase.

I.A.1.2 Electrophysiological properties of beta cells during the late stages in the insulin secretion pathway

During the late stages of the insulin secretion pathway, electrophysiological properties of the beta cell become crucial. At sub-threshold glucose concentrations, ATP-dependent K^+ (K^+_{ATP}) channels are open enabling a free flux of K^+ and, thus, the maintenance of a membrane potential of ca. -70 mV. When blood glucose rises, the beta cell responds with progressive closure of K^+_{ATP}-channels, leading to a depolarization of the membrane (Ashcroft and Rorsman 1989) **[Fig.I-3]**. At stimulatory glucose concentrations, beta cell depolarization reaches a threshold, inducing bursts of action potentials. These bursts are synchronically accompanied by oscillations in intracellular Ca^{2+} concentration, ultimately driving pulsatile insulin secretion (Bergsten et al. 1994; Barbosa et al. 1998) [**Fig. I-5].**

Electrical activity and bursts in intracellular [Ca^{2+}] occur in a highly coordinated manner simultaneously in all beta cells in the pancreatic islet, thus rendering it a "secretory syncytium" (Santos et al. 1991). There, cell-to-cell communication through both chemical and electrical pathways augments timely synchronized spikes of insulin secretion from the entire Langerhans islet (Benninger et al. 2008). This is achieved, for instance, through various secreted factors located in insulin granules or other microvesicle populations, which are released over the course of glucose stimulation. Among them are neurotransmitters (ATP and GABA) and Zn^{2+} ions, which induce a rise in $[Ca^{2+}]_i$ in an autocrine manner (Braun et al. 2012). Furthermore, gap junctions mediate the spatiotemporal coupling in electrical activity in the pancreatic islet. Accordingly, synchronization in membrane depolarization, bursting activity and intracellular [Ca^{2+}] waves is achieved through a beta cell network of 6-7 adjacent cells linked through gap junctions (In't Veld et al. 1986; Zhang et al. 2008).

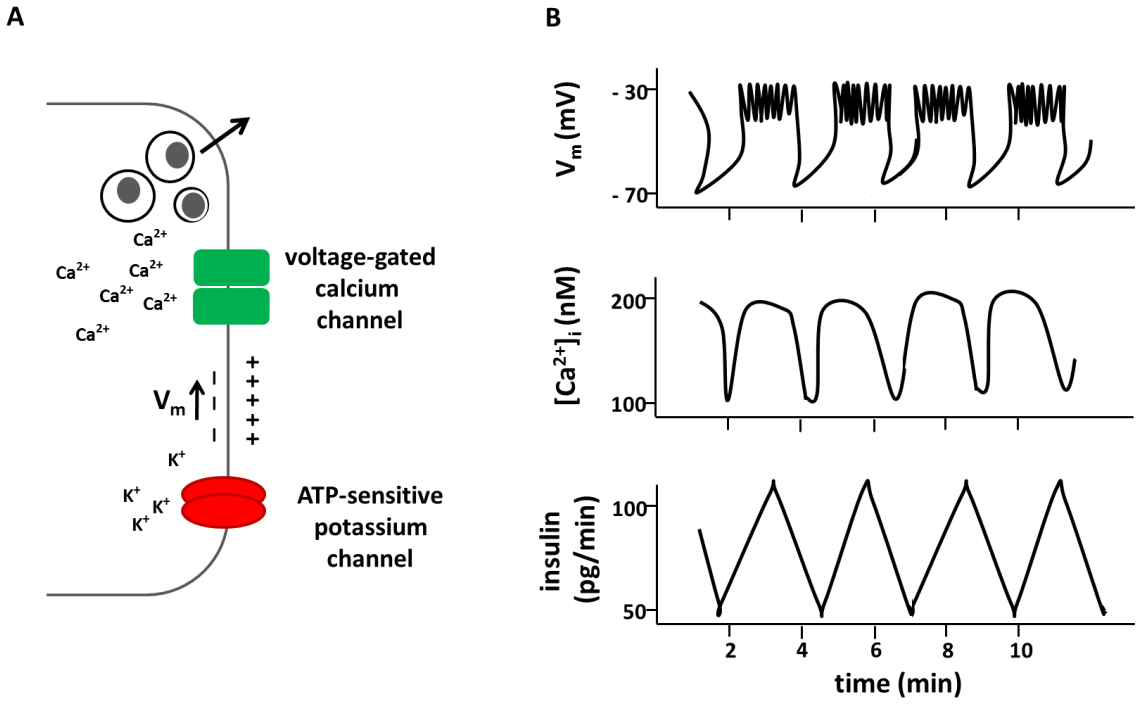


Fig. I-5: Synchronous electrical activity in beta cells
(A) The sequence of events during glucose stimulation, which change the electrical properties of beta cells and lead to insulin secretion: Closure of K^+_{ATP}-channels depolarizes the cell memrane, which opens voltage-gated Ca^{2+}-channels and ultimately triggers exocytosis. (B) Bursts in electrical activity recorded from individual beta cells align with oscillating changes in intracellular calcium and pulsatile insulin secretion (top to bottom). In the islet syncytium, these processes are synchronized on the multicellular level due to intercellular coupling, leading to sustained pulses in insulin release from the entire islet (Note that the concentration range for insulin refers to islets and not to indivudual cells).

I.A.1.3 The mechanism of vesicle fusion

Insulin secretion shares many features with neurotransmitter release at the synapsis – both are Ca^{2+}-dependent and display similar molecular makeup (Lang 1999; Rorsman and Renström 2003).

The "minimal machinery" for exocytosis comprises members of the SNARE (soluble NSF (NH_2-ethylmaleimide-sensitive fusion protein) attachment protein receptor) family, which are located on the vesicle and the outer cell membrane (Söllner et al. 1993; Weber et al. 1998). Plasma lemma-associated SNAREs – SNAP-25 and syntaxin-1 – form complexes located in lipid rafts, in the proximity of voltage-gated L-type Ca^{2+}-channels, and interact with vesicle-associated SNAREs, such as synaptobrevin/VAMP-2, in a zipper-like manner (Vikman et al. 2003; Vikman et al. 2006). The exergonic folding of coiled coil protein domains during this interaction allows a thermodynamically favorable bilayer merge (Baumert et al. 1989; Rothman 1994). The post-fusion unfolding of SNAREs is dependent on ATP and involves the

adenosine triphosphatase NSF, which transforms them into the initial state for a re-cycle of association and exocytosis (Weber et al. 2000) **[Fig. I-6]**.

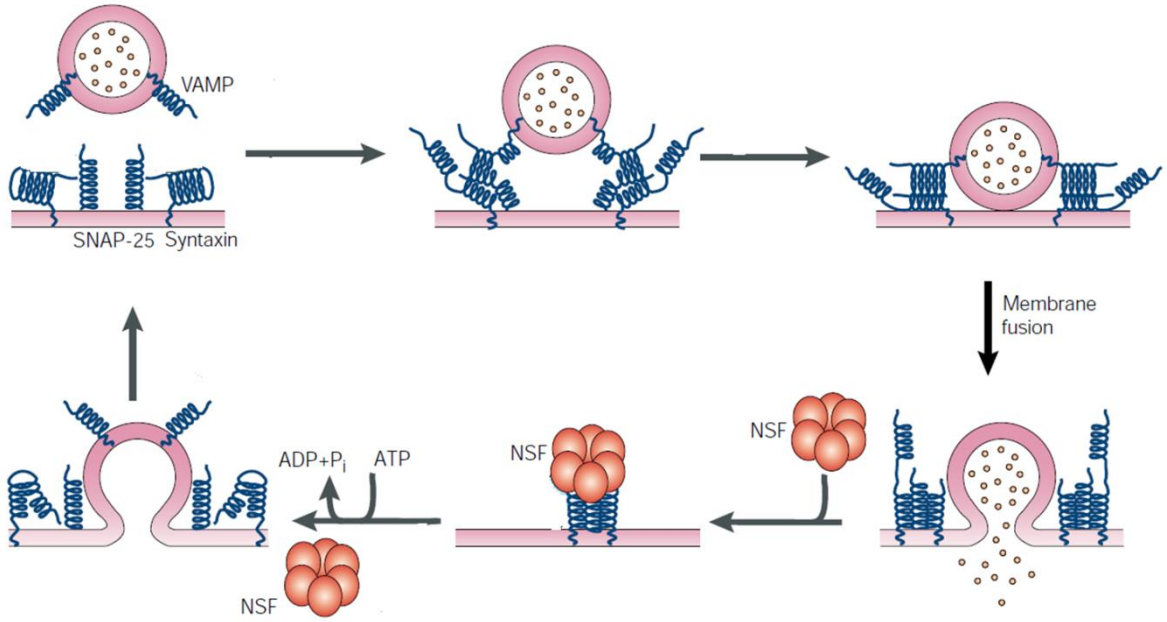


Fig. I-6: The "minimal machinery" for exocytosis – the SNARE cycle
At the initial stage of exocytosis, the free SNAREs (VAMP, SNAP-25 and syntaxin) are located on the vesicle and the plasma membrane (*trans*-SNARE). Their physical engagement and zipping induces a curvature tension of the membranes, forming a fusion pore. The post-fusion *cis*-SNAREs are recycled into the initial *trans*-SNARE condition under ATP-hydrolysis. Note that this model does not imply size and rate of fusion pore formation. Adapted from Chen and Scheller 2001.

Yet, the underlying molecular mechanism postulated by the "SNARE hypothesis" is slow and occurs spontaneously, as demonstrated by *in vitro* liposome fusion assays, while insulin secretion is fast and relies on glucose-stimulation (Söllner et al. 1993). Hence, the fusion machinery described above requires a superimposed dynamic control mechanism, which is responsive to Ca^{2+}. Two of the best studied examples for controllers of SNARE action in neuroendocrine cells include complexins and synaptotagmin (rev. in Südhof and Rothman 2009) **[**Fig.**I-7]**. Complexins act as molecular "clamps" for already built SNARE complexes, preventing the spontaneous merge of membranes (Reim et al. 2001; Giraudo et al. 2006; Tang et al. 2006). In contrast, transmembrane synaptotagmin functions as a Ca^{2+} sensor in beta cell exocytosis. Located in the insulin granule membrane, it responds to a rise of intracellular [Ca^{2+}] through two C2 domains. Ca^{2+}-bound synaptotagmin interacts with and / or displaces complexin, thus allowing the onset of membrane fusion through the action of the zippered SNARE complex (Brose et al. 1992; Barg et al. 2001; Iezzi et al. 2004; Tang et al. 2006; Gustavsson et al. 2008). Collectively, these molecular processes induce an ultrarapid and controlled pore formation underlying insulin exocytosis.

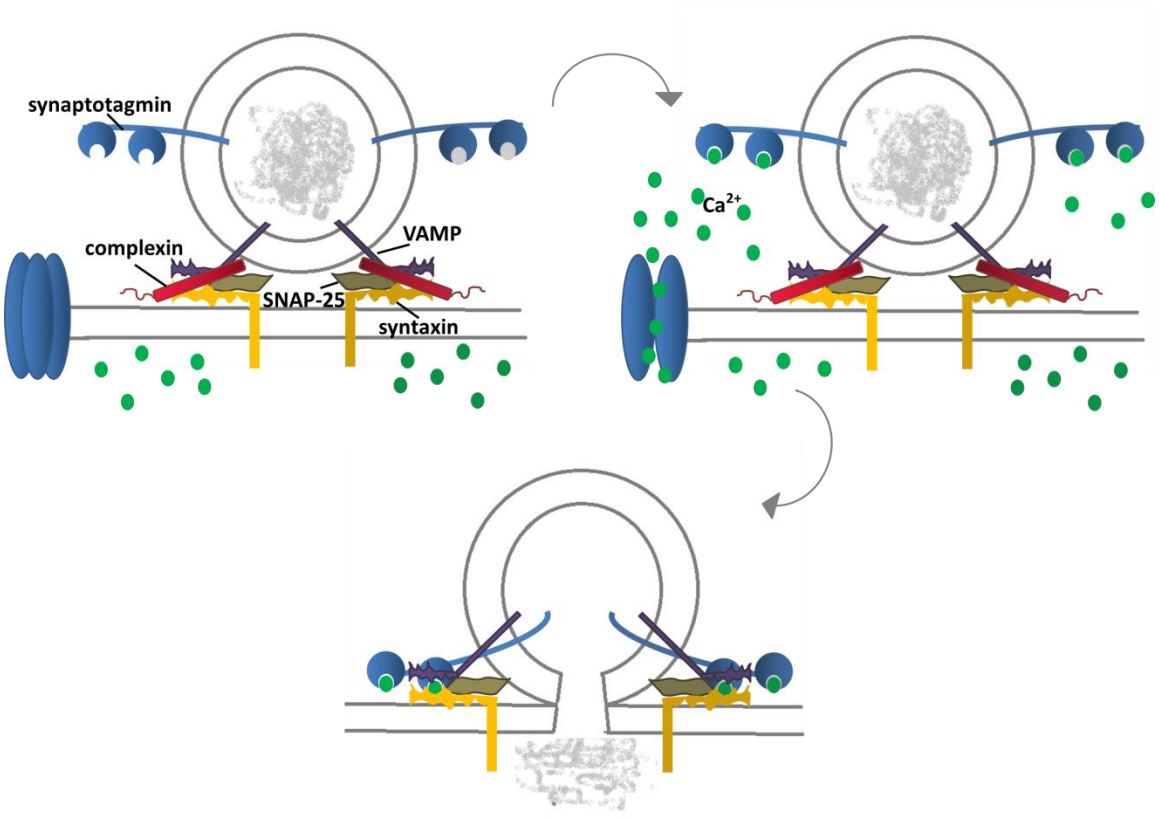


Fig.I-7: Ca^{2+}-controlled insulin granule exocytosis
Rapid and controlled fusion of insulin vesicles is ensured through components beyond the SNARE proteins VAMP, SNAP-25 and syntaxin. The readily-releasable vesicle pool is positioned in direct proximity to L-type Cav1.2 Ca^{2+}-channels (blue). In the absence of Ca^{2+}, the zippered SNARE assembly interacts with the soluble protein complexin (red), which blocks spontaneous membrane merge. As Ca^{2+}-channels open over the course of membrane depolarization, Ca^{2+}-bound synaptotagmin leads presumably to a replacement of complexin in order to activate the zippered SNARE complex. (The graphically represented stoichiometric protein relations are simplified.)

I.A.1.4 Different modes of insulin vesicle release and retrieval – classical vs. kiss-and-run secretion

After fusion of secretory vesicles with the plasma membrane, excess membrane is retrieved by endocytosis (Heuser and Reese 1973; Orci et al. 1973). Currently, two molecularly distinct mechanisms for exo- and endocytosis coupling in neuroendocrine cells exist (rev. in Eliasson et al. 2008).

The classical model relies on full exocytotic fusion during which granule interior is completely emptied into the extracellular space and its bilayer integrated into the plasmalemma **[Fig.I-8A]**. Here, membrane retrieval is *de facto* “uncoupled” from exocytosis since it is spatially and temporally segregated – endocytosis occurs distant from the site of

secretion and delayed by 15 s or more (Takei et al. 1996; Høy et al. 2002). Further, it is mediated by clathrin (Heuser and Reese 1973; Orci et al. 1973).

In contrast, in the alternative "kiss-and-run" model, vesicle retrieval occurs in less than one second post exocytosis and is independent of clathrin (Aravanis et al. 2003; Harata et al. 2006). In this scenario, exo- and endocytosis are temporally and spatially coupled – membrane merge is only partial, followed by immediate vesicle reformation, allowing for a new round of secretion **[Fig.I-8B]**. Here, the transient and incomplete granule collapse with the plasma membrane prevents full vesicular cargo release (Fesce et al. 1994; Klingauf et al. 1998; Klyachko and Jackson 2002; Aravanis et al. 2003; Gandhi and Stevens 2003; Tsuboi and Rutter 2003; Harata et al. 2006). Hence, besides fast vesicle retrieval, kiss-and-run exocytosis represents a mechanism for size-based secretion. It functions as a molecular sieve, which retains insulin in the granule interior since its monomer size comprises ca. 3x3x3 nm, while pore diameter typically is $\leq$ 1,4 nm (Takahashi et al. 2002;Tsuboi et al. 2004). In contrast, it permits extracellular release of low-molecular vesicle compounds, such as ATP, Zn^{2+} and GABA (Lindau et al. 2003; Tsuboi and Rutter 2003; MacDonald et al. 2006).

The decision signal for endorsing classical vs. kiss-and-run exocytosis in beta cells is still under debate. Fusion pore size and membrane reformation kinetics are believed to depend on intracellular cAMP and Ca^{2+} concentrations (MacDonald et al. 2006; Hanna et al. 2009; Elhamdani et al. 2006). In addition, dynamin, complexin II and synaptotagmin IV modulate the stability and reorganization of the zipper-like SNARE complex formed across vesicle and plasma membrane and are involved in the kinetic and type of exocytosis (Tsuboi et al. 2004; Archer, et al. 2002; Wang et al. 2001).

Remarkably, kiss-and-run exocytosis accounts for approximately 30 per cent of all fusion events involving insulin-containing granules in beta cells (MacDonald et al. 2006; Hanna et al. 2009).

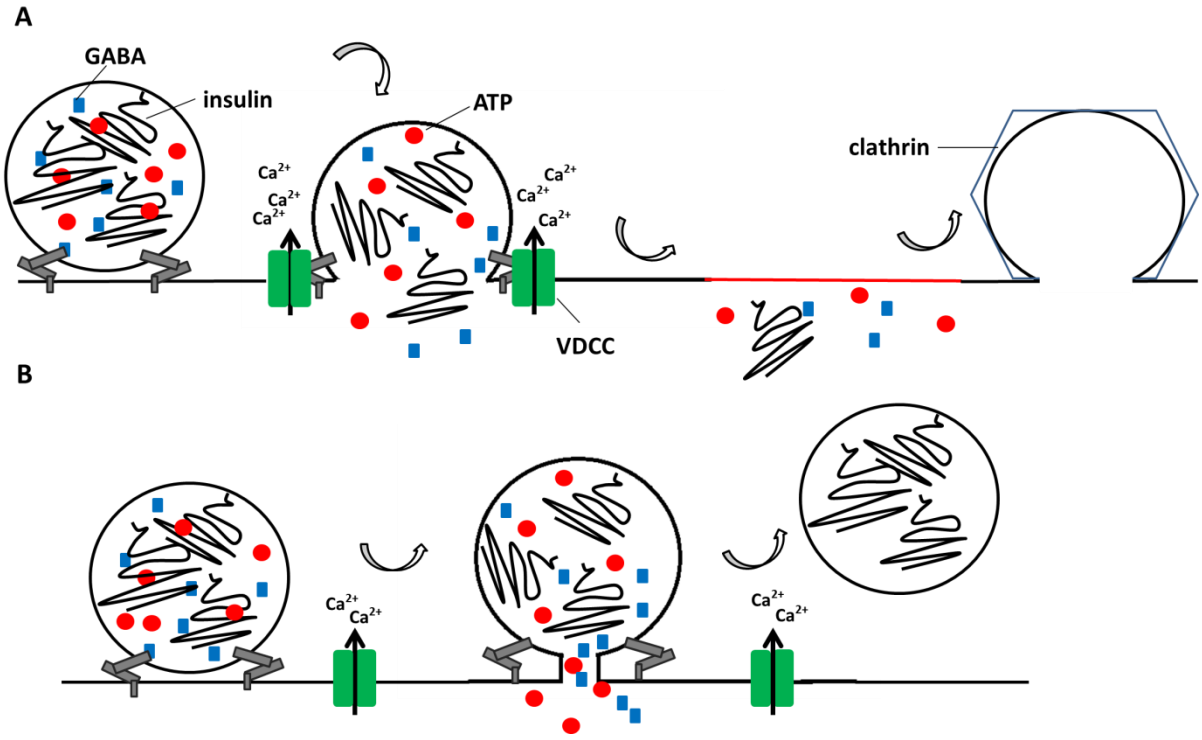


Fig.I-8: Two modes for membrane fusion of insulin-containing vesicles.
(A) Classical insulin granule exocytosis: After a complete vesicle merge and a total cargo release, temporally and spatially separated clathrin-mediated endocytosis occurs. (B) Kiss-and-run exocytosis: The vesicle undergoes a rapid exocytotic-endocytotic cycle, during which vesicle membrane transiently and partially fuses wih the plasma lipid bilayer. As a result, insulin is retained, while small molecular compounds, such as ATP and neurotransmitters are released into the extracellular space. Note that proximity of the docked vesicle to voltage-dependent Ca^{2+}-channels (VDCC) and its exposure to [Ca^{2+}] is considered one factor favouring full over kiss-and-run exocytosis (Adapted from Eliasson et al. 2008).

I.A.1.5 Insulin function

Whereas insulin exerts a mitogenic role during embryogenesis, in postnatal life it controls energy deposition (rev. in Genua et al. 2009). It is a "satiety signal", released when fuel is abundant to drive calorie uptake and storage in target tissues. In the postprandial phase, insulin promotes glucose uptake and synthesis of macromolecular polymers, such as glycogen, lipids and proteins, while blocking their decomposition and release into the blood stream (rev. in Saltiel and Kahn 2001).

The anabolic effects of insulin are mediated through tyrosine kinase receptor signaling and induction of several downstream pathways **[**Fig.I-9**]** (rev. in Taniguchi et al. 2006). In adipocytes and myocytes, ligand binding triggers translocation of glucose transporter 4 (Glut4) to the cell surface and facilitated glucose uptake. In hepatocytes, it leads to inhibition of glycogen synthase kinase 3 beta (GSK3beta) and FOXO1 and, hence, to a stimulation of glycogen synthesis and a blockage of gluconeogenesis, respectively.

Importantly, insulin resistance is pathological and associates with the condition of obesity and type 2 diabetes. There, the insulin signaling cascade is impaired, resulting in a failure in glucose uptake and deposition which contributes to a hyperglycemic state (DeFronzo et al. 1992). It often results from defects downstream of the insulin receptor, such as enhanced activity of phosphatases from the Suppression of Cytokine Signaling (SOCS) protein family, which ablate signaling transduction by insulin (rev. in Taniguchi et al. 2006).

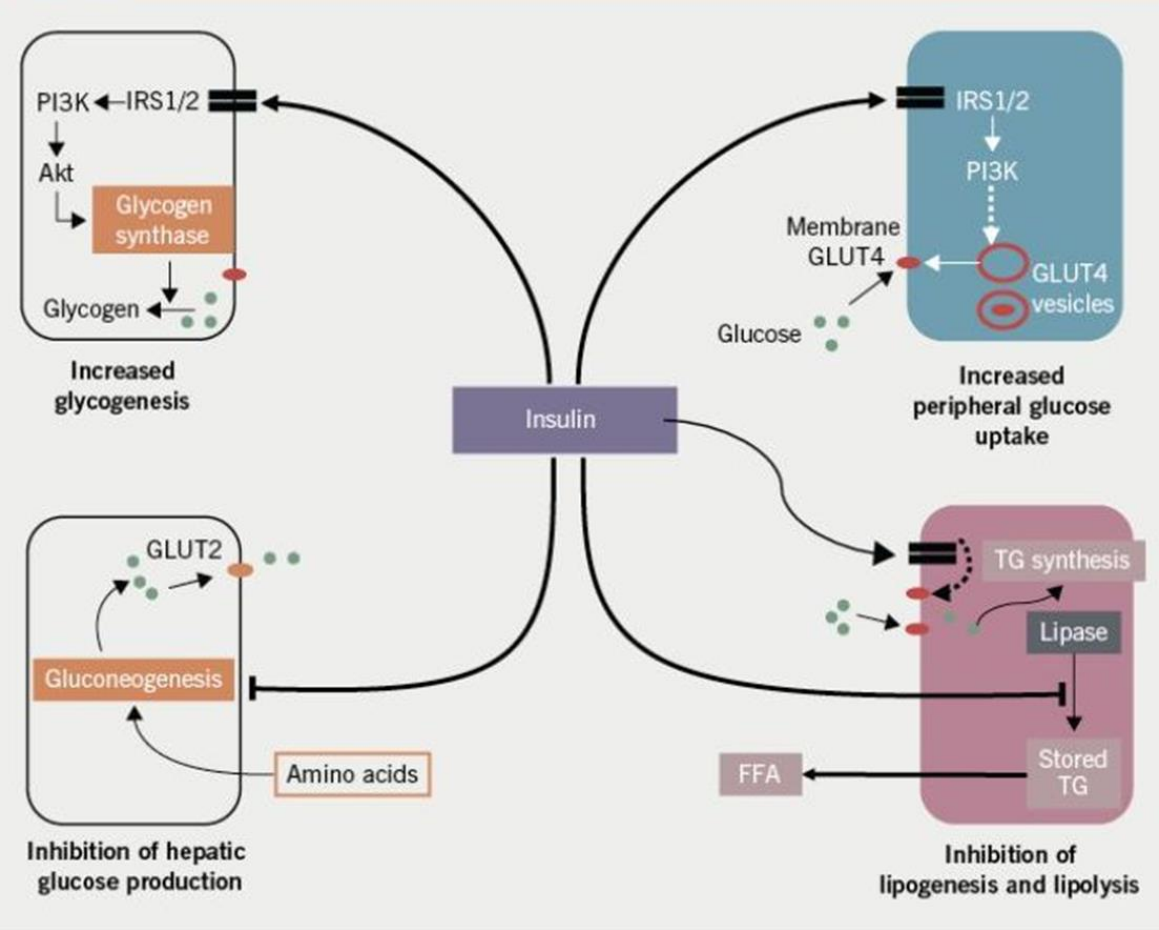


Fig.I-9: Anabolic action of insulin
The main outcomes of insulin binding to its receptor are represented. Most of the downstream effects of insulin are conducted through activation of the phosphatidylinositol 3-kinase (PI3K). In hepatocytes, insulin induces blockage of gluconeogenesis and stimulation of glycogenesis through inhibition of Foxo2 and GSK3beta (left panels). In adipose and muscle cells, it leads to glucose uptake, protein synthesis and lipogenesis. These effects are mediated through upregulation of the GLUT4 receptor and the transcription factor SREBP1 (right panels). (Adapted from Br J Cardiol. 2011;18(5/6):224-228)

I.A.1.6 Diabetes type 2 and the hyperglycaemic state

Insulin is unique in its hypoglycemic and anabolic action and defects in insulin production and/or signaling have detrimental health effects. Hyperglycemia characterizes the pathological state of diabetes mellitus type 2. It is particularly cruel as it disrupts electrolyte balance and, hence, organ function (Tzamaloukas et al. 2008). This is further potentiated

through the reactive nature of the aldehydic group of glucose, which leads to unspecific protein glycosylation, oxidative stress and cellular toxicity (Judzewitsch et al. 1983; Vlassara et al. 1983; Stevens et al. 1995). In fact, glucotoxicity is considered to feedback on beta cell function and progressively deteriorate it over the course of the hyperglycemic state (rev. in Poitout and Robertson 2002).

To over 90 per cent diabetes type 2 is caused by impaired beta cell function and insulin secretion (Stumvoll et al. 2005). The disease is recognized as a multigenic disorder, whose exact molecular mechanisms are still unclear. Low-frequent monogenic diabetes in humans has provided a source for the elucidation of genes, essential for beta cell function. They include transcription factors – HNF-4α, Pdx-1, Foxa2, NeuroD1 – which form cooperative networks to induce "beta cell identity", to activate beta cell maturation, insulin transcription and secretion (Yamagata et al. 1996; Dukes et al. 1998; Frayling et al. 2001; Bell and Polonsky 2001; Wang et al. 2002). Other crucial genes, whose hypomorphic mutation leads to development of diabetes participate in the insulin secretion pathway and involve glucose sensing (Gck, glucokinase) and potassium channeling (K_{ir}6.2, subunit of the ATP-sensitive K^+-channel) (Bell et al. 1996; Gloyn et al. 2001; Gloyn et al. 2003). Recently, genetic manipulation studies in mouse have also highlighted the importance of non-coding RNAs in beta cell function [1] (Lynn et al. 2007; Poy et al. 2009).

Diabetes type 2 has emerged as a major pandemic, affecting over 340 million people worldwide with a tendency for exponential increase (World Health Organization, 2011). Beyond genetic factors, lifestyle and diet are recognized as a strong component for the onset of this disease. Diabetes, obesity and dyslipidemia often co-exist and constitute a complex heterogeneous disorder, termed "metabolic syndrome" (Stumvoll et al. 2005). The etiology of type 2 diabetes is further manifested in increased hepatic glucose production[2] (DeFronzo et al. 1992).

I.A.2 The liver and systemic glucose metabolism

The liver represents the largest internal organ involved in energy homeostasis. It consists to 80% of polyploid hepatocytes and contributes, among other functions, to the maintenance of physiological blood glucose concentrations (Styles 1993). When circulating glucose is abundant, the liver acts as a depot and absorbs the monocarbohydrate in a concentration-

[1] For the role of microRNAs in glucose homeostasis and diabetes refer to section I.C.2
[2] For the role of hepatic glucose production in hyperglycemia and diabetes refer to Section I.A.2

dependent manner in order to convert it into glycogen (rev. in Roach 2002) [**Fig.I-10]**. In contrast, over short periods of fasting, the liver supplies peripheral tissues with glucose derived from glycogenolysis. Further, hepatocytes are unique in their ability to perform *de novo* glucose synthesis (gluconeogenesis) [**Fig.I-10]**. Importantly, elevated hepatic glucose production is often present in diabetic patients and represents the main target of anti-hyperglycemic drug therapies (Bogardus et al. 1984; Stumvoll et al. 1995).

Hepatic glucose output is modulated through systemic hormones, mainly insulin and glucagon, on the transcriptional and post-translational level (rev. in Lin and Accili 2011). The two beta cell-derived peptides exert reciprocal regulation on the metabolic flux of glycolysis and glycogenolysis through activation / deactivation of the respective rate-limiting enzymes (glucokinase, glucose-6-phosphatase, phosphofructokinase, fructose-1,6-bisphosphatase, pyruvate kinase, phosphoenolpyruvate carboxykinase, glycogen synthase and phosphorylase) [**Fig.I-10]** (O'Brien and Granner 1996; Cherrington and Lecture 1999).

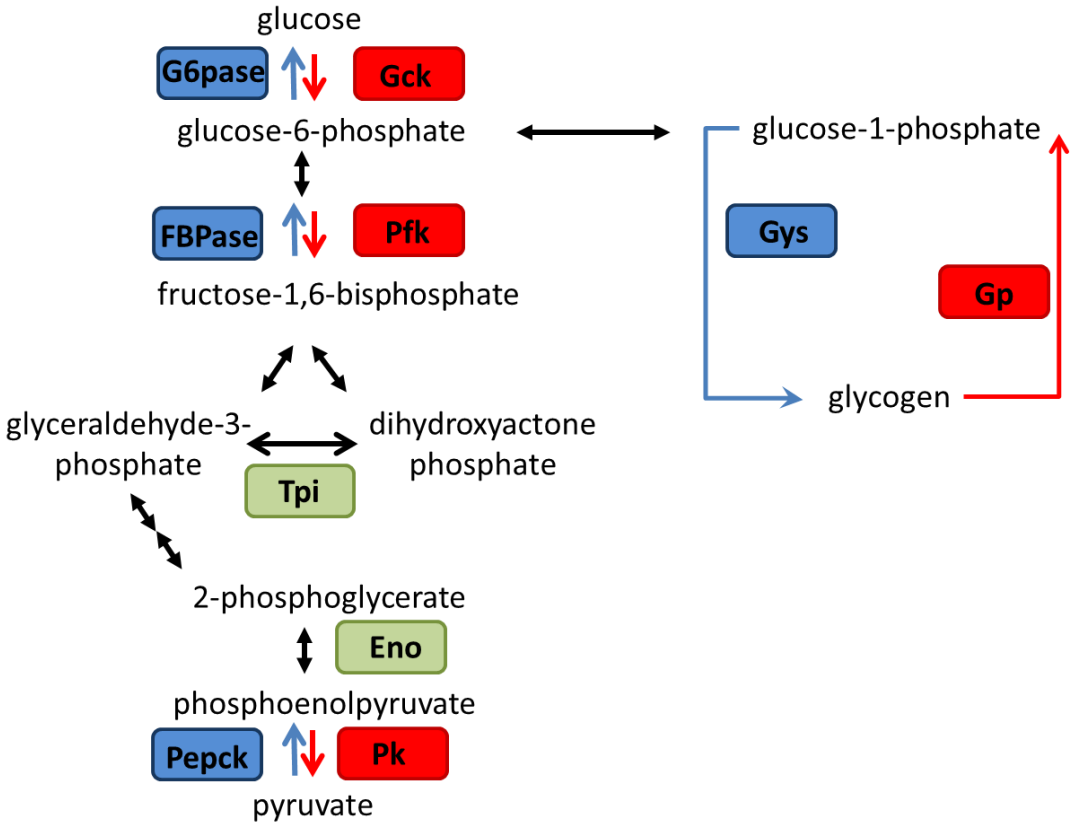


Fig.I-10: Pathways regulating hepatic glucose output
After glucose is absorbed by the liver, it is phosphorylated and processed further to pyruvate in the process of glycolysis (not all biochemical steps are shown). Over the reverse route, glucose is synthesized from pyruvate. All reactions can be reversed except for conversions, catalyzed by the indicated rate-limiting enzymes (blue and red). Alternatively, glucose can serve as precursor for glycogen, which is broken down, when demand for energy

rises. Gpase – glucose-6-phosphatase, Gck – glucokinase, FBPase – fructose-1,6-bisphosphatase, Pfk – Phosphofructokinase, Tpi – triosephosphate isomerase, Eno – enolase, Pepck – phosphoenolpyruvate carboxykinase, Pk – pyruvate kinase, Gys – glycogen synthase, Gp – glycogen phosphorylase

I.A.3 The liver and systemic lipid metabolism

Lipids occur in several forms in the organism - as energy depots in adipocytes, as membrane barriers and determinants of membrane fluidity and as bioactive signaling molecules. Due to the architecture, insolubility and reactive nature of fatty acids and cholesterol, their blood vessel transport is possible only in an esterified form, packaged within phospholipid complexes, stabilized with protein (rev. in Brown and Goldstein 1986). Hepatocytes have evolved the unique ability to generate these lipoprotein complexes and export them to peripheral tissues (Oncley 1956; Fredrickson 1974). The lipids in these lipoprotein particles can be exogenously or endogenously derived.

I.A.3.1 Hepatic transport of endogenous lipids – lipid biosynthesis and VLDL assembly

The fact that only 20% of circulating cholesterol originates from the diet and that *de novo* triglyceride synthesis occurs solely in liver and adipose tissue, emphasizes the central role of hepatic lipogenesis and systemic transport of endogenous lipids (rev. in Kennedy 1961; Hegele 2009).

Triglycerides represent a neutral form of fatty acids esterified with glycerol, which is ca. twofold energy-richer than carbohydrates or proteins **[Fig. I-11A]** (Havel 1972). Fatty acids are generated by the fatty acid synthase (Fasn) in the cytoplasm, elongated and desaturated in the endoplasmic reticulum, and esterified to the glycerol backbone (rev. in Postic and Girard 2008). Cholesterol biosynthesis is initiated from Acetyl-CoA, undergoing a series of 30 enzymatic steps with the rate-limiting conversion catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase (Hmgcr) to yield a bulky steroid ring structure with a hydroxyl group **[Fig. I-11B]** (rev. in Bloch 1965). Free fatty acids are reactive electron donors and contribute to the formation of reactive oxygen species causing mitochondrial and ER stress. On the other hand, excess cholesterol readily precipitates and forms atherogenic extracellular plaques (Robison et al. 1971). Hence, endogenously derived lipids enter the vesicular cycle in form of very-low-density lipoprotein (VLDL) complexes **[Fig. I-11C]** (rev. in Alaupovic 1982).

VLDL assembly is an intrahepatocellular process which requires the function of lipid-binding apoproteins, such as ApoB, and the chaperon microsomal triglyceride transfer protein (MTP)

(Cartwright and Higgins 1995) **[Fig. I-12A]**. As ApoB is translated at the endoplasmic reticulum, MTP binds the peptide, recruiting triglycerides, cholesteryl esters and phopspholipids, allowing ApoB to fold around a lipid core and to form a small emulsion – the pre-VLDL particle. The continuous fusion of the nascent particle with triglyceride-rich lipid droplets over its passage through the Golgi apparatus eventually forms mature, 30-80 nm in diameter, VLDL destined for release (rev. in Kang and Davis 2000).

In the circulation, the triglycerides associated with VLDL are hydrolyzed by lipoprotein lipase (LPL) residing on the luminal surface of capillary endothelium, and taken-up by adipose or muscle tissue [**Fig.I-13]** (rev. in Hegele 2009). The remaining cholesterol-rich remnants (intermediate-density lipoproteins, IDL) are scavenged by hepatocytes or, alternatively, transformed into low-density lipoproteins (LDL) to deliver cholesterol cargo to peripheral tissues via the LDL-receptor.

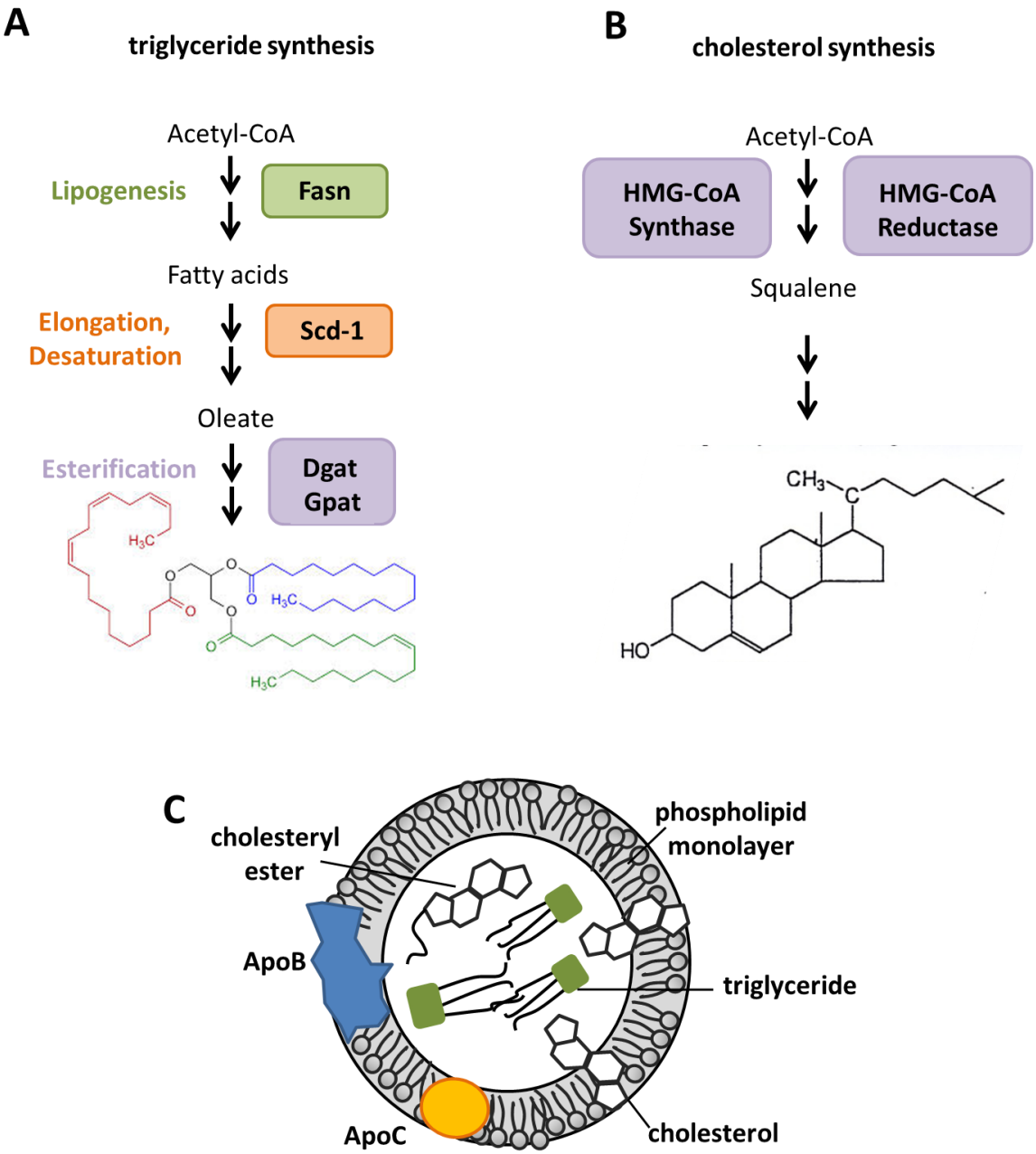


Fig. I-11: Lipogenic pathways and the lipid transporter VLDL
(A) Fatty acids are elongated, desaturated and esterified with a glycerol backbone. Fasn, fatty acid synthase; SCD1, stearoyl-CoA desaturase 1; GPAT, glycerol 3-phosphate acyltransferase; DGAT, diacylglycerol acyltransferase (B) The rate-limiting step in cholesterol synthesis is catalyzed by HMG-CoA reductase. Not all reaction steps are depicted in A and B. (C) The composition of the very-low density lipoprotein (VLDL) complex. VLDL particles are assembled and secreted from the liver. Each particle carries one molecule of apoB as well as apoE (not depicted) and the C apolipoproteins, apoC1, C2, and C3. The particle carries amphipathic lipids (phospholipid and free cholesterol) on its surface and hydrophobic lipids (cholesterol ester and triglyceride) in its inner core.

I.A.3.2 Transcriptional regulation of hepatic lipogenesis and VLDL export

Lipids are important energy suppliers but, at the same time, can evoke detrimental effects on cells. Thus, hepatic lipid synthesis and export are tightly regulated on the transcriptional level.

The transcription factors involved in lipogenesis and lipoprotein generation are often ligand-activated, e.g. through sterols and fatty acids. They act as sensors of the cellular lipid status to stimulate expression of complete genomic sets, bearing responsive elements. The master regulators of lipogenesis are members of the sterol regulatory element binding protein (SREBP) family, which upregulate enzymes in fatty acid and cholesterol synthesis as well as enzymes supplying acetyl-CoA units (Horton 2002). SREBP undergoes posttranslational activation at low sterol concentrations, thus ensuring that transcription of targeted gene sets responds to substrate availability and consumption. Other transcription factors include LXR, ERRα and PPARα (rev. in Blasiole et al. 2007). Apart from upregulating genes involved in triglyceride synthesis, they control assembly and lipidation of VLDL **[Fig. I-12].**

Importantly, lipogenic transcription factors are highly dependent on coactivator proteins to drive transcriptional control, such as Pgc-1α and Pgc-1β (Lin et al. 2005). Pgc-1α controls the adaptive response towards starvation as it stimulates fatty acid oxidation. On the other hand, the homologous Pgc-1β enhances lipid synthesis and lipoprotein production in liver. Pgc-1β mediates its effect through coordinate binding with SREBP, ERRα and Foxa2 **[Fig. I-12B]** (Lin et al. 2005; Wolfrum and Stoffel 2006; Hernandez et al. 2010). Due to its potent action, Pgc-1β might serve as a therapeutic target for treatment of dyslipidemia (Nagai et al. 2009; Hernandez et al. 2010).

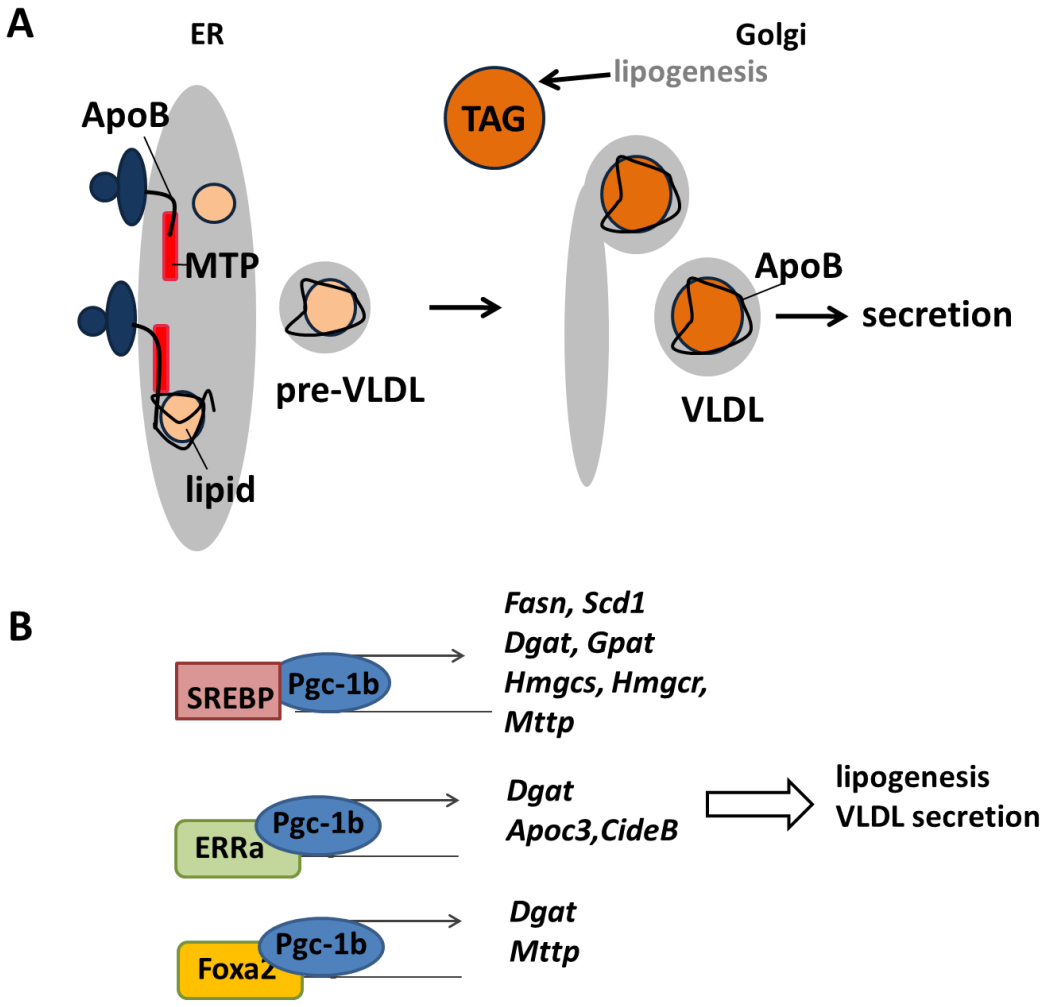


Fig. I-12: Lipoprotein assembly and secretion in hepatocytes
(A) VLDL is generated as the nascent apoB polypeptide is synthesized at the ER. The chaperone microsomal triglyceride transfer protein (Mtp) transfers lipids (yellow), including phospholipids, to stabilize nascent apoB during translation, thereby leading to the formation of an emulsion of apoB and lipid in pre-VLDL particles. Pre-VLDL particles fuse with luminal triglyceride droplets, also formed by MTP activity. The VLDL precursor undergoes association with further triglycerides in the Golgi apparatus, where VLDL is eventually sorted for secretion. The triglyceride content of VLDL is derived from the plasma free fatty acid pool, from uptake of dietary lipoproteins (chylomicron remnants), and from *de novo* lipogenesis. (B) Lipogenic enzyme expression and the genetic program regulating VLDL synthesis and secretion are regulated by the transcriptional coactivator Pgc-1β acting together with SREBP, ERRα or Foxa2.

I.A.3.3 Systemic transport of exogenous lipids

Apart from endogenously synthesized lipids, exogenous lipids can also be shuttled across tissues.

Emulsified dietary fats are hydrolyzed by lipases in the jejunum in order for free fatty acids and cholesterol to get absorbed [**Fig.I-13]**. Subsequently, triglycerides are reconstituted in enterocytes and packaged together with cholesteryl esters on chylomicrons (rev. in Langdon and Phillips 1961). These large-diameter particles are destined for vesicular distribution, so

that free fatty acids can be released into target adipocytes and myocytes under the action of lipoprotein lipase, attached on endothelial cells.

The chylomicron-remnants are taken up by the liver via the hepatic LDL receptor (LDLR) of LDLR –related protein-1 (LRP1) and lysosomally degraded into their basic components – free fatty acids, glycerol, cholesterol, phospholipids and amino acids (Grundy 1983). Unlike adipose tissue, the fat precursors are not re-built and stored, but, instead, employed for VLDL production and re-distribution.

