The cell Cycle
and
Circadian Clock

A Tale of Two Cycles

Eugin Destici

The Cell Cycle & Circadian Clock

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De Celcyclus & Circadiane Clock

Een verhaal van twee cycli

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 voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 22 september 2010 om 11.30 uur

door

Eugin Destici

geboren te Hengelo

ERASMUS UNIVERSITEIT ROTTERDAM

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"Wetenschap is de titanische poging van het menselijk intellect zich uit zijn kosmische isolement te verlossen door te begrijpen."

W.F. Hermans (schrijver en wetenschapper, 1921-1995)

"Science is the titanic attempt of the human intellect to free itself from its cosmic isolation by understanding."

W.F. Hermans (Dutch writer and scientist, 1921-1995)

CHAPTER 1

INTRODUCTION

Chapter 1

The circadian system

> "And God said, "Let there be light," and there
> was light. And God saw that the light was good.
> And God separated the light from the darkness.
> God called the light Day, and the darkness he
> called Night. And there was evening and there
> was morning, the first day". Genesis.

During the first period of its existence, the earth was a hostile environment for the first forms of life due to the extreme temperatures and daily UV exposure, which today we are shielded from by the ozone layer. To cope with this damaging and potentially lethal UV bombardment, early life forms may have developed a mechanism to avoid UV exposure during the process of DNA replication, when cells are most sensitive to UV. This daily UV-exposure may have been of the driving forces underlying the evolution of circadian clock. This hypothesis is referred to as the "flight-from-light" hypothesis (Pittendrigh, 1993). However, it is thought that circadian clocks evolved several times during evolution in the different kingdoms of life (Young and Kay, 2001) and therefore, circadian clocks may have evolved during times when UV radiation was not a big problem anymore due to the presence of the UV-shielding ozone layer. Another driving force for circadian clocks to appear may have been to segregate incompatible processes to different times of the day. For example, in the Cyanobacterium *Oscillatoria* nitrogen fixation and photosynthesis, which are incompatible processes, are temporally separated by the circadian clock allowing both processes to occur in the same cell (Stal and Krumbein, 1987). A similar strategy may have been used for other metabolic processes in other organisms and could therefore have been a driving force for the evolution of circadian clocks.

The rotation of the earth around its axis imposes daily recurring changes to our environment, notably cyclic light-dark and temperature alternations. To anticipate these solar day-night cycles, most organisms have developed an internal clock with a near 24-hour 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 (Pittendrigh, 1960). 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 (Dunlap, 1999). To maintain synchrony between body time and solar time, circadian clocks need to be adjusted every day by light.

Circadian rhythms are defined by their persistence in the absence of any environmental cues (and are therefore self-sustained), their temperature-independence and their close approximation to the period of the earth's rotation (Pittendrigh, 1960). In its simplest form, a circadian clock can be said to be composed of three components: a central clock (an internal oscillator, generating body time), an input (keeping the clock in phase with environmental cues, notably the light-dark cycle), and an output (coupling the clock to biological processes). This basic scheme applies to all known circadian clocks, regardless of the species involved. Given that the work described in thesis only deals with the mammalian system, the remainder of the introduction will focus mainly, but not exclusively, on the mammalian circadian clock.

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 (Stephan and Zucker, 1972; Moore and Eichler, 1972). Moreover, transplantation of fetal SCN tissue to the brain of arrhythmic SCN-lesioned hamsters restored their circadian behavior (Lehman et al., 1987) through a diffusible signal (Silver et al., 1996). Although these experiments already pointed to the SCN as the primary circadian pacemaker, 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 (Ralph and Menaker, 1988). 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 always determined by the donor SCN (Ralph et al., 1990). That is, lesioned wild type hamsters with a mutant SCN now displayed short-period behavioral rhythmicity, while lesioned mutant hamsters with a wild type SCN had their rhythm restored with a period comparable to that of unlesioned wild type hamsters.

On average, the period of the rodent circadian clock is shorter than 24 hour, whereas the human clock has the period slightly longer than 24 hours (Czeisler et al., 1999). Accordingly, the SCN clock needs to be adjusted daily to keep pace with the exact 24 hour 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 (Ralph and Menaker, 1989; Rusak et al., 1989). For instance, when housed in constant darkness, a light pulse at the end of the subjective night (the active 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 for most rodents) light pulses do not exert any effect.

The light information required for photo-entrainment of the mammalian circadian clock, 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 (Provencio et al., 1998; Berson et al., 2002; Hattar et al., 2002). However, as melanopsin-deficient mice still show residual photo-entrainment (Panda et al., 2002; Ruby et al., 2002), other (redundant) photopigments likely also contribute to circadian photoentrainment. Crossing melanopsin-deficient mice with rodless/coneless mice resulted in complete ablation of photo-entrainment, demonstrating a requirement for rods and cones in this process (Hattar et al., 2003; Panda et al., 2003).

Chapter 1

Circadian rhythmicity is generated by a molecular oscillator

Molecular and genetic analysis of the circadian clock in Cyanobacteria, fungi (*Neurospora*), plants (*Arabidopsis*), insects (*Drosophila*), amphibia (*Xenopus*), fish (zebrafish), and mammals (mainly 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 hour periodicity (described in detail below; Dunlap, 1999; Bell-Pedersen et al., 2005).

The first genetic basis for circadian clocks was demonstrated by seminal work from Konopka and Benzer (Konopka and Benzer, 1971). Using a mutagenesis screen in *Drosophila*, they found three different mutant strains that had a short, long or arrhythmic clock. Surprisingly, all three mutations mapped to the same locus, which was named the *period* (*per*) locus. More than a decade later, the gene was mapped and found to be able to rescue rhythmicity in the mutant strains (Bargiello et al., 1984; Zehring, 1984). Later, the first circadian feedback loop was described in which *per* was negatively regulating its own expression (Hardin et al., 1990). A few years after the *Drosophila* screen, a similar screen in the slime mold *Neurospora* also revealed three circadian mutants in that species, which, like in *Drosophila*, mapped to one locus, the *frequency* (*frq*) locus (Feldman and Hoyle, 1973). Like the *Drosophila per* gene, the *frq* gene was found to be involved in a negative feedback loop in which *frq* was inhibiting its own expression (Aronson et al., 1994). Groundbreaking work from the Takahashi lab, involving a large ENU-induced mutagenesis screen in the mouse, identified the first mammalian locus involved in regulating circadian rhythms (Vitaterna et al., 1994), which was named *Clock* (Circadian Locomoter Output Cycle Kaput). A few years later the same lab mapped and positively identified the actual gene, which was found to encode a basis-helix-loop-helix transcription factor, and the mutation in this gene (Antoch et al., 1997; King et al., 1997). Although earlier work identified a (spontaneous) mutation in a different mammalian species, namely the *tau* mutation in the Siberian hamster (Ralph et al., 1988), the actual gene (*Casein kinase 1ε*) and the responsible mutation were identified only several years (again by the Takahashi lab) after the *Clock* gene (Lowrey et al., 2000). As in other species, also the mammalian clock is driven by a negative feedback loop. In fact, to date all the described circadian clocks contain a negative feedback loop at their basis, which is a common phenomenon in most if not all oscillatory cellular mechanisms (Tyson, 2002).

As shown in Figure 1, the mammalian molecular oscillator consists of a feedback loop in which the transcription factor BMAL1 (Brain and muscle Arnt-like protein-1) together with either CLOCK or NPAS2 (Neuronal PAS domain protein 2) 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 (Shearman et al., 2000). 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 (Bunger et al., 2000; Van der Horst et al., 1999; Okamura et al., 1999; Vitaterna et al., 1999; Zheng et al., 1999; Bae et al., 2001), 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 (Preitner et al., 2002; Tsai et al., 2008). In one of these loops, CLOCK/BMAL1 activates

transcription of the *Rev-erbα* orphan nuclear receptor gene. The REV-ERBα protein inhibits ROR (Receptor tyrosine kinase-like orphan receptor)-driven transcription of the *Bmal1* gene by competing with the ROR proteins for binding to ROR elements (RORE) in the *Bmal1* promoter (Preitner et al., 2002). This mechanism generates high-amplitude oscillations in *Bmal1* transcription and, even though this loop is not essential for the clock mechanism, it contributes to precision and robustness of the circadian clock (Preitner et al., 2002). Additionally, it extends the ability of the circadian clock to drive rhythmic transcription to output genes with RORE elements in their promoters.
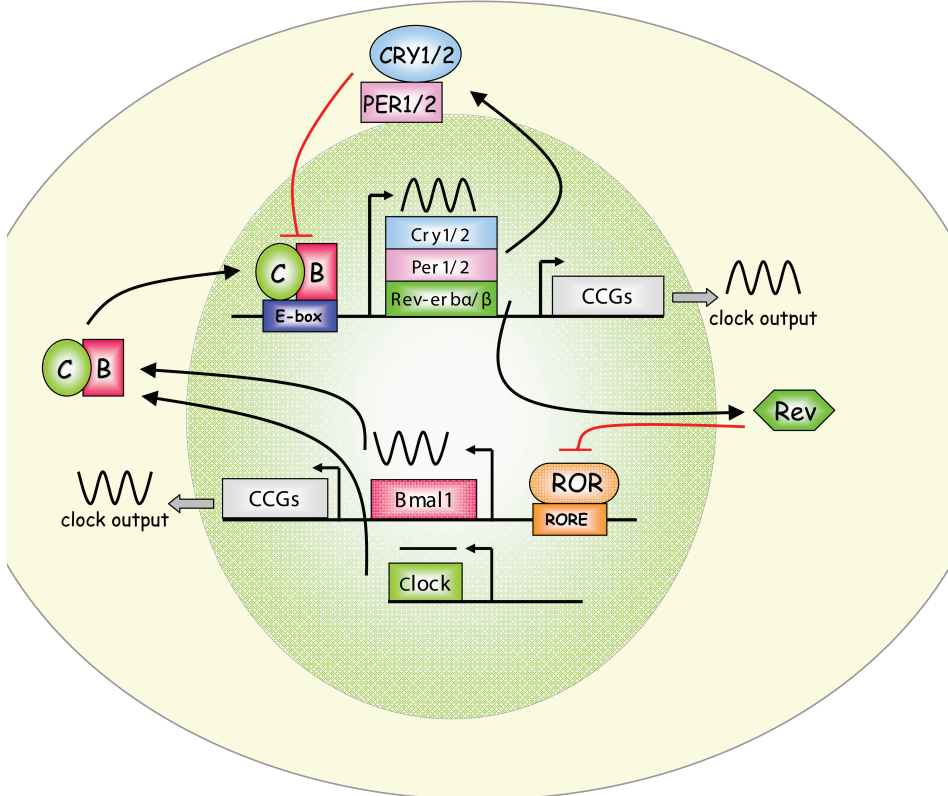

Figure 1. The mammalian circadian oscillator. Simplified scheme showing the molecular mammalian circadian oscillator composed of a negative feedback loop and one auxiliary feedback loop. In this model CLOCK/BMAL1 (components of the positive limb) drive expression of the *Cry* and *Per* genes through 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.

In addition to the core transcription/translation feedback loop, prominent posttranslational modification, in particular phosphorylation, of clock proteins occurs (Gallego and Virshup, 2007; Vanselow and Kramer, 2007). These modifications mainly serve to control the stability and localization of clock proteins, thereby increasing the delay from the start of expression of the *Cry* and *Per* genes to the moment where they inhibit CLOCK/BMAL1-driven transcription of their own genes. Although all core clock proteins have been found to be phosphorylated by a variety of kinases (Lee et al., 2001; Lamia et al., 2009), it is the PER2 protein that has been the most extensively characterized. One study identified 21 phosphorylated residues in PER2 and showed that phosphorylation at one site stabilized and increased nuclear retention of PER2, while phosphorylation at other residues increased degradation (Vanselow et al., 2006). Further underscoring the importance of phosphorylation for the clock and human physiology, a mutation in the human *PER2* gene, which alters a CASEIN KINASE 1 phosphorylation site, was found to underlie familial advanced sleep-phase syndrome (FASPS), an inherited disorder in which patients tend to fall asleep early in the evening (around 7 p.m.) to wake up early in the night (around 4 a.m.) and suffer from profound social, physical (i.e. sleep deprivation, chronic fatigue) and psychological discomfort (Toh et al., 2001). Later, a transgenic mouse model mimicking the human mutation was shown to phenocopy the human syndrome (Xu et al., 2007).

One important question regarding the functioning of the main transcription/translation negative feedback loop is how CRY-mediated inhibition of CLOCK/BMAL1 is turned off to allow a new cycle to start. A major recent breakthrough going far in answering this question came with the identification of FBXL3 as a ubiquitin ligase targeting the CRY proteins for proteasome-mediated degradation (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007). This novel clock player was identified in two independent mutagenesis screens, searching for mice with altered circadian behavior. Conversely, the CRY proteins were found to interact with FBXL3 in a mass-spectrometry screen for proteins interacting with FBXL3. Collectively, the three reports showed that mutations in the *Fbxl3* gene or RNAi-mediated mRNA reduction (in cultured cells) lengthen the period of circadian behavior in constant darkness and strongly impair cellular circadian oscillations in gene expression, respectively, demonstrating that posttranslational modification of the CRY proteins is essential for maintaining proper circadian rhythms (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007).

