Chapter 10
Heat Shock Factors Modulate Circadian Rhythms

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Abstract Temperature changes have a variety of effects on physiological processes including the circadian clock that generates diurnal rhythms of sleep/wake behavior, hormone release, metabolism, and so on. Even in homeothermic mammals, body temperature fluctuates in a circadian manner that transmits the time information to peripheral tissues and cells. The body temperature rhythms cause cyclic activation of the transcription factor HSF1 and its targets such as *HSP* genes and a clock gene *Per2*, resulting in adjustment of the circadian oscillation in peripheral cells for synchronization. Loss of function of HSF1 therefore leads to reduced synchronization of the clock against temperature changes. HSF1 inhibition also slows down the speed of the clock oscillation and impairs the mechanism that maintains the oscillation speed constant under varying temperature. In the chick pineal gland, a photosensitive clock tissue, HSF and *HSP* genes are activated by light pulse at a specific time of the day, suggesting a role of the HSF pathway in light-dependent synchronization of the circadian clock. Together, HSF has substantial roles in modulating the circadian clock function in response to environmental changes.

Keywords Circadian clock • Clock genes • Temperature • Light • Mammals • Chick pineal gland • HSF

10.1 Introduction

We wake up in the morning and sleep at night. Almost all organisms on the earth show daily rhythms of physiology and behavior under the control of an endogenous biological time-keeping mechanism called circadian clock (Mohawk et al. 2012).

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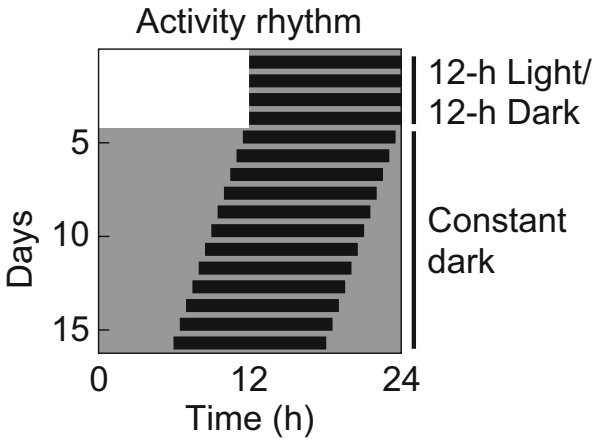

Fig. 10.1 Behavioral circadian rhythm and its entrainment to a light-dark cycle. In the schematic diagram, 1 line represents 1 day (24 h) in which active time is indicated by a *solid bar*. Mouse is a nocturnal animal and hence active at night under a daily cycle of 12-h light (*white area*) and 12-h dark (*gray area*) from day 1 to day 4. When the mouse is transferred to a constant dark condition from day 5, its active time starts to shift day by day with the free-running period of slightly shorter than 24 h (23.5 h in this case). This behavioral rhythm demonstrates the presence of the endogenous circadian clock

The circadian clock oscillates with a period of ~24 h even in the absence of any external time cues. Because the intrinsic period of the circadian clock deviates from exact 24 h that is a rotation period of the light-dark cycle of the earth, the organisms use periodic environmental signals such as light and temperature to adjust the phase of the clock for synchronization, a process called entrainment (Fig. 10.1). In mammals, light information is perceived in the retina and transmitted to the suprachiasmatic nucleus (SCN) of the hypothalamus, a center of the body clock controlling the behavioral rhythms (Welsh et al. 2010). The SCN coordinates peripheral clocks located in most tissues in a hierarchical manner through neuronal, hormonal, and metabolic signals (Fig. 10.2). Although the body temperature of homeothermic animals is kept within a narrow range irrespective of the ambient temperature, it shows daily fluctuations under the control of the circadian clock (1–4 °C, varying among species) and acts as an entrainment signal of the peripheral clocks (Saini et al. 2011). In this chapter, we describe temperature-dependent regulation of the mammalian circadian clock and a role of HSF in this process. We also discuss possible roles of HSF in the light response of the circadian clock in the chick pineal gland, a unique photosensitive clock tissue.