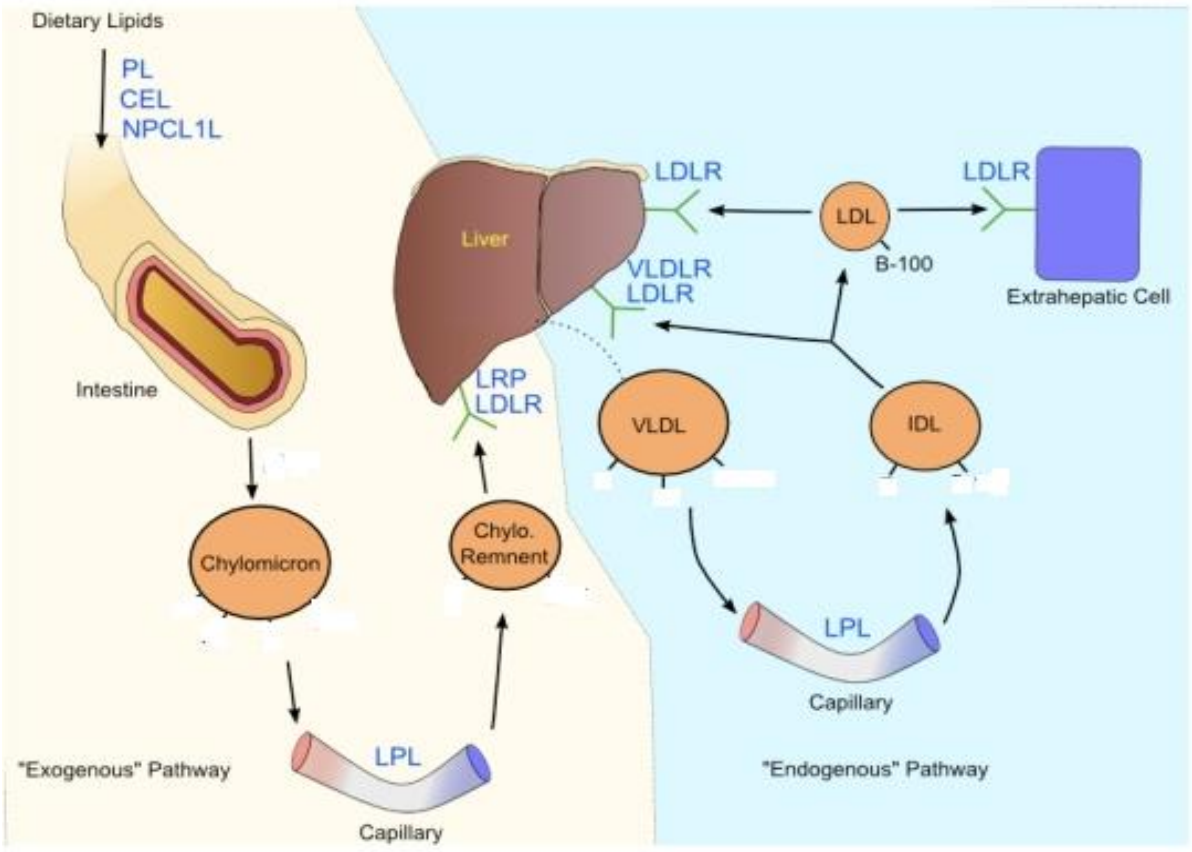


Fig.I-13: The lipoprotein cycle
Triglyceride (TG)-rich lipoproteins are secreted by intestine (chylomicrons) and liver (VLDL). Whereas chylomicrons are exclusively carrying exogenous fat and cholesterol, VLDLs are predominantly enriched in endogenously synthesized triglycerides. Chylomicrons and VLDL undergo lipolysis in the circulation at endothelial cells, thereby delivering fatty acids to tissues. Chylomicron-remnants are taken up by the liver and their residual lipid content is further used for assembly of hepatic VLDL. VLDL remnants are further metabolized to cholesterol-rich LDL. HDL serves as a vehicle for excess cholesterol from peripheral cells to hepatocytes (adapted from Daniels 2009).

B. Circadian rhythms

Circadian rhythms pervade life as virtually all physiological processes are adapted to the alternating day and night cycle (Szymanski 1920; Hastings 1991; Kriegsfeld et al. 2002). The time-keeping system is highly conserved from cyanobacteria to mammals and equips the organism with the ability to anticipate environmental changes, thus improving fitness and survival (Young and Kay 2001; Bell-Pedersen et al. 2005). It is hierarchically structured, with the anatomical center located within the suprachiasmatic nucleus (SCN), a hypothalamic area comprising 10 000 – 15 000 neurons [**Fig.I-14]** (Moore and Eichler 1972; Ralph et al. 1990). The SCN receives photic input from nonvisual photoreceptors in the retina conferring precise entrainment with a period of 24 hours (Rusak and Zucker 1979; Hankins et al. 2008). Importantly, circadian rhythms are preserved in environment lacking periodic input, such as constant darkness, indicating the presence of an intrinsic timing mechanism, which only requires adjustment i.e. entrainment by exterior stimuli (Szymanski 1920; Welsh et al. 1995). The central clock in the SCN controls neuroendocrine function of various brain regions to ultimately regulate cycles in behavioral processes, such as sleep/wake activity, locomotion and food intake (Namihira et al. 1999; Masubuchi et al. 2000; Abe et al. 2002; Guilding and Piggins 2007). Beyond the central pacemaker, the existence of extra-neuronal molecular clocks has recently emerged [**Fig.I-14]** (Kohsaka and Bass 2007). Peripheral tissues, such as liver, adipose tissue, macrophages and skin, have been reported to possess oscillators (Yoo et al. 2004; Shimba et al. 2005; Lamia et al. 2008; Keller et al. 2009; Spörl et al. 2012). They control local functions, ranging from metabolic pathways to cell proliferation and differentiation. The timing accuracy of these "slave" oscillators is achieved through SCN-derived innervation and humoral factors, as well as by periodic reoccurrence of environmental cues, such as food intake or temperature (Meyer-Bernstein et al. 1999; Silver et al. 1996; Kramer et al. 2001; Cheng et al. 2002; Stokkan et al. 2001; Brown et al. 2002; see I.B.3.5). Circadian oscillations are cell-autonomous, as they are maintained in peripheral tissues *ex vivo* or even in some immortalized cell lines (Balsalobre et al. 1998; Yoo et al. 2004). Importantly, intrinsic pacemakers of individual cells are coupled intercellularly in order to confer synchronization of circadian rhythms in the entire tissue (rev. in Aton et al. 2005).

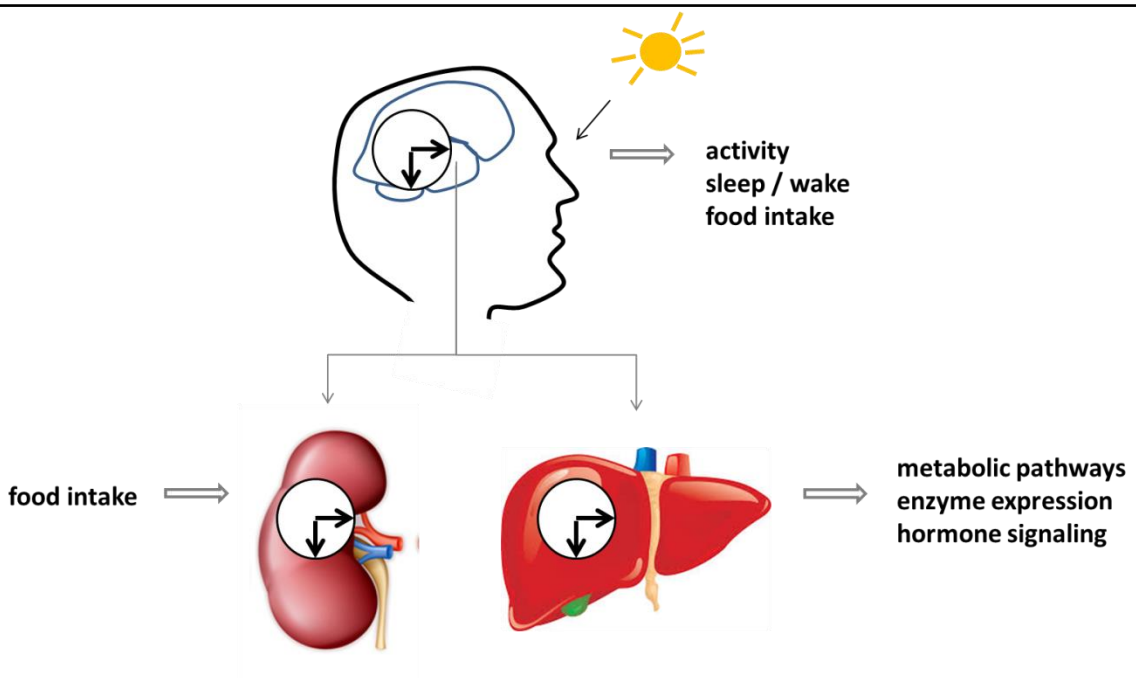


Fig.I-14: Hierarchical anatomical organization of the mammalian circadian system.
The master molecular clock is located within hypothalamic neurons of the suprachiasmatic nuclei (SCN), whose phase of electrical activity exhibits 24-hour period oscillations, entrained by photic input. Circadian rhythms in the SCN and other brain regions regulate behavioral output, such as sleep and wakefulness, food intake and locomotion. The slave oscillators located in peripheral tissues are controlled, on the one hand, by neuroendocrine stimulation of the SCN and, on the other, by repetitive occurrence of external stimuli, such as food. Peripheral clocks regulate the timing of metabolic pathways, such as the alternating response during fasting and feeding.

I.B.1 The molecular mechanism of the clock

Rhythmic gene expression underlies rhythmic physiological output by the endogenous timing system (Jin et al. 1999; Panda et al. 2002; Storch et al. 2002). The circadian pathway is ubiquitously present and its core molecular machinery is identical irrespective of its location in SCN neurons or periphery. The classical circadian model is based on a transcriptional-translational autoregulatory loop, whereby clock proteins feedback to negatively regulate their own transcription, thereby producing rhythmic clock gene expression **[Fig.I-15]** (Dunlap 1999; Reppert and Weaver 2002). At its center is a heterodimeric transcription factor Clock-Bmal1, which associates with E-box elements in promoter regions. Clock and Bmal1 activate *period* and *cryptochrome* genes to become negatively regulated by their cognate protein products **[Fig.I-15]**. Period and Cryptochrome translocate into the nucleus, dimerize and prevent Clock-Bmal1 DNA binding through recruitment of auxiliary proteins, such as casein kinase I (CKI) (Lee et al. 2001). Nuclear accumulation of the inhibitors undergoes posttranslational modifications and proteasomal degradation in an oscillating manner (Lee et al. 2001; Eide et al. 2005). In addition, other clock-controlled targets, such as the nuclear receptor Rev-Erb, inhibit Bmal1 transcription **[Fig.I-15]** (Preitner et al. 2002). In this way,

delayed suppression of the obligate components of the activating arm leads to a transcriptional-translational feedback loop with a 24-hour period and to rhythmic transactivation of output genes, carrying an E-box response element.

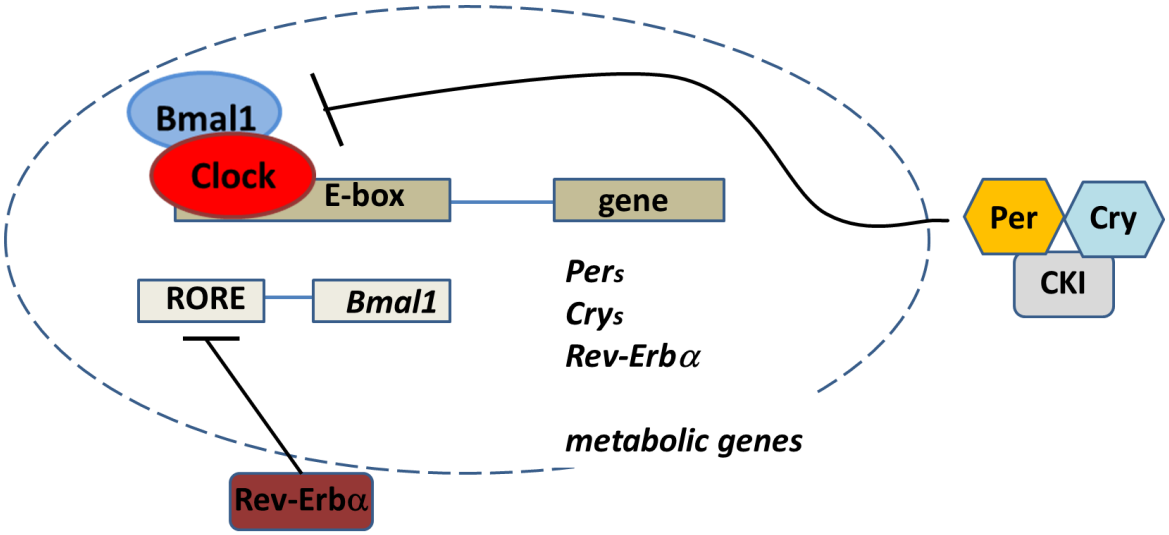


Fig.I-15: Molecular organization of the molecular clock.
The core circadian transcriptional mechanism is based on the Clock-Bmal1 heterodimeric complex, which binds to E-box promoter/enhancer sequences. The clock-controlled genes are either directly involved in metabolic output or constitute components of the feedback circadian loop, such as Per, Cry and Rev-Erb. The major arm of the circadian transcriptional translational feedback mechanism is composed of Per-Cry complexes, which block Clock-Bmal1 function. Their activity is modulated by the kinases CKI epsilon/delta, which guide their time-dependent translocation to the nucleus or proteasomal degradation. Other clock-controlled genes, such as Rev-Erb negatively regulate Bmal1-transcription.

I.B.2 Clock-Bmal1-mediated transcriptional activation

Importantly, the above outlined basic molecular clockwork, driven by Clock (Circadian Locomotor Output Kaput) and Bmal1 (Brain and Muscle Aryl hydrocarbon receptor nuclear translocator-like 1), is sufficient to induce oscillatory transcription of targeted genes (Dunlap 1999). Both proteins belong to the family of bHLH-PAS (basic helix–loop–helix Period–Arnt–Single minded) transcription factors. Whereas their bHLH protein domain confers DNA binding, the PAS regions are responsible for heterodimerization and nuclear translocation (Kondratov et al. 2003).

Clock possesses an intrinsic histone acetyltransferase activity, which is enhanced by Bmal1, thus, rendering the Clock-Bmal1 heterodimer a transcriptional activator (Doi et al. 2006). Beyond lysine acetylation of histones, Clock also acetylates its own partner Bmal1, which is essential for the transcriptional activity of the complex (Hirayama et al. 2007).

In the hypothalamic SCN, Clock is functionally substituted by Npas2 – a paralogue, which bears 84% - 90% identity in the amino acid sequence of the bHLH / PAS domains (King et al. 1997a; Zhou et al. 1997; Rutter et al. 2001; Debruyne et al. 2006).

Depending on the association of Clock-Bmal1 with accessory proteins, Clock-Bmal1 heterodimers are present in two alternating modes **[Fig. I-16]**. At ZT 6 – 8 [3], the "timesome" is in its active state, characterized through occupation of E-box DNA elements and maximal histone acetyl transferase activity (Ripperger and Schibler 2006). During this period, additional histone acetyltransferases dock to induce a fully transcriptionally permissive status at the targeted chromatin loci (Ripperger and Merrow 2011). In contrast, at the exact antiphase timepoint – ZT 18 – 20 – the complex reaches a nadir in transcriptional activity (Ripperger and Schibler 2006). Then, protein components of the multimeric "timesome" are hyperphosphorylated to counteract Clock-Bmal1 function. Most likely, phosphorylation of the two (bHLH)-PAS transcription factors leads to their dissociation from DNA, as occurring in orthologous genetic repertoires, such as of fly (Schafmeier et al. 2005; Kim and Edery 2006). Moreover, at this timepoint, the inhibitory complex recruits histone deacetylases and polycomb group methylases to actively mediate transcriptional repression and heterochromatin formation (Etchegaray et al. 2006; Ripperger and Schibler 2006). Hence, circadian regulation of transcription involves periodic histone acetyl transferase activity and chromatin remodeling, shaped through Clock-Bmal1 together with auxiliary clockwork components **[Fig. I-16]**.

Importantly, circadian rhythms are not solely manifested in transcription. Posttranslational modifications, such as phosphorylation, acetylation, protein oligomerization and redox activity, have also been reported to obey 24-hour periodicity (Lowrey and Takahashi 2000; O'Neill and Reddy 2011; Masri et al. 2013). Sustained clockwork mechanisms have been even highlighted for catalytic protein activity in biological systems devoid of transcription, such as erythrocytes or cell-free cyanobacterial lysates (Nakajima et al. 2005; O'Neill and Reddy 2011).

[3] ZT = Zeitgeber Time in hours; a standard dimension used to indicate time during the subjective day or night on a 12-hour light/ 12-hour dark cycle; ZT0 is defined as the onset of the light (day) phase, ZT12 – as the start of the dark (night) phase

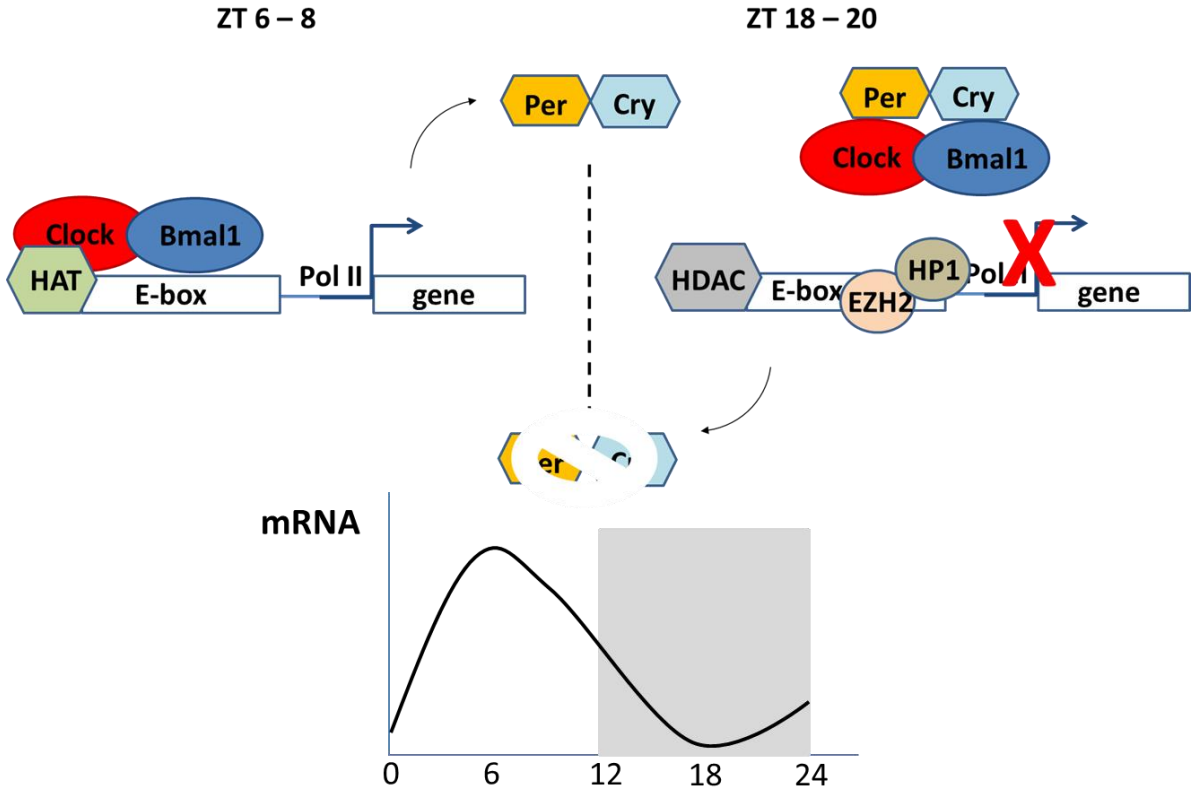


Fig. I-16: Rhythmic chromatin remodeling at Clock-Bmal1 targeted regions
In mouse, the Clock-Bmal1 transcriptional complex reaches highest activity at ZT 6-8, due to its association with histone acetyltransferase (HAT), such as p300, and due to maximal intrinsic HAT-activity of Clock. From this time onwards, the products of Per, Cry and other clock-controlled genes rise. Per and Cry associate with Clock-Bmal1 and lead to the formation of a transcriptional repressor multi-protein complex, whose activity is highest during night, at ZT 18-20. Through their binding, Per and Cry decrease transcriptional activity of Clock-Bmal1 and their association to DNA. In addition, they recruit chromatin remodelling factors, such as histone deacetylases, polycomb group proteins (EZH2) and heterochromatin-promoting proteins (HP1). The periodic epigenetic modulation results in cycling transcription at the targeted region. At the end of the cycle, Per and Cry proteins are ubiquitinated and degraded to initiate a new phase of Clock-Bmal1 activation.

I.B.3 Interconnection between circadian and metabolic networks

I.B.3.1 General physiological / epidemiological evidence

The general manifestation of circadian rhythms in physiology is reflected in recurrent phases of fuel utilization, corresponding to feeding / fasting cycles, and the associated oscillations in circulating hormones and metabolites, observed in mice and man (Hastings 1991; Minami et al. 2009; Dallmann et al. 2012). Some of them include the appetite regulator leptin, the stress hormone cortisol, insulin and plasma lipids (Herpertz et al. 2000; Pan and Hussain 2007). Likewise, glucose tolerance, determined through insulin secretion and insulin sensitivity, varies dramatically depending on the time of the day. In humans, it is highest just before the onset of the activity period in the morning (Carroll and Nestel 1973; Whichelow et al. 1974; Bolli et al. 1984; Lee et al. 1992). Importantly, circadian alterations of these physiological

parameters are abolished in patients suffering type 2 diabetes (Asplin et al. 1979; Lee et al. 1992).

The link between the biological clock and metabolism is further emphasized by epidemiological data. Perturbation of environmental zeitgebers and alteration in the light / dark cycle, such as the extensive exposure to artificial light in modern lifestyle, seem to negatively affect human health: Shift workers exhibit an increased incidence of obesity, hypertension and type 2 diabetes (Karlsson et al. 2001; Scheer et al. 2009). Likewise, rodents, exposed to light or enforced activity during the resting phase, develop features of the metabolic syndrome, such as glucose intolerance and obesity (Fonken et al. 2010; Salgado-Delgado et al. 2010). Importantly, already two weeks of circadian desynchrony induce signs of abnormal glucose homeostasis and ablation of circadian rhythms in metabolic gene expression (Barclay et al. 2012).

Finally, the importance of the heterodimeric duo Clock-Bmal1 in metabolic processes is underscored by means of genetic association studies. In humans, polymorphic variation in *clock* and *bmal1* correlates with predisposition for diabetes, overweight and cardiovascular complications (Woon et al. 2007; Scott et al. 2008). In rats, *bmal1* is part of a hypertension susceptibility locus: Single nucleotide polymorphisms (SNPs) within the *bmal1* promoter, associated with decreased *bmal1* expression, are significantly enriched in a rat model for spontaneous metabolic and cardiovascular complications (Woon et al. 2007). Furthermore, genome-wide association studies have revealed the relationship between other clockwork components and type 2 diabetes – *cry2* polymorphic variants are indicative of fasting hyperglycemia (Dupuis et al. 2010).

I.B.3.2 Genome-wide expression analyses on circadian metabolic output

So far, the best-studied example for metabolic output of the pacemaker is derived from liver. There, approximately 5-20 percent of the genome exhibit oscillating expression, as assessed by time-resolved analyses (Panda et al. 2002; Storch et al. 2002; Reddy et al. 2006; Hughes et al. 2009). This phenomenon is independent of light stimulation since it persists for several days in constant darkness, thus, pinpointing the cell-autonomy of the hepatic pacemaker (Asher and Schibler 2011). Furthermore, oscillation of the transcriptome is dominated by gene categories, whose expression peaks within the phases ZT 7 – ZT 12 and ZT 18 – ZT 22 (Storch et al. 2002). These two time intervals correspond to dusk and dawn, respectively, and represent the associated changes in energy utilization and the anticipation of food availability.

Hence, circadian metabolic output provides optimal enzyme levels when they are required and enable the temporal sequestration of energetically incompatible anabolic and catabolic reactions (Panda et al. 2002; Asher and Schibler 2011; Eckel-Mahan et al. 2012).

Moreover, large-scale studies highlight the extensive impact of circadian rhythms on various metabolic pathways, e.g. through regulation of rate-limiting enzymes in glycolysis, gluconeogenesis, fatty acid, cholesterol and xenobiotic metabolism (Panda et al. 2002; Storch et al. 2002; Reddy et al. 2006). In addition, expression profiling in different organs have revealed that circadian output is highly tissue-specific (Panda et al. 2002; Storch et al. 2002).

Analyses under conditional inactivation of the hepatic pacemaker reveal that 90 % of the cycling transcriptome are controlled by the intrinsic tissue pacemaker, while only 10 % are ascribed to rhythmic systemic cues (Kornmann et al. 2007). Still, despite the impressive amount of oscillating genes involved in metabolic output, the exact mechanism for their circadian regulation, e.g. indirect or directly mediated by Clock-Bmal1 is largely unexplored. So far, only a handful of genes, whose expression is driven by pacemaker components, have been described (see below) (Guillaumond et al. 2010; Doi et al. 2010; Ripperger et al. 2000; Anzulovich et al. 2006).

I.B.3.3 Circadian output in glucose metabolism

A more elaborate support for the tight interconnection between the endogenous timing system and metabolism is provided by mouse models of circadian arrhythmia, which carry genetic mutations in core circadian components **[Tab. I-1]**.

Several studies propose that disruption of the pacemaker in all or certain metabolic tissues impairs glucose metabolism. Some of the evidence is derived from Clock mutant mice (*Clock*^{mut}), in which an antimorphic *clock* allele renders Clock-Bmal1 transcriptional complexes functionally inactive in the SCN and peripheral organs (King et al. 1997a, b; Jin et al. 1999; Panda et al. 2002). Clock^{mut} animals develop hyperglycaemia; however, it is not clear to what extent CNS-associated defects, e.g. ablated feeding rhythms with a pronounced hyperphagia during the inactive period, contribute to this effect (Turek et al. 2005).

Meanwhile, the role of circadian rhythms in the pancreas has been also suggested: pancreatic *bmal1* deletion leads to hyperglycemia and glucose intolerance; however, the exact contribution of the beta-cell pacemaker in this effect remains elusive (Marcheva et al. 2010; Sadacca et al. 2011) **[Tab. I-1]**.

In respect to hepatic function, whole-body and liver-specific Bmal1-disruption have indicated defects in blood glucose buffering **[Tab. I-1]** (Rudic et al. 2004; Lamia et al. 2008). Accordingly, numerous components involved in glucose transport, breakdown and glycogen metabolism have been identified as circadian in liver (Panda et al. 2002; Storch et al. 2002; Reddy et al. 2006; Hughes et al. 2009). However, so far, only one enzyme in this respect – glycogen synthase 2 –has been elucidated as a Clock-Bmal1 target (Doi et al. 2010).

I.B.3.4 Circadian output in lipid metabolism

Animal studies also provide evidence for a tight link between the circadian pacemaker and lipid metabolism. Both *in vitro* and *in vivo* analyses in rodents using incorporation assays of radiolabeled metabolites demonstrate that cholesterol biosynthesis rate varies strongly over day and night, even in the fasted state (Back et al. 1969; Hamprecht et al. 1969; Edwards et al. 1972; Mayer 1976). It reaches a peak during the activity phase, corresponding to the phase of food consumption. Further, the concentration of lipoproteins, triglycerides and free fatty acids in plasma also peak during the active period (Pan and Hussain 2007; Pan et al. 2010; Escobar et al. 1998).

Genetic mouse models of circadian arrhythmia emphasize the circadian output in lipid metabolism **[Tab. I-1].** For instance, *Clock*^{mut} mice display dyslipidemia and elevated circulating triglycerides, while animals lacking Rev-Erbα – a transcriptional repressor and core component of the circadian pathway – exhibit impaired lipid biosynthesis (Pan et al. 2010; Le Martelot et al. 2009).

Importantly, most reports on the mechanism linking pacemaker disruption and lipid metabolism consider an indirect regulation through downstream metabolic transcription factors rather than directly by Clock-Bmal1. For instance, approximately half of all 50 murine nuclear receptors, which regulate the transcription of hundreds of metabolic genes, are expressed in an oscillating manner (Yang et al. 2006). Among them, PPARα and PPARγ, are potent modulators of fatty acid oxidation and lipid synthesis, respectively. Furthermore, SREBP nuclear accumulation has been also reported to cycle, thus, regulating downstream lipogenic genes in a circadian manner (Anzulovich et al. 2006; Le Martelot et al. 2009). Another transcription factor, which is a potent regulator of hepatic energy metabolism and a mediator between circadian and metabolic networks, is the Clock-Bmal1 target Klf10 (Guillaumond et al. 2010).

Despite the impressive amount of oscillating enzymes in cholesterol / triglyceride biosynthesis, information on discrete nodes linking the core circadian transactivators Clock-Bmal1 to these processes is scarce (Pan et al. 2010; Panda et al. 2002).

Tab. I-1: Metabolic phenotypes of circadian gene mutants

mouse model	mutation	metabolic phenotype	reference
$Clock^{mut}$	antimorphic *clock* in all tissues	hyperglycemia, obesity, altered feeding rhythms	Turek et al. 2005
$Bmal1^{-/-}$	whole-body *bmal1* deletion	altered gluconeogenesis and insulin tolerance	Rudic et al. 2004
L-$Bmal1^{-/-}$	*bmal1* deletion in liver	decreased gluconeogenesis	Lamia et al. 2008
PdxCre-$Bmal1^{fl/fl}$	*bmal1* deletion in pancreas	hyperglycemia, glucose intolerance	Marcheva et al. 2010, Sadacca et al. 2011
Rev-KO	whole-body Rev-Erbα deletion	impaired SREBP signaling; impaired lipogenesis and bile acid synthesis	Le Martelot et al. 2009

I.B.3.5 Metabolic input integrated into the circadian system

The endogenous timing mechanism not only controls physiological output but is also itself regulated by metabolic cues. Feeding/fasting cycles represent a potent zeitgeber for the adjustment of the biological clock in liver. Remarkably, the phase of hepatic circadian rhythms can be completely reversed in rodents whose food intake is restricted to the day instead of the night period, without any perturbation of the SCN central pacemaker (Stokkan et al. 2001).

Peripheral clocks integrate local metabolic stimuli in several ways. For instance, they sense the redox status of the cell, i.e. the NAD^+/NADH ratio, through the NAD^+-dependent deacetylase Sirtuin 1 (Sirt1). Sirt1 associates with Clock-Bmal1 to rhythmically drive transcriptional activation (Imai et al. 2000; Nakahata et al. 2008; Belden and Dunlap 2008). Furthermore, energy status, represented by the AMP/ATP ratio, modulates the stability of core clockwork components: The degradation rate of Cryptochrome and Period is determined through the activity of adenosine monophosphate (AMP)-activated kinase (AMPK) (Lamia et al. 2009; Um et al. 2011).

Metabolic perception is also present through the integration of nuclear receptors into the core pacemaker mechanism: The activity of the transcriptional repressor Rev-Erbα is modulated by heme availability, which oscillates according to periods of feeding and fasting (Handschin et al. 2005; Yin et al. 2010).

C. MicroRNAs in circadian and metabolic systems

I.C.1 MicroRNAs – biogenesis and function

Over the last decade, microRNAs (miRNAs) have emerged as key *trans*-acting regulators of gene expression in metazoa (Lee et al. 1993; Reinhart et al. 2000; Lagos-Quintana et al. 2001; Lee and Ambros 2001; Brennecke et al. 2003; Griffiths-Jones et al. 2008). Approximately 30 to 60 percent of the genome obey, often modest, posttranscriptional repression by miRNAs, thus, unraveling their function as rheostats, fine-tuning protein output (Lewis et al. 2005; Baek et al. 2008; Selbach et al. 2008; Friedman et al. 2009). The endogenous 21-23 nt-long RNAs bind predominantly within the 3' untranslated region of mRNAs to direct silencing (Lee et al. 1993; Lai 2002). In animals, base-pairing is imperfect and constitutes only nucleotides at position 2-7 in the 5' end of the microRNA sequence (Lewis et al. 2003; Lewis et al. 2005) **[**Fig.I-18**]**. The consecutive Watson-Crick complementarity in this hexamer region is the primary determinant for miRNA binding and is termed a "seed". Weak complementarity in the miRNA-mRNA interaction is in accordance with the fact that one small RNA controls the expression of dozens to hundreds of transcripts (Kiriakidou et al. 2004; Selbach et al. 2008; Baek et al. 2008). The targeted site sequence exhibits strong evolutionary conservation, highlighting the significance of this regulatory mechanism (Stark et al. 2005; Lee et al. 2007).

The majority of miRNA genes form independent transcription units, situated in intergenic regions or in antisense orientation to annotated genes (Lagos-Quintana et al. 2001; Lee and Ambros 2001; Lee et al. 2002). However, many miRNAs are encoded in introns, which are cotranscribed with the respective gene (Rodriguez et al. 2004). In both cases, transcription is accomplished by RNA polymerase II, forming long primary transcripts (pri-miRNAs), equipped with a Cap-structure and a polyA-tail (Lee et al. 2002; Lee et al. 2004) **[**Fig.I-17**, 18]**. Further, the molecules are processed by the nuclear RNase III Drosha irrespective of the diversity in their primary sequence to yield ca. 70 nt long hairpin-shaped structures (Lee et al. 2003; Wang et al. 2007). These pre-miRNA intermediates are exported into the cytosol to become finally processed by Dicer (Kim 2004). The RNase III endonuclease produces a 21-23 nt-long RNA duplex, characterized by a 5' phosphate and a 3' nucleotide overhang (Hutvágner et al. 2001; Ketting et al. 2001).

At the heart of the miRNA silencing pathway is the microRNA ribonucleoprotein complex (miRNP) (Hammond et al. 2001; Hutvágner et al. 2001; Martinez et al. 2002) **[**Fig.I-17**]**. The

RNA strand, whose 5' end is thermodynamically less stable, is selected and incorporated into the miRNP, while the antisense strand is degraded (Sontheimer 2005). In mammals, Argonaut (Ago) is associated with the miRNP to mediate repression (rev. in Filipowicz et al. 2008). Most of the Ago proteins induce translational repression: Ago competes with the eukaryotic initiation factors eIF4E and eIF4G, thus, inducing their disassembly from the m7G-cap and precluding recruitment of ribosomes to the translational start (Kiriakidou et al. 2007; Wang et al. 2006; Mathonnet et al. 2007; Wakiyama et al. 2007) **[**Fig.I-17**]**. In another, most probably parallel mechanism, the miRNP complex triggers mRNA transport in processing bodies, where transcripts are stored to escape translation and / or are degraded (Wang et al. 2006; Wakiyama et al. 2007).

I.C.2 MicroRNAs in beta cell function – mir-375

Since the initial discovery of the founding member of the microRNA family in *Caenorhabditis elegans* – lin-4 – ca. thousand miRNAs have been identified, accounting for 3 % the mammalian genome (Lee et al. 1993; Bentwich et al. 2005; Landgraf et al. 2007; Griffiths-Jones et al. 2008). They have been implicated in the regulation of development, differentiation, cell death, metabolism and circadian function (Alvarez-Garcia and Miska 2005; Krützfeldt et al. 2006; Hansen et al. 2011). On average, microRNAs are estimated to control hundreds of genes, albeit with a weak (less than twofold) repression (Selbach et al. 2008; Baek et al. 2008). While the identification of microRNAs has been largely completed, the reliable definition of their targets is still outstanding (Rajewsky 2006; Thomas et al. 2010). Bioinformatic algorithms can predict targeted genes based on seed nucleotide pairing and its evolutionary conservation (Krek et al. 2005). However, experimental candidate validation is still scarce and only a handful of targets have been disclosed so far (Rajewsky 2006; Thomas et al. 2010).

Gene manipulation studies in mice point towards a key function of microRNAs in beta cell glucose homeostasis. Dicer-deficient animals lacking mature miRNA processing display defective pancreatic development (Lynn et al. 2007). The importance of one microRNA – mir-375 – reaches far beyond developmental stages into the adult life (Kloosterman et al. 2007; Poy et al. 2009). mir-375 is the most abundant small RNA in the beta-cell endocrine lineage and is primarily involved in regulation of insulin secretion *in vitro* and *in vivo* (Poy et al. 2004; Poy et al. 2009). Its adenoviral overexpression in Langerhans islets inhibits insulin release, whereas islets from mir-375$^{-/-}$ mice hypersecrete the hormone. Importantly, the

negative effect of mir-375 on insulin secretion is exerted through membrane-proximal events. It is believed that this involves the granule fusion machinery, for instance, through the direct miRNA-target myotrophin – a protein which associates with actin filaments (Poy et al. 2004; Bhattacharya et al. 2006).

In addition, mir-375 has been implicated in cell survival and proliferation of the adult endocrine pancreas (Poy et al. 2009). Mice devoid of the small RNA exhibit a drastic reduction in the number of beta cells due to mir-375-mediated suppression of pro-apoptotic genes (Poy et al. 2009).

I.C.3 MicroRNAs in hepatic lipid metabolism

In spite of thorough research on the regulation of hepatic lipid metabolism due to its relevance for cardiovascular disease, only a few miRNAs have been implicated in this process so far. For instance, the most abundant hepatic small RNA – mir-122 – enhances fatty acid and cholesterol synthesis through indirect regulation of genes, such as *hmgcr* (*hmgCoA-reductase*) and *fasn* (*fatty acid synthase*) (Lagos-Quintana et al. 2001; Krützfeldt et al. 2005*;* Esau et al. 2006).

Other key regulators include mir-33a and mir-33b, which are encoded in introns of the master transcription factor in lipogenesis *srebp*. The microRNAs are upregulated under conditions of fat depletion to supress lipid catabolism (Rayner et al. 2010; Najafi-Shoushtari et al. 2010; Gerin et al. 2010b).

I.C.4 MicroRNAs and circadian rhythms

In *Drosophila*, thorough large-scale analyses have identified cycling microRNAs or such, which are differentially expressed in circadian mutant flies (Kadener et al. 2009; Vodala et al. 2012). In mammalian tissues, this void has not been filled yet **[Tab. I-2]**. So far, the expression of only two microRNAs has been demonstrated to depend on the circadian pacemaker: In SCN, mir-219 has been recognized as a Clock-Bmal1-target (Cheng et al. 2007). It is further proposed to feedback on the "gears" of the molecular clockwork through indirect regulation of its period. Secondly, the most abundant hepatic small RNA – mir-122 – is transcribed in a highly circadian manner, probably through the circadian repressor Rev-Erbα (Gatfield et al. 2009). Nevertheless, this interaction lacks any impact on mature miRNA expression as mature mir-122 accumulates in a non-circadian manner.

Over the recent years, the role of microRNAs in the posttranscriptional control of the molecular clockwork has also emerged **[Tab. I-2]**. In the NIH3T3 fibroblast cell line, the dicistronic mir-192/194 cluster and mir-185 have been shown to target the *period* gene family and *cry*, respectively (Nagel et al. 2009; Lee et al. 2013). In mouse embryonic fibroblasts, *period* genes are further regulated by mir-24, mir-29a and 30a (Chen et al. 2013). However, this evidence has arisen from *in vitro* systems and the physiological relevance of microRNA control of the core pacemaker is still unclear.

Tab. I-2: Prominent microRNAs in beta cell function, hepatic lipid metabolism and the circadian system

microRNA	Involved in:	Mechanism of action / targets	Reference
mir-375	beta cell function	negative regulation of insulin secretion / proliferation (*myotrophin* / *aifm1, rasd1, eef1e1, elavl4*)	Poy et al. 2004, Poy et al. 2009
mir-122	hepatic lipid metabolism	indirect regulation of lipogenic and cholesterogenic genes (*hmgcr, fasn, scd-1*)	Esau et al. 2006
mir-122	circadian rhythms / liver	circadian transcription; mature miR-122 does not cycle	Gatfield et al. 2009
mir-33a/b	hepatic lipid metabolism	co-transcribed with SREBP, blocks fatty acid oxidation	Najafi-Shoushtari et al. 2010; Gerin et al. 2010b
mir-219	circadian rhythms / SCN	transcriptional regulation by Clock-Bmal1, regulates circadian period	Cheng et al. 2007
mir-192/194	circadian rhythms / NIH3T3 cells	targets *period 1,2,3*	Nagel et al. 2009
mir-185	circadian rhythms / NIH3T3 cells	targets *cryptochrome1*	Lee et al. 2013
mir-24, 29a, 30a	circadian rhythms /mouse embryonic fibroblasts	targets *period 1,2*	Chen et al. 2013

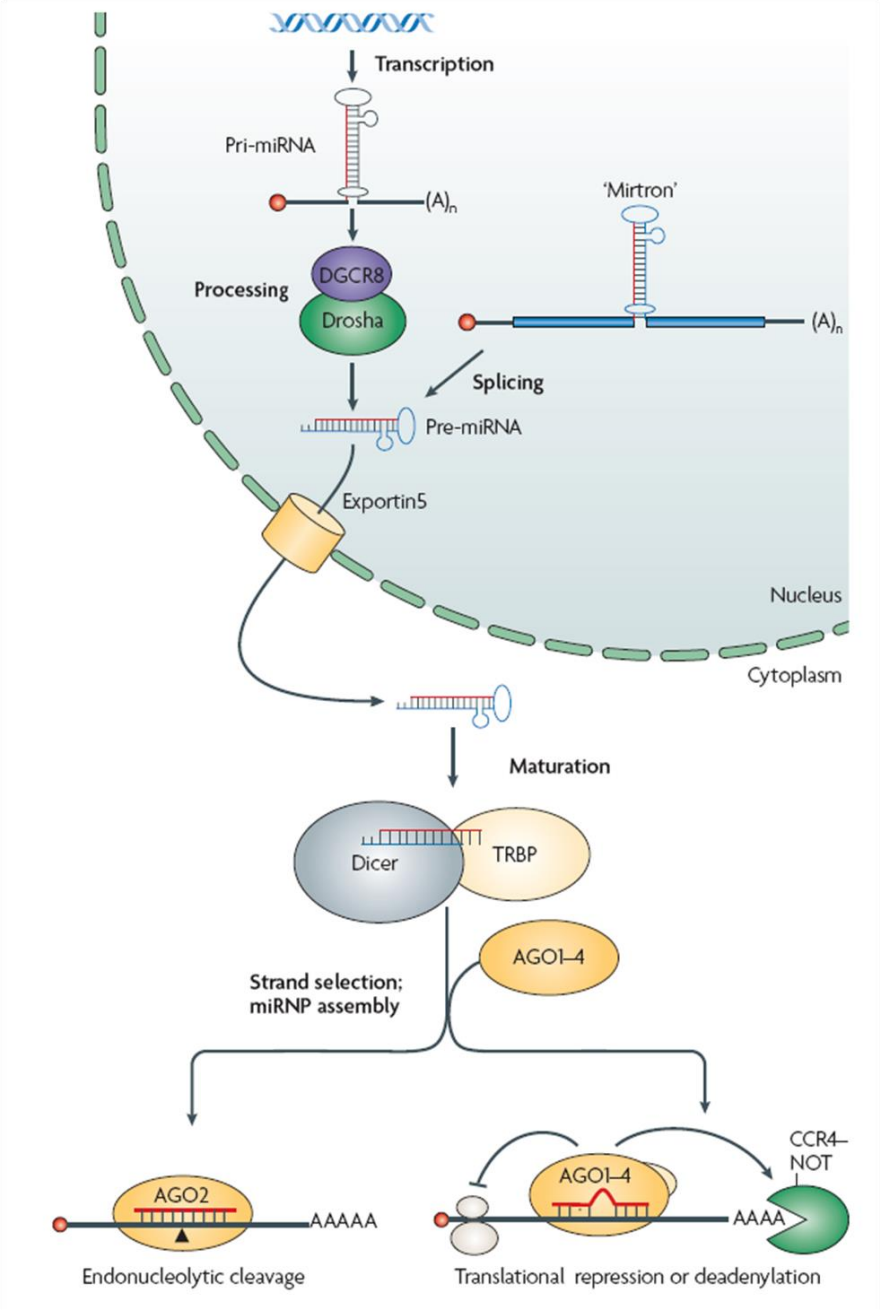


Fig.I-17: Biogenesis of microRNAs
Primary miRNA transcripts are transcribed either from independent miRNA genes or from introns of protein-coding genes by the RNA polymerase II. Pri-miRNAs are further processed in two steps, catalyzed by the RNase III type endonuclease Drosha and Dicer, which act in concert with additional dsRNA-binding proteins. The Drosha-DGCR8 complex cleaves the precursors into ca. 60 nt long pre-miRNAs. Upon export into the cytoplasm, the final cleavage by Dicer takes place. One strand of the ca. 20-bp miRNA duplex is incorporated into the Ago-miRNP, while the remaining is degraded. In mammals, Ago1, 3 and 4 function mainly in miRNA-mediated translational repression, while Ago2 mediates cleavage of the targeted mRNA. (Adapted from Filipowicz et al. 2008.)

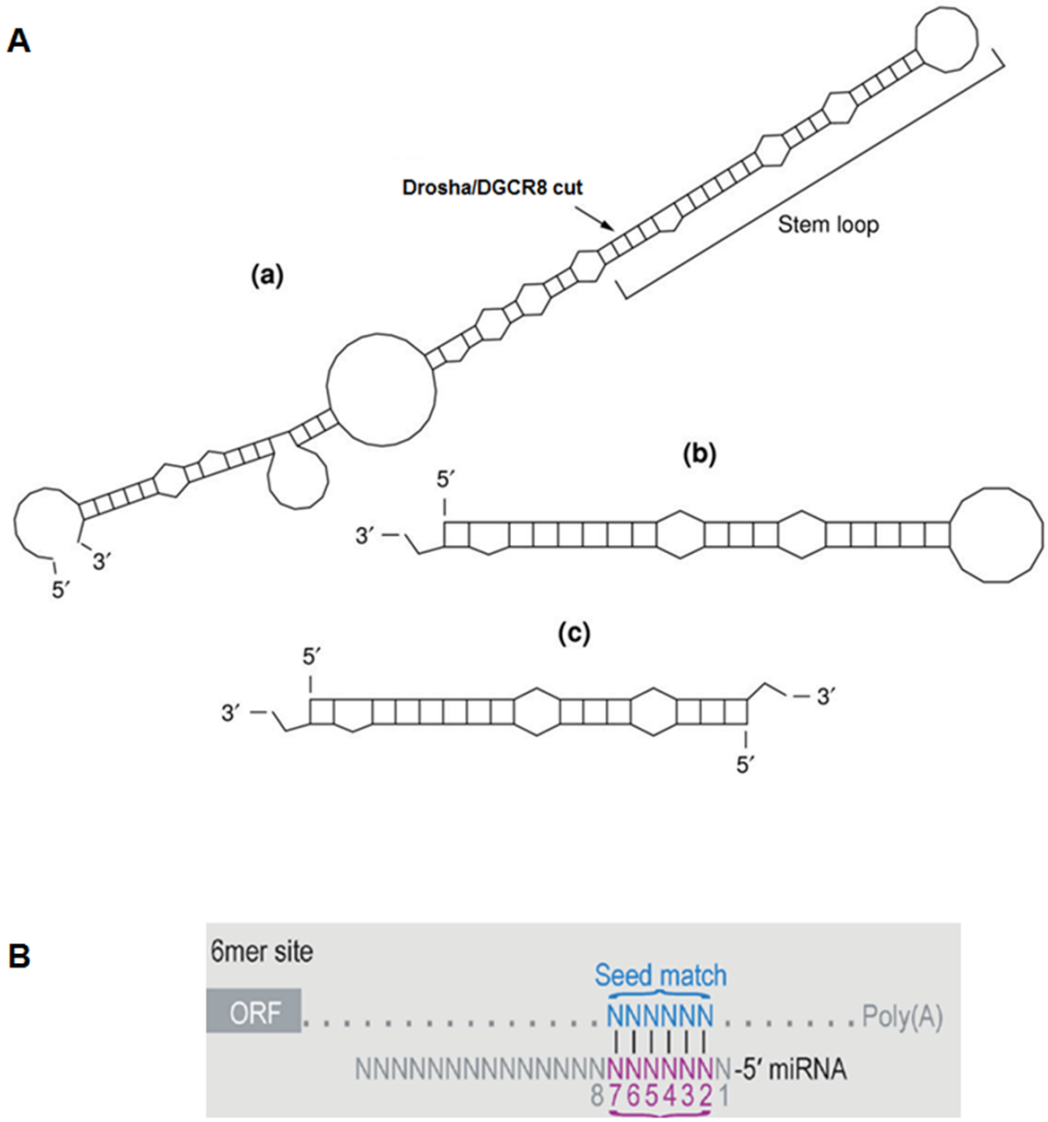


Fig.I-18: Structural characteristics of microRNAs.
(A) Schematic depiction of the intermediates in microRNA processing: (a) primary microRNA transcript, folding into hairpin structures containing imperfectly base-paired stems (b) pre-microRNA formed after Drosha processing and transported into the cytosol (c) mature microRNA duplex after Dicer cleavage (Adapted from Snøve and Rossi 2006) (B) The 'seed' region - the minimal requirement for a microRNA-mRNA interaction – comprises a hexamer from position 2 to 7 in the miRNA sequence and binds to a complementary region in the targeted 3' UTR (adapted from Bartel 2009)

D. Aim of the thesis

The tight interconnection between the endogenous timing system and metabolism is widely accepted; however the molecular basis for these interactions largely relies on the fact that enzymes regulating glucose and lipid metabolism oscillate and lacks an in-depth understanding of critical nodes, which integrate the core circadian transactivator Clock-Bmal1 into metabolic output. Furthermore, while 5-20% of the protein-coding genome are estimated to exhibit circadian expression, evidence for clock-controlled non-coding RNAs is scarce. In addition, miRNA-mediated regulation of core pacemaker components in metabolic tissues is also largely unexplored. In order to shed light on the role of the circadian transactivator Clock-Bmal1 in glucose and lipid homeostasis and its potential crosstalk to miRNAs, the following questions were pursued:

i) What is the role of Clock-Bmal1 in beta cell function? Previous reports using genetic models of circadian arrhythmia indicate that Clock-Bmal1 is involved in systemic glucose homeostasis; however, they do not provide information on the existence of a beta cell specific pacemaker.

ii) What is the impact of Clock-Bmal1 disruption on hepatic glucose and lipid metabolism? Can concrete mechanisms be dissected, which link Clock-Bmal1 to metabolic control?

iii) Are there miRNAs, which crosstalk to the molecular pacemaker in pancreatic beta cells or liver? Do circadian components obey posttranscriptional regulation by miRNAs and, on the other hand, are miRNAs directly transactivated by Clock-Bmal1?

