

The Impact of the Circadian Clock on Toxic and Carcinogenic Responses:

It's about time!

Romana Mercelyne Nijman

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The Impact of the Circadian Clock on Toxic and Carcinogenic Responses:

It's about time!

Het effect van de circadiane klok op toxische en carcinogene responsen:

Het werd eens tijd!

proefschrift

ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus

Prof.dr. H.G. Schmidt

en volgens besluit van het College van Promoties.

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Prof.dr. P.J. van der Spek
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For my hero, sidekicks and everyone who has been told you cannot....

"The greatest pleasure in life is doing what people say you cannot do."

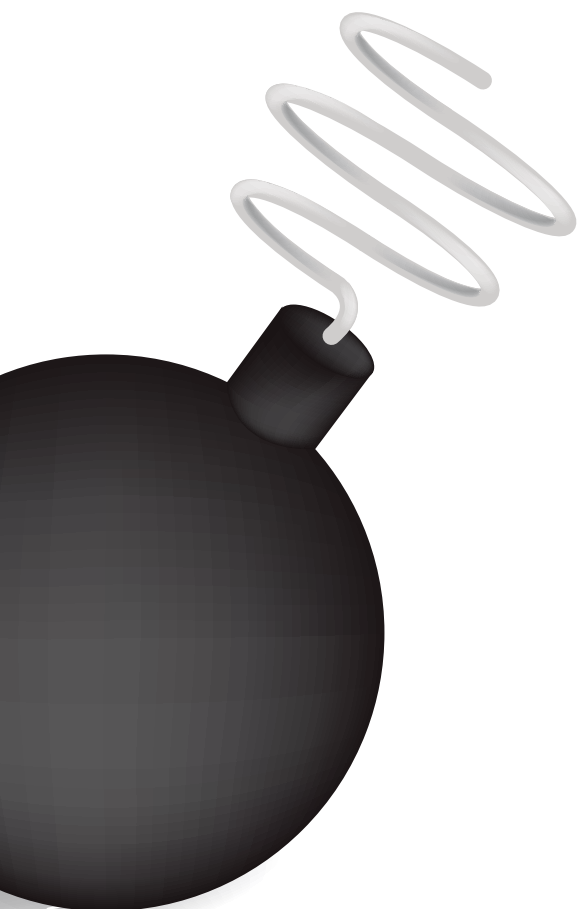
Walter Bagehot

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Chapter 1

General introduction



Annelieke S. de Wit, **Romana M. Nijman**, Eugin Destici, Ines Chaves, and Gijsbertus T.J. van der Horst

Part of this introduction will be published in

"Toxicogenomics-Based Cellular Models: Alternatives to Animal Testing for Safety Assessment"

(Ed. J Kleinjans, Elsevier).

In preparation

Chapter 2

**Toxicogenomics and the circadian clock:
Impact of time of day of exposure on the hepatic
transcriptome of cyclophosphamide exposed mice**



Kirsten C.G. van Dycke*, **Romana M. Nijman***, Paul F.K. Wackers, Martijs Jonker, Wendy Rodenburg, Conny T.M. van Oostrom, Timo M. Breit, Harry Van Steeg, Mirjam Luijten, and Gijsbertus T.J. van der Horst

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In preparation

Chapter 3

**Chronotoxic effects of benzo[a]pyrene,
assessed in cultured liver slices**



Romana M. Nijman, Cesar E. Payan Gomez, Paul Wackers, Annelieke S. de Wit,
António Carvalho da Silva, Inês Chaves and Gijsbertus T.J. van der Horst

In preparation

Chapter 4

Influence of circadian core clock genes on the cancer properties of H1299 human non-small lung carcinoma cells

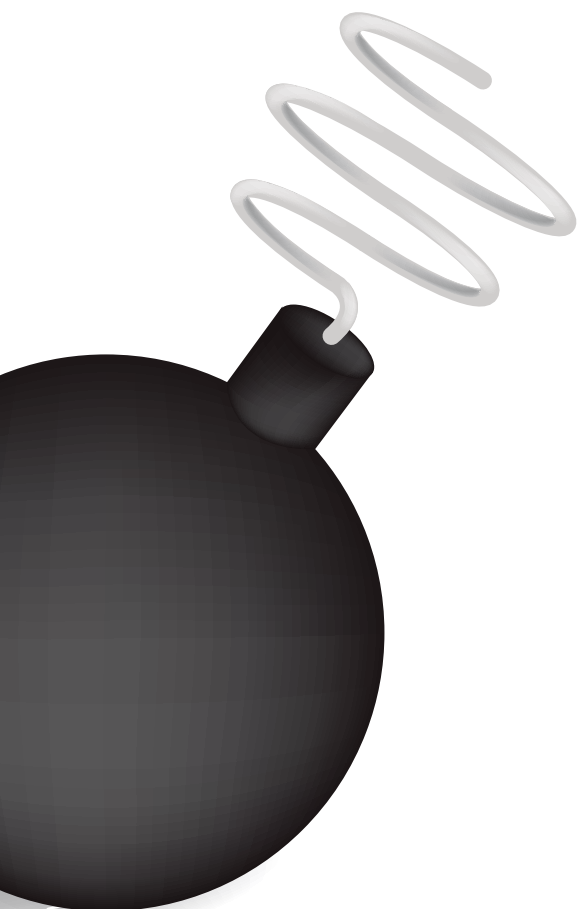


Romana M. Nijman, Cintia R. Bombardieri, Roel Janssens, Stefanie Vester, Yanto Ridwan, Filippo Tamanini and Gijsbertus T.J. van der Horst

In preparation

Chapter 5

Discussion



Discussion

The circadian clock is responsible for generating daily rhythms in our behavior, physiology and metabolism (Albrecht and Eichele, 2003; Pittendrigh, 1993). Many biological processes such as cell cycle regulation, apoptosis, cell metabolism and detoxification are under circadian control (Matsuo et al., 2003; Miller et al., 2007; Lavery et al., 1999). Therefore it might not come as a surprise that the toxic response of cells to compounds may depend on the moment of exposure (e.g. morning vs. evening), a phenomenon that is referred to as chronotoxicity (Lévi and Okyar, 2011; Lévi and Schibler, 2007). Nowadays, many toxicity studies and risk assessment assays use modern omics-based technologies to investigate and identify differences in the biological response of cells exposed to environmental chemical compounds. However, despite the fact that temporal (i.e. daily) variations in gene expression, protein and metabolite levels can be expected to influence the outcome of an omics-based experiment, the circadian clock is usually not considered.

In **Chapter 2** we demonstrated the impact of the circadian clock on transcriptomics experiments by demonstrating that the time of exposure has a dramatic effect on the transcriptional response. To achieve this, we used cyclophosphamide (CP), a chemotherapeutic agent with known chronotoxic properties, as a model compound (Gorbacheva et al., 2005). Previous studies demonstrated that the functional status of the CLOCK/BMAL1 complex (activation of the E-box promoter element-containing clock and clock-controlled genes) was responsible for the sensitivity or tolerance seen in mice after exposure to CP (Gorbacheva et al., 2005). Transcriptome analysis performed on the livers of mice exposed to CP at the moments where animals were found to be sensitive and tolerant (ZT8 and ZT20, respectively), revealed large differences in the number of differentially expressed genes (DEGs) and pathways, with the strongest and weakest response correlating with tolerance and sensitivity towards the drug, respectively. In addition, we have shown that the use of time-matched control samples dramatically reduces the number of false positive DEGs by eliminating clock controlled non-CP responsive genes. This highlights the importance of the circadian clock when performing omics-based experiments to identify biomarkers for biological and toxic response.

The circadian system is known to activate the transcription of clock controlled genes (CCGs), such as genes involved in metabolism and detoxification processes, (e.g. phase I and II and III enzymes) (Lévi and Schibler, 2007), as well as DNA repair, apoptosis, and DNA damage checkpoint control genes (Fu et al., 2002; Gorbacheva et al., 2005; Gery et al., 2006; Kang et al., 2009; Sancar et al., 2010). These biological processes might play a role in the chronotoxic properties of CP or other (chemotherapeutic) compounds. Studies performed on the cerebellum and liver revealed that one of the nucleotide excision repair (NER)

factors that recognize the DNA damage, XPA, is under circadian control and is directly regulated by the CLOCK/BMAL1 complex (Kang et al., 2009; Sancar et al., 2010). The XPA protein was demonstrated to show a robust circadian oscillation, which is anti-phase to CRY1 (Kang et al., 2010). The maximal CP sensitivity was seen at the moment where XPA activity was at its lowest, which coincides with the phase at which CLOCK/BMAL1 transcriptional activation levels were at its lowest (early morning/afternoon) (Gorbacheva et al., 2005; Kang et al., 2009, 2010). This might suggest that the maximum and minimum repair capacities of NER are responsible for the sensitivity and tolerance to CP at defined moments during the day.

In our study, mice were exposed to a single dose of CP for 4 hours and immediately sacrificed afterwards. Gorbacheva and coworkers demonstrated a clear difference in the survival rate of mice that were chronically exposed to CP for 3 days at defined time points. It is not known what kind of damage CP exposure may cause within 4 hours and whether this is enough to already see any chronotoxic effect. To understand more about the chronotoxic effects of CP, it might be useful to study the CP-induced transcriptional response in circadian clock-deficient mice, for instance in *Cry1/Cry2* double knockout animals. These mice, in which the E-box element containing genes are constitutively active, have been demonstrated to be resistant to CP at any moment of the day (Gorbacheva et al., 2005), and might therefore provide more information about the chronotoxic properties of CP.

To get a full understanding of the chronotoxic properties of any compound it might be useful to not only study the liver, but also other organs and tissues, such as kidney, stomach, lungs, blood and bone marrow. Moreover, in addition to transcriptomic analysis, one might even consider using metabolic cages, making it possible to collect the feces and urine of the animals in order to study the detoxification processes and metabolites that are secreted from the body. As animal exposure studies in chemical risk assessment are considered undesirable due to high cost and the use of many laboratory animals, society wants us to replace these *in vivo* assays by *in vitro* systems. To date many of these alternative systems make use of modern omics technology. However, in most of these *in vitro* toxicogenomic studies, the circadian clock is also not considered. In order to obey the 3R principle of legislation (replacement, refinement and reduction of animal testing), we decided to develop an *in vitro* assay that allows us to study the chronotoxic properties of chemical compounds, and that might provide us with a quick screening assay for risk assessment. In **Chapter 3** we designed a liver-based *ex vivo* chronotoxicity assay that allows us to screen (hepato)toxic compounds for their (chrono)toxic properties. In order to set up an *in vitro* or *ex vivo* chrono(hepato)toxicity assay, these systems must fit a couple of requirements, such as the ability to follow the circadian clock and the capacity to metabolize compounds into their active form (*i.e.* expressing cytochrome P450 (CYP-450) phase

I, II and III enzymes) (Xu et al., 2005). In our study, we used liver slices from the *Per2::Luc* clock reporter mouse model. Liver slices have been demonstrated to be a successful model system for toxicogenomics studies (Staal et al., 2007; Elferink et al., 2008). Moreover, liver slices maintain tissue structure, cell-cell and cell-matrix interactions, as well as expression of many xenobiotic enzymes (de Graaf et al., 2010; Boess et al., 2003; Moronville-Halley et al., 2005), making this system more beneficial compared to hepatocytes or established hepatoma cell lines (e.g. HepG2), which lose their differentiation in culture and were demonstrated to express low levels of xenobiotic enzymes (Ogino et al., 2002; Wilkening et al., 2003; de Graaf et al., 2010).

We have shown that liver slices display robust circadian oscillations that can be followed in time by real-time recording of the *Per2::Luciferase* reporter. To investigate whether liver slices can be used in a chronotoxicity assay, we have used benzo[a]pyrene (B[a]P) as a test compound. In previous studies, exposure to polycyclic aromatic hydrocarbons such as B[a]P and tetrachlorodibenzo-p-dioxin (TCDD), was shown to induce *Cyp1a1* in a circadian clock-dependent manner, with highest and lowest induction at the peak and trough of *Per2* (Tanimura et al., 2011; Qu et al., 2010; Tischkau et al., 2011). The aryl hydrocarbon receptor (AhR), responsible for the induction of many CYP-450 enzymes including *Cyp1a1*, contains a bHLH/PAS domain and is known to interact with the core clock component BMAL1 and has been shown to be rhythmically expressed in the lungs, liver and SCN (Hogenesch et al., 1997; Richardson et al., 1998). We showed that liver slices are a valuable tool to study chronotoxic effects of chemical compounds, and that in liver slices, the induction of *Cyp1a1* after B[a]P exposure is dependent on the moment of exposure (time of day), with the highest induction at CTr12 (peak *mPer2*) and the lowest at CTr24 /0 (trough of *mPer2*). By performing microarray analysis of liver slices exposed to B(a)P at those time points, we found differences in gene expression patterns, related biological functions and pathways, demonstrating the involvement of the circadian clock in the biological response of B[a]P. Chronotoxicity assays in either *in vivo*, *in vitro* or *ex vivo* systems might be very useful for risk assessment studies and may provide more insight determining the moment of highest risk when exposed to (geno)toxic compounds.

The circadian clock has demonstrated to be involved in many disorders and diseases such as cancer. Several epidemiological studies demonstrated that disruption of the circadian rhythm may lead to cancer in humans and in rodents (Schernhammer et al., 2001; Lee et al., 2010; Filipinski and Lévi, 2009). However, the involvement of the core clock genes in cancer progression is still poorly understood. In **Chapter 4** we investigated how downregulation of the circadian core clock genes affects the cancer properties (*i.e.* proliferation, migration and invasion capacity) and the response to genotoxic stress. We therefore systematically downregulated the core clock genes in the well-characterized non-small-cell lung carcinoma (NSCLC) cell line H1299. Our results demonstrate that knockdown of the

core clock genes has a significant effect on cancer properties. However, our results contradict with previous published studies, revealing that disruption of the clock is harmful and may lead to cancer (Filipski et al., 2003; Lee et al., 2010; Fu et al., 2002). The differences seen might be explained by the type of cell line used (e.g. cancer vs. normal cells) specific cell characteristics (e.g. status of cell cycle related genes), as well as on gene inactivation procedure (e.g. transient shRNA or siRNA transfection).

We also found significant differences in the genotoxic sensitivity of H1299 cells after stable downregulation of core clock genes. The circadian system is known to influence the DNA damage response and the core clock gene *Per1* is known to play a role in therein (Gery et al., 2006; Borgs et al., 2009). PER1 is shown to be directly involved in the cellular stress response, where it activates the DNA damage proteins ATM and phosphorylates CHK2 (Gery et al., 2006; Borgs, et al., 2009). This will eventually lead to a cell cycle arrest and DNA repair (Borgs et al., 2009). However, little is known about the involvement of the other core clock genes and proteins in the DNA damage response. Previous studies demonstrated that the loss of both *Cry* genes reduced cancer progression and improved the response to chemotherapeutic agents. However, this was only seen in a P53-deficient background (Ozturk et al., 2009; Lee et al., 2011). This phenotype is also seen after knockdown of both *Cry* genes in the H1299 cell line, since H1299 cells do not express the P53 protein. This suggests that in the absence of P53, the CRY proteins may play a significant role in the DNA damage response. Furthermore, after downregulating *Cry1* and *Cry2* in the H1299 cell line, we found a difference in the genotoxic stress response. After genotoxic exposure, *Cry1* knockdown cells appeared to be resistant when exposed to cisplatin and doxorubicin, while after *Cry2* knockdown, the cell appeared to be extremely sensitive. Our results suggest that the CRY2 protein plays a prominent role in the genotoxic stress response. These studies reveal a specific function for the CRY proteins outside the context of the clock, suggesting that this might also be the case for the other core clocks genes. By performing omics-based analysis of our panel of clock gene knockdown H1299 cells, we may be able to gain a better understanding of the involvement of the core clock genes on cancer progression in a particular cell line or mouse model.