Another posttranscriptional mechanism that has quite recently been implicated in the regulation of the circadian clock is microRNA-mediated control (Chekulaeva and Filipowicz, 2009) of clock gene expression. This has been demonstrated in *Drosophila* were the *Drosophila*-specific microRNA *bantam* was found to regulate translation of the *clock* gene and thereby regulate circadian behavior (Kadener et al., 2009). Two different microRNAs have also been implicated in mammalian circadian behavior and photo-entrainment (Cheng et al., 2007), and although the exact mechanism has not been fully elucidated, this appears to be, at least in part, due to their effect on neuronal excitability (Cheng et al., 2007). To what extent microRNAs play a role in the mammalian core circadian oscillator is still unclear, but one report at least indicates that expression of all three *Per* genes is regulated by the miR-192/194 family (Nagel et al., 2009).

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 (Yamazaki et al., 2000). In all rat and mouse peripheral tissues analyzed thus far, such peripheral clocks oscillate with a 7 to 11 hours phase delay (depending on the organ) compared to the master oscillator in the SCN. Initially, peripheral circadian oscillators appeared not self-sustaining as the amplitude of the luciferase signal quickly damps *ex vivo*, when tissues are disconnected from the SCN (Yamazaki et al., 2000). 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 (Yoo et al., 2004). Importantly, SCN-lesioning caused a phase desynchrony of peripheral clocks in these animals (i.e. that tissues taken from the same animal displayed altered phase relationships compared to non-lesioned mice) and from animal to animal, suggesting that peripheral clocks are self-sustained and desynchronize, rather than dampen in the absence of the SCN (Yoo et al., 2004). 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 (Yoo et al., 2004). Peripheral oscillators are thought to confer circadian rhythmicity to biological processes specific to the function of that particular tissue or organ. Recent reports using a conditional *Bmal1* knock-out mouse model support this notion. Deleting *Bmal1* in the liver led to an impairment of glucose homeostasis (Lamia et al., 2008), while deletion of *Bmal1* within the retina led to impairment of retinal visual processing (Storch et al., 2007). In contrast to the SCN core oscillator, peripheral oscillators are not directly entrained by light. However, rodent peripheral clocks (in e.g. kidney and liver) have been shown to be able to entrain to food availability (Damiola et al., 2000; Stokkan et al., 2001). For instance, when a light-entrained laboratory mouse only has access to food during the day, when the animal normally sleeps, the phase of the circadian clock in the liver changes 180 degrees. As the SCN clock does not respond to altered feeding patterns, restricted feeding thus causes a phase uncoupling of master and peripheral clocks (Damiola et al., 2000; Stokkan et al., 2001). This food-entrainment appears to work through glucocorticoid signaling (Balsalobre et al., 2000).

Cultured fibroblasts serve as excellent tools to study circadian clock performance at the molecular level. Whereas cell cultures normally do not show circadian oscillations in gene expression at the population level, the Schibler group has shown that robust oscillations can be induced by exposing confluent, serum-starved cell cultures to 50% serum (Balsalobre et al., 1998). 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 (Balsalobre et al., 2000; Akashi and Nishida, 2000; Yagita and Okamura, 2000). 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 (Nagoshi et al., 2004). 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 (Nagoshi et al., 2004).

The genetic composition and molecular mechanism of the circadian oscillator in

peripheral cells is essentially the same as that of the SCN neurons and, as shown for mouse embryonic and dermal fibroblasts from clock mutant mice, genetic defects that affect period length or cause arrhythmicity of behavior, elicit the same effect on the peripheral molecular oscillator *in vitro* (Balsalobre et al., 1998; Yagita et al., 2001). More recently, additional results indicate that the phenotypes in peripheral clocks tend to be more extreme compared to the SCN or behavioral phenotypes (Brown et al., 2005; Liu et al., 2007).For example, the free-running period of *Cry2*$^{-/-}$ mice is about 1 hour longer, which is also reflected in the period of gene expression in an explanted SCN. In contrast, peripheral clocks of cells and tissues *in vitro* of these same mice have a period lengthening of about 4 hours. It seems that the SCN can compensate for mutations in clock genes through intercellular coupling of neurons (Liu et al., 2007).

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 (Figure 1). Amongst 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 (Ueda et al., 2002; Kumaki et al., 2008). 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 (Ripperger et al., 2000; Ripperger and Schibler, 2006). Two other related PAR-bZIP transcription factors, TEF (Thyrotroph Embryonic Factor) and HLF (Hepatic Leukemia Factor), have also been shown to oscillate, presumably trhough a similar mechanism as *Dbp*. Together with DBP, they generate circadian expression of many genes involved in detoxification and drug metabolism (Gachon et al., 2006). Other examples of rhythmically expressed transcription factors include PPARα (Peroxisome Proliferator-Activated Receptor α) and a variety of other nuclear receptors that link the circadian clock to metabolism and possibly other processes (Oishi et al., 2005; Yang et al., 2006). Through these combined mechanisms the clock generates rhythms in gene expression of ~10% of the genome (Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002; Miller et al., 2007; Hughes et al., 2009). The genes that oscillate vary from tissue to tissue, possibly reflecting the specific requirements for each tissue (Storch et al., 2007; Lamia et al., 2008).

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 (Kornmann et al., 2007). The circadian expression of these genes was suggested to be driven by circadian systemic cues and/or body temperature cycles. In fact, a later study revealed that, in the absence of the clock, hundreds of genes can be expressed in a circadian manner driven by food ingestion (Vollmers et al., 2009). Additionally, there are genes that oscillate at the protein level without oscillations of the corresponding mRNAs, as found in the liver by a mass spectrometry approach (Reddy et al., 2006).

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 (Matsuo et al., 2003). More recently, the activity of the NAD$^+$-dependent deactylase SIRT1 (Sirtuin 1) 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 (Nakahata et al., 2009; Ramsey et al., 2009)[65, 66]. SIRT1 deacytelates proteins such as histones, p53, FOXO, PGC1α, PER2 and BMAL1, and as such acts in a wide range of processes such as cell cycle control, metabolism, and the circadian clock itself (Finkel et al., 2009). 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 factor1 (HSF1) (Reinke et al., 2008).

The Cell Cycle and the DNA damage response

One interesting pathway thought to be under circadian control is the cell cycle. Like the circadian clock, the cell cycle is an oscillatory mechanism driven by several interlocked negative and positive feedback loops (Tyson and Novak, 2008). However, unlike the circadian oscillator, the cell cycle is not self-sustained and it can be halted after exposure to exogenous or endogenous DNA damage. This latter process is controlled by the DNA damage response (DDR) (Bakkenist and Kastan, 2004; Harper and Elledge, 2007). Also the DDR response is connected to the circadian clock (see below). Broadly, the cell cycle consists of four phases. In the G_1-phase cells increase their biomass until they reach a certain threshold. During this phase cells also start preparing for the next phase by synthesizing enzymes and other proteins involved in DNA-replication. After this, the cells start replicating their DNA in the S-phase to produce an additional copy of each chromosome, which later will be divided over two cells. In the G_2-phase cells again increase biosynthesis, mainly to produce microtubules and other proteins required for the final phase, mitosis (M-phase). During the M-phase, the shortest phase of the cycle, one cell splits into two and divides the chromosomes equally over the two daughter nuclei, which will then go on to form two individual cells.

The two most important protein classes involved in the cell cycle are the cyclins and the cyclin-dependent kinases (CDKs). These CDKs are normally inactive until they interact with a cyclin, whose expression is driven by transcription factors. The CDK enzymes then regulate the activity and/or stability of other cell cycle proteins by phosphorylating them. The cyclins and CDKs themselves are also often regulated by phosphorylation. This phosphorylation is counteracted by protein phosphatases like for example WEE1. This very complex set of processes is additionally controlled by the DDR. When cells are exposed to exogenous (e.g. UV-light) or endogenous (e.g. oxygen radicals or mistakes during replication) genotoxic stress, cells halt cell cycle progression to prevent cells from replicating damaged DNA, which might otherwise be converted to deleterious mutations. One of the main steps in the DDR is the activation of the kinases ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia and Rad3-related) (Bakkenist

et al., 2004). These kinases phosphorylate a large set of proteins that are involved in regulating cell cycle progression (Matsuoka et al., 2007). One of the main reasons for halting cell cycle progression in the presence of DNA damage is to give cells sufficient time to repair DNA damage through various pathways depending on the type of damage (Hoeijmakers, 2001). Usually when cells, especially in metazoans, are unable to repair their DNA sufficiently, they start a programmed cell death pathway, apoptosis (Taylor et al., 2008). This is to prevent mutated cells from persisting in the organism.
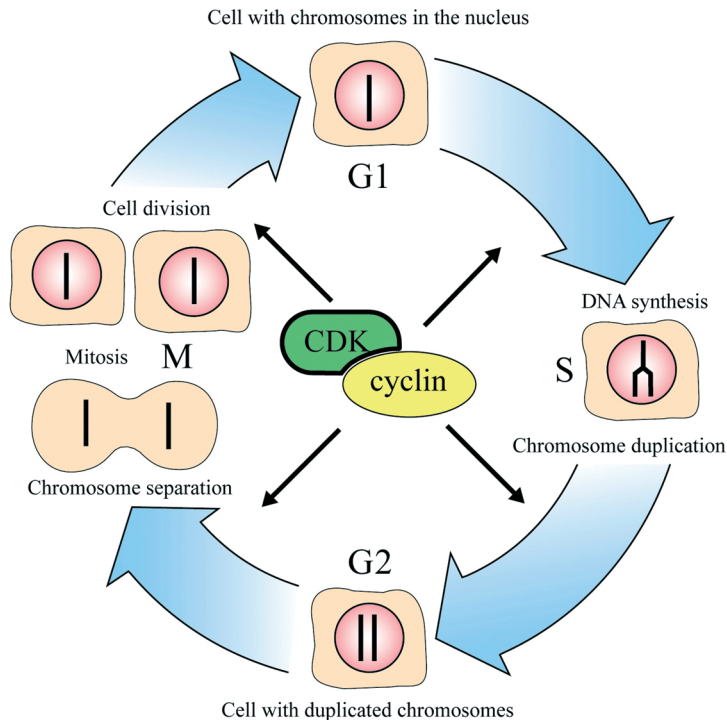

Figure 1. Simplified model of the cell cycle. The mammalian cell cycle consists of four different phases (see main text for details) and is manly driven by the oscillating activity of Cyclin-dependent kinases (CDKs). These oscillations are driven by cyclic transcription of cyclins, with out which the CDKs are inactive, and degradation by the proteasome. Another important regulatory mechanism essential to oscillating CDK activity comes from other kinases and phoshpatases that phosphorylate and dephosphorylate, respectively, the CDKs and thereby control their activity. (Image taken from Nobelprize.org)

The circadian clock, cell cycle and DNA damage sensitivity

As mentioned above, 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. The 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 (Pittendrigh,

1993; Roenneberg and Merrow, 2002; Tauber et al., 2004). Starting almost a century ago with observations in cats (Fortuyn-van Leyden, 1917), there have been many reports on circadian variation in cell cycle division in a wide range of mammalian species (including humans) and single-cell organisms (Bjarnason and Jordan, 2000; Brown, 1991; D'Autilia et al., 2010; Dekens et al., 2003; Edmunds, 1964; Goto and Johnson, 1995; Mori et al., 1996; Pilgrim et al., 1963; Scheving et al., 1978; Smaaland et al., 1991; Tamai et al., 2008). To what extent these observations are indicative for direct links between the cell-autonomous clock and the cell cycle in mammals is still not clear. In zebrafish, there is data indicating that the cell cycle is gated through a systemic mechanism involving circadian glucocorticoid secretion (Dickmeis et al., 2007).

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 (Fu et al., 2002). 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 *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 (Gauger and Sancar, 2005).

Some mechanistic insight into how the circadian clock gates cell cycle progression came from a study of liver regeneration after partial hepatectomy (Matsuo et al., 2003). In wild type mice, partial hepatectomy causes hepatocytes (normally in a G_0 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 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 (Matsuo et al., 2003). Although this study is very elegant and informative, it does not resolve the issue of whether the observed phenotype is caused by the absence of the clock in the liver or elsewhere in the body. 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 (Fu et al., 2005). Using cultured osteoblasts (bone-forming cells) from mice lacking *Per1* and *Per2* and a conditional *Per2* knock-out mouse model, this was demonstrated to be caused by a cell-autonomous increase of osteoblast proliferation (Fu et al., 2005).