II. Results

A. The role of Clock and Bmal1 in pancreatic beta cells

II.A.1 Phenotypic characterization of mice deficient for Clock and Bmal1

First, I focused on the putative relevance of the beta cell pacemaker for the maintenance of postprandial glucose homeostasis.

II.A.1.1 Whole-body Clock-deficiency leads to impaired glucose homeostasis

In an initial step, the phenotype of mice lacking Clock in all tissues was assessed. Failure to identify physiological defects in this animal model would render potential significance of Clock in the endocrine beta cell lineage unlikely. For this purpose, Clock knock-out ($Clock^{-/-}$) mice were used, carrying a genetic deletion of *clock* exons 5 and 6, encoding the basic helix-loop-helix (bHLH) domain required for DNA binding (Debruyne et al. 2006). Although this model is characterized by total loss of Clock protein, its SCN pacemaker function is intact. This is due to the fact that in the SCN Bmal1 dimerizes with the Clock paralogue Npas2, building a fully transcriptionally active complex (Debruyne et al. 2006, DeBruyne et al. 2007) (see I.B.2). Accordingly, $Clock^{-/-}$ mice exhibit robust and persisting day/night rhythms in locomotor activity, illustrating that behavioral and activity rhythms are unaffected (Debruyne et al. 2006).

Prior to investigating the role of Clock in glucose homeostasis, I wanted to rule out that Clock-deficiency in the brain induces metabolic abnormalities, which mask putative effects in peripheral organs. The CNS exerts control over fuel homeostasis through autonomic regulation of appetite, energy expenditure and activity (Migrenne et al. 2006). Hence, disruption of neuronal circuits under conditions of Clock-deficiency could affect downstream metabolic output. To estimate this, food intake, movement and energy expenditure were tested in the circadian mutants **[Fig. II-1]**

Locomotor activity, as indicated by cumulative running distance over 12 hours, was indistinguishable between wildtype and $Clock^{-/-}$ mice (9339±2569 / 11644±1128 cm (day) and 29684±7755 / 29976±6806 cm (night)) **[Fig. II-1A].** This was consistent with previous behavioral reports on the circadian mutants (Debruyne et al. 2006). In addition, their food intake was similar to control mice, with ca. 70 % occuring during the nocturnal period **[Fig. II-1B]**. Furthermore, caloric expenditure in panspecific $Clock^{-/-}$ mice was highly circadian

with a peak during the active phase. This parameter was indirectly measured, on the one hand, through heat production, and on the other, through the respiratory quotient, characterizing O_2/CO_2 exchange **[Fig. II-1C, D].**

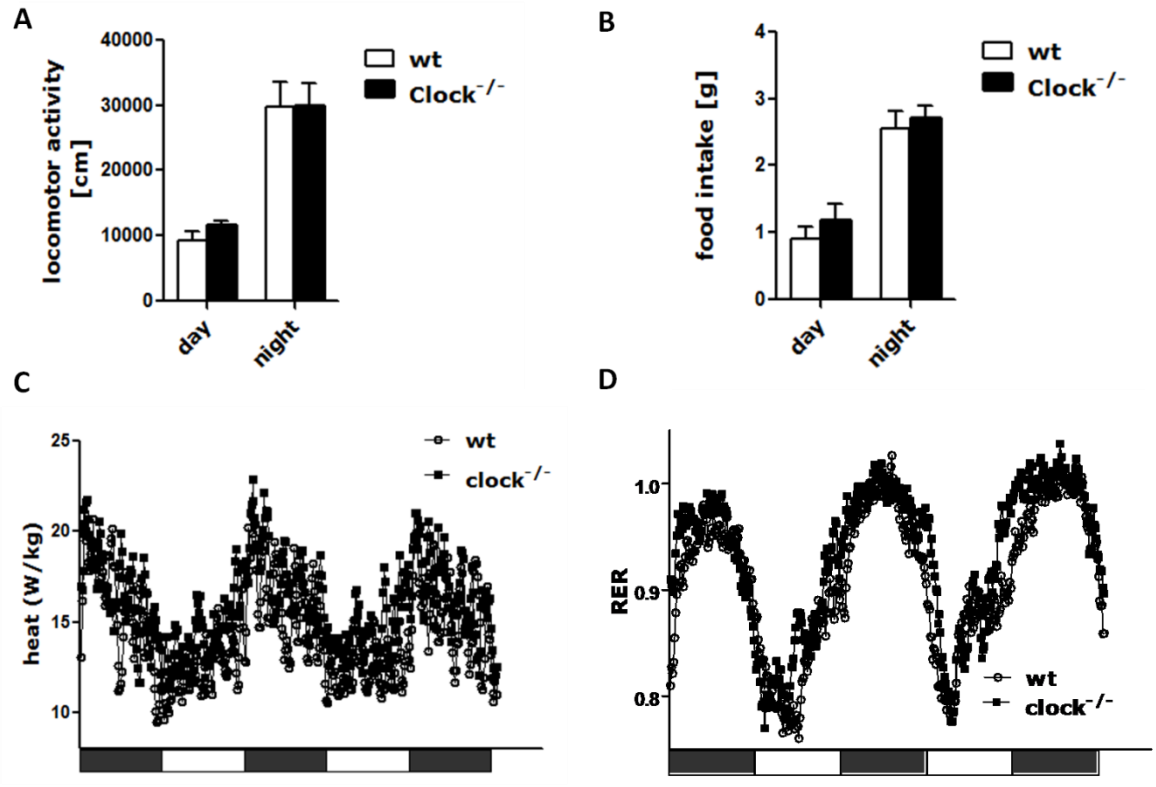


Fig. II-1: CNS-controlled metabolic output in Clock$^{-/-}$ mice.
Essential metabolic functions in Clock$^{-/-}$ mice were screened in order to estimate whether Clock-deficiency in the central nervous system causes primary metabolic defects. On a 12-hour light/dark (white/black bars) cycle locomotor activity (A), food intake (B), heat production per body weight (C) and respiratory exchange ratio of exhaled CO_2 and inhaled O_2 (RER) (D) were recorded. Mean representation over the course of 4 days and standard error, n =4.

Furthermore, the sensitivity of Clock$^{-/-}$ mice towards the appetite supressor leptin was assessed since this hormone represents the central regulator of energy expenditure and the primary satiety signal for the hypothalamus (Schwartz et al. 2000). Intraperitoneal leptin administration induced an anorexic response comparable to control animals, as measured by the drop in food intake and body weight **[Fig. II-2]**. This indicated that the leptin-induced signaling cascade in Clock-deficient hypothalamus is not compromised and supported the results demonstrating normal food intake and energy expenditure.

Taken together, these findings claim that SCN or extra-SCN-controlled functions, such as feeding behavior, activity and energy consumption are intact and their cycling pattern is

preserved under conditions of Clock-disruption. The data confirmed that Clock$^{-/-}$ mice are a suitable model to investigate the role of Clock in peripheral metabolic tissues.

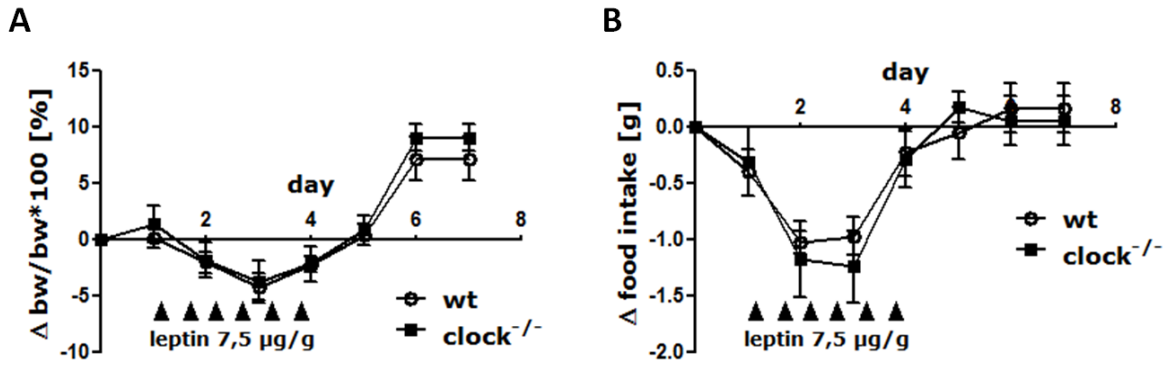


Fig. II-2: Normal leptin sensitivity in Clock$^{-/-}$ mice
Leptin was i.p. administrated on 3 successive days, twice per day (black arrows). Its effects on body weight change (Δbw) (A) and food intake (B) was measured daily (N=6 per group).

II.A.1.1.1 Impaired glucose tolerance in Clock$^{-/-}$ mice

Mice homozygous for the Clock null allele were phenotypically characterized in respect to systemic glucose homeostasis.

First of all, blood sugar concentrations were assessed. They were measured, when regular chow was provided *ad libitum*, and, when food was deprived after an overnight fast **[Fig. II-3A]**. The first scenario represents a biological steady-state, while the latter resembles a hunger response, over which sugar balance is adjusted. In both cases, Clock$^{-/-}$ mice exhibited blood glucose concentrations within the physiological range typical for C57Bl/6 mice (Berglund et al. 2008). Yet, the parameters were slightly but significantly higher than the ones of controls – 98±18, 155±29 mg/dL (Clock$^{-/-}$), and 67±10, 120±7 mg/dL (wildtype) – for the fasted and the random-fed state, respectively **[Fig. II-3A]**. In addition, both conditions displayed normal circulating insulin levels, arguing that Clock$^{-/-}$ mice are diabetic *per se* (0.156±0.032 and 0.537±0.285 ng/ml (Clock$^{-/-}$); 0.161±0.085 and 0.730±0.171 ng/ml (wildtype)) **[Fig. II-3B]**.

However, a more detailed analysis revealed a pronounced difference between Clock$^{-/-}$ and wild-type mice. After a challenge with a high i.p. dosage of sugar, the circadian mutants responded with abnormally elevated blood glucose levels, failing to reach euglycemia as fast as controls **[Fig. II-3C]**. Importantly, ca. 2.5 min after glucose stimulation, circulating insulin concentrations were profoundly decreased (0.304±0.093 (wildtype) and 0.163±0.064 ng/ml (Clock$^{-/-}$)) **[Fig. II-3D]**. This suggested that Clock-deficiency leads to attenuation in the first

phase of insulin release, thus, explaining the impaired hexose clearance during the glucose tolerance test.

As glucose tolerance exhibits circadian variation, next it was addressed whether deterioration of this parameter is time-dependant in Clock$^{-/-}$ mice (Lee et al. 1992). For this purpose, sugar administration was performed at two opposing times of the day, ZT0 and ZT12, corresponding to the onset of the inactive and active period of nocturnal rodents, respectively [**Fig.II-4]**. In the wild-type scenario, glucose clearance was enhanced at ZT12, compared to ZT0, as indicated by lower maximal blood sugar concentrations (291±55 and 221±68 mg/dL, respectively). This reflected that at the onset of the active / awake phase glucose tolerance is improved, corresponding to findings deduced in humans (Lee et al. 1992).

On the contrary, the onset of the active / dark phase did not induce any improvement in Clock$^{-/-}$ glucose tolerance since maximal blood sugar levels were similarly high or even higher at ZT12 (392±44 and 477±91 mg/dL). Thus, whole-body disruption of the molecular pacemaker leads to a general defect in glucose clearance irrespective of time of the day and to an abolishment of its circadian variation.

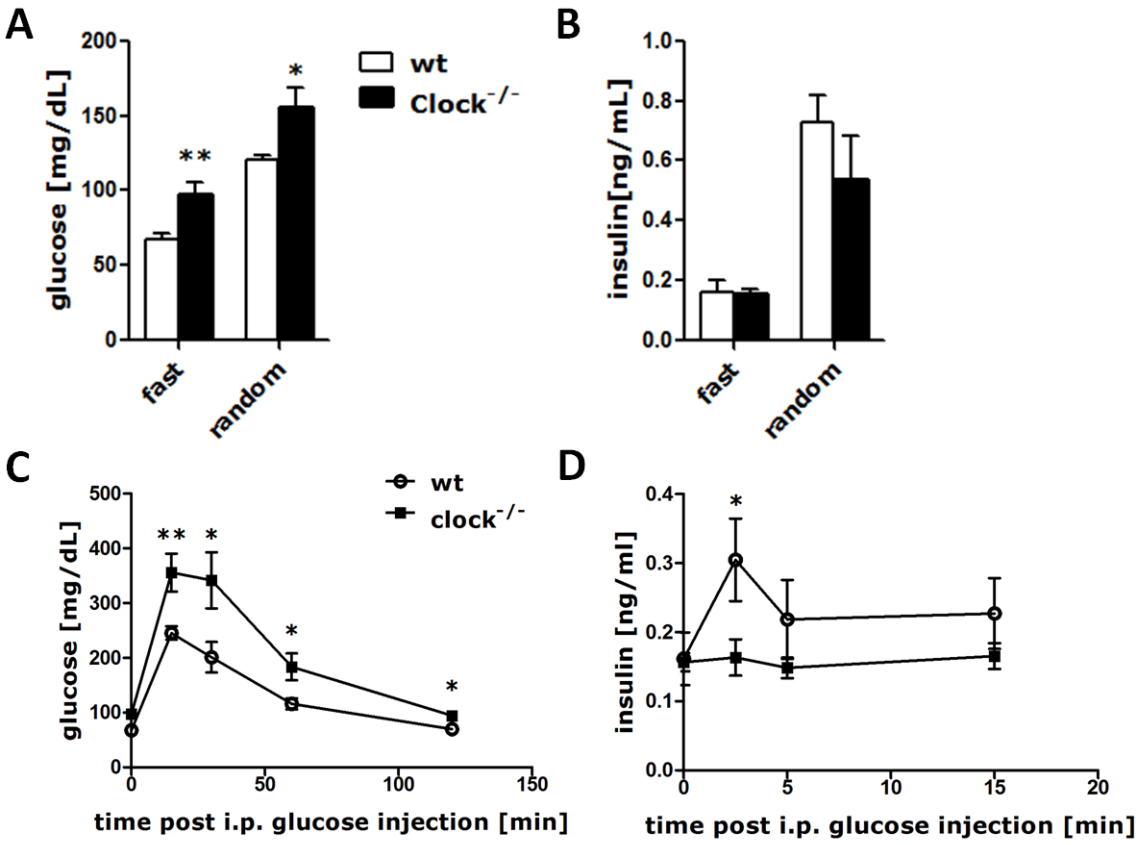


Fig. II-3: Whole-body loss of Clock leads to disturbed glucose homeostasis
(A) Blood glucose levels and (B) insulin plasma levels at fasted or random-fed state (n=11). (C) Glucose tolerance test. After an i.p. glucose injection, blood was collected at the indicated time points and glucose was

measured (n=6). (D) *In vivo* insulin secretion assay. After an i.p. glucose injection, plasma was collected at the indicated time points and insulin was quantified (n=5).

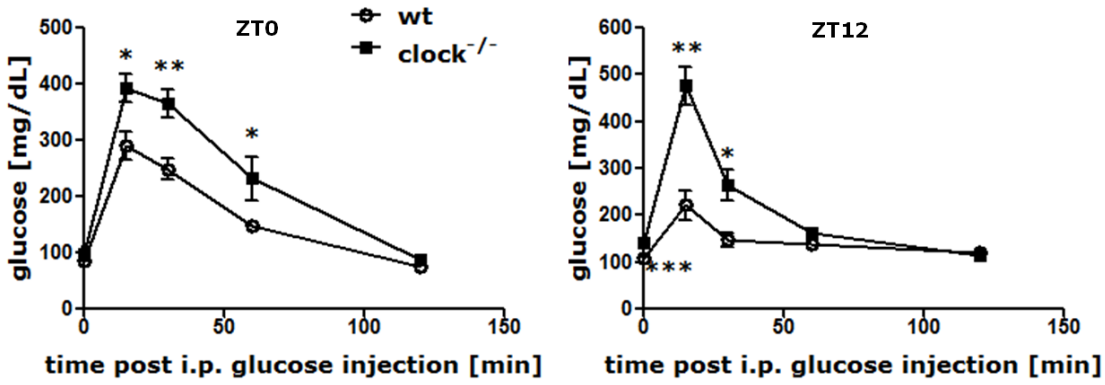


Fig.II-4: Glucose tolerance in Clock$^{-/-}$ mice is impaired irrespective of time of the day
Mice were maintained on a 12-hour light/dark regime and a glucose tolerance test was performed 0 and 12 hours after lights have been shut on (ZT0 and ZT12). N=6 per group

II.A.1.1.2 Normal peripheral insulin sensitivity in Clock$^{-/-}$ mice

Glucose intolerance might result from impaired insulin secretion as well as impaired insulin sensitivity. Under the pathological conditions of type 2 diabetes both effects often co-exist: The increasing demand for insulin in an insulin resistance state cannot be compensated due to deteriorated beta-cell function (DeFronzo et al. 1992). Generally, impaired signalling downstream of the insulin receptor in muscle, adipose tissue and liver associates with overweight (Taniguchi et al. 2006). Hence, I questioned whether obesity and insulin resistance contribute, at least partly, to the glucose intolerance in Clock null mutants.

Indeed, body weights of Clock$^{-/-}$ and littermate controls diverged after 5 weeks of age, with the mutants becoming heavier (e.g. at 10 weeks of age – 24.3±2,1 (wt) and 26.5±1,2 g (Clock$^{-/-}$)) **[Fig.II-5A]**. However, this gain in weight was not associated with an increase in fat: Body composition analysis revealed a significant drop in adipose tissue (19±1 % (wt) and 17±0.9 % (Clock$^{-/-}$)), while lean mass had increased (71.8±0.7 (wt) and 73.8±1.4 % (Clock$^{-/-}$)) **[Fig.II-5B]**. Moreover, administration of exogenous insulin induced a decrease in circulating glucose concentration similar to wild-type controls, with a tendency for an even stronger response, indicating that insulin signalling is not compromised in Clock$^{-/-}$ mice **[Fig.II-5C, D]**. In addition, proper sensitivity towards the hormone was maintained at different times of the day – ZT 1 and ZT 12.

Taken together, the findings excluded that Clock$^{-/-}$ mice are prone to obesity and insulin resistance and that these effects are responsible for glucose intolerance and impaired insulin

secretion. This prompted further investigations focusing on beta cell function under conditions of *clock* deletion.

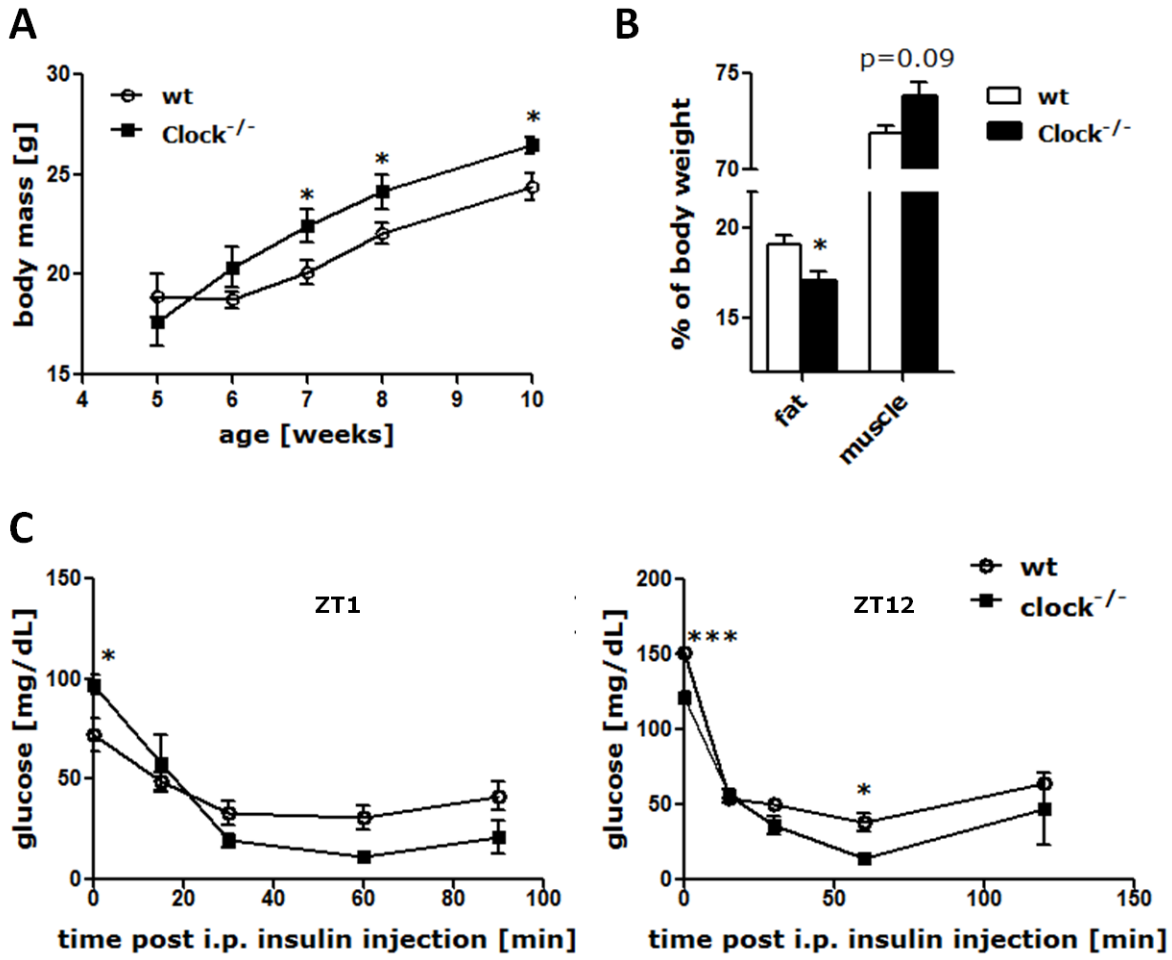


Fig.II-5:Whole-body loss of Clock does not impair insulin signaling in peripheral tissue.
(A) Growth curve of wild-type and Clock$^{-/-}$ mice (n=12) **(B)** Body composition: Percentage of adipose and muscle tissue was assessed by nuclear magnetic resonance (n=5) **(C)** Insulin tolerance test, performed at two different time-points (ZT1 and ZT12): After i.p. insulin administration, blood glucose concentrations were measured at the indicated timepoints (n=4).

II.A.1.2 Conditional inactivation of Clock / Bmal1 in pancreatic beta cells impairs insulin secretion

So far, phenotypic characterization of Clock null mutants pointed towards a potential role for the molecular clock in insulin secretion. In order to elucidate whether beta cells possess an intrinsic oscillator and what its physiological function is, site-specific inactivation of the molecular clockwork in insulin-producing cells was utilized. Genetic disruption of either Clock or Bmal1 exclusively in beta cells was accomplished by means of the cre/loxP recombination system (Sauer and Henderson 1988). Mice carrying a conditional floxed *clock* or *bmal1* allele were crossed with animals expressing the *Cre recombinase* transgene under

control of the rat *insulin* promoter (RIP) in order to generate the two mouse lines InsCre Clock$^{-/-}$ and InsCre Bmal1$^{-/-}$ **[Fig.II-6A, Fig.II-7A]** (Herrera 2000; Debruyne et al. 2006; Storch et al. 2007). The following studies were controlled with littermates expressing the Cre recombinase to verify that the experimental outcome is not influenced by the presence of the transgene.

The site-specific recombination strategy was confirmed through a strong decrease in Clock protein in Langerhans islets of InsCre Clock$^{-/-}$ mice **[Fig.II-6B]**. Residual expression was most likely derived from non-beta cell lineages, which lack transactivation of RIP.

The phenotypic screening of InsCre Clock$^{-/-}$ and InsCre Bmal1$^{-/-}$ mice at a random-fed state revealed a weak increase in resting blood glucose levels, without any pronounced change in circulating insulin, in analogy to total Clock$^{-/-}$ mice (InsCre Clock$^{-/-}$ 175±29, InsCre Bmal1$^{-/-}$ 195±21 mg/dL, controls 124±7 and 153±19 mg/dL, respectively) [**Fig. II-8]**. Furthermore, both conditional mouse models displayed intolerance towards an intraperitoneal glucose challenge since they were unable to clear circulating sugar as rapidly and effectively as control mice **[Fig.II-6C, Fig.II-7B]**.

Moreover, conditional *clock* / *bmal1* inactivation led to a severe impairment in insulin secretion. Firstly, immediate insulin release upon glucose stimulation was abolished in InsCre Bmal1$^{-/-}$ mice [**Fig.II-7C]**. Secondly, the capacity of islets, derived from InsCre Clock$^{-/-}$ mice, to secrete the hormone *ex vivo* was also drastically reduced **[Fig.II-6D].** The latter was assessed in a perifusion system, in which released insulin can be quantified over the course of a glucose stimulation.

Taken together, these experiments clearly indicated that disruption of the circadian duo Clock-Bmal1 exclusively in pancreatic beta cells impairs insulin release.

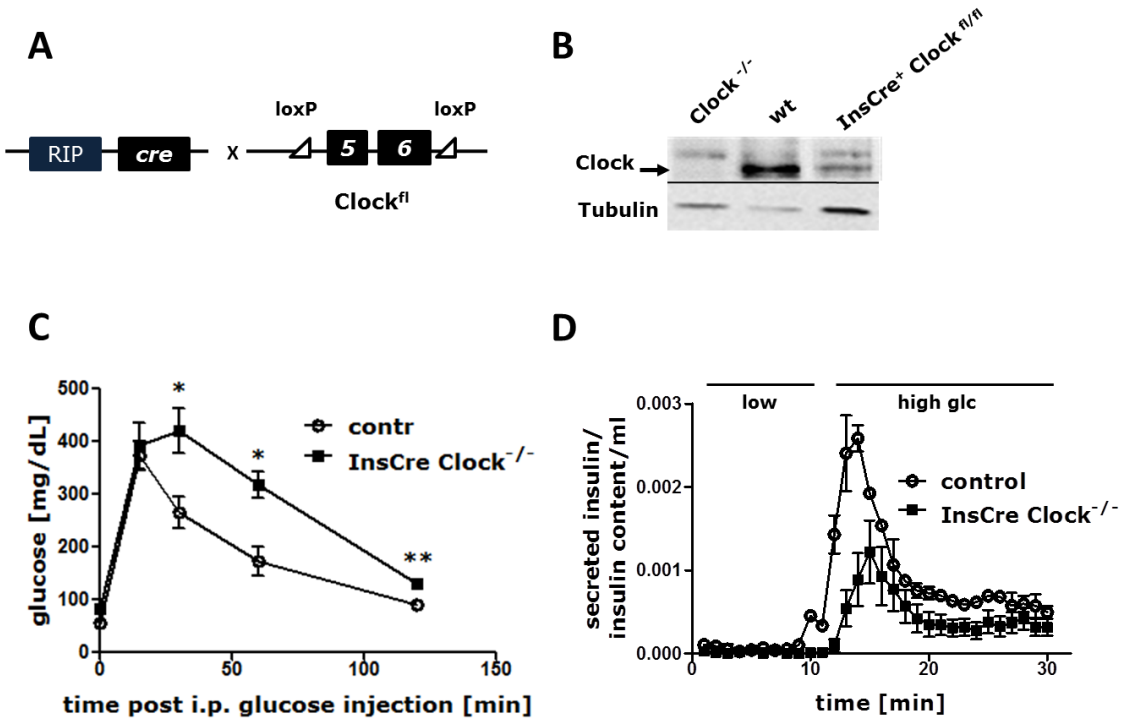


Fig.II-6: Mice deficient for Clock in pancreatic β-cells have impaired glucose tolerance.
(A) Scheme of conditional disruption and pairing strategy for the generation of β-cell specific Clock knock-outs. Transgenic mice expressing *Cre recombinase* under control of the rat *insulin* promoter (RIP) were crossed with mice with a floxed *clock* allele (Clock^{fl}), in which exons 5 and 6 are flanked by loxP sites. (B) Islets from InsCre Clock$^{-/-}$ mice were immunoblotted for Clock, confirming the recombination event in β-cells and the resulting Clock deficiency. (C) Glucose tolerance test in InsCre Clock$^{-/-}$ mice: After i.p. glucose administration, circulating glucose was monitored at the indicated time points (n=4). (D) insulin secretion of InsCre Clock$^{-/-}$ islets *ex vivo*. Isolated pancreatic islets were perifused with low (3.3 mM) or high (16.7 mM) glucose solution for 10 and 30 min, respectively and insulin was measured in the eluate.

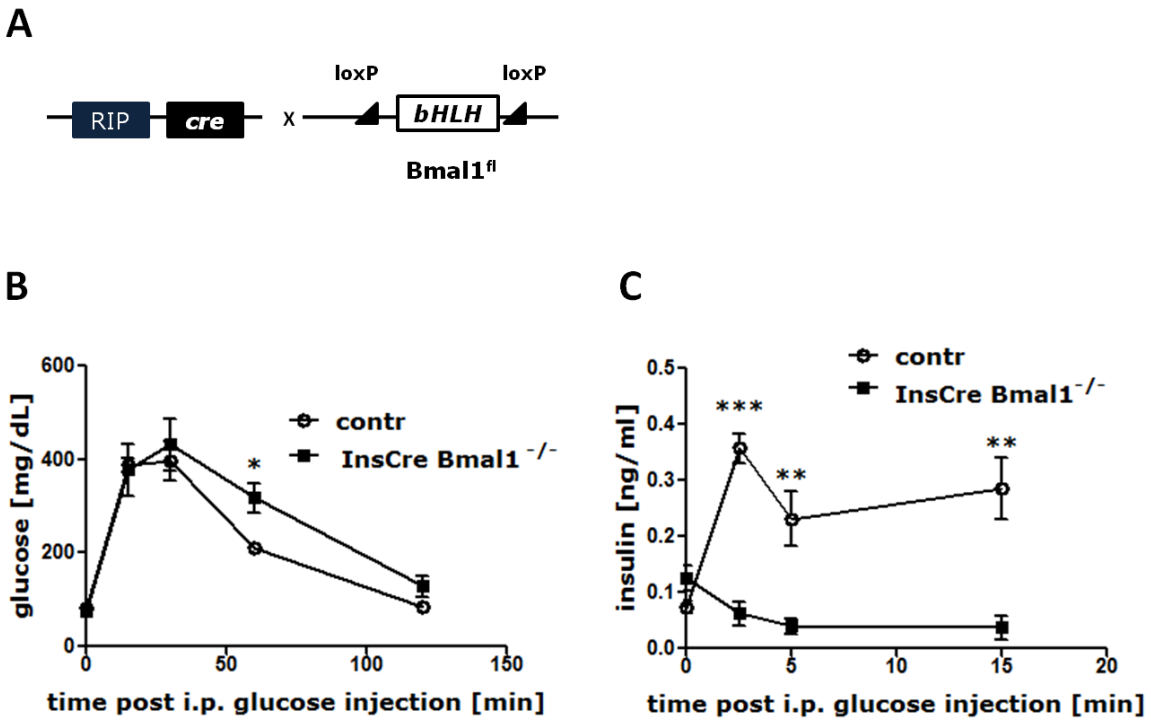


Fig.II-7: InsCre Bmal1$^{-/-}$ mice display defective glucose clearance.
(A) Generation of conditional Bmal1 knock-outs - mice expressing the *Cre* transgene under control of the rat *insulin* promoter were bred with mice with a conditional Bmal1^{fl} allele, where exon 8 encoding the basic helix-loop-helix (bHLH) domain is flanked by loxP sites. (B) Glucose tolerance test in InsCre Bmal1$^{-/-}$: After i.p. glucose administration, circulating glucose was monitored at the indicated time points (n=5). (C) *In vivo* insulin secretion test in InsCre Bmal1$^{-/-}$ after an i.p. glucose challenge (n=6).

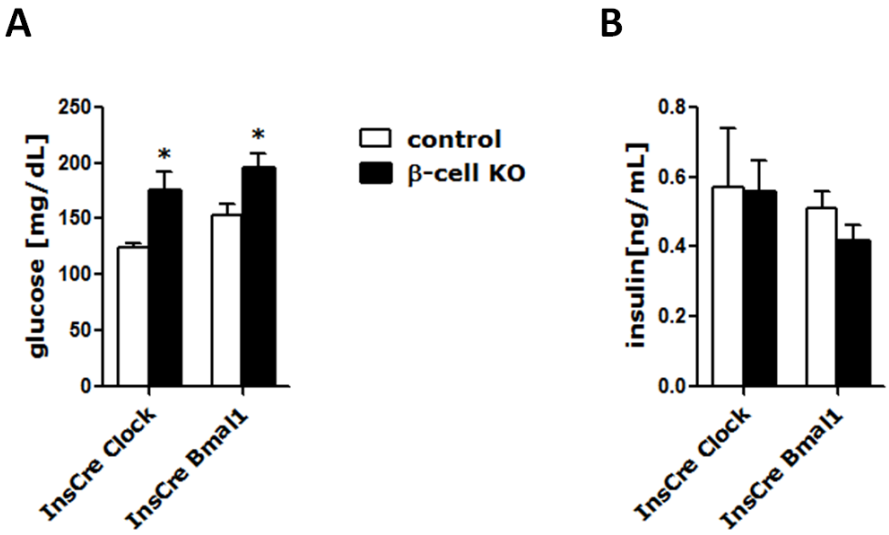


Fig. II-8: Steady-state blood parameters in InsCre Clock$^{-/-}$ and InsCre Bmal1$^{-/-}$ mice
(A) Circulating glucose in InsCre Clock$^{-/-}$ and InsCre Bmal1$^{-/-}$ mice at a random-fed state (B) Plasma insulin concentrations in InsCre Clock$^{-/-}$ and InsCre Bmal1$^{-/-}$ mice at a random-fed state (N=6-8 per group)

II.A.2 Mechanism of impaired insulin secretion in mice deficient for Clock / Bmal1

Next, the reason for impaired insulin secretion in total Clock$^{-/-}$ and beta cell-specific Clock / Bmal1 knockout mice was investigated. Deteriorated insulin release might originate from defective *de novo* hormone synthesis, granulogenesis or secretion. Hence, these putative beta cell dysfunctions were addressed in the circadian mutants.

II.A.2.1 Loss of Clock / Bmal1 does not alter insulin biogenesis

Insulin biogenesis is initiated with transcription of the *ins* gene and simultaneous translation at the ER (see I.A.1.1). Proinsulin is further proteolytically cleaved by endo- and exopeptidases to yield the biologically active hormone. Monogenic defects in transcription, translation and posttranslational processing result in severe beta cell dysfunctions, exemplifying their importance (Kahn and Halban 1997; Bell and Polonsky 2001; Støy et al. 2007).

To test whether some of these processes are affected, the insulin content in pancreata from all three mouse lines – Clock$^{-/-}$, InsCre Clock$^{-/-}$ and InsCre Bmal1$^{-/-}$ – was assessed [**Fig. II-9A]**. Importantly, this experiment employed an immuno-based assay with an antibody detecting the proteolytically end-processed form of the hormone. This method revealed that pancreatic insulin content was comparable between mutants and littermate controls, irrespective whether circadian gene deletion was pan- or beta cell-specific (knockouts: 6.33±2.33, 7.08±1.58, 5.17±0.69; controls: 6.04±2.64, 6.88±1.88, 5.79±2.2 µg/g pancreas). This suggested that *clock* / *bmal1* deletion did not affect the rate of *ins* expression nor downstream zymogen processing.

Further, the generation of insulin-containing granules was investigated. Over the course of insulin granulogenesis, the inner and outer of vesicles is transformed to form mature dense-core granules destined for Ca^{2+}-regulated secretion (see I.A.1.1) (rev. in Kim et al. 2006). Their amount was assessed by means of ultramicroscopic analysis in pancreata derived from random-fed Clock$^{-/-}$ and wildtype mice.

Morphological inspection did not reveal any gross abnormalities in the islets from Clock$^{-/-}$ mice: the overall architecture and appearance of beta cells was preserved [**Fig. II-9C]**. They were identified based on the pronounced cytoplasmic organization and the presence of characteristic vesicles loaded with crystalline insulin, as described elsewhere (Olofsson et al. 2002). Importantly, the average number of dense-core granules per cell area was comparable

between both genotypes (2.8±0.2 (Clock$^{-/-}$) and 2.9±0.6 (wildtype) vesicles /µm^2) [**Fig. II-9B].** Furthermore, it aligned with other reports on cytoplasmic vesicle content (Speidel et al. 2008).

Taken together, the findings indicated that loss of Clock / Bmal1 does not affect the general ability of the neuroendocrine cells for insulin production and granulogenesis. Furthermore, they suggested that insulin vesicle release rather than synthesis might be altered.

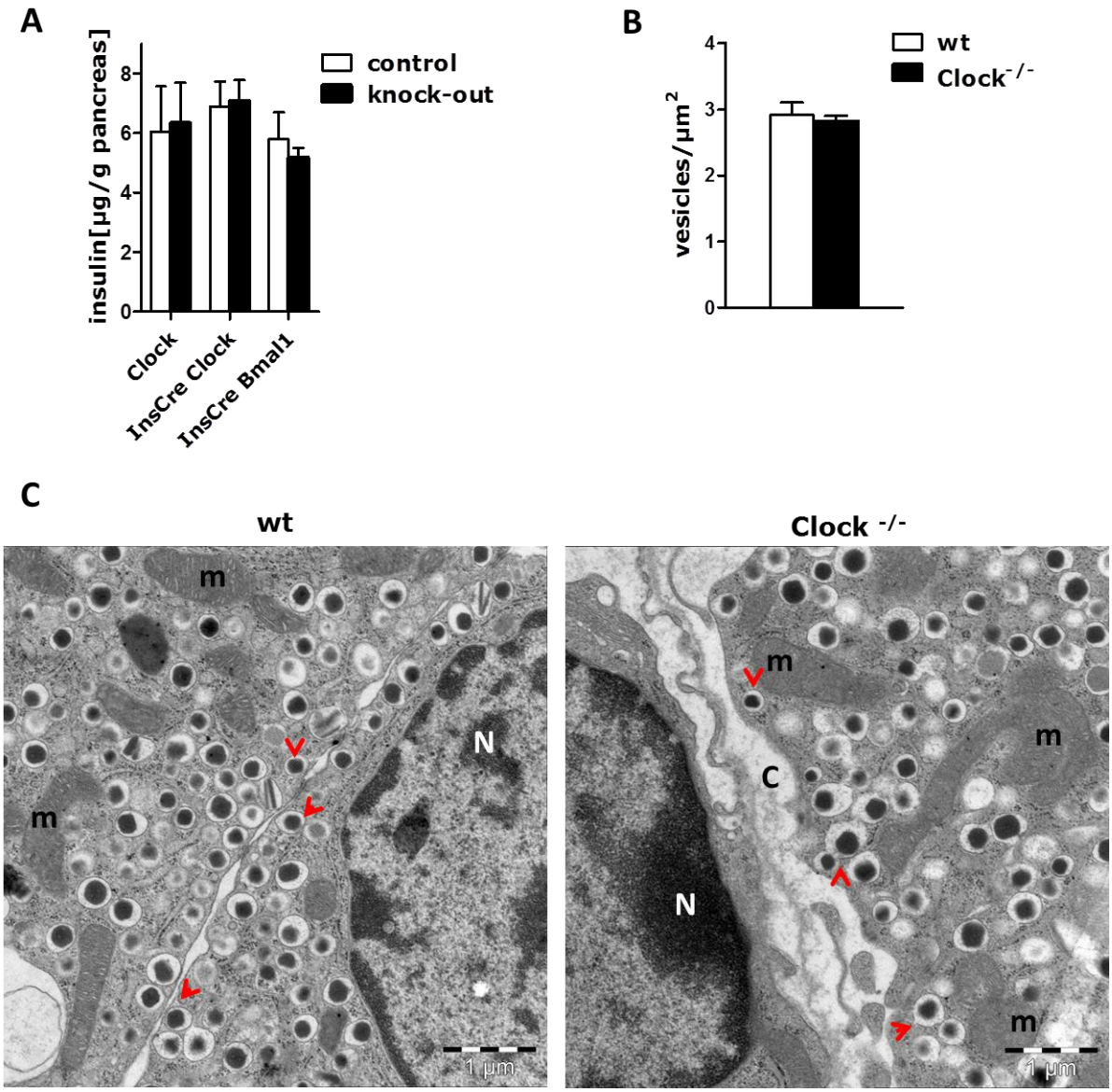


Fig.II-9: Loss of Clock or Bmal1 does not affect insulin biogenesis and granulogenesis.

(A) Pancreatic insulin content from Clock$^{-/-}$, InsCre Clock$^{-/-}$ and InsCre Bmal1$^{-/-}$ mice and littermate controls (N=6 per genotype) (B) Density of insulin-containing granules in Clock$^{-/-}$ beta cells quantified by electron microscopy imaging. β-cell-containing regions derived from 8-10 sections from 3 animals per genotype were used. (C) Ultrastructure micrographs of pancreatic islets of wild-type (left panel) and Clock$^{-/-}$ (right panel) random-fed animals. Few insulin granules are indicated with arrows; Nucleus (N), mitochondria (m), capillary (C). Scale bar 1µm

II.A.2.2 Clock-Bmal1 affect the late but not the early stage of insulin secretion

The results so far prompted investigation of the insulin secretion pathway in beta cells devoid of Clock or Bmal1.

At stimulatory glucose concentrations, sugar breakdown triggers a rise in intracellular Ca^{2+} ($[Ca^{2+}]_i$), leading to fusion of hormone-containing granules. The pathway can be divided into two hypothetic portions – upstream or downstream of $[Ca^{2+}]$-influx (see I.A.1.1). In order to allocate putative defects in the circadian mutants both parts were empirically tested.

Normal rise in $[Ca^{2+}]_i$ upon glucose stimulation in Clock/Bmal1-deficient beta cells

Firstly, the early stage of the insulin secretion pathway was tested. In this part, the increase in ATP upon glucose uptake and oxidation induces membrane depolarization and opening of voltage-gated Ca^{2+}-channels, leading to Ca^{2+}influx **[Fig.I-3].** To evaluate whether the circadian system controls any step in this sequence, the rise in $[Ca^{2+}]_i$ was assessed in pancreatic islets *ex vivo* (in collaboration with the group of Prof. Patrik Rorsman, The Oxford Centre for Diabetes, Endocrinology and Metabolism).

Intact Langerhans islets were superfused consequentially with low (1 mM), medium (12 mM) and high (20 mM) glucose-supplemented buffer, over the course of which $[Ca^{2+}]_i$ was recorded by dual-wavelength microfluorimetry **[Fig.II-10]**. In both wild-type and Clock$^{-/-}$ islets rising sugar concentrations induced a gradual increase in $[Ca^{2+}]_i$. This is a typical feature of the glucose concentration-dependent stimulation of electrical activity in the coupled islet: Membrane depolarization / repolarization intervals lead to a proportional Ca^{2+}-influx at glucose levels above 7-8 mmol/l, reaching a plateau at 20 mmol/l **[Fig.II-10]** (Dean and Matthews 1968; Bergsten et al. 1994; Barbosa et al. 1998)**.**

Another major hallmark of glucose stimulation is characterized by the oscillatory pattern of $[Ca^{2+}]_i$, which underlies pulsatile hormone secretion from the intact islet **[Fig. I-5]**. Importantly, these activation dynamics were also preserved in the Clock$^{-/-}$ islet **[Fig.II-10]**.

Moreover, the glucose stimulus evoked a similar rise in $[Ca^{2+}]_i$ in $Clock^{-/-}$ and wildtype islet at both 12 mM and 20 mM concentrations (0.91±0.03 / 1.11±0.04 ($Clock^{-/-}$) and 0.86±0.02 / 1.09±0.04 (wildtype)) **[Fig.II-10]**.

Taken together, these results demonstrated that loss of Clock does not impair the activation efficiency of voltage-contingent Ca^{2+}-channels upon glucose stimulation, suggesting that intracellular signalling up to this point is intact.

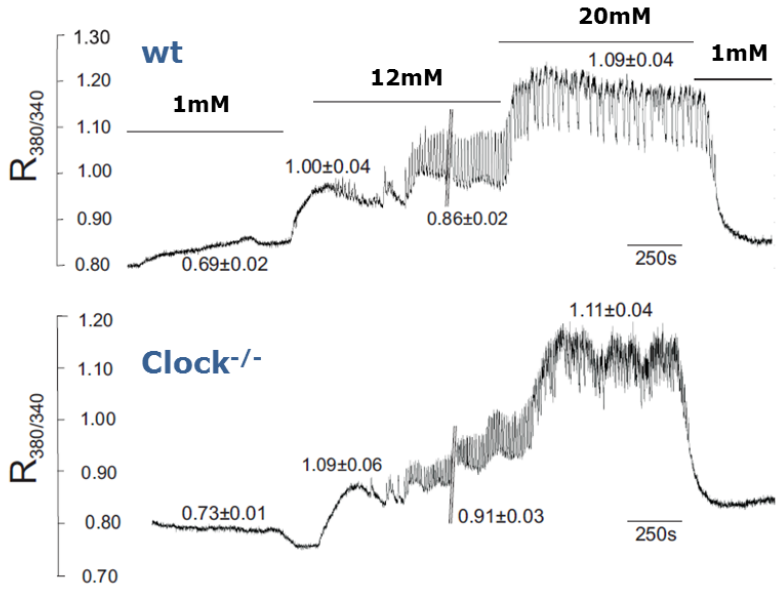


Fig.II-10: Clock deficiency does not influence $[Ca^{2+}]_i$ upon glucose stimulation.
Three different glucose concentrations (1mM, 12mM and 20mM) were used to evoke changes in $[Ca^{2+}]_i$ in islets from wildtype (upper panel) and $Clock^{-/-}$ (lower panel) animals, as measured by the fluorophore emission ratio at 380 and 340 nm. The x-axis represents time, scale bar = 250 s. Representative traces are shown for each genotype. Values above the traces indicate mean and SEM (n=14 islets from 6 wildtype mice; n=12 from 8 $Clock^{-/-}$ mice). Data was contributed by Dr. Stephan Collins, group of Prof. Dr. Patrik Rorsman (The Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital, Oxford, UK).

Increased exocytotic rate in Clock/Bmal1-deficient beta cells

In a next step, the late stage of the insulin secretion pathway encompassing events downstream of Ca^{2+}-channel opening, i.e. exocytosis of insulin-containing granules, was addressed. At the core of this process lies the interaction between transmembrane SNARE proteins and the activity of Ca^{2+}-sensing proteins and auxiliary regulators [see **I.A.1.3]**.

Putative defects in the exocytotic properties of beta cells deficient in either component of the transcriptional duo Clock-Bmal1 were investigated at single cell level and high temporal resolution by means of electrophysiological capacitance measurement (in collaboration with the group of Prof. Patrik Rorsman, The Oxford Centre for Diabetes, Endocrinology and Metabolism). Changes in membrane capacitance are proportional to cumulative changes in cell surface area, as occurring during granule insertion, and represent a reliable indicator for net exocytosis in single cells (Neher and Sakmann 1976; Neher and Marty 1982).

