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Modulation of ATR-mediated DNA damage checkpoint response by cryptochrome 1

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ABSTRACT

Mammalian cryptochromes (Crys) are essential circadian clock factors implicated in diverse clock-independent physiological functions, including DNA damage responses. Here we show that Cry1 modulates the ATR-mediated DNA damage checkpoint (DDC) response by interacting with Timeless (Tim) in a time-of-day-dependent manner. The DDC capacity in response to UV irradiation showed a circadian rhythm. Interestingly, clock-deficient Cry1 and Cry2 double knockout (Cry^{DKO}) cells retained substantial DDC capacity compared with clock-proficient wild-type cells, although the Cry1-modulated oscillation of the DDC capacity was abolished in Cry^{DKO} cells. We found temporal interaction of Cry1 and Tim in the nucleus. When Cry1 was expressed in the nucleus, it was critical for circadian ATR activity. We regenerated rhythmic DDC responses by ectopically expressing Cry1 in Cry^{DKO} cells. In addition, we also investigated the DDC capacity in the liver of mice that were intraperitoneally injected with cisplatin at different circadian times (CT). When mice were injected at CT20, about 2-fold higher expression of phosphorylated minichromosome maintenance protein 2 (p-MCM2) was detected compared with mice injected at CT08, which consequently affected the removal rate of cisplatin-DNA adducts from genomic DNA. Taken together, our data demonstrate the intimate interaction between the circadian clock and the DDC system during genotoxic stress in clock-ticking cells.

INTRODUCTION

The circadian clock is a molecular time-keeping mechanism that orchestrates daily oscillation of behavior and biochemical processes (1–3). The mammalian circadian clock is composed of an autoregulatory transcriptional network in which Clock–Bmal1 heterodimers activate transcription of cryptochromes (Cry1 and Cry2) and periods (Per1–3) (2,4). After a time delay, Cry–Per heterodimers enter the nucleus to repress Clock–Bmal1 activity, forming a transcriptional and translational negative feedback loop (5,6) that confers daily rhythmicity of physiological functions for homeostatic maintenance of the organism.

Apart from their canonical clock function, the mammalian clock factors have also been implicated in diverse non-canonical physiological functions such as the DNA damage response (DDR), which includes cell cycle checkpoint(7–10) and DNA repair(11,12). The Clock–Bmal1 complex controls transcription of genes involved in genotoxic responses (10,13). In addition, clock proteins directly interact with and activate DNA damage checkpoint (DDC) factors for maintaining genomic integrity (8,14). At the core of the DDC signaling is a pair of checkpoint kinases, ataxia telangiectasia mutated (ATM)-Chk2 and ATM and Rad3-related (ATR)-Chk1, which are activated by DNA double-strand breaks (DSBs) and single-strand breaks, respectively.

Ultraviolet (UV) light mainly produces two types of DNA lesions: cyclobutane pyrimidine dimers and 6,4-photoproducts. These lesions primarily activate the ATR-mediated DDC pathway, and nucleotide excision repair (NER) is the sole mechanism in mammals to restore the lesions via an error-free mechanism. Recently, we and others demonstrated that xeroderma pigmentosum group A (XPA), the rate-limiting factor in the NER process, is a clock-controlled gene in which the high-amplitude oscillation of mRNA and protein expression confers daily oscillation of NER activity over the course of a day in various mouse organs (11,12). In addition, recent results showed that phosphorylation of XPA by ATR is critical for XPA stability and subsequent NER activity (15,16). Because the clock modulates temporal NER activity, the ATR pathway may also be affected by clock activity. However, this possibility has not been assessed yet.

Mammalian *Timeless* (*Tim*) shares sequence homology with *Drosophila Tim*, which is a dedicated clock factor in insects. In mammals, however, *Tim* plays roles in cell cycle regulation and DDR (9,17–20). First, the Tim-Tipin (Tim-interacting protein) complex has been shown to be a crucial mediator of the ATR-Chk1 pathway; depletion

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of *Tim* results in compromised Chk1 phosphorylation(9,21,22). Second, Tim also mediates the ATM-Chk2 pathway and activates the subsequent G2/M checkpoint in response to DNA DSBs (23). Consequently, down-regulation or gene knockout of *Tim* results in chromosome instability and defects in cell growth and development, leading to embryonic lethality (23–25). However, the engagement of the circadian clock in the Tim-ATR-Chk1 axis has not been determined in mammalian cells.

