ROLES OF DEC1 AND DEC2 IN THE CORE LOOP OF THE CIRCADIAN CLOCK, AND CLOCK OUTPUTS TO METABOLISM

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Abstract We cloned *Dec1*（*Differentiated embryonic chondrocyte-1*）and a similar gene, *Dec2*, in 1997 and 2000, respectively. DEC structure is similar to that of HES1 and HAIRY, and we observed circadian rhythms of *Dec1* mRNA levels in chondrocytes *in vitro* and rat liver *in vivo*. We then attempted to find out whether these genes are implicated in the circadian pacemaker: We found that a mutation of *Clock* abolishes or shifts circadian expression of *Dec1* and/or *Dec2* in most tissues, including the suprachiasmatic nucleus（SCN）, and that DEC1 and DEC2 modulate their own circadian expression and that of some other clock genes by auto-regulatory and interlocked feedback mechanisms. Studies on deficiencies of these genes indicate that *Dec1* and *Dec2* play roles in the phase shift and maintenance of accurate circadian rhythms and clock outputs to some physiological activities. We speculate that clock genes, including *Dec*, are involved in the pathology of various diseases and aging.

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Circadian rhythms and diseases

Most organisms on the earth, from photosynthetic bacteria to humans, have circadian rhythms in metabolism and behavior with a cycle of about 24 h. A prominent manifestation of circadian rhythms is the sleep-wake cycle, which is essential for health: No sleep for an extended period of time causes death. Nonetheless, whether circadian rhythms are essential for the maintenance of life is a matter of controversy, since disruption of circadian rhythms for a short time does not cause serious diseases. Recent studies however, have demonstrated that chronic circadian rhythm disorders cause various diseases in addition to sleep disorders and depression: Shift workers have higher risks for breast or prostate cancer, ischemic heart disease, diabetes and hypertension, although night workers not on shifts have lower risks than rotated shift workers[1,2]. Circadian disorders may be linked to some other diseases, and i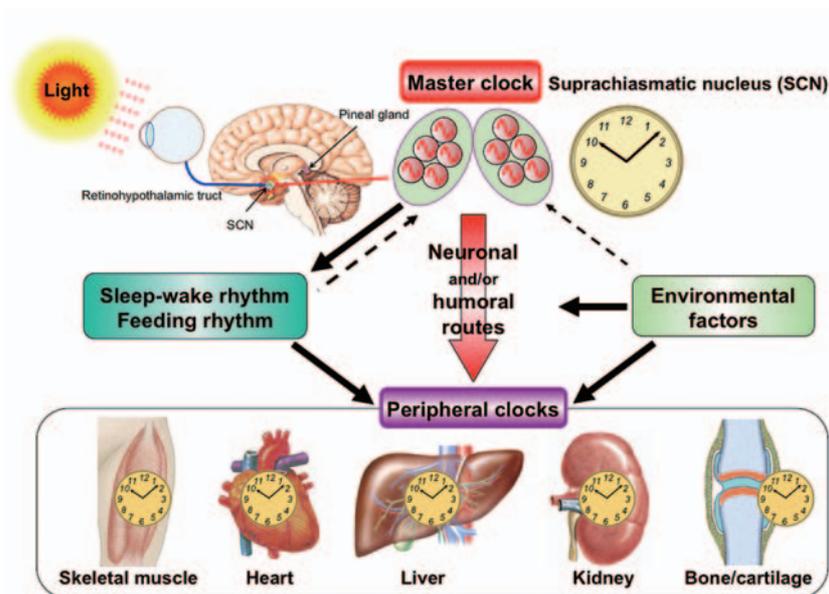n modern life, humans are active late at night, so studies on circadian rhythms are necessary to extend our understanding of disease.

Master and peripheral clocks

Destruction and transplantation experiments have shown that a master circadian clock is located in the hypothalamic suprachiasmatic nucleus（SCN）, and that it involves various clock genes with specific temporal patterns of expression[3]. The SCN clockwork is mainly entrained by the light-dark cycle based on Earth rotation, and it in turn entrains peripheral clocks（"slave" oscillators）in most cells of the body through neuronal (e.g., the autonomic nervous system) and/or humoral routes (Fig. 1). In addition, peripheral clocks can be affected by non-photic stimuli such as daily feeding cycles, which have little effect on the master clock in the SCN[4].