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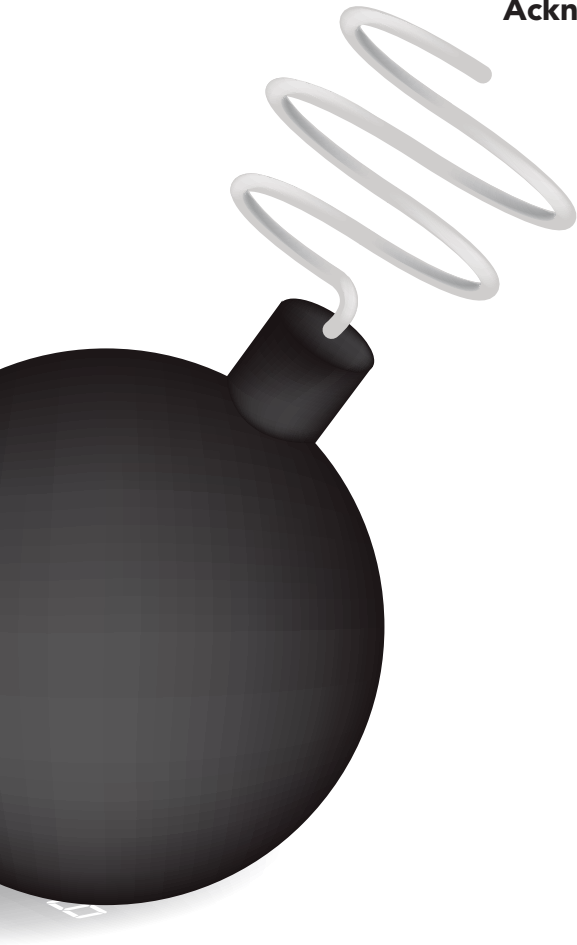
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Chapter 6

Summary
Samenvatting
Curriculum vitae
List of publications
Acknowledgement



Summary

Life on Earth is constantly exposed to annual, monthly and daily (24-hour) geophysical cycles. These cycles may cause temporal changes in the environment and affect most, if not all life forms. Of these cycles, the daily 24-hour day/night cycle has the most profound impact. To adapt to the day/night cycle, most organisms have developed an internal clock, the circadian clock (Latin, *circa*= about, *diem*= day), to impinge 24-hour rhythmic cycles to behavioral, physiological and metabolic processes (e.g., sleep-wake cycle, blood pressure, body temperature, hormone secretion) and allows to anticipate to the momentum of the day. In mammals, the circadian system is organized in a master clock the Suprachiasmatic nuclei (SCN), and peripheral clocks located in all organs and tissues throughout the body. The SCN is located in the hypothalamus, in the brain, and is responsible for the generation of circadian rhythms. The SCN is a small bilateral pair structure of 10.000 neurons that receive light signals from the environment via the retina and is therefore able to entrain the circadian system to the day/night cycle in order to keep the same phase as the environment. Via hormonal and neural factors, the SCN is able to synchronize the peripheral clocks to maintain a 24 hour rhythm.

At cellular level, circadian rhythms are generated by a molecular oscillator, composed of positive and negative transcription translation feedback loops. The CLOCK and BMAL1 protein, which are part of the positive loop, are responsible for the activation of the genes involved in the negative loop, *Period* (*Per*) and *Cryptochrome* (*Cry*) genes. The PER and CRY proteins are able to inhibit CLOCK/BMAL1 and thereby also repress their own transcription, which results in a circadian oscillation in gene expression with a periodicity of approximately 24 hours. The CLOCK/BMAL1 protein complex also activates cyclic transcription of genes that are not part of the molecular oscillator, but couple it to output processes. These clock controlled genes (CCGs) account for approximately 10% of the whole genome and is can be under direct or indirect control of the circadian oscillator. Many of these CCGs are involved in the cell-cycle progression, apoptosis, metabolism and detoxification, suggesting the involvement of the circadian clock in the (in)activation of genotoxic compounds and the DNA damage response. Indeed, the magnitude of the cellular response to (geno)toxic chemical compounds can depend on the moment (day/night) of exposure, a phenomenon nowadays referred to as chronotoxicity. Likewise, the efficacy and the severity of toxic side-effects of several chemotherapeutic agents has been shown to be more pronounced at defined moments over the day which may minimize the side effects and might therefore be very useful to treat cancer. However, many toxicological studies do not take the circadian clock (and therefore the daily variation in gene and protein levels) in consideration, which can influence the outcome of these studies. In **Chapter 2** we performed an exposure study in which mice were treated with cyclophosphamide (CP), a widely used chemotherapeutic

agent, at two define moments throughout the day (day/night). We found remarkable difference in the amount of differentially expressed genes (DEGs) and pathways when treated at those define time-points, demonstrating the importance of the circadian clock and its role in (geno)toxic stress response. These kinds of *in vivo* toxicological studies are very time and cost consuming, are requiring many animals, for which reason society wants us to replace, reduce and refine animal studies. To obey societies wishes, we decided to set up an *ex vivo* chronotoxicity assay. In **Chapter 3** we demonstrate that liver slices are an ideal model system to set up an *ex vivo* chronotoxicity assay. The use of liver slices has many advantages, it maintains similar structure as the liver *in vivo*, and liver slice express genes and proteins involved in xenobiotic activation such as Cytochrome P-450 enzymes (CYP-450). We decided to use Benzo[a]pyrene (B[a]P) a known environmental carcinogenic prodrug, which metabolic activation is under circadian control as test compound. The main P-450 enzyme involved in the metabolism and detoxification of B[a]P, *Cyp1a1*, is rhythmic expressed after B[a]P exposure that follows the circadian oscillation of *mPer2*. The liver slices were exposed to B[a]P and collected at the moment of maximal and minimal induction of *Cyp1a1*. Our results revealed differences in pathways and DEGs that were specific to the time of treatment. These results demonstrate that liver slices make a very useful system to study the chronotoxicity of compounds and that B[a]P has chronotoxic properties.

Disruption of the circadian rhythm by either genetic defects or by chronic disruption such as jet lag or shift work, has significant effects on human health and can lead to many disorders and diseases, such as cancer. Furthermore, the core circadian clock genes have been demonstrated to be involved in cancer progression. In **Chapter 4** we focus on the involvement of the core clock genes on cancer cell properties and tumor progression of the well characterize human non-small-cell lung carcinoma cell-line, H1299. By systematically downregulating the core circadian clock genes we analyzed specific cancer characteristics (proliferation, migration and invasion). Knockdown of the core circadian clock genes in the H1299 cell line had a significant effect on cell growth, cell migration and invasion, and genotoxic stress response. Our study suggests that disruption of the circadian clock in the H1299 cell line might be useful for the treatment of this type of cancer. This however our results contradicts with previous studies and suggests that there is still a lot unknown about the involvement of the core circadian clock genes and cancer.

Overall, we demonstrated that the circadian clock plays an important role in the toxicity and efficiency of (gen)toxic compounds, and that downregulation of the core clock genes in the H1299 cell line has a significant effect of the cancer characteristics and genotoxic stress response.

Samenvatting

Leven op aarde wordt continue blootgesteld aan jaarlijkse, maandelijks, en dagelijkse (24-uur) geofysische cycli. Deze cycli veroorzaken tijdelijke veranderingen in de omgeving die vrijwel alle levensvormen op aarde beïnvloeden. Van deze cycli heeft de dagelijkse dag/nacht cyclus het grootste effect. Om zich te kunnen aanpassen aan deze dagelijkse veranderingen hebben de meeste organismen een interne klok, circadiane klok (Latijns circa= ongeveer, diem= dag), ontwikkeld die zorgt voor een ongeveer 24 uur durende cyclus in gedrag, fysiologische en metabolische processen (bijvoorbeeld, slaap/wakkeritme, bloeddruk, lichaamstemperatuur, hormoon uitscheiding) die nodig zijn gedurende dag. Het circadiane systeem in zoogdieren is opgedeeld in een centrale klok in de suprachiasmatische nucleus (SCN), en in perifere klokken die zich bevinden in vrijwel alle organen en weefsels. De SCN bevindt zich in de hypothalamus in het hersengebied, en is verantwoordelijk voor het genereren van circadiane ritmes. De SCN bestaat uit een tweetal kleine bilaterale structuren van ongeveer 10.000 neuronen en ontvangt lichtsignalen uit de omgeving (dag/nacht cyclus) via de retina om zo dezelfde fase/ tijdsindicatie te behouden als de omgeving. Via hormonale en neuronale factoren kan de SCN de perifere circadiane klokken synchroniseren om zo ook een 24 uur ritme aan te houden.

Op cellulair niveau worden circadiane ritmes in zoogdieren gegenereerd door een moleculaire oscillator, bestaande uit positieve en negatieve transcriptie/translatie terugkoppelings cycli. Hier zijn de CLOCK en BMAL1 eiwitten, die deel uitmaken van de positieve cyclus, verantwoordelijk voor het activeren van de expressie van de *Period (Per)* en *Cryptochrome (Cry)* genen, die deel uitmaken van de negatieve loop. De PER en CRY eiwitten kunnen vervolgens hun eigen activiteit remmen door CLOCK/BMAL1 te onderdrukken, hierdoor ontstaan er genexpressies met circadiane oscillaties van ongeveer 24 uur. De CLOCK/BMAL1 eiwitten activeren ook genen die geen deel uitmaken van de moleculaire oscillator, maar die wel circadiane oscillaties vertonen. Deze klok gereguleerde genen zijn goed voor ongeveer 10% van alle genen van het gehele genoom, staan onder directe of indirecte controle van CLOCK/BMAL1, en koppelen de klok aan circadiane output processen, zoals metabolische en fysiologische processen. Veel van deze klok gereguleerde genen zijn betrokken bij een aantal biologische processen zoals de voortgang van de celdelingscyclus, apoptose (celdood), stofwisseling en ontgiftiging, en laat dus zodanig de betrokkenheid van de circadiane klok zien bij de (de)activatie van (geno)toxische stoffen en bij DNA schade response. Bovendien is gebleken dat van veel (geno)toxische stoffen het moment van blootstelling (dag/nacht) een grote invloed heeft op de cellulaire response, een fenomeen wat tegenwoordig chronotoxiciteit wordt genoemd. Daarnaast, is bewezen dat de doeltreffendheid en de

schadelijke bijwerkingen van chemotherapeutische middelen afhankelijk zijn van het moment (dag/nacht) van blootstelling. Door deze medicijnen op het juiste moment van de dag toe te dienen kunnen de bijwerkingen geminimaliseerd worden en kan de behandelingsmethode effectiever zijn. Vele toxicologische studies houden geen rekening met de circadiane klok en de dagelijkse veranderingen in de activiteit van genen en de hoeveelheid eiwit in de cel, wat de uitkomst van deze studies enorm kan beïnvloeden. Om de betrokkenheid van de circadiane klok bij het uitvoeren van toxicologische experimenten aan te tonen, hebben wij in **hoofdstuk 2** muizen behandeld op twee specifieke momenten van de dag (dag/ nacht) met cyclophosphamide (CP), een gebruikelijk chemotherapeutisch middel met chronotoxische eigenschappen. Uit ons onderzoek is gebleken dat er duidelijke verschillen zijn in differentiële genexpressie niveaus en pathways tijdens blootstelling op de twee tijdstippen. Deze resultaten laten duidelijk het belang zien van de circadiane klok en de rol die het speelt in (geno)toxische stress. Het uitvoeren van *in vivo* experimenten is erg tijdrovend, duur en vereist veel proefdieren. Omdat de maatschappij/samenleving aandringt op vervanging, vermindering en verfijnen van dierproeven, hebben wij besloten om een *ex vivo* chronotoxiciteit assay op te zetten. In **hoofdstuk 3** laten we zien dat leverplakjes een ideaal *ex vivo* model system vormen voor het opzetten van een chronotoxiciteitsassay. Het gebruik van leverplakjes heeft vele voordelen, het is vergelijkbaar met de intacte lever (heeft vergelijkbare structuur als de lever), en vele genen en eiwitten betrokken bij xenobiotische activatie, zoals cytochroom P-450 enzymen (CYP-450) komen tot expressie. Voor het opzetten van de *ex vivo* chronotoxiciteit assay hebben we Benzo[a]pyrene (B[a]P), een bekend milieu verontreinigd carcinogene als test stof gebruikt, waarvan het metabolisme onder circadiane controle staat. Het CYP-450 enzym, *Cyp1a1* is voornamelijk verantwoordelijk voor de metabolisatie en detoxificatie proces van B(a)P, en komt ritmisch tot expressie na B[a]P blootstelling en is vergelijkbaar met de circadiane oscillatie van *mPer2*. Lever plakjes werden blootgesteld aan B[a]P en geïnduceerd op de tijdstippen waar *Cyp1a1* maximaal en minimaal geïnduceerd werd. Onze studie laat duidelijke verschillen zien in differentiële genexpressie waarden en pathways op de verschillende tijdstippen. Uit deze resultaten concluderen wij dat het mogelijk is om lever plakjes te gebruiken voor het bestuderen van de chronotoxiciteit van stoffen, en dat B[a]P chronotoxische eigenschappen bevat.

Het verstoren van het circadiane ritme door genetische veranderingen of chronische verstoring van het ritme, zoals jet lag en shift werken, heeft aanzienlijk veel effect op gezondheid en kan voor vele aandoeningen en ziektes veroorzaken zoals kanker. Daarnaast zijn de essentiële circadiane klokgenen betrokken bij kankerprogressie. In **hoofdstuk 4** laten we zien wat voor effect de essentiële circadiane klokgenen hebben op de kankerprogressie in de goed gekarakteriseerde humane long carcinoma cellijn, H1299. Door

systematisch de circadiane klok genen uit te schakelen hebben wij de specifieke kankereigenschappen (celgroei, -migratie en -invasie) geanalyseerd. Het uitschakelen van de circadiane klokgenen in de H1299 cellijn heeft een significant effect op de celgroei, celmigratie, celinvasie en op de genotoxische stress na blootstelling aan chemotherapeutische middelen. Onze studie suggereert dat verstoring van de circadiane klok in deze cellijn waarschijnlijk erg nuttig kan zijn bij de behandeling van deze vorm van kanker. Hoewel onze resultaten voorgaande studies zowat tegenspreken, suggereert dit dat er nog veel onbekend is over de betrokkenheid van de circadiane klokgenen in het ontstaan en behandelen van kanker.

Samenvattend hebben wij aangetoond dat de circadiane klok een belangrijke rol speelt bij de toxiciteit en werkzaamheid van (geno)toxische stoffen. En dat de uitschakeling van de circadiane klok genen in de H299 cellijn significant effecten hebben op de kanker eigenschappen en genotoxische stress respons.

Curriculum Vitae

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List of Publications

de Wit AS, **Nijman RM**, Eugin Destici E, Chaves I, van der Horst GT. Hepatotoxicity and the circadian clock, a timely matter.

In preparation

van Dycke KCG*, **Nijman RM***, Wackers PFK, Jonker M, Rodenburg W, van Oostrom CTM, Breit, TM, van Steeg H, Luijten M, van der Horst, GT. Toxicogenomics and the circadian clock: Impact of time of day of exposure on the hepatic transcriptome of cyclophosphamide exposed mice.

In preparation *These authors contributed equally to this work.

