Pennington Symposium Supplement: Adipose tissue, adipocytes and the circadian timing system

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Keywords: adipogenesis, adipokines, metabolic syndrome, entrainment

Running title: Adipose and adipocyte clocks

Acknowledgements: Adipose rhythm work conducted by the authors is funded by research grants from the UK Biotechnology and Biological Sciences Research Council (BBSRC; BB/D526853/1) and Diabetes-UK (08/0003607).

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Conflict of interest statement: The authors are aware of no conflicts of interest.

Abstract

Circadian clocks time the daily occurrence of multiple aspects of behaviour and physiology. Through studies of chronic misalignment between our internal clocks and the environment (e.g. during shift work), it has long been postulated that disruption of circadian rhythms is detrimental to human health. Recent advances in understanding of the cellular and molecular basis of mammalian circadian timing mechanisms have identified many key genes involved in circadian rhythm generation and demonstrated the presence of clocks throughout the body. Furthermore, clear links between sleep, circadian rhythms and metabolic function have been revealed and much current research is studying these links in more detail. Here, we review the current evidence linking circadian rhythms, clock genes and adipose biology. We also highlight gaps in our understanding and finally suggest avenues for future research.

Introduction

Circadian rhythms are endogenously controlled changes in physiology and behaviour that enable organisms to regulate their biology in anticipation of predictable daily changes in the environment. These rhythms occur with a periodicity of approximately 24 hours and, in order to maintain an appropriate temporal relationship with the outside world, the phase of endogenous clocks is synchronised (entrained) by external time cues, termed zeitgebers (1).

In mammals, the master circadian clock resides within the suprachiasmatic nuclei (SCN) of the anterior hypothalamus (2,3). For a number of years, it was believed that the SCN represented one of only a few tissues capable of generating circadian rhythms; indeed, the SCN clock was believed to ultimately drive most rhythmic physiological and behavioural rhythms. However, circadian clocks have now been identified in nearly all tissues (4,5) and even in immortalised cell lines (6). These findings have necessitated a change in the perception of the cellular organisation of the clock, which is now conceptualised as an integrated circadian timing system (7) (Figure 1). In this complex system, clocks present in peripheral tissues drive local aspects of physiology. These so-called peripheral clocks are synchronised by multiple output pathways from the SCN (8), which thus retains its position as the master clock but acts by co-ordinating daily rhythms throughout the body.

The molecular basis of the mammalian circadian clock has been reviewed in detail elsewhere (1). In brief, the clock is based on transcriptional-translational feedback loops. In the central clock loop, the basic helix-loop-helix transcription factors BMAL1 and CLOCK (or its paralog NPAS2) stimulate transcription of three *Period* (*Per1-3*) and two *Cryptochrome* (*Cry1-2*) genes via E-box elements within their promoter regions. The translated PER and CRY proteins then form the basis of inhibitory complexes that translocate back into the nucleus and bind to CLOCK-BMAL1 proteins to inhibit their activity and thus the transcription of the *Per* and *Cry* genes. Within this cyclical process, the stability of PER and CRY proteins is tightly controlled by casein kinases (CK1δ/ε) and the F-box protein FBXL3, respectively (9-15). Such post-translational control is believed to be critical in determining the length of the molecular oscillations (16).

Secondary feedback loops also exist within the circadian mechanism. The best characterised of these drives rhythmical expression of *Bmal1* via retinoic acid-related orphan receptor response elements (ROREs) present within its promoter (17). In this loop, CLOCK-BMAL1 dimers stimulate the rhythmic transcription of *Rev-erbα,* which represses *Bmal1* transcription following translation into REV-ERBα protein. The purpose of these secondary loops is unclear but may provide additional robustness to the primary loop or perhaps provide mechanisms through which external processes (e.g. metabolic state) may interact with circadian biology.

In order for the molecular clock mechanism described above to regulate cellular physiology, it must be able to communicate temporal information to other intracellular pathways. It is now recognised that core clock proteins not only regulate the expression of other clock genes but also of output (or 'clock-controlled') genes by binding to circadian promoter elements, including E-boxes and ROREs (18-20). Many of these clock-controlled genes are themselves transcription factors that then regulate the expression of further downstream target genes; for example, the D-element binding protein gene (*Dbp*) is rhythmically expressed via E-boxes in its promoter and the resulting circadian profile of DBP protein then rhythmically induces transcription of downstream genes via activation of D-boxes. Through such mechanisms, the circadian clock is believed to drive rhythmical expression of approximately 10% of the transcriptome of most tissues (21).

Following from these advances in our knowledge of basic circadian mechanisms, importance is now being attached to understanding how the circadian timing system

interacts with other physiological systems. Indeed, one of the key challenges of the field is to determine the functional role of circadian timing processes in health and disease.

Linking the clock to adipose function

There are multiple lines of evidence that suggest a close relationship between circadian rhythms and adipose biology. Indeed, multiple aspects of adipose-related physiology display daily variation. For instance, in humans 24-hour rhythms have been reported in the circulating blood-borne concentration of leptin and adiponectin (22,23), which are members of the fat-derived hormone family, the adipokines (24). Most of these studies have been performed with the experimental subjects being kept in fluctuating external conditions, including a light-dark cycle. As a result, the reported rhythms are not necessarily circadian in nature, as they may be in part driven by the presence of rhythmic zeitgebers. However, the importance of endogenous circadian mechanisms in driving circadian adipokine secretion has been demonstrated in rodent models, in which leptin and adiponectin secretion is dependent on a functional SCN and persists in constant darkness (25,26). Whether human adipokine rhythms persist in the absence of environmental cues remains to be determined.

