Cytosolic and Transcriptional Cycles Underlying Circadian Oscillations

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1.1 Introduction

Circadian (circa- approximately, -dian a day) clocks are the internal pacemakers that drive the daily rhythms in our physiology and behavior that adapt us to the 24-hour world (Duffy et al., 2011). They thereby maintain temporal coherence in our core metabolism, even when individuals are held in isolation, experimentally deprived of external timing cues such as light–dark (LD) cycles. As a result of this ability of our endogenous circadian system to define internal time and use it to drive daily rhythms, our brains and bodies can be viewed as 24-hour machines, alternating between states of wakefulness and sleep, catabolism and anabolism, growth/repair and physical activity. It is now widely recognized that disturbance of this daily program can carry significant costs for morbidity and even mortality (Hastings et al., 2003). Some personal insights into this can come from the subjective experiences of jet lag. More insidiously, however, the disturbance of nocturnal sleep, and consequent disaffected mood, loss of mental capacity and social disruption, is a common element of neurodegenerative and psychiatric conditions (Hatfield et al., 2004; Wulff et al., 2010; Bliwise et al., 2011) (This volume, several chapters). Moreover, epidemiological evidence now associates increased risk of cancer as well as cardiovascular and metabolic diseases with extensive experience of rotational shift-work (Knutsson, 1989; Viswanathan et al., 2007; Huang et al., 2011) (This volume, Chapter 13), a life-style that will inevitably compromise circadian coherence, and which represents a major and growing hazard to public health. Evolution has programmed us to live by a 24-hour day and where genetic, pathological, environmental or social factors drive us against this program, we pay a heavy price. Conversely, the recognition that our body is a 24-hour machine, with different metabolic and physiological states across day and night, provides a route into enhancing therapeutic efficacy by administering medicines on a schedule that maximizes their bioavailability and by targeting disease states at their most critical and vulnerable phases of the day (Levi and Schibler, 2007).

Key to appreciating the role of the circadian clock in both health and illness, and thereby identifying novel therapeutic strategies, is the unravelling of its molecular and cellular bases. Whilst the formal properties of circadian clocks have been understood for over 60 years, and the identification in 1972
of the suprachiasmatic nucleus (SCN) as the brain’s principal pacemaker provided a neuroanatomical focus to circadian biology (Weaver, 1998; Chapter 3) (Fig. 1.1a–e), proper mechanistic understanding of the timing process proved to be elusive. This changed dramatically from the late 1970s onwards, when “circadian clock genes” and their mechanisms of action were identified: firstly in Drosophila, then in Neurospora, and more recently in mouse (Takahashi et al., 2008). The outcome of
these studies was to reveal that an autoregulatory negative feedback oscillator, based on sequential transcriptional and posttranslational processes, lies at the heart of the circadian timepieces of these divergent groups. Even though the molecular components may differ, the “logic” of the mechanism is conserved. But things move on, and there is growing realization that these transcriptionally based clocks do not operate in isolation; rather, they are mutually dependent upon intrinsically rhythmic cytosolic signals (cAMP, Ca\(^{2+}\), kinases), such that the cell as a whole has a resonant structure, tuned to 24-hour operation (Hastings \textit{et al.}, 2008). Finally, the most recent development has been to show that even in cells lacking transcriptional apparatus (most notably mammalian erythrocytes), circadian cycles of metabolic state can be sustained (O’Neill and Reddy, 2011). The purpose of this chapter is to review the development of this molecular and cellular model of the circadian clockwork of mammals.

### 1.2 Assembling the transcriptional feedback loop

#### 1.2.1 Discovering clock genes and their actions in lower species

The idea that a complex behavioral trait such as the circadian cycle of rest and activity could be understood from the viewpoint of single gene actions was, for some time, contentious in both the circadian field and also more widely. Nevertheless, the creation by Ron Konopka and Seymour Benzer of mutant \textit{Drosophila} with atypically short or long periods to their circadian behavior, and the subsequent cloning of the \textit{Period} gene as the molecular target of these mutations, initiated a revolution in clock biology (Konopka, 1987). Alongside the \textit{Frq} gene of \textit{Neurospora}, cloned by Jay Dunlap and colleagues (Loros \textit{et al.}, 1989), \textit{Period} (\textit{Per}) provided an entry point into the molecular mechanisms of clocks: changes in the encoded proteins could make the clock run faster, or slower or not at all. They therefore MUST be an intrinsic part of the clockwork. Moreover, it became apparent that the key action of the encoded proteins was to inhibit the expression of their cognate genes. Given that there is an inevitable time lag between transcriptional activation and nuclear entry of the fully formed protein, an oscillation is bound to ensue, as in any other delayed negative feedback system (Hardin \textit{et al.}, 1990; Aronson \textit{et al.} 1994). Indeed, autoregulation of this type is well recognized in molecular biology, with oscillations commonly occurring over a couple of hours. The critical property here, however, is that the dynamics of the contributory stages (gene activation, protein synthesis, intracellular transport, protein degradation) are extended such that the cycle runs for approximately 24 hours. Subsequent mutational and biochemical studies revealed that \textit{Per} and \textit{Frq} are components of dynamic, multiprotein complexes, the assembly of which is facilitated in part by their protein interaction domains (Hardin, 2005; Crosthwaite \textit{et al.}, 1997). Of particular note were the so-called PAS (Per–Arnt–Sim) interaction domains of \textit{Per}. The positive drives to the feedback loops that stimulate expression of \textit{Per} and \textit{Frq}, comes from additional PAS-containing proteins: CLOCK and CYCLE in flies (Allada \textit{et al.}, 1998; Rutila \textit{et al.}, 1998), and WHITE COLLAR 1 and 2 in \textit{Neurospora} (Crosthwaite \textit{et al.}, 1997; de Paula \textit{et al.}, 2007). After forming heteromers, these positive factors activate transcription via specific regulatory sequences in the enhancer regions of \textit{Per} and \textit{Frq}, respectively. Thus, positive factors drive the expression of negative factors, which in turn oppose the positive drive leading to a decline in negative factor abundance, which allows the cycle to start again approximately 24 hours after the previous point of initiation.