Here, we generated a robust circadian rhythm in mouse fibroblast cells and also in mouse liver to investigate the kinetics of the ATR-mediated checkpoint response by measuring p-Chk1 and p-p53 levels as a function of circadian time (CT). We found that the DDC activity oscillated for a 24-hour period with ~2- to 3-fold difference between the zenith and nadir time, based on Cry1 expression. In mouse liver, p-Chk1 and p-p53 were below the level of detection following a sub-lethal dose of cisplatin. Instead, we were able to detect the level of phosphorylated minichromosome maintenance protein 2 (p-MCM2), another well-characterized substrate of ATR, which was affected by the timing of the cisplatin injection. Moreover, we found that such temporal ATR activation was altered, depending on the nuclear Cry1-Tim interaction that was augmented by DNA damage. Taken together, our results demonstrate that Cry1 is a time-gated modulator of the ATR-mediated DDC response.

MATERIALS AND METHODS

Cell culture, circadian synchronization, UV irradiation and transfection

NIH3T3 (American Type Culture Collection), wild-type mouse embryonic fibroblasts (WT MEFs) and Cry^{DKO} MEFs (gift from Dr KJ Kim, Seoul National University) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin. For circadian synchronization, confluent cells were maintained for an additional 3 days and treated with 10-μM forskolin (Sigma) until harvest, as previously described (26). For UV-C irradiation, confluent cells were exposed to 5 J/m^2 dose using a germicidal lamp (GE) emitting primarily UV-C light. A UV-C sensor (UV Products) was used to calibrate the fluence rate of the incident light. Lipofectamine 2000 (Invitrogen) was used according to the manufacturer's directions for transfection of DNA constructs and ON-TARGETplus SMARTpool small interfering RNA duplexes {Dharmacon; [*Cry1* (L-040485), *Cry2* (L-040486), *Per2* (L-040489) and *cyclophilin-B* (D-001820) as a control)]}.

Animal handling and preparation of liver tissue

C57BL/6J male mice (8–10-week-old) were maintained on a light and dark (LD) 12:12 schedule for at least 2 weeks before killing. ZT0 is the time of lights on (07:00 h), and ZT12 is the time of lights off (19:00 h). For the DD experiments, mice that were maintained under LD were kept in constant darkness for an additional day. For animals under DD, 0700 was considered CT0 (35 h in DD) and 1900 was considered CT12 (47 h in DD). The mice were handled in accordance with the guidelines of Dong-A University. At the indicated times, the mice were killed by exposure to carbon dioxide, and their livers were removed, diced into pieces smaller than 2 mm, washed extensively with cold phosphate buffer saline (PBS) and then frozen in liquid nitrogen. Frozen liver was pulverized using a porcelain mortar and pestle in liquid nitrogen, and total cell lysates were prepared as described previously (11).

Immunoblotting and immunoprecipitation

Whole-cell lysates (27) and cytoplasmic and nucleoplasmic extracts (28) were isolated as described previously. A chromatin-enriched fraction was prepared using the pellet obtained following extraction of the nucleoplasm by treatment with 10-U DNase I (Promega) and 100-U micrococcal nuclease for 30 min at 30°C. Each fraction was analyzed with SDS–PAGE followed by Ponceau S staining and immunoblotting. Conventional immunoblotting procedures were used to determine the levels of proteins. Quantification of immunoblotting was performed using ImageQuant 5.2 software (Molecular Dynamics). Antibodies used in this study include GAPDH, p-Chk1 S345, p53, p-p53 S15 (Cell Signaling Technology), ATR, RPA70, Chk1, Actin (Santa Cruz Biotechnology), Clock, Cry1, Cry2, p-MCM2 S108, Timeless, Tipin (Bethyl Laboratory), histone H3 (Millipore), Per2 (BD Biosciences) and platinum GpG (Oncolyze). For analyzing the interaction between Tim and Cry1, Tim protein complexes were precipitated using anti-Tim antibody preassembled with protein A/G-coupled sepharose (Sigma), followed by immunoblotting to detect Cry1 that was coprecipitated with Tim.

Immunocytochemistry

For immunofluorescence staining, WT MEFs were cultured to 50–60% confluency on 15-mm glass chips coated with poly-D-lysine and laminin (BD Biosciences). Cells were treated with 10-μM forskolin for the indicated times in Figure 3B, followed by standard procedures as previously described (29).

Immunoslot blotting

Genomic DNA from liver tissue was prepared using a QIAamp DNA Mini kit (Qiagen), and 1 μg DNA was vacuum-transferred to nitrocellulose membrane using a BioDot SF Microfiltration apparatus (BioRad). DNA was cross-linked to the membrane by incubation at 80°C for 2 h under vacuum. A monoclonal antibody that recognizes platinum-GpG adducts (Oncolyze) was used to detect the remaining amount of platinum in the genomic DNA. After the immunoslot blot assay, the total DNA amounts loaded onto the membrane were visualized with Sybr-gold staining, and these values were used for normalization.