Nijman RM, Payan Gomez CE, Wackers PFK, de Wit AS, da Silva AC, Chaves I, van der Horst GT. Chronotoxic effects of benzo[a]pyrene, assessed in cultured liver slices.

In preparation

Nijman RM, Bombardieri CR, Roel Janssens R, Vester S, Ridwan Y, Tamanini F, van der Horst GT. Influence of circadian core clock genes on the cancer properties of H1299 human non-small lung carcinoma cells.

In preparation

Bajek MI, **Nijman RM**, Barnhoorn S van der Horst GT, Chaves I. Uncoupling two functions of mammalian cryptochrome 1.

In preparation.

Chaves I, van der Horst GT, Schellevis R, **Nijman RM**, Groot Koerkamp M, Holstege FCP, Smidt MP, Hoekman MFM. FOXO3 in control of circadian rhythms via direct regulation of Clock.

Submitted

Chaves I, **Nijman RM**, Biernat MA, Bajek MI, Brand K, da Silva AC, Saito S, Yagita K, Eker AP, van der Horst GT. The Potorous CPD photolyase rescues a cryptochrome-deficient mammalian circadian clock. PLoS One. 2011

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Hirokawa G, **Nijman RM**, Raj VS, Kaji H, Igarashi K, Kaji A. The role of ribosome recycling factor in dissociation of 70S ribosomes into subunits. RNA. 2005

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General academic skills		
- Molecular and Cell Biology	2009	168
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- Biomedical English Writing and Communication	2011	112
General research courses		
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- Biomedical Research Techniques	2008	35
- Transgenesis, Gene Targeting and <i>in vivo</i> Imaging	2009	8
- Technical Facilities	2009	21
- Basic & Translational Oncology	2009	35
- Epigenetic Regulation	2009	14
- Practical and Theoretical course on Sens-it-iv <i>in vitro</i> methods (Hogeschool Utrecht)	2009	28
- Genome Maintenance and Cancer	2009	14
- Epigenetic Regulation	2010	14
- The Basic Course on ‘R’	2011	35
- Photoshop and Illustrator	2012	8
(Inter)national conferences and meetings		
- 2 nd Annual NTC meeting Amsterdam Rode Hoed (Oral presentation)	2010	32
- 3 rd Annual NTC meeting Amsterdam Rode Hoed (Poster presentation)	2011	24
- Center for Timing Research (CTR) meeting 2011 (Oral presentation)	2011	32
- 12 th European Biological Rhythms Society (ERBS) meeting Oxford Poster (Poster presentation)	2011	50
- Society for Research on Biological Rhythms (SRBR) Florida Poster (Poster presentation)	2012	50
- Thursday morning meetings and work discussion	2008 - 2012	250
Retreats and workshops		
- MGC PhD student workshop Brugge (Poster presentation)	2009	40
- MGC PhD student workshop Cologne (Oral presentation)	2010	50
- MGC PhD student workshop Maastricht (Poster presentation)	2011	40
- Molecular Medicine: Get Out of the Lab days	2011	24
Other		
- Cluster 15 Party Committee 2008-2009	2008 - 2009	150 Hours

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It always seems impossible until it is done – Nelson Mandela

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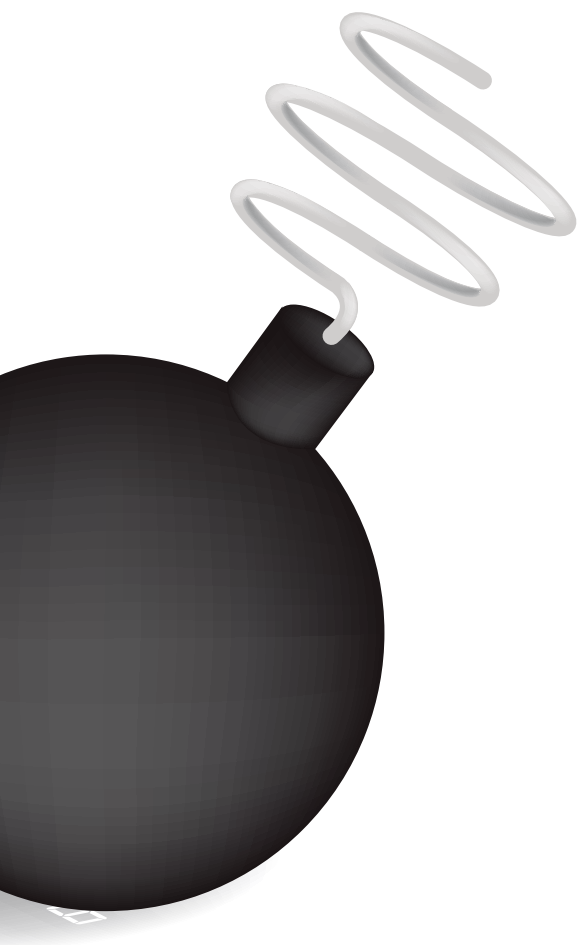
Mi familia, Oma, ooms, tantes, neef(jes) en nicht(jes) te veel om bij naam te noemen, bedankt voor jullie enthousiasme en steun!

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Thank you <3!

Liefs, Romana

Appendix 1



Impact of the circadian clock on *in vitro* genotoxic risk assessment assays

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ABSTRACT

Our society expects safety assessment for drugs, chemicals, cosmetics, and foods, which to date cannot be achieved without the use of laboratory animals. At the same time, society aims at refining, reducing, and (ultimately) replacing animal testing. As a consequence, much effort is taken to establish alternatives, such as toxicogenomics-based risk assessment assays on cultured cells and tissues. Evidently, the properties of cells *in vitro* will considerably differ from the *in vivo* situation. This review will discuss the impact of the circadian clock, an internal time keeping system that drives 24-h rhythms in metabolism, physiology and behavior, on *in vitro* genotoxic risk assessment. Our recent observation that DNA damaging agents can synchronize the circadian clock of individual cells in culture (and as a consequence the cyclic expression of clock-controlled genes, comprising up to 10% of the transcriptome) implies that the circadian clock should not be neglected when developing cell or tissue-based alternatives for chronic rodent toxicity assays.

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1. Introduction

Carcinogenesis is a multi-step process in which genetic and epigenetic alterations enable cells to escape from regulated cell proliferation programs. A selective growth advantage of such cells (allowing clonal expansion), along with a loss of genomic stability (resulting in neoplastic transformation) ultimately results in the formation of primary and secondary (metastatic) tumors [1]. It is generally believed that the driving force behind tumor induction and progression is the induction of deletions and mutations, originating from DNA damage instigated by the exposure

of the genome to dispense endogenous (i.e. reactive metabolic byproducts) and environmental (i.e. ionizing radiation, ultraviolet light, chemical compounds) agents [2]. To counteract the deleterious effects of DNA damage and faithfully duplicate their genome, cells have evolved an intricate network of genome care taking mechanisms, which include DNA damage signaling, cell cycle arrest, DNA repair, replicative senescence, and apoptosis, and which are collectively referred to as the DNA damage response (DDR) [3–5]. Malfunction of DDR pathways causes a mutator phenotype and predisposes to cancer, as well illustrated by genetic disorders like ataxia telangiectasia (AT; defective DNA damage signaling), xeroderma pigmentosum (XP; defective nucleotide excision repair), Fanconi anemia (defective cross-link repair), Li-Fraumeni, and familial retinoblastoma (defective cell cycle control) [6].

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As cancer is the second leading cause of death in the Western society, and since pharmaceutical, chemical, cosmetic, and food industries are continuously developing new chemical compounds (adding up to the vast amount of natural environmental toxicants and carcinogens), it does not come as a surprise that our society demands such synthetic compounds to be tested for (geno)toxic and carcinogenic potential. To date, the only direct test for carcinogenic risk assessment of chemicals involves chronic exposure of rodents (or other animals, e.g. dogs) to various doses of the test compound (18–24 months; at least 50 animals per sex per dose) and analysis of tumor incidence and tumor type [7,8]. As this chronic *in vivo* bioassay (apart from being time-consuming, expensive, and having limited sensitivity) uses large numbers of animals and has a severe impact on animal welfare, it also does not come as a surprise that the same society urges the scientific community to invest in the development of assays that reduce and refine, and preferably replace animal use (3-R principle).

An important step towards refinement has been made with the establishment of transgenic animal models such as the *Xpa*^{-/-}/*p53*^{+/-} mouse (deficient in nucleotide excision DNA repair and lacking one allele of the p53 tumor suppressor gene), which robustly responds in a very sensitive and discriminative manner to human genotoxic and non-genotoxic carcinogens within 9 months following initiation of treatment [9,10]. More recently, the *Xpa*^{-/-}/*p53*^{+/-} mouse model has also been used in a short-term (14 days) exposure study, combined with a genome wide expression profiling using micro-array technology. This toxicogenomics approach allowed the identification of multigene gene-expression signatures as a highly predictive and discriminative biomarker for genotoxic and non-genotoxic carcinogens [11].

The ultimate aim is to entirely replace animal-based carcinogenic risk assessment assays by *in vitro* assays that make use of cultured tissue explants and/or cell lines, the latter requiring no animals at all. To this end, many efforts are undertaken worldwide to establish and validate transcriptomics-based high-throughput cellular assays for carcinogenic risk assessment. Evidently, *in vitro* culture systems have their own limitations and *in vivo-in vitro* differences should be taken into account. For example, whereas in the intact organism, mammalian cells usually are exposed to oxygen levels ranging from 2 to 8% [12], cell culturing is often routinely performed at atmospheric oxygen levels (20%). Under such conditions, primary embryonic mouse fibroblasts will undergo a limited number of population doublings and enter a state of replicative senescence, after which some cells may escape and grow into a spontaneously immortalized cell line. This process is accompanied by an increase in spontaneous mutation frequency and a robust DNA damage response, which can be prevented by culturing the cells at 3% oxygen [13–15].

Another potential source of *in vivo-in vitro* differences is the presence of an internal time keeping system, known as the circadian clock. Mammals are equipped with an internal light-entrained circadian clock that drives 24-h rhythms in metabolism, physiology, and behavior, and allows them to optimally anticipate the momentum of the day. Evidence is increasing that the intensity of adverse effects of exposure to environmental (e.g. pollutants) or therapeutic (e.g. anti-cancer drugs) genotoxic agents can depend on the time of exposure (e.g. morning vs. evening). The phenomenon of time-dependent changes in an organism's sensitivity to toxicants is referred to as chronotoxicity [16,17]. Conversely, we have recently shown that genotoxic agents impinge on the circadian clock [18]. Below, following a general introduction on the circadian system, we will discuss the implications of the reciprocal link between genotoxic stress induced by DNA damage and the circadian clock, as well as its potential impact on *in vitro* genotoxic risk assessment assays.

2. The circadian system

The rotation of the earth around its axis imposes daily recurring changes to our environment, notably cyclic light-dark and temperature alternations. To anticipate to these solar day-night cycles, most organisms have developed an internal clock with a near 24-h periodicity, allowing them to optimally tune metabolic, physiological, and behavioral functions (e.g. the sleep-wake cycle, body temperature, blood pressure, hormone levels) to the special physiological needs the organism will have at specific times during the day [19]. The importance of circadian (Latin for “approximately one day”) clocks is well illustrated by the fact they have evolved multiple times during evolution and are found across all three kingdoms of life [20]. To maintain synchrony between body time and solar time, circadian clocks need to be adjusted every day by light.

2.1. The light-entrained central clock in the SCN

A key feature of circadian rhythms is that they are self-sustained and persist in the absence of light-dark cycles. When housed in constant darkness, laboratory mice will maintain a “free-running” rest-activity pattern with a period length (τ , tau) that is determined by the internal clock. In search for the location of the mammalian circadian clock, rodents have been subjected to lesion experiments in which defined brain areas were selectively damaged. Ablation of a small region in the hypothalamus, known as the suprachiasmatic nucleus (SCN), was shown to cause behavioral arrhythmicity [21,22]. Moreover, transplantation of fetal SCN tissue to the brain of arrhythmic SCN-lesioned hamsters restored their circadian behavior [23] through a diffusible signal [24]. Although these experiments already pointed to the SCN as the primary clock organ, the pivotal evidence putting the SCN at the top of the circadian hierarchy came from transplantation experiments with the first mammalian circadian mutant, the tau hamster, displaying a behavioral period of 22 and 20 h in the hetero- and homozygote state, respectively [25]. By transplanting a mutant SCN to SCN-lesioned wild type hamsters (and vice versa), the Menaker group showed that behavioral rhythms were restored and that the circadian period of the recipient animal was determined by the mutant donor SCN [26].

On average, the period of the rodent circadian clock is shorter than 24 h, whereas the human clock has the period slightly longer than 24 h [27]. Accordingly, the SCN clock needs to be adjusted daily to keep pace with the exact 24-h solar light-dark cycle. Although various stimuli can reset the circadian clock, nature has chosen light (representing the most reliable and predictable environmental change) as entraining stimulus [28,29]. For instance, when housed in constant darkness, a light pulse at the end of the subjective night (the activity phase in rodents) will phase advance the clock of mice, resulting in an earlier onset of activity and other clock-controlled processes. Oppositely, exposing the same animals to a light pulse at the beginning of the subjective night will result in a phase delay. During the subjective day (the resting phase in rodents) light pulses do not exert any effect.

The light information, to achieve the photoentrainment, is perceived by the eye and transmitted to the SCN through the retino-hypothalamic tract. To this end, the inner nuclear layer of the retina is equipped with a subset of ganglion cells that contain the photopigment melanopsin and project specifically to the SCN [30–32]. However, as melanopsin deficient mice still show residual photoentrainment (unless crossed with rodless/coneless mice), other photopigments likely also contribute to circadian photoentrainment [33–36].

2.2. Circadian rhythmicity is generated by a molecular oscillator

Molecular and genetic analysis of the circadian clock in plants (*Arabidopsis*), fungi (*Neurospora*), insects (*Drosophila*), amphibia

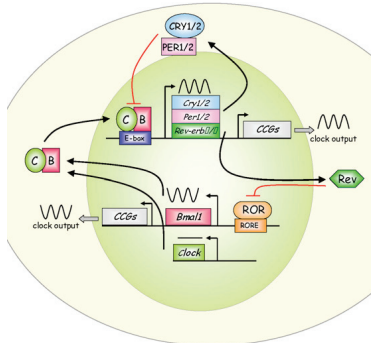


Fig. 1. The mammalian circadian oscillator. Simplified scheme showing the molecular mammalian circadian oscillator composed of a negative feedback loop and one auxiliary feedback loop. In this model CLOCK/BMAL1 (components of the positive limb) drive expression of the *Cry* and *Per* genes through E-box elements in their promoters. Following synthesis and accumulation of the CRY and PER proteins in the cytoplasm (delayed by extensive posttranslational modification of the proteins, resulting in degradation by the ubiquitin-proteasome system), CRY and PER proteins form complexes that shuttle between cytoplasm and nucleus. When the nuclear concentration of CRY/PER complexes reaches a critical level, they inhibit CLOCK/BMAL1 driven transcription. This same mechanism also generates rhythmic transcription of E-box containing clock-controlled output genes with a phase similar to the *Cry* and *Per* genes. At the same time CLOCK/BMAL1 activate *Rev-erbα/β* gene expression. The REV-ERBα/β proteins bind to ROR-elements in the *Bmal1* promoter and inhibit *Bmal1* expression by displacing the ROR transcriptional activators from the ROR-elements. This causes circadian expression of *Bmal1*, as well as output genes with ROR-elements with a phase that is opposite to that of the E-box element containing genes. Among the first order output genes are other transcription factors that drive rhythmic expression of their target genes with a phase different from the E-box or RORE containing genes. These second order genes usually have lower amplitude oscillations than the first order output genes, which are direct targets of CLOCK/BMAL1 or ROR.

