

# Phase Resetting of the Mammalian Circadian Clock by DNA Damage

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## Summary

To anticipate the momentum of the day, most organisms have developed an internal clock that drives circadian rhythms in metabolism, physiology, and behavior [1]. Recent studies indicate that cell-cycle progression and DNA-damage-response pathways are under circadian control [2–4]. Because circadian output processes can feed back into the clock, we investigated whether DNA damage affects the mammalian circadian clock. By using Rat-1 fibroblasts expressing an *mPer2* promoter-driven luciferase reporter, we show that ionizing radiation exclusively phase advances circadian rhythms in a dose- and time-dependent manner. Notably, this *in vitro* finding translates to the living animal, because ionizing radiation also phase advanced behavioral rhythms in mice. The underlying mechanism involves ATM-mediated damage signaling as radiation-induced phase shifting was suppressed in fibroblasts from cancer-predisposed ataxia telangiectasia and Nijmegen breakage syndrome patients. Ionizing radiation-induced phase shifting depends on neither upregulation or downregulation of clock gene expression nor on *de novo* protein synthesis and, thus, differs mechanistically from dexamethasone- and forskolin-provoked clock resetting [5]. Interestingly, ultraviolet light and tert-butyl hydroperoxide also elicited a phase-advancing effect. Taken together, our data provide evidence that the mammalian circadian clock, like that of the lower eukaryote *Neurospora* [6], responds to DNA damage and suggest that clock resetting is a universal property of DNA damage.

## Results and Discussion

### Ionizing Radiation Phase Advances the Circadian Clock

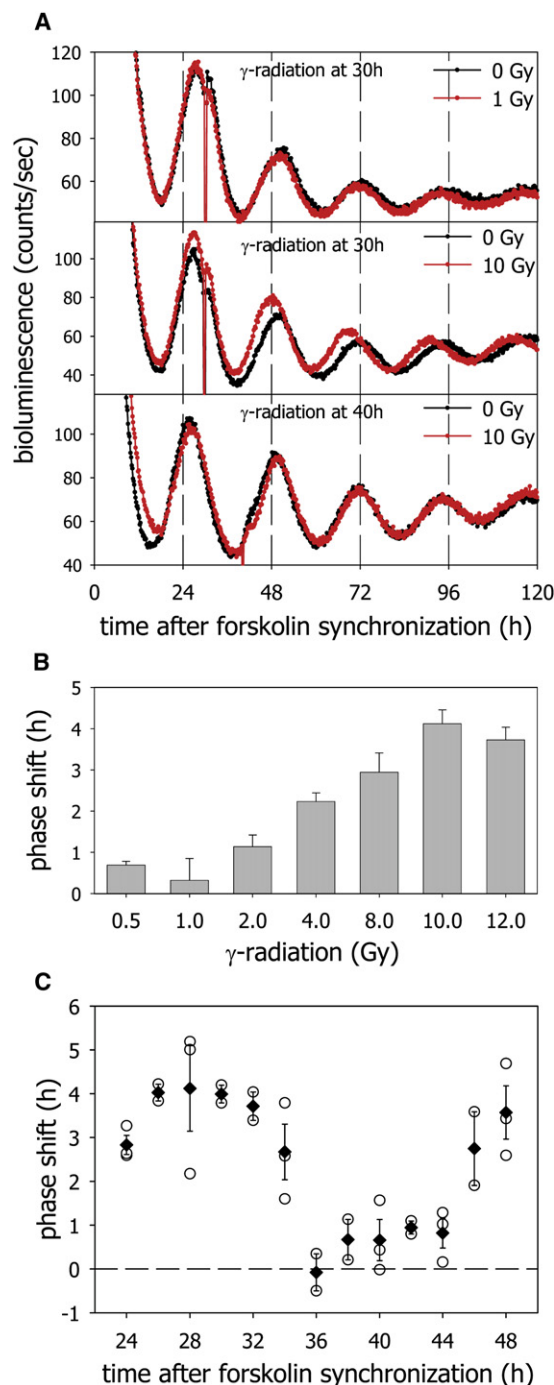
To study the effect of DNA damage on the circadian system, we used Rat-1 fibroblasts stably expressing an *mPer2* promoter-driven luciferase reporter gene, (Rat-1 *mPer2:luc* cells [7]). The *mPer2* promoter is activated by the CLOCK/BMAL1 heterodimer and repressed by the activity of the PER/CRY complex and allows real-time monitoring of circadian oscillations [1]. Confluent Rat-1 *mPer2:luc* cells (arrested in the