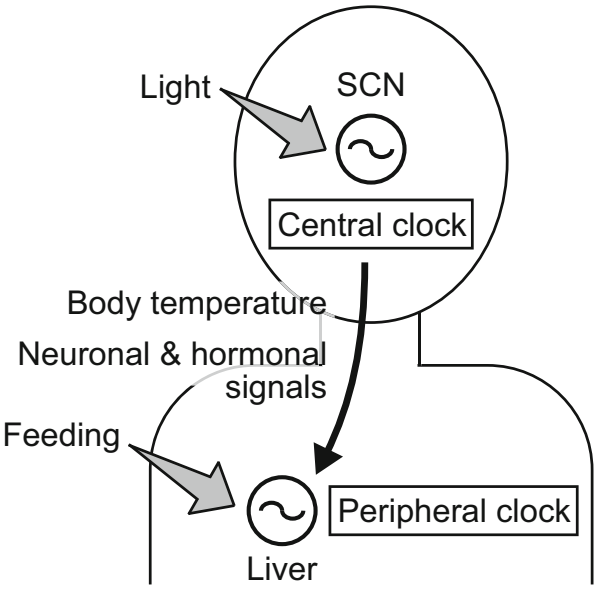

Fig. 10.2 Hierarchical organization of the mammalian circadian clock. The central clock resides in the hypothalamic SCN and orchestrates organismal circadian rhythms including behavior, body temperature, neuronal activity, hormone release, and feeding time that control peripheral clocks in many tissues such as the liver. Light is a dominant time cue for the central clock, while the peripheral clocks can be entrained to feeding time independent of the central clock

10.2 Molecular Mechanism of the Circadian Clock in Mammals

Genetic and molecular biological studies in the past two decades identified more than a dozen of "clock genes" that are required for normal oscillation of the circadian clock with ~24-h period in mammals (Takahashi et al. 2008; Mohawk et al. 2012). The clock genes form transcription- and translation-based feedback loops that cycle once a day (Fig. 10.3). A heterodimer of bHLH-PAS transcription factors CLOCK and BMAL1 activates transcription of *Period* (*Per*) and *Cryptochrome* (*Cry*) genes by binding to E box *cis*-regulatory elements in their promoter region. Translated PER (PER1 and PER2) and CRY (CRY1 and CRY2) that are PAS domain-containing proteins and photolyase homologs, respectively, form a complex to enter the nucleus and repress CLOCK-BMAL1-dependent transcriptional activation. Degradation of PER and CRY through ubiquitin-proteasome pathway releases CLOCK-BMAL1 from repression, resulting in restart of the cycle. This "core loop" couples with "sub-loops" consisting of other transcription factors such as DBP, E4BP4, ROR, and REV-ERB to generate rhythmic expression of a variety of output genes regulating physiological processes. As examples of the clock output, CLOCK-BMAL1 regulates rhythmic expression of *Nampt* gene encoding rate-limiting enzyme of NAD^+ biosynthesis to drive circadian change in NAD^+ levels (Nakahata et al. 2009; Ramsey et al. 2009), and REB-ERBα controls *Ucp1* gene encoding uncoupling protein 1 in brown adipose tissue to generate body temperature rhythms (Gerhart-Hines et al. 2013).