Three components of circadian clocks

Both master and peripheral clocks are composed of input pathways, a pacemaker and

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Figure 1 Direct and indirect controls of peripheral clocks by the master clock, various physiological activity rhythms and some environmental factors via neuronal and humoral routes.

output pathways (Fig. 2). The pacemaker oscillates the expression of clock genes in a period of about 24 h, and the phase of the rhythms is adjusted by various time cues via input pathways. The clock outputs regulate circadian expression of a group of genes tissue-dependently: In each examined tissue, 4-9% of transcripts showed circadian expression, and only a few of these transcripts showed circadian expression in other examined tissues. So numerous genes (>3000) are thought to have circadian expression somewhere in the body. The coordinated circadian gene expression generates circadian rhythms for various physiological activities, including the sleep-wake cycle, blood pressure, body temperature, metabolism and cell cycle.

Light and feeding are the physiological cues that entrain the phase of circadian rhythms. However, hypoxia, X-ray, UV-ray, inflammation, some drugs, hormones and aging alter the phase and/or amplitude of circadian rhythms. In diabetes adipose tissues, for example, circadian expression of clock genes showed lower amplitude or poor rhythmicity. Hypoxia, X-ray, UV-ray, inflammation and some drugs can reset or desynchronize circadian expression of clock genes, which increases the risks of tumor, hypertension and diabetes. Mutation or SNP in some clock genes also increases these risks[5,6]. "Chronotherapy" is still in its earl stages, although appropriate dosing schedules of antitumor drugs and interferon are known to reduce side effects and improve outcomes.

Clock genes

The first clock gene "*Period*" was discovered in a fruit fly, *Drosophila melanogaster*, in 1971[7] and cloned in 1984[8]. In 1997, the first mammalian clock gene, "*Clock*" was identified[9], and it opened the way to understanding the molecular mechanism of the mammalian clock system. Considerable progress has since been made in identifying the molecular components of the clock system in mammals and other animals: They involve *Clock/Npas2, Bmal1/Bmal2, Per1/Per2/Per3, Cry1/Cry2, Rev-erbα/β, Rorα/β/γ, Dbp/Tef/Hlf, E4bp4* and *Dec1/Dec2*[10,11]. Recent genome
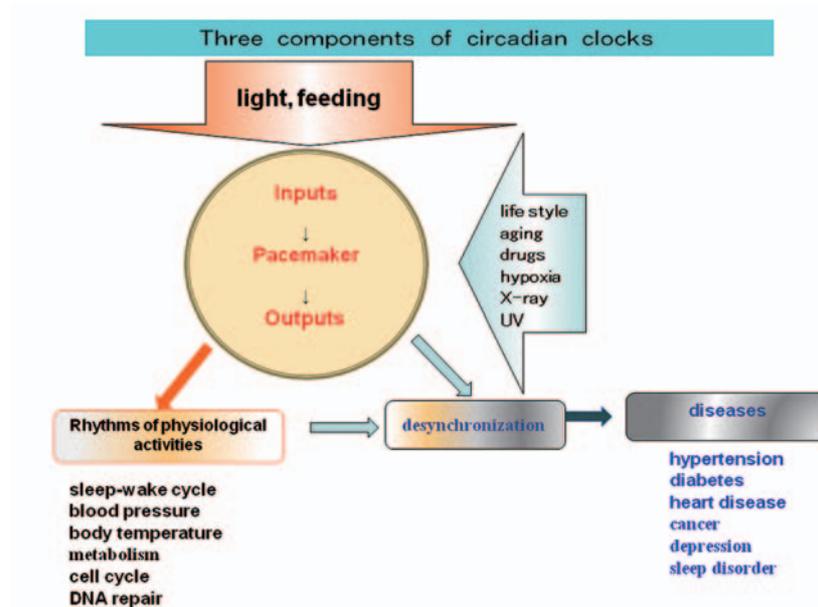
Figure 2 Three components of circadian clocks synchronized or desynchronized by physiological and pathological factors

projects suggest that most of the clock genes have already been identified.