RESULTS

The recent finding that the clock controls DDRs including DNA repair, prompted us to investigate the role of the

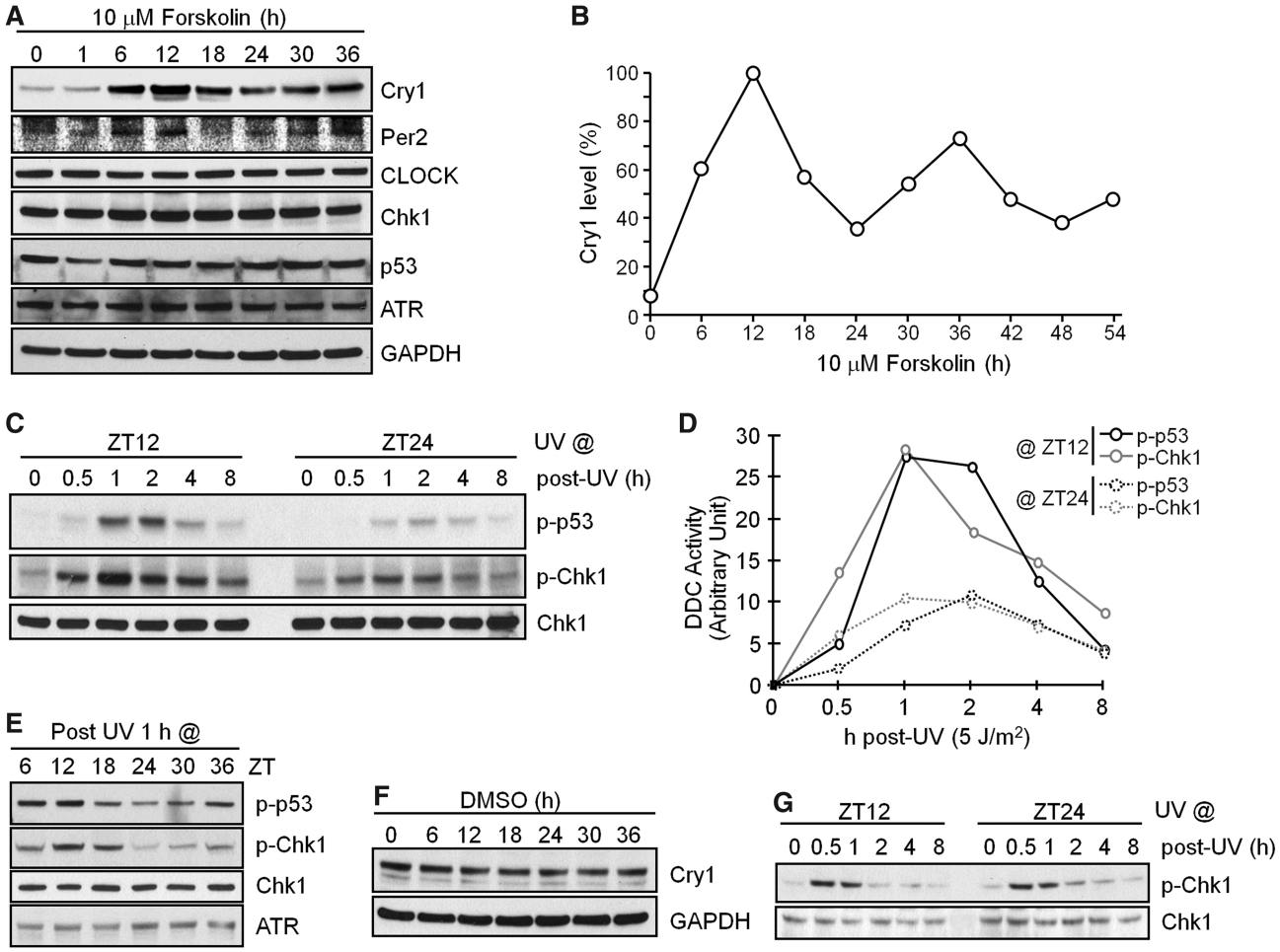

Figure 1. Circadian control of UV-induced checkpoint response. (**A**) Protein levels from forskolin-induced circadian-synchronized NIH3T3 cells were analyzed with the indicated antibodies. (**B**) Quantitative analysis of Cry1 expression measured in (A). Data points represent averages from three independent experiments. (**C**) Kinetic analysis of p-p53 and p-Chk1 after UV exposure at the indicated ZTs. (**D**) DDC activity was plotted using data obtained in (C). Data points represent averages from three independent experiments. (**E**) DDC activity was measured at the indicated ZTs. ATR and Chk1 were used as loading controls. (**F**) Dimethylsulfoxide, the solvent control for forskolin, was used as in (A), and the Cry1 levels at the indicated times were analyzed. (**G**) p-Chk1 kinetics were measured in dimethylsulfoxide -treated cells at the indicated ZTs.