Further links between circadian and adipose physiology lie at the molecular level, in cellular, animal and human models. There is now in vitro evidence to suggest that multiple clock genes play a role in adipocyte differentiation. Not only does the expression of both *Rev-erbα* and *Bmal1* mRNA increase approximately 3-4 days

following the onset of differentiation of cultured adipocytes (27-28), but manipulation of gene expression in cultured cells indicates that they both promote the differentiation process (28-29). However, their expression is apparently not necessary for adipocyte differentiation in vivo as juvenile *Bmal* $^{-/-}$ mice and adult *Rev-erbα*$^{-/-}$ mice do not exhibit any gross adipose abnormalities compared to their respective wild type controls (30-31).

Despite the lack of gross adipose abnormalities in juvenile *Bmal*$^{-/-}$ mice and adult *Rev-erbα*$^{-/-}$ mice, many mutant mouse lines bearing genetic lesions of clock or clock-related genes exhibit abnormal lipid processing. Mice expressing the dominant negative allele *Clock*Δ19 on a C57BL/6J background display many of the symptoms of metabolic syndrome, including adipocyte hypertrophy, hyperleptinemia, hyperlipidemia, hepatic steatosis, hyperglycemia and hypoinsulinemia (32). In addition, serum VLDL triglycerides and apolipoprotein C-III concentration are elevated in *Rev-erbα*$^{-/-}$ mice (33), and mice with targeted disruption of the *Nocturnin* gene, which encodes a clock-controlled deadenylase, are resistant to diet-induced obesity (34). Adult *Bmal1*$^{-/-}$ mice exhibit a reduction of fat and other tissues, although this may be due to premature ageing, rather than a specific abnormality of adipose physiology per se (31).

One caveat when considering the metabolic phenotype of genetically altered mice is the effect of genetic background on expression of the phenotype. As has been reported in other models (35), the background genotype of mice appears to influence the expressed metabolic phenotype of the *Clock*Δ19 mutation onto other mouse lines (36-37). After crossing the *Clock*Δ19 mutation onto an ICR background, Oishi *et al*

reported a reduction in dietary fat absorption and subsequent protection against obesity induced by a high fat diet. Kennaway *et al* crossed the *Clock*Δ19 mutation onto a melatonin-proficient CBA line and reported a metabolic phenotype including elevated plasma adiponectin and reduced plasma free fatty acid concentration. However, despite these phenotypic differences, a common thread through the studies described above is that disruption of clock and clock-related genes induces metabolic disruption and lipid homeostasis.

Building on the rodent studies, a number of groups have now reported associations between metabolic disruption and polymorphisms of human clock genes. Variants of *BMAL1*, *NPAS2* and *PER2* have been linked to components of metabolic syndrome, including hypertension and glucose dysregulation (38-39). Moreover, studies of both British (40) and Argentinean (41) populations have investigated polymorphisms of the *CLOCK* gene and found significant relationships between *CLOCK* haplotypes and the presence of obesity and metabolic syndrome. These data therefore complement the animal studies and are consistent with an interaction between clock genes and lipid metabolism.

Finally, consistent with the existence of a localised peripheral clock within WAT, rhythmical clock gene expression has been reported in adipose tissue. These studies are discussed in detail below.

Adipose tissue rhythms

Rhythms in adipose tissue and the effect of diet

Analysis of murine adipose gene expression has revealed 24-hour rhythms of adipokine clock gene expression. The relative phasing of gene expression in WAT is consistent with SCN rhythms and the molecular model of the circadian clock suggesting the presence of a circadian clock within WAT (42-43). Moreover, use of microarrays suggests that up to 20% of the murine adipose transcriptome is expressed according to a diurnal rhythm, with many of the identified genes being involved in metabolism (43-44). Similar data have also been reported with human WAT arrays, albeit with a limited number of diurnal sampling time points (45). It therefore appears that many aspects of mammalian adipose biology are regulated on a 24 hour basis.

Interestingly, WAT tissue rhythms are altered in obese individuals and are sensitive to diet. Ando et al compared rhythms of clock gene expression in WAT taken from three mouse lines; one was lean, another was obese with mild type 2 diabetes mellitus (T2DM) and a third was obese with sever T2DM. The data revealed markedly reduced rhythm amplitude in obese mouse lines, compared to lean mice (42). This finding has notable similarities with previous reports of reduced amplitude rhythms of plasma adipokines in obese human subjects (22,46-48), suggesting that rhythms of both gene expression and endocrine activity may be attenuated in WAT of obese individuals. The role of diet on adipose rhythms has been investigated using different experimental paradigms. Administration of a high fat diet to mice for 6 weeks had multiple effects on circadian physiology, including a lengthening of the period of behavioural rhythms and also altered diurnal gene expression rhythms in both WAT and liver (49). Consistent with data relating to other peripheral tissues,

restriction of food availability to the light phase regulates the phase of adipose rhythms in mice (43). Finally, in overweight human subjects, short-term fasting also alters diurnal human sub-cutaneous WAT rhythms (45). Together, these data not only indicate a key role of daily rhythms in adipose function, but that there is a functional relationship between metabolic state and the phasing and/or robustness of adipose rhythms.