Although both systems are light sensitive – a prerequisite for synchronization with solar cycles and, thereby, environmental time, their molecular basis to entrainment is different. In flies, the stability of \textit{PER} is dependent on association with another circadian protein, TIMELESS (Myers \textit{et al.}, 1996; Koh \textit{et al.}, 2006), which in turn is subject to degradation by CRY, a light-dependent factor with similarity to photolyase DNA repair proteins (Cashmore, 2003). Consequently, PER protein can only accumulate in the night, thereby stably entraining the entire molecular cycle to solar time. In contrast, the light-sensitive component in the \textit{Neurospora} loop is the positive factor White Collar-1, which binds FAD (flavin adenine dinucleotide) as a chromophore (Crosthwaite \textit{et al.}, 1997; de Paula \textit{et al.}, 2007). Thus, expression of \textit{Frq} is activated at the start of the day, again linking the phase of the molecular cycle to environmental (solar) time.
1.2.2 Discovering clock genes and their actions in mammals

Knowledge of the clock in flies and fungus played a large role in deciphering the mammalian clockwork (Fig. 1.1f). Homology cloning or sequence alignment of novel transcripts, based heavily on knowledge of the PAS domains of Drosophila Period, led ultimately to the discovery of three Per genes in mammals (Tei et al., 1997; Reppert and Weaver, 2002). Mammalian Cryptochromes (Cry1 and Cry2) had previously been studied in the context of DNA repair, but findings from flies focused attention on their potential circadian role, which was confirmed by the demonstration that Cry-deficient mice cannot exhibit circadian behavior (van der Horst et al., 1999). Initial description of a mammalian Timeless gene was subsequently shown to be misleading, as the mammalian gene in question is, in fact, a homologue to Timeout, a different, noncircadian fly gene. A further difference, consistent with the absence of Timeless, is that mammalian CRY proteins are not the light-sensitive component of the cycle: resetting in mammals is mediated by the activation of Per1 and Per2 expression (see below) (Shigeyoshi et al., 1997; Albrecht et al., 1997), an echo of the light-dependent induction of Frq expression in Neurospora. Critically, both Per and Cry mRNA and proteins are expressed rhythmically in the SCN, with respective phases that are consistent with negative feedback action (Field et al., 2000). But what of the positive factors that would drive such a negative feedback system? The identification of mammalian Clock, by mutagenesis and subsequent transgenic rescue, was a landmark achievement by the laboratory of Joe Takahashi – it preceded the discovery of Drosophila Clock, and was dependent upon classical positional cloning, pre-dating the mouse genome era (King et al., 1997). As in flies, CLOCK forms heteromeric complexes to activate expression of Per and other circadian genes, including Cry. The partner to CLOCK is BMAL1, a homologue of Drosophila Cycle, which was initially identified by co-expression screens (Hogenesch et al., 1998).

Both CLOCK and BMAL1 contain PAS dimerization domains, but only CLOCK carries a poly-Q transactivation domain. Loss of BMAL1 leads to circadian incompetence at both molecular and behavioral levels, whereas loss of CLOCK has mixed effects that vary between tissues, depending upon whether or not NPAS2, a parologue of CLOCK, can compensate (DeBruyne et al., 2007). Nevertheless, the original Clockδ219 mutation generated by Takahashi has compromised transactivation; consequently, circadian period is lengthened in the heterozygote and completely disorganized in homozygous mutants because of insufficient transcriptional drive to the Per and Cry genes. Finally, the negative feedback loop has been closed experimentally by the demonstration that CLOCK/BMAL1 heterodimers can acutely activate E-box mediated transcription and that this effect is suppressed by co-expression with PER and CRY (Kume et al., 1999). The details of this transcriptional repression are unclear, but both PER and CRY contribute (see below). In the established transcriptional model in mammals, therefore, the start of circadian day sees CLOCK/BMAL1 activation of Per and Cry expression via their E-box regulatory sequences (Fig. 1.1f). The accumulating mRNAs are translated into protein and by the end of circadian day SCN neurons have high levels of nuclear PER and CRY proteins. This is followed by a progressive decline in mRNA levels, reflecting the negative feedback action of the accumulated PER/CRY complexes. By late circadian night the existing PER/CRY complexes, no longer replenished in the absence of mRNA, are finally cleared from the nucleus such that CLOCK/BMAL1 activity is de-repressed and the cycle starts anew at circadian dawn. The application of this basic model to humans is described in Chapter 2.

1.2.3 Imaging the transcriptional clock in real time: a multitude of cellular oscillators appears

Circadian timing is an intrinsically dynamic process and major advances in analyzing circadian gene expression have come about with the development of real-time reporter genes in which circadian regulatory sequences are coupled to bioluminescent (firefly luciferase) or fluorescent proteins. Although recording of circadian rhythms of intrinsic bioluminescence in unicellular organisms has a long pedigree in clock research (Hastings, 2007), this approach found greater application when directed towards the newly discovered clock genes, firstly in plants and flies and more recently in mammals. Early examples are transgenic lines of mouse and rat in which upstream sequences of Per1 (carrying five E-boxes) are used to drive luciferase. Organotypic slice cultures of SCN from such animals express robust, clearly defined bioluminescence rhythms arising from individual neurons (Fig. 1.2a–c), the phases of which are synchronized but exhibit a complex, wave-like progression across the SCN (Yamaguchi et al., 2003),...
Fig. 1.2 Molecular pacemaking in SCN and other tissues and cells. (a) View of PER2::LUC SCN organotypic slice culture under phase illumination. V = third ventricle, oc = location of optic chiasm, scale bar = 500 um. (b) Serial bioluminescent images from same field of view as in (a), collected for 1 h every 6 h over 2 days in culture. Note stable and synchronized circadian oscillation in both SCN, with regionally specific phases of PER2 expression. CT = circadian time, CT12 = projected time of lights off. (c) Graphical plots of circadian bioluminescence from PER2::LUC organotypic SCN slices that are wild type (dark) or homozygote VIP-null (pale). Note stable molecular oscillation (with progressively smaller peaks due to luciferin substrate utilization) in wild type slices, but rapid loss of circadian organization in SCN lacking VIP. (d) Representative image of bioluminescent PER2::LUC fibroblasts (above) and plots of circadian rhythms of bioluminescence from individual cells in the culture (below). Note very stable molecular pacemaking, but no synchrony between cells. (e) Schematic representation of the internal circadian hierarchy in mammals, whereby local circadian clocks distributed across all major organs are governed by a variety of synchronizing cues ultimately derived from the SCN. In this way, daily rhythms across the body are synchronized to each other and also to solar time.
reflecting the transcriptional cycle of the clock. A second mutant mouse has in-frame luciferase coding sequences inserted into the endogenous Per2 locus to generate an allele encoding a PERIOD::LUCIFERASE fusion protein (Yoo et al., 2004), and again individual SCN neurons express bioluminescence rhythms, this time in-phase with predicted native PER2 protein expression and, thereby, providing a posttranslational report of the clock mechanism.