clock in other types of DDRs, in particular, the cell cycle checkpoint. Because the ATR-Chk1 pathway is the key signaling event activated by any type of DNA damage for cell cycle arrest or a delay to secure repair time, we focused on the ATR pathway to see whether it is affected by clock activity. To generate a circadian rhythm of gene expression in fibroblasts, we used the forskolin-induced synchronization method as established previously (26). As shown in Figure 1A and B, forskolin treatment generated rhythmic Clock/Bmal1 activity in NIH3T3 cells, which resulted in a robust circadian oscillation of Cry1 and Per2 as readouts for clock activity. Other proteins we analyzed displayed no such rhythmicity (Figure 1A). To determine whether clock activity affects the ATR pathway, cells were irradiated with UV at ZT12 (ZT0: the time of forskolin treatment) and ZT24 when the clock showed maximal and minimal activity, respectively, and measured the phosphorylation levels of p53 and Chk1 as surrogate markers for the ATR-mediated checkpoint response. Interestingly, distinct phosphorylation levels were observed according to the time of UV exposure (Figure 1C). Cells irradiated at ZT12 showed ~2- to 3-fold higher phosphorylation than cells irradiated at ZT24 (Figure 1D). To confirm whether this response is indeed mediated by rhythmic clock activity, we measured the intensity of the phosphorylation at various time points. Because both p-p53 and p-Chk1 showed their maximal intensity nearly 1 h after 5 J/m^2 of UV exposure, we collected cells 1 hour after UV exposure and analyzed the phosphorylation levels. We observed approximate circadian oscillation of p-p53 and p-Chk1 (Figure 1E) as a function of time, which suggests that clock activity is involved in the output of ATR signaling on genotoxic stress. Because ATR (Figure 1A and E), p53 and Chk1 (Figure 1A) levels had not changed during the circadian synchronization, we assumed that the oscillatory phosphorylation was not caused by rhythmic expression of these proteins. In addition, when we analyzed the ATR signaling in cells treated with dimethylsulfoxide, the solvent control for forskolin (Figure 1F) as in Figure 1A