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 (Miller et al., 2007). 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 (Miller et al., 2007). *Bmal1*-deficient hepatocytes, cultured *in vitro*, showed a phenotype similar to *Clock* mutant fibroblasts (Gréchez-Cassiau et al., 2008). The reduced proliferation in *Bmal1* deficient hepatocytes was linked to increased expression level of *p21* (also known as *Cdkn1a*), which was shown to be inderctly driven by CLOCK/BMAL1 through regulation of *Rev-erbα*.

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 (Levi and Schibler, 2007). 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.

Scope of the thesis

Although many advances have made been in recent years in understanding how circadian oscillations are generated at the cellular level and how these oscillations are connected to circadian output processes, many questions still remain. This thesis will address the involvement of microRNAs in generating circadian oscillations through regulation of clock gene expression and investigate how the cellular circadian clock is connected to the cell cycle and DNA damage response at the cell-autonomous level. Another important aspect within the clock field is the physiological relevance of the circadian clock. The work described in this thesis will look at the impact of timing of exposure to a mutagenic compound on mutagenesis and the consequences of having no clock or a clock with an altered gene expression-environment phase relationship for survival of mice under normal and challenging conditions.

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Chapter 1

Panda, S., Provencio, I., Tu, D. C., Pires, S. S., Rollag, M. D., Castrucci, A. M., Pletcher, M. T., Sato, T. K., Wiltshire, T., Andahazy, M., et al. (2003). Melanopsin Is Required for Non-Image-Forming Photic Responses in Blind Mice. Science *301*, 525-527.

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CHAPTER 2

Assessing ENU-induced spleen mutation frequency and spectrum after exposure of mice at different times-of-day

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Chapter 2

ABSTRACT

The circadian system is an internal time-keeper that allows an organism to anticipate behavior, physiology and metabolism to the momentum of the day. By controlling xenobiotic metabolism, cell cycle progression and DNA repair, the circadian clock is considered a potential factor determining the (geno)toxicity and carcinogenicity of chemical compounds (e.g. environmental pollutants, chemotherapeutics). To determine whether mutation frequency and/or spectrum vary with the time-of-day of exposure, we treated *lacZ* mutation marker mice with a single dose of ethyl nitrosurea (ENU, a known model compound for mutagenesis) at defined moments over the 24 h light-dark cycle. We observed that time-of-day is not influencing mutation frequency or mutation spectrum in the mouse spleen.

INTRODUCTION

Like most organisms, mammals possess an ingenious internal circadian clockwork with a periodicity of approximately 24 hour (circa dies = about one day) that adjusts behavior, physiology and metabolism (e.g. sleep-wake cycle, body temperature, cell division, hormone secretion) to the day-night cycle and allow the body to anticipate environmental changes (Reppert and Weaver, 2002; Takahashi et al., 2008). The mammalian circadian system is composed of a central clock located in the neurons of the suprachiasmatic nucleus (SCN) of the brain, and peripheral clocks, present in virtually all other tissues and cells. The SCN clock is crucial in receiving light information to keep the circadian system entrained to the light-dark cycle, as well as in maintaining phase coherence between the individual peripheral cellular clocks that serve to generate physiological harmony in tissue functioning in relation to time of the day (Lamia et al., 2008; Storch et al., 2007). Independently from the SCN, the circadian clocks of some peripheral tissues can be entrained by other stimuli, such as food (Stokkan et al., 2001; Yamazaki et al., 2000).

Circadian rhythms are generated by a molecular oscillator, composed of interlocked negative and positive transcription/translation feedback loops in which a set of clock genes rhythmically turn on or switch down their (own) expression, and in which posttranslational modifications that affect the stability of core clock proteins serves to tune the periodicity of the oscillator (Gallego and Virshup, 2007; Ueda, 2007). These molecular oscillators of the SCN and peripheral tissues subsequently drive rhythmic expression of a wide range of so called clock-controlled genes (CCGs) that are involved in driving circadian output processes in a tissue-specific manner (Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002). Taking into consideration that up to 10 % of a tissues transcriptome oscillates in a circadian manner (Panda et al., 2002, etc.) a wide range of biological processes is under control of this biological time keeper trough transcriptional regulation.

The integrity of our genome is continuously threatened by physical and chemical agents that damage our DNA (e.g. UV light, ionizing radiation, chemical pollutants. When not repaired in time, such DNA lesions can trigger programmed cell death (apoptosis) or, upon replication of the genome, can give rise to mutations that ultimately may trigger carcinogenesis (Hoeijmakers, 2001). An immediate consequence of the presence of a circadian system is that an organism may respond differently to (geno)toxic agents, depending on the moment of exposure, a phenomenon known as chronotoxicity. Furthermore, the toxicity and efficacy of many chemotherapeutic agents has been shown to depend on the time of administration (Levi and Schibler, 2007) and, interestingly,

animal studies have shown that the therapeutic efficacy of anticancer drugs is highest when they are least toxic (Lévi, 2006). Chemo- and radiotherapeutical protocols that take into account the presence of the circadian system and deliberately make use of chronotoxic effects of radiation and pharmaceutica are referred to as chronotherapy (Levi et al., 2007).

Among the mechanisms that underlie chronotoxicity and chronotherapy are circadian rhythms in cellular metabolism, cell proliferation and drug pharmaco-kinetics. For instance, many chemotherapeutic compounds need to be metabolically activated and the activity of some components of these metabolization pathways fluctuates throughout the day. In at least some cases this is due to direct transcriptional control by the circadian clock. For example, the PAR-bZIP transcription factors DBP, TEF and HLF are expressed in a circadian manner and, in turn, generate circadian expression of many genes involved in detoxification drug metabolism. Mice lacking all three factors become hypersensitive to xenobiotic compounds (Gachon et al., 2006). Likewise, the response of wild-type mice to cyclophosphamide (CP) fluctuates throughout the day in a manner correlating with CLOCK/BMAL1 activity (Gorbacheva et al., 2005). In this case, rather than circadian control over metabolic activation, the time-of-day differences in the survival of the mice upon CP treatment seem to be caused by differences in the response of B-cells in the bone marrow. Moreover, mice (and cells) lacking core components of the circadian clock show a difference in survival upon CP treatment, with $Bmal1^{-/-}$ and $Clock^{mut/mut}$ mice (constitutive low expression of CCGs) being more sensitive and $Cry1^{-/-}$|$Cry2^{-/-}$ mice (constitutive high expression of CCGs) and cells being more resistant than wild type mice (rhythmic expression CCGs) (Gorbacheva et al., 2005). Understanding why certain compounds are less toxic at different times-of-day is important for the success and implementation of chronotherapy.

Many chemical compounds are genotoxic, and therefore potentially mutagenic and carcinogenic. For such compounds, chronotoxic effects may not only depend on time-of-day variation in DNA damage induction (e.g. through circadian clock-mediated rhythms in metabolisation or pharmacokinetic parameters), but also on circadian fluctuations in the efficiency by which a cell can clear the genome from DNA lesions. In this respect, it is interesting to note that the activity of O-6-methylguanine-DNA methyltransferase (MGMT), also known as O-6-alkylguanine-DNA alkyltransferase (AGT), an enzyme that repairs alkylated DNA, has been shown to oscillate in liver extracts (Martineau-Pivoteau et al., 1996).

To investigate whether chronotoxic effects of genotoxic chemical compounds extend to mutation induction, a phenomenon we propose to be referred to as chronogenotoxicity) we used a plasmid-based transgenic *lacZ* mutation reporter mouse model that allows determination of mutation frequency (MF) and mutation spectrum (ranging from point mutations to insertions and deletions) in any tissue of *in vivo* exposed animals (Boerrigter et al., 1995). To induce mutations, we used the alkylating agent ethyl nitrosurea (ENU), which mainly, but not exclusively, gives rise to point mutations (Shibuya and Morimoto, 1993). The rationale for choosing ENU is that (i) in mice it is the most potent mutagenic compound (Russell et al., 1979), (ii) it has a short *in vivo* half-life of less than 1 hour at pH7.4 (Johansson-Brittebo and Tjälve, 1979), (iii) it does not need to be metabolized and therefore is immediately active (Shank, 1975), and (iv) it is repaired by MGMT ((Bronstein et al., 1992), the activity of which has been reported to be under circadian control.

RESULTS

In a pilot experiment, *lacZ* mice entrained to a 12h light-12h dark cycle received a single treatment with ENU (150 mg/kg body weight), intraperitoneally injected at a defined moment during the day (ZT0, ZT4, ZT8, etc. in which ZT0 refers to the start of the light period; 6 animals per time point). In addition, at two different time points mice (6 animals per time point) were treated with the solvent (DMSO) only in order to determine the background MF. Two weeks after treatment, allowing fixation of mutations, the surviving mice (3-6 per time point) were sacrificed and tissues were collected for genomic DNA isolation and *lacZ* plasmid rescuing. First, we analyzed the ENU-induced MF in the liver, because this organ harbors the most well characterized peripheral clock. However, independent of the time of injection, the MF in the liver was not significantly above background level (data not shown). Next, we analyzed the MF in the spleen, as this tissue is known to be susceptible to ENU-induced mutations (Boerrigter et al., 1995). As shown in Figure 1A, we observed that the spleen of mice exposed to ENU at ZT20 had a lower MF as compared to that of mice treated at the other time points. Although the previous experiment suggests that mutation induction by ENU might depend on time-of-day of exposure, the number of surviving mice in the ZT20 group is only three, making it difficult to draw firm conclusions.
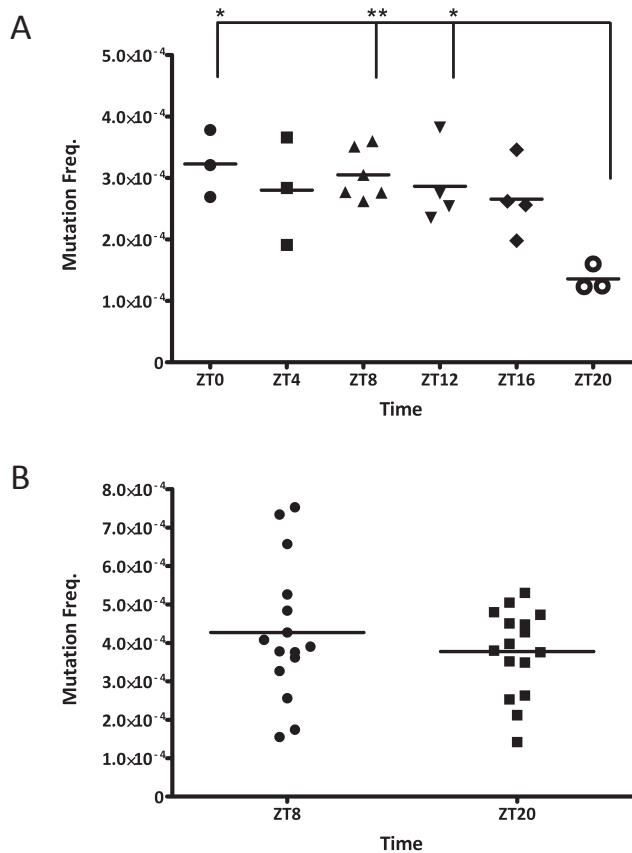

Figure 1: Determination of the ENU-induced mutation frequency in the spleen.
(**A**) Six groups of *lacZ* mutation reporter mice were intraperitoneally injected with ENU (150 mg/kg body weight) at defined time points spread around the day (6 animals per time point). Time is indicated at Zeitgeber Time (ZT) in which ZT0 refers to lights-on and (under a 12h light-12h dark cycle) ZT12 corresponds to lights-off. Mutation frequencies (MF) were determined as outlined in the Methods section. To determine the background MF, two groups of mice were injected with the solvent (DMSO) only at ZT4 or ZT12. Data are presented as a scatter plot, with each symbol representing the background-subtracted (1×10^{-4}) MF of an individual mouse (n=3-6) and horizontal lines indicating the mean of each group. Most DNA samples were analyzed 2-3 times. ANOVA with Bonferroni correction was used to compare all groups with each other. * $p < 0.05$, ** $p < 0.01$, compared with

(**B**) As panel A, except that mice were treated at ZT8 or ZT20 only (16 mice per time point treated, 1 mouse in the ZT20 groupd died). Groups were compared using ANOVA with Bonferroni correction or Student's t-test.

Therefore, we performed a second experiment with a larger number of animals per time point (n=16). Given the results in Figure 1A, we only treated mice at ZT8 or ZT20. For technical reasons, the experiment was spread over two consecutive days (8 animals per time point per day). Data was pooled, since for both time points there was no statistically significant difference the injection days (not shown). Unlike in the pilot experiment, we did not find a difference in MF following treatment at ZT8 or ZT20 (Figure 1B). This suggests that time of day of injection does not have an impact on the ENU-induced mutation frequency.