G0/G1 phase of the cell cycle; see Figure S1 available online) were first treated with forskolin (30  $\mu$ M) to synchronize clock gene expression between individual cells. When applied 30 hr after synchronization, a single dose of gamma ( $\gamma$ )-radiation elicited a clear phase advance of bioluminescence rhythms in a dose-dependent manner (Figure 1A), with a maximum shift of about 4 hr being reached at doses of 10 Gy and higher (Figure 1B). At this dose, we did not observe significant cell death, as further illustrated by the comparable levels of bioluminescence signals in irradiated and mock-treated cells (Figure 1A).

Because the phase of circadian oscillations hardly changed when cells were  $\gamma$ -irradiated 40 hr after synchronization (Figure 1A, bottom), we next examined whether the magnitude and direction (i.e., advance versus delay) of ionizing radiation-induced phase shifts were dependent on the phase of the circadian clock. As evident from the phase response curve (PRC), obtained by exposing Rat-1 *mPer2:luc* cells to 10 Gy of  $\gamma$ -radiation at various phases of the circadian cycle (Figure 1C), a maximal phase advance was elicited between 26 and 32 hr after forskolin synchronization. This shift was intermediate at 34 hr and negligible between 36 and 44 hr after synchronization. At 48 hr, cells again displayed an intermediate phase advance, similar to that observed 24 hr earlier. Interestingly, the PRC for ionizing radiation exclusively shows phase advances. This finding markedly contrasts the reported Rat-1 cell PRCs for forskolin and dexamethasone, which exhibit both phase advances and delays [5]. The lack of phase delays in the PRC for ionizing radiation is not due to an unforeseen artifact in our batch of Rat-1 *mPer2:luc* cells because forskolin instigated a phase delay 32 hr after synchronization of cells with horse serum (Figure S2). Interestingly, forskolin and dexamethasone PRCs [5] provoke phase delays at the time when ionizing radiation produced maximum phase advances (Figure 1C).

Having shown that  $\gamma$ -radiation can phase advance peripheral oscillators *in vitro*, we next investigated its impact on the master circadian clock in the suprachiasmatic nuclei (SCN), as visualized by voluntary wheel-running-behavior recordings. Free-running C57BL/6J male mice were subjected to a nonlethal dose of  $\gamma$ -radiation (4 Gy) given at circadian time (CT) 6 or 22. This choice is based on behavioral PRCs for nonphotic stimuli, showing maximum phase advances and delays at CT6 (middle of the subjective day) and CT22 (end of the subjective night), respectively [8–10]. Exposure to  $\gamma$ -radiation at CT6 significantly phase advanced locomotor activity rhythms by  $1.2 \pm 0.2$  hr (as compared to the  $-0.6 \pm 0.2$  hr shift elicited by sham-treatment;  $p < 0.001$ ; Figure 2). In contrast, radiation of mice at CT22 did not produce a significant response ( $0.3 \pm 0.3$  hr and  $-0.4 \pm 0.3$  hr in exposed and sham-treated mice, respectively;  $p = 0.08$ ). Importantly, neither the period of circadian rhythms nor the overall wheel-running activity per day was affected by ionizing radiation (Figure S3), excluding that the observed effect was influenced by changes in core oscillator performance. This animal study shows that the phase-advancing properties of ionizing radiation are not limited to peripheral oscillators but extend to the master clock in the SCN. In line with the notion that the mechanism and molecular makeup of the circadian clock in cultured cells and the SCN are alike [11, 12], ionizing radiation exerted its effect at the same circadian phase (i.e., when