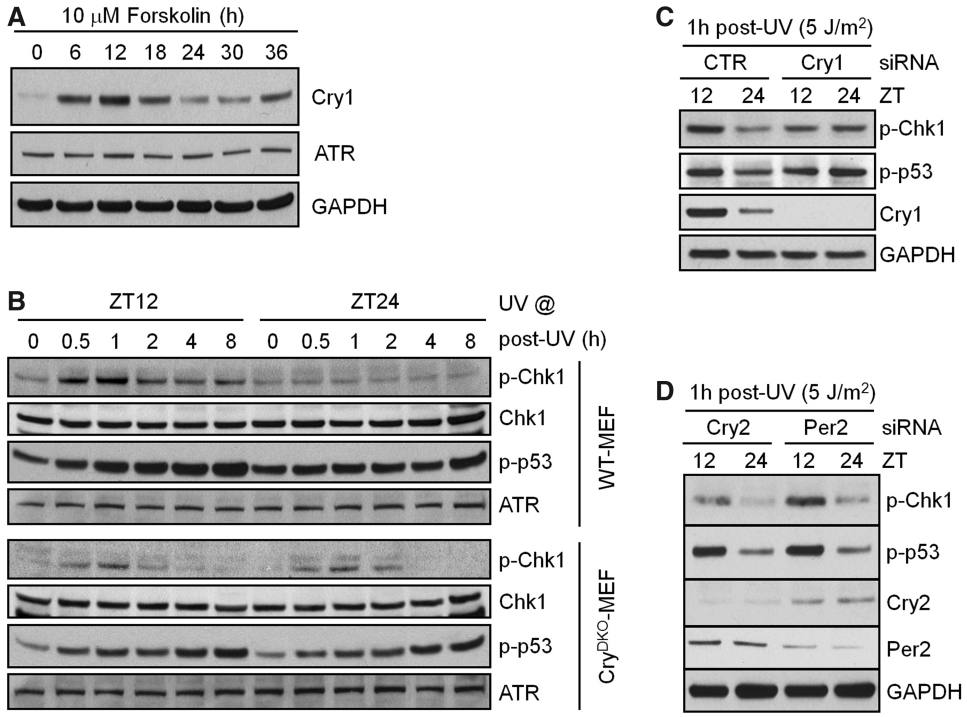

Figure 2. Cry1 mediates circadian ATR activity. (**A**) Protein levels from forskolin-induced circadian-synchronized MEFs were analyzed with the indicated antibodies. (**B**) Kinetic analysis of p-p53 and p-Chk1 after UV exposure at the indicated ZTs in WT MEFs and Cry^{DKO} MEFs. (**C**) DDC activity on UV damage was measured at the indicated ZTs in cells treated with either siControl or siCry1. (**D**) DDC activity on UV damage was measured as in (**C**) in cells treated with either siCry2 or siPer2.

and C, we did not detect distinct phosphorylation at different times after UV exposure (Figure 1G). Thus, clock activity appears to modulate ATR signaling evoked by UV damage.

To ascertain whether ATR signaling is indeed affected by the clock, we compared the phosphorylation rates in clock-proficient wild-type (WT) mouse embryonic fibroblasts (MEFs) and in clock-deficient Cry1 and 2 double knockout (Cry^{DKO}) MEFs. Similar to NIH3T3 cells, WT MEFs exhibited robust circadian Cry1 expression in response to forskolin at the zenith at ZT12 and nadir at ZT24 (Figure 2A), and showed distinct ATR activity according to the time of UV exposure (Figure 2B). In contrast, Cry^{DKO} MEFs showed constant ATR activity regardless of the time of DNA damage, although substantial ATR activity was detected (Figure 2B). Thus, rhythmic clock activity is required for temporal ATR activity. Intriguingly, depletion of *Cry1* alone using small interfering RNA in WT MEFs was sufficient to abolish clock-dependent ATR activity (Figure 2C), implying that Cry1 is the mediator in the clock-modulated ATR activation. Because depletion of other core clock components, including *Cry2* and *Per2*, had little effect on the temporal ATR activation (Figure 2D), we conclude that in response to UV damage, Cry1 mediates temporal ATR activation in clock-ticking cells.

Next, we wanted to understand the underlying mechanism of Cry1 modulation of the ATR pathway. A recent report showed that Cry2 interacts with Tim, leading to ATR-dependent phosphorylation of Chk1 and subsequent cell cycle arrest (9). Here we performed immunoprecipitation analysis to check for an interaction between Tim and Cry1 in the absence and presence of DNA damage at different times after damage. In the absence of DNA damage, the amount of Cry1 that was pulled down with Tim was similar in WT MEFs, regardless of the CT (Figure 3A). However, on genotoxic damage, the amount of Cry1 interacting with Tim was increased relative to the no-damage condition. In addition, the amount of Cry1 differed according to the time after damage (Figure 3A). At ZT12 when Cry1 expression was high, more Cry1 interacted with Tim compared with the amount of Cry1 at ZT24 when the Cry1 level was low. Because Tim is required for proper ATR activation, we hypothesized that Cry1 may function as a time-gated linker for the Tim-ATR interaction. We analyzed the subcellular localization of Cry1 and Tim, and detected Tim expression exclusively in the nucleus, regardless of the CT, whereas Cry1 was localized in the nucleus at ZT12 and mostly in the cytoplasm at ZT24 (Figure 3B). Thus, the interaction between Cry1 and Tim in the nucleus may be involved in temporal ATR activation. To address this possibility, we ectopically expressed Cry1 in Cry^{DKO} cells and analyzed the localization of Cry1 and ATR activity on UV damage. As expected, more nuclear Cry1 was detected at ZT12 than at ZT24, and more Cry1 was coprecipitated with Tim at ZT12 than at ZT24 (Figure 3C). Concomitantly, ATR activity was upregulated at ZT12 compared with ZT24 as determined by the level of p-Chk1 and p-p53 (Figure 3D). Taken