Clock genes generate circadian oscillations by forming an auto-regulatory transcription-translation feedback loop, and in mammals, multiple sets of clock genes form the primary negative feedback loop (*Clock/Npas2, Bmal1, Per1/Per2/Per3, Cry1/Cry2, Rev-erbα/β*, and *Dec1/Dec2*). In these loops, clock gene products -CLOCK/BMAL1 heterodimer- serve as a positive limb, and -PERs, CRYs, and DECs- as negative limbs. CLOCK/BMAL1 heterodimer binds E/E'-box elements (CACGTG or CACG TT) in the promoter of the negative components *Per, Cry*, and *Dec* genes, and then activates the transcription of these genes[10]. The PER and CRY proteins repress the activity of CLOCK/BMAL1 heterodimer by protein-protein interaction; DEC proteins bind both E-box and BMAL1 protein and repress the transcription of their target genes[12]. In addition, the turnover time of the gene products allows the loop to form rhythms of clock gene expression with a basal period of about 24 hours (Fig. 3A). In the case of Per-loop, casein kinase Iε is thought to phosphorylate PER proteins and regulate PER stability and subcellular localization. In contrast, *Bmal1* and Npas2 are positively regulated by RORα and negatively by REV-ERBα via ROR response element (RORE), so their transcripts display nearly opposite phases to those of the negative regulators (*Per, Dec, and Rev-erbα*)[13]. The current model for how these factors are organized into an auto-regulatory interlocked feedback loop, is summarized in Fig. 3B.

## Distinct roles of DEC and PER/CRY in the pacemaker

DEC1 and DEC2 form a heterodimer or homodimer that binds to E-box elements in clock genes, thereby becoming a target sequence of BMAL1/CLOCK heterodimer[14-16]. The ability of DEC1 and DEC2 to bind E-boxes leads to competition with BMAL1/CLOCK for DNA binding: BMAL1/CLOCK can bind to E'-box sequences in addition to E-box sequences, while the binding of DEC1 and DEC2 to E'-boxes is much weaker than that to E-boxes[17]. On the other hand, PER/CRY heterodimer down-regulates transcription from some clock
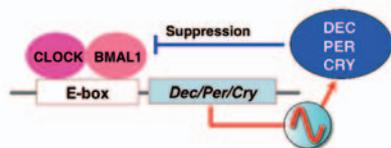
Figure 3A The core auto-regulatory negative feedback loop of clock gene expression includes a positive limb（CLOCK/BMAL）and negative limbs（DEC and PER/CRY）.
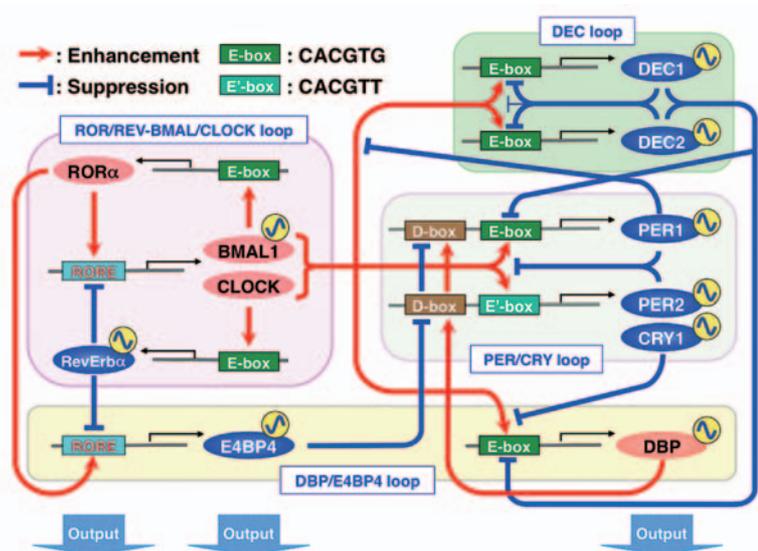
Figure 3B Three loops of circadian clocks are interlocked with each other.

genes through protein-protein interaction with BMAL1/CLOCK, regardless of E-box- or E'-box-mediated transcription. Although DEC1 and DEC2 also have weak binding affinity for BMAL1, their repressive ability is primarily due to selective binding to E-box sequences. These findings may explain why the expression of the *Per2* gene, which contains E'-boxes instead of E-boxes in its promoter region, is repressed by PER but is hardly affected by DEC1. Furthermore, ChIP analysis has demonstrated that the binding phase of DEC1 to E-boxes in clock genes is several hours earlier than that of PER1, indicating that DEC has a role in the early phase of circadian regulation of clock gene expression, while PER acts as a repressor in the late phase（Fig. 4）[17]. Thus, DEC and PER/CRY suppress clock gene expression by discrete mechanisms in discrete circadian phases.