(*Xenopus*), fish (zebrafish), and mammals (rodents) revealed that circadian rhythms are generated by a molecular oscillator, consisting of an ingeniously designed auto-regulatory transcription-translation feedback loop (TTFL) in which cyclically expressed clock gene products regulate their own expression with an approximate 24-h periodicity [20].

As shown in Fig. 1, the mammalian molecular oscillator consists of a feedback loop in which the transcription factor Brain and muscle Arnt-like protein-1 (BMAL1) together with either CLOCK or Neuronal PAS domain protein 2 (NPAS2) drives transcription of the Cryptochrome (*Cry1* and *Cry2*) and Period (*Per1* and *Per2*) genes through E-box enhancer elements in their promoters. After a delay of several hours, the gene products accumulate and form CRY/PER heterodimers that move to the nucleus and shut down their own expression (negative feedback) by inhibiting BMAL1–CLOCK-mediated transcription [37]. Inactivation of *Bmal1* or simultaneous inactivation of *Cry1* and *Cry2*, or *Per1* and *Per2* results in an immediate loss of rhythmicity at the behavioral and molecular level [38–43], demonstrating the importance of this negative feedback loop. Additionally, other feedback loops exist that are thought to confer robustness and precision to negative feedback driven oscillations [44]. In one of these loops, CLOCK/BMAL1 activates transcription of the *Rev-erbα* orphan nuclear receptor gene. The REV-ERBα protein inhibits Receptor tyrosine kinase-like orphan receptor (ROR)-driven transcription of the *Bmal1* gene by competing with the ROR proteins for binding to ROR-elements (RORE) in the *Bmal1* promoter [45]. This mechanism generates high-amplitude oscillations in *Bmal1* transcription and, even though this

loop is not essential for the clock mechanism, it extends rhythmic transcription to genes with RORE elements in their promoters.

In addition, prominent posttranslational modification of clock proteins occurs [46]. Particularly, phosphorylation and ubiquitination of the PER and CRY proteins (determining the rate of degradation of these proteins) is important to establish the delay in *Cry* and *Per* mRNA and protein peaks.

2.3. Peripheral clocks in other tissues and cells

Initially, neurons of the central circadian pacemaker in the SCN were thought to be the only cell type capable of generating long-term self-sustained circadian rhythms. However, using transgenic rats expressing the luciferase reporter gene under control of the *Per1* promoter, it was shown that circadian oscillators also exist in peripheral tissues [47]. In all rat and mouse peripheral tissues analyzed thus far, such peripheral clocks oscillate with a 7–11 h phase delay (depending on the organ) compared to the master oscillator in the SCN. However, peripheral circadian oscillators appeared not self-sustaining as the luciferase signal gradually damps *ex vivo*, when tissues are disconnected from the SCN [47]. However, using a knock-in mouse model expressing a PERIOD2::LUCIFERASE fusion protein as a real-time reporter of circadian dynamics, the Takahashi laboratory later showed that explanted peripheral tissues were capable of maintaining self-sustained rhythms [48]. Importantly, SCN-lesioning caused a phase desynchrony of peripheral clocks in these animals, suggesting that peripheral clocks are self-sustained and desynchronize, rather than dampen in the absence of the SCN [48]. Moreover, these findings also show that the SCN functions to maintain proper phase relationships between tissues, rather than being the driving force for peripheral clocks [48]. Peripheral oscillators are thought to regulate the circadian output of the functions specific to that particular tissue or organ. In contrast to the SCN core oscillator, peripheral oscillators are not entrained by light. However, rodent kidney and liver peripheral clocks have been shown to be able to entrain to restricted food availability during the subjective day, when the rodents normally sleep. As the SCN clock does not respond to alter feeding patterns, restricted feeding thus causes a phase uncoupling of master and peripheral clocks [49].

Cultured fibroblasts serve as excellent tools to study circadian clock performance at the molecular level. Whereas cell cultures provided with a clock reporter gene do not show circadian oscillations, the Schibler group has shown that robust oscillations can be induced by exposing confluent, serum-starved cell cultures to 50% serum [50]. It soon became clear that a variety of compounds acting on different pathways (e.g. cAMP/PKA and MAPK) were able to elicit the same response [51–53]. Later, it was shown that aforementioned treatments cause a synchronization of individual self-sustained cellular oscillators rather than a reinitiation of damped cellular oscillators. To achieve this synchronization, the clock of the individual cells must be reset to the same phase [54]. For the glucocorticoid receptor agonist dexamethasone, for example, this synchronization is achieved by resetting all phases of the individual cells to the same, new phase [54].

The composition and molecular mechanism of the circadian oscillator in peripheral cells is essentially the same as that of the SCN clock and, as shown for mouse embryonic and dermal fibroblasts from clock mutant mice, genetic defects that affect period length or cause arrhythmicity of behavior, have the same effect on the peripheral molecular oscillator *in vitro* [50,55].

2.4. Clock-controlled output processes

The circadian core oscillator is coupled to behavioral, physiological, and metabolic output processes through clock-controlled genes, which may contain E-box or ROR-elements in their promoter

(Fig. 1). Among these clock-controlled genes are transcription factor encoding genes, which further add to the complexity and dynamics of circadian gene expression by rhythmically driving output genes with a phase different from that obtained with E-box or ROR-elements alone [56,57]. One well-studied example of a clock-controlled transcription factor is the PAR-bZIP transcription factor DBP (D-site albumin promoter Binding Protein), expression of which is directly activated by CLOCK/BMAL1 [58]. Two other related PAR-bZIP transcription factors, Thyrotroph Embryonic Factor (TEF) and Hepatic Leukemia Factor (HLF), have also been shown to oscillate. Together with DBP, they generate circadian expression of many genes involved in detoxification and drug metabolism [59]. Other examples of rhythmically expressed transcription factors include Peroxisome Proliferator-Activated Receptor α (PPAR α) and a variety of other nuclear receptors that link the circadian clock to metabolism and possibly other processes [60,61]. Through these combined mechanisms the clock generates rhythms in gene expression of up to 10% of the genome [62]. The genes that oscillate vary from tissue to tissue, possibly reflecting the specific requirements for each tissue.

Surprisingly, as shown using a mouse model with a conditional active/inactive liver clock, a portion of the rhythmically expressed genes (including the clock gene *Per2*), keep oscillating even in the absence of a functional molecular liver clock [63]. The circadian expression of these genes is probably driven by circadian systemic cues and/or body temperature cycles.

By cyclically expressing genes encoding posttranslational modification enzymes, the circadian clock may also control the function of output proteins that are constitutively expressed (and thus escape notice in transcriptome profiling or other gene expression studies). For example, the expression of the cell cycle kinase *Wee1* is controlled through E-box elements in its promoter, leading to circadian oscillations of *Wee1* protein and kinase activity and resulting in circadian phosphorylation of its constitutively expressed target proteins [64]. More recently, the activity of the NAD⁺-dependent deacetylase Sirtuin 1 (SIRT1) was shown to be controlled by the circadian clock through transcriptional control of the *Nampt* gene, encoding nicotinamide phosphoribosyltransferase, which acts as a SIRT1 cofactor and rate-limiting enzyme in the NAD⁺ salvage pathway [65,66]. SIRT1 deacetylates proteins such as histones, p53, FOXO, PGC1 α , PER2 and BMAL1, and as such acts in a wide range of processes such as cell cycle control, metabolism, and the circadian clock itself [67]. Thus, the circadian clock may influence these processes through control of SIRT1 activity, which may have important implications for toxicological and therapeutic studies.

Besides these intra-cellular mechanisms, the clock in the central nervous system governs the secretion of many hormones and peptides, through which it affects cellular processes in the periphery. Even the circadian variation in body temperature has an impact on peripheral oscillations by regulating the activity of the transcription factor heat-shock factor 1 (HSF1) [68].

3. The circadian clock, cell cycle and DNA damage sensitivity

Interestingly, recent studies have shown that the cell cycle, as well as the DNA damage response that controls cell cycle progression under conditions of genotoxic stress, are connected to the circadian clock [5,69,70]. A circadian clock mediates an additional layer of control over cell cycle progression and it may well originate from the need of organisms to restrict replication of their genome (i.e. S-phase) to the moment of the day where the risk of exposure to environmental and endogenous DNA damaging agents (i.e. ultraviolet light during the day; reactive oxygen species and other harmful metabolic side products generated during respiratory metabolism)

is at its lowest [71,72]. There have been several reports on circadian variation in cell cycle division in human and rodent tissues [73–75]. For instance, human epidermal cells have been shown to follow a diurnal pattern of periodic entrance from G1 into S-phase, with higher levels of DNA synthesis noted between 2 and 6 p.m. [74] with p53 and cyclin E, A, and D1 gene expression exhibiting a circadian pattern [75].

To date, many of the core clock genes have been linked to cell cycle related phenotypes, both *in vitro* and *in vivo*. The first demonstration came from experiments with *Per2* mutant mice. When exposed to ionizing radiation (IR), mutant animals displayed a marked increase in tumor development (as compared to wild type animals) and an impaired DDR in thymocytes [76]. Under normal conditions and after IR, the expression of several cell cycle and DDR related genes (including *c-Myc* and several *p53* target genes) was altered in the *Per2* mutant liver. This impaired DDR response and IR-induced tumorigenesis was not observed in fibroblasts and mice deficient in other components of the negative regulatory feedback loop, such as the *Cry1* and *Cry2* genes [77].

Some mechanistic insight into how the circadian clock gates cell cycle progression came from a study of liver regeneration after partial hepatectomy [64]. In wild type mice, partial hepatectomy causes hepatocytes (normally in a G₀ state of non-division) to start proliferating *en masse* and entry into M-phase was shown to be time-of-day dependent. In contrast, arrhythmic *Cry1/Cry2*-deficient mice did not display such gating of cell cycle progression. Additionally, *Cry*-deficient hepatocytes also took longer to restore the liver to its original mass, a phenotype that was linked to constitutively high expression and activity of the clock-controlled *WEE1* kinase that governs entry to M-phase [64]. In another study, mice lacking components of the negative limb of the feedback loop (i.e. *Cry1/Cry2* or *Per1/Per2* animals) were shown to have increased bone mass due to accelerated proliferation of osteoblasts in a cell-autonomous manner [78].

Components of the positive limb of the circadian oscillator have also been implicated in cell cycle control. Proliferation of arrested, serum-starved *Clock* mutant mouse embryonic fibroblasts was impaired after serum-induced cell cycle re-entry [79]. Microarray analysis of the liver transcriptome revealed that a variety of cell cycle related genes are under circadian control and have altered expression levels in *Clock* mutant tissues, some but not all of these genes were also deregulated in *Clock* mutant mouse embryonic fibroblasts [79]. *Bmal1*-deficient hepatocytes, cultured *in vitro*, showed a phenotype similar to *Clock* mutant fibroblasts [80]. The reduced proliferation in *Bmal1*-deficient hepatocytes was linked to increased expression level of p21 (also known as *Cdkn1a*).

Energy metabolism (producing reactive radicals) and xenobiotic metabolism are prominently controlled by the circadian clock, implying that the sensitivity of tissues to environmental (e.g. pollutants) or therapeutic (e.g. anti-cancer drugs) genotoxic agents can depend on the time of exposure (e.g. morning vs. evening). Indeed, it is known for long that the effectiveness of radiotherapy and chemotherapeutic agents and the extent of toxic side effects depend on the time-of-day of administration [81]. This phenomenon is referred to as chronotoxicity, and its intended use in the clinic chronotherapy. Investigating how the circadian clock is connected to the cell cycle and DDR is of prime importance for understanding why and how chronotoxicity and chronotherapy work, especially when it involves genotoxic agents.

4. DNA damage and the circadian clock

Interestingly, we and others [18,82] have shown that the connection between the mammalian clock and the DDR is reciprocal. When studying the response of cultured fibroblasts to DNA dam-

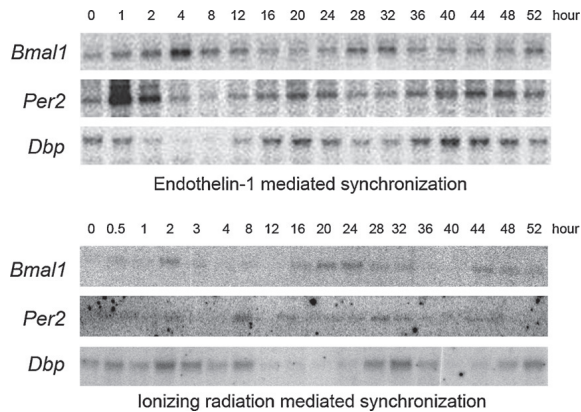


Fig. 2. DNA damage synchronizes the circadian clock *in vitro*. Northern blot analysis of *Bmal1*, *Per2* and *Dbp* mRNA levels following treatment of Rat-1 fibroblasts with endothelin-1 (known to synchronize individual cellular circadian oscillators) and primary mouse fibroblasts with ionizing radiation. Note that ionizing radiation synchronizes the circadian clock with a 12 h phase difference as compared to endothelin-1, implying different underlying clock resetting mechanisms. Upper panel adapted from Yagita et al. [55] and reprinted with permission from AAS.

age, we noticed to our great surprise that ionizing radiation is able to synchronize the circadian clock with an opposite phase angle, as compared to the rhythms elicited upon treatment of cells with known synchronizers such as serum shock, forskolin and dexamethasone (Fig. 2). This finding prompted us to investigate whether ionizing radiation could phase shift the circadian clock of synchronized cell cultures. As shown in Fig. 3, using confluent forskolin-synchronized Rat-1 fibroblasts, expressing a luciferase reporter gene under control of the *Per2* promoter, we observed that ionizing radiation (IR) phase-advanced the circadian clock in a dose- and time-dependent manner [18]. A similar response could be provoked by ultraviolet light and oxidative stress, suggesting that clock resetting may be a universal property of DNA damage. Moreover, a non-lethal dose of ionizing radiation was also able to phase advance the running-wheel-behavior of free-running C57BL6/J mice when treatment was performed in the middle of the subjective day (CT6), but not at the end of the subjective night (CT22) [18]. This type of clock resetting is very different from light-mediated phase shifting: photic pulses are known to phase advance or delay the clock only during the subjective night, and not during the subjective day. The phase response curve (PRC) for ionizing radiation in cultured mammalian cells [18,82] is remarkably similar to the PRC for the genotoxic agent MMS in the mold *Neurospora* [83] and ultraviolet light in the flagellate *Gonyaulax* [84]. In both cases, only phase advances were observed, and only when applied during the subjective day.

Noteworthy, ionizing radiation-induced phase shifting of the mammalian circadian clock does not involve temporary changes in the expression level of known clock genes (i.e. *Per1*, *Per2*, *Cry1*, *Cry2*, *Bmal1*, *Clock*) and does not require *de novo* protein synthesis, suggesting that some posttranslational modification mechanism might be at work. Our observation that three different genotoxic agents (i.e. ionizing radiation, ultraviolet light and tert-butyl peroxide) were able to reset the mammalian circadian clock not only points to DNA damage as the causative trigger, but also suggests that a clue to the underlying mechanism might be found among the DNA damage response pathways. Indeed, we have shown that ionizing radiation-induced phase shifting of the mammalian molecular clock is abrogated in cultured fibroblasts from cancer-predisposed ataxia-telangiectasia and Nijmegen breakage syndrome patients

(carrying mutations in the *ATM* and *NBS* gene, respectively) and in Rat-1 cells treated with an ATM-specific inhibitor [18]. The ATM (ataxia telangiectasia mutated) protein belongs to the phosphatidylinositol 3-kinase-related kinase (PIKK) family of protein kinases and initiates the DNA damage signaling cascade by phosphorylating cell cycle regulator and DNA repair proteins [85].