Despite their importance in identifying molecular rhythms in adipose tissue, there are some limitations with the above studies. As with the reports of adipokine rhythms in human plasma, they were conducted in changing light-dark cycle and so the contribution of endogenous timing mechanisms cannot be readily distinguished from possible masking effects of environmental rhythms. Also, the complex composition of adipose tissue and its input pathways makes the cellular basis of adipose rhythms unclear. These factors are discussed in detail below.

WAT cellular heterogeneity

As with other tissues, WAT is comprised of multiple different cell types and this heterogeneity causes difficulty interpreting which cells contribute to rhythmicity at the tissue level. In addition to adipocytes and pre-adipocytes, WAT contains a wide variety of cells that are believed to contain endogenous clocks (Table 1).

Other potential confounding factors when interpreting rhythmicity in WAT are 1) the inflammatory state of the tissue and 2) the origin of the WAT explant. One aspect of

obesity is the chronic inflammatory state of adipose tissue (50). During this time, the balance of the WAT cellular composition changes, notably including an increase in the macrophage content of the tissue. It is now clearly understood that different adipose depots have markedly different influences over body metabolism; for example, visceral WAT is a much better predictor of metabolic syndrome than subcutaneous fat (51). This differential function of adipose depots also appears to extend to circadian rhythmicity, as the phase of clock gene rhythms in adipose tissue is dependent upon anatomical location (52-53).

Rhythmical inputs

As with other peripheral tissues, WAT is subject to a variety of rhythmical input signals (Figure 2). Many of these inputs are neuroendocrine in origin; neuronal inputs include autonomic innervation; endocrine inputs include melatonin and glucocorticoids. Other putative rhythmical inputs include feeding and paracrine factors from other WAT cells, e.g. macrophage cytokines. Although the likelihood of physiological redundancy makes it difficult to identify the role of individual signalling pathways in the synchronisation of WAT rhythms in vivo, there is evidence that these neuroendocrine and behavioural rhythms are involved in the entrainment of at least some peripheral clocks.

Feeding behaviour

It has been recognised for a number of years that temporal restriction of food availability acts as a powerful environmental time cue that regulates circadian rhythms (54). In nocturnal rodents, restriction of food availability to the day time synchronises rhythms of food anticipatory activity and gene expression in peripheral tissues, including WAT (43). In most experimental models, the SCN remain entrained to the light-dark cycle, resulting in an uncoupling with many peripheral tissues. However, in some mouse strains and during both temporal and caloric food restriction, SCN rhythms also entrain to food availability (55-57).

The mechanisms through which temporal food availability regulate circadian clocks are poorly understood. Changes in metabolite concentration are able to synchronise cultured cells in vitro (58) and so may also contribute to synchronisation in vivo. Furthermore, there is a wealth of evidence supporting the existence of a food-entrainable oscillator (FEO), which lies outside of the SCN and is capable of driving physiological and behavioural rhythms (54). Although the location(s) of the FEO is the subject of much current debate (59,60), its role within the circadian timing system is likely to be of great physiological importance.

Autonomic innervation

The autonomic nervous system is a major physiological regulator of many peripheral tissues. Neuronal outputs from the SCN directly regulate the activity of hypothalamic pre-autonomic neurones, and thus the activity of the autonomic nervous system exhibits circadian changes (61). In particular, sympathetic innervation has a key role in driving many peripheral rhythms, including pineal melatonin synthesis, hepatic

glucose metabolism and adrenal corticosteroid secretion. Interestingly, denervation of the sympathetic input to the submaxillary glands made these tissues more receptive to the synchronising effects of temporal food availability (62), indicating that a complex interaction between different physiological entraining stimuli may exist for peripheral clocks.

There is some controversy in the literature regarding the parasympathetic control of WAT (63,64). However, clear evidence for sympathetic innervation of WAT function has been available for many years (65). This sympathetic innervation exerts physiological control over WAT, for example increasing lipolysis, and may conceivably also entrain circadian rhythms in this tissue.

Melatonin

Melatonin is the primary product of the pineal gland and exhibits an extremely robust daily rhythm of secretion, with elevated plasma melatonin concentration at night (66). In many model systems, melatonin has proven to be a powerful entraining signal for circadian rhythms. At the molecular level, much of our understanding of melatonin signalling on circadian rhythms comes from studies of the pars tuberalis of the anterior pituitary. In this tissue, melatonin signals through its MT1 receptor subtype to regulate the expression of multiple clock genes (67-69). Furthermore, recent array studies have demonstrated that melatonin is likely to have acute stimulatory and inhibitory effects on a wide range of signalling pathways (70,71).

Pinealectomy modifies sensitivity of rats to insulin, suggesting a role for melatonin in energy balance in vivo (72). Studies of cultured cells indicate that melatonin receptors are expressed on adipocytes (73,74) and that melatonin may directly regulate lipolysis and leptin expression (74,75). However, the ability of melatonin to regulate adipose rhythms is currently unknown.

Glucocorticoids

The secretion of glucocorticoids from the adrenal cortex also exhibits a clear circadian rhythm, controlled by a combination of 1) SCN regulation of the hypothalamo-pituitary-adrenal axis, 2) SCN regulation of autonomic input to the adrenal gland and 3) rhythmic sensitivity to ACTH driven by a local adrenal clock (76). Administration of the glucocorticoid receptor agonist dexamethasone entrains liver clock gene rhythms in vivo (77) and can synchronise cultured cells, including fibroblasts and adipocytes, in vitro (78,79). Interestingly, although physiological glucocorticoid signalling does not appear to regulate liver expression of core clock genes, rhythmic expression of a large proportion of cycling liver genes appears to be dependent upon a functional adrenal gland (80). Moreover, liver-specific loss of glucocorticoid signalling accelerates the entrainment of mice to a temporally restricted feeding paradigm (81). Thus, there is evidence that both exogenous and endogenous glucocorticoid hormones are capable of regulating peripheral clock function.