Although we did not observe quantitative differences in the mutation frequency after ENU-treatment at different times over the day, it is well possible that qualitative differences, i.e. time-of-day dependent changes in the mutation spectra, occur. We therefore isolated plasmid DNA from mutant colonies obtained from mice exposed to ENU at ZT8 and ZT20 and subjected the DNA to *Ava*I and *Pst*I restriction analysis to determine the whether the *lacZ* reporter gene contains "no-change" or "size-change" mutations (Dollé et al., 1999). No-change mutant plasmids (containing only point mutations or very small insertions/deletions) have a distinct restriction pattern (representative examples of which are shown in Figure 2A, lanes 1-5), which is altered if the mutant plasmid contains a large insertion or deletion (Figure 2A, lanes 6-10). We observed that 13.3% of the mutant plasmids rescued from the spleen of animals of the ZT8 group (8 out of 60 mutant colonies) contained size-changes (Figure 2B), a finding that is in full agreement with previously reported frequency of size-change mutations (14%) in the spleen of ENU-treated mice (Boerrigter et al., 1995). For the ZT20 group we found a frequency of 12.1% size-change mutant plasmids (8 out of 66), which is not significantly different (p=1, Fischer's exact test) from the results obtained for the ZT8 group.

Taken together, our data suggest that time-of-day of exposure does not have a major impact on the frequency and spectrum of mutations in spleen, provoked by the model mutagen ENU.
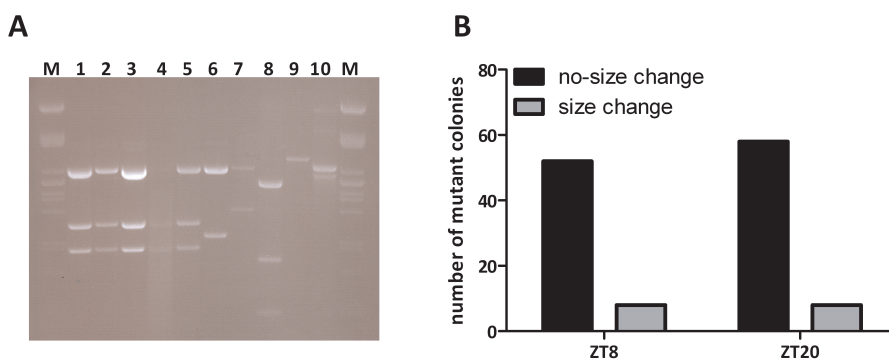

Figure 2: Determination of the ENU-induced mutation spectrum in the spleen.

(A) Mutant plasmids, rescued from mice exposed to ENU at ZT8 and ZT20 (see Fig. 1B) were digested with *Pst*I and *Ava*II to determine the presence of no-change mutations (i.e point mutations or very small deletions/insertions) or size-change mutations (large insertions/deletions). Shown are restricition patterns of representative examples of mutant plasmids with no-change mutations (lanes 1-5) and size-change mutations (lanes 6-10). M, marker (λ DNA cut with *Pst*I) (B) Quantification of the number of size-change and no-change mutant plasmids. The fraction of size-change mutants is not different between the two time points (p=1, Fischer's Exact test).

DISCUSSION

Chronotoxicity has been defined as the changes in an organism's sensitivity to toxic agents in relation to time of exposure. Well-known examples of chronotoxic compounds are found among the class of anti-cancer drugs and many chemotherapeutical agents have been show to exert less side effects and/or higher therapeutic efficacy when given at a certain time of the day (Lévi, 2006). In the present study we investigated whether mutagenesis, provoked by exposure of mice to the genotoxic chemical ethyl nitrosurea (ENU), is affected by the time of the day of exposure (chronogenotoxicity).

In a pilot study, we observed that the mutation frequency in the spleen of *lacZ* reporter mice, when exposed to the model mutagen ENU at different times of the day, was lowest at ZT20. Interestingly, this coincides with the peak of MGMT activity in the liver (Martineau-Pivoteau et al., 1996). This finding prompted us to perform a second study, in which a larger group of animals was exposed to ENU at ZT8 (light phase) and ZT20 (dark phase). In contrast to the pilot study, the second experiment suggests that neither mutation frequency nor mutation spectrum is modulated by the time-of-day of exposure. Whereas at first sight, these experiments point to the absence of chronogenotoxic effects of ENU, several considerations can be made.

First, while this study was in progress, nucleotide excision repair (NER), another repair pathway for removal of alkylated DNA lesions (Bronstein et al., 1992), was shown to be directly controlled by the circadian clock in brain and liver (Kang et al., 2009, 2010). As mentioned before, highest NER activity in the liver was observed between ZT6 and ZT14 (Kang et al., 2009, 2010), which partially overlaps with MGMT activity peaking around ZT19 (Martineau-Pivoteau et al., 1996). Assuming that this partial overlap in repair activity also translates to the spleen, a broadening of the time window in which alkylated DNA lesions is more efficiently repaired might cause a flattening of the expected sinusoidal pattern of mutation frequencies and rather give a sharp peak or trough at a time point when repair capacity is at its lowest. Moreover, subtle differences in ENU-mediated genotoxicity may be difficult to reveal in single exposure studies and it is well conceivable that repeated exposures (i.e. at the same time on consecutive days) may amplify a subtle difference as seen in the pilot study.

Furthermore, it has been shown that the response to DNA damage is clock-controlled *in vivo* and *in vitro* (Fu et al., 2002; Gery et al., 2006). It therefore can not be excluded that time-of-day differences in the DNA damage response, notably in DNA damage induced apoptosis, have masked differences in mutation frequency as cells with a high level of unrepaired damage were lost, rather than given the chance to convert the damage into mutations. Such chrono(cyto)toxic effect could have resulted in an underestimation of the mutation frequency in animals exposed to ENU around ZT0.

In conclusion, the present study neither provides evidence for, nor excludes chronocytotoxic and chronogenotoxic effects of ENU and requires a follow-up study, in which in addition to mutation frequency and spectrum, ENU-induced damage load and apoptosis are included as biological endpoints. Moreover, subtle differences in time-of-day-dependent ENU-induced mutation frequencies may be difficult to reveal in single exposure studies and it is well conceivable that repeated exposures (i.e. at the same time on consecutive days) will amplify such small genotoxic effects. Preferably, follow-up experiments should not only be performed with wild type mice, but also with NER-deficient *Xpa* and methyltransferase-deficient *Mgmt* (double) knockout mice (De Vries et al., 1995; Glassner et al., 1999) to allow discrimination between the contribution of

each of the alkylated DNA damage repair pathways.

METHODS

Animal experiments

Transgenic *LacZ* mice (IM30 mouse model in a C57BL/6J background) (Dolle et al., 1996) were housed in a controlled environment under a 12h-light:12h-dark cycle with food and water *ad libitum*, at the Animal Resource Center of the National Institute of Public Health and the Environment (RIVM), which operates in compliance with the ''Animal Welfare Act'' of the Dutch government and uses the ''Guide for the Care and Use of Laboratory Animals'' as its standard. All animal studies were approved by an independent Animal Ethical Committee (Dutch equivalent of the IACUC). At the age of 8 weeks, animals (6 males per time point) received a single intraperitoneal injection of ethyl nitrosurea (ENU, Sigma), dissolved in DMSO at a dose of 150 mg per kg body weight, and given at Zeitgeber Time 0, 4, 8, 12, 16, or 20 (in which ZT refers to Zeitgeber Time and ZT0 corresponds to the time at which lights are switched on). Likewise, mice were injected with the solvent alone (ZT4 and ZT12 only; 6 males per time point). Note that mice injected at ZT12, ZT16 and ZT20 were housed in a reversed LD cycle from weaning on, and accordingly were treated at the same external time as mice injected at ZT0, ZT4 and ZT8, respectively.

Two weeks after injection, allowing fixation of mutations (Dolle et al., 1996), animals were sacrificed and all major tissue were isolated, snap-frozen in liquid nitrogen and then stored at -80° C until further use.

Plasmid rescue and mutation frequency determination

The *lacZ* mutation frequency was determined essentially as described (Boerrigter et al., 1995). In brief, upon digestion of Ultra-thorax prepared tissue homogenates with proteinase K 16 h at 37°C), genomic DNA was isolated using standard phenol/chloroform extractions and subsequently precipitated with ethanol. Next, genomic DNA was digested with *Hin*dIII after which the plasmid was rescued using magnetic beads coated with the LacZ/LacI fusion protein. Plasmids were eluted using isopropyl β-D-1-thiogalactopyranoside (IPTG) and circularized using T4 DNA ligase. Next, these plasmids were subsequently electroporated into the *E. coli* strain C (*lacZ⁻galE⁻*). From each bacterial culture, 2 out of 2000 µl (1/1000) was plated on a non-selective 5-bromo-4-chloro-3-indolyl-β-d-galactoside (X-gal) plate to determine the total number of plasmids. The remainder of the sample was used to determine the number of mutant plasmids by plating onto a selective phenyl-β-d-galactoside (P-gal) on which only bacteria with a mutated *LacZ* plasmid can grow. The *lacZ* mutation frequency was calculated by dividing the number of mutants by the total number of rescued colonies × 1000.

Analysis of mutation spectra

To determine the number of "size-change" (carrying large deletions/insertions) and "no-change" mutations (carrying very small deletions/insertions or point mutations), plasmid DNA was isolated from 6 ml overnight cultures of mutant colonies, digested with *Ava*I and *Pst*I, and analyzed by agarose gel electrophoresis as described before (Dollé et al., 1999).

Statistics

Group differences in MF were analyzed by a Student's t-test or ANOVA with Bonferroni correction, as indicated in the figure legends. The proportion of size-change mutants vs. no-change mutants at the two different time points was analyzed by a Fischer's exact test. All tests were performed using GraphPad Prism 5 software.

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CHAPTER 3

ALTERED PHASE-RELATIONSHIP BETWEEN PERIPHERAL OSCILLATORS AND THE ENVIRONMENT IN MICE LACKING CRY1 OR CRY2: ASSESSING THE IMPACT OF INTERNAL DISSONANCE ON LIFE SPAN

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Chapter 3

ABSTRACT

The circadian system in mammals generates day-night rhythms in e.g. behavior, physiology and metabolism. According to the resonance theory, as demonstrated for Cyanobacteria and plants maintained under competitive conditions, such rhythms are only advantageous to an organism when in tune with the environment. In mammals, there is data from the mutant *tau* hamster suggesting that having an internal clock with a period that is different from the environment, has negative consequences for survival. Here, we show that under regular light-dark cycles, and despite normal circadian behavior, the peripheral clocks in the liver and kidney of short-period *Cry1* and long-period *Cry2* knock-out mice are out of phase with that of wild type mice. These mice may therefore represent animal models that mimic extreme human early and late chronotypes ("larks" and "owls"). Also, we demonstrate that this internal dissonance has no negative effect on the survival of these mice under normal or challenging (i.e. exposure to ionizing radiation) conditions. However, we did find that mice lacking *Cry2* were more sensitive to the acute effects of ionizing radiation when given at a specific time point. The implications of these findings for human health are discussed.

INTRODUCTION

Circadian clocks provide an organism with a timing-mechanism to adjust a wide range of behavioral, physiological processes to the momentum of the day and are found in diverse species, ranging from single-cell cyanobacteria and fungi, to multi-cellular plants and animals (Bell-Pedersen et al., 2005). The mammalian circadian clock is composed of a molecular oscillator that generates near 24 hour (circa dies = approximately one day) rhythms in the expression of a set of clock genes that act through interlocked negative and positive transcription/translation feedback loops. In short, the heterodimeric transcription activator CLOCK/BMAL1 activates transcription of the *Cryptochrome* (*Cry1* and *Cry2*) and *Period* (*Per1* and *Per2*) genes through E-box elements in their promoters. After being synthesized in the cytoplasm, CRY and PER proteins heterodimerize and subsequently enter the nucleus, where they shut down expression of their own genes by inhibiting CLOCK/BMAL-mediated transcription (reviewed by Ueda, 2007). Likewise the CLOCK/BMAL heterodimer and CRY/PER complexes drive cyclic expression of a set of clock controlled genes (CCGs) that couple the molecular oscillator to rhythmic output processes. Amongst the CCGs are transcription factor genes, cyclic expression of which allows circadian expression of certain output genes in an E-box-independent manner.

The central circadian clock is housed in the neurons of the suprachiasmatic nucleus (SCN), which is located in the hypothalamus (Moore and Eichler, 1972; Stephan and Zucker, 1972). To keep in phase with the light-dark cycle, the SCN clock receives light input from the environment through the retino-hypothalamic tract (RHT) (Sousa-Pinto and Castro-Correia, 1970; Moore and Lenn, 1972). Besides the SCN also other parts of the brain and almost all peripheral tissues have their own circadian clock. These peripheral clocks are entrained by the SCN through humoral and neuronal signals (Reppert and Weaver, 2002), which act in a tissue-specific manner (Guo et al., 2005). Transcription profiling studies with SCN and peripheral tissues have shown that the circadian clock confers rhythmicity to up to 10% of the genome in a tissue-specific manner (Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002; Hughes et al., 2009). Tissue-specific inactivation of *Bmal1* in liver and retina has shown that non-rhythmic expression of CCGs, impacts the physiology

of these tissues (Storch et al., 2007; Lamia et al., 2008), thereby further illustrating the importance of peripheral circadian clocks.