Extensive studies using these and other reporter lines have revolutionized circadian biology because they, quite literally, provide a “window” on the SCN clock mechanism as it progresses through real time. They have, however, provided an even more profound understanding when applied to peripheral tissues and organs. A remarkable discovery based on Northern blot analyses of intermittent samples of cell culture extracts was that circadian genes are not only expressed in such cultures, but they are expressed with a circadian period: the transcriptional clock is active not only in the SCN but also in fibroblasts (Balsalobre et al., 1998) (Fig. 1.2d). Indeed, primary cultures of organs and tissues from circadian reporter animals could also exhibit self-sustained circadian transcriptional and posttranslational rhythms that can be imaged in real time by bioluminescent (Welsh et al., 2004) or fluorescent reporters (Nagoshi et al., 2004). Importantly, these rhythms lack the “staying power” of the SCN, progressively damping out over a week or so. Nevertheless, circadian gene expression is sustained at the single cell level, but in the absence of any synchronising cues in vitro, the phases of individual cells within the culture dish or tissue gradually disperse and so the rhythm at the population level loses definition. The role of the SCN, therefore, is not to impose rhythms upon the rest of the brain and viscera. Rather, it is to coordinate the activity of the intrinsic transcriptional/posttranslational clocks distributed across innumerable cells in all of the major organs and tissues. The presence of such a complex spatio-temporal network underpinning metabolism and behavior has obvious relevance to health and disease. Moreover, it provides novel approaches for sophisticated diagnostic and therapeutic applications.

### 1.2.4 Elaborating the core transcriptional clockwork

Elucidation of the feedback actions mediated by PER/TIM in flies, FRQ in fungi and PER/CRY in mammals led to the idea of the “core” feedback loop, but developments in all three of the model organisms saw a gradual elaboration, adding additional rhythmic components and identifying rate-limiting enzymes. Importantly, in all three systems it became evident that some positive factors were rhythmically expressed due to the influence of their targets. In the case of mammals, this advance was facilitated by the tractable analysis of the transcriptional clockwork of peripheral tissues and cell lines, and the strongest early evidence came from identification of Rev-Erba. This is a highly rhythmic circadian output gene driven by CLOCK/BMAL1 that encodes an orphan nuclear receptor that, in turn, inhibits Bmal1 expression via its retinoic acid receptor-related orphan receptors response elements (RORE) regulatory sequences (Preitner et al., 2002). Thus, output of the “core” loop becomes its input. Further elaboration showed how a second circadian-controlled gene, Rora, acts as a positive factor to Bmal1, opposing the effect of Rev-Erbα at the RORE and thereby sculpting Bmal1 expression. Whereas single-mutant mice show limited effects on the clock, mice lacking both Rev-Erbα and the closely related Rev-Erbβ have major disruptions of metabolic and behavioral rhythms (Cho et al., 2012). Consequently, definition of the “core” clockwork progressively loses its focus as a network of transcriptional interactions develops. A further pair of basic helix–loop–helix transcription factors, DEC-1 and DEC-2, has also been implicated in the clock, insofar as they are expressed rhythmically in the SCN and also interfere with CLOCK/BMAL1 mediated transactivation. A final auxiliary loop consists of Dbp and E4BP4, which respectively activate and suppress transcription mediated by so-called D-boxes present in the Per, Rev-Erbβ and Rora genes. The clock-driven, rhythmic activities of DBP/E4BP4 will, therefore, feed back to influence the clock, generating a further autoregulatory group. The significance of this architecture of internested transcriptional loops is twofold (Ueda et al., 2005). First, it confers robustness to the overall behavior of the molecular oscillator and likely also boosts its amplitude. Second, because of the time constants of the various interlocking stages, the network establishes a phase map defined by serial episodes of activation and suppression of a number of genes, thereby providing more precise and definitive temporal resolution within the composite oscillation.
The discovery that cells and tissues contain transcriptional clocks very similar to those of the SCN was transformational for the experimental analysis of their regulatory mechanisms. The utility of cell cultures as a proxy for SCN pacemaking and the use of abundant tissues such as liver for biochemical analysis have made it possible to conduct studies that would be extremely difficult to perform on SCN. This has allowed a more comprehensive decoding of the molecular events associated with transcriptional activation. For example, with the description of the CLOCK/BMAL1 heterodimer to 2.3 Å it is now possible to define the roles of the basic helix–loop–helix and PAS domains in dimerization and DNA binding, and reveal key residues in the protein interfaces, mutations of which can alter transcriptional activity and the period of circadian pacemaking in fibroblasts (Huang et al., 2012). ChIP-seq and other biochemical analyses of liver have been able to track the various components of the CLOCK/BMAL1 complex (including RNA polymerase II, CRY, PER and associated factors) as it progresses through activated and suppressed states, associating with E-box-containing (and other) sequences (Koike et al., 2012). This cycle is accompanied by pronounced rhythms of histone modifications, including differentially phased cycles of methylation and acetylation as the oscillation progresses through times of transcriptional activation and suppression. Careful analysis of this molecular procession will likely provide important information regarding the general mechanisms of transcriptional coordination, with relevance well beyond the field of circadian clocks.

1.3 Keeping the transcriptional clockworks in tune

1.3.1 Entrainment of the SCN transcriptional clockwork

Retinal innervation of the SCN, carried via the retino-hypothalamic tract (RHT) is the means by which the transcriptional program of the SCN is synchronized to solar and seasonal time, as represented by the cycle of light and darkness (Reppert and Weaver, 2002). This pathway is described in detail in Chapter 3. The RHT consists of the axons of retinal ganglion cells (RGCs) and enters the ventro-lateral subdivision of the SCN, which contains neurons that express the neuropeptides vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP). The nonretinorecipient zone surrounding the core, termed the shell, is characterized by neurons expressing arginine vasopressin (AVP). Until recently it was assumed that conventional rods and cones are the circadian photoreceptors but a remarkable recent discovery was that a subclass of RGCs expresses a novel invertebrate-like opsin, melanopsin, that confers upon them intrinsic photoreceptivity (Rollag et al., 2003) (This volume, Chapter 3). These intrinsically photoreceptive RGCs (iPRGCs) are sufficient for circadian entrainment of the SCN, and they also mediate numerous other subliminal aspects of vision (Guler et al., 2008). They have broad receptive fields and act as luminance detectors rather than feature detectors: properties clearly adapted to their circadian role.