Adipocyte rhythmicity in vitro

A recent study has investigated rhythmic gene expression in explants of human WAT. Here, visceral and subcutaneous adipose tissue was removed from obese subjects, kept in tissue culture conditions overnight and then analysed every 6 hours over a 24 hour period (53). Rhythmic expression was observed for selected clock genes and also other adipose-related genes, consistent with the hypothesis that WAT contains its own autonomous clock. However, it is still unclear from this study whether the clock lies in the adipocytes, or some other cell type(s). In order to address this question, some groups have begun to study cultures of adipose-derived cells.

The ability to use cultured cells to analyse circadian rhythms was first demonstrated using hepatic fibroblast cultures (6). Application of a 'pulse' of high serum concentration or one of many pharmacological stimuli has been shown to induce molecular rhythms within a cell population using many different cell types, including immortalised cell lines (78). Elegant analysis of single cell fibroblast rhythms using reporter constructs shows that the effect of the pulse is not to induce rhythmicity within cells, but rather to synchronise existing cellular rhythms that have drifted out of phase with one another (82,83).

The first study to apply the pulse technique to adipose physiology utilised human subcutaneous adipose-derived stem cells (ASCs). Following a 2-hour pulse with either dexamethasone, rosiglitazone or 30% foetal bovine serum, circadian expression of selected clock genes was observed in both undifferentiated and adipocyte-differentiated ASCs (79). Similarly, a later study reported rhythms of *Per1* and *Bmal1* mRNA in undifferentiated ASCs following a 1-hour pulse with 50% foetal bovine serum (84).

We have taken a similar approach to investigate murine adipocyte rhythms and have studied the well-characterised 3T3-L1 cell model (Otway, Frost, Johnston unpublished observations). Although these cells are known to express clock genes (28,85), there are no available quantitative data describing temporal changes in their physiology. Our data are consistent with some of the ASC findings described above, but also add extra levels of analysis and reveal some interesting differences. Consistent with the ASC data (79,84), we observed rhythmical variation of *Per2*, *Dbp* and *Rev-erbα* expression in pre-adipocytes. However, we failed to observe circadian rhythmicity of other genes involved in the circadian clock (*Per1*, *Cry1*, *Bmal1*) or other aspects of adipocyte biology (*PPARα*, *PPARγ*, *SREBP1*). This result was surprising given that all of the genes are rhythmic in murine WAT (42-44,86) and therefore suggest that some of the reported rhythmicity in murine WAT may derive from non-adipocyte cell types and/or rhythmical input signal(s).

In differentiated 3T3-L1 adipocytes, we found that expression of *Per2*, *Dbp* and *Rev-erbα* were again rhythmic, but the other genes tested exhibited no obvious circadian pattern. Although the rhythmic expression of *Rev-erbα* was similar to that in the pre-adipocytes, the amplitude of *Per2* and *Dbp* rhythms was markedly attenuated. The reason for this could potentially be due to attenuated intra-cellular rhythm amplitude or a decreased ability of the serum pulse to synchronise the cell population. Finally, in contrast to a lack of circadian *Leptin* and *Adiponectin* mRNA expression, our data indicate that adipocyte clocks may drive circadian secretion of leptin, but not adiponectin. Although the mechanism of circadian leptin secretion is currently

unclear, this is the first indication of an adipocyte-based mechanism driving the plasma leptin rhythms described above.

Conclusion

There is an increasing body of evidence linking circadian rhythms and clock genes with adipose physiology and pathophysiology. Despite the progress made in understanding adipose rhythms over the past few years, there are a number of important research avenues that remain to be explored. Of particular importance will be an understanding of rhythmic input pathways and intercellular communication, the relationship between circadian and metabolic dysfunction, and the generation of cell- and tissue-specific clock knockout models. Given the importance of obesity and metabolic disease in contemporary society, a clearer understanding of the function of adipose clocks has great potential therapeutic value.

References

1. Takahashi JS, Hong HK, Ko CH, McDearmon EL. The genetics of mammalian circadian order and disorder: implications for physiology and disease. *Nat Rev Genet.* 2008; 9: 764-775.

2. Moore RY, Eichler VB. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. *Brain Res.* 1972; 42: 201-620.

3. Stephan FK, Zucker I. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. *Proc Natl Acad Sci USA.* 1972; 69: 1583-1586.

4. Yamazaki S, Numano R, Abe M, Hida A, Takahashi R, Ueda M, Block GD, Sakaki Y, Menaker M, Tei H. Resetting central and peripheral circadian oscillators in transgenic rats. *Science* 2000; 288: 682-685.

5. Yoo SH, Yamazaki S, Lowrey PL, Shimomura K, Ko CH, Buhr ED, Siepka SM, Hong HK, Oh WJ, Yoo OJ, Menaker M, Takahashi JS. PERIOD2::LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. *Proc Natl Acad Sci USA.* 2004; 101: 5339-5346.

6. Balsalobre A, Damiola F, Schibler U. A serum shock induces circadian gene expression in mammalian tissue culture cells. *Cell* 1998; 93: 929-937.

7. Schibler U. The 2008 Pittendrigh/Aschoff lecture: peripheral phase coordination in the mammalian circadian timing system. *J. Biol. Rhythms* 2009; 24: 3-15.