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**Figure 1. Ionizing Radiation Phase Advances Circadian Rhythms in Fibroblasts**

(A) Representative examples of luminescence rhythms in confluent Rat-1 *mPer2:luc* cell cultures, either exposed to a single pulse of  $\gamma$ -radiation (red lines) at a dose of 1 Gy (top) or 10 Gy (middle) or mock-treated (black lines) 30 hr after forskolin synchronization. The bottom panel presents an example of confluent Rat-1 *mPer2:luc* cell cultures exposed to 10 Gy of  $\gamma$ -radiation 40 hr after forskolin synchronization.

(B) The graph illustrates dose dependency of the  $\gamma$ -radiation-induced phase advance of luminescence rhythms in Rat-1 *mPer2:luc* cell cultures. Each bar represents the mean of three independent experiments. Error bars represent the SEM.

(C) Phase response curve of ionizing radiation-induced phase shifts. Forskolin-synchronized Rat-1 *mPer2:luc* cell cultures were exposed to 10 Gy of  $\gamma$ -radiation at different phases of the second circadian cycle. Individual

the CLOCK/BMAL1 complex is engaged in transcription activation of E-boxes containing genes like *Per2*) [13].

#### **Ionizing Radiation-Mediated Phase Resetting Involves the ATM/ATR Pathway**

DNA double-strand breaks (DSBs) form the primary type of DNA lesion introduced by ionizing radiation and trigger DNA damage signaling pathways mainly through members of the phosphatidylinositol 3-kinase-related kinase (PIKK) family of protein kinases, notably ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related) [14]. These kinases initiate the signaling cascade by phosphorylating a wide spectrum of cell-cycle regulators and DNA-repair proteins [15, 16]. Treatment of synchronized Rat-1 *mPer2:luc* cell cultures with the ATM-specific inhibitor Ku-55933 [17] reduced the  $\gamma$ -radiation-induced phase advance in a dose-dependent manner, whereas mock treatment did not have any effect (Figure 3A, right). Similarly, the nonselective ATM/ATR inhibitor caffeine [17, 18] caused a dose-dependent reduction of the ionizing radiation-induced phase advance (Figure 3A, left). These findings strongly suggest that ATM is a mediator of the clock-resetting properties of ionizing radiation and point to DNA damage as the ultimate trigger. Nonetheless, as caffeine inhibits ATM and ATR at reported  $IC_{50}$  levels (half inhibitory dose) of 0.2 mM and 1.1 mM, respectively [18, 19], the magnitude of the radiation-induced phase advance at 1.1 mM caffeine ( $>5 \times IC_{50}$  for ATM) suggests that other kinases (i.e., ATR) also may contribute to the response.

To further evaluate the involvement of ATM in ionizing radiation-mediated resetting of the circadian clock, we extended our analysis to ATM- and NBS1-deficient human primary dermal fibroblasts (HDFs) obtained from patients with the ionizing radiation-sensitive, cancer-predisposing disorder ataxia telangiectasia (AT) and Nijmegen breakage syndrome (NBS), respectively [reviewed in 20]. NBS1 (also termed nibrin) is a component of the MRN complex (MRE11-RAD50-NBS1), which recruits ATM to the proximity of DSBs and activates the latter protein [21]. To visualize circadian clock performance, we first stably infected control ( $n = 4$ ), ATM-deficient ( $n = 3$ ), and NBS1-deficient ( $n = 3$ ) HDFs with a lentiviral *mPer2:luc* reporter construct. Interestingly, forskolin-synchronized patient cell lines were oscillating with a circadian period shorter than that of control cell lines ( $24.4 \pm 0.3$  hr and  $25.8 \pm 0.4$  hr, respectively,  $p = 0.01$ ; Figure 3B). Importantly, however, all ATM- and NBS1-deficient cells were moderately to severely impaired in their phase-advancing response upon ionizing radiation exposure, whereas rhythms shifted as expected in control cells ( $p < 0.001$ ; Figures 3B and 3C). These results provide definite evidence that ATM (directly or indirectly) communicates DNA damage information to the core clock machinery. Given the notion that ATM-mediated damage-signaling pathways are active in neuronal tissues [22], which likely includes the SCN, and given the shorter circadian periodicity of the patient lines (final proof requiring analysis of larger numbers of cell lines), it would be of interest to investigate the chronotypes of AT and NBS patients.