The principal neurotransmitter of RGCs is glutamate and the terminals of the RHT act upon the NMDA- and AMPA-type glutamate receptors expressed by retinorecipient SCN neurons. The subsequent influx of Ca\(^{2+}\) mediated by NMDAR increases the rate of firing of the neurons, which is otherwise low in circadian night (Kuhlman et al., 2003). It also activates a signaling cascade leading to increased gene expression mediated by the cAMP/Ca\(^{2+}\) response element (CRE) regulatory sequences in target genes (Obrietan et al., 1999; Schurov et al., 1999). Importantly, both Per1 and Per2 carry CREs, additional to their E-boxes (Travnickova-Bendova et al., 2002), so nocturnal light pulses acutely induce Per expression in the core SCN at a circadian time when it is otherwise very low (Albrecht et al., 1997; Shigeyoshi et al., 1997). This can be followed a few hours later by an increase in Per expression in the shell – likely triggered by the increased firing of action potentials by the core neurons and subsequent release of VIP, GRP and other transmitters onto the shell. These neuropeptides act via G-protein coupled receptors to activate cAMP and Ca\(^{2+}\) signaling, so will, in turn, increase Per expression via the CREs.

Thus, during circadian night, when spontaneous E-box-mediated expression of Per in the SCN is low, a light pulse will activate it and the additional pulse of PER protein will feed through the core loop and reset it to a new phase. If this occurs in the early subjective night when PER levels are falling, progression of the SCN molecular cycle is delayed, whereas light delivered in late circadian night when Per expression
is beginning to rise, will accelerate the rise and shift the molecular cycle forwards. During circadian daytime, when Per expression is already high, light has little impact on the molecular cycle. Thus, in the “real world,” small phase adjustments to the molecular cycle at dusk (delay) and dawn (advance) will keep it synchronized to, and predictive of, the solar cycle, thereby ensuring appropriate phasing of the behavioral and physiological rhythms it controls. It is important to note that this entrainment by photic induction of Per expression is equally applicable to both diurnal and nocturnal species because the cycle of Per expression in the SCN is the same in both: high in circadian day, low in circadian night, regardless of the animal’s behavioral habits (Maywood and Mrosovsky, 2001). This transcriptional effect of light upon the SCN clock also enables the molecular cycle to encode season – the longer days of summer drive a broader peak of Per expression (Messager et al., 1999; Nuesslein-Hildesheim et al., 2000), which is ultimately decoded by brain and pituitary to engage adaptive seasonal changes such as altered appetite and nutrient utilization, reproductive status and migratory behavior (Dardente et al., 2010). The SCN can also be synchronized by cues other than light, principally behavioral arousal mediated by serotonergic and neuropeptidergic cues from the brain stem (Hastings et al., 1997). In this case, the clock is most sensitive during circadian daytime, when Per expression is high and the cycle is acutely reset (advanced) by early suppression of Per – the mirror image of the effect of light. Indeed, because they have convergent but opposite molecular actions, light can block the resetting effect of arousal on the SCN and vice versa (Mead et al., 1992; Maywood et al., 2002).

### 1.3.2 Entrainment of transcriptional clocks in peripheral tissues

In contrast to the relatively limited number of mechanisms serving the SCN, the entrainment of the clocks within other brain regions, peripheral organs and cells is dependent upon a kaleidoscope of stimuli, some general and others specific to the cell types involved (Fig. 1.2e). The starting point is the SCN efferent innervation, which is distributed to a variety of target nuclei in the hypothalamus, brain stem and beyond, indirectly, to spinal cord and pituitary. The intrinsic clockwork of the SCN will enable it to convey time cues encoded as firing rate and very probably changes in the neurotransmitter types being released onto target cells. Although a small number of SCN neuropeptides have been implicated in transmitting circadian cues to the brain, we are far away from understanding how, at a systems-level such information is used to time fundamental neural processes, not least the alternation between states of sleep and wakefulness. In coordinating rhythms across the body, three general mechanisms are evident: behavior and the consequential cycle of feeding and fast; endocrine cues, especially the daily surge of corticosteroid hormones from the adrenal glands; and cues derived from the autonomic nervous system, such as the daily cycle of body temperature (Fig. 1.2e). Taking the liver clock as an example, under normal circumstances the SCN will determine the phases of feeding, core body temperature and corticosteroid secretion, which, in turn, will affect the local molecular pacemakers in the liver to ensure its various functions are appropriately timed to match the needs of the animal across day and night. This internal phasing can be altered experimentally by, for example, restricting the time of food availability or by injecting exogenous corticosteroids. Under such experimental conditions, the SCN remains phase locked to solar time but the liver clockwork can be advanced or delayed by the new cues, thereby interfering with internal temporal coordination and, thus, metabolic efficiency.

The molecular basis of such entrainment is inevitably varied, although in the case of corticosteroid hormones (which are secreted at the start of the respective activity phase of nocturnal and diurnal species) the presence of glucocorticoid-response elements in Per, Cry and Bmal1 genes provides a direct entry point to shifting the core clockwork. In contrast, entrainment by temperature cycles can involve transcriptional effects of heat shock factor (HSF 1), acting via response elements in the Per2 gene, and posttranscriptionally via cold-inducible RNA-binding protein (CIRP), which appears to be necessary for normal expression and function of CLOCK protein. Indeed, the pivotal role of Per2 as a sensor of entraining cues to the liver is demonstrated by the fact that in mice with a genetically compromised liver clock, Per2 expression nevertheless continues to oscillate under the influence of systemic cues.

One clear demonstration of the functional importance of internal circadian tuning comes from the observation that mice lacking a liver clock but with otherwise normal circadian behavior are prone to
daily episodes of hypoglycemia because they are unable to perform the usual circadian up-regulation of hepatic gluconeogenesis to maintain glucose homeostasis during the fasting day. Notwithstanding these successes in identifying molecular pathways that entrain the local hepatic clock, the scale of the remaining problem remains enormous: the complexities of intercellular signaling between different tissues are already evident anatomically and biochemically, but now it must be taken to a new level by factoring in biological time. Solving this problem, however, will provide new therapeutic opportunities by the exploitation of circadian-based cues to regulate vital functions. One immediate example comes from the circadian morning surge in cardiovascular output (Fig. 1.3). Under normal circumstances this is adaptive, preparing the individual to engage with the world, but in those suffering from cardiovascular disease it represents a point of vulnerability – as reflected in the increased incidence of sudden cardiac death in hours immediately after awakening. Knowledge of the circadian signaling cascades from SCN to brain stem, to myocardium and to vascular endothelium that generate the morning surge could be used to develop time-based therapies to ameliorate the point of vulnerability without affecting baseline ongoing cardiovascular regulation (This volume, Chapters 8 & 9). This principle of circadian targeting applies to any number of systemic illnesses, not least metabolic syndrome and diabetes (This volume, Chapter 11).