8. Kalsbeek A, Palm IF, La Fleur SE, Scheer FA, Perreau-Lenz S, Ruiter M, Kreier F, Cailotto C, Buijs RM. SCN outputs and the hypothalamic balance of life. *J. Biol. Rhythms* 2006; 21: 458-469.

9. Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR, Menaker M, Takahashi JS. Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. *Science* 2000; 288: 483-492.

10. Xu Y, Padiath QS, Shapiro RE, Jones CR, Wu SC, Saigoh N, Saigoh K, Ptácek LJ, Fu YH. Functional consequences of a CKIdelta mutation causing familial advanced sleep phase syndrome. *Nature* 2005; 434: 640-644.

11. Meng QJ, Logunova L, Maywood ES, Gallego M, Lebiecki J, Brown TM, Sládek M, Semikhodskii AS, Glossop NR, Piggins HD, Chesham JE, Bechtold DA, Yoo SH, Takahashi JS, Virshup DM, Boot-Handford RP, Hastings MH, Loudon AS. Setting clock speed in mammals: the CK1 epsilon tau mutation in mice accelerates circadian pacemakers by selectively destabilizing PERIOD proteins. *Neuron* 2008; 58: 78-88.

12. Walton KM, Fisher K, Rubitski D, Marconi M, Meng QJ, Sladek M, Adams J, Bass M, Chandrasekaran R, Butler T, Griffor M, Rajamohan F, Serpa M, Chen Y, Claffey M, Hastings M, Loudon A, Maywood E, Ohren J, Doran A, Wager TT.

Selective Inhibition of Casein Kinase 1 Epsilon Minimally Alters Circadian Clock Period. *J Pharmacol Exp Ther*. 2009; in press.

13. Siepka SM, Yoo SH, Park J, Song W, Kumar V, Hu Y, Lee C, Takahashi JS. Circadian mutant Overtime reveals F-box protein FBXL3 regulation of cryptochrome and period gene expression. *Cell* 2007; 129: 1011-1023.

14. Busino L, Bassermann F, Maiolica A, Lee C, Nolan PM, Godinho SI, Draetta GF, Pagano M. SCFFbxl3 controls the oscillation of the circadian clock by directing the degradation of cryptochrome proteins. *Science* 2007; 316: 900-904.

15. Godinho SI, Maywood ES, Shaw L, Tucci V, Barnard AR, Busino L, Pagano M, Kendall R, Quwailid MM, Romero MR, O'Neill J, Chesham JE, Brooker D, Lalanne Z, Hastings MH, Nolan PM. The after-hours mutant reveals a role for Fbxl3 in determining mammalian circadian period. *Science* 2007; 316: 897-900.

16. Gallego M, Virshup DM. Post-translational modifications regulate the ticking of the circadian clock. *Nat Rev Mol Cell Biol.* 2007; 8: 139-148.

17. Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, Schibler U. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell* 2002; 110: 251-260.

18. Jin X, Shearman LP, Weaver DR, Zylka MJ, de Vries GJ, Reppert SM. A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. *Cell* 1999; 96: 57-68.

19. Ripperger JA, Shearman LP, Reppert SM, Schibler U. CLOCK, an essential pacemaker component, controls expression of the circadian transcription factor DBP. *Genes Dev.* 2000; 14: 679-689.

20. Ueda HR, Hayashi S, Chen W, Sano M, Machida M, Shigeyoshi Y, Iino M, Hashimoto S. System-level identification of transcriptional circuits underlying mammalian circadian clocks. *Nat. Genet.* 2005; 37: 187-192.

21. Duffield GE. DNA microarray analyses of circadian timing: the genomic basis of biological time. *J. Neuroendocrinol.* 2003; 15: 991-1002.

22. Sinha MK, Ohanneslan JP, Heiman ML, Kriauclunas A, Stephens TW, Magosin S, Marco C, Caro JF. Nocturnal rise of leptin in lean, obese, and non-insulin-dependent diabetes mellitus subjects. *J. Clin. Invest.* 1996; 97: 1344-1347.

23. Gavrila A, Peng CK, Chan JL, Mietus JE, Goldberger AL, Mantzoros CS. Diurnal and ultradian dynamics of serum adiponectin in healthy men: comparison with leptin, circulating soluble leptin receptor, and cortisol patterns. *J. Clin. Endocrinol. Metab.* 2003; 88: 2838-2843.

24. Trujillo ME, Scherer PE. Adipose tissue-derived factors: impact on health and disease. *Endocrine Rev.* 2006; 27: 762-778.

25. Kalsbeek A, Fliers E, Romijn JA, La Fleur SE, Wortel J, Bakker O, Endert E, Buijs RM. The suprachiasmatic nucleus generates the diurnal changes in plasma leptin levels. *Endocrinology* 2001; 142: 2677-2685.

26. Rudic RD, McNamara P, Curtis A-M, Boston RC, Panda S, Hogenesch JB, FitzGerald GA. BMAL1 and CLOCK, two essential components of the circadian clock, are involved in glucose homeostasis. *PLoS Biology* 2004; 2: e377.

27. Chawla A, Lazar MA. Induction of Rev-ErbAα, an orphan receptor encoded on the opposite strand of the α-thyroid hormone receptor gene, during adipocyte differentiation. *J. Biol. Chem.* 1993; 268: 16265-16269.