#### **Induction of Clock Gene Expression Is Not Required for Ionizing Radiation-Mediated Resetting**

The mechanism of clock resetting in the SCN involves early induction (after photic stimuli) or repression (after nonphotic

and averaged data are represented by open and closed symbols, respectively. Error bars represent the SEM.

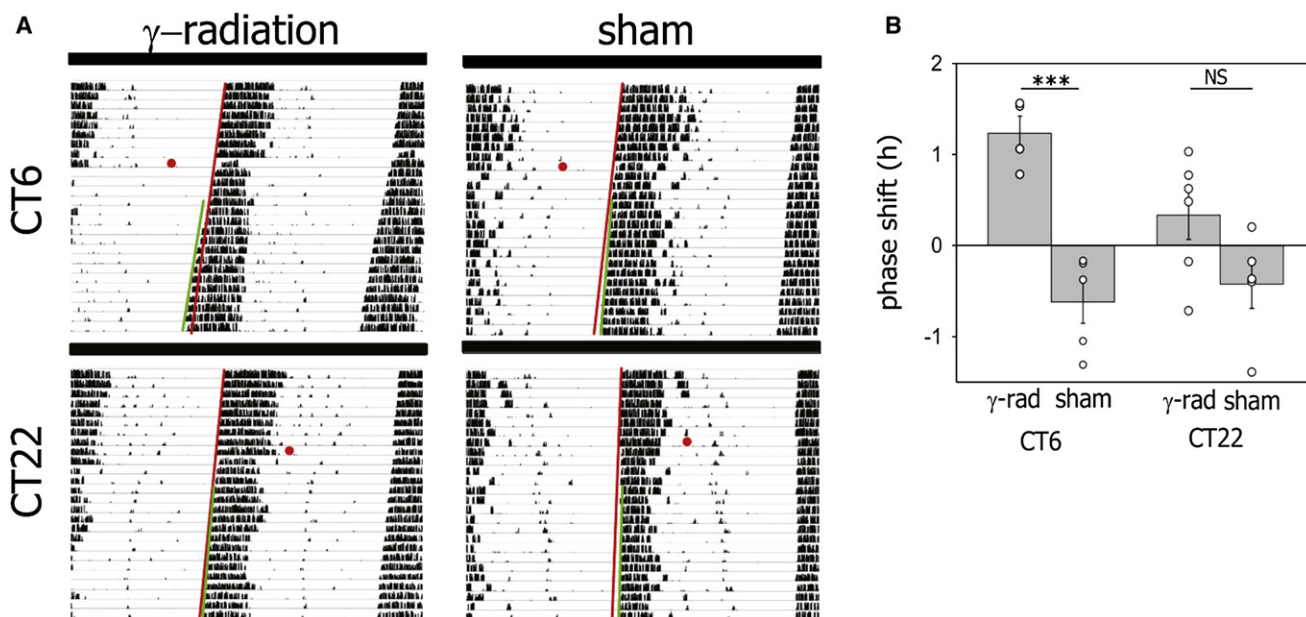


Figure 2. Ionizing Radiation Can Phase Advance Behavioral Rhythms in Free-Running Mice

(A) Shown are representative examples of double-plotted activity records of C57BL/6J male mice,  $\gamma$ -radiated (left) or sham-treated (right) at CT6 (top) and CT22 (bottom). Red dots indicate the moment of treatment. Lines through the onsets of activity indicate the phase of rhythmicity before (red line) and after treatment (green line).