Circadian clocks allow an organism to properly anticipate the momentum of the day. For cyanobacteria and plants, it has been demonstrated that being in phase with the environment (a phenomenon referred to as "resonance") is beneficial to the organism. Under regular (i.e. 24 hour) light dark (LD) cycles, mutant plants and bacteria that contain a circadian clock with a longer or shorter period turned out to perform worse than their wild type counterparts. Conversely, the same mutants performed better than wild type organisms in LD cycles that matched the period length of their endogenous clock (Ouyang et al., 1998; Dodd et al., 2005). These findings clearly demonstrate that the suboptimal performance of the mutants under normal 24-h day-night cycles can not be attributed to clock gene mutations per sé, but rather originate from the organism being out of phase with the environment (dissonance). In view of this, it is not surprising that animal and epidemiological studies have provided evidence that disturbance of the mammalian circadian clock (e.g. by clock gene mutations or by chronic jet lag/shift work conditions) has been associated with a variety of pathologies such as cancer, metabolic syndrome and cardiovascular disease (Fu and Lee, 2003; Froy, 2010).

Studies with the *tau* hamster (carrying a natural mutation in the CKIɛ gene, rendering a clock with a 22- or 20-h period in heterozygous or homozygous state, respectively (Ralph and Menaker, 1988), indicate that the shorter life span (Hurd and Ralph, 1998) and cardiac and renal pathology of heterozygous *tau* hamsters in a 24-h LD cycle can be corrected when animals are housed under a 22-h LD environment (Martino et al., 2008). This provides evidence that the resonance hypothesis also applies to mammals. Added to this, studies with clock mutant mice suggest that that some of the observed phenotypes are not due to altered rhythmicity, but rather to non-core oscillator related functions of clock proteins (Antoch and Kondratov, 2010).

Unlike inbred mouse colonies, the genetic heterogeneity within the human population causes a wide range of circadian periods. Extreme late and early chronotypes (reflecting a person's tendency to be more active and alert early or late in the day) in the average population are encountered in so called "owls" and "larks", the eveningness and morningness phenotype of which has been attributed to period length differences, as well as amplitude and phase changes (Roenneberg et al., 2004; Brown et al., 2008). Work or study obligations often force such individuals to wake up together with the general population, and accordingly, to live out of phase with their internal clock, a situation referred to as "social jetlag" (Wittmann et al., 2006). The opposite situation is encountered in shiftworkers. Rather than being faced with a genetically predisposed desynchrony between body time and environmental time (as in owls, larks and sleep phase syndrome patients), shiftworkers voluntarily and temporarily live out of phase with environmental time. An even more dramatic desynchrony between internal and external time is encountered in patients with Familial Advanced Sleep Phase Syndrome (FASPS) and Delayed Sleep Phase Syndromes (DSPS), who present with dramatically shifted sleep-wake cycles and suffer from chronic fatique and a strong tendency to developing depression (Ptáček et al., 2007). FASPS mutations have been found in the human clock genes *PER2* and *Casein Kinase 1δ* (Toh et al., 2001; Xu et al., 2005) and, when mimicked in the mouse, were shown to shorten the period of circadian behavior (Xu et al., 2005, 2007).

Mice with inactivated *Cry1* or *Cry2* gene possess a circadian clock with a shorter ($\tau =$

22.5 h) or longer period (τ = 24.6 h), respectively (as compared to control littermate mice; τ = 23.7 h), while mice lacking both genes are arrhythmic, as revealed by running-wheel behavior under constant dark conditions (Van der Horst et al., 1999). Explanted tissues and cells from these mice display similar circadian phenotypes *in vitro*, although the period changes are more extreme (Yagita et al., 2001; Liu et al., 2007). In contrast, under regular 12:12h LD cycles, *Cry1* and *Cry2* knock-out mice entrain normally to the LD cycle as measured by running-wheel behavior (Van der Horst et al., 1999; Spoelstra et al., 2004), except for a very small (~10 min) phase advance in activity onset for *Cry1*^{-/-} mice (Spoelstra et al., 2004). However, information on the performance of peripheral oscillators of *Cry1*^{-/-} and *Cry2*^{-/-} mice under LD cycles is thus far lacking. In the present study, we analyzed the peripheral clock in the liver and kidney of *Cry1* and *Cry2* knock-out mice and show that they may serve as animal models for human larks and owls (extreme human chronotypes), respectively. To start determining the impact of "social jetlag" on health, we determined to what extent the daily internal dissonance in the *Cry1*^{-/-} and *Cry2*^{-/-} mice would affect life span under normal and under challenging conditions.

RESULTS
Characterization of the peripheral circadian clock in light-entrained *Cry1*^{-/-} and *Cry2*^{-/-} mice
To determine the state of the clock in peripheral tissues *in vivo*, we analyzed the mRNA levels of several oscillating core clock genes (*Per1*, *Per2* and *Bmal1*) and clock controlled genes (*Dbp*, *Ak4*, *Por*) in liver and kidney taken from mice entrained to a LD12:12 cycle and euthanized at 4-hour intervals. Despite the normal behavior of the animals in LD12:12, we found that *Per1* and *Bmal1* mRNA rhythms in the liver are phase advanced and phase delayed (as compared to wild type liver), in *Cry1*^{-/-} and *Cry2*^{-/-} mice, respectively (Figure 1A and B). This difference was more pronounced in *Cry2*^{-/-} mice. In contrast, we did not observe marked phase differences in *Per2* expression, although we did find differences during the rest of the cycle (Figure 1C). Recently, it was shown that *Per2* is one of 31 genes that are still cyclically expressed in the liver after hepatocyte-specific inactivation of the circadian clock (Kornmann et al., 2007) and that expression of *Per2* is in fact food-driven (Vollmers et al., 2009). Although there are differences in *Per2* expression in the *Cry1*^{-/-} and *Cry2*^{-/-} livers during the day (ZT0-12, Figure 1C), the food-driven transcription of the *Per2* gene in liver presumably aligns the timing of peak expression of *Per2* in all three genotypes. The expression of *Dbp* (involved in detoxification and drug metabolism (Gachon et al., 2006)), a well-characterized CLOCK/BMAL1-target gene and thus a good readout for the activity of this transcription factor (Ripperger et al., 2000; Ripperger and Schibler, 2006), was phase shifted, especially in the *Cry2*^{-/-} mice (Figure 1D). We next analyzed the mRNA profiles of *adenylate kinase 4* (*Ak4*) and *P450 oxidoreductase* (*Por*), two clock-controlled metabolic output genes that oscillate in wild type liver, but expression of which is constitutively low in liver-specific *Bmal1* knock-out mice (Lamia et al., 2008). Figure 1E shows that *Ak4* expression is phase advanced and delayed in *Cry1*^{-/-} and *Cry2*^{-/-} mice, respectively, while *Por* expression is only different in *Cry2*^{-/-} mice (Figure 1F). This lends further support to the notion that the circadian gene expression program in liver is out of phase, especially in *Cry2*^{-/-} mice.

To extend our findings to another tissue, we next analyzed clock performance in kidneys from the same set of mice. As shown in Figure 2, overall circadian gene expression profiles in the kidney show similar phase changes as observed in the liver, except that

for the core clock genes and *Dbp* the shift in peak expression appears to be somewhat more pronounced than in the liver, especially in the *Cry1⁻/⁻* mice. Interestingly, the *Per2* expression profile in the kidney is in better agreement with the mRNA rhythm of the other CLOCK/BMAL1-target genes, *Per1* and *Dbp*, suggesting that, unlike liver, *Per2* oscillation in kidney is predominantly BMAL/CLOCK driven. Apparently, system-driven (BMAL1-independent) expression of *Per2*, as described for the liver (Kornmann et al., 2007), may not be a general feature. The oscillations of *Ak4* and especially *Por* are less pronounced in kidneys (Figure 2E and F) making it difficult to draw conclusions, but at least *Ak4* expression is shifted in the *Cry2⁻/⁻* mice (Figure 2E).
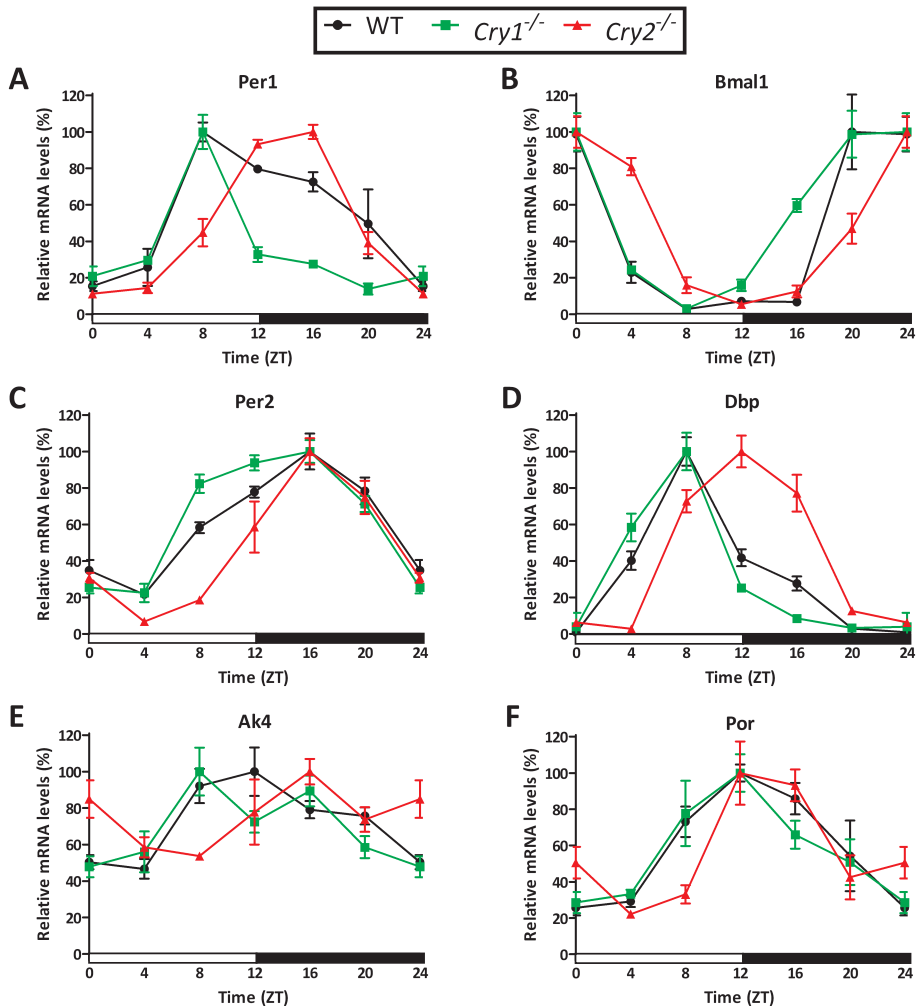

Figure 1: Circadian gene expression in the liver of *Cry1⁻/⁻* and *Cry2⁻/⁻* mice. Relative mRNA expression profiles of 3 core clock genes (panels A-C) and 3 clock-controlled genes (panels D-F) in the liver of LD12:12 entrained WT (black), *Cry1⁻/⁻* (green) and *Cry2⁻/⁻* (red) mice, as measured by RT-qPCR. Data at ZT0 and 24 is double-plotted to more easily visualize diurnal expression. Expression was normalized using *beta-2-microglobulin* mRNA levels and expressed relative to the peak of expression (set to 100 %) for each genotype. The error bars indicate the standard error of the mean (n=3-4 mice per time point).

Taken together, these data show that at least part of the circadian gene expression program in peripheral tissues of *Cry1*^{-/-} and *Cry2*^{-/-} mice shows a phase difference with the light-dark cycle, as compared to wild type animals.