Deficiency of *Dec* or its Drosophila ortholog (*cwo*) impairs circadian rhythmicity

Recently, a Drosophila ortholog of *Dec*（*cwo*）was identified and shown to be expressed in

a circadian fashion, regulated by the CLOCK and CYCLE, Drosophila counterpart of BMAL1, through its E-box. Disruption of *cwo* results in reduced amplitude of various clock gene expression and lengthened periods or loss of behavioral rhythms[18]. Similarly, *Dec1* knockout mice show a slightly but significantly longer period of circadian rhythms under constant darkness and faster re-entrainment to 6-h phase-advanced shift of a 12:12 h light-dark cycle compared with wild-type mice[17]. In addition, Double knockout of *Dec1* and *Dec2* causes a more dramatic lengthening of the period[19]. Thus, *Dec* as well as *cwo*, participates in the control of circadian rhythms by modulating the circadian phase and period length.

Role of DEC in clock inputs

In rat SCN, *Dec1* expression is enhanced by light pulse within 1 h only during the subjective night, whereas *Dec2* expression is suppressed[11]. In other parts of the brain, *Dec1* expression is enhanced by the same light pulse time-independently, suggesting that *Dec1* plays a unique role in the entrainment by light in
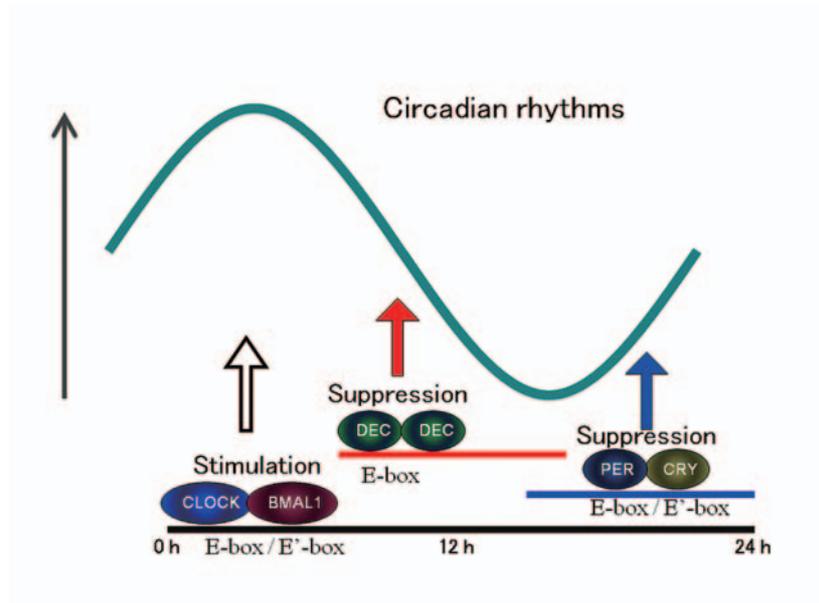
Figure 4 Distinct actions of DEC and PER/CRY: DEC suppresses CLOCK/BMAL1 actions on the E-box selectively only in the early circadian phase. DEC suppresses CLOCK/BMAL1 actions in the early stage, and RER/CRY does it in the late stage. Furthermore, DEC acts on the E-box selectively, while PER/CRY acts on both the E-and E'-boxes. These distinct actions of DEC and PER/CRY may be necessary for the precise control of circadian rhythms.

the SCN. Interestingly, *Dec1* and *Dec2* double knockout markedly suppressed the light-pulse-induced expression of *c-fos*, *c-jun* and *Per1* in the SCN[19].