How does the DNA damage response (notably DNA damage signaling and the ATM kinase) connect to the circadian system? In *Neurospora*, the DNA damage-induced clock resetting is mediated by phosphorylation of the Frequency (FRQ) clock protein (one of the core proteins of the *Neurospora* clock; see [86]) by PRD-4, an ortholog of the mammalian checkpoint kinase CHK2 [83]. Thus, the question arises which mammalian clock protein would serve as a candidate for bridging DNA damage signaling and the circadian clock. Recently, in a large-scale mass spectrometry analysis, the PER1 clock protein was identified as a potential target for ionizing radiation-induced phosphorylation by ATM/ATR [85]. Furthermore, PER1 was shown to physically interact with the ATM and CHK2 proteins [87]. Although other clock proteins should not be excluded, these findings make PER1 a good candidate to reciprocally connect the DDR and circadian clock pathways.

5. Consequences for *in vitro* genotoxic risk assessment assays

Evidently, our recent observation that DNA damaging agents can synchronize the circadian clock of individual cells in culture (and as a consequence the cyclic expression of clock-controlled genes, comprising up to 10% of the transcriptome) has implications for the development of transcription profiling based *in vitro* risk assessment assays.

Let us consider the set-up of a typical experiment for determination of the transcriptional response of cultured cells (e.g. fibroblasts, primary hepatocytes, HepG2 cells, etc.) upon exposure to genotoxic stress (Fig. 4A). In this experiment, cell cultures are treated with the DNA damaging agent of interest at $t=0$ and mRNA levels are measured before, and at several time points after exposure. In this example, gene X shows a higher expression level 12 h after exposure of the cells, while transcript levels at $t=4$ and $t=24$ are close

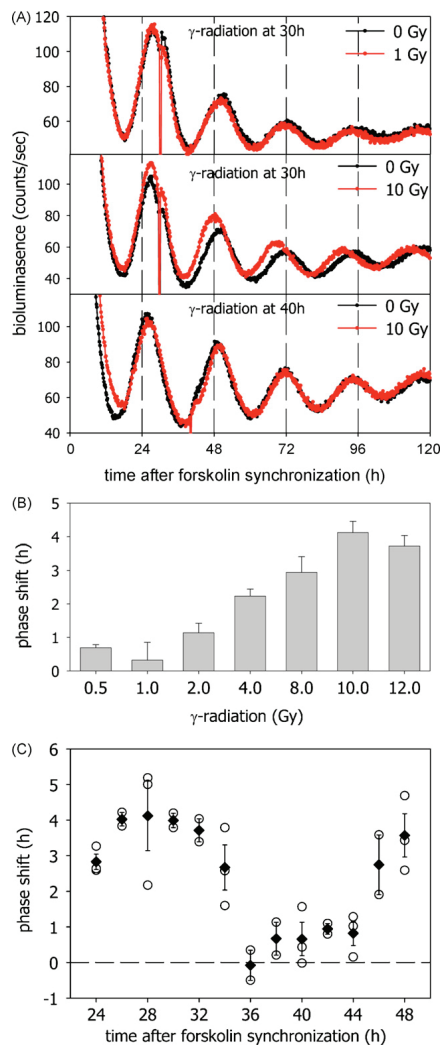


Fig. 3. DNA damage resets the circadian clock in a time- and dose-dependent manner. (A) Examples of circadian clock performance of forskolin synchronized Rat-1 *Per2:luc* cells (stably expressing a luciferase reporter gene under control of the *mPer2* promoter) after treatment with different doses of IR (1 or 10 Gy) at different times (30 h vs. 40 h) after synchronization. (B) Dose dependency of the magnitude of ionizing radiation induced phase shifts in Rat-1 *Per2:luc* cells irradiated 30 h after forskolin synchronization. (C) Phase response curve of ionizing radiation (10 Gy) induced phase shifts in Rat-1 *Per2:luc* cells. Note that ionizing radiation only induces phase advances, and as such differs from the PRC for forskolin and dexamethasone (containing phase advances and delays). Reprinted with permission from Elsevier, Oklejewicz et al. [18].

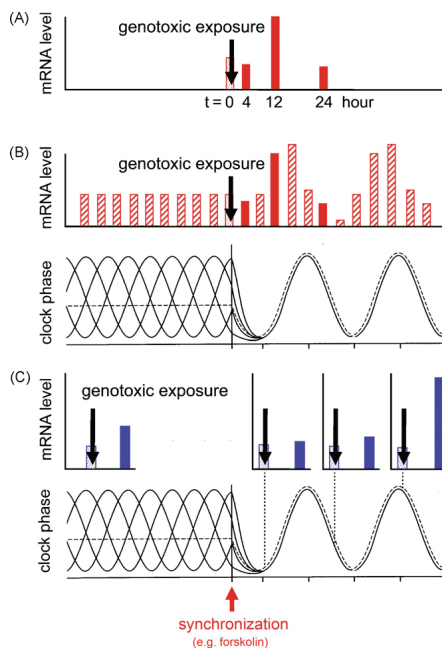


Fig. 4. Implications of DNA damage resetting the circadian clock for *in vitro* genotoxic risk assessment assays. (A) Imaginary *in vitro* experiment for identification of genotoxic stress markers. Cultured cells are exposed to a DNA damaging agent. mRNA profiles are determined at various times after exposure (solid bars) and compared to those obtained from mock-treated cells (not shown) or cells harvested prior to treatment (dashed bars). The data obtained for gene X suggest that this gene is a DNA damage responsive gene. (B) Same experiment as described under (A) except that mRNA levels were analyzed at 4-h intervals (additional data represented by hatched bars). Note that exposure to the DNA damaging agent is synchronizing the individual cellular circadian clocks, and accordingly transcription of clock (output) genes. On the basis of this experiment, gene X is likely a clock-controlled output gene, rather than DNA damage responsive gene. (C) Modified version of the *in vitro* experiment for identification of genotoxic stress markers. The circadian clock of the individual cells is synchronized by treatment with forskolin (or another known synchronizing compound), after which cells are exposed to the DNA damaging agent at a defined phase of the circadian day. Expression profiles are determined 12 h after treatment (solid bars) and compared to those obtained prior to treatment (dashed bars). Note the time-of-day differences in the response of gene Y, as well as the stronger response at a defined moment of the day in circadian clock synchronized cell cultures, as compared to non-synchronized cultures, indicative for chronotoxic effects of the DNA damaging agent.

to baseline expression level. On the basis of these data one might easily conclude that gene X is a DNA damage responsive gene that could well serve as a biomarker for the tested compound. However, in view of our finding that DNA damage resets the cellular clock of mammalian cells [18], the measured changes in transcript levels could well represent rhythmic expression of a clock gene or clock-controlled output gene (Fig. 4B). We therefore envisage that a large percentage of responsive genes (as identified by microarray analysis) in non-synchronized genotoxic stress-exposed cell and tissue cultures might not represent DNA damage responsive genes. Given the purpose of the experiment (identification of biomarkers for genotoxic stress), such responsive genes can even be considered as false positives. Evidently, such genes will blur the biomarker pro-

file, and although bioinformatics approaches can be taken to filter out clock-controlled genes, one should bear in mind that many of the DDR genes are under circadian control (e.g. c-myc, p53, p21, cyclins: A, B, D, wee1) [64,76,80,87–89].

We therefore propose an alternative approach in which (i) intracellular clocks are synchronized prior to exposure and (ii) exposure is carried out at various specific phases of the circadian day (Fig. 4C). Analysis of transcriptome changes at various time points after treatment will allow identification of DNA damage responsive genes, irrespective of whether these genes are under circadian control or not. Importantly, this approach will also recognize chronotoxic characteristics of chemical agents, as in such cases the magnitude of the response will depend on the circadian phase at which cells were treated. Moreover, the magnitude of the change in expression levels of DNA damage responsive genes, as induced by treatment of cells with a genotoxic agent at a defined circadian phase, might even be stronger than in non-synchronized cells, thereby adding to the sensitivity of the assay.

We are currently performing a comparative *in vivo/in vitro* study in which animals, tissues, and cells are exposed to (geno)toxic/carcinogenic agents at defined circadian times, and transcriptome changes are determined by omics approaches. Apart from delivering predictive gene expression fingerprints for chemical risk assessment, we believe that the obtained knowledge will provide insight into the mechanisms underlying chronotoxicity. This information will not only be of prime importance to further optimize chronotherapeutic treatment of cancer patients, but can also be used to advise workers to restrict handling of such compounds to the moment of the day (i.e. body time) where the biological consequence of accidental exposure is at its lowest.

Conflicts of interest

None.

Acknowledgements

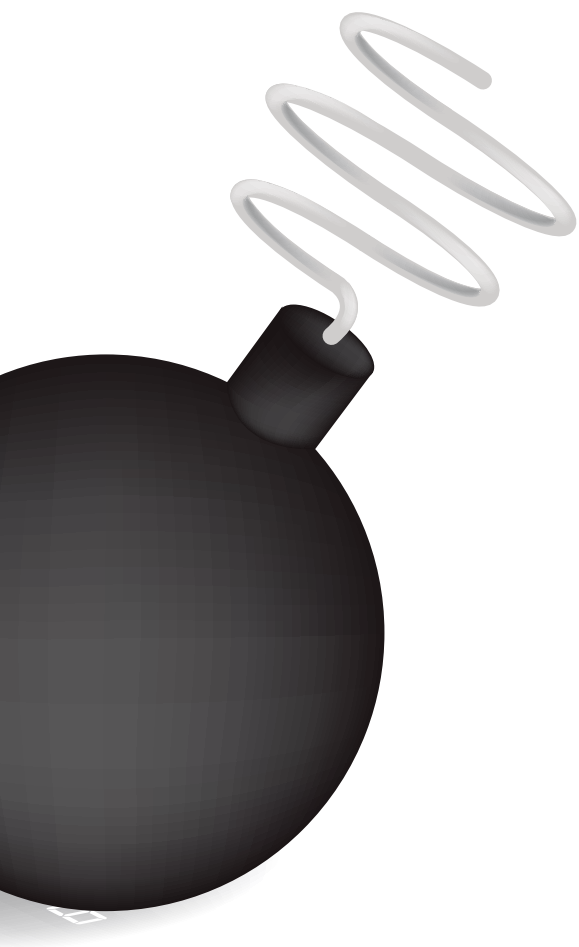
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Appendix 2



The *Potorous* CPD Photolyase Rescues a Cryptochrome-Deficient Mammalian Circadian Clock

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Abstract

Despite the sequence and structural conservation between cryptochromes and photolyases, members of the cryptochrome/photolyase (flavo)protein family, their functions are divergent. Whereas photolyases are DNA repair enzymes that use visible light to lesion-specifically remove UV-induced DNA damage, cryptochromes act as photoreceptors and circadian clock proteins. To address the functional diversity of cryptochromes and photolyases, we investigated the effect of ectopically expressed *Arabidopsis thaliana* (6-4)PP photolyase and *Potorous tridactylus* CPD-photolyase (close and distant relatives of mammalian cryptochromes, respectively), on the performance of the mammalian cryptochromes in the mammalian circadian clock. Using photolyase transgenic mice, we show that *Potorous* CPD-photolyase affects the clock by shortening the period of behavioral rhythms. Furthermore, constitutively expressed CPD-photolyase is shown to reduce the amplitude of circadian oscillations in cultured cells and to inhibit CLOCK/BMAL1 driven transcription by interacting with CLOCK. Importantly, we show that *Potorous* CPD-photolyase can restore the molecular oscillator in the liver of (clock-deficient) *Cry1/Cry2* double knockout mice. These data demonstrate that a photolyase can act as a true cryptochrome. These findings shed new light on the importance of the core structure of mammalian cryptochromes in relation to its function in the circadian clock and contribute to our further understanding of the evolution of the cryptochrome/photolyase protein family.

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Introduction

Life is subject to the 24-hour rotation cycle of the earth, which imposes rhythmic changes in light and temperature conditions. In order to anticipate these environmental changes, most organisms have developed a circadian clock with a period of approximately 24 hours that allows them to adjust behavior, physiology and metabolism to the momentum of the day. To keep pace with the day/night cycle, this internal clock needs to be reset every day, using light (the most predictable environmental cue) as the strongest Zeitgeber (German for “time giver” or synchronizer). The mammalian master clock is located in the suprachiasmatic nuclei (SCN) of the hypothalamus, and receives light-induced signals from the retina via the retino-hypothalamic tract [1]. In turn, this master clock sends humoral and neuronal signals that synchronize peripheral oscillators, located in virtually every cell or tissue [2–5].

The mammalian cryptochrome proteins (CRY1 and CRY2) belong to the cryptochrome/photolyase family (CPF) of flavoproteins and were initially identified as homologues of photolyase [6,7]. In view of their strong resemblance to plant cryptochrome proteins, which act as blue light photoreceptors, the mammalian CRY proteins were hypothesized to act as photoreceptors for resetting of

the circadian clock [6,8]. Unexpectedly however, inactivation of the *Cry1* and *Cry2* genes in the mouse was shown to shorten or lengthen the period length of the circadian clock respectively, whereas in the absence of both genes circadian rhythmicity was completely lost [9–11]. This observation, together with the finding that the *Cry* genes encode the most potent inhibitors of the circadian transcription activator CLOCK/BMAL1 [12], positioned the mammalian CRY proteins at the heart of the circadian core oscillator.

The mammalian circadian clock consists of a molecular oscillator, composed of a set of clock genes that act in transcription-translation-based feedback loops. The CLOCK/BMAL1 heterodimer activates transcription of the *Period* (*Per1*, *Per2*) and *Cryptochrome* (*Cry1*, *Cry2*) clock genes through E-box elements in their promoter. Following synthesis, the PER and CRY proteins will gradually accumulate in the nucleus and ultimately repress CLOCK/BMAL1, and thereby transcription of their own gene [13–15]. A second loop is formed by REV-ERB α , which cyclically inhibits ROR α -driven transcription of the *Bmal1* gene [16–19]. Adding to this transcription/translation feedback loop mechanism is a network of post-translational modifications of clock proteins (phosphorylation, (de)acetylation, sumoylation and ubiquitylation) that fine-tune the period length of the circadian oscillator and confer robustness and persistence to the molecular clock [20–27].

Photolyases, the other members of the CPF, are DNA repair enzymes that use visible light to lesion-specifically remove ultraviolet light-induced cyclobutane pyrimidine dimers (CPDs) or (6-4) pyrimidine-pyrimidone photoproducts ((6-4)PPs) from the DNA in a reaction called photoreactivation [28,29]. Placental mammals have lost photolyase genes during evolution and solely rely on nucleotide excision repair for removal of CPDs and (6-4)PPs [30]. Nevertheless, when expressed in the mouse, CPD and (6-4)PP photolyases rapidly remove these UV-induced lesions in a light-dependent manner and protect the animal from sunburn, mutation induction, and skin cancer development [31–33].