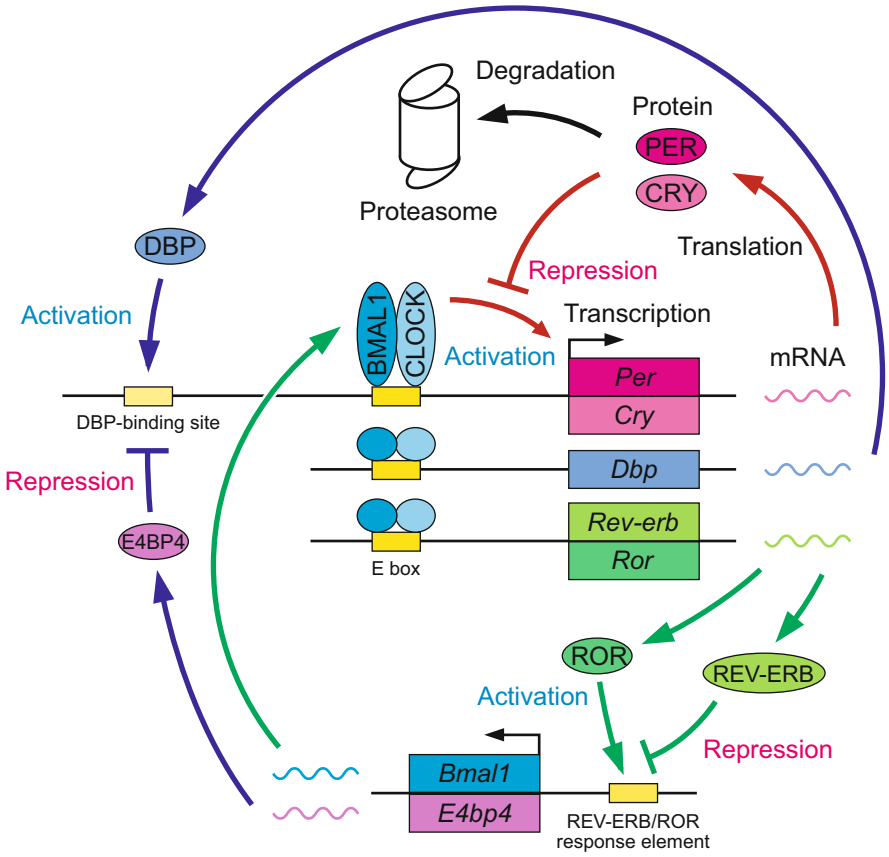

Fig. 10.3 Feedback loops of the mammalian circadian clock. In the core loop (*red arrows*), BMAL1-CLOCK transcription factor complex activates expression of *Per* and *Cry* genes. Translated PER and CRY proteins in turn repress their own transcription to form negative feedback loop. Degradation of PER and CRY through the proteasomal pathway reinitiates transcription of *Per* and *Cry* genes. The core loop is tightly connected with sub-loops composed of nuclear hormone receptors ROR and REV-ERB (*green arrows*) and bZip transcription factors DBP and E4BP4 (*blue arrows*)

In order to facilitate understanding of this review, here we explain several technical terms of the circadian clock research. The circadian rhythms can be characterized by a combination of three parameters: period, phase, and amplitude (Fig. 10.4). The period is the time required for the clock to oscillate one cycle under a free-running condition without any external signals, and it is close but not equal to exact 24 h. The phase is the angle of the rhythm relative to a reference rhythm such as the external day–night cycle. Phase shift is an important property of the circadian clock to synchronize with the environmental 24-h rhythm. The amplitude is the difference between the peak (or trough) and the mean value of the rhythm that

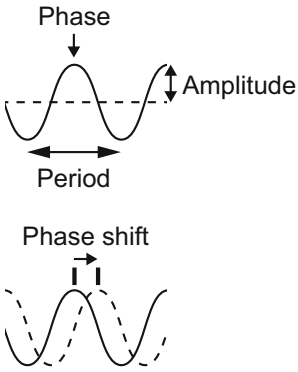

Fig. 10.4 Parameters of the circadian rhythm. See the text for details

represents the strength of fluctuation. A decrease of the amplitude with progressing cycles is called damping.