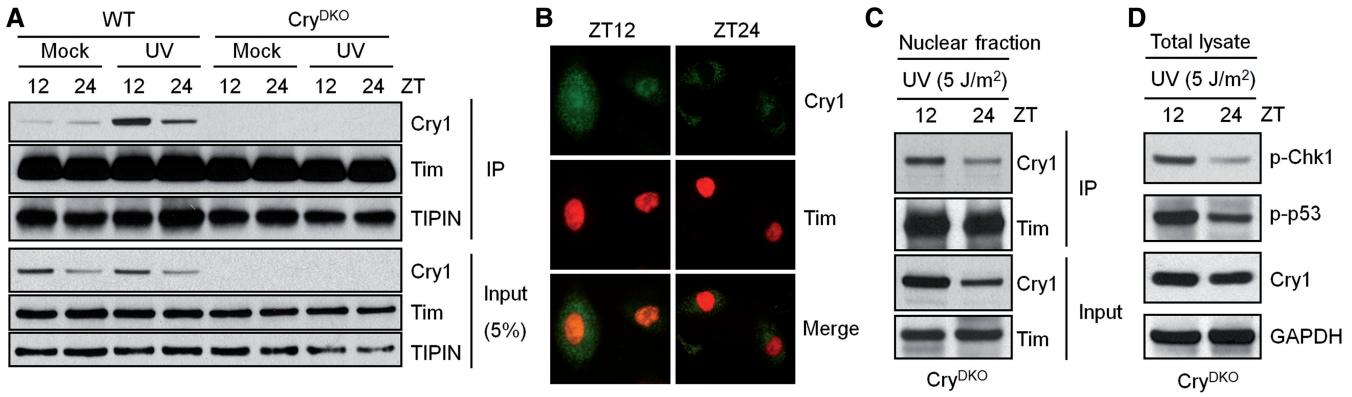

Figure 3. Physical interaction between Cry1 and Tim on DNA damage is critical for circadian checkpoint activation. (**A**) The amount of Cry1 and Tim complexes was analyzed using immunoprecipitation in the presence and absence of UV damage at the indicated ZTs. Five percent of total protein lysates used for immunoprecipitation was analyzed to detect the indicated protein level. (**B**) Subcellular localization of Cry1 and Tim at the indicated ZTs was analyzed using immunofluorescence. (**C**) Cry1 was ectopically expressed in Cry^{DKO} cells, and then the Cry1 level coimmunoprecipitated with Tim at ZT12 and ZT24 was analyzed in the nuclear fraction. (**D**) DDC activity at the indicated ZTs was measured after Cry1 overexpression in Cry^{DKO} cells.

together, we conclude that nuclear Cry1 mediates clock-controlled ATR activation through temporal interaction with Tim.

Next we examined the Cry1-modulated DDC response in mouse liver. The liver metabolizes the chemotherapeutic agent cisplatin, which activates the ATR pathway (30). In mice, the circadian rhythm was induced using a light/dark lighting regimen, resulting in a robust circadian rhythm of Cry1 expression (Figure 4A). We chose CT08 (CT, where CT0 = subjective dawn and CT12 = subjective dusk) and CT20 for cisplatin injection because pharmacodynamic data for cisplatin indicate that ~2 h is needed for the liver to take up the drug following intraperitoneal injection (31,32). Thus, cisplatin is expected to affect the liver at CT10 and CT22 when Cry1 expression is the lowest and highest, respectively (Figure 4B). However, we did not detect any significant signal for p-Chk1 and p-p53, presumably because of the low levels of expression of these two proteins in mouse liver (Figure 4C). Instead, we detected the other ATR substrate, p-MCM2 S108, which showed a significantly different profile according to the time of cisplatin injection (Figure 4C). Quantitative analysis indicated that after 8 h of cisplatin treatment, the mice treated at CT20 showed ~2-fold stronger p-MCM2 signal than those treated at CT08 (Figure 4D). Thus, according to the Cry1 levels, liver cells responded more sensitively to genotoxic stress through the ATR pathway.

Because the ATR pathway participates in DNA repair, especially NER (33,34), we measured NER activity using an immunoslot blot assay. As shown in Figure 4E, the removal kinetics of platinum-GpG adducts at CT08 was faster than that at CT20, although the shape of the two plots appeared similar (Figure 4F). Consistent with these data, we have previously shown that NER activity of cisplatin-DNA adducts in mouse liver exhibited a robust circadian rhythm with the zenith near CT10 and the nadir near CT22(12). We further showed that this oscillation is caused by the circadian rhythmicity of XPA expression that was antiphase with Cry1 expression (12). In the meantime, replication protein A (RPA) accumulation was detected on chromatin after cisplatin injection at CT20 (Figure 4G), which implicates sustained ATR activation at CT20 that was presumably because of the longer exposure of NER-processed RPA-coated single-strand gaps, which are the unique substrates of ATR in normal cells(35,36).