Phylogenetic analysis has shown that the CPF is divided in two major subgroups. The first subgroup encompasses (i) class I CPD photolyases, (ii) (6-4)PP photolyases and animal cryptochromes, (iii) plant cryptochromes, and (iv) DASH cryptochromes, whereas the second subgroup is solely composed of class II CPD photolyases [29]. It is accepted that all members of the photolyase/cryptochrome protein family evolved from a common ancestor CPD photolyase by multiple gene duplications [34]. Cryptochromes and photolyases on the one hand share a common backbone, the core domain, which binds two chromophoric cofactors (i.e. FAD and either 5,10-methenyl-tetrahydrofolate or 8-hydroxy-5-deazaflavin), but on the other hand differ in the presence of N- and C-terminal extensions (see Figure 1). Whereas eukaryotic photolyases have an N-terminal extension, containing nuclear and mitochondrial localization signals, cryptochromes contain a unique C-terminal extension of variable length and amino acid composition. It is currently accepted that the functional diversity among cryptochromes (i.e. photoreceptor, circadian photoreceptor, or core clock protein) is achieved by the diversity of their C-terminal extensions.

Detailed structure/function analysis of the C-terminal region of mammalian CRY1 allowed us to identify a putative coiled-coil domain at the beginning of the C-terminal extension as a potential PER and BMAL1 binding site [35]. Deletion of the complete C-terminal extension (aa 471–606 of mouse CRY1) abolished the CLOCK/BMAL1 transcription inhibitory potential of CRY1. Similarly, Green and co-workers have demonstrated that the C-terminal extension of *Xenopus laevis* CRY proteins is crucial for transcription repression [36]. Interestingly, specific deletion of either the coiled-coil domain (aa 471–493) or the downstream tail

region (aa 494–606) of mammalian CRY1 failed to eliminate its ability to inhibit CLOCK/BMAL1-mediated transcription [35], likely because these mutant proteins can still bind to CLOCK via a yet unidentified region of the core domain. This finding lead us to suggest that an interaction between the C-terminal extension and the core domain is mandatory for the clock function of mammalian cryptochromes, possibly by providing structure to the latter [35].

In the present study, we have explored the importance of the core domain of mammalian CRY proteins for core oscillator function by addressing the question to what extent photolyase enzymes affect circadian core oscillator function. Using *in vivo* (photolyase transgenic mice) and *in vitro* (cellular clock reporter and CLOCK/BMAL1 transcription assays) approaches, we show that *Potorous tridactylus* CPD photolyase (hereafter referred to as *PtCPD-PL*) not only displays cryptochrome-associated functions, but also can replace the CRY proteins in the mammalian circadian core oscillator.

Materials and Methods

Ethics statement

Mice were kept at the Animal Resource Center (Erasmus University Medical Center), which operates in compliance with the European guidelines (European Community 1986) and The Netherlands legislation for the protection of animals used for research, including ethical review. Animal studies at Erasmus University Medical Center were approved by DEC Consult, an independent Animal Ethical Committee (Dutch equivalent of the IACUC) under permit numbers 139-09-02 (EUR1702) 139-09-11 (EUR1760) and 139-09-12 (EUR1761).

Mouse lines and monitoring of circadian behavior

β -actin::*At*(6-4)PP-PL [33], β -actin::*PtCPD-PL* [31], and *Per2::Luc* transgenic mice (generation described below), as well as *Cry1^{-/-}/Cry2^{-/-}* knockout mice [9], all in a C57BL/6J background, were housed under standard conditions and fed *ad libitum*. All mouse lines were backcrossed at least 11 times to a C57BL/6J background. For the monitoring of locomotor activity rhythms, male mice (12–16 weeks) were individually housed in a light-proof

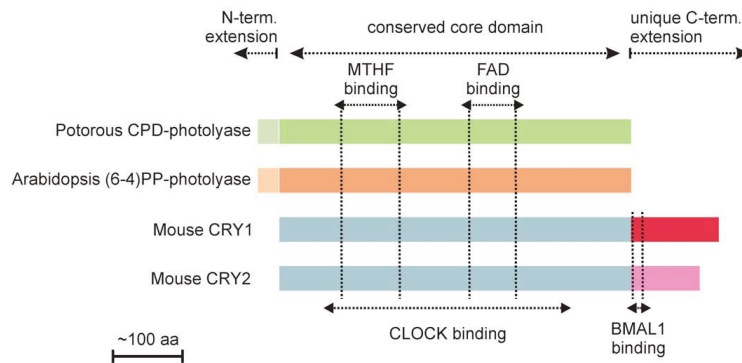


Figure 1. Cryptochromes and photolyases. Schematic representation of *PtCPD-PL*, *At*(6-4)PP-PL and mouse CRY1 and 2. Conserved and unique domain are indicated above, chromophore binding site are indicated by vertical dotted lines. CLOCK and BMAL1 binding regions in mouse CRY are indicated below.

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chamber in cages (30×45 cm) equipped with a running wheel (14 cm in diameter) and a sensor system to detect wheel revolutions. Animals were maintained in a cycle of 12 h light (150 lux) and 12 h darkness (LD) or in continuous darkness (DD) in constant ambient temperature with water and food available *ad libitum*. Voluntary wheel running (wheel revolutions per unit of time) was continuously recorded by an online computer using the ERS program. Activity records were plotted as actograms and the period of locomotor activity was determined by the chi-square method. Unpaired Student's t-tests were used to make statistical comparisons between the different genotypes.

Generation of *Per2::Luc* transgenic mice

The construct used to generate the *Per2::Luc* transgenic mice consists of the luciferase gene under control of the *mPer2* promoter, cloned in pBS (Figure S1). The primers used to amplify the 4.2 kb *mPer2* promoter fragment are indicated in Figure S1. Intronic sequences from the rabbit β -globin locus were included in the expression construct for messenger stability. The expression construct fragment was excised from the plasmid using appropriate restriction enzymes, separated from the vector DNA by agarose gel electrophoresis, isolated from the gel with the GeneClean II kit (Bio101), and further purified using Elutip-D-mini columns (Schleicher and Schuell, Dassel, Germany). The fragment was dissolved in injection buffer (10 mM Tris-HCl pH 7.5, 0.08 mM EDTA) and injected in the pronucleus of fertilized eggs derived from FVB/N intercrosses as described [37]. Animals were backcrossed in a C57BL/6J background. Genotyping was performed by PCR using primers located in the luciferase gene (Figure S1). Annealing was performed at 55°C. DNA derived from transgenic mice rendered a PCR product of 475 bp, whereas no product was detected using DNA from wild type litter mates.

RNA isolation and quantitative PCR

Coronal cryosections (25 μ m) mounted on 1 mm PALM pen-membrane™ slides were rapidly thawed, fixed for 30 seconds in 70% EtOH and immediately stained with haematoxylin for 3 minutes. Following staining sections were rinsed in DEPC treated dH₂O and dehydrated by several rinses in 100% EtOH. Laser catapult microdissection (LCM) of the SCN was accomplished using the PALM Microlaser system on freshly prepared sections. Isolated SCN was dissolved immediately in Lysis Buffer (Qiagen) and stored at -80°C for subsequent RNA purification. RNA was purified with the inclusion of 'on-column' DNase treatment using the Qiagen RNeasy 'Micro' kit according to the manufacturers protocol, except that an additional elution was performed in the final step to maximize RNA yield. RNA eluted with RNase-free dH₂O was vacuum evaporated for immediate amplification and cDNA generation. Quality of the freshly purified RNA was assayed using the Agilent BioAnalyzer in combination with the RNA 'Pico' chip. When intact 18S/28S ribosomal RNA peaks were evident, the sample was considered worthy of assay by Q-PCR.

Amplification was accomplished using the Ovation™ RNA Amplification System V2 according to the manufacturers protocols (Nugen Technologies Inc). Efficiency of the amplification was assayed quantitatively by 260/280 nm estimation of cDNA concentration, where a yield of at least 4.8 μ g cDNA was deemed sufficient for specific amplification of the RNA template; and qualitatively using the BioAnalyzer with the RNA 'Nano' chip to confirm that the majority of unfragmented, amplified cDNA is approximately of 900 bp in length. Quantitative PCR for the determination of *At(6-4)PP-PL* and *PICPD-PL* mRNA levels was performed in triplicate using an iCycler iQ™ Real-Time PCR

Detection System (BioRad), SYBR-green and primers [31,33] generating intron-spanning products of 150-300 bp. Expression levels were normalized to *Hprt* (hypoxanthine guanine phosphoribosyl transferase) mRNA levels. The generation of specific products was confirmed by melting curve analysis, and primer pairs were tested with a logarithmic dilution of a cDNA mix to generate a linear standard curve, which was used to calculate primer pair efficiencies.

Cell culture and transfection

COS7 [35], NIH3T3 (American Type Culture Collection), and HEK293T (American Type Culture Collection) cells, as well as primary wild type and *PICPD-PL* mouse dermal fibroblasts (MDFs) and immortalized *Cry1^{-/-}/Cry2^{-/-}* MDFs, were cultured in Dulbecco's modified Eagle's medium-F10-Pen/Strep-10% fetal calf serum. To generate MDFs, mice were sacrificed by cervical dislocation, and a small piece of back skin of the mouse was removed and cut into pieces with a razor blade. Skin pieces were washed in ethanol, rinsed in phosphate-buffered saline, and incubated overnight in medium supplemented with 1.6 mg/ml collagenase type II. Single cells were obtained by passing through a cell strainer, and collected by centrifugation for 5 min at 500 g, resuspended in culture medium, and seeded onto a 10 cm dish. MDFs were cultured in a low-oxygen incubator (5% CO₂, 3% O₂). Transient expression studies were performed by transfecting cells with plasmids using Fugene reagent (Boehringer) according to the manufacturer's instructions. The following pCDNA3-based plasmids (Invitrogen) were used: pCDNA-HA-mCry1, pCDNA-*PICPD-PL*, pCDNA-Bmal1 and pCDNA-Clock. pCDNA-*PICPD-PL* is based on the construct used to generate the transgenic mice [31]. For luminescence measurements pG4.11-Bmal1:luciferase (kindly provided by Dr. U. Schibler, Geneva) was used as a reporter.

Real time bioluminescence monitoring

To monitor circadian oscillations in cultured cells in real time, cells were cultured in medium buffered with 25 mM HEPES and containing 0.1 mM luciferin (Sigma). After synchronization of intracellular clocks by treatment of confluent cultures with forskolin (dissolved in 100% ethanol, added to the culture medium at a final concentration of 30 μ M), bioluminescence was recorded for 7 days (75 sec measurements at 10 min intervals) with a LumiCycle 32-channel automated luminometer (Actimetrics) placed in a dry, temperature-controlled incubator at 37°C. Data was analyzed with the Actimetrics software and two sample comparisons were done using a Student's T-test. Amplitudes were calculated both with Actimetrics software, on base-line subtracted data, and by comparing peak versus trough values for RAW data. Control amplitude was set at 100%. Both methods were comparable.

CLOCK/BMAL1 transcription reporter assay

To determine the capacity of *PICPD-PL* to inhibit CLOCK/BMAL1 driven transcription, we used a luciferase reporter assay as previously described [12,35]. COS7 cells were transfected with 200 ng of the *mPer1::luciferase* reporter construct and 15 ng of null-*Renilla luc*, which was used as an internal control. Clock, Bmal1, Cry1 and *PICPD-PL* plasmids were added as indicated in the figure legend. The total amount of DNA transfected was kept constant at 2 μ g by supplementing with empty pCDNA3.1 vector (Invitrogen). Transcriptional activity was assessed with the Dual-Luciferase 10 Reporter Assay System (Promega) by measuring a ratio of firefly luciferase activity to *Renilla* luciferase activity in each cellular lysate.

Co-immunoprecipitation experiments

Co-immunoprecipitation studies were performed as described previously [21]. In short, we transiently expressed a *P*CPD-PL and either Flag-Bmal1 and/or Flag-Clock in HEK 293T cells and used anti-FLAG antibodies (Sigma) and anti-*P*CPD-PL [31] antibodies for the immunoprecipitation and immunoblot analysis step (1:1000 dilution). As secondary antibody, we used horseradish peroxidase conjugated anti-mouse IgG (DAKO) and anti-rabbit IgG (BioSource) at a 1:1000 dilution. Chemoluminescence was detected using the ECL system (Pharmacia Biotech).

Hydroporation experiments

Hydrodynamic tail vein injection experiments were performed as described [38]. In brief, a sterile Ringers Solution (0.9% NaCl, 0.3% KCl, 0.13% CaCl_2) containing a total of 10 μg plasmid DNA was rapidly injected (8–10 sec) in the tail vein of the mouse under isoflurane anesthesia. This induces uptake of DNA by the liver, which is initially transient and a small proportion will integrate upon regeneration of the liver. Expression of the hydroporated constructs was non-invasively analyzed using an IVIS[®] Spectrum imaging device (Caliper/Xenogen) (Figure S2), and positive mice were selected for liver isolation and slicing. Animals were sacrificed 24 h after injection (transient expression) and the livers were rapidly removed and placed in ice cold Hank's balanced salt solution supplemented with 50 mM glucose, 4 mM sodium bicarbonate, 10 mM HEPES, 10,000 unit/ml penicillin/10,000 $\mu\text{g}/\text{ml}$ streptomycin [5]. Liver slices (200 μm) were prepared using an automated Krundieck tissue slicer (Alabama R&D). Individual slices were placed on a membrane insert (Millipore) in a 35-mm dish in imaging medium (DMEM supplemented with 0.1 mM luciferin, 2% B27 supplement, 4 mM sodium bicarbonate, 10 mM HEPES, 2.5 ml 10,000 units/ml penicillin/10,000 $\mu\text{g}/\text{ml}$ streptomycin). Real time imaging and synchronization were performed as described above. The plasmids used were pG4.11-Bmal1:luciferase and pCry1::P*CPD*-PL. Cloning and characterization of the *Cry1* promoter will be described elsewhere (Saito and van der Horst, unpublished data).

Results

Potorous tridactylus CPD photolyase transgenic mice have a short period circadian clock

To investigate whether their strong structural resemblance to cryptochromes (see Figure 1) allows photolyases to interfere with mammalian circadian core oscillator function, we took advantage of the availability of β -actin promoter-driven *Potorous tridactylus* CPD photolyase and *Arabidopsis thaliana* (6-4)PP photolyase transgenic mice (hereafter referred to as *P*CPD-PL and *At*(6-4)PP-PL mice), previously generated in our laboratory. These animals carry 3 copies of the *P*CPD-PL and *At*(6-4)PP-PL transgene, respectively, and express an active photolyase, capable of removing DNA lesions from the DNA in a light-dependent manner [31,33]. As shown in Figure 2A, and as could be expected on the basis of the ubiquitous expression of the β -actin promoter, quantitative RT-PCR analysis of mRNA derived from laser microdissected SCN revealed that *P*CPD-PL and *At*(6-4)PP-PL mice express the photolyase transgene in the SCN at comparable levels to the *Hprt* gene.

We next addressed the question whether expression of photolyase in the SCN would affect the circadian behavior of the mouse. To this end, we measured circadian wheel-running behavior of photolyase transgenic mice and sex and age-matched control littermates under normal light/dark (LD) cycles and in constant darkness (dark/dark; DD). As shown in Figure 2B, the period length (τ or τ) of circadian behavior of *At*(6-4)PP-PL transgenic mice is indistinguishable from that of the corresponding

wild type littermates. In marked contrast, *P*CPD-PL transgenic mice revealed a small but significant shortening of the period length of (15 to 20 min, $p < 0.05$), as compared to wild type littermates (Figure 2C). In addition, the tau of the *P*CPD-PL mice has a larger (2-fold) variation than that of control littermates, possibly derived from small individual differences in expression levels.

These findings strongly suggest that expression of *Potorous tridactylus* CPD photolyase in the SCN interferes with circadian clock performance. In contrast, *Arabidopsis thaliana* (6-4)PP photolyase does not appear to influence the circadian clock. This observation is in agreement with our finding that *At*(6-4)PP-PL is not able to inhibit CLOCK/BMAL1 [35]. We therefore further will focus on the effect of *P*CPD-PL on circadian rhythms.