Life span of *Cry1*^{-/-} and *Cry2*^{-/-} mice

To determine the physiological impact of this internal dissonance on life span, we monitored survival and development of pathological events in a large cohort of *Cry1*^{-/-} (n=41), *Cry2*^{-/-} mice (n=83), and wild type mice (n=52). Animals were scored as dead either when found dead, or when they had to be sacrificed, according to the local bio-ethical standards (criteria applied: severe weight loss (>20%), no food-intake, no movement, no response to external stimuli and hunchbacked appearance).
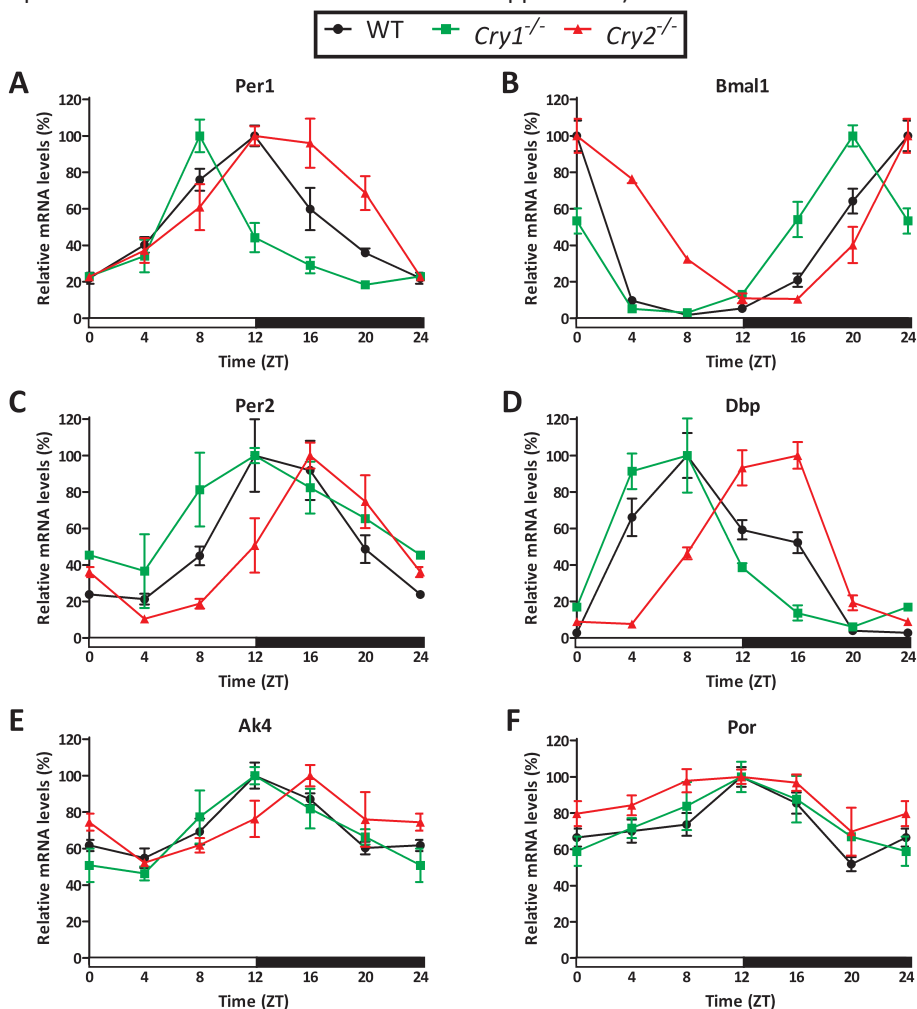

Figure 2: Circadian gene expression in the kidney of *Cry1*^{-/-} and *Cry2*^{-/-} mice. Relative mRNA expression profiles of 3 core clock genes (panels A-C) and 3 clock-controlled genes (panels D-F) in the kidney of LD12:12 entrained WT (black), *Cry1*^{-/-} (green) and *Cry2*^{-/-} (red) mice, as measured by RT-qPCR. Data at ZT0 and 24 is double-plotted to more easily visualize diurnal expression. Expression was normalized using *beta-2-microglobulin* mRNA levels and expressed relative to the peak of expression (set to 100 %) for each genotype. The error bars indicate the standard error of the mean (n=3-4 mice per time point).

In line with our previous observation that *Cry1*^{-/-} and *Cry2*^{-/-} mice develop normally and do not display any overt pathology up to the age of 14 and 7 months, respectively (Van der Horst et al., 1999), we did not observe any difference in body weight between young wild type and *Cry* knock-out animals up to the age of 1 year (data not shown). Also during the remainder of their life the mice did not develop any noticeable pathological phenotype (other than those also observed in WT mice). Moreover, the survival curves for *Cry1*^{-/-} and *Cry2*^{-/-} mice are not significantly different from that of wild type control mice (Figure 3A and B; Kaplan-Meier with Log-rank test p = 0.3 and p = 0.5, for *Cry1*^{-/-} and *Cry2*^{-/-}, respectively). In addition, analysis of both sexes seperately did not reveal differences either (data not shown). From these data, we conclude that advanced and delayed phase of peripheral oscillators in *Cry1*^{-/-} and *Cry2*^{-/-} mice (as compared to wild type animals), does not affect life span, nor triggers aberrant visible pathological events under regular 12h light-12h dark cycles.
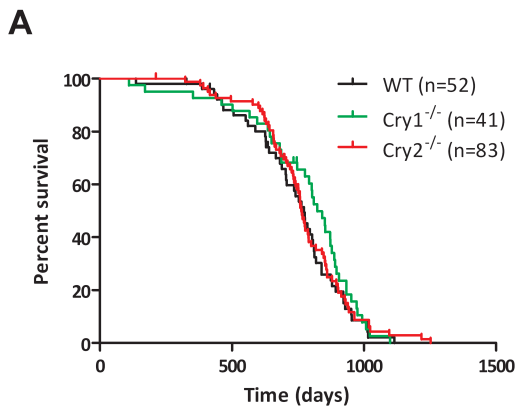
A

Figure 3: Life span of *Cry1*^{-/-} and *Cry2*^{-/-} mice. (A) Kaplan-Meier survival plots for WT (black), *Cry1*^{-/-} (green) and *Cry2*^{-/-} (red) mice. Animals were scored as dead from the cohort when found dead or when appearing moribund. Log-rank test analysis indicated no significant differences between the curves. Tick marks on the curves indicate censored events (e.g. mice that had to be killed due fighting wounds). **(B)** Mean, median, standard error and 95% confidence interval (upper and lower bound) for data presented in panel A.

B

Means and Medians for Survival Time								
	Mean				Median			
			95%				95%	
Genotype	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
WT	741.7	26.7	689.3	794.1	773	26.0	722.0	824.0
Cry1-/-	773.5	34.1	706.7	840.4	824	35.6	754.1	893.9
Cry2-/-	770.3	20.0	731.1	809.5	763	9.5	744.4	781.6

Life span of ionizing radiation-exposed *Cry1*^{-/-} and *Cry2*^{-/-} mice

Since the effect of circadian desynchronization may only occur in under challenging conditions (Filipski et al., 2004; Preuss et al., 2008), we next exposed a cohort of 8-10-week old mice to a single dose (6 Gy, given at ZT10) of ionizing radiation (IR). This treatment has been shown to shorten life span and induce tumorigenesis (mainly lymphomas in C57Bl/6 mice), as well as increased tumorigenesis in clock mutant mice (Fu et al., 2002; Antoch et al., 2008). Animals were analyzed as described for Figure 3. Figure 4A shows a Kaplan-Meier plot of the survival of ionizing radiation exposed *Cry1*^{-/-}, *Cry2*^{-/-} and wild type control mice. Like the untreated mice, the survival of *Cry1*^{-/-} mice does not significantly deviate from that of wild type animals (p=0.89, Log-rank test). However, the survival of *Cry2*^{-/-} mice was significantly reduced compared to treated wild type mice (p=0.033, Log-rank-test). From the survival curves it appears that the *Cry2*^{-/-} mice die more frequently from the acute (first month after treatment) effects of IR. In

this period mortality is known to be caused by radiation-induced hematopoietic and/or intestinal damage.. To determine whether the *Cry2*-/- mice die more frequently from the acute effects of IR, we analyzed the mice that died within the first month after treatment separately. Although there are no significant differences between the genotypes in the survival timing within this initial period after treatment (Figure 4C), Figure 4D shows that the number of *Cry2*-/- mice that either died acutely (acute) or survived the first month after treatment (long) is significantly higher (Chi-square test, p=0.031). This finding clearly points to an increased acute radiosensitivity of *Cry2*-/- mice. When the mice that die acutely are excluded from the analysis there are no differences in the survival curves (p>0.4, Log-rank test), indicating that the long-term survival is not different from wild type mice (Figure 4E). From these data, we conclude that *Cry2*-/-, but not *Cry1*-/- mice display increased acute radiosensitivity, while the long-term survival after IR-exposure of both *Cry1*-/- and *Cry2*-/- mice is indistinguishable from wild type mice.

A
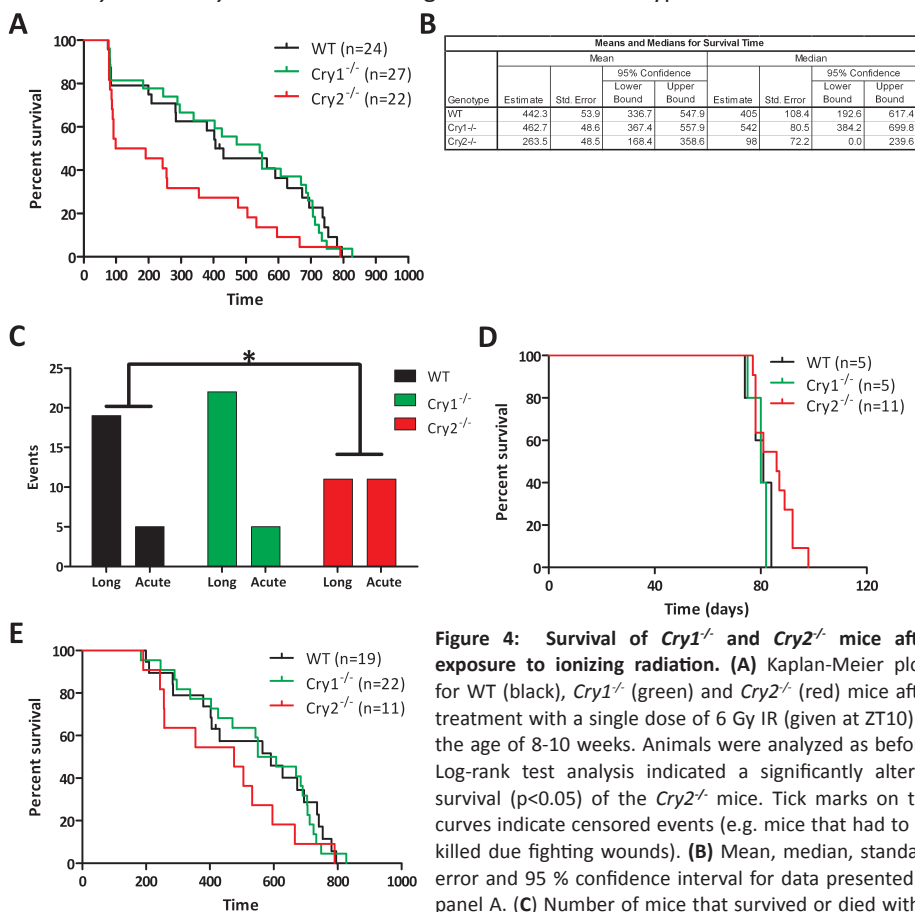

B

	Means and Medians for Survival Time								
	Mean				Median				
			95% Confidence					95% Confidence	
Genotype	Estimate	Std. Error	Lower Bound	Upper Bound	Estimate	Std. Error	Lower Bound	Upper Bound
WT	442.3	53.9	336.7	547.9	405	108.4	192.6	617.4
Cry1-/-	462.7	48.6	367.4	557.9	542	80.5	384.2	699.8
Cry2-/-	263.5	48.5	168.4	358.6	98	72.2	0.0	239.6

C

D

E

Figure 4: Survival of *Cry1*-/- and *Cry2*-/- mice after exposure to ionizing radiation. (A) Kaplan-Meier plots for WT (black), *Cry1*-/- (green) and *Cry2*-/- (red) mice after treatment with a single dose of 6 Gy IR (given at ZT10) at the age of 8-10 weeks. Animals were analyzed as before. Log-rank test analysis indicated a significantly altered survival (p<0.05) of the *Cry2*-/- mice. Tick marks on the curves indicate censored events (e.g. mice that had to be killed due fighting wounds). **(B)** Mean, median, standard error and 95 % confidence interval for data presented in panel A. **(C)** Number of mice that survived or died within the first month after IR treatment. The proportion of *Cry2*-/- mice that died acutely is higher compared with WT mice (* p < 0.05, Chi-square test).

(**D**) As panel A, except that only animals that died from the acute effects of IR are included. Log-rank test analysis indicated no significant differences between the curves. (**E**) As panel A, except that animals that died from the acute effects of IR are excluded. The long-term survival of *Cry1*-/- and *Cry2*-/- mice after IR treatment is not significantly different compared to IR-exposed wild type mice (p=0.7 and 0.4, respectively, Log-rank test).