(B) Shown is a quantitative representation of ionizing radiation-induced phase shifts in ionizing radiation exposed or sham-treated C57BL/6J mice. Individual data points and mean values are represented by open circles and gray bars, respectively. Error bars represent the SEM. \*\*\* $p < 0.001$ . Abbreviations: NS, not significant.

stimuli) of *Per* gene expression [23–25]. Because the molecular mechanism of clock resetting in cultured cells by either chemical synchronizers or ionizing radiation is not documented, we next analyzed clock mRNA levels in oscillating Rat-1 *mPer2:luc* cells exposed to either dexamethasone or  $\gamma$ -radiation 30 hr after synchronization. Remarkably, whereas dexamethasone strongly stimulated transcription of *Per1* and *Per2* genes up to 7-fold (peaking 2–4 hr after the pulse), ionizing radiation neither up- nor downregulated transcript levels of these genes (Figure 3). The levels of other clock gene transcripts like *Clock*, *Bmal1* (Figure 4), *Cry1*, and *Cry2* (data not shown) were unaffected by both treatments. The opposite direction of forskolin and dexamethasone versus ionizing radiation-induced phase shifts at the same circadian phase (i.e. phase delay and phase advance, respectively [5; this study], as well as the nonresponsiveness of *Per* genes upon  $\gamma$ -radiation, further points to differences in the underlying resetting mechanism.

Additionally, to investigate whether de novo protein synthesis may be required for clock resetting by ionizing radiation, we treated Rat-1 *mPer2:luc* cells with cycloheximide (CHX) prior to and after  $\gamma$ -radiation. Inhibition of translation by CHX did not significantly prevent phase resetting ( $3.6 \pm 0.6$  and  $3.0 \pm 0.6$  hr for solvent and CHX treatment, respectively;  $p > 0.05$ , *t* test), which implies the involvement of a posttranslational regulation mechanism. Noteworthy, PER1 has been identified as one of the many substrates phosphorylated by ATM/ATR after DNA damage [15]. Moreover, PER1 and TIM proteins are engaged in complex formation with the ATM and ATR kinases, respectively [26, 27]. Therefore, these clock proteins may be the primary targets for posttranslational modifications to subsequently change their abundance and activity in a fast and controllable manner.

Finally, we assessed whether clock-resetting potential is restricted to ionizing radiation-induced genotoxic stress or

whether it extends to other types of DNA-damaging agents. Interestingly, ultraviolet light (crosslinking the base moiety of adjacent pyrimidines) and tert-butyl hydroperoxide (causing oxidative DNA damage), when applied to Rat-2 *mPer2:luc* cells 30 hr after forskolin synchronization, also elicited a dose-dependent, phase-advancing effect on the circadian clock (Figure 5). This suggests that clock resetting could be a universal property of DNA damage.

## Conclusions

In summary, whereas the mammalian circadian clock has been reported to control cell-cycle progression and DNA-damage-response pathways [26–29], the current study provides the first evidence that, conversely, DNA damage can act as a resetting cue for the mammalian circadian clock in vitro and in vivo. The underlying mechanism differs from that of known resetting agents. Thus far, the only other example of DNA damage impinging on the circadian clock is represented by the lower eukaryote *Neurospora crassa*, in which the radiomimetic MMS caused phase advances through a mechanism involving Check2-mediated phosphorylation of the clock protein FRQ [6]. In view of the hypothesis that circadian clockworks might have originated from protective mechanisms to escape from DNA/RNA damage (as imposed by diurnal exposure to ultraviolet light and ultradian respiratory metabolic cycles) that ultimately evolved in a self-sustained oscillator [30, 31], the question remains open whether DNA-damage sensitivity of the circadian clock is a remnant of clock evolution or whether it is the immediate consequence (or by-product) of the close coupling between the circadian clock and cell-cycle control. Alternatively, as recently put forward by Chen and McKnight [32], circadian and metabolic cycling might have coevolved with cell-cycle gating. Considering DNA-damage avoidance