In this whole-cell patch-clamp technique voltage depolarization of the cell membrane of intact beta cells was induced from -70 mV to 0 mV. Two different depolarization durations were applied: Short (50 ms) electrical stimulations resembled a physiological trigger, whereas, longer (500 ms) depolarization trains induced a higher signal to noise ratio in the recorded capacitance. The response was represented as change in membrane capacitance (ΔC_m) between two depolarization trains (n, n-1) at the respective depolarization duration of 50 ms or 500 ms **[Fig. II-11]**. Overall, 50 ms stimulation trains were accompanied by high experimental variation **[Fig. II-11C,G].** This depolarization duration did not evoke significant changes in capacitance of single stimulations in Clock or Bmal1-deficient cells as compared to control**.** However, when observing the cumulative increase in membrane capacitance ΔC_m over all twenty depolarization trains, $Bmal1^{-/-}$ cells displayed a significant elevation, whereas $Clock^{-/-}$ cells exhibited no difference **[Fig. II-11D,H]** (171±39 fF (wildtype) / 218±42 fF ($Clock^{-/-}$) and 62±12 (control) / 113±19 fF (InsCre $Bmal1^{-/-}$)).

In contrast, longer electrical stimulation of 500 ms resulted in a clearly pronounced increase in ΔC_m of mutant beta cells, which persisted over the individual depolarization trains **[Fig. II-11A,E].** This was also reflected by the cumulative increase in ΔC_m over all 500 ms depolarization trains, which was on average twofold higher in Clock- and Bmal1-deficient beta cells (233±32 fF (wildtype) / 471±84 fF ($Clock^{-/-}$) and 110±15 (control) / 257±45 fF (InsCre $Bmal1^{-/-}$)) **[Fig. II-11B, F].**

Taken together, functional analyses of the electrophysiological properties of pancreatic beta cells *in vitro* indicated an enhanced exocytotic rate upon Clock-Bmal1 disruption, which was unexpected to biochemical and physiological data, demonstrating a diminished first phase of glucose-induced insulin secretion. These experiments complemented *in vivo* studies and proved that the Clock-Bmal1 transcriptional complex is indispensable for the final stages of insulin secretion.

Further attempts were undertaken in order to pursue exocytotic properties upon pacemaker disruption *in vitro*, in the transformed beta cell line MIN6, derived from murine insulinoma cells. However, this system did not represent a suitable model to study the impact of clockwork components **[see Appendix, Fig. V-4]**. Firstly, MIN6 cells lacked cycling in circadian components after a serum-shock activation known to synchronize cells *in vitro* and induce their oscillations [**Fig. V-4A]** (Balsalobre et al. 1998). Secondly, the immortalized cells were refractory to siRNA-mediated *clock* knockdown as their capacity for insulin secretion was not affected [**Fig. V-4B]**. Hence, this cell line has lost its intrinsic ability to perform circadian rhythms and hence, unlike primary beta cells, clock components are dispensable for insulin secretion there.

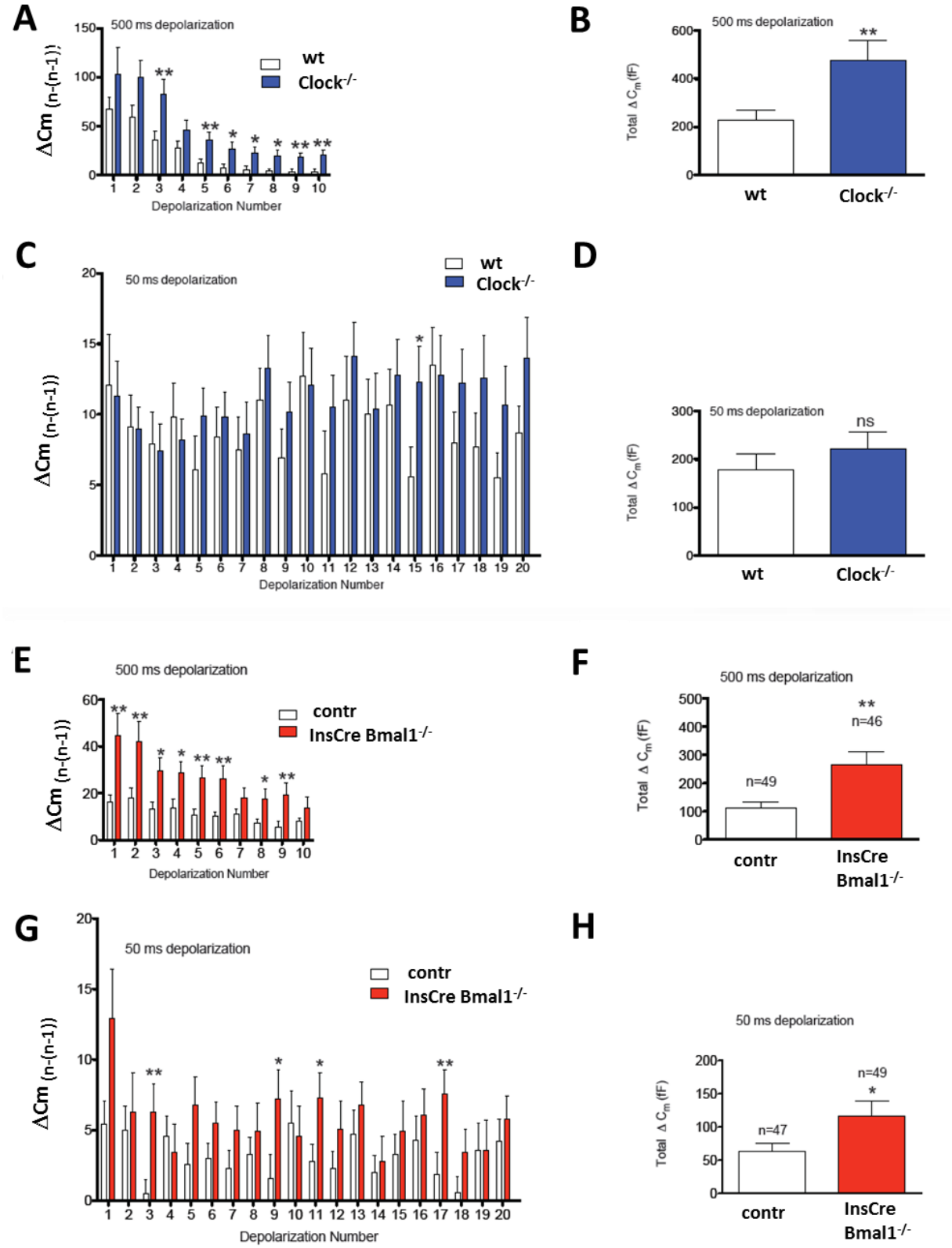


Fig. II-11: Loss of Clock or Bmal1 enhances exocytotic activity in pancreatic beta cells
Increase in cell capacitance (ΔCm,n-(n-1)) displayed against depolarization number measured in response to a train consisting of ten 500-ms (1 Hz) or twenty 50-ms (10 Hz) depolarizations in functionally identified pancreatic β-cells from wildtypt/control (white bars), or Clock$^{-/-}$/ InsCre Bmal1$^{-/-}$ (filled bars) mice ((A)/(E) and (C)/(G)). Total exocytosis (ΔCm) elicited by 500-ms and 50-ms depolarizations from -70 mV to 0 mV in β-cells from wildtype/control (white bars), or Clock $^{-/-}$/ InsCre Bmal1$^{-/-}$ (filled bars) mice ((B)/(F) and (D)/(H)). Data

presented as mean values ± S.E.M. of 55-70 β-cells from ca. 6-8 animals of each genotype. Figure was contributed by the group of Prof. Patrik Rorsman (The Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital, Oxford, UK).

II.A.2.3 Clock-Bmal1 affects expression of genes involved in vesicle trafficking and release

So far, physiological investigations revealed that Clock-Bmal1-disruption in beta cells induces impaired insulin release and an enhanced exocytotic rate. In order to shed light on the mechanism for the coexistence of these phenomena, an unbiased high-throughput expression analysis was undertaken.

In order to circumvent cost-intensive temporal profiles and, yet, obtain a reliable comparative analysis between Clock-proficient and Clock-deficient islets, a one-time-point analysis at ZT 9 was performed. This zeitgeber time would allow to identify the greatest expression discrepancies since shortly prior to it, at ZT 6-8, Clock-Bmal1 reach zenith in transcriptional activity (Ripperger and Schibler 2006).

Comparative microarray analysis was performed using Affymetrix chips hybridized with islet RNA obtained from 8-10 animals per genotype in order to obtain sufficient RNA amounts and account for biological variability. Due to the high abundance of beta cells, comprising ca. 90 % of the endocrine pancreas, the islet expression analysis represents a good estimation of the transcriptional status in insulin-producing cells (Elayat et al. 1995).

Downstream bioinformatic processing (conducted at The National Institute of Diabetes & Digestive & Kidney Diseases, USA) detected in total 14 057 transcripts, of which 3782 (27 %) were differentially expressed in $Clock^{-/-}$ islets, when a threshold of at least 30 per cent regulation was considered. Of them 2304 were up- and 1478 – downregulated **[Fig.II-12A, Appendix V.B.1]**. Subsequently, the data sets were processed through the **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (**DAVID**) in order to identify biological pathways, as opposed to individual genes, regulated in islets from the circadian mutants (Huang et al. 2009).

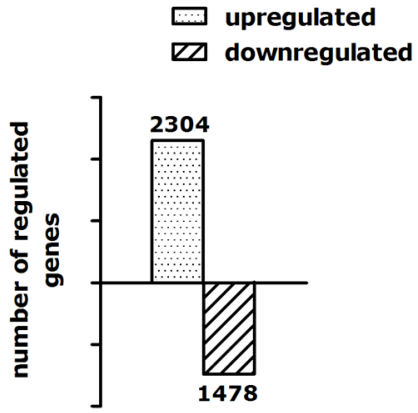
Gene ontology analysis revealed the following biological themes among the downregulated genes in $Clock^{-/-}$ islets: transcriptional regulation, splicing, circadian rhythms, cell junction and nuclear receptor signaling **[Fig.II-12B].** As expected, components of the circadian pathway – *per1, per2, per3, nr1d1, dbp, tef, hlf* – were overrepresented **[Fig.II-12B, V.B.1]**. These genes are established Clock-regulated targets, whose downregulation is in alignment

with the genetic ablation of the circadian transactivator (Jin et al. 1999; Yoo et al. 2005; Preitner et al. 2002; Gachon et al. 2006).

Further, the downregulated data set was particularly enriched in DNA-binding proteins and transcription factors, such as members of the zinc finger and basic leucine zipper (bZIP) protein families – *dnmt3a, dbp, tef, hlf, foxo3, gata6, hus1, jun, klf11, rad18, sirt1, snai1, tgif2, tgfb1* **[Fig.II-12B, V.B.1]**. These genes are implicated in a broad spectrum of cellular and physiological functions, including epigenetic control, organogenesis, DNA repair, metabolism, insulin signaling, beta cell identity and insulin production (Yokochi and Robertson 2002; Allaman-Pillet et al. 2004; Accili and Arden 2004; Decker et al. 2006; Weiss et al. 2003; Yang et al. 2012; Fernandez-Zapico et al. 2009; Tateishi et al. 2003; Imai et al. 2000; Rukstalis et al. 2006; Spagnoli and Brivanlou 2008; Lin et al. 2009).

Additional gene ontology themes, which were downregulated represented gene clusters, involved in mRNA splicing (*sf1, sf3a, sf3b, sfrs2*), cell junction (*actb, cldn6, cldn23, gjd2*) and nuclear receptor signalling (*esrrg, hnf4a, nr4a1, thra*) **[Fig.II-12B, V.B.1]**.

A



B

GO downregulated (enrichment score; p-value)	GO upregulated (enrichment score; p-value)
Transcription regulation, DNA-binding, transcription factor activity ($13,61; 1,4*10^{-27}$)	Vesicle-mediated transport, membrane invagination, endocytosis ($4,95; 3,5*10^{-9}$)
Basic-leucine zipper (bZIP) transcription factor ($5,11; 1,7*10^{-8}$)	Lysosome pathway ($4,83; 5,8*10^{-8}$)
mRNA metabolic processes, splicing ($4,07; 7,5*10^{-7}$)	Cytoskeletal protein binding, actin binding ($3,97; 9,9*10^{-7}$)
Circadian rhythms ($2,56; 3,7*10^{-4}$)	Cell adhesion ($3,47; 2,2*10^{-5}$)
Cell-cell junction, tight junction ($1,72; 1,1*10^{-3}$)	Cytoplasmic vesicle ($2,86; 3,5*10^{-4}$)
Nuclear receptors ($1,49; 1,2*10^{-2}$)	Secretion, exocytosis ($1,75; 1,1*10^{-2}$)

Fig.II-12: Microarray transcriptional analysis in islets from Clock$^{-/-}$ mice.
(A) The analysis revealed significant, greater than 30%, regulation in 3782 genes, of which 2304 were up- and 1478 downregulated. (B) The top seven Gene Ontology clusters, obtained through a DAVID analysis performed across the data set according to A. The significance of the enrichment in biological terms is characterized through the enrichment score and the p-value.

In respect to upregulated genes in Clock$^{-/-}$ islets, the data set was particularly abundant in the following biological terms: vesicle-mediated transport, membrane invagination, endo- / exocytosis, lysosome pathway, cytoskeletal binding, cell adhesion and vesicle **[Fig.II-12B]**. **Tab. II-1** summarizes a selection of genes in the respective categories and their precise function, preferably elucidated in pancreatic beta cells or other neuroendocrine lineages.

Some of these genes mediate vesicle trafficking between the ER and Golgi and along the cortical cellular network of actin filaments, participating in both constitutive and regulated secretion (*Ergic1, Spire1, Stx18, Scfd1, Sec16b, Rint1, Zw10, Bet1*) [**Tab. II-1]**.

Other components are related to granulogenesis [**Tab. II-1]**. Among them are proteases, inducing proinsulin to insulin conversion (*Pcsk2, Cpe*), but also vesicle proteins associated with constitutive secretion, which are sorted out over the course of insulin granulogenesis (*Vamp4, Clta*) (rev. in Kim et al. 2006).

Additional upregulated genes participate in processes downstream of granulogenesis, such as vesicle priming (*Unc13b*) and Ca^{2+}-regulated exocytosis (*Rims2, Nsf, Syt4, Cplx2*) **[Tab. II-1]**. For some of them, e.g. *pclo, rab27a, gsn, rims2, unc13b,* enhanced expression associates with improved hormone secretion, whereas, their experimental knockdown induces the opposite effect (ref. in **Tab. II-1**). However, others are inversely correlated with insulin release [**Tab. II-1]**. Of note, complexin 2 (*Cplx2)* and synaptotagmin IV (*Syt4)*, act as critical modulators of membrane fusion kinetics, promoting kiss-and-run exocytosis at the expense of insulin secretion (Archer et al. 2002; Wang et al. 2003; Bhalla et al. 2008). Complexin 2 represents a molecular "clamp" arresting SNARE-mediated membrane merge (Reim et al. 2001; Malsam et al. 2012). In a similar manner, synaptotagmin IV has been found to preclude insulin release (Wang et al. 2001; Wang et al. 2003; Bhalla et al. 2008).

Further upregulated transcripts in Clock$^{-/-}$ islets correspond to endocytosis **[Fig.II-12B, Tab. II-1]**. Particularly, they comprise the protein scaffold of clathrin-coated vesicles and are, thus, facilitating classical membrane retrieval (*Arf6, Ap1s1, Ap2a1, Ap2b1, Hip1r, Clta)* (ref. in **Tab. II-1**).

In addition, the data revealed upregulation in numerous components of the lysosomal pathway, such as enzymes involved in lumen acidification and macromolecular decomposition **[Fig.II-12B, Tab. II-1]**.

In sum, the large-scale expression analysis yielded a valuable snap-shot into transcriptome changes, induced by Clock-Bmal1 disruption in pancreatic beta cells. Importantly, this molecular footprint was characterized through differentially regulated gene sets involved in vesicle trafficking and exo- / endocytosis.

Tab. II-1: Upregulated genes in Clock$^{-/-}$ pancreatic islets
List of upregulated genes according to Gene Ontology themes from Fig.II-12B. The genes were divided into subgroups related to vesicle-mediated transport, exo- and endocytosis, and lysosomal components. The representative function was preferably referenced in insulin-secreting or neuroendocrine cells. For source microarray gene list see **V.B.1**

Gene symbol	fold change increase	Full name	Process (reference)
ERGIC1	1.51	endoplasmic reticulum-golgi intermediate compartment 1	vesicle-mediated transport (Breuza et al. 2004)
Spire1	1.88	spire homolog 1 (Drosophila)	vesicle transport along actin filaments (Schuh 2011)
Stx18	1.38	Syntaxin 18	vesicle-mediated transport between ER and Golgi (Hatsuzawa et al. 2000)
Vamp4	2.24	vesicle-associated membrane protein 4	vesicle-mediated transport, removed during insulin granule maturation (Eaton et al. 2000)
Scfd1	2.66	Sec1 family domain containing 1	vesicle trafficking, transport between ER and Golgi (Williams et al. 2004)
SEC16B	1.51	SEC16 homolog B (S. cerevisiae)	vesicle-mediated transport, membrane trafficking between ER and Golgi, peroxisome biogenesis (Tani et al. 2011)
Rint1	1.48	RAD50 interactor 1	vesicle-mediated transport, membrane trafficking between ER and Golgi (Arasaki et al. 2006)
ZW10	1.44	ZW10 homolog (Drosophila), centromere/kinetochore protein	vesicle-mediated transport, membrane trafficking between ER and Golgi (Hirose et al. 2004)
Bet1	1.35	Bet1 blocked early in transport 1 homolog (S. cerevisiae)	vesicle-mediated transport between ER and Golgi , v-SNARE (Springer and Schekman 1998)
Cpe	1.32	carboxypeptidase E	insulin secretion, insulin vesicle maturation (rev. in Steiner et al. 2009)
Pcsk2	2.6	proprotein convertase subtilisin/kexin type 2	insulin secretion, insulin vesicle maturation (rev. in Steiner et al. 2009)
Rab27a	3.14	member RAS oncogene family	exocytosis, vesicle association to actin filaments (Waselle et al. 2003; Kasai et al. 2005)
Pclo	1.5	piccolo	exocytosis, Ca^{2+}-sensor (Fujimoto et al. 2002)
Gsn	1.3	gelsolin	exocytosis, vesicle association to actin filaments (Kalwat et al. 2012; Tomas et al. 2010)
Rims2	1.3	regulating synaptic membrane exocytosis 2	exocytosis, late stages of secretion (Ozaki et al. 2000)
Unc13b	1.4	unc-13 homolog B (C. elegans)	exocytosis, vesicle priming (Sheu et al. 2003)

Nsf	2.22	N-ethylmaleimide sensitive fusion protein	exocytosis, ATPase returning the trans-SNARE complex into cis-SNARE conformation for a new round of fusion (Weber et al. 2000)
Syt4	1.31	Synaptotagmin 4	exocytosis, inhibitor of membrane fusion, promoter of kiss-and-run exocytosis (Wang et al. 2003; Bhalla et al. 2008)
Cplx2	1.34	Complexin 2	exocytosis, inhibitor of membrane fusion, promoter of kiss-and-run exocytosis (Archer et al. 2002)
Arf6	2.68	ADP-ribosylation factor 6	endocytosis, vesicle-mediated transport (Tomas et al. 2010)
Ap1s1	1.53	adaptor-related protein complex 1, sigma 1 subunit	endocytosis, vesicle-mediated transport, clathrin adaptors (Owen et al. 2004)
Ap2a1, Ap2b1	1.4 / 1.6	adaptor-related protein complex 2, alpha/beta sub.	endocytosis, vesicle-mediated transport, clathrin adaptors (Owen et al. 2004)
Htt	2.62	huntingtin	endocytosis, vesicle-mediated transport , microtubule association (Smith et al. 2009)
Hip1r	2	huntingtin interacting protein 1 related	endocytosis, vesicle-mediated transport , clathrin adaptor, (Owen et al. 2004)
Ankhzn	1.47	ankyrin repeat and FYVE domain containing 1	endocytosis, endosomal protein (Ito et al. 1999)
Cav1/2	1.58 / 1.4	caveolin 1/2	endocytosis (Matveev et al. 2001)
Clta	1.5	clathrin, light polypeptide	endocytosis, removed during insulin granule maturation (rev. in Kim et al. 2006)
ATP6V 0A1/1H	1.4 / 2	ATPase, H+ transporting, lysosomal V0 subunit a1 / H	lysosome acidification (Schröder et al. 2010)
NagA	1.4	N-acetyl galactosaminidase, alpha	lysosome degradation enzyme (Schröder et al. 2010)
Acp5	1.83	acid phosphatase 5, tartrate resistant	lysosome degradation enzyme (Schröder et al. 2010)
Naglu	1.47	N-acetylglucosaminidase, alpha	lysosome degradation enzyme (Schröder et al. 2010)
CtsA/C /H/O/S	1.4-1.9	cathepsin A/C/H/O/S	lysosome degradation enzyme(Schröder et al. 2010)
Gaa	1.6	glucosidase, alpha, acid	lysosome degradation enzyme (Schröder et al. 2010)
Lamp2	1.4	lysosomal-associated membrane protein 2	lysosome protein (Schröder et al. 2010)

B. Posttranscriptional regulation of the molecular clock in pancreatic beta cells by mir-375

So far, cumulative evidence pointed towards a pivotal role for the circadian duo Clock-Bmal1 in the promotion of insulin secretion.

mir-375 is the most abundant microRNA in pancreatic beta cells, which is implicated in negative regulation of the late stages in the insulin secretion pathway (Poy et al. 2004; Poy et al. 2009). As most miRNAs, mir-375 potentially inhibits hundreds of genes (Poy et al. 2004; Krek et al. 2005; Selbach et al. 2008; Baek et al. 2008). Hence, in a next step, it was investigated whether pacemaker components undergo posttranslational regulation by this microRNA, thus mediating part of its effect on insulin secretion.

II.B.1 mir-375 regulates components of the circadian pathway *in vitro* and *in vivo*

MicroRNAs (miRNAs) are ca. 22 nt-long RNAs inhibiting expression through base-pairing with the 3' UTR of the targeted mRNA (Lewis et al. 2005). In order to estimate whether components of the circadian pathway bear complementary seed regions for miR-375, an *in silico* approach was applied **[Fig.II-13]**. Two bioinformatics algorithms – PicTar and TargetScan – were employed to detect putative interactions (Krek et al. 2005; Lewis et al. 2005). These computational methods were chosen based on their high stringency: They consider only perfect Watson-Crick base-pairing, which accounts for the majority of miRNA–target interactions (rev. in Rajewsky 2006).

Both algorithms identified *mClock*, *mPer2* and *mTef* to carry one complementary seed region for mir-375 in their 3'UTR **[Fig.II-13].** Whereas *clock* and *per2* are core pacemaker circuit components, *tef* is a downstream transcription factor, involved in metabolic output of the circadian system (Gachon et al. 2006). As a criterion for confident target prediction, evolutionary sequence conservation of the respective miRNA binding sites was taken into consideration (rev. in Rajewsky 2006). Essentially, *bona fide* miRNA-targeted regions are likelier to exhibit conservation across orthologous sequences, reflecting the biological relevance of the interaction. The heptamers of *tef* and *per2* lacked sequence conservation across the vertebrate phylum **[Fig.II-13B]**. In case of the master circadian regulator *clock*, the putative miR-375 binding region was broadly conserved across primates, including humans, but was absent in other rodent orthologs **[Fig.II-13B]**.

Fig.II-13: Prediction algorithms suggest that circadian genes are targeted by mir-375
(A) Sequence of mature mir-375 and its seed region. (B) The 3' UTRs of mouse *clock*, *per2* and *tef* contain binding sites for mir-375. Evolutionary conservation across mammals of the putative miR-375 binding site – alignment match is marked in red. Mmu – mouse (*Mus musculus*); Rno – rat (*Rattus norvegicus*); Hsa – human (*Homo sapiens*); Ptr – chimpanzee (*Pan troglodytes*); Mml – rhesus (*Macaca mulatta*); Bta – cow (*Bos taurus*)

Putative miRNA binding, revealed by genome-wide alignments, do not indicate whether these sites are indeed occupied by the microRNA ribonucleoprotein silencing complex; hence, their biological functionality required further experimental validation by means of several methods.

The first experiment comprised a cell-based luciferase reporter assay, in which the 3' UTR of the respective gene – *clock, per2* or *tef* – was cloned into a vector, downstream of the *Renilla luciferase* open reading frame (Kuhn et al. 2008). Upon transient co-transfection of the recombinant plasmid and the miRNA into a host cell, specific miRNA-target interaction could be easily quantified based on reporter protein repression. Importantly, mir-375 introduction into HEK293 cells resulted in a robust decrease in luciferase activity for all three constructs, as compared to transfection with a control miRNA, not predicted to target the respective

UTRs **[Fig.II-14A]**. This suggested that mir-375 exerts a functionally inhibiting effect over *clock*, *per2* and *tef* expression.

Further experiments validating putative posttranscriptional regulation were restricted to the core transactivator *clock*. The rationale for this was based on the fact that *per2* and *tef* exhibited poor phylogenetic conservation in their mir-375 seed region across mammals, referring a biological function rather unlikely **[Fig.II-13B]**.

In a second experiment, the capacity of mir-375 to regulate endogenous *clock* expression was tested in a cell line *in vitro*. In Hepg2 cells, mir-375 overexpression, achieved through introduction of a *mir-375*-encoding plasmid, evoked approximately 40 % reduction in Clock protein **[Fig.II-14B].**

Lastly, in order to avoid cellular delivery of supraphysiological mir-375 duplex concentrations, in which Clock mRNA can be engaged in an unphysiological interaction, the regulation was assessed in mouse pancreatic islets *in vivo*, where both microRNA and its putative target are coexpressed (Poy et al. 2004, Fig.II-6B). Hence, endogenous Clock expression was evaluated in islets derived from mir-375$^{-/-}$ mice **[Fig.II-14C]**. Importantly, under conditions of microRNA deletion, Clock protein has increased by approximately 60 %.

Taken together, cumulative *in vitro* and *in vivo* evidence from mir-375 overexpression and knockdown suggested a functional miRNA–mRNA interaction, suggesting a first example for posttranscriptional inhibition of *clock* in a peripheral tissue.

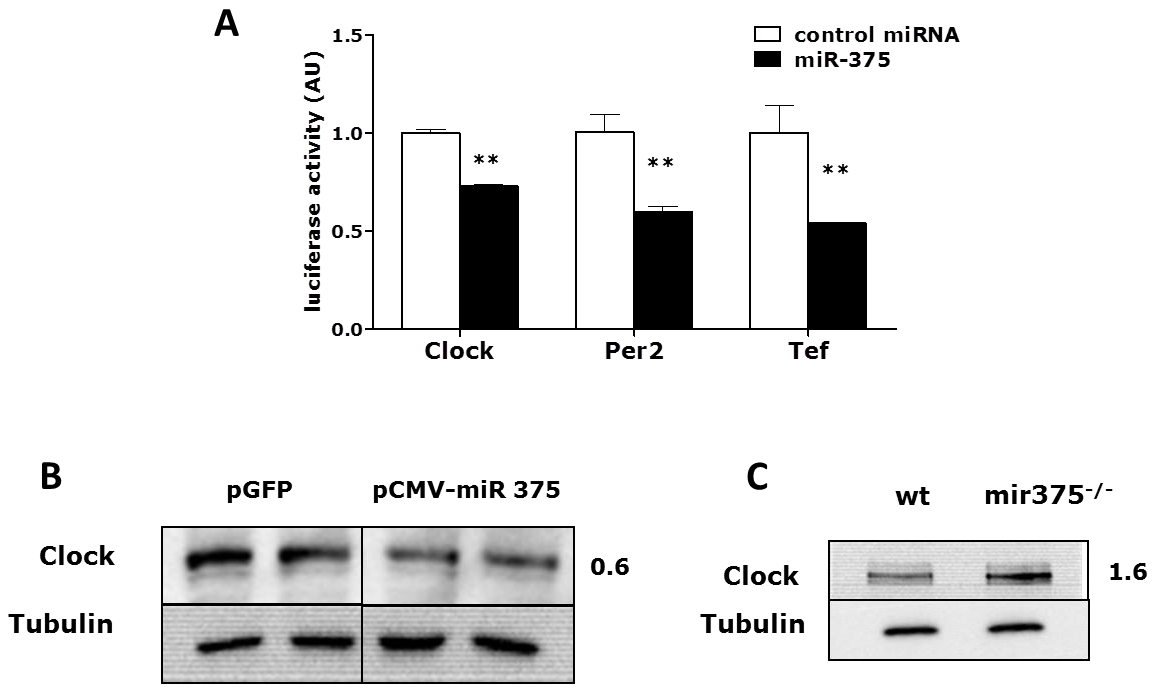


Fig.II-14: Clock is regulated by mir-375 *in vitro* and *in vivo*
(A) miRNA target reporter assay: Luciferase activity, as determined by luminescence emission, was assessed after cells were transfected with 3' UTRs (complete or truncated) of Clock, Per2 or Tef downstream of *Renilla* luciferase and co-transfected with mir-375 or a control miRNA. (B) Western Blot showing Clock expression in HepG2 cells after mir-375 overexpression. (C) Western Blot showing Clock expression in pancreatic islets isolated from a mir-375-deficient mouse. Figures indicate relative Clock expression normalized to Tubulin expression according to densitometry analysis. One out of three representative experiments is shown.

II.B.2 miR-375 regulates the damping ratio of circadian oscillations in the islet

Next, it was investigated whether mir-375 regulation over Clock exerts a physiological effect on circadian rhythms in beta cells. Particularly, it was tested whether loss of mir-375 alters the dynamics of oscillations (experiments were conducted in collaboration with the group of Prof. Achim Kramer, Charite Berlin).

For this, a circadian reporter mouse model lacking mir-375 was generated. The bioluminescence reporter rodent line expressing *Period2*^{luciferase} (Per2^{luc}) is widely accepted as a model for real-time monitoring of circadian oscillations (Yoo et al. 2004). In this mouse knock-in strain, endogenous *per2* is fused with firefly *luciferase* (*per2::luciferase*), thus, preserving its transcriptional and posttranscriptional regulation. The reporter protein displays robust circadian oscillations indistinguishable from wild-type Per2 (Yoo et al. 2004). This mouse strain was bred with mir375-deficient animals (mir-375$^{-/-}$) to obtain offspring, expressing the luciferase reporter together with homo- or heterozygous deficiency for *mir375* **[Fig.II-15]**.

Importantly, in cultured *per2::luc* expressing islets, real-time bioluminescence could be continuously measured over 24 hours for several weeks, confirming the presence of an autonomous islet pacemaker and its preservation *ex vivo* [**Fig.II-16]**. The period of the islet pacemaker comprised 24.47±0.51 hours, consistent with results obtained in other peripheral organs (Yoo et al. 2004).

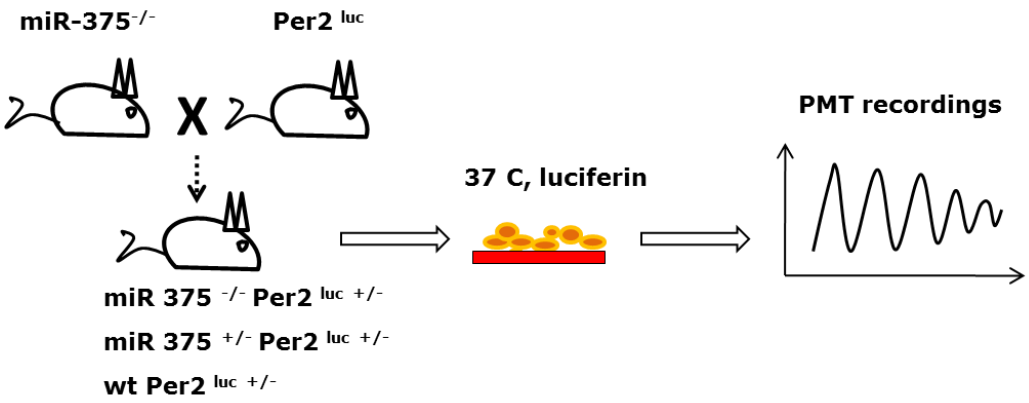


Fig.II-15: Experimental set-up for the recording of circadian bioluminescence in mir-375$^{-/-}$ mice.
Experimental mice expressing the luciferase knock-in Per2 reporter (Per2::luc) were generated by breeding of the respective parental strains – Per2^{luc} and mir-375$^{-/-}$. Islets from Per2::luc expressing animals carrying a hetero- or homozygous deficiency for mir-375 were isolated and a comparable number was plated with D-luciferin and incubated for 7-14 days. Real time bioluminescence was recorded using photomultiplier tube detectors (PMT). Data was subsequently fitted and analyzed.

Next, period, amplitude and persistence of the self-sustained rhythms under conditions of mir-375 deficiency were evaluated based on bioluminescence readings.

Firstly, homo- or heterozygous deficiency for *mir-375* did not display any difference in circadian period as it was comparable to wildtype (wt - 24.47±0.51, 375$^{+/-}$ - 24.3±0.34, 375$^{-/-}$ 24.24±0.26 hours) [**Fig.II-16C]**.

Secondly, phase of Per2-cycling in all three genotypes was similar, reflecting that zenith and nadir in bioluminescence were reached at the same time of the day (46.63±3.19, 42.79±2.1, 42.03±2.53 hours) [**Fig.II-16D]**.

In respect to amplitude of bioluminescence expression, miR-375-deficiency seemed to result in a minor, albeit not significant, decrease (0.15±0.05, 0.11±0.04, 0.096±0.04) [**Fig.II-16E].**

Finally, the persistence of *ex vivo* oscillations in the islet was addressed [**Fig.II-16F]**. Generally, explanted nonneural tissues can sustain intrinsic circadian rhythms for several days before they damp out (Yoo et al. 2004). The latter does not result from cell death but, instead, from the lack of SCN-derived synchronization *ex vivo*. In the absence of such entrainment, intercellular coupling in the oscillator is gradually lost, i.e. individual cell pacemakers keep running but develop phase desynchrony relative to each other, eventually damping rhythms in the overall multicellular population (Nagoshi et al. 2004). Damping was quantified based on the decay of amplitude half-live, with a damping ratio of one corresponding to an undamped oscillator (Westermark et al. 2009).

Remarkably, loss of mir-375 induced a pronounced effect on the persistence of circadian Per2::luc oscillations. Islets, deficient for the microRNA, displayed a ca. 50 % reduction in damping rate as compared to control, indicating that bioluminescence rhythms damp out much slower under these conditions [**Fig.II-16F]**. Importantly, mir-375 expression seemed to exert a dose-dependent effect on oscillatory persistence as the heterozygous mir-375$^{+/-}$ state resulted in intermediate reduction of this parameter (wt - 1.78±0.29, 375$^{+/-}$ - 1.56±0.03, 375$^{-/-}$ - 1.31±0.11). Of note, these alterations in cycling pattern are not associated with differences in cell survival, as tissue explants from all genotypes were regularly inspected and no impairment in cell viability was detected (data not shown).

Taken together, mir-375-deficiency did not affect the precision of the islet pacemaker, as circadian period and phase were unaffected. However, loss of the miRNA rendered oscillations more long-lived, thus, suggesting a role for mir-375 in sustaining intercellular coupling.

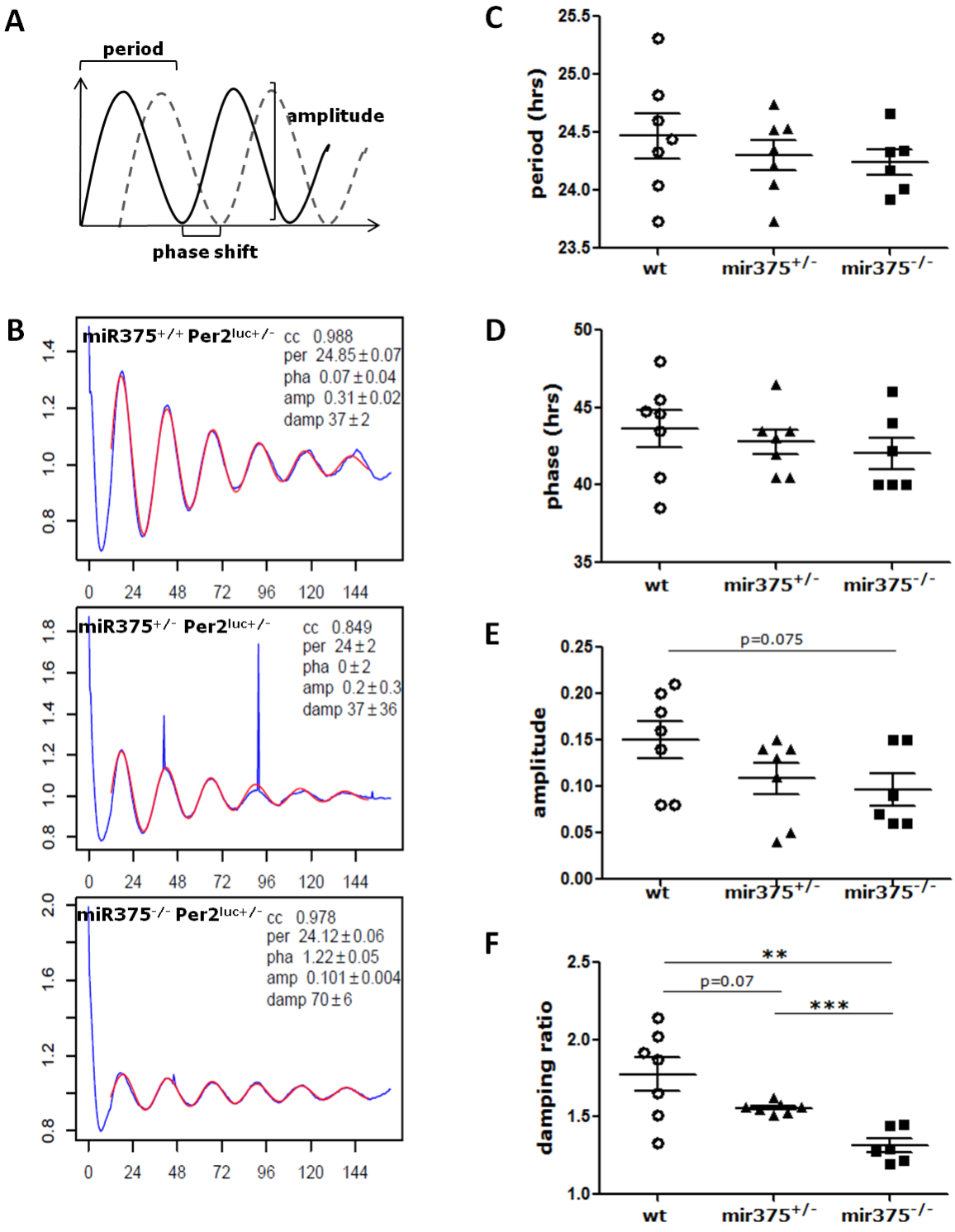


Fig.II-16: Per2::luc bioluminescence is altered in mir375-deficient islets.
(A) Schematic representation of parameters characterizing circadian oscillations. (B) Representative records of bioluminescence oscillations for the three respective genotypes – wt, mir-375$^{+/-}$ and mir-375$^{-/-}$. Data has been

curve-fitted and normalized to account for varying emission magnitude through a 24-hour running average. Spike peaks indicate artefacts, such as short light exposure of the PMT detectors due to opening of the incubator. (C) period, (D) phase and (E) amplitude of Per2::luc oscillations. (F) Damping ratio, characterized by the exponential decay of the oscillations. A ratio of 1 corresponds to an undamped oscillator. Bioluminescence emission was recorded from ca. 100 islets per sample, derived from 1-2 mice. N=6-7. Data represents mean and standard error; standard deviation is referred in text.

C. The role of Clock in hepatic function

To shed light on the role of the molecular clock in energy metabolism, its impact was further elucidated in liver – a pivotal organ in systemic glucose and lipid homeostasis. Here, I aimed to identify discrete molecular pathways, which link Clock-Bmal1 to metabolic output and to assess whether the heterodimeric duo influences miRNA expression.

These goals were addressed in the panspecific Clock$^{-/-}$ mouse model, which was chosen based on two facts. Firstly, it is characterized through complete loss of Clock protein (Debruyne et al. 2006). Secondly, Clock$^{-/-}$ mice display intact circadian function in the central nervous system and lack any abnormalities in sleep, locomotor activity and feeding behavior, which could mask the pacemaker function in liver (see Fig. II-1, Debruyne et al. 2006).

II.C.1 Clock-deficiency leads to increased hepatic glucose production

The initial phenotypical characterization of Clock$^{-/-}$ mice revealed mild hyperglycaemia after starvation, which was not accompanied by decreased insulin concentration, suggesting that, beyond beta cell dysfunction, increased hepatic glucose production might be also present [**Fig. II-3]**. This prompted a detailed investigation of the impact of Clock-disruption in liver carbohydrate balance.

Hepatic glucose output is turned on over the postabsorptive catabolic phase in the inactive period to derive circulating sugar via two distinct routes: On the one hand, glucose is released from stored glycogen. On the other, it is formed *de novo* from non-carbohydrate metabolites, such as amino acids, pyruvate, glycerol and tricarboxylic acid cycle intermediates (rev. in Nuttall et al. 2008). In the fasted state both pathways contribute to the maintenance of normal glycemia; once glycogen stores are depleted, gluconeogenesis predominates. Both processes were assessed in Clock$^{-/-}$ mice.

Gluconeogenesis rate was determined by means of a pyruvate tolerance test. After i.p. administration of pyruvate, the carbon substrate is taken up by the liver to serve metabolic conversion to glucose. In addition, an overnight starvation of the animals prior to the challenge depletes glycogen depots in the liver and stimulates gluconeogenesis.

Importantly, Clock$^{-/-}$ mice responded with elevated glucose release during the pyruvate tolerance test **[Fig.II-17A]**. Moreover, gluconeogenesis was boosted irrespective of circadian time as pyruvate administration at the onset of the subjective day and night, respectively, yielded similar results.

Secondly, the capacity for glycogen storage was assessed. Importantly, the carbohydrate polymer was decreased by over 50 per cent in liver tissue of Clock$^{-/-}$ mice **[Fig.II-17B]** (2.4±0.6 and 1.0±0.3 mg/g liver). This effect was further confirmed in primary hepatocytes derived from Clock-deficient animals, cultured for 24 hours *ex vivo* **[Fig.II-17C].** There, glycogen concentration exhibited a similarly drastic reduction as in wet tissue, indicating that impairment in glycogen metabolism is cell-autonomous and irrespective of systemic physiological stimuli (4.1±1.7 and 2.3±0.9 AU).

Next, I wanted to confirm whether these defects are liver-born or arise as a secondary effect from an altered hormone homeostasis due to panspecific pacemaker disruption. The counteraction of islet-derived insulin and glucagon is critical: Elevated glucagon and / or decreased insulin signalling could act stimulatory on hepatic glucose output and misrepresent a liver-born defect resulting from *clock*-deletion **[**Fig.**II-18A]** (Barthel and Schmoll 2003; Jiang and Zhang 2003). To rule out this possibility the following experiments were undertaken.

Firstly, alpha-cell function in Clock$^{-/-}$ mice was addressed. Circulating glucagon concentrations were comparable to wild-type controls, excluding that an altered secretion of the hormone affects liver glucose metabolism in Clock$^{-/-}$ mice **[**Fig.**II-18B]** (56±6 and 61 ±13 pg/ml).

Secondly, to evaluate whether reduced postprandial insulin release under conditions of panspecific *clock* deletion can account for an increase in hepatic glucose output, liver carbohydrate metabolism was determined in InsCre Clock$^{-/-}$ mice, characterized solely through a beta cell secretion defect. Importantly, there, gluconeogenesis rate and hepatic glycogen content were comparable to littermate controls [Fig.**II-18C, D]** (0.69±0.18 and 0.72±0.13 mg/g). This illustrated that the reduced insulin release characterizing InsCre Clock$^{-/-}$ and Clock$^{-/-}$ mice, was not sufficient to compromise liver physiology.

Collectively, these results excluded that alterations in insulin and / or glucagon signalling might boost glycogenolysis and gluconeogenesis in whole-body Clock$^{-/-}$ animals. Together with the finding that glycogen metabolism is changed in cultured Clock$^{-/-}$ hepatocytes in the

absence of any systemic signals *ex vivo*, the data indicated that the increase in hepatic glucose output is a liver-born and cell-autonomous defect.

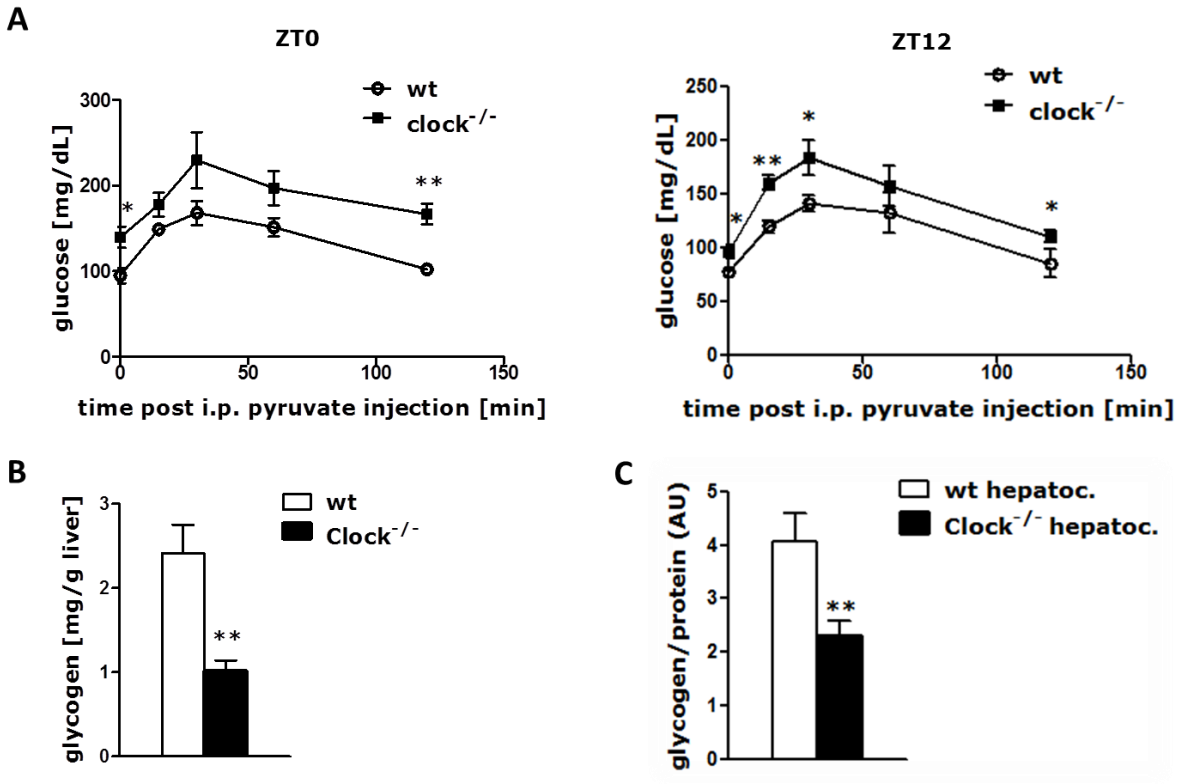


Fig.II-17: Loss of Clock in liver increases hepatic glucose output
(A) Pyruvate tolerance test in Clock$^{-/-}$ mice at ZT0 and ZT12. After an i.p. pyruvate challenge, blood glucose was measured at the indicated timepoints, n=5. (B) Liver glycogen content in Clock$^{-/-}$ and wildtype mice normalized to wet tissue mass, n=10. (C) Intracellular glycogen content in primary hepatocytes isolated from Clock$^{-/-}$ or wildtype mice and cultured 24 h prior to the assay. The values are normalized to intracellular protein amount and depicted as arbitrary units (AU), n=10.