DISCUSSION

In the present study, we have provided evidence that the peripheral circadian clock as well as the expression pattern of clock-controlled output genes in the liver and kidney of short period $Cry1^{-/-}$ mice, housed under a regular light-dark regime (i.e. LD12:12) are phase advanced in comparison to that of wild type animals, while oppositely, these peripheral clocks are phase delayed in long period $Cry2^{-/-}$ mice. The resulting dissonance between body time and environmental time likely mimics the situation encountered by human "larks" and "owls". We therefore propose that $Cry1$ and $Cry2$ knock-out mice may well serve as animal models for morningness and eveningness. Additionally, we observed that the phase of $Per2$ peak expression in livers of $Cry1$ and $Cry2$ knock-out mice remains comparable to that of wild type animals when animals are kept under a regular light-dark regime. This is presumably due to the food-driven expression of the $Per2$ gene in the liver, (Vollmers et al., 2009), which apparently dominates over CLOCK/BMAL1-drive transcription. Interestingly, the $Per2$ mRNA rhythm in the kidney remains coupled to the circadian core oscillator, suggesting that the stringency of food-driven $Per2$ transcription is tissue-dependent. The absence of phase changes in $Per2$ expression in the liver of the Cry knock-out mice also implies that the feeding pattern of the animals is not phase-shifted and that, in line with the locomotor activity in running-wheels (Van der Horst et al., 1999), animals entrain normally to the light-dark cycle, even in the absence of a running-wheel. As running-wheel behavior is subject to masking (suppression of wheel running activity in the light phase; Mrosovsky, 1999) this needs to confirmed by monitoring the behavior of $Cry1$ and $Cry2$ knock-out mice using infrared beams in cages without running-wheels.

The resonance theory, first postulated based on experiments with wildtype *Drosophila* placed in LD-cycles with different periods (Pittendrigh and Minis, 1972), and later firmly established using mutant models in Cyanobacteria, plants and hamsters (Hurd et al., 1998; Ouyang et al., 1998; Dodd et al., 2005; Martino et al., 2008) states that being in phase is beneficial for an organism. Consistent with this, continuous body/environmental time desynchrony, resulting from e.g. repeated shift-work or redoents exposed to chronic jetlag protocols, has an adverse effect on the health of humans and rodents (Fu et al., 2003). Therefor, we investigated whether inactivation of the $Cry1$ or $Cry2$ gene affects the life span of the mouse under regular light-dark cycles. In contrast to our expectations, we did not find a significant impact on the life expectancy of $Cry1^{-/-}$ and $Cry2^{-/-}$ mice, despite the fact that their (peripheral) body clocks are continuously out of phase. Our findings also contrast data obtained with heterozygote *tau* hamsters that display decreased survival under 24-h LD conditions (Hurd et al., 1998; Martino et al., 2008). One explanation could be that, the *tau* mutant hamsters, unlike the $Cry1^{-/-}$ and $Cry2^{-/-}$ mice, do not show a normal activity onset, even though they do entrain to the LD cycle (Hurd et al., 1998). Instead, the active phase of the *tau* hamsters already starts 4 h ahead of lights-off. Another explanation could be that the period change in the Cry knock-out mice (~ 1 h, Van der Horst et al., 1999) is not large enough, as compared to that of the *tau* hamster (~ 2 h, Ralph et al., 1988). Nevertheless, despite the normal behavior of the Cry knock-out mice, the phase difference in peripheral circadian gene expression is quite profound, especially in the $Cry2^{-/-}$ mice.

Even though the $Cry1^{-/-}$ and $Cry2^{-/-}$ mice do not show a long-term survival disadvantage under our LD12:12 laboratory conditions, they could have a disadvantage under more competitive conditions in the wild or under different photoperiod conditions (e.g.

LD16:8 or LD8:16) where differences between peripheral clocks and the environment might become even larger in the *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice. Furthermore, it might be that the *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice display other phenotypes that do not necessarily affect long-term survival or that only become obvious when specifically measured. In this context it is interesting to mention that functional magnetic resonance imaging of the brain of human "owls" and "larks" brains has revealed that maintaining attention in the evening was associated with higher activity in "owls" than "larks" in a few brain regions, including the SCN (Schmidt et al., 2009). It therefore would be interesting to measure gene expression in non-SCN brain regions of LD12:12 entrained *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice to determine whether these peripheral brain clocks, like the liver and kidney clocks, are out of phase with those of wild type animals, housed under the same conditions. If this is the case, the *Cry* mouse models could also be used to mimic "owls" and "larks" in behavioral assays that address neurological parameters (e.g. learning and memory).

Although we did not find an effect of ionizing radiation on the long-term survival, the *Cry2*$^{-/-}$ mice did have a higher chance of dying from the acute effects of IR exposure. The reason why this only occurs in the *Cry2*$^{-/-}$ mice and not the *Cry1*$^{-/-}$ mice is not clear, but could be due to the fact that the phase changes in gene expression seem to be more pronounced in the *Cry2*$^{-/-}$ mice. Alternatively, it could somehow be a property specifically related to delayed peripheral gene expression. One way of distinguishing between these two scenarios would be to determine 30-day survival in relation to time-of-day of IR treatment. It could well be that *Cry1*$^{-/-}$ mice also show increased acute radiosensitivity when IR exposure would have been performed at another time of the day.

The finding that the *Cry2*$^{-/-}$ mice show increased acute mortality after IR may also have important implications for chronotherapy. IR is frequently used to treat tumors and there is increasing interest in applying therapy at a time were the treatment is most effective or is showing the least side-effects (Lévi et al., 2010). Our results suggest that not only should the time-of-day be considered a factor in radiotherapy (or possibly also other treatments), but also the individual chronotype (i.e. morningness or eveningness) of each patient.

In conclusion, we show that under LD conditions, circadian gene expression in peripheral tissues of *Cry1*$^{-/-}$ and especially *Cry2*$^{-/-}$ mice has an altered phase-relationship with behavior, as compared to wild-type mice, leading to internal dissonance. Although this does not appear to affect the long-term survival under normal and cancer-predisposing conditions, this phase difference does affect the acute survival after ionizing radiation exposure in the *Cry2*$^{-/-}$ mice.

MATERALS AND METHODS
Mice

The generation of *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice has been described previously (Van der Horst et al., 1999) and where more than 99 % C57BL/6J genetic background. Mice were kept at the Animal Resource Center (Erasmus University Medical Center), which operates in compliance with and used in compliance with the European guidelines (European Community 1986) and The Netherlands legislation for the protection of animals used for research, including ethical review. Mice were housed socially (up to 4 animals per cage) at ambient temperature (19–22°C) and humidity (53–63%) under a 12 h light /12 h dark cycle (LD12:12) with standard chow and water available *ad libitum*. All animal studies were approved by an independent Animal Ethical Committee (Dutch equivalent of the

IACUC).

Life span study

To determine life span, a cohort of *Cry1⁻/⁻* mice (21 males, 20 females) and *Cry2⁻/⁻* mice (45 males, 38 females) was formed, along with a cohort of wild type controls (22 males, 30 females) was followed in time. The health state of the animals was checked 2-3 times per week, starting at the day of weaning. Individual animals were initially weighed weekly to determine body weights and with longer intervals at later age. During the entire study, all animals were kept in the same and stringently controlled environment as described above. The microbiological status of the cohorts was monitored quarterly. Mice were scored as dead either when found dead, or when they had to be sacrificed, according to the local bio-ethical standards (criteria applied: severe weight loss (>20%), no food-intake, no movement, no response to external stimuli and hunchbacked appearance). Most animals were fixed in 1% formalin for future pathology. Statistical analysis of survival curves was performed using the Kaplan-Meier method, followed by a Log-rank test.

Ionizing radiation exposure study

To determine the life span under challenging conditions a cohort of *Cry1⁻/⁻* (13 males, 14 females), *Cry2⁻/⁻* (9 males, 13 females) and wild type (13 males, 11 females) mice was formed. At the age of 8-10 weeks, animals were exposed to a single dose of 6 Gy of γ-radiation, using a ^{137}Cs source. The mice were housed, scored and analyzed in the same way as described for the life span study. Statistical analysis of 30-day and overall survival curves was performed using the Kaplan-Meier method, followed by a Log-rank test.

Quantitative RT-PCR analysis

Tissues were isolated from light-entrained (LD12:12) wild type (n=4 per time point) and *Cry1⁻/⁻* and *Cry2⁻/⁻* mice (n=3 per time point for each genotype), sacrificed around the clock at four hour time intervals. Tissues were isolated and snap-frozen in liquid nitrogen. For RNA isolation, tissues were ground on dry-ice to a fine powder using a mortar and pestle. The powder was lysed in Trizol (Invitrogen), after which total RNA was isolated following standard procedures. For first-strand cDNA synthesis, 1 µg of RNA was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad), following the manufacturer's protocol and subsequently diluted to 200 µl. Real-time qPCR analysis of clock and clock-controlled genes was performed (5 µl of the cDNA mixture in a final volume of 25 µl), using SYBR green and an iCyclerIQ detection system (Bio-Rad). All samples were analyzed twice. Primers sequences are available on request. Expression levels were normalized using *beta-2-microglobulin* (*β2m*) mRNA levels and plotted with the maximum expression level for each gene set at 100%. The generation of specific PCR products was confirmed by melting curve analysis and each primer pair was tested with a serial dilution of a cDNA mix which was used to calculate the primer pair efficiency.

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CHAPTER 4

Cryptochromes impinge on cell cycle progression in a circadian-oscillator-independent manner

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Chapter 4

ABSTRACT

The circadian system is known to control or modulate a wide range of physiological processes. By gating cell cycle progression to certain times of the day, the circadian clock is thought to reduce the exposure of replicating cells to potentially hazardous compounds that arise during cellular metabolism or UV-radiation from the sun. Cells and tissues lacking core clock components show various cell-cycle-related phenotypes, including an altered *in vivo* response to genotoxic stress. To what extent these phenotypes are due to a cell-autonomous lack of circadian oscillations is not well-defined. Using several genotoxic agents and readouts, we found no alterations in the *in vitro* response to DNA damage in primary cells lacking the core clock components *Cry1* and *Cry2*. However, we observed that primary *Cry1|Cry2*-deficient cells progressed faster through the cell cycle and provide evidence that this increased proliferation is not caused by the absence of a circadian clock *per se*. Instead, our data suggest that widespread deregulation of CLOCK-/BMAL1-target genes underlies the increased proliferation of *Cry*-deficient cells. Together, our results provide new insight into how clock components are connected to the cell cycle and suggest that the strong cell cycle-clock connections reported on the basis of *in vivo* experiments may be attributed to systemic effects, rather than a direct cell-autonomous control of the circadian clock over cell cycle-related processes.

INTRODUCTION

Oscillatory systems are recurring phenomena in biology. Some well-studied examples are the circadian clock, driving daily rhythms in behavior, physiology and metabolism (Reppert and Weaver, 2002), and the cell cycle, driving the periodic duplication of the genome and subsequent cell division (Tyson and Novak, 2008).

Circadian clocks are present in most organisms and have a periodicity of about (*circa*) one day (*dies*). In mammals, the central clock is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. To keep pace with the exact 24 hour solar cycle, the SCN clock is entrained every day by light stimuli, received via the retina (reviewed by Dibner et al., 2010). In turn, the SCN transmits information to peripheral organs (e.g. through control of hormonal secretion) to synchronize cell-autonomous clocks in peripheral tissues (Reppert et al., 2002; Stratmann and Schibler, 2006a). Remarkably, even cultured cell lines display circadian oscillations that are asynchronous in a population, but can be synchronised by various compounds that impinge on different pathways (e.g. cAMPand glucocorticoid signaling (Balsalobre et al., 2000; Stratmann and Schibler, 2006b). The circadian system orchestrates various physiological processes, e.g. sleep-wake cycle, metabolism, blood pressure, hormone secretion and cell cycle division (Reppert et al., 2002; Takahashi et al., 2008). In the human population and in animal models, genetic disruption of circadian rhythmicity or chronic desynchrony with the light-dark cycle (e.g. repeated jetlag or frequent shift work) has been linked to various pathologies such as cancer, obesity, cardiovascular and mental disorders (Fu and Lee, 2003; Froy, 2009; Rudic, 2009; McClung, 2007).

At the molecular level, the circadian clock is composed of clock genes and proteins that act in interlocked transcription/(post-)translational feedback loops. The only essential transcription/translation loop for circadian rhythmicity in this molecular oscillator, is a negative feedback loop composed of CLOCK/BMAL1 driven transcriptional activation of the *Cryptochrome* (*Cry1* and *Cry2*) and *Period* (*Per1* and *Per2*) genes via E-box promoter elements. After a delay, the CRY and PER proteins accumulate and heterodimerize in

the cytoplasm and subsequently enter the nucleus, where they shut down transcription of their own genes by inhibiting the CLOCK/BMAL1 complex (Kume et al., 1999; Griffin et al., 1999; Sato et al., 2006). The delay in CRY and PER protein rhythms (as compared to the mRNA rhythms, is controlled by various posttranslational modifications (e.g. phosphorylation and ubiquitylation) that modulate the stability and subcellular localization of the clock proteins (Gallego and Virshup, 2007; Vanselow and Kramer, 2007). In addition, auxiliary feedback loops such as cyclic expression of the *Bmal1* gene, confer robustness and precision to the core negative feedback loop (Preitner et al., 2002; Mukherji and van Oudenaarden, 2009). The CLOCK/BMAL1 and PER/CRY complexes also underlie cyclic expression of a series of E-box containing clock-controlled genes (CCGs), These CCGs vary from tissue to tissue, probably reflecting the specific requirements of each tissue (Storch et al., 2007; Lamia et al., 2008). Amongst the CCGs are transcription activators and inhibitors, circadian expression of which indirectly drives cyclic expression of other genes. Transcription profiling studies have shown that the circadian clock allows up to 10% of a tissues transcriptome to oscillate (Panda et al., 2002; Storch et al., 2002; Ueda et al., 2002; Hughes et al., 2009). For a small proportion of these circadian genes in the liver (10%), the oscillations are not driven by the cell-autonomous clock, but through system-driven signals (Kornmann et al 2007). In relation to this, a recent report indicated that in the absence of the circadian clock, a few hundred liver genes can display circadian expression, driven by the feeding pattern of mice alone (Vollmers et al., 2009).