28. Shimba S, Ishii N, Ohno T, Watabe Y, Hayashi M, Wada T, Aoyagi T, Tezuka M. Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. *Proc. Natl. Acad. Sci. USA* 2005; 102: 12071-12076.

29. Fontaine C, Dubois G, Duguay Y, Helledie T, Vu-Dac N, Gervois P, Soncin F, Mandrup S, Fruchart J-C, Fruchart-Najib J, Staels B. The orphan nuclear receptor Rev-Erbα is a peroxisome proliferator-activated receptor (PPAR) γ target gene and PPARγ-induced adipocyte differentiation. *J. Biol. Chem.* 2003; 278: 37672-37680.

30. Chomez P, Neveu I, Mansen A, Kiesler E, Larsson L, Vennstrom B, Arenas E. Increased cell death and delayed development in the cerebellum of mice lacking the rev-erbAα orphan receptor. *Development* 2000; 127: 1489-1498.

31. Kondratov RV, Kondratova AA, Gorbacheva VY, Vykhovanets OV, Antoch MP. Early aging and age-related pathologies in mice deficient in BMAL1, the core component of the circadian clock. *Genes Dev.* 2006; 20: 1868-1873.

32. Turek FW, Joshu C, Kohsaka A, Lin E, Ivanova G, McDearmon E, Laposky A, Losee-Olson S, Easton A, Jensen DR, Eckel RH, Takahashi JS, Bass J. Obesity and metabolic syndrome in circadian Clock mutant mice. *Science* 2005; 308: 1043-1045.

33. Raspe E, Duez H, Mansen A, Fontaine C, Fievet C, Fruchart J-C, Vennstrom B, Staels B. Identification of Rev-erbα as a physiological repressor of apoC-III gene transcription. *J. Lipid Res.* 2002; 43: 2172-2179.

34. Green CB, Douris N, Kojima S, Strayer CA, Fogerty J, Lourim D, Keller SR, Besharse JC. Loss of nocturnin, a circadian deadenylase, confers resistance to hepatic steatosis and diet-induced obesity. *Proc. Natl. Acad. Sci. USA* 2007; 104: 9888-9893.

35. Barthold SW. Genetically altered mice: phenotypes, no phenotypes, and Faux phenotypes. *Genetica* 2004; 122: 75-88.

36. Oishi K, Atsumi G, Sugiyama S, Kodomari I, Kasamatsu M, Machida K, Ishida N. Disrupted fat absorption attenuates obesity induced by a high-fat diet in Clock mutant mice. *FEBS Lett* 2006; 580: 127-130.

37. Kennaway DJ, Owens JA, Voultsios A, Boden MJ, Varcoe TJ. Metabolic homeostasis in mice with disrupted Clock gene expression in peripheral tissues. *Am J Physiol Regul Integr Comp Physiol.* 2007; 293: R1528-R1537.

38. Woon PY, Kaisaki PJ, Bragança J, Bihoreau MT, Levy JC, Farrall M, Gauguier D. Aryl hydrocarbon receptor nuclear translocator-like (BMAL1) is associated with susceptibility to hypertension and type 2 diabetes. *Proc. Natl. Acad. Sci. USA* 2007; 104: 14412-14417.

39. Englund A, Kovanen L, Saarikoski ST, Haukka J, Reunanen A, Aromaa A, Lönnqvist J, Partonen T. *J Circadian Rhythms.* 2009; 7: 5.

40. Scott EM, Carter AM, Grant PJ. Association between polymorphisms in the Clock gene, obesity and the metabolic syndrome in man. *Int J Obes (Lond).* 2008; 32: 658-662.

41. Sookoian S, Gemma C, Gianotti TF, Burgueño A, Castaño G, Pirola CJ. Genetic variants of Clock transcription factor are associated with individual susceptibility to obesity. *Am J Clin Nutr.* 2008; 87: 1606-1615.

42. Ando H, Yanagihara H, Hayashi Y, Obi Y, Tsuruoka S, Takamura T, Kaneko S, Fujimura A. Rhythmic messenger ribonucleic acid expression of clock genes and adipocytokines in mouse visceral adipose tissue. *Endocrinology* 2005; 146: 5631-5636.

43. Zvonic S, Ptitsyn AA, Conrad SA, Scott LK, Floyd ZE, Kilroy G, Wu X, Goh BC, Mynatt RL, Gimble JM. Characterization of peripheral circadian clocks in adipose tissues. *Diabetes* 2006; 55: 962-970.

44. Ptitsyn AA, Zvonic S, Conrad SA, Scott LK, Mynatt RL, Gimble JM. Circadian clocks are resounding in peripheral tissues. *PLoS Comput. Biol.* 2006; 2: e16.

45. Loboda A, Kraft WK, Fine B, Joseph J, Nebozhyn M, Zhang C, He Y, Yang X, Wright C, Morris M, Chalikonda I, Ferguson M, Emilsson V, Leonardson A, Lamb J, Dai H, Schadt E, Greenberg HE, Lum PY. Diurnal variation of the human adipose transcriptome and the link to metabolic disease. *BMC Med Genomics.* 2009; 2: 7.

46. Saad MF, Riad-Gabriel MG, Khan A, Sharma A, Michael R, Jinagouda SD, Boyadjian R, Steil GM. Diurnal and ultradian rhythmicity of plasma leptin: effects of gender and adiposity. *J. Clin. Endocrinol. Metab.* 1998; 83: 453-459.