The oscillatory mechanism of the circadian clock resides in each cell of the entire body and generates long-lasting rhythms at the single cell level (Leise et al. 2012). In the central SCN clock, the cellular clocks communicate with each other by using neural signals such as neurotransmitters and electric activities to form a tightly connected network that produces robust circadian rhythms at the tissue level (Welsh et al. 2010). In contrast, peripheral clocks in most tissues and cultured cell lines do not have such a strong coupling and show rapid damping as a population because of desynchronization among individual cells (Nagoshi et al. 2004; Welsh et al. 2004). It was found that serum shock induces (or resets) rhythmic gene expression in cultured cell lines (Balsalobre et al. 1998), and this discovery led to identification of a variety of resetting stimuli such as a synthetic glucocorticoid dexamethasone, an adenylate cyclase activator forskolin, a PKC activator phorbol 12-myristate 13-acetate, growth factors FGF and TGF-β, glucose, and pH (Akashi and Nishida 2000; Balsalobre et al. 2000a, b; Yagita and Okamura 2000; Hirota et al. 2002; Kon et al. 2008). The cell lines therefore have been used as a model system to study the resetting mechanism of the peripheral clocks.

10.3 Effect of Temperature on Circadian Clock Function

One of the hallmarks of the circadian clock is "temperature compensation." Most biochemical reactions depend on temperature, and their reaction rate varies two to threefold with a temperature change of 10 °C ($Q_{10} = 2–3$). In contrast, the period of the circadian clock oscillation is much less affected by temperature. Q_{10} is 0.99 in cultured rat SCN (Ruby et al. 1999), 0.85–0.88 in rat-1 fibroblasts (Izumo et al. 2003), and 0.88 in NIH3T3 fibroblasts (Tsuchiya et al. 2003), indicating that the oscillation speed of the mammalian circadian clock is affected only slightly by the change of the temperature (few-hour period change in response to 10 °C difference).

While the period length is temperature compensated, the clock system responds to temperature changes to adjust its phase. In cultured fibroblasts, imposing a square temperature cycle (a repeat of 12 h at 33 °C and 12 h at 37 °C) or a simulated cycle of mouse body temperature (35.5–38.5 °C) results in rhythmic expression of the clock genes (Brown et al. 2002; Saini et al. 2012), reflecting synchronization of individual cellular clocks. Although efficiency of the synchronization becomes reduced when the amplitude of the temperature cycle decreases, a difference as small as 1 °C (36.5–37.5 °C) is sufficient to entrain the rhythms (Saini et al. 2012). Peripheral clocks of mouse pituitary glands and lungs in culture are also entrained to a square temperature cycle (a repeat of 12 h at 36 °C and 12 h at 38.5 °C), whereas the central clock of the mouse SCN is unaffected (Buhr et al. 2010). These results suggest that physiological body temperature cycles act as a time cue for the peripheral clocks but not for the SCN clock.

In addition to temperature cycles, a single down- or upshift change of temperature (37–33 °C, 33–37 °C, or 37–42 °C) can induce rhythmic expression of clock genes in fibroblasts (Brown et al. 2002; Tsuchiya et al. 2003), suggesting an acute resetting of individual cellular clock in response to such a temperature shift. Indeed, a brief pulse of high temperature (38.5 °C for 1 or 6 h from 36 °C) strongly phase-shifts or resets preexisting rhythms of mouse pituitary glands and lungs in culture (Buhr et al. 2010). In contrast, the central clock exhibits much smaller responses to temperature pulse in the rat SCN (Ruby et al. 1999) or is mostly unaffected in the mouse SCN (Buhr et al. 2010). Interestingly, the mouse SCN becomes to show peripheral clock-like properties of strong resetting when the intercellular SCN communication is reduced by treatment with a voltage-gated Na^+ channel blocker tetrodotoxin or an L-type calcium channel blocker nimodipine, as well as by physical separation of the dorsomedial and ventrolateral SCN regions (Buhr et al. 2010). Therefore, the strong coupling among the SCN neurons appears to confer resistance against temperature changes. This property is reminiscent of the coupling-mediated robustness against mutation of the clock genes (Liu et al. 2007).

Evaluating the effect of temperature on the circadian clock in vivo is complicated because altered ambient temperature affects the patterns of not only the body temperature but also the feeding that is a major time cue for the peripheral clock (Damiola et al. 2000; Stokkan et al. 2001). Nonetheless, exposing mice to an ambient temperature cycle (24 °C during the day and 37 °C during the night) synchronizes the phase of the clock gene expression in the liver without affecting the central clock in the SCN (Brown et al. 2002). Furthermore, a 2-h treatment of mice with 41 °C water bath causes phase shift by several hours in the kidney, liver, and submandibular gland as revealed by in vivo imaging of the clock gene expression (Ohnishi et al. 2014).