1.3.3 Local tissue clocks direct local transcriptional and posttranscriptional programs

The relevance of transcription to circadian coordination extends far beyond the core feedback loops. A variety of DNA microarray studies and, more recently, RNA sequencing have shown that (depending on the algorithms used to detect significant rhythms) between 5 and 20% of the local transcriptome is subject to circadian modulation. Importantly in tissues such as the liver, this circadian modulation is most pronounced for transcripts involved in metabolic and signaling pathways (Akhtar et al., 2002; Koike et al., 2012; Menet et al., 2012), as well as cell cycle regulators. Characteristically, it is the enzymatic components of the cell that are clock-regulated rather than structural genes, such that the clock up- and down-regulates the “software” of the tissue, rather than its “hardware”. The most immediate point of regulation of the circadian transcriptome is provided by the rhythmic activity of the proteins of the core oscillation, which periodically activates/suppresses the expression of target genes carrying E-boxes, D-boxes and ROREs. Recent ChIP-seq studies have established the genome-wide extent of such circadian control (and also highlighted the numerous targets of “clock” genes that are not circadian in their activity). Furthermore, some of the rhythmic targets of the core loop factors are themselves transcriptional regulators, for example, PPAR and HNF4a, so further tiers of circadian gene expression will be driven in a cascading effect. In addition, cues that entrain the core clock can also act upon clock-controlled genes directly, most obviously corticosteroids, which may act via glucocorticoid response elements (GREs) either independently or in concert with E-boxes, ROREs and other “circadian motifs.” The transcriptome can, therefore, be viewed as a resonant network, enabling tissues to prepare to perform night- and day-specific metabolic and other functions in a timely manner, thereby supporting the individual’s daily cycle of rest and activity.

The control of transcription is not, however, the only means to achieve temporal adaptation. Analysis of the cytosolic proteome of liver and SCN has revealed numerous proteins that are regulated at the level of protein abundance but not at steady-state transcript level. Furthermore, several isoforms of the same protein can be rhythmic but with contrasting phases of peak abundance, and recent RNA-sequencing studies suggest that only about 20% of rhythmically expressed genes in the liver are driven by de novo transcription. Clearly, posttranscriptional and posttranslational modifications are also important avenues for the clock to sculpt the functions of a tissue. Many RNA-binding proteins are circadian in their expression, and for example in liver and lung the clock and clock-related cues can influence the splicing of primary transcripts into different isoforms with contrasting temporal profiles. Posttranslational modifications, not least phosphorylation, are an additional circadian influence on the proteome, generating temporal diversity in cellular function. The prevailing view, however, remains one in which circadian cycles of gene expression drive rhythmic regulation of metabolism and signaling networks.
Fig. 1.3 Schematic view of systems-level circadian organization. Schematic view of circadian coordination across the individual in which the primary pacemaker of the SCN, entrained to solar time by retinal afferents, maintains and synchronises tissue-based clocks in the major organ systems by a blend of endocrine, autonomic and behavioral (feeding-related) cues. Disruption of these timing cues can result in pathology throughout the body. In this way, a robust circadian system contributes to our health and well-being while disrupting these rhythms as wide-ranging negative consequences. Redrawn with permission from Hastings et al. 2003.
1.4 Building posttranslational mechanisms into the circadian pacemaker

1.4.1 Posttranslational control of the clock: localization and stability of clock proteins

The obvious sophistication of posttranscriptional mechanisms in coordinating clock outputs raises the question of their potential role in the core pacemaking loop itself. For the nested transcriptional loops to oscillate effectively it is necessary for them to incorporate delays, which cannot be generated by the (inherently noisy) process of transcription itself (Suter et al., 2011). Rather, they can arise from regulation of the localization, activity and stability of the transcription factors that exert rhythmic transcriptional regulation (Hastings et al., 2007; Zheng and Sehgal, 2008; Asher and Schibler, 2011). These properties are themselves points of regulation by such mechanisms as phosphorylation and ubiquitinylation, and a conserved feature of the clock in fungi, flies and mammals is the role that dynamic protein phosphorylation plays in supporting rhythmicity per se and setting the clock’s period (Hastings et al., 2008).

A good example is the ubiquitously expressed, highly conserved, and multifunctional serine/threonine-phosphorylating CASEIN KINASE 1 (CK1). In mammals both CK1e and CK1d (which are encoded by different genes) complex with and phosphorylate the PER proteins, directing their nuclear localization and stability. In the absence of both enzymes PER cannot be degraded, so the core transcriptional oscillation ceases (Etchegaray et al., 2010; Meng et al., 2010). Pharmacological inhibition of the enzymes slows down the rate of PER degradation, so progressively lengthens the period of the core loop and, thereby, slows down the behavioral activity rhythm. Conversely the gain-of-function Tau mutation in CK1e destabilizes PER protein and accelerates the pacemaker to 20 hours in homozygous mice and hamsters (Meng et al., 2008; Lowrey et al., 2000). In humans, mutations in both CK1d and the CK1 binding domain of PER2 are associated with a pronounced sleep disturbance, specifically advanced sleep phase, which is indicative of an accelerated circadian cycle consistent with the observations in rodents (Toh et al., 2001). The importance of CK1-dependent phosphorylation of PER is in its dual roles of licensing nuclear localization and yet also targeting the protein for ubiquitinylation by bTRCP and proteasomal degradation (Eide et al., 2005; Shirogane et al., 2005). In the case of CRY, stability is regulated in part by AMP kinase-mediated phosphorylation, which in turn licenses it for ubiquitinylation by the ligase FBXL3 (Godinho et al., 2007; Lamia et al., 2009). Various pharmacological and genetic manipulations of bTRCP and FBXL3 in mice, SCN and cell cultures can enhance PER and CRY stability, respectively, and thereby lengthen circadian period both in vitro and in vivo (Hiroti et al., 2012).