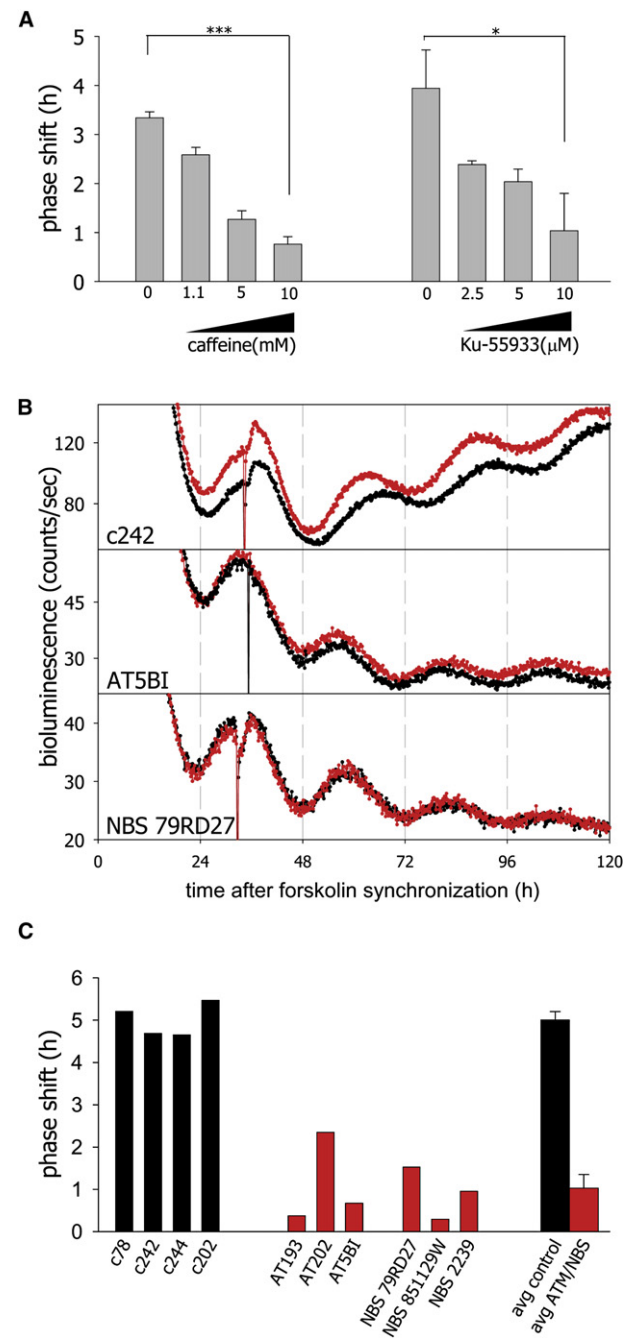


Figure 3. ATM/ATR Kinases Are Involved in Ionizing Radiation-Induced Phase Advances

(A) Caffeine (left) and Ku-55933 dose dependently inhibit ionizing radiation-induced phase advances in forskolin-synchronized confluent Rat-1 *mPer2:luc* cell cultures. Up to 1 hr prior to  $\gamma$ -radiation (10 Gy) or mock treatment (administered 28 hr after synchronization), cells were exposed to caffeine or Ku-55933 at the indicated dose. Bars represent the average of three independent experiments. Error bars represent the SEM.

(B) Shown are representative examples of bioluminescence rhythms in human primary dermal fibroblasts from control subjects and AT, and NBS patients exposed to  $\gamma$ -radiation (5 Gy, red lines) or mock-treated (black lines) approximately 34 hr after initial synchronization with forskolin.

(C) Shown is a quantitative analysis of the magnitude of ionizing radiation-induced phase advances in human control, AT, and NBS primary dermal fibroblasts. The overall mean values are shown on the right. Error bars represent the SEM.