Potorous tridactylus CPD photolyase dampens the circadian core oscillator

As photolyases structurally resemble cryptochromes [29,34], and given the observation that transient constitutive overexpression of the CRY1 protein suppresses the rhythmic expression of a cotransfected Bmal1::Luc reporter gene (Figure 3A), we next investigated the effect of transient overexpression of *P*CPD-PL on the circadian clock of cultured fibroblasts. After synchronization of the individual intracellular circadian clocks with forskolin [39], cells cotransfected with the Bmal1::Luc reporter construct and empty pcDNA3 vector (used as a negative control) were shown to oscillate with a period of 25.6 ± 0.2 hr ($n = 11$). Interestingly, overexpression of *P*CPD-PL reduces the amplitude of the oscillations in a dose-dependent manner (Figure 3B; Table 1).

To study the influence of CPD photolyase on core oscillator performance under physiological conditions, *P*CPD-PL mice were interbred with *Per2::Luc* mice to obtain primary CPD-photolyase mouse dermal fibroblast (MDF) lines containing a clock reporter. Bioluminescence rhythms in *P*CPD-PL/*Per2::Luc* MDFs show a reduction in amplitude ($38 \pm 5\%$) when compared to those in *Per2::Luc* (control) fibroblasts (Figure 3C; Table 1), thus confirming the data obtained in the transient expression studies. Interestingly, and in line with the animal studies, the period of oscillations in *P*CPD-PL/*Per2::Luc* MDFs (22.7 ± 0.4 hr; $n = 4$) is approximately 50 min shorter ($p < 0.05$) than that of *Per2::Luc* fibroblasts that do not carry the CPD photolyase transgene (23.8 ± 0.2 hr; $n = 4$).

Taken together, these data demonstrate that the *P*CPD-PL exerts a dominant negative effect on the circadian clock by dampening the oscillations and shortening the period length, likely by interfering with CRY mediated functions.

Potorous tridactylus CPD photolyase inhibits CLOCK/BMAL1-driven transcription

The dominant negative effect of CPD photolyase on cellular clock performance and circadian behavior, as evident from the *in vivo* and *in vitro* studies, prompted us to investigate the underlying mode of action. Since CRY proteins are strong inhibitors of the CLOCK/BMAL1 transcription activator [12], we used a COS7 cell based reporter assay to analyze the ability of *P*CPD-PL to inhibit CLOCK/BMAL1-driven transcription of the *mPer1* promoter-driven luciferase reporter gene. Consistent with previous studies [12,35] simultaneous expression of CLOCK and BMAL1 causes a 30-fold induction in transcription of the luciferase gene, which is strongly repressed in the presence of CRY1 (Figure 4A). Interestingly, notwithstanding the fact that the protein should be expressed at high level, the *P*CPD-PL is also capable of significantly suppressing CLOCK/BMAL1 activity.

From these data we conclude that *P*CPD-PL is able to interfere with mammalian core oscillator performance by exerting a

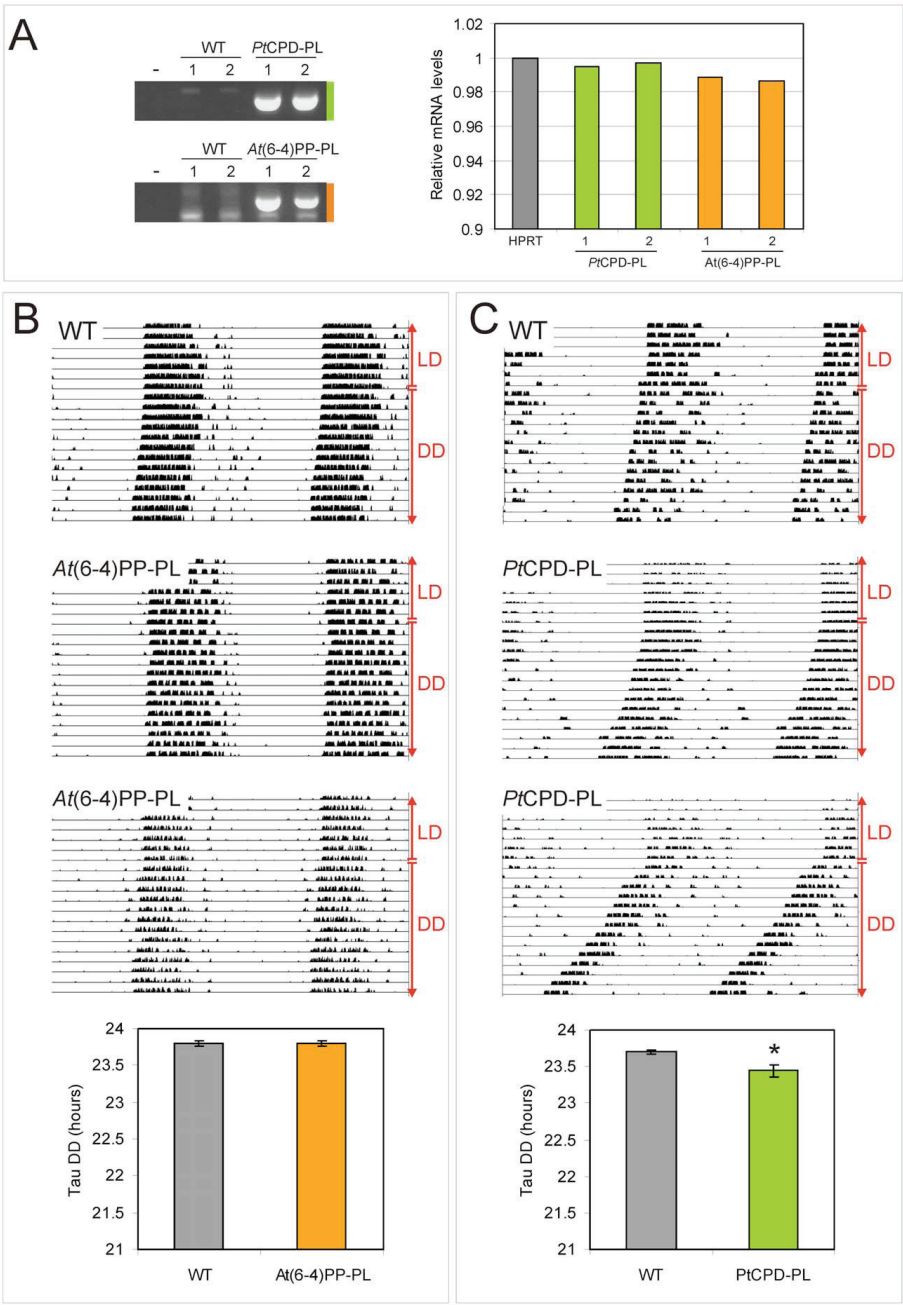


Figure 2. Circadian behavior of photolyase transgenic mice. (A) Quantitative RT-PCR analysis of photolyase mRNA levels in the laser-microdissected SCN of *PtCPD-PL* and *At(6-4)PP-PL* transgenic mice. Left panel: Ethidium bromide stained gel of PCR amplified cDNA, obtained from two independent transgenic mice and corresponding wild type littermates (sacrificed at ZT3). Right panel: graphic representation of quantitative RT-PCR amplification data from two independent animals per genotype (see Experimental procedures for details). The Y-axis represents the *PtCPD-PL* and *At(6-4)PP-PL* mRNA levels relative to that of *Hprt*. (B, C) Circadian behavior of *At(6-4)PP-PL* (B) and *PtCPD-PL* (C) transgenic mice and corresponding littermates ($n = 10$ per genotype). Animals were kept under normal light conditions (LD 12:12 h) and subsequently exposed to constant darkness (DD) (indicated on the right side of the panels). Shown are representative examples of double-plotted actograms and graphic representations of the free-running period (τ) in constant darkness (bottom panels). Error bars represent the standard error of the mean (SEM); the asterisk indicates significance ($p = 0.03$). doi:10.1371/journal.pone.0023447.g002

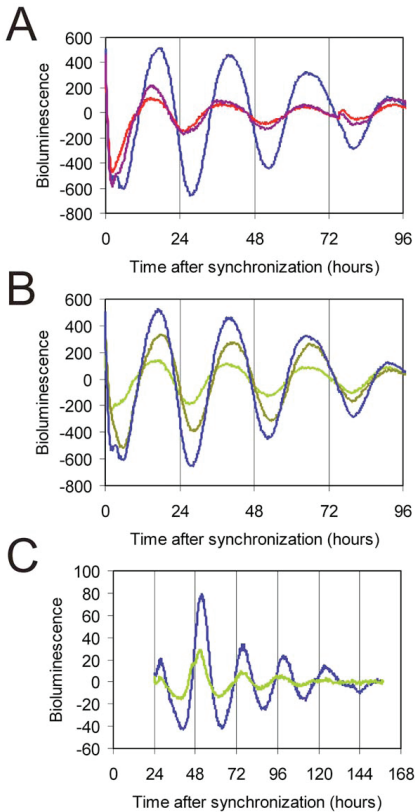


Figure 3. *PtCPD* photolyase dampens circadian oscillations. (A, B) Representative examples of bioluminescence rhythms in NIH3T3 cells co-transfected with a mBmal1:luciferase reporter construct and either empty pcDNA3 (blue line), pcDNA-Cry1 (100 ng, purple line; 200 ng, red line) or pcDNA-CPD photolyase (200 ng, olive line; 400 ng, green line). Empty pcDNA3 vector was added to correct for the amount of DNA transfected. (C) Representative example of bioluminescence rhythms in primary MDFs, derived from *PtCPD* photolyase transgenic mice (green line) and wild type littermates (blue line), transiently expressing the Bmal1:luciferase reporter gene. Bioluminescence recordings were started immediately after forskolin synchronization of the individual cellular clocks. The Y-axis represents base line subtracted bioluminescence values. doi:10.1371/journal.pone.0023447.g003

cryptochrome-like function, i.e. inhibition of CLOCK/ BMAL1-mediated transcription.

Potorous tridactylus CPD photolyase interacts with CLOCK

We have previously shown that the inhibition of CLOCK/ BMAL1 mediated transcription by CRY1 requires a complex network of interactions with CLOCK and BMAL1, involving the CRY1 core domain and C-terminal extension [35] (see also Figure 1). We therefore next asked the question whether *PtCPD-PL*, like CRY proteins, can physically interact with CLOCK and BMAL1. To this end, we performed a co-immunoprecipitation experiment using HEK293T cells overexpressing the FLAG-CLOCK or FLAG-BMAL1 proteins alone, or in combination with *PtCPD-PL* (Figure 4B). In the absence of *PtCPD-PL* neither CLOCK nor BMAL1 were pulled down with anti-*PtCPD-PL* antibodies, which excludes non-specific binding of the antibodies to these proteins. However, in the presence of *PtCPD-PL*, the CLOCK protein is shown to co-precipitate with *PtCPD-PL*, whereas the BMAL1 protein is not. This result indicates that *PtCPD* photolyase inhibits CLOCK/BMAL1-mediated transcription through direct interaction with CLOCK.

Potorous tridactylus CPD photolyase can replace cryptochromes in the mammalian circadian oscillator

Having shown that *PtCPD-PL* can interact with CLOCK, leading to inhibition of CLOCK/BMAL1-driven transcription, the intriguing question arises whether this photolyase can actually replace the CRY proteins in the mammalian circadian oscillator and rescue the arrhythmicity of *Cry1^{-/-}/Cry2^{-/-}* mice. Generation of a new *Cry1* promoter-driven *PtCPD-PL* transgenic mouse line in a *Cry*-deficient background and subsequent behavioral analysis would be extremely time-consuming. As peripheral circadian clocks form a good model for the master clock in the SCN [40,41], we chose to generate stable fibroblast lines, derived from *Cry1^{-/-}/Cry2^{-/-}* mice, transfected with pCry1::*PtCPD-PL*, pCry1::Cry1 or the empty vector. These cells were then transiently transfected with Bmal1::Luc reporter construct, synchronized with forskolin,

Table 1.

NIH 3T3 cells		
	Tau (hours)	Amplitude (%)
control	25.60 ± 0.16	100
<i>PtCPD-PL</i>	25.57 ± 0.16	65 ± 7
MDF lines		
	Tau (hours)	Amplitude (%)
WT	23.83 ± 0.18	100
<i>PtCPD-PL</i>	22.70 ± 0.43	62 ± 5

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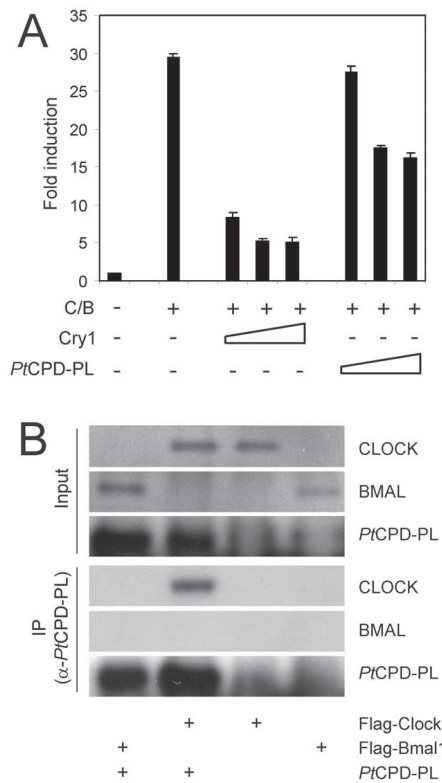


Figure 4. PiCPD photolyase represses CLOCK/BMAL1-driven transcription and interacts with CLOCK. (A) COS7 cell-based CLOCK/BMAL1 transcription assay using a *mPer1* E-box promoter-luciferase reporter construct. Luminescence, shown as x-fold induction from the basal expression level (set to 1), is indicated on the Y axis. pcDNA3, pRL-CMV, and the *mPer2::luc* were added in all reactions. The presence or absence of Cry1 (10–100 ng) and PiCPD-PL (100–300 ng) expression plasmids is indicated below the graph. Empty pcDNA3 vector was added to correct for the amount of DNA transfected. Mean and standard deviation of triplicate samples are shown. (B) Identification of photolyase-binding proteins. PiCPD-PL was precipitated from HEK293T cells, transfected with PiCPD-PL, Flag-Clock or Flag-Bmal1 or double transfected with PiCPD-PL and either Flag-Clock or Flag-Bmal1. Upper panels: Immunoblot analysis of total cell lysates, confirming the presence of the various transiently expressed proteins. Lower panels (IP): Immunoblot analysis of precipitated PiCPD-PL (anti-PiCPD-PL antibodies) and CLOCK and BMAL1 (anti-FLAG antibodies). doi:10.1371/journal.pone.0023447.g004

and subjected to real time luminescence monitoring. As expected, expression of pCry1::Cry1 (C, D) restores circadian rhythms in arrhythmic *Cry1^{-/-}/Cry2^{-/-}* fibroblasts (Figure 5C and D), whereas the empty vector has no effect (Figure 5A and B). Interestingly, pCry1::PiCPD-PL induces oscillations (Figure 5E and F). Although the period appears in the circadian range, the irregular character of the oscillations precluded calculation of tau.