47. Heptulla R, Smitten A, Teague B, Tamborlane WV, Ma Y-Z, Caprio S. Temporal patterns of circulating leptin levels in lean and obese adolescents: relationships to insulin, growth hormone, and free fatty acids rhythmicity. *J. Clin. Endocrinol. Metab.* 2001; 86: 90-96.

48. Perfetto F, Tarquini R, Cornelissen G, Mello G, Tempestini A, Gaudiano P, Mancuso F, Halberg F. Circadian phase difference of leptin in android versus gynoid obesity. *Peptides* 2004; 25: 1297-1306.

49. Kohsaka A, Laposky AD, Ramsey KM, Estrada C, Joshu C, Kobayashi Y, Turek FW, Bass J. High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab.* 2007; 6: 414-421.

50. Odegaard JI, Chawla A. Mechanisms of macrophage activation in obesity-induced insulin resistance. *Nat Clin Pract Endocrinol Metab.* 2008; 4: 619-626.

51. Després JP, Lemieux I. Abdominal obesity and metabolic syndrome. *Nature* 2006; 444: 881-887.

52. Bray MS, Young ME. Circadian rhythms in the development of obesity: potential role for the circadian clock within the adipocyte. *Obes. Rev.* 2007; 8: 169-181.

53. Gómez-Santos C, Gómez-Abellán P, Madrid JA, Hernández-Morante JJ, Lujan JA, Ordovas JM, Garaulet M. Circadian Rhythm of Clock Genes in Human Adipose Explants. *Obesity* 2009; in press.

54. Stephan FK. The "other" circadian system: food as a Zeitgeber. *J. Biol. Rhythms* 2002; 17: 284-292.

55. Challet E, Solberg LC, Turek FW. Entrainment in calorie-restricted mice: conflicting zeitgebers and free-running conditions. *Am J Physiol.* 1998; 274: R1751-R1761.

56. Mendoza J, Graff C, Dardente H, Pevet P, Challet E. Feeding cues alter clock gene oscillations and photic responses in the suprachiasmatic nuclei of mice exposed to a light/dark cycle. *J. Neurosci.* 2005; 25: 1514-1522.

57. Abe H, Honma S, Honma K. Daily restricted feeding resets the circadian clock in the suprachiasmatic nucleus of CS mice. *Am J Physiol Regul Integr Comp Physiol.* 2007; 292: R607-R615.

58. Hirota T, Okano T, Kokame K, Shirotani-Ikejima H, Miyata T, Fukada Y. Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. *J Biol Chem.* 2002; 277: 44244-44251.

59. Fuller PM, Lu J, Saper CB. Differential rescue of light- and food-entrainable circadian rhythms. *Science* 2008; 320: 1074-1077.

60. Mistlberger RE, Buijs RM, Challet E, Escobar C, Landry GJ, Kalsbeek A, Pevet P, Shibata S. Standards of evidence in chronobiology: critical review of a report that restoration of Bmal1 expression in the dorsomedial hypothalamus is sufficient to restore circadian food anticipatory rhythms in Bmal1-/- mice. *J. Circ. Rhythms* 2009; 7: 3.

61. Kalsbeek A, Perreau-Lenz S, Buijs RM. A network of (autonomic) clock outputs. *Chronobiol. Int.* 2006; 23: 521-535.

62. Vujovic N, Davidson AJ, Menaker M. Sympathetic input modulates, but does not determine, phase of peripheral circadian oscillators. *Am J Physiol Regul Integr Comp Physiol.* 2008; 295: R355-R360.

63. Kreier F, Kap YS, Mettenleiter TC, van Heijningen C, van der Vliet J, Kalsbeek A, Sauerwein HP, Fliers E, Romijn JA, Buijs RM. Tracing from fat tissue, liver, and pancreas: a neuroanatomical framework for the role of the brain in type 2 diabetes. *Endocrinology* 2006; 147: 1140-1147.

64. Giordano A, Song CK, Bowers RR, Ehlen JC, Frontini A, Cinti S, Bartness TJ. White adipose tissue lacks significant vagal innervation and immunohistochemical evidence of parasympathetic innervation. *Am J Physiol Regul Integr Comp Physiol.* 2006; 291: R1243-R1255.

65. Slavin BG, Ballard KW. Morphological studies on the adrenergic innervation of white adipose tissue. *Anat Rec.* 1978; 191: 377-389.

66. Simonneaux V, Ribelayga C. Generation of the melatonin endocrine message in mammals: a review of the complex regulation of melatonin synthesis by norepinephrine, peptides, and other pineal transmitters. *Pharmacol. Rev.* 2003; 55: 325-395.

67. von Gall C, Weaver DR, Moek J, Jilg A, Stehle JH, Korf HW. Melatonin plays a crucial role in the regulation of rhythmic clock gene expression in the mouse pars tuberalis. *Ann N Y Acad Sci.* 2005; 1040: 508-511.

68. Johnston JD, Tournier BB, Andersson H, Masson-Pévet M, Lincoln GA, Hazlerigg DG. Multiple effects of melatonin on rhythmic clock gene expression in the mammalian pars tuberalis. *Endocrinology* 2006; 147: 959-965.

69. Wagner GC, Johnston JD, Tournier BB, Ebling FJ, Hazlerigg DG. Melatonin induces gene-specific effects on rhythmic mRNA expression in the pars tuberalis of the Siberian hamster (Phodopus sungorus). *Eur. J. Neurosci.* 2007; 25: 485-490.