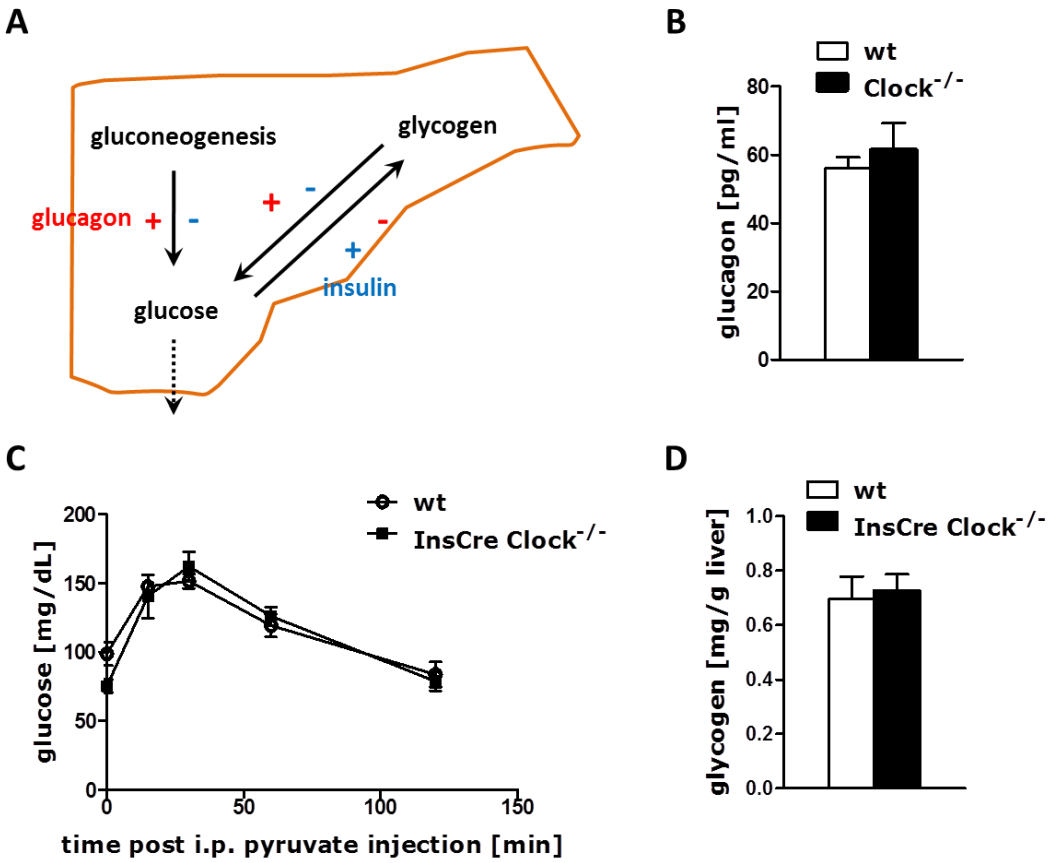


Fig.II-18: Islet-derived hormones do not influence hepatic glucose output in Clock$^{-/-}$ mice
(A) The action of insulin and glucagon on liver. Insulin suppresses hepatic glucose production by blockage of glycogen breakdown and gluconeogenesis (blue –), glucagon stimulates both processes (red +). (B) Circulating glucagon in whole-body Clock$^{-/-}$ and wildtype animals (n=6). (C) Pyruvate tolerance test in InsCre Clock$^{-/-}$ mice (n=5) .(D) Liver glycogen content in InsCre Clock$^{-/-}$ mice, normalized to wet tissue, n=6.

II.C.2 Clock-deficiency alters VLDL metabolism

Besides glucose, the liver promotes metabolism of further macronutrients, such as lipids: It governs systemic trafficking of cholesterol and triglycerides through synthesis and secretion of lipoprotein complexes. Since many lipometabolic processes exhibit circadian regulation in mice and man, I questioned whether hepatic lipoprotein secretion in particular is altered under conditions of Clock deficiency (see I.B.3.4; Edwards et al. 1972; Miettinen 1982; Pan and Hussain 2007; Le Martelot et al. 2009).

Hence, the lipoprotein profile of $Clock^{-/-}$ mice was investigated. Over the course of systemic circulation lipoproteins undergo progressive unidirectional lipolysis, resulting in the formation of differently sized species – very low (VLDL), low (LDL), intermediate (IDL) and high density lipoproteins (HDL) (Alaupovic 1982). By means of size-exclusion chromatography and subsequent protein detection in the eluate, these subpopulations can be reliably assessed and quantified (Yang et al. 2012).

As a start, circulating lipoproteins in plasma were determined at a steady-state condition, when food was provided *ad libitum*, at three time points across the subjective day and night – ZT 3, ZT 12 and ZT 19 **[Fig.II-19]**. Fast protein liquid chromatography (FPLC) revealed an overall preservation of the lipoprotein profile in mice lacking the circadian transactivator. The discrete lipoprotein subpopulations could be assigned to the ascendant FPLC elution peaks – chylomicrons/VLDL, IDL/LDL and HDL [Fig.**II-19]** (Dominiczak and Caslake 2011; Yang et al. 2012). As expected, the predominant class, characterized by an overriding peak in protein absorbance, consisted in the cholesterol-carrier LDL, which is the most abundant lipoprotein in circulation.

Interestingly, in $Clock^{-/-}$ plasma a significantly higher concentration in particles of the first elution peak was determined, which was also confirmed through mathematical calculation of the area under the curve **[Fig.II-19B, C]** (ZT3 65±15 and 100±9; ZT12 116±23 and 175±34; ZT19 171±13 and 219±17 mAU*ml)**.** At a random-fed state, the first peak represents a heterogeneous mixture of two distinct lipoprotein species with identical diameter-range – intestine-derived chylomicrons, loaded with dietary fat, and liver-secreted very-low density lipoprotein (VLDL) – which cannot be separated using a size-based approach (rev. in Schaefer 2010). In alignment with the fact that this fraction is rich in lipoproteins transporting exogenous lipids, in both wildtype and $Clock^{-/-}$ animals, this population exhibited highest concentration during the active nocturnal period, when food intake predominately occurs, with a maximum at ZT19 [**Fig.II-19C]**.

In the absence of dietary fats, *de novo* lipogenesis and VLDL assembly / secretion is triggered in hepatocytes (Thompson et al. 1996). In order to evaluate the intrinsic capacity for VLDL secretion under conditions of Clock-disruption and overcome the presence of circulating chylomicrons in the FPLC profile, Clock$^{-/-}$ mice were subjected to food deprivation over the entire active period prior to plasma lipoprotein profiling at ZT1 **[**Fig.II-20**].**

Remarkably, under this condition, a significant decrease in the first elution peak was determined, whereas other classes remained unaffected **[**Fig.II-20**C]** (VLDL 35±10 and 22±3; LDL 760±85 and 705±62; HDL 46±19 and 50±9 mAU*ml). In contrast to the previous experimental setting, here, the large-molecular particle fraction represented a pure VLDL population, loaded with endogenously derived triglycerides and cholesterol.

Taken together, the circulating lipoprotein profile in Clock$^{-/-}$ mice diverged depending on the nutritional status of the mice. Particularly, Clock-deficiency resulted in a reduced VLDL fraction, loaded with endogenous lipids. Further investigations are required to elucidate whether an impaired ability of hepatocytes to synthesize and/or secrete VLDL underlies this defect.

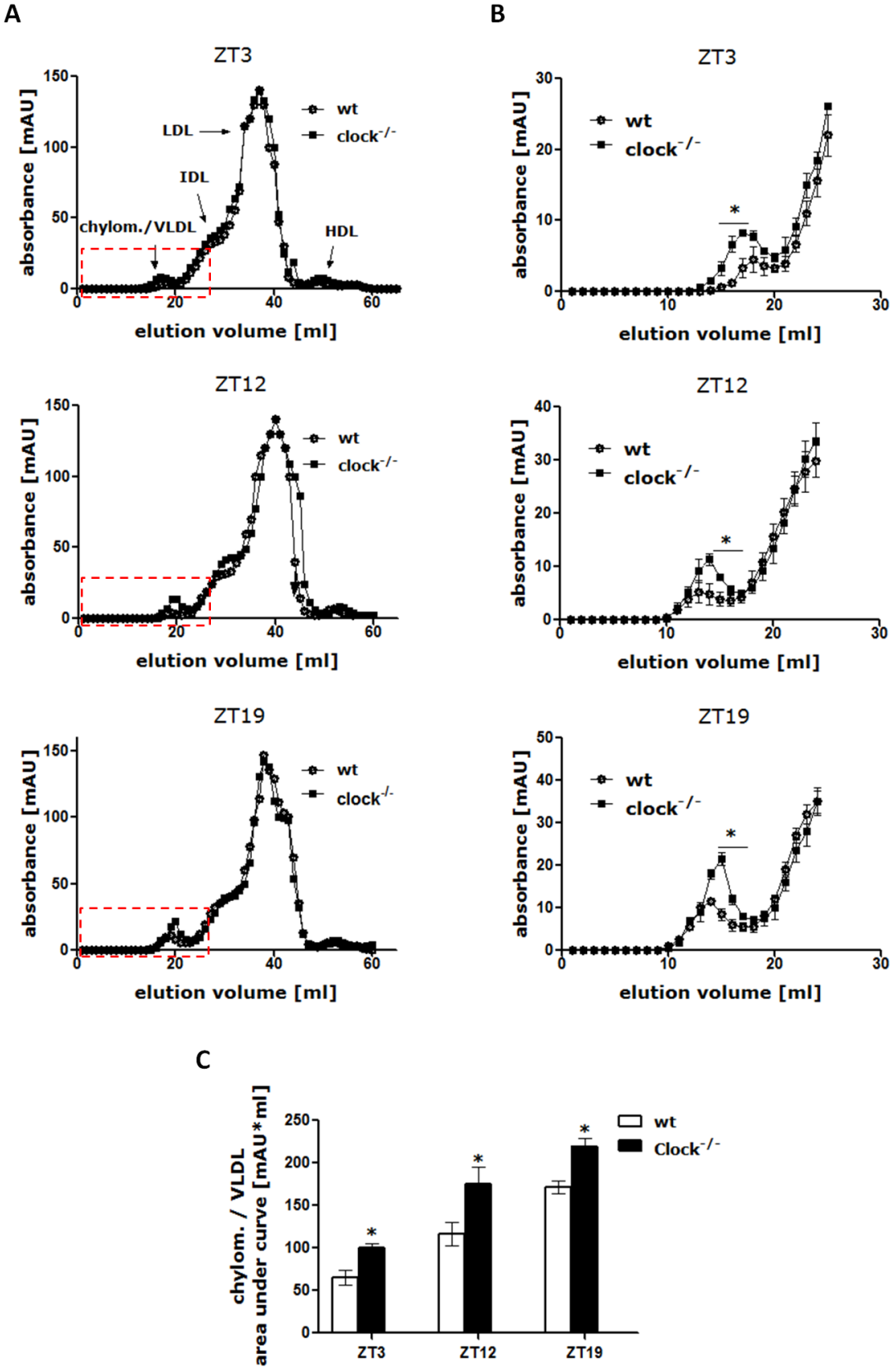


Fig.II-19: Circulating lipoproteins in Clock$^{-/-}$ mice fed *ad libitum*
(A) Representative FPLC lipoprotein profiles in plasma isolated at ZT3, ZT12 and ZT19 from wildtype and Clock$^{-/-}$ mice fed *ad libitum*. The x-axis represents the elution volume in ml, the y-axis – the UV absorbance of lipoprotein at 280 nm in milli absorbance units (mAU). The different lipoprotein subclasses are assigned to the

corresponding peaks. The dashed line square is magnified in B. Chylom. – chylomicrones, VLDL – very low density lipoprotein, IDL – intermediate density lipoprotein, LDL – low density lipoprotein, HDL – high density lipoprotein. (B) Close-up of the elution peak containing the chylomicrone / VLDL fraction from A. Data represents mean and standard error (n=4). In each run, 200 µl of plasma was combined at equal amounts from 4-5 mice (C) Quantification of the chylomicrons / VLDL fraction by means of area under the curve

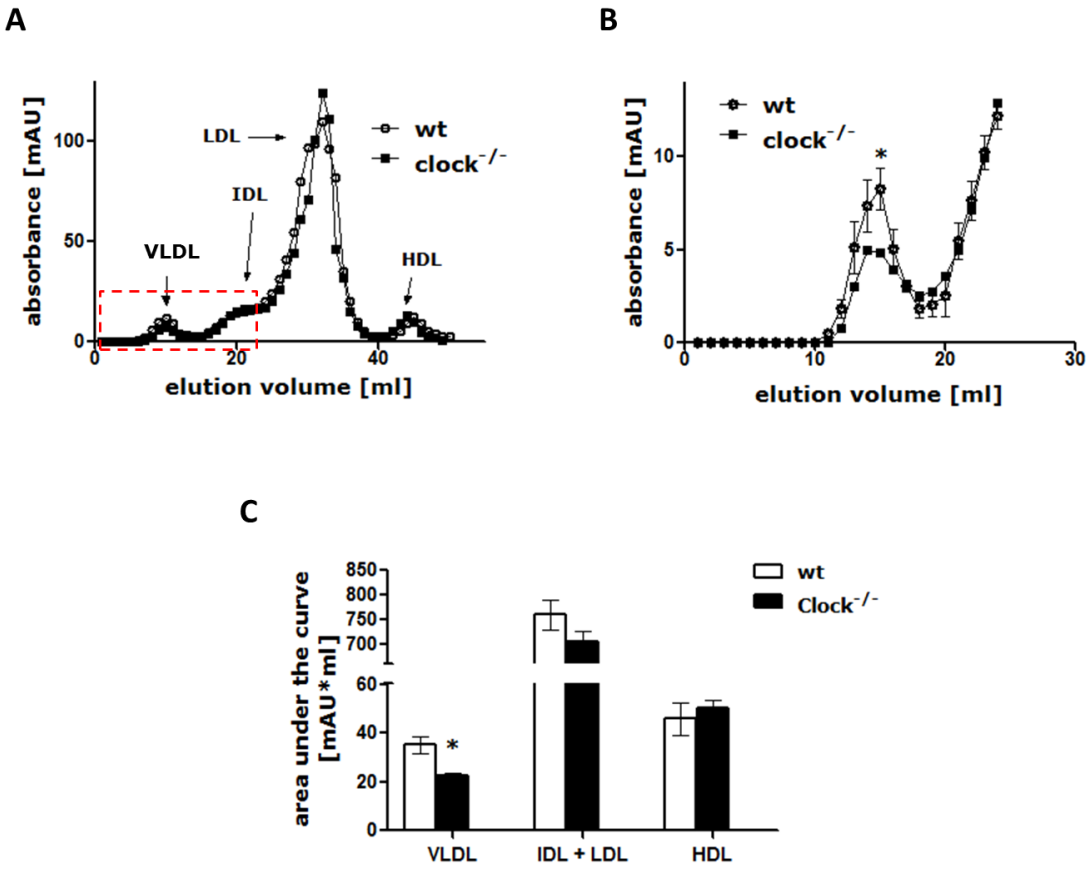


Fig.II-20: Circulating lipoproteins in Clock$^{-/-}$ mice after fasting.
(A) Representative FPLC lipoprotein profile in plasma isolated from wildtype or Clock$^{-/-}$ mice at ZT1 after an overnight fast. The x-axis represents the elution volume in ml, the y-axis – the UV absorbance of lipoprotein at 280 nm in milli absorbance units (mAU). The different lipoprotein subclasses are assigned to the corresponding peaks. The dashed line square is magnified in B. VLDL – very low density lipoprotein, IDL – intermediate density lipoprotein, LDL – low density lipoprotein, HDL – high density lipoprotein. (B) Close-up of the elution peak containing the VLDL fraction from A. Data represents mean and standard error (n=8). In each sample run through the chromatography column, 200 µl of plasma were combined at equal amounts from three mice. (C) Quantification of the elution fractions for VLDL, IDL+LDL and HDL by means of area under the curve.

II.C.3 Gene expression analysis in Clock$^{-/-}$ liver

In order to gain a better understanding of hepatic glucose output and lipoprotein metabolism in Clock$^{-/-}$ mice, in a next step, gene expression was addressed. Large-scale analyses of the transcriptome, proteome as well as the microRNA profile were applied in order to dissect the global impact of the transcription factor Clock on protein- and miRNA-coding genes. The prevailing interest lied in the identification of Clock-Bmal1 targets, which are involved in lipoprotein metabolism.

In analogy to the expression analysis in Clock$^{-/-}$ islet, a one-timepoint snapshot comparison between wild-type and Clock$^{-/-}$ liver was conducted at ZT 9 (see II.A.2.3). Around this timepoint *bona fide* Clock-Bmal1 targets peak at their transcript level, while corresponding protein accumulation also rises with a 3-4 hour phase-delay in oscillation (Lee et al. 2001). Hence, at ZT 9, putative Clock-Bmal1 targets could be identified based on their downregulation under conditions of *clock* deletion.

II.C.3.1 Differential expression of enzymes involved in glucose and lipid metabolism in Clock$^{-/-}$ liver

II.C.3.1.1 Transcriptome analysis in Clock$^{-/-}$ liver

Comparative transcriptome analysis was performed using Affymetrix microarray. Raw RNA-hybridization signals were normalized and subjected to an intensity cut-off to remove low-abundant transcripts and subsequently used to calculate transcript expression data considering hybridization signals from multiple probe sets. Fold change regulation was corrected for multiple hypothesis testing.

Ultimately, the microarray approach led to the detection of 15,663 hepatic transcripts. Considering a minimum of 30 per cent change, the significantly up- and downregulated genes comprised 145 and 122, respectively, corresponding to 1.7% of the hepatic transcriptome **[Fig. II-21, Appendix V.A.1]**. Furthermore, the validity and reliability of the transcriptome data was evaluated: For this, a panel of genes from various metabolic categories was selected and screened by real time PCR. The expression differences captured by this alternative approach aligned largely with the results in the microarray profile, thus, confirming its integrity [**Fig. II-22].** Furthermore, the reproducibility of the transcriptome data was assessed: The expression profiles were characterized through a higher correlation between biological replicates than among genotypes **[Fig. V-1, V3-A]**.

Differentially expressed transcripts were analysed with the **D**atabase for **A**nnotation, **V**isualization and **I**ntegrated **D**iscovery (**DAVID**) in order to identify functional groups and biological pathways, as opposed to individual genes altered in Clock$^{-/-}$ liver (Huang et al. 2009).

The gene ontology analysis revealed the following themes among the downregulated genes in Clock$^{-/-}$ liver: secreted proteins, lipoproteins, innate immunity, circadian rhythms, transcription factors and mitochondrion **[Fig. II-21B]**. As expected, circadian genes were downregulated, highlighting the disruption of the positive transcriptional limb in the clock circuit (*per3, nr1d1, dbp)* [**Fig. II-22]** (Jin et al. 1999; Yoo et al. 2005; Preitner et al. 2002; Gachon et al. 2006).

Importantly, the highest enriched biological terms among the downregulated genes were involved in secretion of extracellular factors and plasma lipoproteins **[Fig. II-21B]**. This group was overrepresented by constituents of lipoprotein components, associated with VLDL (*apoa5*) or transcriptional regulators, promoting VLDL assembly and export (*ppargc1b*, *hes6*) **[Ref. in Tab. II-2]**.

Further downregulated genes in Clock$^{-/-}$ liver were related to mitochondrial function **[Fig. II-21B, Tab. II-2, Fig. II-22]**. Among them were oxidoreductases controlling oxidative phosphorylation, detoxification, drug metabolism, fatty acid and cholesterol biosynthesis – *aldh1b1, cyb5r1, sod1, slc25a5* (Stagos et al. 2010; Neve et al. 2012; de Haan et al. 1994; Yang et al. 2010) [**Appendix V.A.1].**

Finally, the downregulated data set was particularly enriched in chromatin-binding factors (*nr1d1, ppargc1b, hes6, jun-b, cebpb*), implicated in versatile physiological processes, such as lipid metabolism, liver regeneration and apoptosis (Le Martelot et al. 2009; Lin et al. 2005; Shaulian 2010; Martinez-Jimenez et al. 2010; Isoda et al. 2005) **[Fig. II-21B, Tab. II-2, Fig. II-22, Appendix V.A.1].**

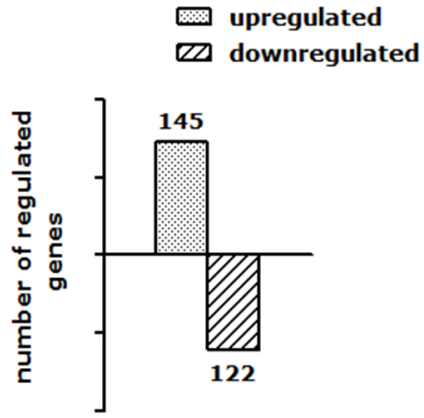
In respect to the upregulated genes in Clock-deficient liver, the data set was enriched in components, involved in lipid metabolism, peroxisome function, apoptosis, DNA-binding and cell adhesion [**Fig. II-21B]**. The highest enriched gene class mediated lipid catabolism, particularly cholesterol and triglyceride breakdown, beta-oxidation, cholesterol conversion to bile acids and preclusion of *de novo* cholesterol synthesis – *hsd3b7, insig2, lipc, osbp, lpl, ehhadh* **[Tab. II-2, Fig. II-22]** (Wang et al. 2011; Yabe et al. 2002; Dichek et al. 1991; Du et al. 2011; Veldhoven 2010). Many of the proteins, encoded by these genes, constitute

components of mitochondria and peroxisomes, where most of the lipid catabolic processes occur (Reddy and Mannaerts 1994).

Additionally, various transcriptional repressors and activators also displayed upregulation in Clock$^{-/-}$ liver (*atf5*, *cry1*, *ncoa5*, *grhl1*) [**Fig. II-21B]**.

Taken together, transcriptome analysis revealed broad expression changes in Clock$^{-/-}$ liver, particularly, in respect to lipid and lipoprotein metabolism.

A



B

GO downregulated (enrichment score; p-value)	GO upregulated (enrichment score; p-value)
Extracellular space, secreted proteins (2,57; 5*10^{-5})	Catabolic lipid metabolism (1,72; 2,3*10^{-3})
Plasma lipoprotein particle (1,84; 7,6*10^{-3})	Peroxisome (1,2; 6*10^{-2})
Acute inflammatory response (1,45; 3,1*10^{-4})	Anti-apoptosis (0,7; 3,9*10^{-3})
Circadian rhythms (1,19; 3*10^{-2})	Transmembrane glycoproteins (0,72; 1,2*10^{-1})
Transcription factors, bZIP family (0,95; 9,8*10^{-4})	Cell junction (0,34; 3*10^{-2})
Mitochondrion, oxidation reduction (0,46; 1,2*10^{-2})	Transcription factors (0,21; 8*10^{-2})

Fig. II-21: Microarray transcriptional analysis in Clock$^{-/-}$ liver
(A) The analysis revealed significant, greater than 30%, regulation of 267 genes, of which 145 and 122 genes were up- and downregulated, respectively (B) The top seven gene ontology clusters, obtained through a DAVID analysis performed across the data set in (A). The significance in enrichement is characterized through enrichment score and p-value.

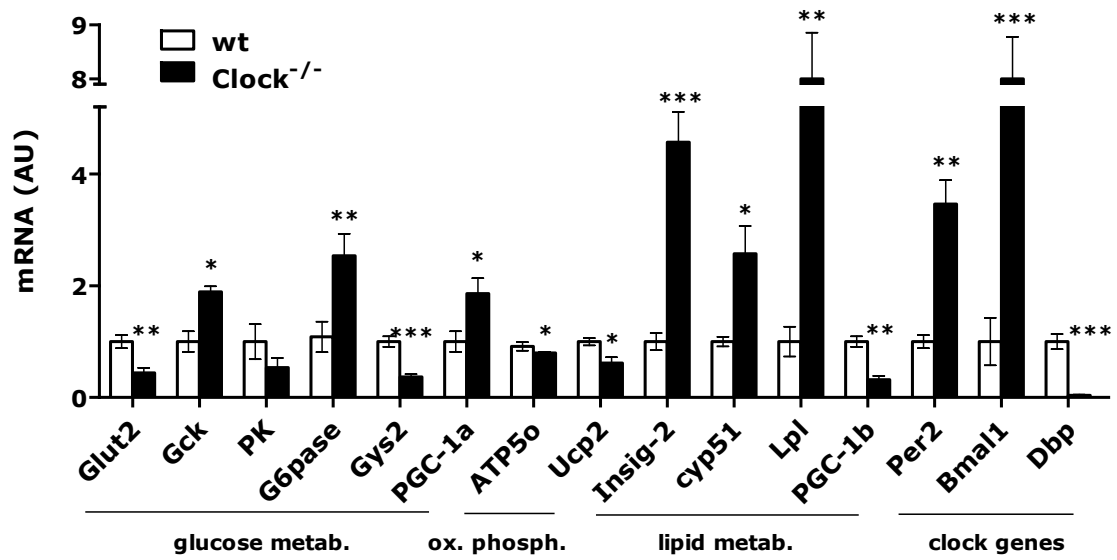


Fig. II-22: Validation of hepatic gene expression in Clock$^{-/-}$ mice by Real Time PCR
Selected genes from the microarray analysis, involved in glucose metabolism, oxidative phosphorylation, lipid metabolism and circadian rhythms, were tested by Real Time PCR (4-6 animals per genotype; data represents mean and standard error).

Tab. II-2: Differentially regulated transcripts in Clock$^{-/-}$ liver
A selection of downregulated (white) and upregulated (grey) genes in Clock$^{-/-}$ liver involved in lipoprotein metabolism, lipid catabolism, cholesterol biogenesis and gluconeogenesis. For source microarray gene list see V.A.1

Gene symbol	Fold change regulation	Full name	Process
Nr1d1	-2.18	nuclear receptor subfamily 1, group D, member 1	circadian regulation of cholesterol synthesis, bile acid production (Le Martelot et al. 2009)
Ppargc1b	-1.41	peroxisome proliferator-activated receptor gamma, coactivator 1 beta	regulator of lipoprotein metabolism, mitochondrial function (Lin et al. 2005)
Hes6	-1.61	hairy and enhancer of split 6 (Drosophila)	promoter of lipoprotein secretion in liver, repressor of gluconeogenesis (Martinez-Jimenez et al. 2010; Lee et al. 2012)
Apoa5	-1.1	apolipoprotein A-V	lipoprotein secretion by liver, associates with VLDL particles (Sharma et al. 2012)
Saa1	-5	serum amyloid A 1	lipoprotein metabolism, cholesterol efflux, HDL metabolism (Westhuyzen et al. 2005)
Saa4	-1.98	serum amyloid A 4	lipoprotein metabolism, cholesterol efflux, HDL metabolism (Beer et al. 1995)
Hsd3b7	+1.5	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 7	cholesterol catabolism, cholesterol conversion to bile acids (Wang et al. 2011)
Insig2	+4	insulin induced gene 2	inhibitor of lipid synthesis (Yabe et al. 2002)

Lipc	+1.6	hepatic lipase	uptake of chylomicrons and VLDL in liver, lipolytic breakdown of their content (Dichek et al. 1991)
Lpl	+4.6	lipoprotein lipase	uptake of chylomicrons and VLDL in liver, lipolytic breakdown of their content (Dichek et al. 1991)
Osbp	+1.3	oxysterol binding protein	inhibitor of cholesterol synthesis (Du et al. 2011)
Ehhadh	+1.3	enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase	peroxisomal beta-oxidation pathway (Veldhoven 2010)
Atf5	+2.84	activating transcription factor 5	promoter of gluconeogenesis (Shimizu et al. 2009)

II.C.3.1.2 Proteome analysis in Clock$^{-/-}$ liver

Gene expression analysis in Clock$^{-/-}$ liver was further complemented by quantitative proteomics (performed in collaboration with Dr. Guido Mastrobuoni, group of Dr. Stefan Kempa, MDC). In-depth identification of proteins was achieved through high-resolution "bottom up proteomics", in which peptides derived by enzymatic digestion are identified according to their mass-to-charge ratio and eventually mapped to the fragment spectra of a protein database (Mann et al. 2001). In addition, relative quantification of protein abundance was accomplished through stable isotope labeling with amino acid in cell culture (SILAC), extended to an *in vivo* mouse model, in which labeled "heavy" tissue lysate is used as a normalization control (Ong and Mann 2007; Krüger et al. 2008).

Shotgun proteomics identified 1319 hepatic proteins in total, unambiguously ascertained based on a minimum of 8 matching peptides (peptide "light" vs. "heavy" ratios) **[see Appendix V.A]**. Technical reproducibility of the data was further confirmed by biostatistical experiments, which demonstrated a higher variation among genotypes than biological replicates **[Fig. V-2]**.

Eventually, 5.5% of the detected proteins displayed differential regulation considering a false discovery rate < 0.05 and fold change > 0.3, corresponding to a set of 23 downregulated and 49 upregulated proteins **[Fig. II-23A, Fig. V-3]**. The data set was enriched in biological themes, such as oxidoreductase reactions, glucose and xenobiotic metabolism, and peroxisome function **[Fig. II-23B].**

Downregulated proteins in livers of Clock$^{-/-}$ mice were particularly enriched in components related to glucose metabolism, specifically glycolysis/gluconeogenesis and glycogen synthesis (*Eno1, Tpi, Gys2*) **[Fig. II-23B, Fig. II-24, Fig. II-25, Fig.I-10]**.

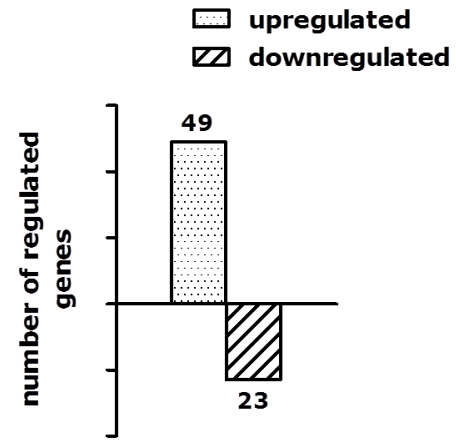
Moreover, some of the downregulated genes from the category "glycoproteins" were unravelled as pivotal components in lipoprotein secretion: For instance, the microsomal triacylglycerol hydrolase *Ces1* is essential for VLDL-assembly over the course of ApoB association with triglycerides at the ER **[Fig. II-23B, Fig. II-24, Fig. V-3]** (Dolinsky et al. 2004). Accordingly, attenuation of *Ces1*-expression has been linked to reduction in plasma VLDL (Wei et al. 2010).

Furthermore, gene ontology analysis revealed clusters categorizing into xenobiotic metabolism / glutathione transferase activity, amino acid metabolism, peroxisomes and membrane components to be upregulated **[Fig. II-23B]**. Interestingly, members of the cytochrome P450 and glutathione S-transferase family were some of the most upregulated proteins across the entire data set **[Fig. V-3]**. Analogously to the transcriptome analysis, the functional term "peroxisome", designating a cellular compartment, specialized in lipid catabolism, was also among the upregulated themes [**Fig. II-23B, Fig. II-21B, Fig. II-25, Tab. II-3].** This finding, along with the fact that expression of numerous proteins related to lipid metabolism was altered, indicated defects in hepatic lipid metabolism under conditions of *clock*-deletion [**Fig. II-24]**.

Furthermore, a comparison between transcriptome and proteome analysis displayed that the overlap between both data sets comprised only 8 genes – *Cyp2a5, Gstm6, Gstm2, Agxt, Hsd3b7, Akr1c19, Ces1, Gys2* [**Fig. II-25A]**.

Moreover, it was investigated to what extent differentially regulated genes in Clock$^{-/-}$ liver exhibit cycling transcription. This was evaluated based on the database CircaDB, which harbors high-density sampled liver transcriptome data around the clock (Hughes et al. 2009). Thereafter, approximately half of all 267 regulated transcripts were revealed as cycling [**Fig. II-25B].** Similarly, 37 out of the 72 regulated proteins exhibited an oscillating mRNA counterpart [**Fig. II-25B].** This finding demonstrated that *clock* deletion evokes expression changes in both circadian and non-circadian genes to a similar extent.

A



B

GO downregulated (enrichment score; p-value)	GO upregulated (enrichment score; p-value)
Oxidation reduction, drug metabolism ($0,89; 7*10^{-3}$)	Xenobiotic metabolism Glutathione transferase activity ($3,91; 1*10^{-9}$)
Ion binding proteins ($0,64; 1,1*10^{-2}$)	Amino acid derivative metabolism ($1,85; 3*10^{-2}$)
Glucose metabolism ($0,55; 5*10^{-2}$)	Peroxisome ($1,39; 5*10^{-3}$)
Glycoproteins ($0,35; 0,1$)	Membrane proteins ($1,32; 2,4*10^{-1}$)

Fig. II-23: Proteome analysis in Clock$^{-/-}$ liver
(A) The analysis revealed significant, greater than 30%, regulation of 72 genes, of which 49 and 23 genes were up- and downregulated, respectively. (B) The top Gene Ontology clusters, obtained through a DAVID analysis performed across the data set in (A). The significance in enrichement is characterized through enrichment score and p-value.

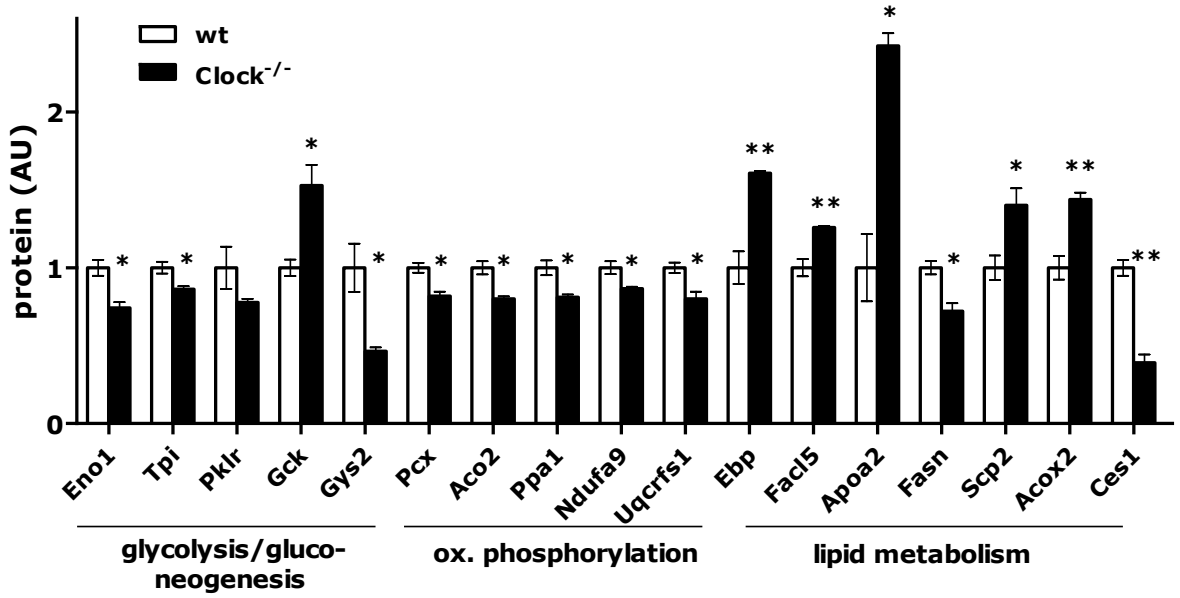


Fig. II-24: Differentially regulated proteins in Clock$^{-/-}$ liver according to proteomic analysis
Representation of relative fold changes in individual genes from the Clock$^{-/-}$ hepatic proteome analysis, related to glucose, lipid metabolism and oxidative phosphorylation. Data represent mean and standard error, n=3

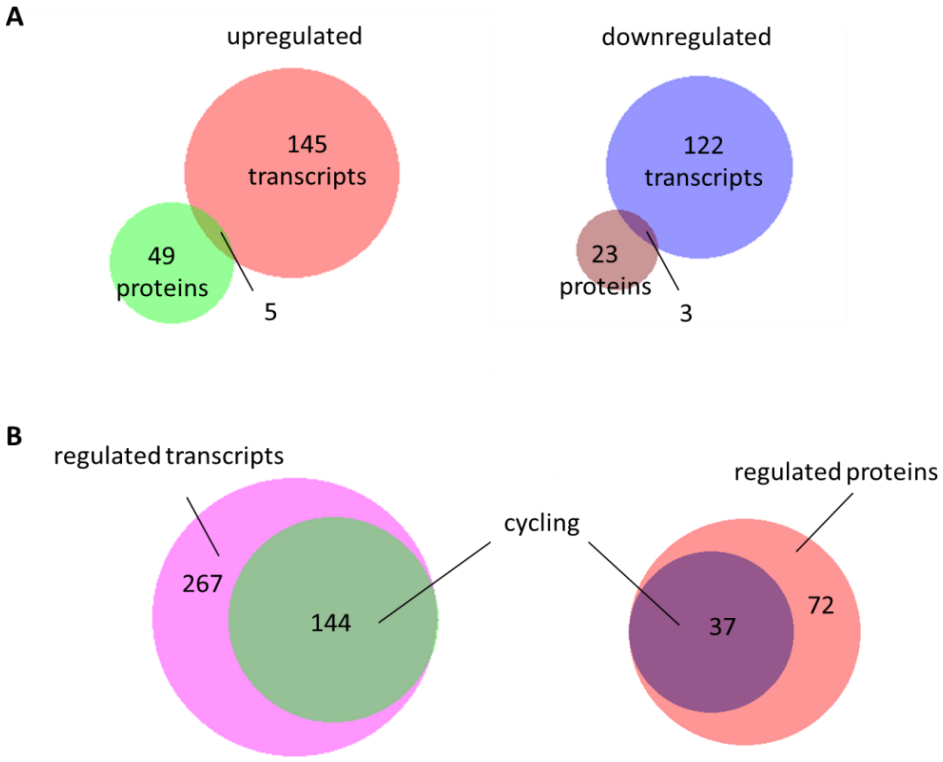


Fig. II-25: Comparison between transcriptome and proteome data in Clock$^{-/-}$ liver
(A) The overlap between up- and downregulated genes from proteome and transcriptome analysis in Clock$^{-/-}$ liver comprised only 5 and 3 genes, respectively. (B) The portion of genes, transcribed in a cycling manner, in the total amount of regulated transcripts / proteins. Cycling genes were identified according to the CircaDB data base of circadian transcription in murine liver (Hughes et al, 2009). 7 out of the 8 mutual genes for transcriptome and proteome data sets exhibited cycling transcripts (not shown). Differential regulation between wildtype and Clock$^{-/-}$ comprises at least 30 % and a significance $p < 0.05$.

Tab. II-3: Differentially regulated proteins in Clock$^{-/-}$ liver
A selection of downregulated (white) and upregulated (grey) genes in Clock$^{-/-}$ liver according to proteome data, related to glucose and lipid homeostasis. For source proteome data refer to V.A.1.

Gene symbol	Fold change regulation	Full name	Process
Gys2	-0.5	glycogen synthase 2	rate-limiting enzyme in gluconeogenesis (Doi et al. 2010)
Pgk1	-0.4	phosphoglycerate kinase 1	glycolysis (Rinaudo 1966)
Ces1	-0.62	carboxylesterase 1	lipoprotein metabolism, VLDL secretion (Dolinsky et al. 2004; Wei et al. 2010)
Hacl1	+0.33	2-hydroxyacyl-CoA lyase 1	peroxisomal enzyme, fatty acid oxidation (Casteels et al. 2007)
Acox2	+0.44	acyl-CoA oxidase 2, branched chain	peroxisomal enzyme, fatty acid beta-oxidation (KEGG pathway database)

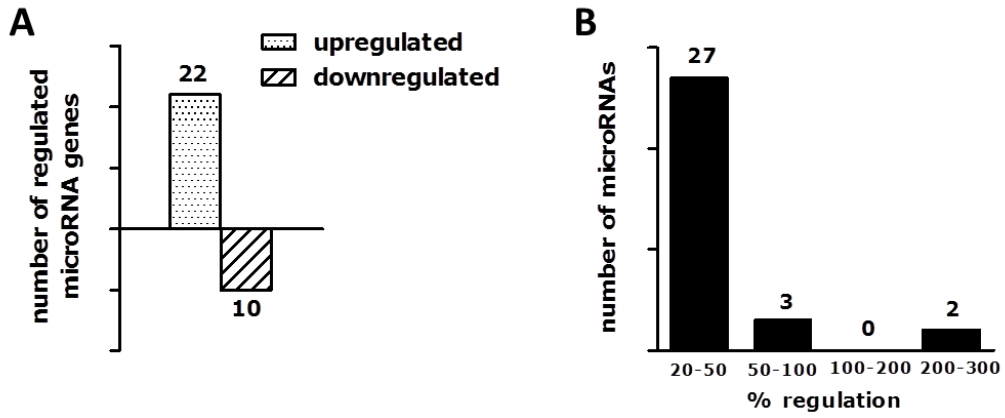
II.C.3.2 The miRNA pool in Clock$^{-/-}$ liver

Finally, expression analysis of Clock$^{-/-}$ liver was extended to the miRNA pool in order to identify the impact of circadian disruption on this non-coding small RNA class.

For this, miRNA deep sequencing was conducted (in collaboration with the group of Dr. Wei Chen, Max Delbrück Center for Molecular Medicine). Small RNA libraries, prepared from 4 livers per genotype in order to account for biological variability, were subjected to high-throughput Illumina sequencing. On average, 13 million sequencing reads per sample were identified, which could be perfectly mapped to 197 unique microRNAs in the genome. To normalize expression, relative miRNA abundance was calculated as ratio of miRNA reads to the total number of reads. miRNA abundance was unequally distributed: mir-122, mir-192 and mir-21 together accounted for 47 % of all sequence reads, while 113 of the remaining small RNAs covered only 10 % **[see Appendix V.A.1]**.

Next, the data set was filtered, taking into account only relatively well expressed miRNAs i.e. comprising at least 0.05 % of all reads **[Appendix V.A.1]**. If one estimates miRNA copy number per cell, this correspond to ca. 140 copies since mir-122 expression has been equalled to 50.000 copies / cell in adult murine liver (Chang et al. 2004). Among the 50 small RNAs meeting these criteria, 32 revealed differential expression when a cut-off of 20 per cent regulation was applied **[Fig. II-26A]**. Remarkably, the majority (84 %) of these sequences exhibited a rather weak, less than 50 % regulation, arguing that loss of Clock induces a striking dysbalance in the microRNA transcriptome **[Fig. II-26B]**. Only 5 miRNAs showed a marking, greater than twofold, regulation.

Yet, moderate alteration in expression occurred for a number of the most abundant miRNAs – mir-122, mir-192, mir-378, mir-143, let-7, mir-126, mir-101a, mir-194 **[Fig. II-26C]**. Interestingly, many of them are implicated in liver function. For instance, mir-122 is involved in cholesterol and amino acid metabolism (Chang et al. 2004; Krützfeldt et al. 2005). The let-7 family controls hepatic insulin sensitivity and glucose homeostasis, whereas the mir192/194 bicistronic cluster and mir-101 control hepatocyte proliferation and apoptosis (Frost and Olson 2011; Krützfeldt et al. 2012; Su et al. 2009).



C

miRNA	% of reads	fold change
mir-122	17,97	1,20
mir-192	16,89	0,80
mir-378	6,99	0,62
mir-143	4,82	0,69
let-7f	4,32	1,43
let-7a	2,89	1,49
mir-126	1,72	1,55
mir-101a	1,43	0,65
mir-30e	1,43	1,32
mir-194	0,98	1,51

Fig. II-26: miRNA expression in Clock$^{-/-}$ liver.
(A) High-throughput sequencing of the miRNA profile in Clock$^{-/-}$ liver at ZT9 revealed 22 up- and 10 downregulated miRNAs with regulation cut-off ≥ 20 % and abundance ≥ 0.05 % of all reads. (B) Most differentially regulated miRNAs (27) exhibited only weak less than 50 % change in expression. (C) The top 10 differentially expressed miRNA sorted according to their abundance. The fifty most abundant miRNAs (≥0.05% of all reads) are listed in Appendix V.A.1.

II.C.4 Clock-Bmal1 regulate Pgc-1β and mir-378 in liver

II.C.4.1 mir-378 and Pgc-1β are transactivated by Clock-Bmal1 *in vivo*

Next, I pursued the extraction of a concrete mechanism, linking Clock-Bmal1 action to hepatic lipoprotein transport.

Particularly, my interest was caught by mir-378, the fourth most abundant hepatic miRNA, accounting for ca. 19,500 copies per cell, which was reduced by ca. 40 % in the circadian mutants **[Fig. II-26C, Appendix V.A.1]**. MiR-378 is implicated in the molecular control of oxidative energy metabolism and lipogenesis (Eichner et al. 2010; Gerin et al. 2010a; Carrer et al. 2012). Expression of the 21 nt-long RNA was explored by means of alternative

detection techniques, such as Northern Blot, which confirmed a significant drop at circadian time ZT9 and ZT19 **[Fig.II-27A, B]** (27±8 and 28±5 %)**.** To further assess the validity of the high-throughput expression data, mir-122 levels were also measured. At both ZT9 and ZT19, the hepatocyte-specific miRNA displayed significant upregulation, which was in accordance with the sequencing results **[Fig.II-27A, C, Fig. II-26C]** (47±16 and 45±8 %).

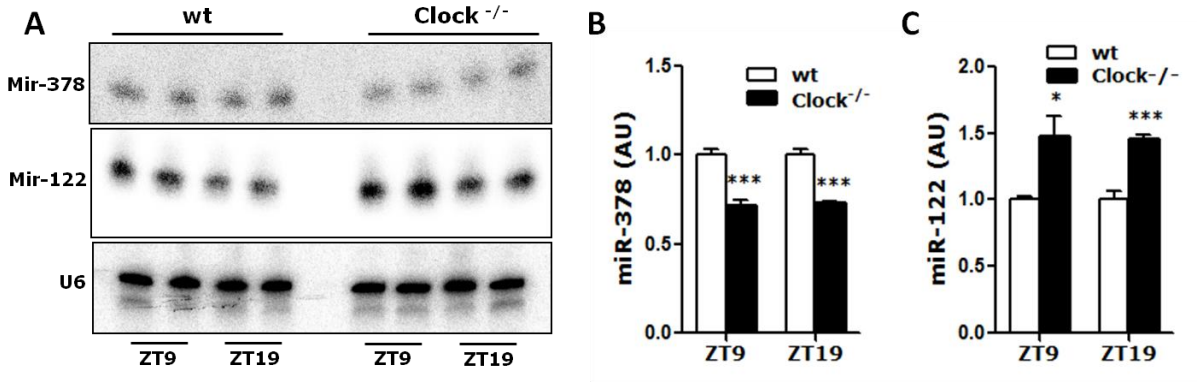


Fig.II-27: Validating miRNA expression changes in Clock$^{-/-}$ liver.
(A) Hepatic RNA from wt and Clock$^{-/-}$liver, collected at ZT9 or ZT19, was blotted and probed for mir-378 and mir-122. The small nucleolar RNA U6 was used as an internal loading control. Expression levels of miR-378 (B) and miR-122 (C) were quantified based on densitometric analysis and normalized to U6 intensity from three independent blots (n=3).

Mir-378 downregulation suggested that it might represent a molecular target of Clock-Bmal1. A closer look at the genomic context of *mir-378* revealed that it is embedded within the first intron of *ppargc1b* **[Fig.II-28**A**]**. The latter encodes **p**eroxisome **p**roliferative **a**ctivated **r**eceptor **g**amma **c**oactivator 1 beta (Pgc1β) – a member of the Pgc1 family, which functions as a transcriptional coactivator of a broad range of nuclear receptors to regulate mitochondrial function, lipid biosynthesis and lipoprotein metabolism (Lin et al. 2005). Interestingly, Pgc1β appeared among the downregulated genes in the large-scale microarray analysis of Clock$^{-/-}$ liver **[Fig. II-22, Tab. II-2]**. Since expression of both mir-378 and its host gene was attenuated in Clock$^{-/-}$ liver, I further pursued whether Clock-Bmal1 might be required for transactivation of the *ppargc1b* locus.

As *bona fide* clock-controlled transcripts are expected to cycle, firstly, the temporal accumulation of *ppargc1b* gene-products was evaluated at 4-hour intervals over one light-dark cycle. Intronic miRNAs are co-transcribed with their host gene and later cropped from the unexcised intron (Kim and Kim 2007). Hence, in real time PCR, primary mir-378 transcript (pri-mir-378) and Pgc1β-mRNA could be differentiated through implementation of

pri-mRNA-specific primers and primers, spanning exon-exon boundaries, respectively. Remarkably, transcription at the *ppargc1b* locus was revealed as highly circadian. Both pri-mir-378 and spliced Pgc1β-mRNA peaked at ZT 9, when amplitude reached a 5-fold increase in expression **[Fig.II-28B].** Importantly, primary miRNA hairpin and host transcript exhibited identical phase, period and amplitude of oscillation.

Notably, under conditions of Clock-deficiency, the expression peak at ZT9 was ablated in both pri-mir-378 and Pgc1β transcripts **[Fig.II-28C].** This indicated that, firstly, the protein-coding gene and its small RNA derivative are cotranscribed in a circadian manner. Secondly, it demonstrated that loss of Clock ablated this shared mechanism for transactivation.

In a next experiment, expression of mature end-processed mir-378 was assessed since it binds targeted mRNA to promote translational inhibition (Kim and Kim 2007). Temporal expression of the 21 nt-long mir-378 sequence was monitored in wildtype liver across the 24-hour cycle. Interestingly, it displayed constant accumulation, confirmed by means of two alternative detection techniques – Northern Blot and Real Time PCR **[Fig.II-29A, B, C]**. As a control, mir-122 levels, which had been reported to be stable over day and night, were also monitored **[Fig.II-29A, D]** (Gatfield et al. 2009).