DNA damage, hypoxia and TGF-beta also shift the phase of circadian rhythms of clock gene expression in some tissues: DNA damage induces DEC1 via p53, hypoxia induces DEC1 via HIF1-alpha, and TGF-beta induces it via Smad3 and 4 within a few hours in various tissues[20-22, 24-26]. It is still unknown whether DEC is involved in the phase shift caused by DNA damage or hypoxia, but an injection of TGF-beta shifted the circadian phase of *Per1* and *Dbp* expression in kidney and adrenal glands after the immediate early induction of *Dec1*, a phase shift not observed in *Dec1*-deficient mice, indicating that *Dec1* induction is a prerequisite for the phase shift of peripheral clocks by growth factors[26].

Dec1 may also be involved in feeding-induced phase shifts: It was induced rapidly by feeding in various tissues, including the liver, and feeding did not enhance, or had less effect

on, the expression of the other clock genes[27]. Food contains glucose and cholesterol, which induce *Dec1* via the carbohydrate-responsive element-binding protein (ChREBP) and LXR, respectively[28, 29].

Role of DEC in clock outputs

DEC1 modulates energy metabolism by suppressing gluconeogenesis and lipid synthesis: DEC1 represses transcription of phospho-enolpyruvate carboxykinase (PEPCK), pyruvate kinase, fatty acid synthase and PPAR-gamma, and it inhibits LXR and SREBP1c[29-32]. DEC2 represses *Cyp7A* expression[28], and DEC1 and/or DEC2 suppress *ID1*, *MLH1*, and *VEGF* expression, and enhance *Survivin* expression[25, 33]. Some of these genes show circadian expression, indicating a role of DEC in clock outputs. However, when DEC1 and DEC2 are induced at high levels under some pathological conditions, such as hypoxia, they may overpower clock controls.

DEC1 also suppresses proliferation of some

cells, including NIH3T3 cells and keratinocytes, partly via suppression of *c-myc* expression[35], but it has little effect on proliferation of many other cell types. DEC1 has opposite effects on apoptosis cell-dependently[33,36], it enhances tumor metastasis, possibly via cell survival at sites of metastasis[23]; and it enhances differentiation to chondrocyes or osteoblasts, and suppresses differentiation to adipocytes or myoblasts via PPAR-gamma or MyoD, respectively[23,30]. It is unknown whether these actions are linked to the clock system.

Regulation of circadian clock gene expression by protein acetylation and deacetylation

It is possible that epigenetic modulations are involved in circadian regulation of gene expression. Histone acetyltransferase (HAT), for example, induces a transcription-permissive state by unfolding the compact chromatin, whereas histone deacetylase (HDAC) induces gene silencing by compacting the chromatin. There are two types of HDAC: HDAC1-10 are NAD-independent, and Sirt1-7 are NAD-dependent.

Intriguingly, CLOCK has intrinsic HAT activity in the C-terminal region, where it acetylates histone H3 and its own partner BMAL1. Acetylation by CLOCK increases the ability of the BMAL1 to bind to CRY1, thereby facilitating CRY1-mediated repression. On the other hand, CRY1, DEC1 and DEC2 associate with HDACs. We previously reported that tricostatin-A, an inhibitor of class I and II HDACs, partially relieves DEC1- and DEC2-mediated transcriptional repression, and that DEC2 binds to HDAC1 and SIRT1 via the C-terminal and bHLH domains, respectively[37]. Therefore, DEC2 may bind to HDAC1 and SIRT1 simultaneously.

SIRT1 regulates diverse cellular processes, including gene silencing, energy metabolism, and aging. Recently, two groups reported that SIRT1 is involved in circadian regulation, suggesting a relationship between circadian rhythms and aging. Nakahata et al. demonstrated that SIRT1 activity is regulated in a circadian manner, and influences the expression level of clock genes including *Dbp, Per2* and *Cry1*[38], and SIRT1 likely induces deacetlylation of Histone H3 on the *DBP* gene and BMAL1 protein[37]. Furthermore, Asher et al. demonstrated that SIRT1 associates with CLOCK-BMAL1 in a circadian manner and promotes deacetylation and degradation of PRE2[39].