Casein kinase 2 (CK2) and glycogen synthase kinase 3 (GSK3) are other well-known examples of ubiquitously expressed, multifunctional, highly conserved eukaryotic serine/threonine kinases that, in addition to their other established roles in the biology of the cell, have been shown to play critical roles in determining the cellular localization and/or stability of circadian transcription factors across a wide range of eukaryotes – even though the transcription factors themselves are not conserved (Yin et al., 2006; Hastings et al., 2008; Maier et al., 2009; O’Neill et al., 2011). As might be expected, the role of protein phosphatases, for example, PP1, is equally well conserved (Yang et al., 2004; Gallego et al., 2006; Fang et al., 2007; Schmutz et al., 2011). In the context of the transcriptional/translational feedback loop (TTFL) that has been proposed to account for cellular circadian rhythms, current data support a general model wherein a dynamic interplay between clock protein phosphorylation and de-phosphorylation by these ubiquitous enzymes acts as an interval timer to regulate the kinetics of complex formation, protein degradation and nuclear entry. Certain specific serine/threonine residues on each clock protein substrate are implicated in tipping the balance between degradation and nuclear localization (Reischl and Kramer, 2011).

1.4.2 Metabolic regulation of the transcriptional clockwork

An important question, therefore, is whether the posttranslational mechanisms discussed above are themselves circadian in nature or whether they are constitutively active and modify PER and CRY proteins as these proteins are generated. Although the expression level of most of these enzymes does not appear to be circadian, it remains possible that their activity is controlled in a rhythmic fashion and they act
coordinately, as in WNT signaling (Del Valle-Perez et al., 2011), to facilitate rhythmic intracellular localization and degradation of PER and CRY. In support of this possibility is the observation that the phosphorylation status, and therefore activity, of GSK3β is spontaneously rhythmic in cultured fibroblasts (Iitaka et al., 2005). Indeed the ubiquitin ligase FBXL21, which also targets CRY for degradation, is expressed in the SCN with high amplitude (Dardente et al., 2008). Even more intriguing is the fact that the kinase activity of AMPK is itself subject to the ratio of AMP and ADP:ATP in the cell (Oakhill et al., 2011). Thus, the ability of the cell to degrade CRY may vary as a function of the metabolic state of the cell, which in the case of the SCN, with its rhythm of electrical activity, is highly circadian. From this perspective a clock output (metabolic state) can be viewed as a clock input, and thus becomes part of the oscillator mechanism.

More generally it has been observed in several contexts that cellular metabolism is intrinsically rhythmic, for example, in mouse liver in vivo (Kaminsky et al., 1984; Eckel-Mahan et al., 2012; Fustin et al., 2012) and isolated mammalian cells in vitro (Radha et al., 1985; O’Neill and Reddy, 2011). This becomes of particular interest in light of observations that the cell’s metabolic state can directly regulate transcription factor activity. For example, the DNA-binding activities of the CLOCK/BMAL1 and NPAS2/BMAL1 complexes is directly regulated by the redox state of nicotinamide adenine dinucleotide (NAD and NADP) cofactors, in vitro (Rutter et al., 2001).

In a still broader context, gene expression at loci bound by many clock gene transcription factors is associated with chromatin remodeling via recruitment of assorted histone methyl- and acetyl-transferases (Etchegaray et al., 2003; Ripperger and Schibler, 2006; Hosoda et al., 2009; Katada and Sassone-Corsi, 2010), all of which are ultimately reliant on the availability of their respective 1- and 2-carbon substrates (S-adenosylmethionine and Acetyl-CoA, respectively). These are generated by primary metabolism, and therefore also probably rhythmic, since intermediates in the pathways that generate them are (Eckel-Mahan et al., 2012). By the same token, the stability of PER2 and activity of BMAL1, are additionally regulated by dynamic lysine acetylation (Asher et al., 2008; Nakahata et al., 2008) and deacetylated via specific recruitment of deacetylase SIRT1, which also targets histone H3, in an NAD+‐dependent manner. This facilitates the transition to transcriptionally repressive/inactive clock protein complexes later in the circadian cycle, again in a manner dependent on primary metabolism, in this case NAD+ availability – which is also clock‐regulated (Kaminsky et al., 1984; Ramsey et al., 2009). Finally, it is highly significant that several of the identified “clock gene” transcription factors are heme-binding proteins and exhibit reciprocal regulation between rhythmic heme metabolism and the heme protein’s redox/ligand status, for example, heme-binding and, thus, activity of the nuclear receptor REV-ERBβ is governed by a redox-sensitive cysteine (Kaasik and Lee, 2004; Gupta and Ragsdale, 2011).

1.4.3 Cause versus effect in circadian transcriptional regulation

There are far more interactions between circadian timekeeping and metabolism than are discussed above; these are covered at length in some excellent reviews (Green et al., 2008; Asher and Schibler, 2011). In the latter, Asher and Schibler make the insight that “the discrimination between metabolic and circadian oscillations may be somewhat arbitrary.” At the level of circadian timekeeping in cell culture or organotypic slice, therefore, there is an issue of cause and effect. The prevailing view is that circadian cycles of gene expression drive cellular rhythms of metabolism; however, as much evidence exists to support the contrary view that circadian cycles of metabolism drive rhythms of gene expression.

Certainly overexpression of clock gene transcription factors does not result in a major detriment to cellular timekeeping (Fan et al., 2007; Yamanaka et al., 2007; Asher and Schibler, 2011) and even gene knock-out does not completely abolish time-keeping in SCN slices (Liu et al., 2007; Ko et al., 2010; Maywood et al., 2011). Thus, whilst it is clear that circadian regulation of transcriptional circuits is essential for normal mammalian physiology and rhythmic behavior, at the cellular level rhythmic gene expression cannot be accounted for without delegating the majority of timekeeping function to rhythmic posttranslational regulation of clock protein localization, stability and activity. These are, in turn, determined by rhythmic enzyme activities and metabolic status – many of which are regulated at the level of transcription or translation: Catch 22 – or seemingly so.
1.5 Is the transcriptional clock paramount?