70. Dupré SM, Burt DW, Talbot R, Downing A, Mouzaki D, Waddington D, Malpaux B, Davis JR, Lincoln GA, Loudon AS. Identification of melatonin-regulated genes in the ovine pituitary pars tuberalis, a target site for seasonal hormone control. *Endocrinology* 2008; 149: 5527-5539.

71. Fustin JM, Dardente H, Wagner GC, Carter DA, Johnston JD, Lincoln GA, Hazlerigg DG. Egr1 involvement in evening gene regulation by melatonin. *FASEB J* 2009; 23: 764-773.

72. Alonso-Vale MI, Borges-Silva CN, Anhê GF, Andreotti S, Machado MA, Cipolla-Neto J, Lima FB. Light/dark cycle-dependent metabolic changes in adipose tissue of pinealectomized rats. *Horm Metab Res.* 2004; 36: 474-479.

73. Brydon L, Petit L, Delagrange P, Strosberg AD, Jockers R. Functional expression of MT2 (Mel1b) melatonin receptors in human PAZ6 adipocytes. *Endocrinology* 2001; 142: 4264-4271.

74. Zalatan F, Krause JA, Blask DE. Inhibition of isoproterenol-induced lipolysis in rat inguinal adipocytes in vitro by physiological melatonin via a receptor-mediated mechanism. *Endocrinology* 2001; 142: 3783-3790.

75. Alonso-Vale MI, Andreotti S, Peres SB, Anhê GF, das Neves Borges-Silva C, Neto JC, Lima FB. Melatonin enhances leptin expression by rat adipocytes in the presence of insulin. *Am J Physiol Endocrinol Metab.* 2005; 288: E805-E812.

76. Dickmeis T. Glucocorticoids and the circadian clock. *J. Endocrinol.* 2009; 200: 3-22.

77. Balsalobre A, Brown SA, Marcacci L, Tronche F, Kellendonk C, Reichardt HM, Schütz G, Schibler U. Resetting of circadian time in peripheral tissues by glucocorticoid signalling. *Science* 2000; 289: 2344-2347.

78. Balsalobre A, Marcacci L, Schibler U. Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. *Curr. Biol.* 2000; 10: 1291-1294.

79. Wu X, Zvonic S, Floyd ZE, Kilroy G, Goh BC, Hernandez TL, Eckel RH, Mynatt RL, Gimble JM. Induction of circadian gene expression in human subcutaneous adipose-derived stem cells. *Obesity* 2007; 15: 2560-2570.

80. Oishi K, Amagai N, Shirai H, Kadota K, Ohkura N & Ishida N. Genome-wide expression analysis reveals 100 adrenal gland-dependent circadian genes in the mouse liver. *DNA Research* 2005; 12: 191–202

81. Le Minh N, Damiola F, Tronche F, Schütz G, Schibler U. Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. *EMBO J.* 2001; 20: 7128-7136.

82. Nagoshi E, Saini C, Bauer C, Laroche T, Naef F, Schibler U. Circadian gene expression in individual fibroblasts: cell-autonomous and self-sustained oscillators pass time to daughter cells. *Cell* 2004; 119: 693-705.

83. Welsh DK, Yoo SH, Liu AC, Takahashi JS, Kay SA. Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. *Curr. Biol.* 14: 2289-2295.

84. Huang TS, Grodeland G, Sleire L, Wang MY, Kvalheim G, Laerum OD. Induction of circadian rhythm in cultured human mesenchymal stem cells by serum shock and cAMP analogs in vitro. *Chronobiol. Int.* 2009; 26: 242-257.

85. Wang J, Lazar MA. Bifunctional role of Rev-erbalpha in adipocyte differentiation. *Mol. Cell. Biol.* 2008; 28: 2213-2220.

86. Yang X, Downes M, Yu RT, Bookout AL, He W, Straume M, Mangelsdorf DJ, Evans RM. Nuclear receptor expression links the circadian clock to metabolism. *Cell* 2006; 126: 801-810.

87. Hayashi M, Shimba S, Tezuka M. Characterization of the molecular clock in mouse peritoneal macrophages. *Biol Pharm Bull.* 2007; 30: 621-626.

88. Takeda N, Maemura K, Horie S, Oishi K, Imai Y, Harada T, Saito T, Shiga T, Amiya E, Manabe I, Ishida N, Nagai R. Thrombomodulin is a clock-controlled gene in vascular endothelial cells. *J. Biol. Chem.* 2007; 282: 32561-32567.

Figure Legends

Figure 1. Modelling the mammalian circadian timing system. (A) The master circadian clock in the hypothalamic suprachiasmatic nuclei (SCN) was previously thought to directly drive rhythms throughout the body. (B) It is now recognised that autonomous clocks are present in most tissues. The role of the SCN is now considered to be synchronisation of these peripheral clocks to ensure that they oscillate in an appropriate phase to each other. This synchronisation occurs through multiple neuroendocrine pathways and temporal regulation of behaviour, such as feeding.

Figure 2. Complexity of adipose rhythms. Interpretation of the underlying mechanisms of adipose tissue rhythms is complicated by both the multiple rhythmic input pathways and also the heterogeneous nature of adipose cells.

Table 1: Presence of circadian clocks in adipose tissue cell types

Interpretation of adipose rhythms is complicated by the identification of circadian clocks in multiple cell types found in adipose tissue.

Cell type	Presence of a circadian clock	References
Pre-adipocyte	Yes	79,84
Adipocyte	Yes	79
Fibroblast	Yes	6,82,83
Macrophage	Yes	87
Vascular endothelial cell	Yes	88