Taking into account the irregular character of the above mentioned oscillations, we took an alternative approach to further to study the capacity of PiCPD-PL to rescue circadian rhythms in tissues. To this end, we used the hydroporation technique [38] to introduce clock reporter and CPD photolyase expression constructs in the mouse liver. Twenty-four hours after co-injection of pGLA.11-Bmal1::Luc and either pCry1::PiCPD-PL or the empty vector in the tail vein of the mouse, liver slices were prepared and clock performance was monitored by real-time imaging of bioluminescence. Co-injection of the Bmal1::Luc reporter plasmid and empty vector in *Cry1^{-/-}/Cry2^{-/-}* mice and *Cry1^{-/-}/Cry2^{-/-}* littermate controls resulted in rhythmic expression of the luciferase reporter gene in *Cry1^{-/-}/Cry2^{-/-}* liver slices (Figure 6A and B), whereas, in line with the absence of a circadian clock, bioluminescence levels remained flat in liver slices from *Cry1^{-/-}/Cry2^{-/-}* mice (Figure 6C and D), thus validating the hydroporation approach. Interestingly, upon co-injection of the Bmal1::Luc reporter plasmid and pCry1::PiCPD-PL and PiCPD-PL expression construct in *Cry1^{-/-}/Cry2^{-/-}* animals, we observed a reinitiation of circadian rhythmicity in *Cry1^{-/-}* liver slices for at least two cycles (Figure 6E and F). This oscillation dampened rapidly, but could be revived for at least one cycle by forskolin treatment of the liver slices (Figure 6G). As in the absence of PiCPD-PL forskolin did not exert any effect on bioluminescence levels in *Cry1^{-/-}/Cry2^{-/-}* slices (Figure 6H), the forskolin-induced bioluminescence rhythm in PiCPD-PL expressing *Cry1^{-/-}/Cry2^{-/-}* slices can only be explained by resynchronization of (running) intracellular clocks.

From these data we conclude that rhythmically expressed PiCPD photolyase can functionally substitute for CRY proteins in the mammalian circadian oscillator and that such a PiCPD-PL-driven molecular oscillator can still respond to non-photic clock-synchronizing stimuli (i.e. forskolin).

Discussion

In the present study, we analyzed the capacity of two different photolyases to interfere with circadian clock performance: the Class II CPD photolyase from *Potorous tridactylus* (PiCPD-PL), which is only distantly related to CRY1, and the (6-4)PP photolyase from *Arabidopsis thaliana* (At(6-4)PP-PL), which is closely related to CRY1. In line with our observation that At(6-4)PP-PL does not inhibit CLOCK/BMAL1 transcriptional activity [35], analysis of the circadian behavior of *β-actin* promoter-driven At(6-4)PP-PL transgenic mice (ubiquitously expressing the transgene, including the SCN) did not reveal any dominant negative effect of the photolyase on circadian period length. In marked contrast, *β-actin* promoter-driven PiCPD-PL transgenic mice showed a small but significant reduction of the period length of circadian behavior. In support of this *in vivo* observation, we also obtained a shortening of the period length of the molecular oscillator in cultured PiCPD-PL dermal fibroblasts. This accelerated clock in PiCPD-PL transgenic mice is unlikely an artifact resulting from unintended inactivation of known (core) clock genes, as analysis of the sequences flanking the integration site of the transgene excluded the presence of such genes (data not shown). Moreover, transiently overexpressed PiCPD-PL was able to dampen the circadian oscillator in NIH3T3 cells. In this respect PiCPD-PL resembles CRY1, which when overexpressed also blunts circadian rhythms. The effect of PiCPD-PL on circadian rhythms is mediated by repression of CLOCK/BMAL1-mediated transcription via direct physical interaction with CLOCK, but not with BMAL1.

Previously, we have shown that removal of the complete C-terminal extension of CRY1 abolishes repressor activity towards

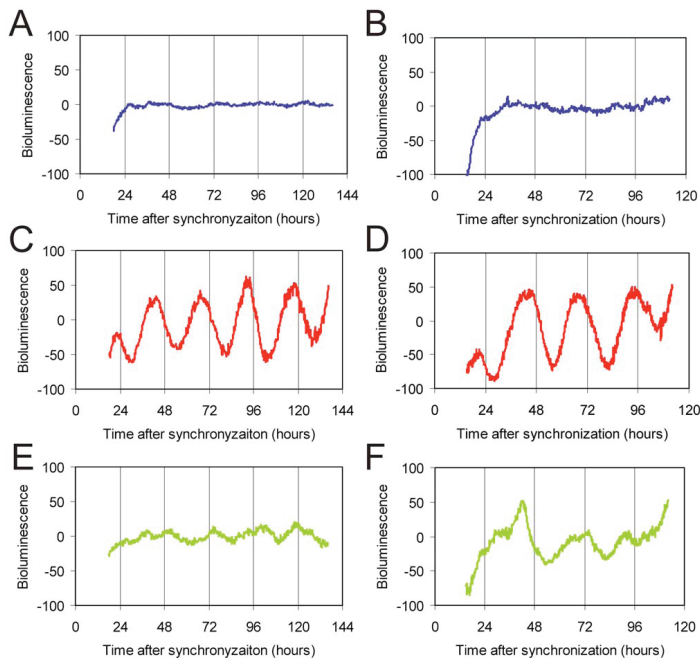


Figure 5. Correction of the circadian clock in fibroblast lines derived from CRY-deficient mice. Representative examples of bioluminescence rhythms in immortalized MDF lines, derived from *Cry1^{-/-}/Cry2^{-/-}* mice, and stably expressing either empty pcDNA3 (A, B), pcCry1::Cry1 (C, D) or pcCry1::PcCPD-PL (E, F), and transfected with the reporter construct. Y-axis represents base line subtracted bioluminescence values.

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CLOCK/BMAL1 driven transcription of E-box containing clock genes and clock-controlled genes [35]. In the same study, we demonstrated that whereas *At(6-4)PP-PL* by itself has no effect on CLOCK/BMAL1, fusion of the last 100 aa of the CRY1 core domain in conjunction with its C-terminal extension (aa 371-606) to *At(6-4)PP-PL* resulted in a chimeric protein which is still able to inhibit CLOCK/BMAL1-mediated transcription. Based on these findings, we hypothesized that acquirement of C-terminal extensions (to the core domain) during evolution functionally separated cryptochromes from photolyase and conferred a clock function to the CRY proteins [35]. We now provide evidence that *PcCPD-PL* harbors core clock features that allow it to repress CLOCK/BMAL1 transcriptional activity and function as a true cryptochrome. Considering that *At(6-4)PP-PL* is more homologous to CRY1 than *PcCPD-PL*, our findings suggest that it is not the primary amino acid sequence per se, but rather the overall structure of the core domain, that makes a photolyase repressing CLOCK/BMAL1-mediated transcription. Our results indicate that the *PcCPD-PL* by itself has the proper structure, whereas *At(6-4)PP-PL* gains such a structure after fusion with a C-terminal extension of mammalian CRY1 [35]. In addition, as *PcCPD-PL* fails to bind BMAL1, interaction with CLOCK is sufficient to inhibit CLOCK-BMAL1-mediated transcription, which is in complete agreement with our previous observation that the BMAL1-binding coiled-coil domain in the C-terminal

extension of CRY1 can be deleted without major consequences [35].

Given the dominant negative effect of constitutive *PcCPD-PL* expression on circadian behavior of the mouse (*in vivo* data), it is tempting to speculate on the underlying molecular mechanism by which *PcCPD-PL* can inhibit CLOCK/BMAL1-driven transcription (*in vitro* data) and its impact on circadian core oscillator performance. By interacting with CLOCK, *PcCPD-PL* may prevent the formation of CLOCK/BMAL1 heterodimers and/or binding of the CLOCK/BMAL1 heterodimer to E-box promoters in the DNA. In this scenario, the photolyase reduces the efficiency at which E-box containing clock (controlled) genes are transcribed by reducing the number of available transcription activators. However, as we have shown that a *Chrysodeixis chalcites* nucleopolyhedroviral photolyase can bind to mammalian CLOCK without affecting CLOCK/BMAL1 transcription potential, binding of a CPF protein per se does not prevent CLOCK/BMAL1 heterodimerization and DNA binding (Biernat and Chaves, submitted for publication). Therefore, a more plausible explanation would be that binding of *PcCPD-PL* to CLOCK inhibits transcription activation of E-box promoter-bound CLOCK/BMAL1 heterodimers in a cryptochrome like manner. Strikingly, using an *in vivo* hydroporation approach, we show that *PcCPD-PL* can rescue the lost circadian oscillator in CRY-deficient cells. When expressed from the *Cry1* promoter, *PcCPD-PL* revived rhythmic expression of

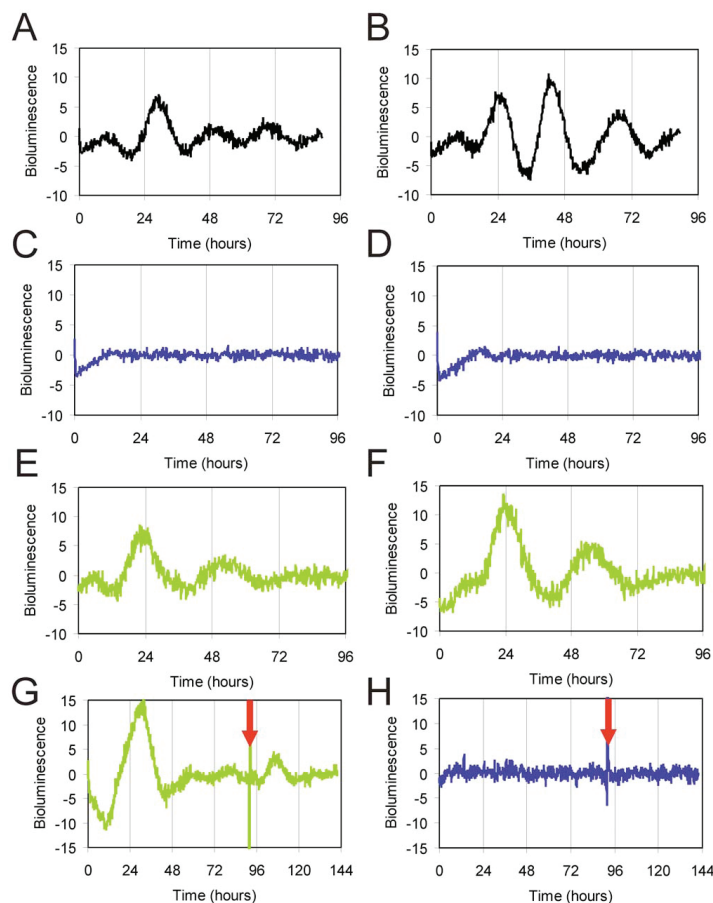


Figure 6. *PcCPD* photolyase corrects the circadian clock in the liver of *CRY*-deficient mice. Representative examples of bioluminescence rhythms in liver slices obtained from mice hydroperated with pGL4.11-Bmal1:Luc (black line), together with either empty pcDNA3 (blue line) or pcDNA-CPD photolyase constructs (green line). (A, B) Liver slices from control mice, injected with the reporter construct only. (C-H) Liver slices from *Cry1^{-/-}/Cry2^{-/-}* mice injected with either empty pcDNA3 (C, D, H) or pcCry1::PcCPD-PL (E-G), in addition to the reporter construct. In some experiments (G and H) the slices were treated with Forskolin at 96 h to resynchronize the circadian clock. The Y-axis represents base line subtracted bioluminescence values.

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the *Bmal1::luciferase* reporter gene in the *Cry1^{-/-}/Cry2^{-/-}* mouse liver explants. Moreover, the period of oscillations was in the same range as that of a CRY-driven oscillator and responds to non-photic phase synchronizing stimuli (i.e. forskolin). We therefore conclude that the *PcCPD-PL* protein has the potential to act as a true mammalian CRY protein.

While this work was in progress, two other members of the CPF have been shown to maintain dual functions: the *PcCPF1* protein from the marine diatom *Phaeodactylum tricornutum* and the *OtCPF1* protein from the green algae *Ostreococcus tauri*. These proteins hold

(6-4)PP photolyase activity and (like *Potorous* CPD-PL) can inhibit CLOCK/BMAL1 driven transcription in a heterologous mammalian system [42,43] and are therefore considered a missing link in evolution. Interestingly, we found that *Arabidopsis thaliana* (6-4)PP photolyase does not inhibit CLOCK/BMAL1 [35] or affect circadian behavior, whereas the distantly related *Potorous* CPD photolyase does. Moreover, we show that this marsupial class II CPD-photolyase can actually substitute for CRY proteins in the mammalian circadian oscillator. In a parallel study, we have shown that the *Chrysoideixis chalcites* nucleopolydiovirus PHR2

protein, another class II CPD photolyase, is able to interact with CLOCK and affect circadian rhythms *in vitro* (Biernat and Chaves, unpublished data).

These findings contribute to understanding the functional evolution of cryptochromes and photolyases. So far, the identified CPF members with a dual function are either (6-4)PP photolyases from lower eukaryotes [42,43] or class II CPD photolyases (this manuscript; Biernat and Chaves, submitted for publication). Ancestral CPF members were likely proteins with both DNA repair and circadian clock function. We propose that after the divergence of classes I and II, class II CPD photolyases have kept this dual function throughout evolution. Class I CPD photolyases and (6-4)PP photolyases, however, have lost the circadian function in time, which was taken by cryptochromes. In view of this hypothesis, it will be challenging to study molecular clocks in organisms that have both a photolyase with a dual function and cryptochromes, as is the case of marsupials, such as *Potorous* and *Monodelphis*. The genome of *Monodelphis domestica* has been sequenced and reveals the presence of cryptochrome genes, as well as photolyase. It will be of interest to determine how the clock of non-placental mammals will respond to the loss of photolyase. On the basis of our data, one would predict a change in tau, suggesting that the circadian clock of placental mammals has adapted to the loss of photolyase by adjusting period. Studying the marsupial circadian system at the cellular and molecular level will answer these questions and shed light on the functional evolution of the CPF.

The present study has identified the *Potorous tridactylus* CPD photolyase as an attractive candidate for further structure-function studies, aiming at understanding the functional diversity between cryptochromes and photolyases. Analogous to our previous study with mammalian CRY1 - *Arabidopsis* (6-4)PP photolyase chimeric proteins, it will be informative to swap domains between *Potorous* CPD photolyase and CRY proteins for functional mapping. Such

studies will ultimately reveal how nature uses the same core sequence for completely different functions (e.g. photoreactivation by photolyases vs clock function of cryptochromes).

Supporting Information

Figure S1 Schematic representation of the mPer2::Luc construct used to generate transgenic clock reporter mice. (A) The luciferase gene is cloned in front of the *mPer2* promoter, using pBS as backbone. Intronic sequences from the rabbit β -globin locus were included in the expression construct for messenger stability. Restriction enzyme sites are indicated. (B) Sequence of the primers used to amplify the 4.2 kb *mPer2* promoter fragment. (C) Sequence of the luciferase primers used to genotype mPer2::Luc mice. (TIF)

Figure S2 Detection of luminescence in the liver of hydrophorated mice. Representative examples of dorsal luminescence images, obtained 24 hour after hydrophoration of mice with either the Bmal1::Luc reporter construct (left) or the empty vector (right). Expression of the hydrophorated constructs was non-invasively monitored in isoflurane anesthetized animals using an IVIS[®] Spectrum imaging device (Caliper/Xenogen). Colors indicate signal intensity. Note that the reporter is prominently expressed in the liver. (TIF)

Author Contributions

Conceived and designed the experiments: IC APME GTJvdH. Performed the experiments: IC RMN MAB MIB KB ACdS. Analyzed the data: IC GTJvdH. Contributed reagents/materials/analysis tools: SS KY. Wrote the paper: IC GTJvdH.

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