DISCUSSION

The importance of the clock has been recognized with increasing enthusiasm during the past decade. Many key physiological mechanisms are subject to clock regulation, ranging from the cell cycle to metabolism. Recent evidence suggests that clock proteins are also implicated in DDRs, and thus, failure of this can result in genomic instability and tumorigenesis (37–39). For instance, mice with a homozygous mutation of *Per2* show deregulated *cyclin D* and *c-Myc* expression and spontaneous tissue hyperplasias and lymphomas (40). However, in cancer-prone mice such as *p53*-null mice, additional deletion of mammalian *Crys* delays the onset of cancer caused by the absence of the tumor suppressor *p53*, making the role of the clock in tumorigenesis controversial. Importantly, when tumor xenografts induced by *p53*-deficient and *p53-Cry*-deficient cells are treated with the chemotherapeutic agent oxaliplatin, tumor growth is suppressed in *p53-Cry*-deficient tumors, but no therapeutic effect is seen on the growth of *p53*-deficient tumors (41). These data provide a plausible mechanism for the sensitization of tumor cells that are often deficient in *p53* function to chemotherapeutics through activation of a *p73*-dependent apoptosis mediated by Clock/Bmal1 transcriptional activators (42).

Although transcriptional regulation by the clock partially affects tumorigenesis, recent findings now suggest that each clock factor may play a specific role in DDR by participating as a key modulator. For instance, Per1 interacts with the ATM/Chk2 complex to regulate DNA damage induced by agents such as ionizing radiation that