10.4 Regulation of Circadian Gene Expression by HSF1

Connection between HSF and the circadian rhythm in mammals has emerged from two unbiased screenings both done by the Schibler laboratory at the University of Geneva to understand the mechanism underlying rhythmic gene expression.

Transcriptome profiling analyses revealed thousands of genes exhibiting rhythmic expression patterns in each tissue (Panda et al. 2002; Storch et al. 2002; Ueda et al. 2002; Yoshitane et al. 2014; Zhang et al. 2014). However, it was not known how much contribution to the gene expression rhythms comes from the local clock in the tissue and from systemic signals such as hormones and body temperature. To address this question, Kornmann and colleagues developed a transgenic mouse strain to stop the circadian clock specifically in the liver in a conditional manner (Kornmann et al. 2007). The transgene consists of a *Rev-erbα* coding sequence under the control of tetracycline-responsive elements and a gene encoding tetracycline-dependent transactivator under the control of a liver-specific promoter. In the absence of doxycycline, a tetracycline analog, mRNA and protein expression of *Rev-erbα* continuously elevates only in the liver and suppresses transcription of the core clock gene *Bmal1*, resulting in liver-specific shutdown of the clock oscillation. By using this animal, hepatic circadian gene expression was analyzed with genome-wide transcriptome profiling. A set of 351 genes showed circadian expression in the liver with functional clock, and more than 90 % of them became arrhythmic when the liver clock was ablated, indicating a dominant role of the local clock in regulating circadian gene expression. In contrast, a set of 31 genes exhibited rhythmic expression even in the absence of the functional clock. These genes contain a variety of heat shock protein (*HSP*) genes with the expression peaking at a time of highest body temperature, suggesting a role of temperature cycles and HSF in mediating systemic signals to impose extra-hepatic rhythms. Interestingly, the core clock gene *Per2* showed circadian rhythms in the clock-deficient liver (Kornmann et al. 2007). Expression of *Per2* is induced by a heat shock (from 37 °C to 40 °C) in the liver explants (Kornmann et al. 2007), and the *Per2* promoter has functional HSEs, heat shock elements (Tamaru et al. 2011), further supporting the potential role of HSF in the rhythmic gene expression.

In contrast, Reinke and colleagues developed a new technique, differential display of DNA-binding proteins (DDDP), in order to identify transcription factors that exhibit rhythmic DNA-binding activity in vitro (Reinke et al. 2008). They constructed a random DNA library and conducted an EMSA screening of 400 probes with liver nuclear extracts prepared at six different time points across a day. Known circadian transcription factors DBP, REV-ERBα, and CLOCK-BMAL1 were found to show cyclic patterns of DNA-binding activities, indicating the suitability of the assay. The screen further identified three rhythmic probes containing HSE. Among the HSF family members, HSF1 is responsible for the DNA-binding rhythms as revealed by supershifting with anti-HSF1 antibody and EMSA with liver nuclear extracts of *Hsf1*-deficient mice. Consistent with the major role of posttranslational regulation in controlling HSF1 activity (Fujimoto and

Nakai 2010), the nuclear HSF1 level and degree of its phosphorylation exhibit circadian rhythms while *Hsf1* mRNA and total HSF1 protein levels are constant throughout the day. A ChIP experiment indicated circadian rhythms of HSF1 binding to the promoter region of *HSP* genes in the liver in vivo with a phase compatible with the target genes. In cultured fibroblasts, a simulated cycle of the mouse body temperature (35–39 °C) drives circadian expression of a luciferase reporter harboring four copies of HSE, suggesting rhythmic activation of HSF1 by physiological temperature changes (Reinke et al. 2008).