1.5.1 Cytosolic rhythms and the SCN pacemaker

To be effective as a central timekeeper, individual SCN neurons have to synchronize their molecular cycles, one to another and also to the light–dark cycle. When dispersed in cell culture, SCN neurons obviously lose synchrony as expected, but they are also less effective circadian pacemakers than when embedded in the usual SCN circuit – the transcriptional rhythms of individual cells lose amplitude and coherence. This dependence upon coupling is even more marked when the cells lack individual Per or Cry genes – single gene mutations that do not affect coherence at the level of the SCN ensemble. Clearly, intercellular signaling is a critical aspect not only in synchronizing the SCN cellular transcriptional clocks but also in maintaining them. Consistent with this, interference with electrophysiological signaling by tetrodotoxin (TTX) not only causes SCN neurons to become desynchronized but also to lose amplitude and definition to their transcriptional cycle (Yamaguchi et al., 2003; Maywood et al., 2007). There are several ways in which altering electrical communication across the circuit may affect the transcriptional clockwork. Suppression of action potential firing may alter intracellular Ca\(^{2+}\) signaling in both pre- and post-synaptic neurons, which in turn will alter transcriptional activation of Per and other genes via their CREs. In addition, the consequently reduced secretion of neuropeptides, including AVP, VIP and GRP, across the SCN will attenuate intracellular cues mediated via their G-protein coupled receptors, dis-regulating, inter alia, Ca\(^{2+}\), cAMP and kinase cascades. Again this will compromise CRE-mediated transcription of Per and, thereby, undermine the core loops. A clear example of this is seen in mice lacking VIP or its cognate receptor, VPAC2. Not only are they behaviorally arrhythmic, but cellular transcriptional cycles in the SCN are also desynchronized and of low amplitude and coherence. These transcriptional cycles in the mutant SCN can be re-activated by paracrine cues, including AVP and VIP, derived from wild-type SCN grafted onto the mutant slice in vitro (Maywood et al., 2011). These peptides act via receptors that activate Gq and Gs signaling respectively, thereby driving Ca\(^{2+}\), cAMP and kinase cascades to “rescue” the transcriptional loop. Under normal circumstances, the core loop drives the circadian rhythms of action potential firing, cAMP and Ca\(^{2+}\) levels, neuropeptide synthesis and secretion. Consequently, nontranscriptional outputs of the core loop within the SCN neuron are also its sustaining inputs, acting both within a neuron and between neurons. It can, therefore, be argued that this intercellular coupling is what makes the SCN special as a sustained pacemaker: the first amongst equals (Liu et al., 2007). Whereas bioluminescence recordings of other tissues (perhaps with the exception of the retina) progressively damp out as component, non-communicative cells lose phase coordination, that of the SCN slice will continue indefinitely (literally for many months, subject to an adequate supply of culture medium) with high amplitude and astonishing precision, as cells drive each other in reciprocal dependence. There is also a converse to this paramount competence – when dissociated from each other, SCN neurons may oscillate actually worse than individual fibroblasts do (Liu et al., 2007; Webb et al., 2009). The dependence upon coupling and intracellular signaling is, therefore, hardwired into the SCN as a condition of its pre-eminent role, and thus under contrived experimental conditions the SCN clockwork appears more vulnerable than that of a simple fibroblast.

The interdependence of intercellular cues, signaling cascades and the expression of clock genes blurs the distinction of a core loop. This distinction is further challenged by the observation that pharmacological manipulations of intracellular cAMP levels can change the canonical oscillatory properties of the transcriptional loop: its phase, amplitude and period (O’Neill et al., 2008). The case can be made, therefore, that the “real” pacemaker consists of both transcriptional and cytosolic components that are mutually dependent and act in concert. Some evidence for autonomous function of the cytosolic components comes from studies in Cry-null and Bmal1-null SCN, where the transcriptional loops are compromised but circadian cycles of Per-driven bioluminescence can still be observed in SCN neurons, albeit with shortened period and poor coherence (Ko et al., 2010; Maywood et al., 2011). This suggests that some capacity for cytosolic oscillation: a “cytoscillator,” can exist independently of the transcriptional timer. Indeed, when transcription of nascent mRNA is compromised by treatment
with alpha-amanitin oleate (a potent transcriptional inhibitor), SCN slices can exhibit at least one and sometimes two further cycles of PER2::LUC- reported bioluminescence (O’Neill et al., 2013).

The possibility of a self-sustained cytosolic clock that normally couples with, but can run in the absence of, cycling clock genes has received further attention following recent observations made using undifferentiated embryonic stem cells (ESCs). ESCs were previously thought not to possess any intrinsic timekeeping because there is no detectable cycling gene expression until differentiation. Paulose et al. report, however, a self-sustained rhythm in ESC glucose uptake prior to, and following, differentiation (Paulose et al., 2012). This again implies the existence of intrinsic timekeeping that is not reliant on any known transcriptional clock mechanism.

These observations reiterate earlier experiments in diverse model organisms that also addressed whether nascent transcription was necessary for cellular timekeeping, the earliest being in the alga Acetabularia mediterranea, where circadian rhythms of chloroplast movement persisted when the nucleus of the cell was removed (Sweeney and Haxo, 1961; Woolum, 1991). The landmark observations, however, were performed in the cyanobacteria Synechococcus elongatus, a prokaryote. Here it was shown that the approximately 24-hour rhythm of KaiA/B/C protein phosphorylation and complex formation that occurs in living cells, and which normally interacts reciprocally with genome-wide transcriptional regulation (Johnson et al., 2008), could be reconstituted in vitro using just the three recombinant proteins (KaiA, B and C) with ATP (Nakajima et al., 2005). Bacterial expression systems tend to work on a 1 protein = 1 function principle, whilst mammalian proteins tend to possess multiple domains with multiple, context-dependent cellular functions. We therefore think it unlikely that a directly equivalent experiment can be performed for mammalian timekeeping. It does raise the possibility, however, that the smallest functional circadian timekeeping unit in mammals may not include the nucleus.

1.5.2 Totally transcription-free pacemaking

Recently the absolute requirement for nascent gene expression in mammalian cells was investigated in vitro. The ultimately cytotoxic effects of chronic inhibition of gene expression often confound pharmacological approaches to this question. To circumvent this, preparations of human red blood cells (which are naturally anucleate) were employed (O’Neill and Reddy, 2011). A rhythmic posttranslational modification of the peroxiredoxin (PRx) family of anti-oxidant proteins, first observed in mouse liver (Reddy et al., 2006), was used as a rhythmic marker. Briefly, the PRX family constitutes a major part of the cellular defense against reactive oxygen species (ROS), specifically H₂O₂, which are an unavoidable byproduct of aerobic metabolism. Erythrocytes express PRX at high levels (approximately 1% total protein), presumably due to the high ROS generation resulting from hemoglobin auto-oxidation. 2-Cys PRxs exist primarily as dimers that catalyze their own oxidation by H₂O₂ at conserved peroxidatic cysteine residues. The resultant sulfenic acid (CysP- SOH) may be reduced by a resolving cysteine on the opposing monomer (Cys₅₋S-S-Cys₅₀), and ultimately reduced to the free thiol (SH) by the thioredoxin system. The kinetics of the resolving cysteine attack is quite slow, however, and in the presence of additional H₂O₂ overoxidation to the sulfinic (Cys₅₋SO₂H) or even sulfonic (Cys₅₋SO₃H) form, occurs (reversible through sulfiredoxin-catalyzed, ATP-dependent mechanisms). By performing anti-2-Cys PRX-SO₂/₃ immunoblots on time-courses of erythrocytes, isolated in a minimal glucose/salt buffer under constant conditions, circadian rhythms of PRX oxidation were observed. These rhythms were temperature-compensated, entrainable by temperature cycles, and (predictably) robust to inhibitors of gene expression. In addition, the concentrations of several cellular metabolites ([ATP], [NADH], [NADPH]) appeared to be rhythmically modulated, as did an indirect fluorescence assay for hemoglobin multimeric state (O’Neill and Reddy, 2011).