In spite of constant accumulation of end-processed miR-378, the fact that transcription of the *ppargc1β* locus peaks in a circadian manner, and this effect is ablated under conditions of Clock-deficiency, strongly suggested that the gene and its intronic miRNA might obey transactivation by Clock-Bmal1. This was further supported by the fact that transcriptional zenith is reached at ZT9, prior to the onset of the dark phase – a typical feature of Clock-Bmal1 molecular targets (Ripperger et al. 2000; Doi et al. 2010). Thus, next it was tested whether the transactivator duo interacts with this chromatin locus.

Importantly, sequence analysis of *ppargc1β* revealed presence of a Clock-Bmal1 binding motif: A consensus circadian E-box sequence – CACGTG – was located in the first intron, ca. 2.3 kB upstream of mir-378 **[Fig. II-30A]** (Gekakis et al. 1998; Hogenesch et al. 1998). This hexamer was evolutionarily conserved, suggesting that it is under purifying selection **[Fig. II-30A].** Putative occupancy of this E-box by Clock-Bmal1 was further tested by chromatin immunoprecipitation (ChIP), which was performed with a Bmal1-specific antibody in primary hepatocytes at ZT6 – the timepoint of maximal DNA occupancy by Clock-Bmal1 (Ripperger and Schibler 2006) **[Fig. II-30B]**. Indeed, Bmal1 associated with the region of interest, while no interaction with a random E-box motif, ca. 3 kb downstream in the

ppargc1β gene, could be detected **[Fig. II-30C]**. Furthermore, the immunoprecipitated DNA was enriched in a *dbp* enhancer element, which is previously known to be functionally occupied by Clock-Bmal1, thus, validating the use of this antibody (Ripperger and Schibler 2006).

In sum, the results indicate that the *ppargc1b* locus is targeted by Clock-Bmal1 through an evolutionary conserved intronic E-box element. The interaction leads to high-amplitude circadian oscillations of pri-mir-378 precursor and its host transcript, while mature mir-378 levels remain non-circadian.

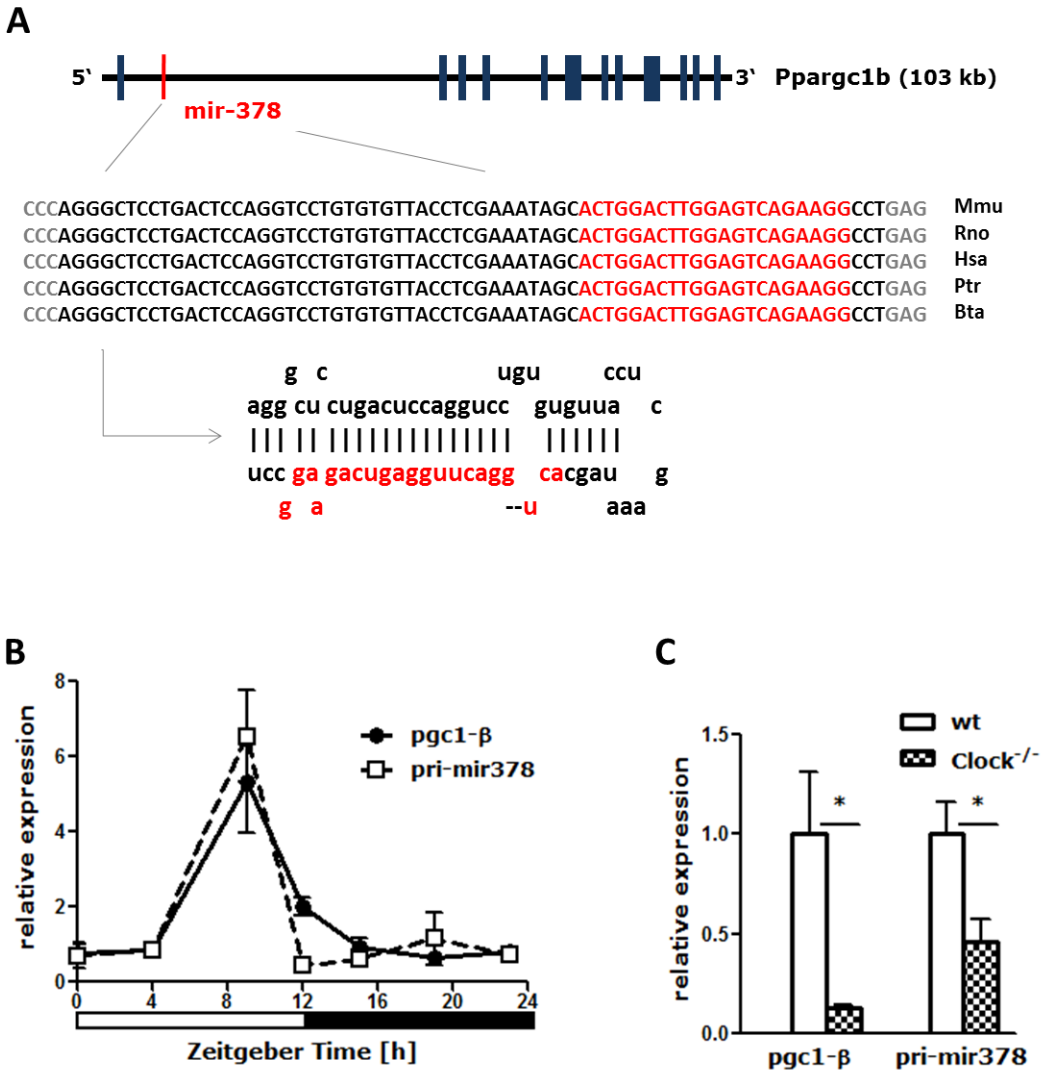


Fig.II-28: Circadian transcription of *ppargc1b* and intronic *mir378*
(A) The first intron of *ppargc1b* gives rise to mir-378, whose nucleotide sequence is conserved across mammals. The mature 21 nt-long sequence within the Drosha-processed pre-miRNA is highlighted in red. (Mmu – *Mus musculus* (mouse), Rno – *Rattus norvegicus* (rat), Hsa – *Homo sapiens* (man), Ptr - *Pan troglodytes* (chimpanzee), Bta - *Bos Taurus* (cattle)) (B) Circadian accumulation of Pgc1β mRNA and pri-mir-378, as assessed by Real Time PCR (Zeitgeber time = hours after lights are switched on). The black and white bars represent subjective day and night, respectively. N = 4-6 mice per genotype and timepoint, data represent mean

and standard error (C) Expression of Pgc-1β and pri-mir-378 at ZT9 in wildtype and Clock$^{-/-}$ liver as assessed by Real Time PCR. n=4 per genotype, data represent mean ans standard error.

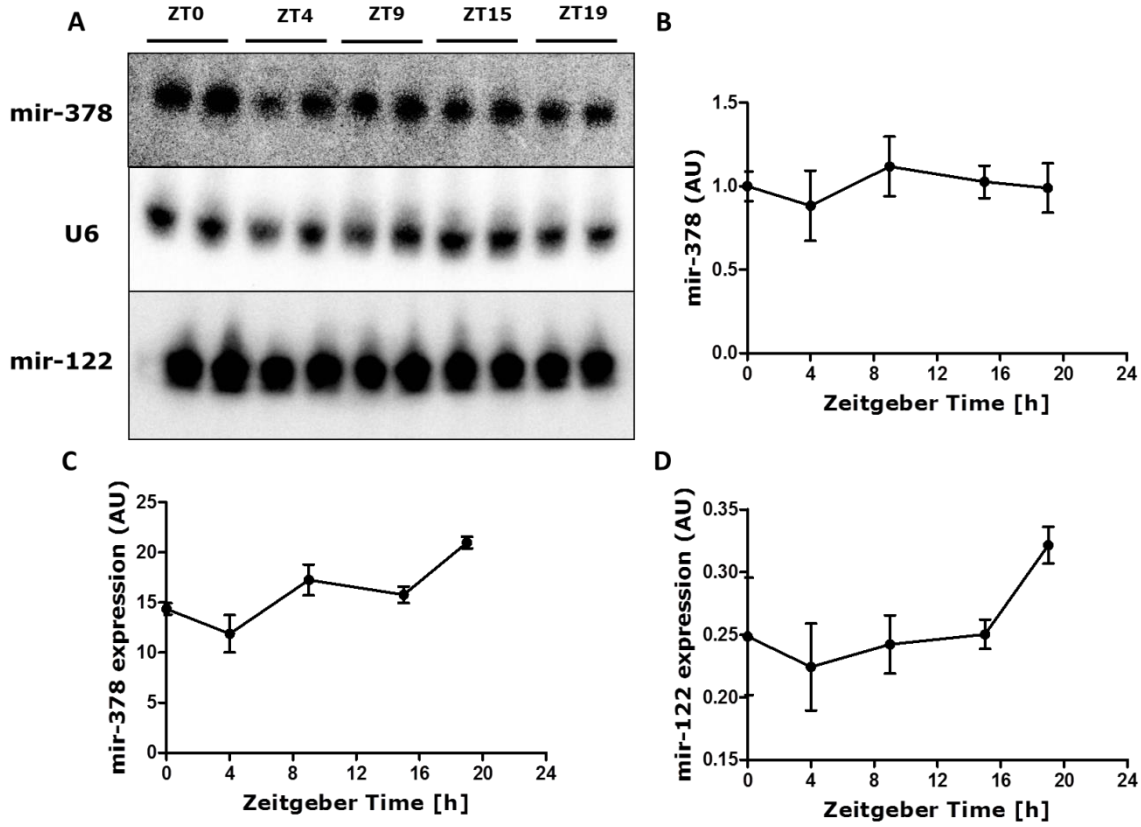


Fig.II-29: Mature mir-378 expression does not oscillate
(A) Murine liver RNA was blotted and probed for mir-378 and mir-122 around the clock (n=2) (B) Densitometric miR-378 analysis from (A) normalized to U6. (C) and (D) Murine liver RNA was sampled around the clock at indicated Zeitgeber Time and analyzed for mir-378 or mir-122 expression by Real Time PCR (n=3), data represents mean and standard error

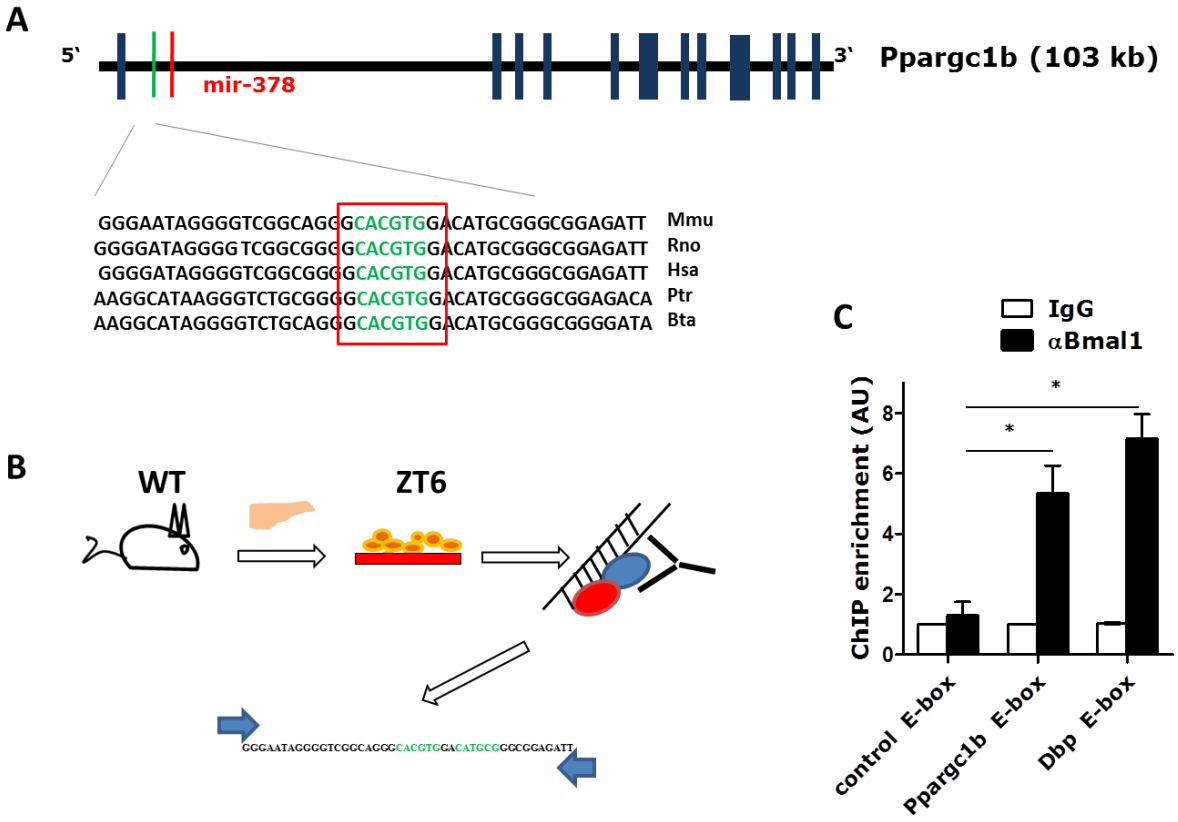


Fig. II-30: Clock-Bmal1 target the *ppargc1β* locus *in vivo*
(A) Location of the E-box element within the intron of *ppargc1β* and its conservation across mammals (Mmu – *Mus musculus* (mouse), Rno – *Rattus norvegicus* (rat), Hsa – *Homo sapiens* (man), Ptr - *Pan troglodytes* (chimpanzee), Bta - *Bos Taurus* (cattle)) (B) Experimental set-up for chromatin immunoprecipitation (ChIP): primary mouse hepatocytes were isolated, let to recover and fixed at ZT6 in order to capture chromatin-protein interactions. After pull-down with an anti-Bmal1 antibody, isolated DNA fragments were analyzed for enrichment of the region of interest using site-specific primers. (C) Relative enrichement of immunoprecipitated genomic regions containing a random control E-box, the intronic E-box of *ppargc1β* or a functional *dbp*-element (filled bars). Unspecific antibody binding was assessed using an IgG-control (white bars).

II.C.4.2 *ppargc1β* mediates Clock-Bmal1 function in hepatic lipoprotein transport

Pgc-1β confers specificity and enhancement of metabolic transcriptional programs. In liver, the transcriptional coactivator acts with SREBP (sterol regulatory element binding transcription factor) and EERα (estrogen-related receptor alpha) to augment lipid biosynthesis and VLDL assembly (Lin et al. 2005; Hernandez et al. 2010) [**Fig. I-12]**. As Pgc-1β was deduced as a molecular target of Clock-Bmal1, next, it was addressed whether its ablated expression leads to the impaired lipoprotein secretion under conditions of *clock* deletion. Hence, lipogenic transcriptional programs, triggered by Pgc-1β, were evaluated in the liver of the circadian mutants.

Particularly, downstream expression of HMG-CoA-reductase (*hmgcr*), fatty acid synthase (*fasn*), stearoyl-Coenzyme A desaturase 1 (*scd1*) and apolipoprotein C-III (*apoc3*) was addressed (Lin et al. 2005, Hernandez et al. 2010). These candidates represent key enzymes, involved in synthesis of triglyceride and cholesterol VLDL-load (Hmgcr, Fasn and Scd-1) [**Fig. I-11]**. Further, Apoc3 is involved in VLDL lipidation, i.e. lipid droplet recruitment to the nascent protein at the ER membrane (Sundaram et al. 2010; Sundaram et al. 2010b) [**Fig. I-12].** Notably, *apoc3* expression positively correlates with circulating VLDL levels in mouse and man, thus, highlighting its crucial role in hepatic lipoprotein transport (Cohn et al. 2004; Cohn et al. 2004b).

Remarkably, all investigated transcripts displayed a downregulation in $Clock^{-/-}$ liver **[Fig. II-31]**. This indicated attenuation in lipogenic pathways triggered by Pgc-1β and its transcriptional partners EERα and SREBP as the underlying cause for impaired VLDL synthesis and secretion under conditions of *clock* deletion.

In sum, this data provided evidence that the *ppargc1b* locus, targeted by Clock-Bmal1 *in vivo*, represents a direct link between the circadian pacemaker and lipoprotein metabolism in liver.

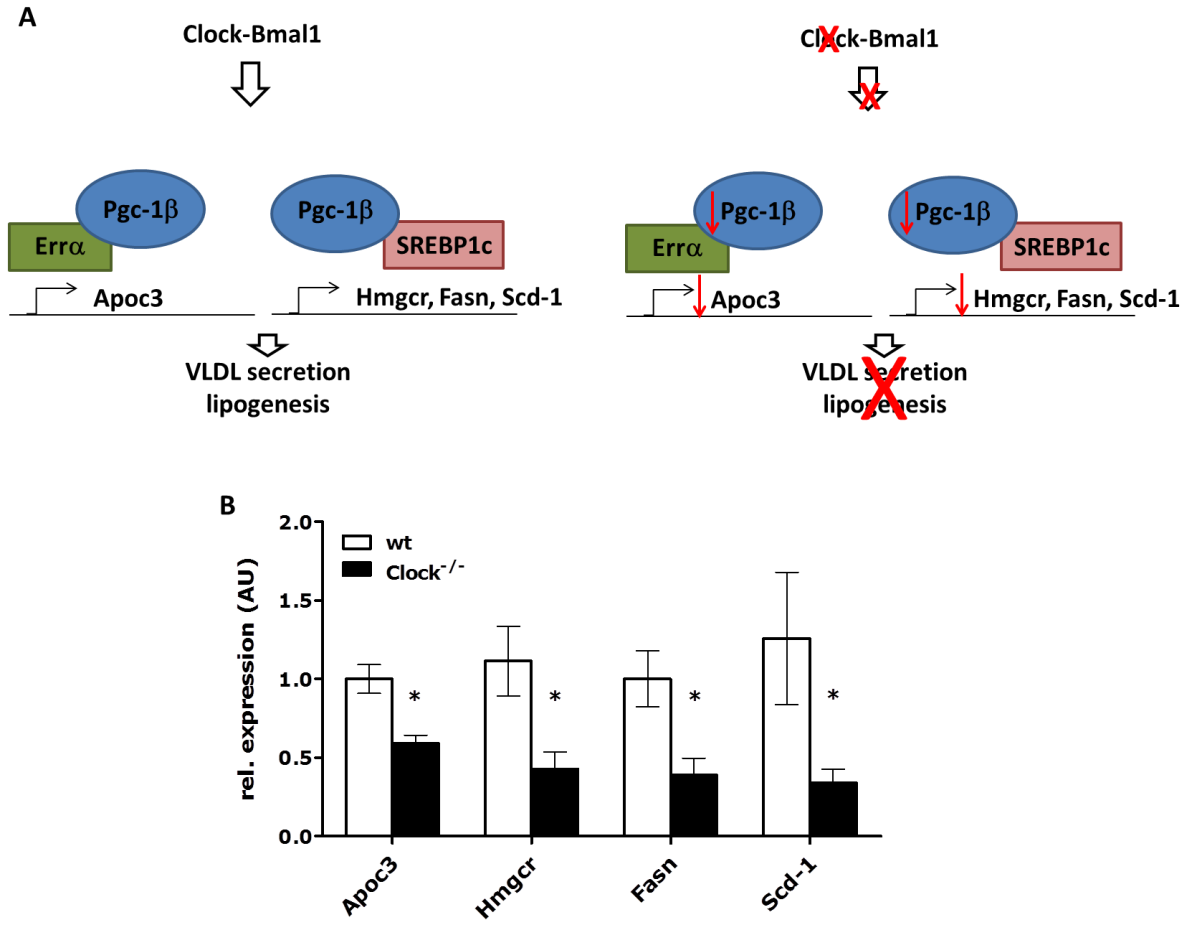


Fig. II-31: Pgc-1β targets promoting VLDL secretion are downregulated in Clock$^{-/-}$ liver
(A) Schematic representation of hepatic lipogenic pathways activated by Pgc-1β together with SREBP and ERRa. **(B)** Expression of targeted genes, activated via the ERRα and SREBP route, respectively, in Clock$^{-/-}$ liver. Real Time PCR was performed in liver samples from 8-9 littermates per genotype, collected at ZT15 or ZT19. Data represents mean and standard error.

III. Discussion

Despite observation-based data on physiological processes obeying day/night variation, the evidence that numerous metabolic genes oscillate and that circadian desynchrony inclines the incidence of diabetes and dyslipidemia, the understanding of the exact molecular role of Clock-Bmal1 in these phenomena is fragmentary. The current work shed light on the role of the transcriptional duo in two aspects of energy metabolism – systemic glucose and lipid homeostasis. Furthermore, it addressed the interconnection between metabolic microRNAs and the core circadian pacemaker.

A. Clock and Bmal1 are required for beta cell function and insulin release

III.A.1 Physiological evidence for the role of Clock-Bmal1 in beta cell function and insulin release

Diabetic hallmarks upon Clock-Bmal1 disruption – glucose intolerance and impaired insulin release

In the present study, phenotypic characterization of three mouse models – whole-body Clock$^{-/-}$, beta cell-specific InsCre Clock$^{-/-}$ and InsCre Bmal1$^{-/-}$ – yielded consistent results, defining a crucial role for Clock-Bmal1 in beta cell function and insulin secretion.

The failure of beta cells to maintain physiological blood glucose levels in spite of a general insulin presence, typically, due to a secretion defect, is a central hallmark of type 2 diabetes mellitus (DeFronzo et al. 1992). The causes for this epidemic are multigenic and heterogeneous: recently, genetic variation in the circadian components *clock* and *bmal1* has been associated with this disease (Woon et al. 2007; Scott et al. 2008). Its major diagnostic marker is hyperglycemia. Glucose intolerance as well as diminished first phase of insulin secretion mark disease onset before its full clinical manifestation (Gerich 2002; LeRoith et al. 2004; Stumvoll et al. 2005). Remarkably, Clock-Bmal1 disruption partially or entirely reflected all these hallmarks.

In mice, standardized criteria for hyperglycemia are lacking, partly due to the natural variation among inbred strains (Clee and Attie 2007). Typically, blood sugar levels in rodent models of spontaneous diabetes, such as the Non-Obese Diabetic (NOD) mouse and the Goto-Kakizaki rat, comprise 250-300 mg/dL (Clee and Attie 2007). Clock- and Bmal1-deficient

animals failed to exhibit exaggerated hyperglycemia and/or a corresponding hypoinsulinemia in that range; hence, they cannot be considered "diabetic" *per se* **[Fig. II-3, Fig. II-8]**.

However, under conditions of glucose challenge, their defective beta cell function became evident – they failed to restore physiological blood sugar concentrations efficiently due to a blunted insulin release in the acute phase after stimulation **[Fig. II-3, Fig.II-6, Fig.II-7]**. Moreover, Clock-Bmal1 disruption impaired glucose tolerance in a general manner, irrespective of time of the day **[Fig.II-4]**. Further, under these conditions, clearance of glucose failed to exhibit the expected enhancement at the onset of the active dark phase (ZT12) (Carroll and Nestel 1973; Lee et al. 1992; Asplin et al. 1979). Notably, abolished circadian variation in glucose tolerance also appears in diabetic patients (Jarrett and Keen 1969).

Clock-Bmal1 does not affect insulin action

Insulin resistance in peripheral tissues is another critical factor, which contributes to the development of type 2 diabetes (DeFronzo et al. 1992). Attenuation of the signalling cascade downstream of the insulin receptor in target tissues deteriorates insulin secretion over time as beta cell cannot compensate for the increasing demand. Impaired insulin signaling often accompanies the obese and hyperphagic state due to the accumulation of toxic nutritional by-products, e.g. reactive-oxygen species and inflammatory cytokines (Yki-Järvinen and Koivisto 1983; Taniguchi et al. 2006; Muoio and Newgard 2008).

In alignment with the fact that $Clock^{-/-}$ mice displayed normal feeding behaviour and their body composition comprised less fat and more lean mass, the animals exhibited normal or even slightly improved insulin sensitivity **[Fig.II-5].** Further, the reduction in adipose tissue might suggest a putative defect in adipogenesis in the whole-body circadian mutants: *In vitro* Clock-Bmal1 have been implicated in adipocyte differentiation (Shimba et al. 2005).

Clock-Bmal1 in beta cells is indispensable for insulin secretion

As described below, the role of peripheral pacemakers can be reliably investigated in the $Clock^{-/-}$ model due to its intact hypothalamic function (see **III.A.4**). Yet, tissue-specific gene deletion is the most optimal method to examine the relevance of the beta cell pacemaker. For this purpose, the two rodent lines – InsCre $Clock^{-/-}$ and InsCre $Bmal1^{-/-}$ – lacking either partner of the heterodimeric duo in pancreatic beta cells, were generated **[Fig.II-6, Fig.II-7]**. In analogy to whole-body null mice, these animals exhibited mild hyperglycaemia,

intolerance towards a sugar challenge and reduced first phase of glucose-stimulated insulin secretion **[Fig.II-6, Fig.II-7]**.

Moreover, the impaired capacity of beta cells with a disrupted molecular clock to release insulin was also confirmed *ex vivo* **[Fig.II-6]**. This highlighted the cell-autonomy of this defect, suggesting its independence of factors, regulating insulin secretion *in situ*, such as neuronal innervation and humoral stimulation (rev. in Thorens 2010; Chandra and Liddle 2012).

III.A.2 Mechanism of Clock-Bmal1 action

The current study provided insights into the mechanism, underlying Clock-Bmal1 control of insulin secretion, through addressing the early and the late stages of the pathway.

III.A.2.1 Clock-Bmal1 does not affect insulin biogenesis and granulogenesis of insulin-containing vesicles

Insulin biosynthesis is tightly regulated – on the transcriptional level, by the coordinate action of HNF-1α/β, HNF-4α and NeuroD1, and posttranslationally, by the principles of the prohormone theory (Bell and Polonsky 2001; Steiner et al. 1996). Proteolytical processing of the endocrine hormone occurs over the course of granule maturation, eventually yielding insulin vesicles competent for Ca^{2+}-regulated secretion.

Genetic defects in transcription factors driving *ins* expression, or the *ins* gene itself, which prevent extracellular transport of the hormone or its proteolytic processing, are rare and account for a severe onset of diabetes even in the heterozygous state (Bell and Polonsky 2001; Fajans et al. 2001; Støy et al. 2007). In contrast, Clock-Bmal1 disruption results in mild hyperglycemia, rendering defects in some of these steps rather unlikely. Accordingly, insulin content and granule density did not exhibit any alterations upon *clock* / *bmal1* deletion **[Fig. II-9]**. This suggested that the heterodimeric duo is not obligatory for proper insulin transcription, translation, posttranslational processing and generation of insulin-containing vesicles.

Furthermore, quantification of the insulin content in pancreatic lysates indicated that beta cell mass is not changed under conditions of Clock-Bmal1 disruption. The latter parameter is proportional to pancreatic insulin content, reflecting size and number of insulin-producing cells and, thus, indicative of beta cell function (Tomita et al. 1992; Poy et al. 2009; Kalis et al. 2011; Tarabra et al. 2012). Hence, it is unlikely that beta cell morphometry is affected by the

molecular pacemaker. This was further supported by ultrastructural microscopic imaging of $Clock^{-/-}$ islets, which did not reveal any eminent beta cell abnormalities in structure or shape **[Fig. II-9]**.

III.A.2.2 Clock / Bmal1-disruption affects the late but not early stages of insulin secretion

Glucose is the most potent secretagogue for insulin release; hence, the beta cell acts both as a sensor and controller of circulating sugar (Dean and Matthews 1968; Nuttall and Gannon 1991). In a first stage of the glucose-triggered pathway, stimulatory concentrations lead to a rise in ATP, inducing membrane depolarization and the subsequent opening of voltage-gated Ca^{2+}-channels. In a second stage, elevation in free intracellular Ca^{2+} ($[Ca^{2+}]_i$) triggers conformational change in the zippered SNARE complexes of docked vesicles and, thus, a rapid membrane fusion. These two phases – upstream or downstream of Ca^{2+}-influx – were investigated in Clock/Bmal1-deficient beta cells by a systematic approach. Functional defects were revealed in the late-stages of the pathway.

Clock-Bmal1 do not affect Ca^{2+}- influx upon glucose stimulation

Elevation of free $[Ca^{2+}]_i$ upon glucose stimulation was comparable between $Clock^{-/-}$ and wildtype islets, displaying that activation of voltage-dependent Ca^{2+}-channels was not impaired upon disruption of the molecular pacemaker **[Fig.II-10]**. Furthermore, two essential characteristics of this activation were preserved in the secretory islet syncytium and hence, independent of Clock-Bmal1: Firstly, increase in $[Ca^{2+}]_i$ was proportional to rise in glucose concentration and, secondly, stimulation induced an oscillatory pattern of $[Ca^{2+}]_i$- bursts, accompanying underlying pulses in electrical excitability (Barbosa et al. 1998). In respect to the latter, it might be worthwhile to quantify Ca^{2+}oscillations in order to assess intercellular synchronization and excitability under conditions of Clock-Bmal1-disruption. Nevertheless, the equal rise in $[Ca^{2+}]_i$ in Clock-proficient and Clock-deficient islets suggests that glucose uptake, breakdown, ATP generation, membrane depolarization and opening of Ca^{2+}-channels is not controlled by the beta cell pacemaker.

Exaggerated exocytosis in Clock/Bmal1-deficient beta cells – an increase in kiss-and-run fusion?

Electrophysiological analysis revealed a difference in the rate of SNARE-mediated exocytosis at the plasma membrane of beta cells devoid of the circadian components: Cell capacitance measurements, induced by individual depolarization trains, indicated a higher net exocytosis **[Fig. II-11]**. Although at shorter physiological stimuli this difference was also detectable, it was particularly pronounced at longer pulses due to the higher signal to noise ratio.

Based on physiological and biochemical data on glucose-stimulated insulin secretion in Clock/Bmal1-deficient animals, exaggerated exocytosis *in vitro* coexists with an attenuated first phase of insulin release *in vivo*. The following two proposed scenarios could explain this paradox.

First, although capacitance measurements represent a sensitive and powerful approach to study membrane dynamics with millisecond resolution, the technique does not detect actual insulin release *per se* (Neher and Marty 1982; Rorsman and Renström 2003). Principally, increase in this electrophysiological parameter can be equated with hormone secretion: Both exhibit the same dependence for Ca^{2+}, temperature and neurotransmitters / hormones as ascertained by voltage-clamp assays, combined with fluorimetric quantification of GFP-tagged insulin (rev. in Rorsman 1997). However, capacitance measurements also overestimate actual hormone release since the technique detects additional exocytotic activity arising from kiss-and-run fusion (Eliasson et al. 2008; Rorsman and Renström 2003). Exocytosis is not an all-or-none effect and a substantial portion of insulin vesicle fusion events – 30-80 % – represent a form of incomplete "kiss-and-run" membrane merge, which does not coincide with insulin cargo emptying. Instead, the constricted fusion pore (ca. 1,4 nm) allows diffusion of low-molecular weight compounds, such as Zn^{2+}, ATP and GABA, into the extracellular space (Takahashi et al. 2002; Barg et al. 2002; Lindau and Alvarez de Toledo 2003; Tsuboi and Rutter 2003; MacDonald et al. 2006; Tsuboi et al. 2006) **[Fig.I-8, Fig. III-1]**.

Notably, an abnormal predominance of kiss-and-run exocytosis in beta cells has been already described as a pathological feature: Its incidence is increased under conditions of long-term exposure to high glucose concentrations and is believed to contribute to the impaired insulin secretion in the diabetic state (Tsuboi et al. 2006; Olofsson et al. 2007). Although the exact mechanism for this deleterious effect has not been resolved, it relies on the premature termination of the fusion pore, as assessed by fluorescence-tagged insulin vesicle imaging

(Tsuboi et al. 2006). Hence, it might seem plausible that a rise of kiss-and-run fusion at the expense of full membrane merge might underlie the defective insulin secretion under conditions of Clock-Bmal1 disruption. To investigate this hypothesis, the impact of the heterodimeric duo on membrane fusion dynamics should be studied **[III.A.5, Fig. III-1]**.

A second possible explanation for the discrepancy between increased cell capacitance recordings and decreased insulin secretion relies on the fact that electrophysiological patch-clamp technique does not discriminate in the type of granules fusing with the plasmalemma. Besides dense-core insulin granules (DCV), beta cells contain small synaptic-like microvesicles (SLV), which are also destined for glucose-stimulated secretion (rev. in Burgoyne and Morgan 2003). The latter are smaller in size (70 nm (SLV) vs. 300 nm (DCV)) and exclusively devoted to the secretion of intraislet signaling molecules, such as GABA (Wendt et al. 2004; MacDonald et al. 2005). So far, little is known about the biogenesis of synaptic-like microvesicles in beta cells. However, SLVs and DCVs are believed to share their Ca^{2+}-dependent exocytotic machinery and, in a similar manner as DCVs, to undergo both full exocytosis and partial kiss-and-run release, with the latter accounting for up to 60 % of all fusion events (MacDonald et al. 2005). Hence, an elevated exocytotic rate might not necessarily correlate with actual insulin secretion in Clock-Bmal1 disrupted beta cells and might potentially result from SLV exocytosis [**Fig. III-1]**.

III.A.3 Transcriptome analysis in Clock$^{-/-}$ islet – alterations in vesicle fusion and endo/exocytosis

Functional studies in Clock-Bmal1-disrupted beta cells were complemented by high-throughput transcriptome analysis in the islet. Due to the high abundance of beta cells, comprising ca. 90 % of the endocrine pancreas, the islet expression analysis reflected predominantly the transcriptional status in insulin-producing cells (Elayat et al. 1995). Ca. 27% of the islet transcriptome displayed a differential expression at ZT 9 – the circadian time, of maximal Clock-Bmal1 transcriptional activity (Ripperger and Schibler 2006). This is in accordance with large-scale data in other peripheral organs, such as liver and heart, where 5 – 20 % of all genes are estimated to obey circadian regulation (Panda et al. 2002; Storch et al. 2002; Reddy et al. 2006).

Gene ontology analysis provided insights into the pleiotropic actions of Clock-Bmal1 and suggested that vesicle trafficking and endo-/exocytosis are altered in “clockless” beta cells **[Fig.II-12, Tab. II-1]**. In particular, the upregulation of gene clusters related to vesicle

trafficking, vesicle components, endo- and exocytosis revealed a molecular footprint, which fits to the alterations in the late stages of the insulin secretion pathway **[Fig.II-12, Tab. II-1].**

Indeed, upregulation of genes, involved in these terms correlates with increased exocytotic capacity **[Fig. II-11]**. Many of the components are required for exocytosis – they interact with the SNARE complex, function as Ca^{2+}-sensors or bind to actin filaments necessary for vesicle priming and docking (*pclo, rab27a, gsn, rims2, unc13b*)**[Fig.II-12, Tab. II-1]**. Some of them have been reported to associate with dense-core vesicles and act as positive regulators of insulin secretion; whether they are also involved in glucose-stimulated secretion of synaptic-like microvesicles, which presumably share identical exocytotic machinery, has not been explored so far (Fujimoto et al. 2002; Waselle et al. 2003; Kasai et al. 2005; Kalwat et al. 2012; Tomas et al. 2010; Ozaki et al. 2000; Sheu et al. 2003).

Of note, some of the upregulated components involved in the SNARE-mediated interactions at the plasma membrane are prominent modulators of kiss-and-run membrane merge – *Syt4, Cplx2* **[Fig.II-12, Tab. II-1]**. Complexins are indispensable components in neurotransmission, which assemble with the zippered trans-SNARE complex (Reim et al. 2001). The function of the prevailing isoform in neuroendocrine tissues – complexin II – is to induce a premature fusion termination (Archer et al. 2002; Liu et al. 2007; Malsam et al. 2012). Accordingly, its overexpression favours rapid kiss-and-run exocytosis and inhibits hormonal release (Archer et al. 2002; Liu et al. 2007).

An additional molecular fusion "clamp", upregulated in $Clock^{-/-}$ beta cells, is represented by synaptotagmin IV **[Fig.II-12, Tab. II-1]**. Whereas other members of the synaptotagmin protein family act as Ca^{2+}-sensors to enhance pore dilation during exocytosis, *syt4* precludes it, leading to increased rate of kiss-and-run exocytosis and inhibition of cargo expulsion in neuroendocrine cells and neurons (Wang et al. 2001;Wang et al. 2003; Machado et al. 2004; Bhalla et al. 2008).

N-ethylmaleimide-sensitive-factor (*Nsf*) is also overexpressed in the circadian mutants **[Fig.II-12, Tab. II-1]**. Although this ATPase has not been linked directly to kiss-and-run exocytosis, it is inevitable for dissociation of the assembled SNARE complex and its recycling for another round of fusion (Söllner et al. 1993; Vikman et al. 2003). Hence, Nsf upregulation might promote rapid detaching and resealing of dense-core granules, thus, precluding insulin release.

Interestingly, the SCN neuronal pacemaker has been recently implicated in vesicle fusion at the plasma membrane: There, proteins controlling granule recapture, such as NSF, SNAP and synapsin, were found to oscillate in a circadian manner (Deery et al. 2009). This study along with the findings in the present work suggests that in beta cells, the exocytotic machinery can be also under control of the circadian pacemaker.

Further upregulated transcripts in Clock$^{-/-}$ islets corresponded to membrane trafficking between ER and Golgi and vesicle constituents (*Ergic1, Spire1, Stx18, Scfd1, Sec16b, Rint1, Zw10, Bet1*) **[Fig.II-12, Tab. II-1]**. This could indicate a potential enhancement in granulogenesis. Since the overall density of insulin-containing granules was not affected in Clock-deficient beta cells, this potential effect could result from increased biosynthesis of synaptic-like microvesicles (SLVs) **[Fig. II-9]**. Furthermore, enhanced exocytosis due to an expanded SLV pool at the expense of DCV release, could explain the impaired insulin secretion in the circadian mutants.

Finally, lysosomal enzymes were also upregulated in the circadian mutants **[Tab. II-1]**. The lysosomal compartment is highly relevant for beta cell physiology: Lysosomal amount and activity have been negatively associated with insulin secretion and the size of the readily-releasable pool (Salehi et al. 1999; Speidel et al. 2008). Hence, a potential abnormal lysosomal dysfunction in "clockless" beta cells might also contribute to their insulin secretion defect.

Taken together, the expression analysis in Clock$^{-/-}$ islet pinpointed potential defects in SNARE-mediated exocytosis and granulogenesis in beta cells devoid of the circadian components. Based on this, a mechanism can be hypothesized according to which an increase in kiss-and-run fusion of DCVs and / or an expanded cytoplasmic SLV pool could result in an elevation in exocytotic rate without insulin release **[Fig. III-1]**.

Moreover, the current analysis added to the concept of circadian regulation obtained in other peripheral tissues, such as liver, heart, skeletal muscle, adipose tissue, lung, skin and macrophages (Panda et al. 2002; Storch et al. 2002; Miller et al. 2007; Shostak et al. 2013; Sukumaran et al. 2011; Geyfman et al. 2012; Keller et al. 2009).

III.A.4 Advantages of Clock$^{-/-}$, InsCre Clock$^{-/-}$ and InsCre Bmal1$^{-/-}$ mice over other "clockless" models

In the present study, the necessity of a functional Clock-Bmal1 transactivator duo for insulin secretion was underlined in both whole-body and beta cell-specific knock-out mice (see II.A.1.1, II.A.1.2). Previous reports have also suggested a role for the pacemaker in glucose homeostasis; however, the genetic nature of the mouse models there has led to inconclusive interpretation.

Clock$^{-/-}$ mice versus Clock^{mut} mice

For instance, mice with an antimorphic *clock* mutant allele (*Clock*^{mut}) have been shown to develop hyperglycaemia (Turek et al. 2005). However, this rodent line also exhibits gross perturbations related to circadian function in the central nervous system, such as blunted circadian rhythms in the SCN and hypothalamic nuclei controlling food intake (Jin et al. 1999; Turek et al. 2005). This most likely results from the presence of Clock^{mut} protein, constantly bound to DNA, thus, precluding the homologous Npas2 to dimerize with Bmal1 and exert its transcriptional activity (King et al. 1997a and b). Accordingly, the altered wakefulness, feeding behavior, as well as energy expenditure and the obese state in Clock^{mut} mice jeopardize the function of peripheral clocks, involved in glucose homeostasis (Turek et al. 2005).

In contrast, whole-body Clock$^{-/-}$ mice possess a functional pacemaker in the SCN and, most probably, in other hypothalamic regions controlling energy homeostasis since their physiological output was intact. Food intake, leptin sensitivity, metabolic heat and O_2-consumption displayed normal circadian variation in Clock$^{-/-}$ animals **[Fig. II-1, Fig. II-2]** (Debruyne et al. 2006)**.** Of note, some of these parameters, represent efferent functions of hypothalamic nuclei, directly innervated through SCN projections and, thus, are under strict circadian regulation (rev. in Buijs et al. 2006). In contrast to the Clock^{mut} mouse model, here, Clock-deficiency in the central pacemaker is compensated by brain-specific Npas2 (Debruyne et al. 2006; DeBruyne et al. 2007). Hence, this mouse model allows for the unambiguous investigation of the role of the transcriptional activator in metabolic organs.

Furthermore, differences in CNS pacemaker function in Clock^{mut} and Clock$^{-/-}$ mice provide, at least partly, explanation for the numerous phenotypical differences between both models. While the former exhibit adiposity and impaired glucose homeostasis only late in life, the latter display reduced fat tissue and defective insulin secretion independent of age (Turek et

al. 2005; Marcheva et al. 2010) **[Fig. II-3, Fig.II-5]. G**lucose intolerance and defective insulin secretion were manifested in young Clock$^{-/-}$ mice at 6 – 8 weeks of age **[Fig. II-3, Fig.II-5]**.

InsCre Clock$^{-/-}$ and InsCre Bmal1$^{-/-}$ versus PdxCre Bmal1$^{-/-}$

Conditional inactivation of Clock/Bmal1 specifically in pancreatic beta cells was directed through *cre recombinase* transgene expression, driven by the rat insulin promoter (Herrera 2000). Initially, this has been described as an efficient and reliable experimental strategy for gene-deletion in pancreatic beta cells. Recent reports, however, have demonstrated ectopic recombinase expression in hypothalamic neurons, probably, due to a low activation rate of the insulin promoter in these cell populations (Song et al. 2010; Wicksteed et al. 2010; Baskin et al. 1987). So far, offsite genetic ablation in the brain has been ascribed to all pancreas-specific Cre recombinases – such as under regulation of the *insulin*, *pdx1* and *ngn3* promoter (Postic et al. 1999; Gannon et al. 2000; Song et al. 2010; Wicksteed et al. 2010). Yet, considering the fact that *clock* is redundant in the brain, the artefact of hypothalamic *clock* deletion is not expected to obscure the results obtained here in the InsCre Clock$^{-/-}$ model.

Meanwhile, two other research groups have suggested the relevance of the beta cell pacemaker for glucose homeostasis (Marcheva et al. 2010; Sadacca et al. 2011). In contrast to the results presented herein, their conclusions rely on *bmal1* deletion in the entire pancreas, encompassing both endocrine and exocrine tissue. For instance, the PdxCre Bmal1$^{-/-}$ mouse lacks Bmal1 in islet glucagon- and somatostatin-producing cells, which are known to crosstalk with the beta cell and affect insulin secretion. Thus, these studies lack proof that disruption of the circadian transactivator Clock-Bmal1 specifically in beta cells is sufficient to deteriorate glucose metabolism. Furthermore, the *Pdx-Cre* used there is expected to associate with artefacts due to ectopic expression and, thus, hypothalamic deletion of non-redundant *bmal1* (Gu et al. 2002; Song et al. 2010). Still, their findings are consistent with the results obtained in total Clock$^{-/-}$, InsCre Clock$^{-/-}$ and InsCre Bmal1$^{-/-}$ mice: The circadian mutants display attenuated insulin secretion *in vivo* and *ex vivo* and this defect is independent of [Ca^{2+}]-signaling (Marcheva et al. 2010).

III.A.5 Conclusion and outlook

The current study provides information on the physiological role of the circadian transactivator tandem Clock-Bmal1 in beta cells and contributes to the emerging understanding of the pacemaker function in tissues beyond the CNS (Lamia et al. 2008; Nikolaeva et al. 2012; Pan et al. 2010; Andrews et al. 2010; Spörl et al. 2012). Genetic disruption of the core pacemaker machinery induces a diminished acute phase of insulin secretion, which marks type 2 diabetes before the overt onset of severe hyperglycaemia (Gerich 2002; LeRoith et al. 2004). The circadian activator duo Clock-Bmal1 is dispensable for glucose-stimulated induction of $[Ca^{2+}]_i$-signaling. Furthermore, electrophysiological and gene expression analyses hint towards an unprecedented role for the beta cell pacemaker in modulation of fusion pore formation. There, an enhanced exocytotic rate is observed, which does not correlate with hormone release.

Based on the current results, two hypothetical scenarios might explain the coexistence of a decreased insulin release *in vivo* with an increased exocytotic rate at the beta cell level *in vitro*. In the first model, Clock-Bmal1 coordinate the molecular decision for complete membrane merge versus kiss-and-run exocytosis **[Fig. III-1]**. In the second, Clock-Bmal1 might be involved in granulogenesis and secretion of non-insulin containing granules, such as synaptic-like microvesicles **[Fig. III-1].**

Several follow-up experiments should be performed in order to investigate both hypotheses and membrane merging in beta cells devoid of Clock/Bmal1. Firstly, in order to differentiate between full and transient release of insulin vesicles, optical techniques and *in vivo* fluorescence tagging can be employed - for instance - lipophilic dyes or fluorescence-tagged proteins in the membrane of dense-core vesicles together with reporter-labeling of luminal insulin (Leung et al. 2002; Tsuboi et al. 2004). Ideally, these methods should be coupled to total internal reflection fluorescence microscopy (TIRF-M) to allow for specific and high-resolution visualization of plasmalemma-proximal molecules (Ohara-Imaizumi and Nagamatsu 2006). This enables the tracking of individual docked insulin granules as they merge with the plasmalemma, monitoring of fusion pore opening and dissolution of granule proteins as well as recapture and vesicle endocytosis. Hence, such real-time imaging techniques could elucidate whether clock disruption facilitates kiss-and-run fusion over full membrane merge.

Alternatively, the incidence of incomplete exocytosis of insulin-containing granules could be assessed indirectly through ultrastructural imaging of beta cells upon a glucose challenge. The

inability of insulin vesicles to integrate into the cell membrane and release their content would lead to excessive accumulation of dense-core granules at the plasma membrane, thus, confirming the secretion defect.

According to the second hypothesis, exaggerated exocytosis in "clockless" beta cells might arise, at least partly, from an increase in small synaptic-like vesicles (SLV) **[Fig. III-1]**. This scenario would comply with the increased cell capacitance demonstrated in patch clamp experiments as well as with the upregulation of genes related to vesicle trafficking and granulogenesis. While ultrastructural imaging of these small, ca. 70 nm in diameter, granules might be challenging due to lack of electron-dense structures, biochemical assays could be employed in order to detect the extracellular release of their content – GABA (Wendt et al. 2004).

Furthermore, the question arises whether in Clock/Bmal1-disrupted beta cells, increased exocytotic capacity is accompanied by changes in the readily-releasable vesicle pool, which typically comprises less than 10 % of the total amount of insulin-containing vesicles (Olofsson et al. 2002). For instance, failure of DCVs to localize in direct proximity to Ca^{2+}-channels is considered to decrease their capacity for complete membrane collapse, thus, favoring kiss-and-run exocytosis and hindering insulin peptide release (Wiser et al. 1999; Elhamdani et al. 2006; Collins et al. 2008; Eliasson et al. 2008).

Finally, it would be important to establish whether Clock-Bmal1 regulate the exocytotic machinery in a 24-hour rhythm. The prevalence of complete exocytosis of dense-core vesicles and, hence, the promotion of insulin secretion could be coupled to feeding cycles. To the best of my knowledge, such mechanism for circadian cargo release has not been reported for any neuroendocrine or neuronal cell type so far. The fact that membrane retrieval proteins in SCN neurons were found to oscillate further supports this hypothesis (Deery et al. 2009). In beta cells, Clock-Bmal1 might provide a unique layer of control, acting at the exocytotic machinery to ensure time-adjusted secretion of the anabolic hormone.