Collectively, HSF1 may function as a key transcription factor that imposes the body temperature cycles to the local transcriptional rhythms of target genes.

10.5 Role of HSF1 in Temperature-Dependent Regulation of the Circadian Clock

Recent studies using a small molecule inhibitor and gene knockout/knockdown demonstrate a central role of HSF1 in mediating the effect of temperature on the circadian clock function.

KNK437, a benzylidene lactam compound, was shown to block heat shock-dependent induction of *HSP* genes and to inhibit interaction of HSF1 with HSE (Yokota et al. 2000; Ohnishi et al. 2004). Buhr and colleagues applied KNK437 to investigate the role of heat shock response pathways in the clock entrainment (Buhr et al. 2010). Interestingly, a 1-h pulse treatment with the compound causes a strong phase shift that is similar to a 1-h cold pulse (from 36 °C to 33.5 °C) and is different from a 1-h warm pulse (from 36 °C to 38.5 °C) in cultured mouse pituitary glands and lungs. Therefore, inhibition of the heat shock response pathway mimics a condition with reduced temperature. Consistently, KNK437 treatment completely blocks the phase-shifting effect of the warm pulse given at the same timing. A flavonoid compound quercetin, another heat shock response inhibitor that acts through HSF1 (Hosokawa et al. 1992), also attenuates the phase shift induced by the warm pulse (Buhr et al. 2010). The role of HSF1 in the temperature resetting is supported by the study of Tamaru and colleagues who demonstrated that the induction of the circadian rhythms by heat shock (43 °C for 30 min) in embryonic fibroblasts from wild-type mice is abolished in the cells from *Hsf1*-deficient mice (Tamaru et al. 2011). Furthermore, Saini and colleagues revealed that the entrainment of circadian gene expression to physiological temperature cycles (35.5–38.5 °C) is delayed by *Hsf1* knockdown in NIH3T3 fibroblasts and severely attenuated in primary fibroblasts of *Hsf1*-deficient mice (Saini et al. 2012). In contrast, *Hsf2* knockdown has almost no effect on the kinetics of the temperature entrainment. Collectively, these results indicate the principal role of HSF1 in the regulation of circadian phases of peripheral clocks in response to temperature change.

In contrast to the peripheral clocks, the phase of the central clock in the SCN is resistant to the pulse treatment with KNK437. Interestingly, however, chronic treatment with KNK437 has a pronounced period lengthening effect in cultured SCN as well as pituitary glands and lungs in a dose-dependent manner (Buhr et al. 2010). Consistently, *Hsf1*-deficient mice show a long free-running period of the behavioral rhythms (Reinke et al. 2008), and their embryonic fibroblasts also exhibit a long period of the gene expression rhythms (Tamaru et al. 2011). Based on the chronic effect, Buhr and colleagues further revealed that KNK437 treatment impairs temperature compensation of the circadian period (Buhr et al. 2010). The period length under control condition shows only a ~1-h difference between the culture temperatures of 30 °C and 38 °C, but the KNK437 treatment expands the period difference to ~7 h in both the SCN and the pituitary. Therefore, the HSF1 pathway also contributes to the mechanism maintaining the period length constant at various temperatures.

10.6 Cross Talk Between HSF and Light Response in the Chick Pineal Gland

In addition to the role of HSF in temperature-dependent regulation of the mammalian circadian clock, a study using the chick pineal gland identified an unexpected link between HSF and the light input pathway of the clock. Light is one of the most prominent signals mediating synchronization of the endogenous clock. Similar to temperature, the light-dark cycle entrains the clock, and a light pulse shifts its phase. Importantly, the phase-shifting effect of light differs depending on the timing when the pulse is provided. A light pulse causes delay and advance of the phase at the early and late night, respectively, while it has no effect during the day in constant dark condition. To approach the molecular mechanism underlying this time-of-day-dependent response of the clock to light, Hatori and colleagues conducted genome-wide expression analysis of light-responsive genes (Hatori et al. 2011). The chick pineal gland provides a unique model system because it contains both the central clock and the light input pathway for entrainment (Takahashi et al. 1989), while in mammals, these functions are separated into the SCN and the retina. Comparison of the gene expression in the chick pineal gland with and without 1-h light pulse at three different time points (early night, late night, and daytime) identified more than 100 light-inducible transcripts (Hatori et al. 2011). Most of the transcripts show stronger induction at one time point than the others, indicating circadian rhythms of these light responses. Of note, many of the genes strongly induced at late night are related to protein folding and known to respond to heat shock or endoplasmic reticulum (ER) stress. Consistent with the gene expression profiles, their upstream regulators are light-activated: HSF1 and HSF2 but not HSF3 accumulate in the nucleus, and expression level of spliced *Xbp1* mRNA increases to produce the activated form of XBP1 transcription factor, the