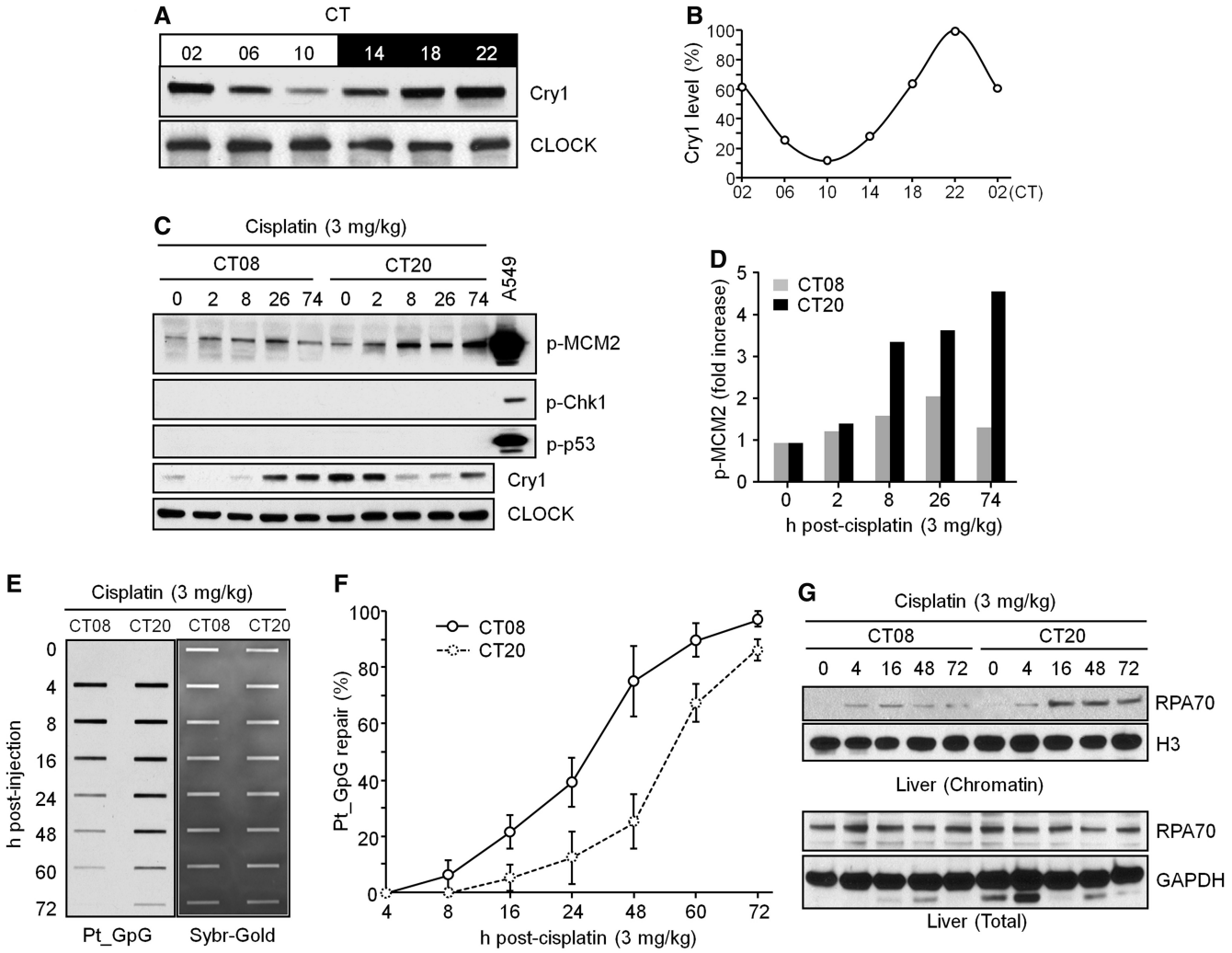

Figure 4. The Cry1 level dictates checkpoint activity and DNA repair. (**A**) Expression of Cry1 and Clock was measured in mouse livers taken at the indicated CTs. (**B**) Quantitative analysis of Cry1 expression measured in (A). Data points represent averages from three independent experiments. (**C**) Kinetic analysis of p-MCM2 after cisplatin injection at the indicated CTs. (**D**) DDC activity was plotted using p-MCM2 data obtained in (C). Data points represent averages from three independent experiments. (**E**) Cisplatin was injected intraperitoneally at the indicated CTs, and the platinum-GpG repair kinetics were analyzed using immunoslot blotting. (**F**) DNA repair activity was plotted using data obtained in (E). Data points represent averages and standard deviations from three independent experiments. (**G**) RPA70 on the chromatin of liver cells after cisplatin treatment at the indicated CTs was analyzed. Histone H3 was used as a loading control for the chromatin fraction.

cause DNA DSBs (8). Importantly, *Per1*-depleted cells are impaired in ATM activation and subsequent Chk2 phosphorylation. Consequently, the downregulation of *Per1* in human tumor cells makes them more resistant to anticancer drugs (8,43). Another report on the interconnection between the components of the circadian clock and DNA damage modulators involves Tim and the ATR/Chk1 complex, which affects DNA replication and cell cycle progression (44). A recent study showed that Per2 competed with Tim for binding to Cry1 to control clock speed and resetting (45). In Figure 2D we found a slight, but significant, increase of Chk1 phosphorylation in the absence of *Per2* in Figure 2D, possibly through increased Cry1–Tim interaction. Because the ATM and ATR pathways are major cancer targets in many pharmaceuticals, specific circadian clock factors may be considered potential targets for pharmacological manipulations directed toward alleviating cellular defects caused by DNA damage.

Recent works have established a distinct connection between the circadian clock and the DNA repair system NER. NER of a UV photoproduct displays daily oscillations in the mouse brain and liver with maximum and minimum values at CT6 and CT18, and CT10 and CT22 for the brain and liver, respectively (11,12). Interestingly, in both tissues, the maximum NER activity coincides with the light phase of the cycle, which may reflect adaptation to UV in sunlight. In the brain, NER activity also coincides with daily oscillations in the levels of reactive oxygen species resulting from brain metabolic activity (46). This is not surprising given the fact that although UV light and reactive oxygen species produce different types of lesions, they both are removed by the NER system. In addition to UV- or oxidative stress-induced lesions, the NER system

is also capable of removing intrastrand diadducts (platinum-GpG and platinum-GpXpG) caused by treatment with cisplatin compounds. Cisplatin is a chemotherapeutic drug widely used to treat various types of cancers (47). The repair of cisplatin-induced DNA damage displays daily oscillations in liver extracts with maximum and minimum activities at CT10 and CT22, respectively (12). Interestingly, NER activity appears to be constant in testis, an organ that does not demonstrate prominent circadian oscillation. NER activity is constitutively high in the livers of Cry-deficient mice. The latter result indicates that this repair system is activated by disruption of the clock through Cry deficiency and suggests that the circadian clock downregulates the activity of NER at certain times of the day. In the current study, we showed that *in vivo* platinum-GpG repair was more efficient at CT08 than CT20, which coincided with previous *in vitro* data showing better repair activity at CT10 than at CT22(12). Because Cry-deficient mice display no rhythmic NER activity (12), we believe that time-dependent NER activity is mainly controlled by Cry expression. Recently, we demonstrated that in lung cancer cells, ATR upregulates NER activity by stabilizing XPA through ATR-mediated phosphorylation of XPA (16). If this kind of regulation also occurred in mouse liver, one can expect stronger NER response at CT20 than at CT08. However, this has not been observed in mouse liver presumably because of the different activity of ATR in cancer cells and normal cells. ATR activity is much stronger in highly proliferating cells than in quiescent cells, and this may explain the reason we could not detect p-Chk1 and p-p53 expression in mouse liver in Figure 4C. Thus, this newly discovered link between the circadian clock factors Crys and the DNA repair system NER provides an invaluable tool for therapeutic applications.

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Conflict of interest statement. None declared.

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