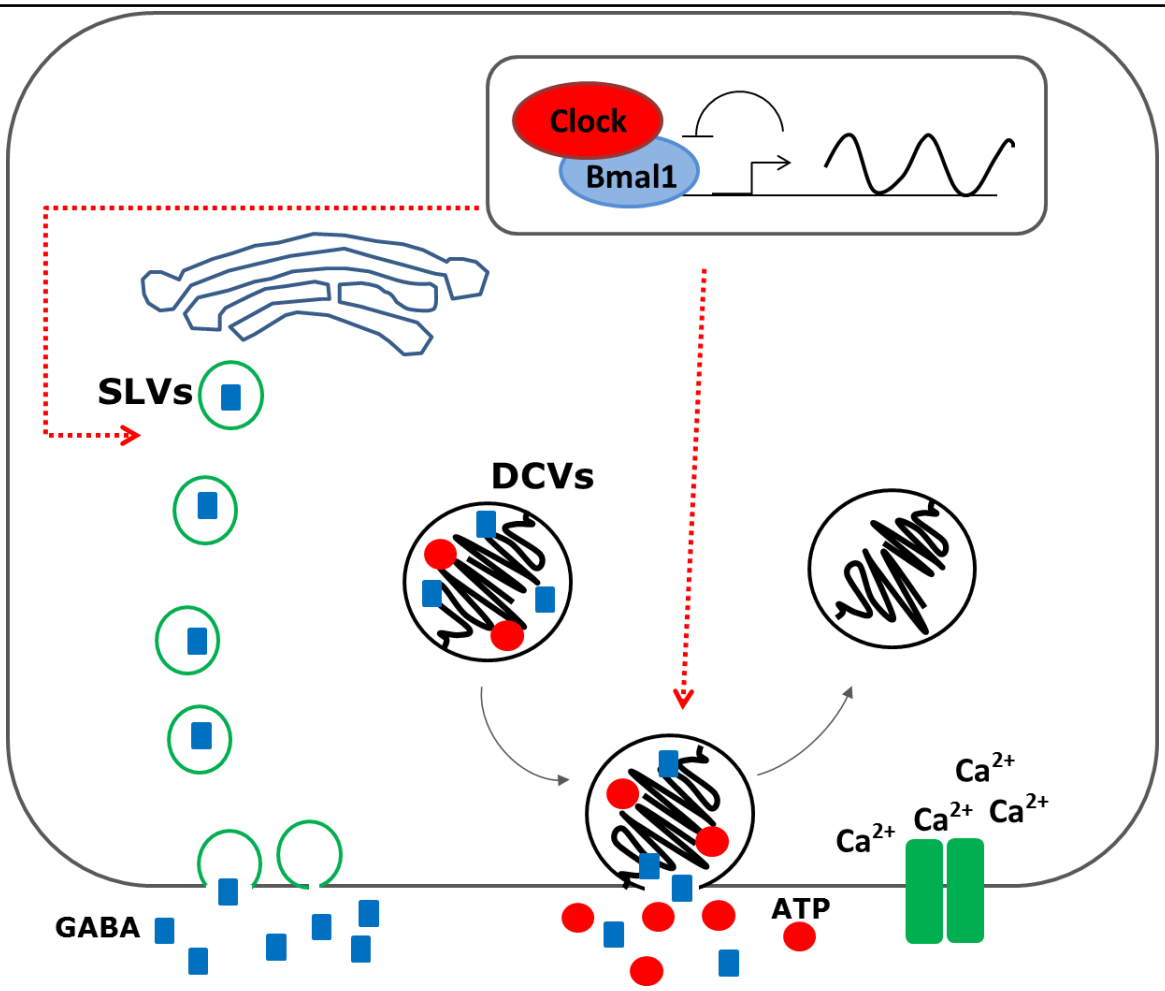


Fig. III-1:Proposed mechanisms for impaired insulin secretion in Clock-Bmal1-deficient pancreatic beta cells

Clock-Bmal1 might promote transcription of components regulating membrane fusion dynamics of dense-core vesicles (DCVs), controlling the balance of full versus transient vesicle merge. As a consequence, in a Clock-Bmal1-deficient state, kiss-and-run exocytosis prevales and insulin cannot be released into the bloodstream, while small-molecular autocrine factors, such as GABA and ATP, can (right arrow). Accordingly, DCVs with retained insulin cargo would accumulate upon glucose stimulation. In the second mechanism, Clock-Bmal1 might control granulogenesis of synaptic-like vesicles (SLVs) transporting GABA (left arrow). In Clock-Bmal1-disrupted beta cells, the SLV pool might be enlarged, accounting for an increased exocytotic rate upon glucose stimulation without a correlation with insulin release.

B. Posttranscriptional regulation of the molecular clock in pancreatic beta cells by mir-375

III.B.1 mir-375 targets Clock expression *in vitro* and *in vivo*

mir-375 is the highest expressed miRNA in beta cells, implicated in the regulation of insulin secretion (Poy et al. 2004, Poy et al. 2009). It is believed to target the end-stages of insulin release through direct suppression of exocytotic machinery components, such as myotrophin. However, small noncoding RNAs silence on average hundreds of genes simultaneously in a mild/moderate manner to confer fine-tuning of cellular processes (Krek et al. 2005; Baek et al. 2008; Selbach et al. 2008). This prompted the investigation, whether mir-375 function in beta cells encompasses regulation of clock components, which are also implicated in insulin secretion.

Indeed, *clock*, *per2* and *tef* were retrieved as molecular targets of mir-375 by both *in silico* prediction algorithms and *in vitro* luciferase reporter assays **[Fig.II-13, Fig.II-14A]**. This is in accordance with the fact that miRNAs often regulate multiple members of the same pathway (Valastyan et al. 2009; Lynch et al. 2012). While Clock and Per2 participate in the positive and negative core circadian loops, respectively, Tef is a metabolic transcription factor of the PAR-domain basic leucine zipper family, downstream of Clock-Bmal1 (Reppert and Weaver 2002;Gachon et al. 2006). Evolutionary conservation of the mir-375 seed was displayed only in the *clock* 3' UTR, suggesting a functional interaction there likelier; hence, further investigations were restricted to *clock* **[Fig.II-13]**.

One has to bear in mind that both bioinformatic prediction algorithms and luciferase reporter validations associate with technical limitations, which can result in identification of "false positives". *In silico* approaches do not consider secondary mRNA structures or protein occupancy, which might preclude microRNA interaction with its complementary sequence (rev. in Rajewsky 2006). In respect to chimeric reporter gene constructs – although they offer a fast and reproducible screening method – the massive overexpression of recombinant plasmid and miRNA mimic, both non-natural to the recipient cell, might trigger nonphysiological interactions. Hence, the strongest support for mir-375 suppression of Clock arose from studies in pancreatic islets, where Clock protein displayed an upregulation under conditions of mir375-deficiency **[Fig.II-14C].** This effect was mild, ca. 30 per cent, in accordance with the current concept that commonly microRNAs exert mild effects on target gene expression (Selbach et al. 2008; Baek et al. 2008).

Taken together, the present study provided experimental evidence for the posttranscriptional regulation of the core circadian component *clock* by mir-375. Consistent with that, *clock* deletion induced an analogous beta cell phenotype as *mir-375* overexpression *in vivo* (see II.A, Poy et al. 2004). Both impair insulin secretion. Further, they were found to affect granule exocytosis, while leaving activation of Ca^{2+}-channels upon glucose stimulation intact. Hence, this raises the hypothesis that regulation of *clock* at least partly contributes to the action of mir-375 on insulin secretion, thus, adding to the complex network of this global regulator in beta cells [**Fig. III-2]**.

III.B.2 mir-375 affects the robustness of the islet pacemaker

Peripheral mammalian pacemakers receive various metabolic input – they can be entrained by nuclear receptors or metabolites, such as ATP and NAD^+ in order to adapt, i.e. to synchronize their intrinsic rhythms with feeding cycles (Nakahata et al. 2009; Lamia et al. 2009; Handschin et al. 2005; Yin et al. 2010). Recently, in *Drosophila melanogaster* and cell-based systems, microRNAs have been also implicated in that process. For instance, bantam regulates the fly ortholog *clock*, whereas in embryonic fibroblasts or NIH3T3 cells, *period* and *cryptochrome* ate targeted by mir-24, mir-29a, mir-30a, mir-192/194 and mir-185 (Kadener et al. 2009; Nagel et al. 2009; Lee et al. 2013; Chen, 2013). Frequently, these regulations account for the adjustment of circadian period – particularly, *clock* suppression results in its prolongation (Kadener et al. 2009).

Since mir-375 targets *clock* in the pancreatic islet, the current work focused on the miRNA impact on pacemaker dynamics. This was achieved through monitoring circadian rhythms under conditions of mir-375 deletion *ex vivo* – in the islets of mir-$375^{-/-}$ mice expressing the period2::luciferase reporter **[**Fig.**II-15]**.

First of all, real-time bioluminescence readings once again confirmed the existence of a beta cell pacemaker through the "visualization" of 24-hour rhythms in Per2 expression, thus, complementing evidence provided by Clock/Bmal1 loss-of-function **[**Fig.**II-16, see II.A]**.

Furthermore, in alignment with other peripheral organs – e.g. liver, pituitary, kidney and lung – islet oscillations demonstrated a phase-delay of ca. 7 hours as compared to the SCN, which is considered a common feature of slave pacemakers (Yoo et al. 2004; Fig.**II-16D**).

Importantly, loss of mir-375 did not exert any effect on entrainment, resetting and precision of the islet pacemaker since circadian period, phase and amplitude did not display any

significant change in mir-375$^{-/-}$ tissue explants [Fig.**II-16]**. Based on reports, demonstrating that miRNA regulation of *clock* prolongs circadian period in fly, whereas *clock* overexpression in a cell-based system shortens it, one could expect that *mir375* deletion in the islet would also result in a shorter period (Kadener et al. 2009; Lee et al. 2011). The fact that this was not observed might be attributed to the high variation in circadian bioluminescence recordings of tissue explants as opposed to immortalized embryonic fibroblasts, rendering detection of subtle changes in some parameters difficult. For instance, the 5-fold *in vitro* overexpression of *clock* altered circadian period by only 0.5 hours, which corresponds to the interindividual variation of this parameter in wildtype murine islets observed in the present study (Fig.**II-16C,** Lee et al. 2011).

Remarkably, mir-375 profoundly affected the self-sustained capacity of the islet to maintain circadian rhythms: mir-375$^{-/-}$ tissue explants exhibited more persistent bioluminescence cycling (Fig.**II-16F)**. This effect was dependent on the level of mir-375 expression, as heterozygous mice displayed an intermediate phenotype. The damping of multicellular pacemakers *ex vivo* characterizes their intrinsic property for synchronization and circadian coupling in the absence of SCN-derived signals (Yoo et al. 2004b; Liu et al. 2007). Damping results from the fact that, on the single cell level, oscillators vary in their phase, so that on the multicellular level, non-uniform rhythms abolish the overall cycling in gene expression (Welsh et al. 1995; Welsh et al. 2004; Herzog et al. 1998). Synchronization within the multicellular oscillator is accomplished through intercellular coupling (Welsh et al. 2004; Herzog et al. 1998).

Unfortunately, the mechanism for circadian coupling in peripheral tissues has not been elucidated so far and evidence is available only for intercellular synchronization within the SCN. There, it is conferred mainly through neurotransmitters and gap junctions (Aton et al. 2005; Liu and Reppert 2000; Lundkvist et al. 2005; Long et al. 2005; Yagita and Okamura 2000). Interestingly, many of the mechanisms described in neurons, are operating also in neuroendocrine beta cells, where they have an established role in insulin secretion. For instance, they include the neurotransmitters GABA and the vasoactive intestinal polypeptide (VIP), membrane electrical activity, Ca^{2+}-influx and gap-junction proteins, such as Connexin-36 (Liu and Reppert 2000; Aton et al. 2005; Reetz et al. 1991; Ahrén and Lundquist 1981; Long et al. 2005; Lundkvist et al. 2005; Benninger et al. 2008; Carvalho et al. 2012). Thereafter, it seems plausible that in beta cells, these pathways potentially also serve

circadian coupling. Hence, mir-375 might attenuate intercellular circadian synchronization through regulation of these mechanisms [**Fig. III-2]**.

A preliminary *in silico* analysis did not reveal any of the coupling mediators GABA, VIP or Connexin-36 as potential mir-375 targets, bearing complementary mir-375 binding sites in their 3' UTRs (data not shown). However, previous reports on gene expression analysis in mir-375$^{-/-}$ islets have shown an upregulation of the neurotransmitter VIP, suggesting an indirect regulation and further supporting the hypothesis that cell-cell coupling might be affected by the miRNA (Poy et al. 2009, data not shown) [**Fig. III-2]**.

Finally, differences in intercellular synchronization, i.e. the intrinsic capacity of a tissue to sustain oscillations, characterize the anatomical and hierarchical specificities between central and slave oscillators. Peripheral pacemakers are typically weakly coupled. Thus, they can quickly adapt their period and phase to changes in activity cycles or feeding schedule (Yoo et al. 2004b; Welsh et al. 2004; Abraham et al. 2010; Stokkan et al. 2001). On the contrary, SCN neurons are synchronized highly efficiently and represent a rigid oscillator, which is resilient towards entrainment and whose rhythms cannot adapt quickly towards environmental alterations (Liu et al. 1997; Herzog et al. 1998; Abraham et al. 2010).

According to this hypothesis, the current results hint towards a possible role of mir-375 in defining the robustness of the islet pacemaker, proposing that *mir-375* deletion improves robustness. This might further explain the fact that circadian period was not affected despite Clock upregulation: As shown in studies by Liu et al., circadian robustness renders the pacemaker resistant towards genetic disturbances in the clockwork machinery (Liu et al. 2007). Moreover, the results speculate that the physiological role of mir-375 might be to suppress the rigidity of the islet pacemaker i.e. to confer enhanced adaptation towards external stimuli. This hypothesis seems tempting since it would provide a mechanism by which the islet clockwork has evolved flexibility, for instance, in the adaptation towards changing feeding / fasting cycles.

Taken together, the current results do not imply a role for mir-375 in entrainment and precision of the islet pacemaker. However, mir-375 was found to regulate the self-sustained capacity of this microorgan to perform circadian rhythms – a property, which is believed to be managed through intercellular coupling. At present, it seems likelier that this effect is exerted through mir-375-targets other than *clock*, which do not influence the cell-autonomous transcriptional-translational loop, but, instead, confer cell-cell communication and, thus, account for circadian synchronization [**Fig. III-2]**.

III.B.3 Conclusion and outlook

The current results elucidate the circadian transactivator *clock* as a direct molecular target of mir-375 – the most abundant beta cell-specific miRNA. This represents an unprecedented posttranscriptional regulatory mechanism in the core clockwork of mammalian peripheral tissues, so far. Furthermore, mir-375 suppression of *clock* – a promoter of insulin release – is consistent with the negative control of the miRNA over hormone secretion (Poy et al. 2004, see II.A). Hence, this mechanism integrates pacemaker components and miRNAs into the current concept of beta cell physiology [**Fig. III-2]**.

Moreover, the study provides insights into the physiological impact of mir-375 on the islet pacemaker. Despite the fact that the small RNA fine-tunes *clock* expression, it is not required for the determination of circadian period, phase and amplitude of oscillations. Importantly, loss of mir-375 enhances the self-sustained capacity of the islet pacemaker to perform rhythms autonomously. This implicates the small RNA as a possible suppressor of circadian coupling, probably through regulation of the pathways underlying intercellular synchronization, e.g. VIP signaling or gap junction coupling, independent of its effect on *clock* expression [**Fig. III-2]**. Furthermore, this raises the hypothesis that *in vivo*, mir-375 attenuates robustness of the islet pacemaker in order to promote its adaptability towards entrainment cues.

Hence, the prevailing question is whether / what intercellular coupling pathways might be inhibited by the miRNA. The greatest challenge is represented by the fact that, so far, synchronization stimuli within the multicellular oscillator have been delineated only in the SCN (Aton and Herzog 2005). Still, the fact that many of the neuronal coupling agents, such as neurotransmitters and gap junctions, are operating in insulin secretion, suggests their putative role for circadian synchronization in the beta cell syncytium (Liu and Reppert 2000; Aton et al. 2005; Reetz et al. 1991; Ahrén and Lundquist 1981; Long et al. 2005; Lundkvist et al. 2005; Benninger et al. 2008; Carvalho et al. 2012).

Thus, first, the relevance of individual coupling pathways in the islet pacemaker should be assessed. Then, in a second step, the effect of mir-375 on intercellular coupling can be studied, e.g. through pharmacological tools. For instance, circadian rhythms in *per2::luc*-expressing islets can be uncoupled through treatment with pathway-specific inhibitors, e.g. for VIP, electrical and Ca^{2+}-mediated signalling (O'Neill et al. 2008; Hagen et al. 2006; Yamaguchi et al. 2003). Subsequent antagomir-based knockdown of mir-375 should reveal

whether the decoupling effect can be abolished, i.e. whether islet rhythms can be prolonged, thus, pinpointing the miRNA mechanism of action (Krützfeldt et al. 2005).

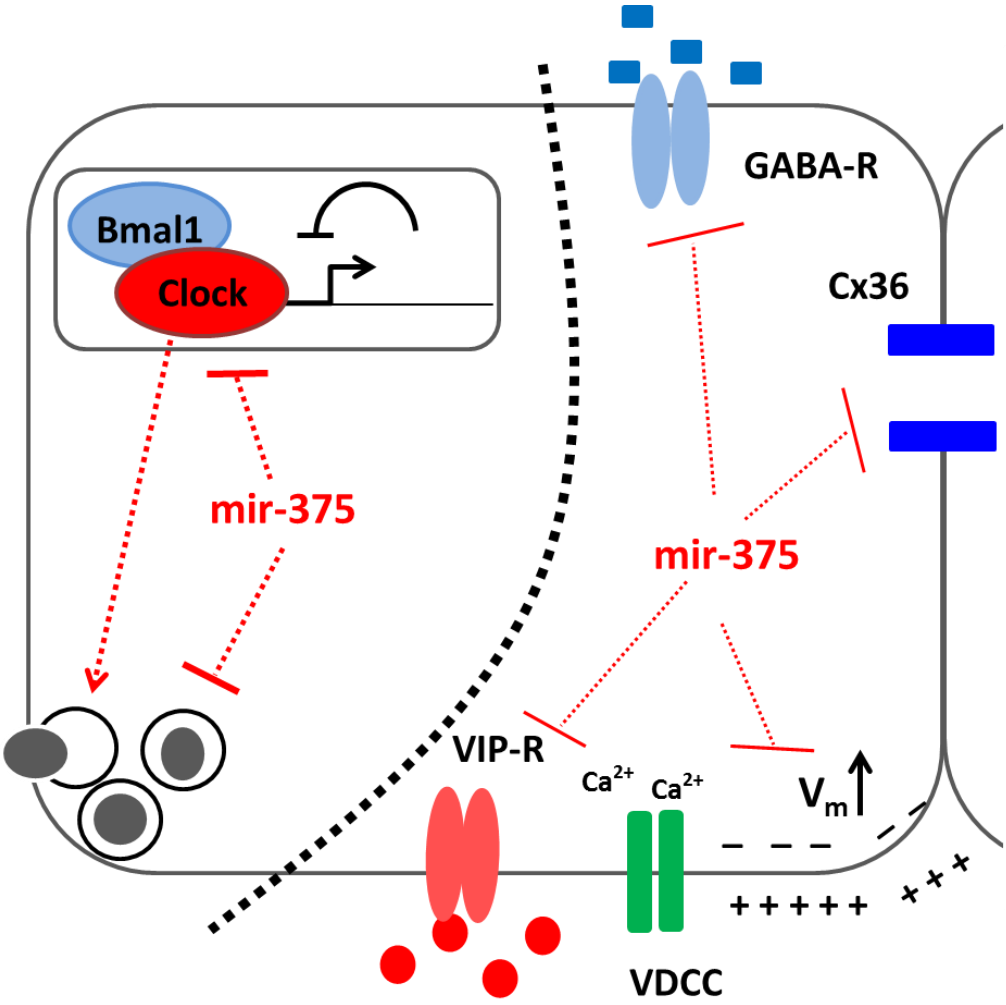


Fig. III-2: Postulated dual mechanism of mir-375 function in the beta cell clock
mir-375 suppresses *clock*, which probably contributes to the negative impact of the miRNA on the late stages of insulin secretion. In addition, mir-375 targets also other components involved in exocytosis (left) (Poy et al. 2004). Beyond its effect on *clock* expression, mir-375 attenuates the intrinsic capacity of the islet to sustain circadian rhythms. This might occur through attenuation of intercellular coupling in the islet syncytium, e.g. though a putative suppression of mir-375 on one or more of the depicted pathways – GABA-mediated signaling, gap-junction protein Connexin 36 (Cx36), membrane electrical firing, Ca^{2+}-signaling, vasoactive intestinal polypeptide (VIP)-stimulation (clockwise order). The respective neurotransmitters can act as autocrine factors and/or could be derived by adjacent neurons.

C. The role of Clock and the molecular pacemaker in hepatic function

In the third part of the thesis, the molecular crosstalk between circadian and metabolic networks in the liver was addressed. Genetic, biochemical and high-throughput approaches were exploited in order to assess the impact of Clock-Bmal1 on lipoprotein metabolism and to elucidate a concrete mechanism, which mediates it.

Studies were performed in mice lacking Clock in all tissues (Debruyne et al. 2006). As described above, these animals represent a suitable model to evaluate the function of local pacemakers since central oscillator function and brain-regulated metabolic processes are intact (Fig. II-1, Fig. II-2, Debruyne et al. 2006).

III.C.1 Clock-deficiency leads to increased hepatic glucose output

The current study expanded the knowledge on the impact of the hepatic molecular clock on glucose output. As the liver can store and release glucose in phases of low or high demand, aligning with feeding and fasting, respectively, metabolic fluxes of glucose production, intracellular hepatic metabolites and rate-limiting enzymes in these processes have been found highly circadian (Bolli et al. 1984; Panda et al. 2002; Reddy et al. 2006 ; Eckel-Mahan et al. 2012). Not surprisingly, the liver is one of the first extra-SCN tissues which has been assigned an endogenous time-keeping system and represents the best studied example of an metabolic oscillator so far (Bass and Takahashi 2010; Eckel-Mahan and Sassone-Corsi 2013).

Here, both routes – *de novo* gluconeogenesis and glycogen turnover – were shown to exhibit alterations under conditions of *clock* deletion, resulting in an uncontrolled glucose production – a critical factor for the development of type 2 diabetes **[Fig.II-17]** (Best et al. 1982; Kahn 2001)**.** Importantly, hepatic glucose output was boosted irrespective of circadian time [**Fig.II-17]**. Moreover, this defect was cell-autonomous since it existed in Clock-deficient hepatocytes *ex vivo* and did not arise as a secondary effect due to altered insulin / glucagon signalling *in vivo* [**Fig.II-17C**; Fig.**II-18].**

Transcriptome and proteome analyses uncovered altered expression of gene clusters involved in glycolysis / gluconeogenesis **[Fig. II-22, Fig. II-24]**. For instance, downregulation of the rate-limiting enzyme in glycolysis and glycogen polymerization – *pyruvate kinase* (*Pk*) and *glycogen synthase 2* (*Gys2*), respectively – and upregulation of *glucose-6-phosphatase* (*G6pase*), support the increased hepatic glucose output in the circadian mutants [**Fig.I-10, Fig. II-22, Fig. II-24].**

Importantly, several models of circadian arrhythmia have demonstrated the relevance of the hepatic pacemaker in gluconeogenesis; however, its physiological manifestation has been conflicting. In alignment with the current report, whole-body Bmal1-deficiency has been described to exhibit enhanced gluconeogenesis (Lamia et al. 2008). In contrast, other mouse models, using panspecific or liver-specific *bmal1* deletion, associated with impaired glucose production and hypoglycemia (Rudic et al. 2004; Lamia et al. 2008) [**Tab. I-1]**. The ambiguous results might arise from different genetic backgrounds as well as the disturbance of the SCN-pacemaker in whole-body Bmal1-deficient mice.

III.C.2 Clock-Bmal1 control hepatic lipoprotein synthesis and export

III.C.2.1 Impaired very-low density lipoprotein (VLDL) secretion in Clock$^{-/-}$ mice

Under conditions of nutrient deprivation, the liver serves a buffering function not only with regard to glucose but also to lipid supply. It is the only organ, capable of triglyceride and cholesterol production, destined for extracellular export in form of very-low density lipoproteins (VLDL) (rev. in Alaupovic 1982). Importantly, Clock-deficiency in hepatocytes impairs this process as levels of circulating VLDL are significantly reduced **[**Fig.II-20**]**.

The profile of circulating lipoproteins is dependent on the nutritional status: Under feeding conditions, plasma is rich in large-molecular chylomicrons, transporting dietary fats, derived from enterocytes, whereas, under fasting, it is abundant in VLDL secreted by the liver and loaded with endogenous lipids (Cohn et al. 1993; Thompson et al. 1996). Thus, although both lipoprotein classes could not be differentiated in the FPLC elution profile, it can be assumed that in mice with free access to food the large-molecular fraction represented mainly chylomicrons, whereas, after an overnight fast, it corresponded to hepatocellular VLDL. Accordingly, levels of the former correlated with periods of nocturnal food intake and were highest at night [Fig.**II-19]**. Interestingly, the fact that this lipoprotein fraction was elevated in Clock$^{-/-}$ mice under fed conditions hints towards a putative postprandial dyslipidemia, a metabolic disorder, in which enterocytic overproduction of chylomicrons often associates with diabetes (rev. in Pang et al. 2012). Indeed, the molecular pacemaker has been already implicated in intestine function in this respect: chylomicron packaging was reported to follow a circadian pattern due to oscillating expression of *microsomal triglyceride transfer protein* (*mttp*) involved in chylomicron assembly at the ER (Edwards et al. 1972; Pan al. 2010).

In contrast, the drop in circulating VLDL in the postabsorptive catabolic phase devoid of calorie intake clearly indicates that Clock-disruption impairs the intrinsic capacity of hepatocytes for lipogenesis and / or assembly of triglyceride-rich particles **[**Fig.II-20**]**.

III.C.2.2 Pgc-1β – the molecular link between Clock-Bmal1 and lipoprotein secretion in liver

Transactivation of *ppargc1b* is controlled by Clock-Bmal1

The current report provides evidence that Clock-Bmal1 exert their effect on VLDL production through transactivation of *ppargc1b*. Pgc-1β is a crucial regulator of lipogenesis and hepatic lipoprotein output, which binds master lipogenic transcription factors, such as SREBP, LXR, EERα and Foxa2, to activate and potentiate their downstream effects (Lin et al. 2005; Wolfrum and Stoffel 2006; Hernandez et al. 2010).

Several indices proved *ppargc1b* a clock-controlled gene: Its mRNA exhibits large, over five-fold variations in expression over a 24-hour cycle with a peak at ZT9 – a characteristic feature of *bona fide* Clock-Bmal1 targets [**Fig.II-28]**(Ripperger and Schibler 2006; Doi et al. 2010). Secondly, circadian transcription is dependent on the pacemaker and does not result from reoccurrence of systemic cues since expression is abrogated under conditions of *clock* deletion [**Fig.II-28]**. Finally, Clock-Bmal1 physically interact with an intronic canonical E-box element in *ppargc1b* **[Fig. II-30]** (Gekakis et al. 1998; Hogenesch et al. 1998).

Unfortunately, the present study could not investigate potential cycling accumulation of Pgc-1β protein due to lack of specific commercially available antibody for immunological detection. Furthermore, quantitative mass-spectrometry failed to detect a potential decrease in Pgc-1β in Clock$^{-/-}$ liver due to the limited dynamic range of this technique, which cannot identify low-abundant proteins, such as transcription factors (Liu 2004, see section III.C.3). Nevertheless, circadian protein expression of this coactivator has been reported in another study, which did not primarily investigate *ppargc1b* regulation: There, Pgc-1β accumulation was found to peak at ZT 9-13, consistent with the mRNA increase described herein (Liu et al. 2007). Hence, Clock-Bmal1 transactivation of *ppargc1b* presumably results in an oscillating coactivation of the transcriptional partners of Pgc-1β [**Fig. III-3].**

This was emphasized by the fact that downstream targets of Pgc-1β displayed reduced expression under conditions of Clock-deficiency **[Fig. II-31]**. On the one hand, this involved

SREBP-responsive genes, such as *HMG-CoA-reductase* (*hmgcr*), *fatty acid synthase* (*fasn*) and *stearoyl-Coenzyme A desaturase 1* (*scd1*), which are involved in biosynthesis of VLDL constituents, i.e. triglycerides and cholesterol (Lin et al. 2005) **[Fig. I-11, Fig. I-12]**. On the other, *apolipoprotein C-III* (*apoc3*), which is transactivated by EERα and Pgc-1β, facilitates VLDL lipidation at the ER and its maturation in Golgi cisternae (Hernandez et al. 2010; Sundaram et al. 2010; Sundaram et al. 2010b). Importantly, *apoc3* correlates positively with circulating VLDL levels in mouse and man, thus, highlighting that the attenuated expression of this apoprotein contributes to the observed defect in lipoprotein secretion (Cohn et al. 2004; Cohn et al. 2004b).

Hence, the results indicate that in Clock$^{-/-}$ liver, abrogated Pgc-1β expression induces impaired activation of lipogenic programs, thus, unraveling Pgc-1β as a critical node between Clock-Bmal1 function and hepatic lipoprotein secretion [**Fig. III-3]**.

Furthermore, it is worth noting that the Pgc-1β homologue – Pgc-1α – also represents an intimate link between pacemaker and metabolic networks in liver. However, unlike Pgc-1β, Pgc-1α integrates metabolic input into the circadian pathway – it drives rhythmic *bmal1* transcription together with the nuclear receptor ROR (Liu et al. 2007).

mir-378 is a Clock-Bmal1 target

The current report further elucidates intronic mir-378, derived from the *ppargc1b* locus, as a Clock-Bmal1 target. Importantly, this is the first example of a microRNA in an extra-SCN mammalian organ, directly regulated by the heterodimeric duo: In the murine hypothalamus, Clock-Bmal1 drive expression of *mir-219* (Cheng et al. 2007). However, unlike the oscillating brain-specific small RNA, mir-378 levels accumulated at a constant rate over day and night, in spite of cycling transcription at the *ppargc1b* locus [**Fig. II-26-29]**.

The fact that mature mir-378 is decreased under conditions of Clock-deficiency, suggests that the Clock-Bmal1 duo is required for gating mir-378 expression, instead of its circadian variation. Thus, mir-378 is not expected to contribute to hepatic circadian output.

At present, the downstream effects of mir-378 reduction in Clock$^{-/-}$ liver remain unclear. Mir-378 is implicated in liver regeneration and oxidative phosphorylation and, interestingly, in adipocyte lipogenesis (Song et al. 2010; Carrer et al. 2012). The miRNA is upregulated during adipogenesis to promote lipid droplet size and lipid synthesis. However, the targets mediating lipogenic effects remain elusive and further investigation is required in order to

elucidate whether mir-378 is also involved in hepatic lipogenesis and VLDL production (Gerin et al. 2010a). The latter hypothesis seems plausible based on the fact that, frequently, intronic miRNAs co-modulate biological processes together with the gene they originate from (van Rooij et al. 2009; Lutter et al. 2010; Dill et al. 2012; Najafi-Shoushtari et al. 2010). In this respect, a preliminary bioinformatic screen suggested *sortilin 1* as a putative mir-378 target (data not shown). Notably, this gene is a prominent regulator of hepatic lipoprotein assembly and secretion (Musunuru et al. 2010; Strong et al. 2012). Remarkably, *sortilin 1* expression correlates inversely with plasma VLDL levels, suggesting that mir-378 might act in synergy with Pgc-1β to promote hepatic lipoprotein secretion through this target [**Fig. III-3]**.

Interestingly, microRNAs, whose mature sequence accumulates in a non-circadian manner but whose transcription oscillates, have been already described. The most abundant liver-specific miRNA – mir-122 – is transcribed antiphase to the circadian repressor Rev-Erbα; however, mature mir-122 does not cycle (Gatfield et al. 2009). This phenomenon is consistent with the current concept that mature miRNAs are long-lived and that their half-life exceeds 24 hours (Winter et al. 2009; Gatfield et al. 2009) (see below).

III.C.3 Hepatic expression analyses in Clock$^{-/-}$ mice

The project added another layer to the existing information on the circadian hepatic transcriptome through proteome and miRNA transcriptome analyses under conditions of *clock*-ablation (Panda et al. 2002; Storch et al. 2002; Reddy et al. 2006; Hughes et al. 2009; Eckel-Mahan et al. 2012). Undoubtedly, it lacked temporal resolution required to detect changes in circadian dynamics, such as phase shifts or period deviations, thus, representing a snap-shot of the molecular footprint under conditions of Clock-deficiency. Yet, the gene expression data delivered valuable insights into alterations in glucose and lipoprotein metabolism and served as a basis for the identification of a Clock-Bmal1-controlled target (see III.C.1, III.C.2.2).

Clock$^{-/-}$ hepatic transcriptome and proteome

Transcriptome and proteome analyses revealed that ca. 1.7 % and 5.5 % of the genes detected, respectively, were differentially regulated in Clock$^{-/-}$ liver. These portions seem relatively low based on reports claiming that 5 – 10% of all transcripts and 10 – 20 % of all proteins in liver cycle (Panda et al. 2002; Storch et al. 2002; Hughes et al. 2009; Reddy et al. 2006). This is

probably due to the fact that the current analyses represent expression changes at Zeitgeber Timepoint (ZT) 9 – shortly after the zenith in Clock-Bmal1 transcriptional activity has been reached – thus, underrepresenting the affected genes under conditions of Clock-disruption. Nevertheless, the transcriptomic and proteomic data sets retrieved several established Clock-Bmal1 targets as downregulated, such as *dbp, gys2* and *klf10,* confirming the reliability of the data (Ripperger and Schibler 2006; Guillaumond et al. 2010; Doi et al. 2010) **[Fig. II-22, Fig. II-24, Appendix V.A.1].**

Importantly, the overlap between differentially regulated transcripts and proteins was low and comprised only 8 genes **[Fig. II-25A]**. This might result from the fact that the comparative analysis was restricted to one circadian timepoint. If protein cycling is phase-delayed to mRNA oscillations, at ZT9 differential regulation might be observed only at one expression level while at the other it escapes detection.

Another aspect associates with the technical limitation of shotgun proteomics. Currently, microarray profiling is far more comprehensive: Although the quantitative SILAC-based approach identified over 1,000 proteins, this number was still relatively low, compared to over 15,000 transcripts detected by standard mRNA hybridization chip technique. This results from the low dynamic range of mass-spectrometry in complex biological mixtures and its bias towards high-abundant proteins (Liu 2004). This constraint was reflected by the fact that low-abundant proteins, such as transcription factors and core circadian components, escaped detection [**Appendix V.A.1]**. Yet, in comparison to other techniques, such as two-dimensional gel electrophoresis, utilized for the assessment of the circadian proteome so far, the current proteomic approach is characterized through superior quantitation and reproducibility (Uitto et al. 2007; Reddy et al. 2006; Deery et al. 2009).

Finally, the fact that many regulated proteins lack a regulated mRNA counterpart suggests that Clock-Bmal1 might be involved in translational / posttranslational control. This is consistent with the emerging role of the pacemaker in mechanisms beyond rhythmic transcription, e.g. circadian control of translation through regulation of ribosome biogenesis, or posttranslational modulation through circadian acetylation (Jouffe et al. 2013; Masri et al. 2013). This is further supported by other large-scale analyses, demonstrating that a substantial portion of cycling proteins lack a corresponding oscillating transcript (Reddy et al. 2006; Deery et al. 2009).

Genes controlling lipoprotein secretion and lipid metabolism are dysregulated in Clock$^{-/-}$ liver

Gene ontology analysis of transcriptomic and proteomic data underscored the defective intrinsic capacity of hepatocytes to synthesize and release lipoproteins: The term "plasma lipoprotein particle" and numerous components involved in VLDL assembly and secretion were downregulated **[Ref. in Tab. II-2**; **Fig. II-21]**. The data sets were also enriched in differentially regulated components involved in lipid metabolism, e.g. inhibitors of *de novo* cholesterol synthesis, and genes associated with peroxisome function – the organelle where lipid catabolism predominantly occurs [**Fig. II-21, Fig. II-22, Fig. II-23, Tab. II-2]**.

Furthermore, additional dysregulated protein classes, such as oxidoreduction and xenobiotic metabolism, confirmed previous reports on the involvement of the pacemaker in drug detoxification [**Fig. II-23]** (Gachon et al. 2006)**.**

Taken together, the integrative high-throughput gene expression analysis in Clock$^{-/-}$ liver underlined the control of the hepatic pacemaker over lipogenesis and lipoprotein transport and mirrored the physiological defect of the circadian mutants for VLDL secretion.

microRNA pool in Clock$^{-/-}$ liver

Further, the current report provided data on expression alterations under *clock*-deletion beyond the protein-coding genome.

32 of the fifty most abundant hepatic miRNAs exhibited mild, less than twofold changes in expression **[Fig. II-26]**. This indicates that loss of the circadian transactivator exerts a large-scaled but subtle effect on the miRNA transcriptome. The latter might result from the fact that mature miRNAs display high stability. For instance, the average half-life of mir-122 has been estimated to exceed 24 hours, rendering it resilient towards circadian variation despite high-amplitude oscillation in transcription (Gatfield et al. 2009). This is in line with the current finding that even a direct Clock-Bmal1-target – mir-378 – lacked oscillation in mature miRNA [**Fig.II-29]**. Furthermore, the high stability of miRNAs is illustrated by examples of acute miRNA-mediated regulation (Bhattacharyya et al. 2006). Indeed, suppression by miRNAs can be quickly reversed, e.g. during a stress response; however, this effect does not result from dynamic miRNA expression, but, instead, from changes in the subcellular localization of targeted mRNA. Suppressed transcripts are exported from processing bodies in order to resume translation.

It is worth noting that recently the concept of high miRNA stability has been challenged. Several reports claim rapid activation / repression of miRNA genes, followed by an acute change in the corresponding mature forms, for instance upon light stimulation in the retina or upon cholesterol fluctuations in macrophages (Krol et al. 2010; Rayner et al. 2010).

Furthermore, reports on high-amplitude circadian oscillation of miRNAs also exist, albeit restricted to non-mammalian systems or the SCN: In the fat body of *Drosophila melanogaster*, which corresponds to a mixture of metabolic and hematopoietic tissue, cycling of the mir-959-964 complex is intertwined with feeding phases (Vodala et al. 2012). In the SCN, brain-specific mir-219 is transcribed in a circadian manner through recruitment of Clock-Bmal1 to its promoter (Cheng et al. 2007). Further evidence originates from nonphysiological cell-based systems: For instance, mir-185 was found to oscillate anti-phase to Cry-1 and to regulate its cytoplasmic accumulation (Lee et al. 2013).

Importantly, in $Clock^{-/-}$ liver, the small magnitude of miRNA regulation is still expected to evoke physiological impact. Mild effects of individual sequences can combine in a synergetic manner, leading to substantial consequences for cellular processes and thus, contributing to perturbation of hepatic homeostasis

III.C.4 Conclusion and outlook

The present work addressed Clock-Bmal1 action in the liver in the context of intact hierarchical circadian regulation by the central SCN pacemaker. Previous reports showing Clock-Bmal1 involvement in carbohydrate metabolism were confirmed, as loss of Clock enhanced hepatic glucose output, manifested in both increased gluconeogenesis and defective glycogen turnover – traits, characterizing type 2 diabetes. Moreover, the pacemaker was found to control lipoprotein secretion. This effect was mediated by the transcriptional coactivator Pgc-1β – a stimulator of lipogenesis and VLDL assembly – whose expression is driven by Clock-Bmal1 **[Fig. III-3]**. Accordingly, in $Clock^{-/-}$ liver, high-throughput expression analyses revealed transcriptome and proteome alterations related to lipoprotein and glucose metabolism.

Moreover, Clock-Bmal1 transactivation of *ppargc1b* regulated the expression of intronic mir-378, which was co-transcribed with Pgc-1β in an oscillating manner but lacked circadian accumulation in its mature form **[Fig. III-3]**. Small RNA deep sequencing revealed that Clock-Bmal1 exert mild but extensive impact on the miRNA transcriptome.

These findings raise several questions. Firstly, is mir-378 involved in hepatic lipoprotein transport? To answer this, mir-378 targets should be elucidated. One interesting candidate in this respect would be Sortilin 1 – an important regulator of VLDL assembly and secretion, whose levels adversely correlate with circulating lipoproteins (Musunuru et al. 2010; Strong et al. 2012). It would be important to investigate whether Sortilin 1 is targeted by mir-378 *in vivo* and whether its potential increase in $Clock^{-/-}$ liver contributes to the lipoprotein secretion defect **[Fig. III-3]**.

Furthermore, it would be important to assess whether other miRNAs beyond mir-378 obey regulation by Clock-Bmal1. For this, a global approach, e.g. chromatin immunoprecipitation coupled with DNA sequencing (ChIP-seq), should be performed. Subsequently it could be verified whether thus elucidated miRNA targets are downregulated in $Clock^{-/-}$ liver. In this way, the global connection between miRNAs and circadian rhythms can be addressed.

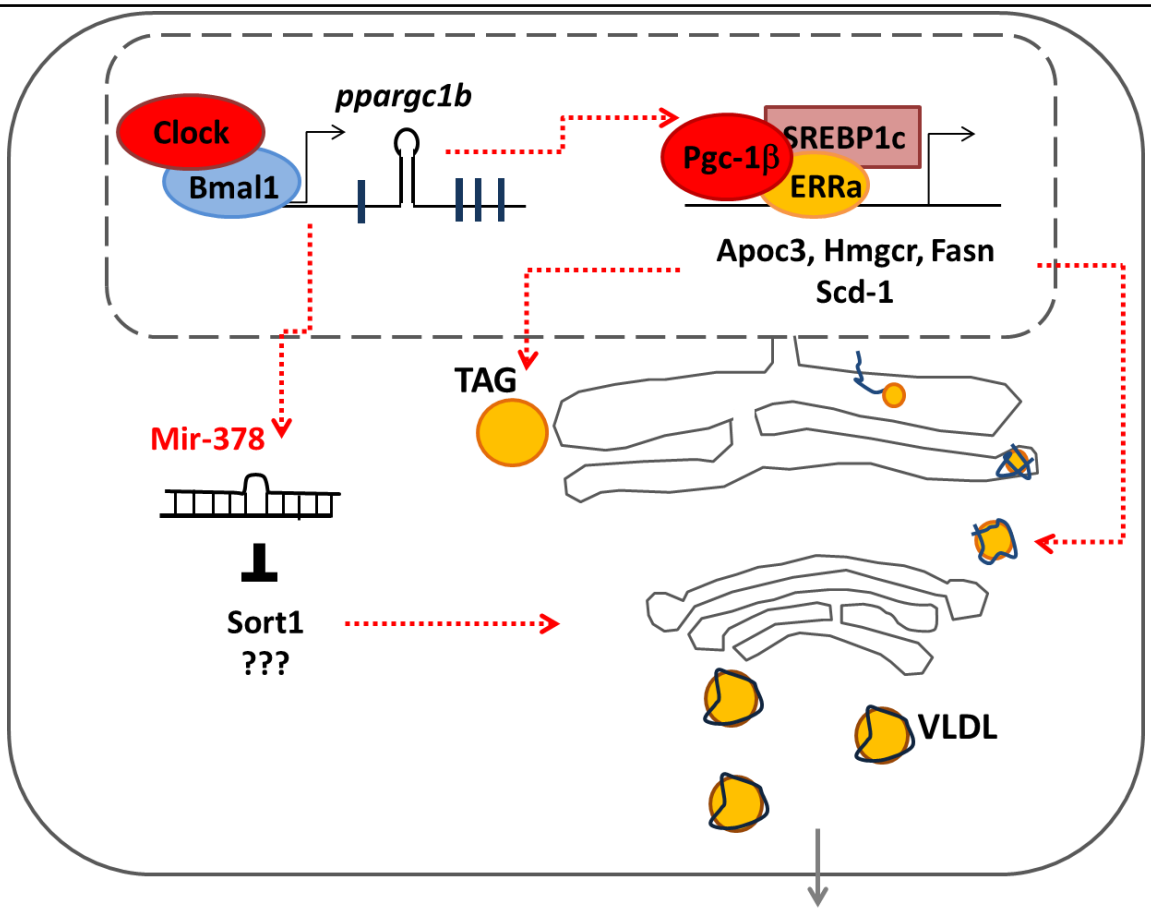


Fig. III-3: Mechanisms of Clock-Bmal1 regulation of hepatic lipoprotein secretion.
Clock-Bmal1 bind a conserved consensus E-box in the *ppargc1b* intron to drive circadian transcription of the host gene and intronic mir-378. Circadian rise in Pgc-1β co-activates other transcription factors to drive expression of downstream effectors, such as *apoc3, hmgcr, fasn and scd1*, promoting lipid synthesis and VLDL assembly. Mature mir-378 accummulates in a constant manner and lacks circadian oscillations, but might be also involved in control of lipoprotein expression, e.g. through its putative target Sortilin 1.

IV. Materials and Methods

A. Mouse line breeding and genotyping

Experiments with mice were performed in accordance with the German standards of animal care.

All mice were maintained on C57BL/6J background. Animals with a panspecific *clock* deletion ($Clock^{-/-}$) and mice with a conditional floxed allele ($Clock^{fl/fl}$) were kindly provided by Dr. David Weaver from University of Massachusetts Medical School (Debruyne et al. 2006). The $Clock^{fl/fl}$ strain was crossed with transgenic mice, overexpressing *cre recombinase* under regulation of the rat *insulin* gene promoter (*InsCre*), in order to obtain beta cell-specific *clock* deletion (InsCre $Clock^{-/-}$) (Herrera 2000). Analogously, $Bmal1^{fl/fl}$ animals, purchased from The Jackson Laboratory (Bar Harbor, ME), were bred with *InsCre*-expressing mice to generate the InsCre $Bmal1^{-/-}$ line (Storch et al. 2007). Breeding of $Clock^{-/-}$, InsCre $Clock^{-/-}$ and InsCre $Bmal1^{-/-}$ mice yielded normal Mendelian ratios.

Animals with a whole-body *mir-375* deletion (mir-$375^{-/-}$) were kindly provided by Prof. Markus Stoffel, ETH Zurich (Poy et al. 2009).

The reporter line for monitoring of circadian rhythms, expressing *luciferase* fused to the endogenous *per2* open reading frame ($Per2^{luc}$), was a generous gift from Prof. Achim Kramer, Charite Berlin (Yoo et al. 2004). It was crossed with the $375^{-/-}$ line to obtain $miR375^{-/-}$ animals carrying the $Per2^{luc}$ allele ($miR375^{-/-}$ $Per2^{luc}$). Initial crossing did not follow Mendelian inheritance as $per2^{luc}$ and the disrupted *mir-375* gene were located on the same chromosome 1. Mice were bread until crossing over segregated the alleles, which occurred at a frequency of ca. 4%, to obtain heterozygous animals, which were further used for breeding.

For mouse genotyping, tail biopsies were proteinase K-digested (PAN-Biotech) in 4 M urea, 10 mM EDTA pH = 8.0, 0.1 M Tris/HCl pH = 8.0, 0.5 % sodium sarcosyl, 0.2 M NaCl at 55°C overnight. Genomic DNA was precipitated in 75% EtOH plus 60 mM sodium acetate and subjected to PCR according to the guidelines in the above listed references.

Mice were housed under a 12-hour light/12-hour dark regimen (7 a.m. lights on, 7 p.m. lights off) with free access to water and standard rodent chow. When fasted, they were deprived from food over their active period (overnight) for ca. 14-16 hours. Experiments were conducted with age-matched littermate-controlled mice at 6-10 or 18-20 weeks of age.

All procedures for the use and care of animals were approved by the German Ethical Committee of Experimental Animal Care.

B. Analysis of blood parameters

IV.B.1 Circulating glucose, insulin and glucagon

For the analysis of parameters at a random-fed and fasting state, whole blood was collected at 5 p.m. and at 10 a.m. after a 16 hour fast, respectively. Blood glucose concentration was determined from whole venous blood from the tail using a Bayer Contour Glucometer according to the manufacturer's protocol. For hormone detection, plasma was extracted from the cellular fraction after two centrifugation rounds at 10.000g, 5 min and processed immediately or stored at -20°C for further analysis. For glucagon analysis, blood collection was conducted at 10 a.m., after an overnight fast. Insulin and glucagon concentrations were determined using radioimmunoassays (Millipore).

IV.B.2 Circulating lipoproteins

Analysis of lipoprotein profiles by fast protein liquid chromatography was done on a single Tricorn 10/300 glass column (GE Healthcare) with Superose 6 resin (GE Healthcare) and Äkta FPLC chromatography system (Amersham Pharmacia Biotech). Plasma from freshly collected blood was extracted through centrifugation (2X 10.000g, 5 min) and combined from several donors, as stated. 200µl-sample was injected and then eluted with PBS at a flow rate of 1 ml per min. Fractions were collected and spectroscopically inspected for protein content based on 280nm-absorption. The resulting elution spectra were used for the relative quantification of lipoprotein subclasses (Sata et al. 1972).

C. Physiological tests

IV.C.1 Glucose, pyruvate and insulin tolerance test

Mice were fasted overnight and injected intraperitoneally with D-glucose (Sigma-Aldrich, 2g/kg body weight), pyruvate (Sigma-Aldrich, 2 g/kg body weight) or recombinant human insulin (Sigma-Aldrich, 0,75 unit/kg body weight) in sterile PBS. Blood from tail cuts was collected before (time 0) and 15, 30, 60 and 120 min after injection and inspected for glucose concentration (see above) (Kulkarni et al. 1999). If not otherwise stated the experiments were performed at ZT3 (10 a.m.).