master regulator of ER stress response (Mori 2009), in the pineal gland after the light pulse (Hatori et al. 2011). While heat shock regulates nuclear accumulation of HSF1 and HSF3 (Fujimoto and Nakai 2010), the light pulse stimulates that of HSF1 and HSF2, suggesting a unique mechanism independent of temperature change to transmit the light information to the clock at late night. Furthermore, the light pulse at early night was discovered to induce posttranslational activation of SREBP transcription factor to stimulate expression of *E4bp4* gene (Hatori et al. 2011). E4BP4 is a transcription factor that represses *Per2* expression (Doi et al. 2001, 2004), connecting light-dependent activation of SREBP at early night to the phase delay of the core clock mechanism. These results together provide a starting point to understand the molecular mechanism of time-of-day-dependent regulation of the circadian phase, in which HSF1, HSF2, XBP1, and SREBP may play key roles.

10.7 Future Perspectives

It is straightforward that high temperature activates HSF to induce expression of the clock gene *Per2* for resetting of the circadian clock. *Per2* induction also plays a key role in light-dependent phase shift of the mammalian clock (Albrecht et al. 2007). Therefore, the light-dependent activation of HSF (Hatori et al. 2011) is likely to induce *Per2* expression to reset the clock in the chick pineal gland. Future studies need to reveal the role of HSF in this process as well as the mechanism how HSF1 and HSF2 are activated by light in a time-of-day-dependent manner. Such studies will lead to identification of a unique regulatory mechanism of HSFs that mediate environmental light information to the pineal circadian clock controlling melatonin release.

In addition to *HSP* genes, temperature cycles drive rhythmic expression of *Cirp* gene encoding cold-inducible RNA-binding protein (Kornmann et al. 2007; Morf et al. 2012). CIRP protein interacts with *Clock* mRNA, and knockdown of *Cirp* causes reduction of cytosolic *Clock* mRNA level and CLOCK protein expression, resulting in a strong decrease of the amplitude of clock gene expression rhythms and faster entrainment to temperature cycles (Morf et al. 2012). Therefore, HSF and CIRP seem to have opposite effect on the temperature entrainment of the clock, and their interplay needs to be addressed to understand how the peripheral clock synchronizes with temperature cycles.

The temperature compensation of the circadian period has been a long-standing question among the researchers of the circadian clock, and its molecular mechanism is still unknown. Phosphorylation of the clock proteins is temperature-compensated and/or plays a key role in the temperature compensation in a variety of organisms such as cyanobacteria, fungi, plants, and mammals (Tosini and Menaker 1998; Nakajima et al. 2005; Isojima et al. 2009; Mehra et al. 2009; Portoles and Mas 2010). It is necessary to reveal the mechanism underlying reduced temperature compensation by HSF inhibitor KNK437 (Buhr et al. 2010) and to explore its potential link with phosphorylation of clock proteins. In order to further identify

essential components of the temperature compensation, it would be a promising strategy to apply recent advances of cell-based RNAi and small molecule screens of circadian clock modifiers (Hirota et al. 2008, 2010, 2012; Hirota and Kay 2009, 2015; Isojima et al. 2009; Maier et al. 2009; Zhang et al. 2009; Lee et al. 2011; Chen et al. 2012, 2013).

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