As a marker for circadian timekeeping, the PRX oxidation rhythm appears to be highly conserved, being observable in representative organisms from across all three domains of life (Bacteria, Archeaea, Eukaryota), unlike any TTFL component (Edgar et al., 2012). Whilst PRX itself does not appear to play a critical timekeeping role, the redox rhythm it reports persists (albeit perturbed) in organisms that are deficient in “core” TTFL components. It is thus plausible that this remarkable conservation reflects either some underlying and ancient metabolic oscillation, which remains deeply embedded in the cellular machinery, or an evolutionary convergence upon rhythmic redox regulation to facilitate temporal segregation of mutually antagonistic metabolic processes.
1.5.3 A general model for mammalian cellular circadian timekeeping

Nascent transcription (cycling or otherwise) is not required for cellular circadian timekeeping (Tomita et al., 2005; O’Neill et al., 2011) but metabolism and signal transduction are required since they sustain life. In “normal” cells and organisms, however, circadian cycles of gene expression are observed and many of these cycling genes influence cell signaling and metabolism; ultimately facilitating rhythmic control of physiology. The activities of most known clock-relevant transcription factors are reliant upon metabolism and redox state, whereas their localization and stability, and in some cases acute induction, are determined posttranslationally and regulated by intracellular signaling systems. Furthermore, there are many established reciprocal pathways connecting redox balance and cellular metabolism with the activity of the various signaling mechanisms discussed above (Cheong and Virshup, 2011; Dickinson and Chang, 2011; Hardie, 2011; Sethi and Vidal-Puig, 2010; Metallo and Vander Heiden, 2010; Montenarh, 2010; Vander Heiden et al., 2009). In order to integrate these observations into a coherent framework (Fig. 1.4), therefore, we speculate that circadian rhythms in the cytoplasm persist through cyclical,

![Diagram of circadian timekeeping](image)

**Fig. 1.4** A general model for mammalian cellular circadian timekeeping. Circadian timekeeping is functionally distributed within the cell’s metabolic and signaling networks (top level) independently of nascent gene expression. In most (nucleated) cells, however, the integrated output from transcriptional networks (lower level) is manifest in the circadian cycles of protein activity/stability/localization (middle level) observed, for example, in canonical clock protein transcription factors which act as coincidence-detecting substrate effectors for network state. Rhythmically modulated chromatin structure facilitates coordinated temporal regulation of downstream networks of gene expression, including their own cognate clock gene circuitry, resulting in signal amplification. Rhythmic modulation of “clock-controlled genes” facilitates coordinated temporal regulation of physiology, and feeds forward into metabolic/signaling networks (right-hand flow), modulating expression of some component mechanisms, e.g., rhythmic NAMPT expression facilitates rhythmic activity of the NAD⁺ salvage pathway (Ramsey et al., 2009), PDE1B degrades cAMP and affects rhythmic amplitude (Zhang et al., 2009). The circadian state of the signaling network modulates communication with local and distant targets, whilst selectively and temporally gating the capacity of relevant extracellular signals to affect circadian phase (left-hand flow).
distributed cross-talk between multiple metabolic and signaling networks, with transcriptional clock components acting as coincidence-detecting, substrate effectors. They thereby integrate the state of the network as a whole to coordinate genome-wide temporal, and cell type-specific, programs of gene expression. In this context, irrelevant network perturbations would be ignored but appropriate extracellular cues responded to in a phase-dependent fashion. Rhythmic licensing of transcription, with its slower kinetics, would impart robustness to the “cytoscillator” by rhythmic modulation of component protein/transcript levels. Critically, a rhythmic transcriptional contribution would not be required for oscillator competence but the additional repression of clock protein activity upon its cognate gene and CCGs would facilitate signal amplification. Similarly, rhythmic protein degradation is not required for cellular timekeeping (fibroblast rhythms are relatively insensitive to proteasomal inhibition) (Stratmann et al., 2012) but where present, it would increase the signal-to-noise ratio and amplify any transcriptional/translational contribution to the following cycle (Fig. 1.3). In essence, we suggest that, in contrast to the discreet clock mechanism in cyanobacteria, circadian timekeeping in mammalian (and by inference all eukaryotic) cells is functionally distributed amongst its component systems, which seem to have been coopted into the clock as soon as, or shortly after, they arose evolutionarily (Edgar et al., 2012).

1.6 Conclusion: cytoscillators, clocks and therapies

Since the acceptance that circadian rhythms are truly endogenous phenomena, driven by an internal timing mechanism rather than a response to undefined cyclical environmental cues, it has been obvious that an understanding of their nature and the mechanisms that govern them would provide a deeper insight into normal physiology and behavior, and thus identify new avenues for therapeutic intervention. There have been many surprises as the clock mechanisms have been unraveled; perhaps the greatest being that almost every cell has the potential to act as a circadian oscillator. This revelation brings complexity and opportunity in equal measure to biology and medicine. Because the paradigm of biology for the last decades has been genes and genomes, it is perhaps unsurprising that analysis of the clock mechanism focused on gene expression, leading to the development of the canonical model of TTFLs. Indeed, transcriptional processes are evident at all levels of the mammalian circadian system – from the core feedback loop, to entrainment by gene induction to orchestration of outputs and ultimate physiological rhythms by circadian transcriptomes. Nevertheless, the idea that the cellular environment within which the feedback loops are embedded influences their behavior has gained ground, leading to the view that transcriptional pacemaking and intrinsically rhythmic cytosolic oscillations are inescapably coupled, conferring precision and robustness. The latest revelations of transcription-free clocks in erythrocytes push this model further to show that cytosolic oscillations can exist independently of the nucleus: an echo of work on Acetabularia 50 years ago. Given the tractable “drugability” of cytosolic signaling in contrast to the dangers of meddling with transcription, it may well be that elucidation of the cytoscillator will provide the best entry point for future chronotherapies seeking to address diseases with a circadian dimension and, hence, circadian vulnerability (Fig. 1.3).

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References


cytosolic and transcriptional cycles underlying circadian oscillations