IV.C.2 Insulin secretion

In vivo insulin secretion was assessed after i.p. injection with D-glucose (Sigma-Aldrich, 2g/kg body weight) in sterile PBS after an overnight fast. Whole blood from the tail vein was collected at 0, 2.5, 5 and 15 minutes and inspected for insulin concentration (see above) (Kulkarni et al. 1999).

IV.C.3 Leptin sensitivity

Leptin sensitivity and corresponding changes in body-weight and food intake were assessed in individually housed mice. Before the start of the experiment, mice were let to accommodate for one week, during which body weight and food intake were measured daily in order to construct the baseline. After this phase, animals received two i.p. injections daily, at 9 a.m. and 6 p.m of recombinant rat leptin (7,5 µg/g body weight, Sigma-Aldrich) over the course of 3 days and their body weight and food intake were recorded daily (Mori et al. 2004).

IV.C.4 Temporal recording of metabolic parameters

Automated metabolic and behavioral monitoring of small laboratory animals was performed with the PhenoMaster lab system from TSE, Bad Homburg, Germany. After an accommodation period of ca. 7 days, individually housed mice were placed in the recording chambers for a period of 5-7 days, under 12-hour light/dark conditions and free access of water and food. The metabolic cages were equipped with automated feeding sensors, detecting food consumption over time. Home cage activity corresponding to the cumulative distance in horizontal and vertical direction, was measured via the crossing of an infrared-laser beam. Metabolic performance – body heat production and respiratory exchange rate (RER) – was assessed via indirect calorimetry, through sensor-based recording of O_2 consumption and CO_2 production.

IV.C.5 Recording of body composition

Measurement of body composition - fat and lean muscle mass – was performed by nuclear magnetic resonance (NMR) technique at the Mouse Phenotyping Platform at Max-Delbrueck Center for Molecular Medicine, Berlin with Dr. Arnd Heuser. Measurements were conducted on Minispec Model LF90 II (6.5 mHz) (Bruker Scientific Instruments). This method enables collection and analysis of proton signals from the entire body and a noninvasive and rapid measurement without anesthetics.

D. Tissue isolation and cell culture

IV.D.1 Isolation of Langerhans islets

Langerhans islets were isolated using the intraductal collagenase technique (Wang et al. 1993). The common bile duct of sacrificed mice was cannulated and the pancreas was perfused with Hanks' buffered salt solution (HBSS). The tissue was minced and digested with 3 mg/ml collagenase (Worthington) for 20 min at 37°C. Islets were isolated from remaining pancreatic exocrine tissue through a Histopaque density gradient (Sigma-Aldrich) and hand-picked under a stereomicroscope (Stereozoom GZ7, Leica).

IV.D.2 Primary hepatocyte cell preparation

Primary hepatocytes were isolated according to an *in situ* two-step perfusion protocol (Berry and Friend 1969). First, livers were perfused with Hanks' buffered salt solution (supplemented with 250 µM EGTA, 4,19 mM Sodium Bicarbonate, 50 mM Hepes) via the portal vein until blood vessels were washed out. Second, the tissue was perfused with Leibovitz-15 medium with 1 mg/ml collagenase type IV (Sigma-Aldrich) for ca. 4 min. Following mechanical tissue disruption, a single-cell suspension was obtained. Hepatocytes were washed twice with Phosphate buffered saline (PBS) and plated onto culture dishes coated with 0.5 mg/ml collagen R (PAN Biotech).

IV.D.3 Cell culture

All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere. Langerhans islets were incubated in Roswell Park Memorial Institute-1640 (RPMI) medium with 10% fetal bovine serum (FBS) supplemented with penicillin (100 U/ml) plus streptomycin (100 m g/ml). Primary hepatocytes were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and antibiotics in collagen-coated plates (0,5 mg/ml, PAN Biotech). Immortalized human hepatocytes (Hepg2) and kidney epithelial cells (HEK 293) were grown in DMEM with 10% FBS. The SV40 T-antigen stably transformed mouse insulinoma beta cell line (MIN6) was culture in DMEM plus 15% FBS and 79 µM beta-mercaptoethanol in order to denature disulphide bonds of secreted insulin and avoid its mitogenic action.

For serum shock activation of MIN6 cells, aiming to synchronize circadian oscillations *in vitro*, culture medium was replaced with medium containing 50 % FBS for 2 hours, after which it was changed back to normal growth medium (Balsalobre et al. 1998).

E. Cell / Tissue assays

IV.E.1 Insulin secretion assay in isolated Langerhans islets

Timed kinetics of glucose-stimulated insulin secretion were assessed using a home-made perifusion system, based on the one described in Panten et al. 1977. 80-120 islets per animal were isolated and cultured overnight to allow replacement of receptors that have undergone proteolysis during collagenase treatment. The islets were placed on a gel of porous polyacrylamide beads (Biorad) in a chamber at 37°C. They were equilibrated for ca. 15 min with Gey & Gey buffer and subsequently for 30 min with 5,5 mM glucose so that an uniform and complete docking of insulin granules can occur. After that, the chamber was perifused with Gey & Gey buffer containing low (3,3mM) and high (16,7 mM) glucose concentration at a rate of 1ml/min. Eluate was collected every 1 min using a fraction collector (Amersham). Insulin concentration per fraction was determined and divided by cellular insulin content in order to compensate for differences in islet counts.

IV.E.2 Insulin secretion assay in MIN6 cells

MIN6 cell were seeded in 24-well plates at a density of 60-70 percent. Prior to the functional experiment they were pre-equilibrated for 30 min with Gey & Gey secretion buffer with 5,5 mM glucose in order to induce pre-docking of the insulin containing granules to the plasma membrane. Cells were subsequently washed with Gey & Gey buffer without glucose and incubated in 2 mM glucose (low) or 20 mM glucose (high) for 1 hour. Supernatants were collected for insulin quantification and cells were lysed in ethanol with 5% HCl. Hormone quantification was performed using rat insulin radioimmunoassay (Millipore). For normalization, the release of insulin was divided by the cellular insulin content.

IV.E.3 Pancreatic insulin content

Total pancreata were excised and lysed in ethanol with 5% HCl overnight at -20°C. Extracts were diluted in PBS and subjected to rat insulin radioimmunoassay (Millipore). Hormone concentration was normalized to tissue mass.

IV.E.4 Hepatic glycogen content

Liver tissue or cultured primary hepatocytes were lysed in 1 M NaOH for 1h at 65°C and tissue extracts were spun at 14.000 g to remove cell debris. Lysates were spotted onto

Whatmann paper (Bio-Rad), precipitated in 66% ice-cold ethanol and the glycogen polymer was hydrolysed using amyloglucosidase (Sigma-Aldrich). The released glucose was quantified by a glucose oxidase-based photometric reaction (WAKO). The glucose concentration was used to estimate the glycogen content, which was normalized by wet liver mass or protein concentration. The latter was quantified spectrophotometrically using a bicinchoninic acid assay and copper (II) sulfate solution from Sigma-Aldrich.

IV.E.5 $[Ca^{2+}]_i$ imaging in pancreatic islets

Intracellular Ca^{2+} changes were measured by means of dual-wavelength microfluorimetry in collaboration with Prof. Patrik Rorsman, Oxford Centre for Diabetes, Endocrinology and Metabolism. Prior to measurement islets were loaded with 3 µM fluorescence indicator fura-2 (Molecular Probes, Leiden, Netherlands) for 40 min at 37°C. During the experiment, the islets were held in place by a glass pipette and superfused continuously with a medium containing 140 mM NaCl, 3,6 mM KCl, 2 mM $NaHCO_3$, 0,5 mM NaH_2PO_4, 0,5 mM $MgSO_4$, 5 mM Hepes (pH 7,4), 2,5 mM $CaCl_2$ and glucose, as indicated – 1mM / 12mM / 20mM. Fluorescence measurements were carried out on a microfluorimetry system (D104, PTI, Monmouth Junction, NJ, USA) in an optical plane of beta cells. The fluorophore was excited at 380 and 340 nm corresponding to peak excitation changes for unbound and Ca^{2+}-bound state, respectively. The emission ratio represents a reliable method to estimate $[Ca2+]_i$ independent of dye concentration and cell thickness. Furthermore, $[Ca2+]_i$ was monitored in intact islets, as opposed to dispersed cells, in order to avoid profound changes in $[Ca2+]_i$ oscillations, occurring upon beta cell-dissociation (Grynkiewicz et a. 1985; Jonkers et al. 1999).

IV.E.6 Membrane capacitance measurement

Electrophysiological studies were conducted in collaboration with Prof. Patrik Rorsman, Oxford Centre for Diabetes, Endocrinology and Metabolism. Changes in cell capacitance as a result of exocytosis in individual beta cells was monitored in response to membrane depolarization, circumventing closure of ATP-dependent K^+-channels in the absence of physiological stimulus.

Recordings were performed on dissociated beta cells, obtained from pancreatic islets, using conventional patch-clamp technique. The extracellular bath solution contained 138 mM NaCl, 5,6 mM KCl, 2,6 mM $CaCl_2$, 1,2 mM $MgCl_2$, 5 mM glucose and 5 mM HEPES (pH 7,4).

Patch pipette resistance was around 2 MΩ when filled with 76 mM Cs_2SO_4, 10 mM NaCl, 10 mM KCl, 1 mM $MgCl_2$ and 5 mM Hepes (pH 7.35). The measurements were conducted in a standard whole-cell configuration using an EPC-7 patch–clamp amplifier in conjunction with the software Pulse (version 8.53; HEKA Elektronik).

Changes in membrane capacitance were elicited by train of depolarization pulses from −70 to 0 mV. Each train consisted of either 10 pulses 500-ms pulse duration (1-Hz stimulation frequency) or 20 pulses 50-ms pulse duration at 10-Hz stimulation frequency. All electrophysiological measurements were performed at ≈33°C.

IV.E.7 Bioluminescence recordings of circadian oscillations

Bioluminescence recordings of *per2::luciferase*-expressing islets were conducted in collaboration with the chronobiology lab of Prof. Achim Kramer, Charité Universitätsmedizin (Yoo et al. 2004). Ca. 100 islets per mouse genotype, isolated the previous day, were incubated in sealed culture flasks without gas exchange in 1 ml growing medium supplemented with 0.1 mM beetle D-luciferin (Biothema). They were placed under photomultiplier tubes (Hamamatsu P7360-02, Hamamatsu) in a recording chamber at 37°C and photon emission was integrated for 30 min over a period of 7 to 14 days. Using the software ChronoStar (Dr. Stephan Lorenzen, Universitätsmedizin Göttingen), raw bioluminescence series data was detrended by dividing values through a 24-hour running average, thereby normalizing the magnitude to 1, independent of sensitivity/background of the photomultiplier or efficacy of the luciferase. Circadian parameters – amplitude, period, phase and damping ratio – were estimated by fitting the cosine wave curve.

F. Electron microscopy

Ultrasructural microscopic analysis was conducted at the electron microscopy core facility at MDC with (Dr. Bettina Purfürst). Dissected pancreata were fixed in 4% formaldehyde plus 2% glutaraldehyde in 0,2 M phosphate buffer (pH 7,4) over night. Tissue was minced, incubated with 1% OsO_4 for 2 h at room temperature to provide contrast to the image and dehydrated in ascending alcohol series. Samples were embedded in Poly/Bed 812 (Polysciences, Inc.). Ultrathin 80 nm-sections were cut and contrasted with uranyl acetate and lead citrate, and analyzed with an electron microscope (model EM 910; Carl Zeiss

MicroImaging, Inc.) with a CDD camera (Proscan). Number of secretory beta-cell granules in micrographs was counted using the iTEM Software (Olympus Soft Imaging Solutions).

G. Molecular biology techniques

IV.G.1 Isolation of RNA

RNA was prepared using Trizol reagent (Invitrogen) according to the manufacturer's protocol from snap-frozen tissue / cells. Tissue lysates were processed with an automatic homogenizer (IKA) prior to extraction of nucleic acids. RNA integrity was inspected after electrophoretic separation on a 6% denaturing formaldehyde gel. RNA concentration was determined at 260 nm with a NanoDrop ND-1000 UV-Vis spectrophotometer (Peqlab) according to manufacturer's guidelines. The ratio of adsorptions at 260 nm versus 280 nm was used as control for nucleic acid purity.

IV.G.2 Reverse transcription

cDNA was synthesized using random hexamer and oligo(dT) primers and Superscript III reverse transcriptase (Invitrogen) according the manufacturer's protocol .

IV.G.3 Real Time PCR

For mRNA expression analysis relative RNA levels were quantified by real-time PCR technology using SYBR Green reagents (Roche) on a StepOnePlus system (Roche). Gene-specific primers with annealing temperature of ca. 60°C were designed as to span intron boundaries (see oligonucleotide list for details). The PCR reaction consisted of the following steps: 1) 95°C 5 min denaturation, 2) 40 cycles 95°C 15 s → 60°C 30 s → 72°C 30 s primer annealing and extension, and 3) 72°C 10 min. Relative quantification of gene expression was performed according to the $\Delta\Delta C_t$ method (Winer et al. 1999). Data was normalized to endogenous levels of the ribosomal component 36B4.

For assessment of mature miRNA expression, a commercially-designed kit (TaqMan MicroRNA Assays, Applied Biosciences) was used. Targeted cDNA synthesis from total RNA was achieved through miRNA-specific stem-looped primers. Subsequently, quantitative PCR based on specific TaqMan probes was conducted. As an internal normalizer, expression of the small nucleolar RNA U6 was used.

IV.G.4 Northern Blot

20-40µg of total RNA were separated electrophoretically on a 12% Polyacrylamid denaturing gel using the Polyacrylamid/Urea SequaGel system (National Diagnostics) and electroblotted to a Hybond N^+ membrane (Amersham Biosciences). RNA crosslinking and immobilization was achieved through UV irradiation (0,12J, 30 sec) and baking in an oven for 1h at 80°C. Membranes were blocked for one hour at 50°C in 5× SSC, 20 mM Na phosphate at pH 7.2, 7% SDS, 2× Denhardt's solution (Invitrogen) and 0,1 mg/ml sonicated salmon sperm DNA (Invitrogen). Hybridizations with radioactively labeled oligonucleotide probes were performed overnight in blocking solution at 50°C. Subsequently, blots were washed twice for 10 min in 5× SSC, 5% SDS and once for 5 min in 1× SSC, 1% SDS at 50°C.

DNA probes complementary to the miRNA were end-radiolabeled using ^{32}P-γATP and polynucleotide kinase (NEB) according to the manufacturer's protocol. Blots were exposed to autoradiography films (Kodak) and scanned on a Typhoon Trio (GE Healthcare). Evaluation of 2D densitometry was performed using the AIDA software tool (Raytest).

IV.G.5 Western Blot

For imunoblotting snap-frozen tissue or cells were lysed in 20 mM Tris-HCl (pH 7,5), 150 mM NaCl, 0,25 % NP-40, 1,5mM $MgCl_2$. Protein concentration was determined spectrophotometrically using a bicinchoninic acid (BCA) assay and copper (II) sulfate solution (Sigma-Aldrich) according to the manufacturer's protocol, using a standard curve with bovine serum albumin. Protein was denatured using 5 X SDS loading buffer (310 mM Tris, 10 % (w/v) SDS, 50 % (v/v) glycerol, 5 mM EDTA, 0.05 % (w/v) bromophenol blue, 5 % (v/v) 2-mercaptoethanol) and incubated for 5 min at 95 °C. Subsequently, samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad) on 7 – 10 % (v/v) SDS gels in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1 % (w/v) SDS, pH = 8.3) at room temperature. Samples were transferred onto nitrocellulose membrane (Roth) in mini trans-blot cell system (Bio-Rad Laboratories, CA, USA) in transfer buffer (25 mM Tris-HCl pH = 8.4, 192 mM glycine, 20 % (v/v) methanol) for 100 min at 80 V at 4 °C. Blots were blocked in 5% milk in TBS for 1h at room temperature and then incubated with anti-Clock (Santa Cruz) or anti-γ-Tubulin antibodies at 4°C over night (Sigma). After a final incubation with a secondary HRP-conjugated antibody (Calbiochem) for 1h at RT, chemiluminescence was detected with SuperSignal West Femto (Thermo Fisher Scientific) using a camera (LAS-1000/ Intelligent Dark Box, Fujifilm). Between antibody-binding steps, blots were washed 3

times á 5 min with TBS+0,05% Tween. Evaluation of 2D densitometry was performed using the AIDA software tool (Raytest).

IV.G.6 Luciferase reporter assay

miRNA targets were validated using a dual luciferase reporter assay (Promega) (Kuhn et al. 2008). In this method, the relative expression of putative targets is assessed using the reporter pRL-TK vector (Promega), carrying the *Renilla* luciferase gene. Cotransfection with the pGL3 vector (Promega), containing the firefly luciferase gene serves as internal control for transfection efficiency and technical variability. A ca. 1 kb fragment derived from the 3'UTR of *clock* and *per2*, and the complete 3' UTR of *tef*, containing the binding site for mir375, were cloned into the pRL-TK vector (Promega). 100 nmol reporter construct and 100 nmol pGL3 control vector were cotransfected with 20 pmol siRNA, mimicking miR-375 or a control miRNA which is not bound by the respective 3' UTR, into HEK 293 cells using Lipofectamin 2000 (Invtrogen) according to manufacturer's guidelines. 36 hours later, cells were lysed and the activities of renilla and firefly luciferase were measured using the Dual-Luciferase assay kit (Promega) on a Tecan Infinite M200 luminescence reader. Relative inhibition of the respective 3' UTR sequences by the microRNA was expressed as a decrease in the ratio of renilla to firefly luminescence.

IV.G.7 miR-375 overexpression

A mammalian CMV-driven vector encoding pre-mir-375 was transfected into Hepg2 cells using Lipofectamin 2000. 36 hours post transfection, cells were lysed and processed for Western Blot.

IV.G.8 siRNA transfection

0,2 pmol siRNAs against Clock (siClock) or a scrambled control (siControl), purchased from Dharmacon, were electroporated in MIN6 cells using the Amaxa Nucleofector electroporation kit V (Lonza Cologne GmbH) according to manufacturer's guidelines. 48 hours after transfection, cells were processed for insulin secretion assay or Western Blot.

IV.G.9 Chromatin immunoprecipitation (ChIP)

Primary hepatocytes from C57BL/6J wild-type mice were isolated, let to attach to the collagen-coated plates and processed further for ChIP the same day. At ZT 7, cells were fixed

using 1% formaldehyde for 10 min at room temperature. Cross-linking was precluded by adding 0,125 M glycine for 5 min at room temperature. Cells were washed with ice-cold PBS and cell nuclei were isolated using a two-step lysis procedure: Cells were first treated with buffer 1 (0,05M Hepes-KOH (pH 7,5), 0,14 M NaCl, 1mM EDTA, 10% glycerol, 0,5% NP-40, 0,25% Triton X-100, with protease inhibitor cocktail (Roche)) for 15 min at 4°C and subsequently lysed with buffer 2 (0,2 M NaCl, 1mM EDTA, 0,5 mM EGTA, 10 mM Tris (pH 8), protease inhibitors) for 15 min at 4°C and spun down at 500 g for 5 min to pellet nuclei. Nuclear extracts were sheared in sonication buffer (Diagenode) for 26 cycles 30 sec on/off using a Biorupter sonicator (Diagenode). DNA from ca. 2*10^6 hepatocytes was incubated with 4 µg of rabbit anti-BMAL1 (Bethyl) or control rabbit IgG (Abcam) plus 30 µl of Protein A-coupled agarose beads (Diagenode) at 4°C overnight. Coimmunoprecipitated DNA-protein complexes were washed consecutively with low salt buffer (20 mM Tris-HCl (pH 8,1), 150 mM NaCl, 2 mM EDTA, 1 % Triton-X, 0,1% SDS), twice with high salt buffer (20mM Tris-HCl, pH 8,1, 500mM NaCl, 2mM EDTA, 1% Triton-X, 0,1% SDS), once with LiCl wash buffer (10mM Tris-HCl, pH 8,1, 0,25M LiCl, 1% IGEPAL, 1% sodium deoxycholate) and twice with TE buffer. Finally, DNA-protein complexes were eluted from agarose beads with buffer containing 1% SDS and 0,1 M $NaHCO_3$. To reverse cross-linking, samples were incubated for 4h at 65°C and subjected to proteinase K (Roche) and RNase A/T1 digestion (Invitrogen). DNA was purified using phenol/chloroform extraction and ethanol precipitation (Roth), and used for quantitative Real Time PCR (see table for oligonucleotide sequences). Sheared chromatin from ca. 2*10^6 cells which was not subjected to immunoprecipitation served as an input control.

H. High-throughput expression analysis

IV.H.1 miRNA sequencing

Massive parallel sequencing of miRNAs was conducted in collaboration with Dr. Wei Chen, MDC. Small RNA libraries, ligated at the 3' and 5' end with synthetic RNA adapter, were prepared using Illumina small RNA library preparation kits according to the manufacturer's protocol. Subsequently, the small RNA fractions were reverse transcribed and amplified using Illumina sequencing primers, and purified by polyacrylamide gel electrophoresis (PAGE) according to the expected product size. The libraries were sequenced using Illumina HiSeq 2000. In order to map miRNA sequences to the genome, an in-house Perl script was used to

remove oligonucleotide adapters from the sequencing reads, and the data was aligned using the mouse UCSC genome browser.

IV.H.2 Microarray analysis

mRNA expression profiling was conducted using the Affymetrix microarray hybridization platform. Total RNA was purified with the RNeasy kit (Qiagen) and converted into double-stranded cDNA using oligo $(dT)_{24}$ primer containing a T7 RNA polymerase recognition sequence. Eventually, the product was transcribed into biotin-labeled cRNA by the T7 RNA polymerase and hybridized to AffymetrixGeneChipMouse Genome 430_2 array, which was analysed on the AffymetrixGeneChip Instrument system at the National Institute for Diabetes and Digestive and Kidney Diseases (NIDDK), USA. Scanned chip images were processed using GeneChip algorithms.

For islet mRNA expression analysis, raw data was evaluated through the Affymetrix pipeline at NIDDK to obtain fold change regulation.

For the hepatic transcriptome, raw data was bioinformatically processed through a multi-stage pipeline using the AltAnalyze software (University of California, San Francisco). Firstly, raw signal RNA-hybridization data was normalized and subjected to an intensity cut-off to remove low-abundant transcripts. Secondly, gene expression levels were calculated, taking into account hybridization signals from multiple probe sets for the same transcript. Eventually, fold change regulation in biological replicates were quantified, subjected to statistical analysis and corrected for multiple hypothesis testing (see below).

IV.H.3 Quantitative proteomics

Studies of the hepatic proteome were executed in collaboration with Dr. Stefan Kempa, MDC. Protein identification was performed by shotgun “bottom up” mass-spectrometry, in which detection of proteins in complex biological mixtures, undergone enzymatic cleavage, is achieved through high performance liquid chromatography combined with mass spectrometry. To confer relative abundance of identified proteins, this technique was coupled with **S**table **I**sotope **L**abeling with **A**mino acid in **C**ell culture (SILAC) expanded to an *in vivo* animal model (Ong and Mann 2007; Krüger et al. 2008).

Livers from C57BL/6 mice, fully labeled with $^{13}C_6$-lysine served as a reference for relative quantification and were generously provided by the group of Prof. Matthias Mann, Max-Planck-Institute for Biochemistry, Martinsried. The SILAC-mouse had been fed over two

generations with a SILAC-diet supplemented with $^{13}C_6$-lysine, to achieve a complete near 97% labeling of liver and other organs.

Liver proteins from unlabeled (wild-type, Clock$^{-/-}$) and labeled $^{13}C_6$-SILAC mouse were extracted in Urea buffer (8 M Urea, 100 mM Tris-HCl, pH 8.5). After reduction and alkylation of disulfide bridges, samples were enzymatically digested with the two endoproteases trypsin and Lys-C (Promega). SILAC and non-SILAC lysate were mixed in a 1:1 ratio and subjected to LC-MS/MS-based shotgun sequencing on a LTQ-Orbitrap hybrid mass spectrometer (ThermoFisher). The liquid chromatography separation step prior to the mass spectrometry run (LC-MS/MS), ensured a highly resolved detection of peptides in the complex mixture of the liver lysate. Proteomics raw data was analyzed through the MaxQuant pipeline (Cox and Mann 2008). Proteins were considered reliably identified if at least 8 light to heavy peptide ratios were detected. Downstream data processing was performed with the Perseus platform.

I. Statistics and data analysis

Graphical data represent mean ± standard error of the mean for the indicated (n) number of experiments. Data mentioned in text represent mean ± standard deviation.

Physiological experiments presented herein are representative of at least three independent experiments.

One-tailed unpaired Student's t-test was applied in order to evaluate statistical significance for two-group comparison with unequal variance. All p-values below or equal 0,05 were considered significant (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$). High-throughput expression data and respective fold change regulations were subjected to Student’s t-test, indicating false discovery rate and multiple hypothesis testing (Benjamini and Hochberg 1995).

The majority of data analysis was performed by GraphPad Prism. Transcriptomic analysis of replicate reproducibility and volcano plots were executed with R statistical programming language. Transcriptome and proteome data were subjected to the DAVID Gene Ontology tool (Huang et al. 2009). Oscillating liver transcripts were determined using the CircaDB database (Hughes et al. 2009). Overlap between differentially regulated genes in transcriptomic and proteomic data sets was assessed using the web application tool BioVenn and depicted as area-proportional Venn diagrams. Potential miRNA targets were determined

using the bioinformatic prediction tools PicTar and TargetScan (Krek et al. 2005; Bartel et al. 2005).

J. Buffers and oligonucleotides

The following buffers were used:

Gey&Gey buffer – 111 mM $NaCl_2$, 25,2 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 0,3 mM $MgSO_4$, 0,4 mM Na_2HPO_4, 0,3 mM KH_2PO_4, 1 mM $CaCl_2$

HBSS (**Hanks' balanced salt solution)** – 135 mM NaCl, 20 mM Hepes, 4 mM KCl, 1 mM Na_2HPO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose

PBS (Phosphate buffered saline) – 136,8 mM NaCl, 2,68 mM KCl, 10 mM Na_2HPO_4, 1,76 mM KH_2PO_4, pH = 7.4

SSC (saline-sodium citrate) buffer – 150 mM NaCl, 15 mM $Na_3C_6H_5O_7$

TBS (tris-buffered saline) – 20 mM Tris, 137 mM NaCl, pH = 7.6

Tab. IV-1 summarizes all custom-designed oligonucleotides, purchased from Eurofins.

Tab. IV-1: List of oligonucleotides in 5'-3' orientation
(forward (for) and reverse (rev) primers for PCR and oligonucleotide miRNA probes)

36B4 for	GGC CCT GCA CTC TCG CTT TC	**Hmgcr_for**	TTTGATAGCACCAGCAGATTTGCTCGTC
36B4 rev	TGC CAG GAC GCG CTT GT	**Hmgcr_rev**	GCAGTGCTTTCTCCGTACCCTTAGAGATC
Apoc3_for	CTCCTGGCATCTGCCCGAGCTGAAG	**Insig-2_RT_for**	CAGTGCTAAAGTAGACTTCGAC
Apoc3_rev	GTCCATCCAGCCCCTGGCCACCAC	**Insig-2_RT_rev**	GCATTCATACATTGCCAGTTGAC
ATP5o for	GCGTAGGGCAACTCCTGAA	**Lpl for**	GTGATCCATGGATGGACGGTAACG
ATP5o rev	CTAGAGGAGATGCTGTGGTC	**Lpl rev**	AGTTAAACTCCTCCTCCATCCAG
BMAL1_for	TGACCCTCATGGAAGGTTAGA	**miR-122 NB**	CAAACACCATTGTCACACTCCA
BMAL1_rev	GTTGGTGGCACCTCTCAAAGT	**miR-378 NB**	CCTTCTGACTCCAAGTCCAGT
Clock_RT_for	agatcagttcaatgtcctca	**Per2 for**	CACAAAGAACTGATAAGGACCCTGAAG
Clock_RT_rev	tgtcgaatctcactagcatc	**Per2 rev**	ACGATATACCGGAGGTAATGCCCT
con_Pgc1b_ChIP_for	CAATCTGCTTGAACTCTCACC	**per2_UTR_for**	CCCTGTCCCCCAGCCAGAGGT
con_Pgc1b_ChIP_rev	GACTAAAGGTTCTCTCAGGAC	**per2_UTR_rev**	GCAGCAGTGTGGGTGCCCTC
cyp51_RT_for	CTAGATTCTACATACAAGGACGG	**ppargc-1a_RT_for**	TTGAAATGTGCTGCTCTGGTTG
cyp51_RT_rev	AATTCAGATCCTTCAACTGGTCG	**ppargc-1a_RT_rev**	CTTCTCAAATATGTTCGCAGGC
Dbp for RT	GGCTCTTGCAGCTCCTCTT	**ppargc1b_ChIP for**	CCTAGCCACATGACCGAGGAAA
Dbp rev RT	ATTAGCACCTCCACGGTGTC	**ppargc1b_ChIP rev**	GGAAGACCCACAGGCTGAGG
DBP_ChIP_for	TGGGACGCCTGGGTACAC	**ppargc-1b_RT_for**	GCTCTTCCAGATTGACAGTGA
DBP_ChIP_rev	GGGAATGTGCAGCACTGGTT	**ppargc-1b_RT_rev**	GAGCTTCTGTAGCAGTGAAAG
Fasn_for	GCAAGGTGTAACATTCCCCTCTGGAGA	**Rev-Erba for**	GACTCCAATAACAACACAGGTGGTGT
Fasn_rev	CTGGGGGTCACCCACCTTGGTG	**Rev-Erba rev**	AGCTATCATCACTGAGACTGGGTG
Gck for	CGTTGACTCTGGTAGAGCAGATC	**Scd1_for**	ACACCTGCCTCTTCGGGATTTTCTACTACA
Gck rev	GAGAAAGTCTCCAACTTCTGAGC	**Scd1_rev**	CATTCGTACACGTCATTCTGGAACGC
Gys2 for	GAGGTGACAGACCACGCAGAC	**ucp2_RT_for**	CTGCAGATCCAAGGGGAGAGT
Gys2_rev	CCTCTTTGTCAATGTCGAACTTGTC	**ucp2_RT_rev**	CCTTGGTGTAGAACTGTTTGACAG

V. Appendix

A. Transcriptome, proteome and miRNA expression profile in Clock$^{-/-}$ liver

V.A.1 Gene expression data

For complete gene expression data in Clock$^{-/-}$ liver please refer to the following links:

Clock$^{-/-}$ transcriptome at ZT9:

https://mega.co.nz/#!xxwCmABK!DexnKJ5JT0HeUF58UsoZbmJW8J9v_QLzlh1q3AOZvBk

Proteome data at ZT9:

https://mega.co.nz/#!FhhgzBDb!QmI2Dgbj8B1tAXorvG-4uMeoezytPVPCGz51s-NISf0

miRNA data at ZT9:

https://mega.co.nz/#!J85kWYzT!4pzE6bNNqvmss0ogGMnFfD4izApNyaFwmIMYEzIdcIk

V.A.2 Biostatistical analysis of transcriptome and proteome data

V.A.2.1 Variation and reproducibility of high-throughput data

Reproducibility of the respective methodology (Affymetrix microarray or SILAC-based mass spectrometry) was tested across samples derived from biological replicates i.e. mice with identical genetic makeup. For this purpose, quantified gene expression levels (transcript hybridization intensities or peptide ratios) identified for each sample were depicted pairwise in a scatter plot matrix **[Fig. V-1, Fig. V-2]**.

V.A.2.2 Graphical representation of expression changes

The most "meaningful" gene expression changes in the Clock$^{-/-}$ "omics" data sets were visualized using a volcano plot. This type of scatter plot combines significance according to the p-value and magnitude of change. Accordingly, the majority of detected transcripts / proteins referred to datapoints, which were either unchanged or not significantly changed [**Fig. V-3]**. Genes exhibiting significant differential expression ($p<0,05$; at least 0,3 fold change) were easily identified graphically.

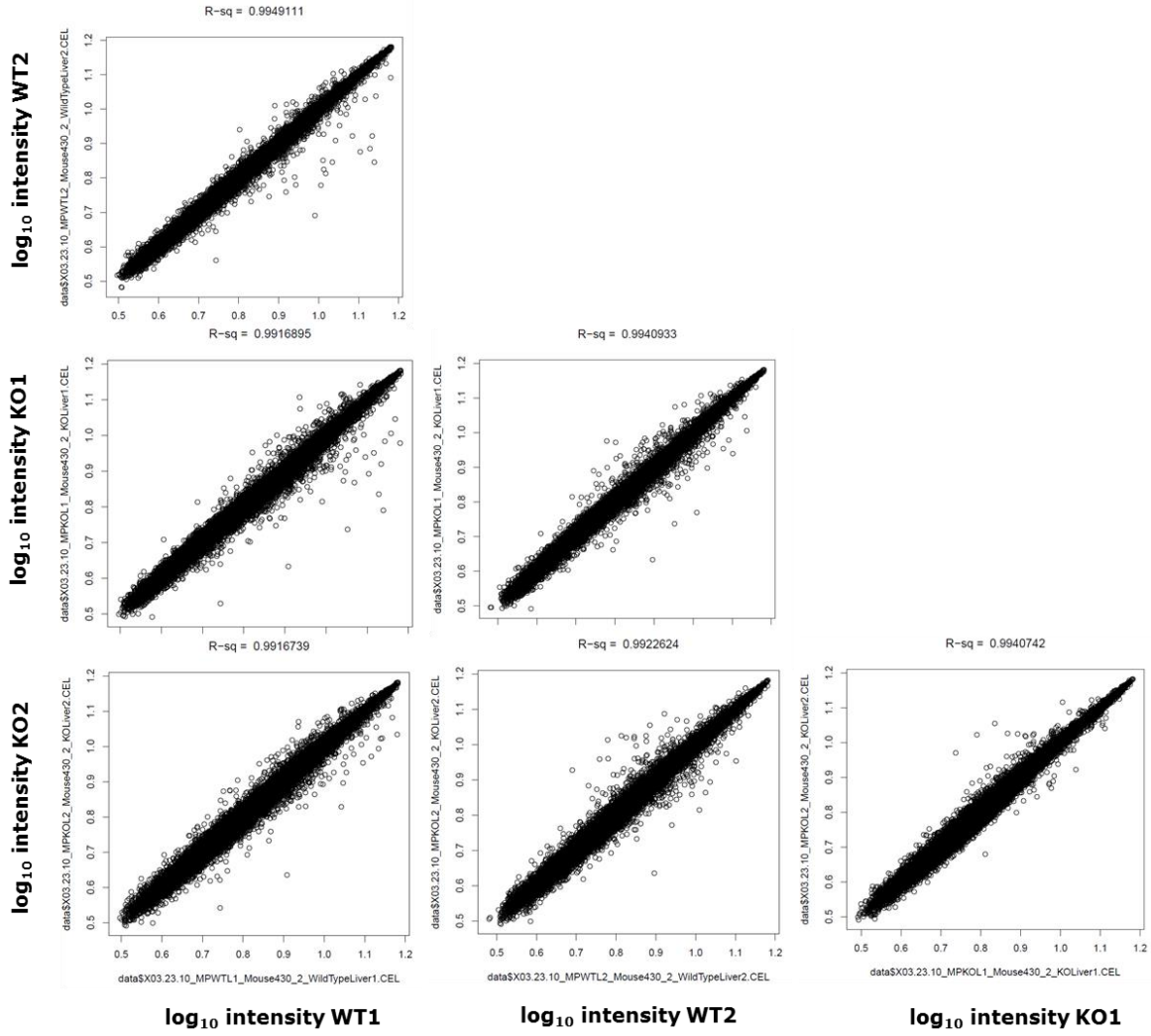


Fig. V-1: Variability in transcriptome expression data
A scatter plot matrix visualizes the intensity of hybridization signal for each transcript, plotted against every sample (wildtype or Clock$^{-/-}$ liver). The central four panels represent differences in intensity in wildtype vs Clock$^{-/-}$ samples. The two side panels depict the variation between biological replicates in the respective genotype.

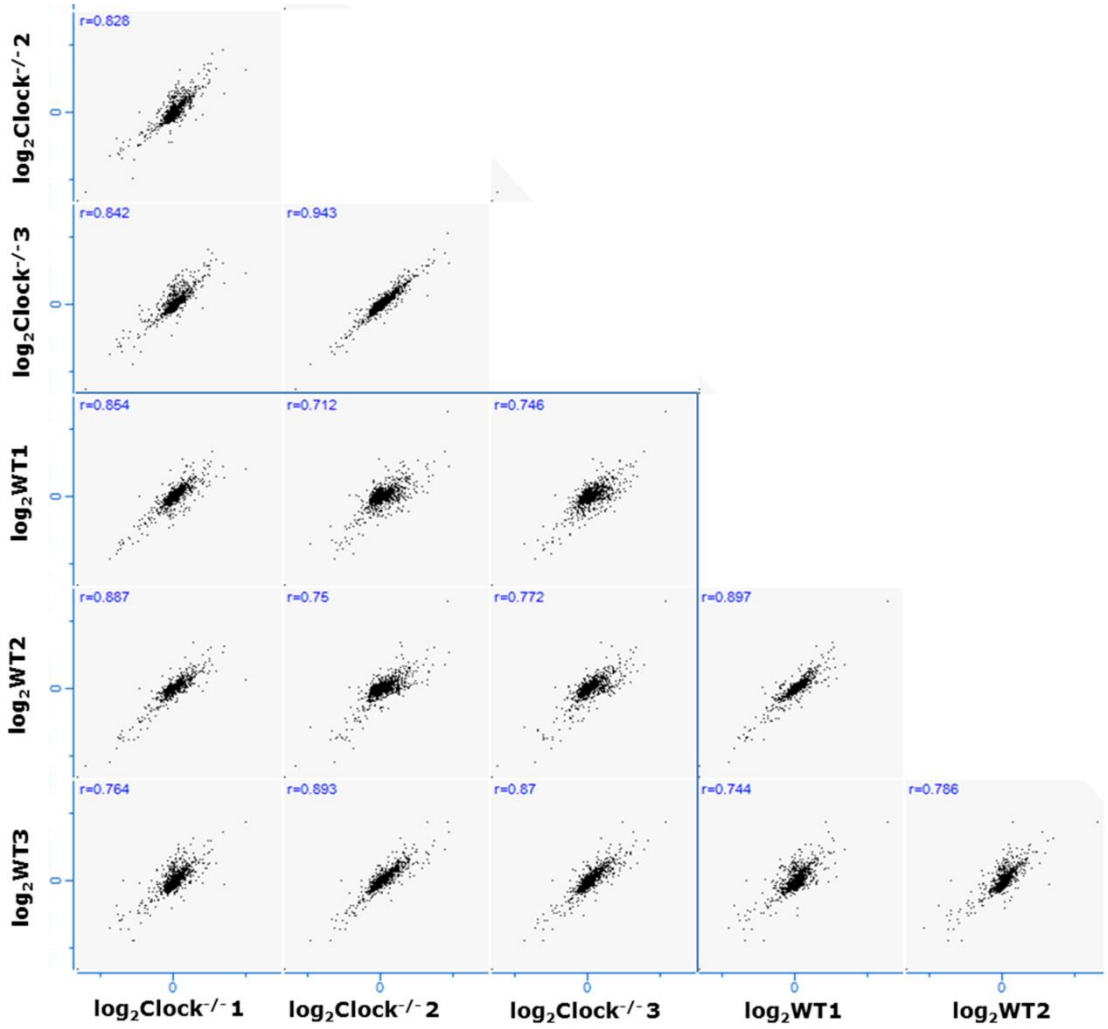


Fig. V-2: Variability in proteome expression data
Scatter plot matrix visualizing expression value (mass spectrometry ratio of "light" to "heavy" SILAC-labeled peptide) for each gene, plotted against every sample (wildtype or Clock$^{-/-}$ liver). The central 9 graphs represent wildtype vs. Clock$^{-/-}$ samples. The 3 panels on every side depict the variation between biological replicates in the respective genotype (data analysis was performed by Dr. Guido Mastrobuoni, MDC).

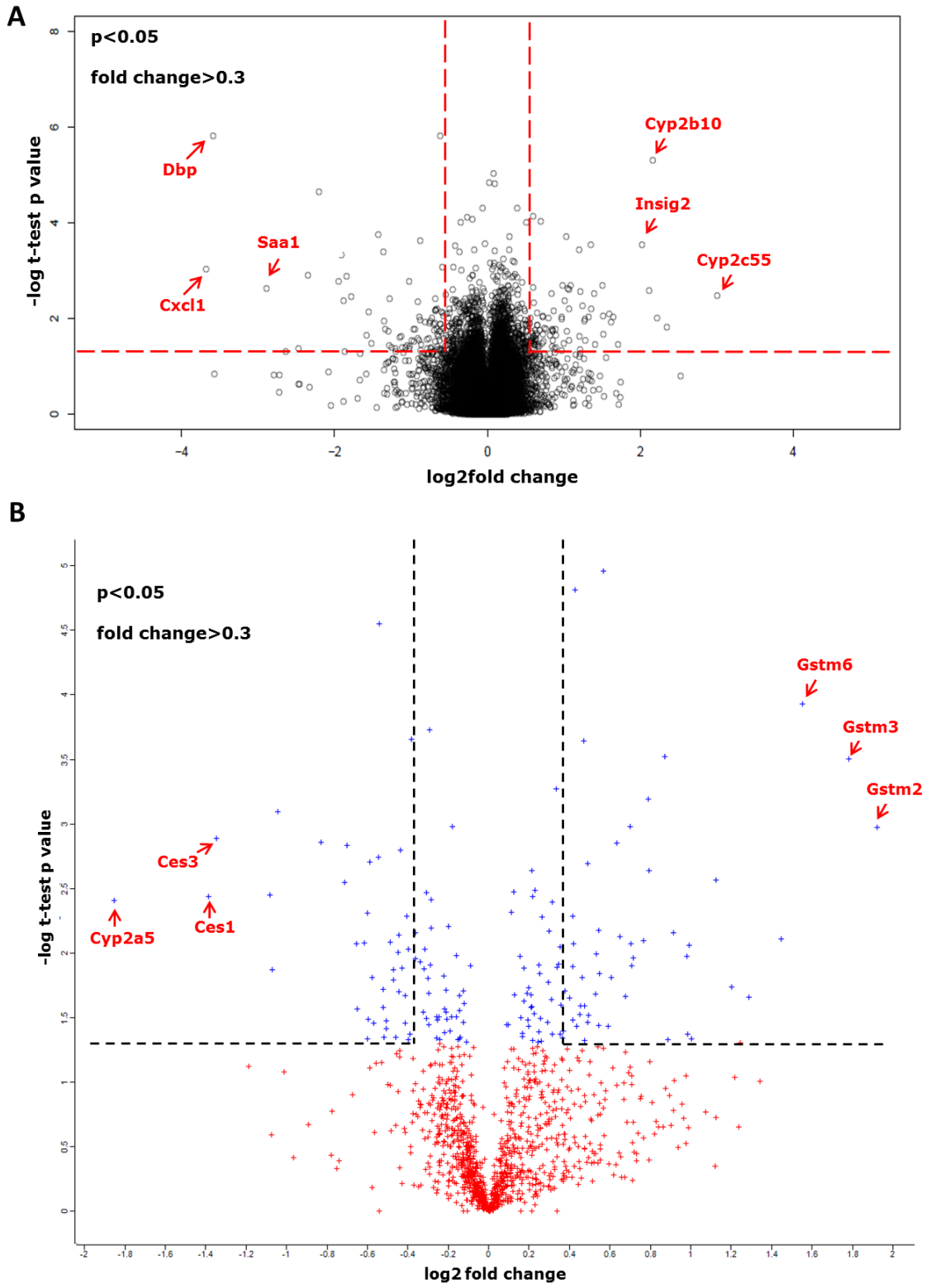


Fig. V-3: Differentially expressed genes in Clock$^{-/-}$ liver
Volcano scatter plot for transcriptome (A) and proteome (B) expression data. The negative log10 of the p-value is plotted on the y-axis, the log2 fold change on the x-axis. Highly significant data sets appear towards the top of the plot, up- or downregulation appears equidistant from the center. The dashed squares visualize data points of interest according to the selection criteria ($p < 0,05$; fold change > 0,3). (B) was contributed by Dr. G. Mastrobuoni, MDC)

B. The role of Clock-Bmal1 in insulin secretion

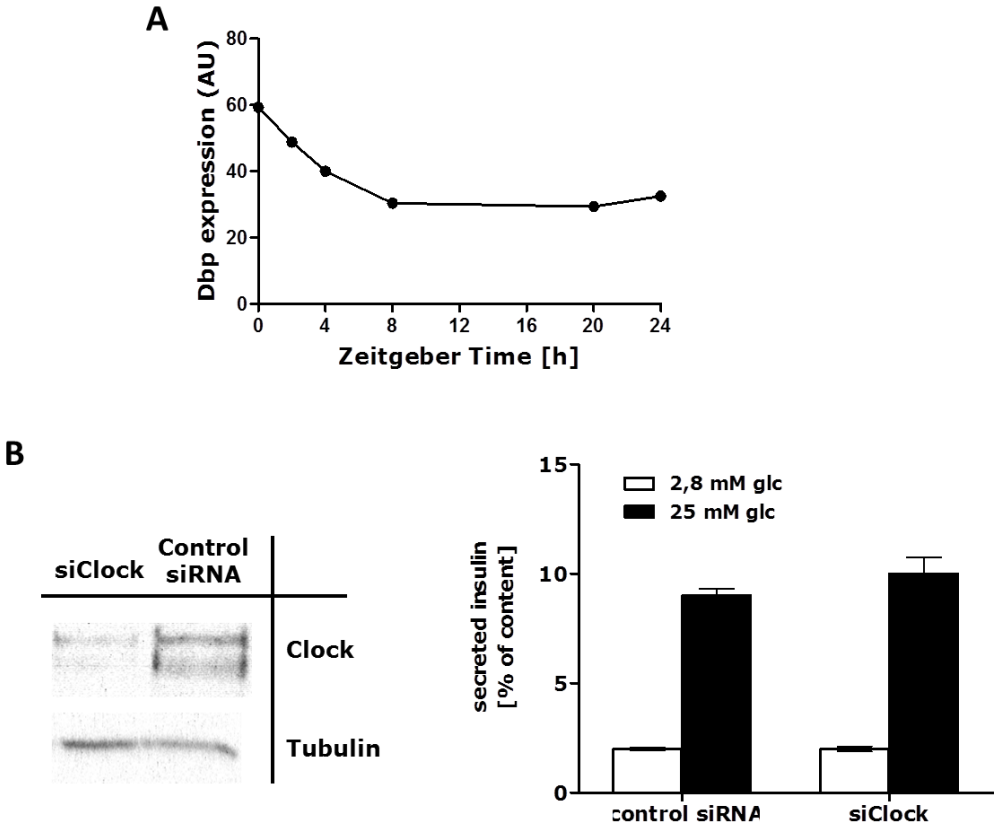


Fig. V-4: The immortalized pancreatic beta cell line MIN6 does not posess a circadian pacemaker
(A) mRNA levels of the Clock-Bmal1 target Dbp after serum-activation, known to induce the oscillator *in vitro* (Balsalobre et al. 1998). (B) Insulin secretion in MIN6 cells upon transfection with a scrambled or Clock-soecific siRNA (right panel). siRNA-mediated knockdown in MIN6 cells is depicted on the left.

V.B.1 Transcriptome analysis in Clock$^{-/-}$ islet

Complete Affymetrix microarray data in Clock$^{-/-}$ islet can be found on the Internet:

https://mega.co.nz/#!At4WVCbL!Vkn0WdGK_9NoAeMnPF5hLSb1bjzkf8UXUVak_CwPW5c

C. Abbreviation List

ATP	adenosine triphosphate
cAMP	3'-5'-cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
DNA	deoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ERRa	estrogen-related receptor alpha
Foxa2	forkhead box A2
Foxo1	forkhead box O1
GABA	gamma-Aminobutyric acid
GLP-1	Glucagon-like peptide-1
HNF	hepatic nuclear factor
i.p.	intraperitoneal
Klf	Kruppel-like factor
LXR	liver X receptor
NAD+/NADH	nicotinamide adenine dinucleotide
Neurod1	neurogenic differentiation 1
Npas2	neuronal PAS domain protein 2
PCR	polymerase chain reaction
Pdx-1	pancreatic and duodenal homeobox 1
polyA	poly-adenosine
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SNAP-25	synaptosomal-associated protein 25
SREBP	Sterol Regulatory Element-Binding Protein
UV	ultraviolet
VAMP-2	vesicle-associated membrane protein 2

VI. References

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