So we have a revised model: CLOCK/BMAL1 induces acetylation of histone and BMAL1, and stimulates *PER*, *CRY* and *DEC* expression. As DEC protein accumulates, DEC binds to E-boxes, which are the target sites of CLOCK/BMAL1, and recruits HDAC1 and SIRT1. Then the DEC/HDAC/Sirt1 complex deacetylates histones and represses transcription, and therefore the CLOCK/BMAL/PER/CRY/ SIRT1/HDAC complex suppresses transcription in the late stage.

Clocks and aging

We are interested in the mysterious triangle of clocks, energy metabolism and lifespan (Fig. 5): Calorie restriction extends lifespan by reducing energy metabolism and oxygen radicals, and activates SIRT1 activity. Circannual clocks also extend lifespan, possibly by reducing energy metabolism: Squirrels that hibernate have a lifespan of 10 years, whereas those do not have a lifespan of 3 years[40]. Circadian clocks, which ensure deep sleep of animals, may also play a part in decreasing oxygen radicals by synchronizing energy metabolism. Furthermore, the interaction between circadian clocks and SIRT1 may modulate the aging process. Accordingly, *Bmal1*-deficient mice, like *Sirt1*-deficient mice, have shorter lifespans. Further studies are necessary to investigate the precise relationship between clocks and aging.
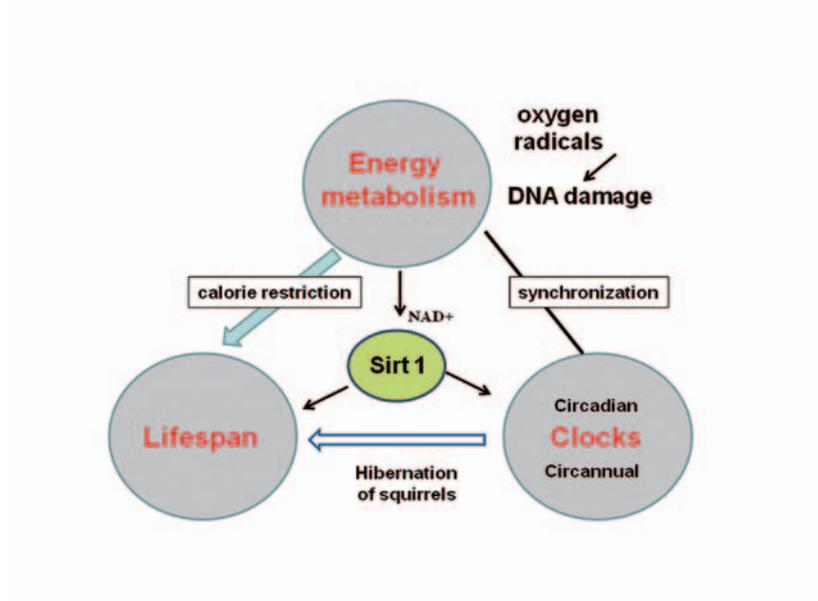
Figure 5 The mysterious triangle of clocks, energy metabolism and lifespan. Calorie restriction reduces energy metabolism and oxygen radicals, and elevates the NAD+ level, which activates SIRT1. The circadian clocks also decrease oxygen radicals by synchronization of energy metabolism, and some clock molecules, including DEC2, bind SIRT1. The circadian clocks also ensure deep sleep at night, which reduces energy metabolism. The circannual clock induces hibernation, which reduces energy metabolism, and extends lifespan of some squirrels. Thus, Bmal1-deficient mice show higher levels of cellular oxygen radicals and a shorter lifespan. And Bmal1 and Clock mutant mice display energy metabolism disorders. It is therefore essential to understand precisely how clock molecules modulate energy metabolism and aging.

Conclusion

DEC1 and DEC2 play roles in clock input pathways, pacemakers and clock output pathways, although a deficiency of DEC in mice had less effect on circadian rhythms of various physiological activities than did deficiencies of PER and/or CRY proteins. On the other hand, DEC-deficient flies showed prominent changes in circadian rhythms of locomotion activity, at least, when in constant darkness. Most actions of DEC depend upon binding to the E-box of clock and non-clock genes, whereas PER/CRY mainly acts on target genes via protein-protein interactions with CLOCK/BMAL. How these clock molecules synchronize various biochemical pathways is a vital issue in human physiology and pathology.

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