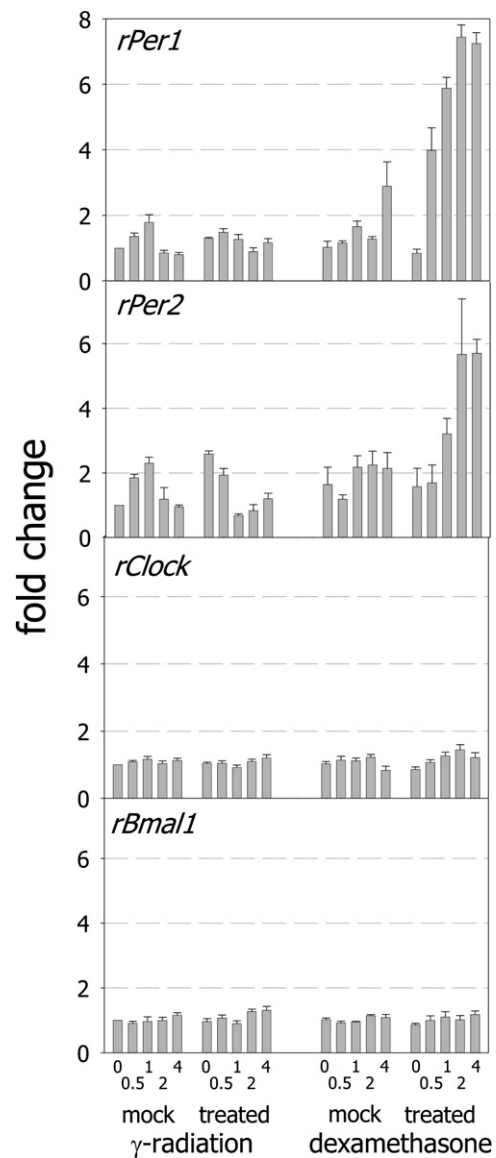


Figure 4. Clock Gene Expression in Ionizing Radiation- and Dexamethasone-exposed Rat-1 *mPer2:luc* Cells

Graphs illustrate the quantitative RT-PCR analysis of *Per1*, *Per2*, *Clock*, and *Bmal1* expression 0, 0.5, 1, 2, and 4 hr after  $\gamma$ -radiation (10 Gy) or dexamethasone (100 nM) and mock treatment of forskolin-synchronized Rat-1 *mPer2:luc* cell cultures. The values represent the average and the SEM.

as the underlying unifying evolutionary driver, it was hypothesized that DNA damage might act as Zeitgeber. The present data, showing that physical and chemical genotoxins can phase shift the clock, well support this idea. Yet, except for radiotherapeutic purposes, organisms are unlikely to be exposed to the ionizing radiation doses used in this study. It would be interesting, therefore, to investigate to what extent daily exposure to low but timed doses of genotoxins might affect the circadian system.

Supplemental Data

Supplemental Experimental Procedures and Three Figures are available at <http://www.current-biology.com/cgi/content/full/18/4/286/DC1/>.



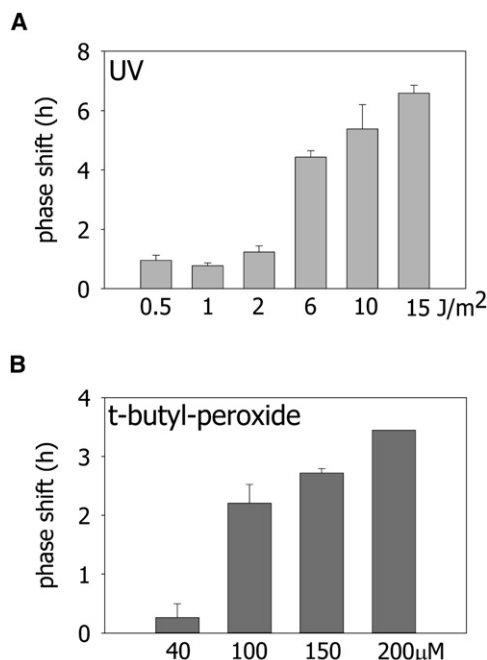


Figure 5. UV and Oxidative Stress Phase Advances Circadian Rhythm in Fibroblasts

Treatment of confluent Rat-1 *mPer2:luc* cell cultures with UV-light (A) or t-butyl-peroxide (B) 30 hr after forskolin synchronization, dose dependently phase advances bioluminescence rhythms. Each bar represents the average of two independent experiments. Error bars represent the SEM.

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