Another oscillatory process is the cell division cycle, in which cells cyclically duplicate their genome (S phase, preceded by the G1 phase) and divide into two daughter cells (M phase, preceded by the G2 phase) (Tyson et al., 2008). The cell division cycle has several similarities with the circadian cycle. Both are composed of several interlocked positive and negative feedback loops and both are controlled at various levels of gene expression. Yet, (at least) two important distinctions can be distinguished. First, the cell cycle is not a self-sustained oscillator as a cell needs to receive external information to start and continue through the different phases. Second, cell cycle progression can be halted, as is the case after exposure to genotoxic stress. Following detection of DNA damage, cells activate the DNA damage response (DDR) pathway that ensures that the injured genome gets repaired before replication and cell division occur (Bartek and Lukas, 2007; Zhou and Elledge, 2000).

Interestingly, an additional layer of control over cell division has been postulated to emanate from the circadian clock, which may ensure that certain steps of the cell cycle happen at a defined moment during the day. In fact, the evolutionary advantage for an organism to replicate its genome when the change of induction of DNA damage (e.g. UV-radiation from the sun or by oxidative stress generated during metabolism) is lowest, has been postulated to constitute the driving factor for the naissance of the circadian molecular oscillator (Pittendrigh, 1993). After the initial observation that *in vivo* cell cycle progression in epithelial cells in e.g. human and rodent skin and intestinal tract is synchronized between cells and with time of the day, known as cell cycle gating (Bjarnason and Jordan, 2000; Smaaland, 1996), recent studies linking core clock genes to cell cycle related phenotypes *in vitro* or *in vivo* have concentrated on knockout or mutant mouse models and cells/tissues thereof (Fu et al., 2002, 2005; Matsuo et al., 2003; Gorbacheva et al., 2005; Miller et al., 2007; Grechez-Cassiau et al., 2008; Ozturk et al., 2009). Moreover, most of the core clock genes, but especially *Per1* and *Per2*, have been shown to display altered expression levels in tumors and affect the proliferation

rate and apoptosis sensitivity of cancer cells (Chen-Goodspeed and Cheng Chi Lee, 2007). Surprisingly, recent studies have shown that the connection between the clock and the DDR is reciprocal: activation of the DDR by genotoxic exposure leads to phase shifting of the circadian clock in *Neurospora*, (cultured) mammalian cells, as well as free-running mice (Pregueiro et al., 2006; Oklejewicz et al., 2008; Gamsby et al., 2009). While the full implications of the latter observations have not yet been elucidated, they are further indications strengthening the intertwinement of the circadian clock and cell cycle related processes.

Despite recent progress, several fundamental questions with respect to the coupling of the circadian clock and the cell cycle remain largely unanswered. For instance, it is not clear whether the observed cell cycle alterations in clock mutant cells or tumor cells with altered clock gene expression levels are due to alterations in the clock itself or due to clock-independent functions of core clock proteins. Furthermore, it remains to be resolved whether *in vivo* the circadian clock controls cell cycle progression cell-autonomously or systemically, as it is clear that peripheral clock output processes are not only controlled by a local circadian clock (Storch et al., 2007; Lamia et al., 2008), but also (in)directly by the SCN central clock via hormones, peptides and other compounds (including growth factors) that are released in a circadian manner and may affect peripheral clock output pathways (e.g. Mendez-Ferrer et al., 2008), including potentially cell cycle related processes.

In the present study, we investigated the DNA damage sensitivity and proliferative capacity of cultured primary $Cry1^{-/-}|Cry2^{-/-}$ mouse embryonic fibroblasts MEFs and showed that whereas the absence of CRY proteins does not affect genotoxic stress sensitivity, it causes cells to proliferate faster under normal conditions. We show that the CRY proteins affect cell cycle progression in a cell-autonomous, but circadian clock-independent manner. Instead, our results suggest that the accelerated cell cycle progression of *Cry*-deficient cells is caused by global deregulation of *Bmal1*-dependent gene expression, regardless of whether these genes still oscillate or not. Together, these results suggest that the inconsistency between *in vivo* and *in vitro* observations might be attributed to systemic circadian control rather than a direct cell-autonomous control.

RESULTS

Normal genotoxic stress response in circadian clock-deficient $Cry1^{-/-}|Cry2^{-/-}$ cells

To investigate the impact of the circadian clock on the DDR, we isolated isogenic (C57BL/6J) primary mouse embryonic fibroblasts (MEFs) from wild type (WT) and circadian clock-deficient $Cry1^{-/-}|Cry2^{-/-}$ embryos (Van der Horst et al., 1999; Okamura et al., 1999) and maintained them at physiological (3%) O_2-concentration. When exposed to increasing doses of UV light, $Cry1^{-/-}|Cry2^{-/-}$ cells showed normal survival (Figure 1A). Likewise, the sensitivity of $Cry1^{-/-}|Cry2^{-/-}$ MEFs to chronic oxidative stress (induced by placing culture dishes in a normal 20% O_2 incubator; Figure 1B) or ionizing radiation (IR; Figure 1C), determined by measuring the relative proliferation capacity of cells following treatment, is in the WT range. We included primary $p53^{-/-}$ MEFs (isolated from $p53^{-/-}$ mice in a C57BL/6J background, (Donehower et al., 1992)), which are known to be more resistant to various genotoxic agents (Kastan et al., 1992), as a positive control in some of these assays. As expected, $p53^{-/-}$ cells showed higher cellular survival after exposure to ionizing radiation and oxidative stress (data not shown) making it unlikely that the observed normal genotoxic stress sensitivity of $Cry1^{-/-}|Cry2^{-/-}$ MEFs is due to technical

issues. Next, using FACS analysis, we looked at cell cycle arrest after exposure of cells to 4 Gy of IR. After treatment, cells were pulsed with BrdU for 1 h to label replicating cells. The *Cry1^-/-|Cry2^-/-* MEFs showed a response that was not consistently different from WT cells (Figure 1D). Likewise, checkpoint activation (as determined by measuring the induction of the key p53-target gene *p21*), was normal in *Cry1^-/-|Cry2^-/-* MEFs (Figure 1E). Collectively, these data show that there is no major impairment of the genotoxic stress sensitivity and DDR in clock-deficient *Cry1^-/-|Cry2^-/-* fibroblasts *in vitro*.
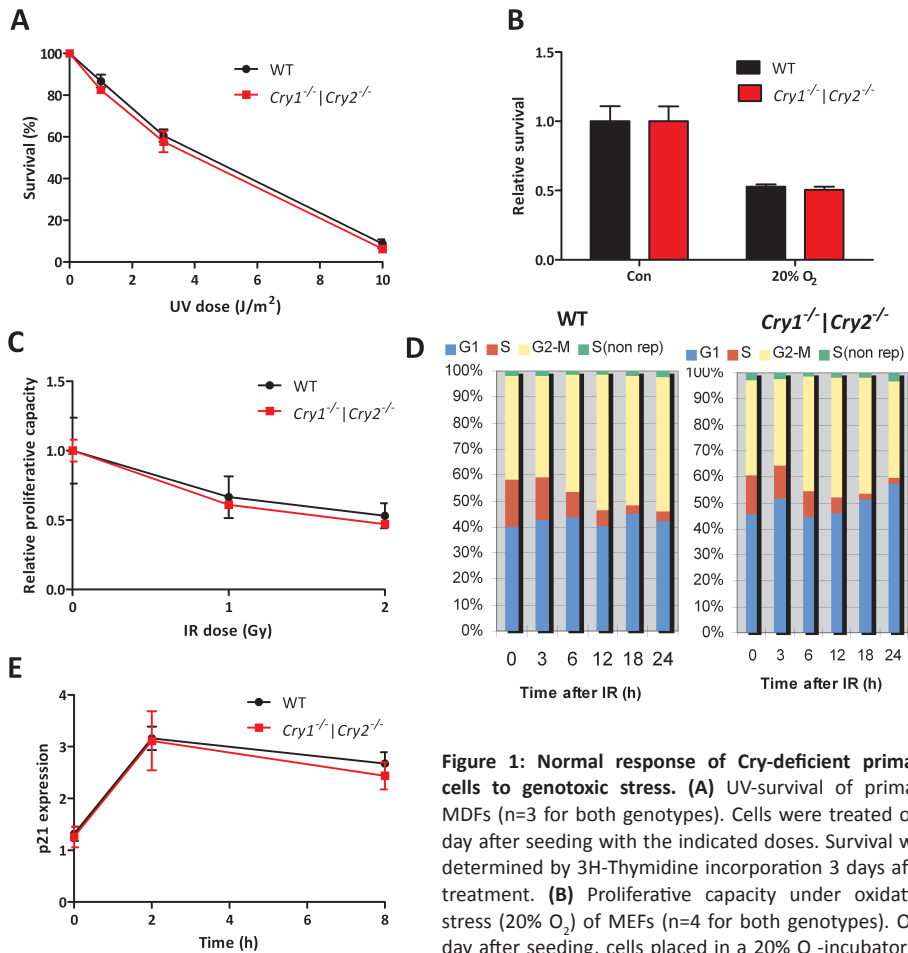

Figure 1: Normal response of Cry-deficient primary cells to genotoxic stress. (A) UV-survival of primary MDFs (n=3 for both genotypes). Cells were treated one day after seeding with the indicated doses. Survival was determined by 3H-Thymidine incorporation 3 days after treatment. **(B)** Proliferative capacity under oxidative stress (20% O$_2$) of MEFs (n=4 for both genotypes). One day after seeding, cells placed in a 20% O$_2$-incubator or left in the 3%-incubator for 3 days.

(C) Proliferative capacity of WT (n=2) and *Cry1^-/-|Cry2^-/-* (n=2) MEFs after IR-exposure. Cells were treated one day after seeding with the indicated doses. Another experiment with a single, different cell line for each genotype gave similar results; higher doses in that experiment did not substantially further reduce proliferation (not shown). **(D)** FACS analysis of IR-treated MEFs. Cells were plated in 6-cm dishes and then treated the next day with 4 Gy IR. At the indicated time points cells were incubated with BrdU for 1h to measure the amount of replication. Cells with an S-phase DNA amount, but that did not incorprotate BrdU, are indicated as S (non rep), for non-replicating. **(E)** Activation of ATM-signaling as determined by qPCR of *p21* gene induction at the indicated time points after 4 Gy IR of WT (n=4) and *Cry1^-/-|Cry2^-/-* (n=3) MEFs.

For each cell line, the untreated cells were set to 1 or 100% and the treated cells are expressed relative to that. The error bars indicate standard error of the mean (SEM) in all cases.

Accelerated cell cycle progression of circadian clock-deficient *Cry1⁻/⁻|Cry2⁻/⁻* cells

Interestingly, while we did not observe any difference in their ability to respond to genotoxic stress, primary *Cry1⁻/⁻|Cry2⁻/⁻* MEFs displayed a higher proliferation rate than primary WT MEFs under non-stressed (low-oxygen) culture conditions (p<0.05, Figure 2A), although not as profoundly as, for example, the very rapidly growing *p53⁻/⁻* MEFs ((Xu et al., 1998) and Figure 3C). This difference was consistently observed in 4 independent experiments, using MEFs from different litters or with the same lines at a different passage (Figure 2B). Similar results were obtained using primary mouse dermal fibroblasts (MDFs) from adult WT and *Cry1⁻/⁻|Cry2⁻/⁻* mice (data not shown). To proliferate faster, cells must shorten one or more phases of the cell cycle (mainly G1 or G2). We therefore compared the cell cycle distribution of proliferating cultures of the WT, *Cry1⁻/⁻|Cry2⁻/⁻* and *p53⁻/⁻* cell lines by FACS analysis. In line with their reported shorter G1 phase (Lowe et al., 1993), *p53⁻/⁻* cell cultures contain a reduced percentage of cells in G1 phase (Figure 2C). In contrast, *Cry1⁻/⁻|Cry2⁻/⁻* MEFs showed a phase distribution that strongly resembled that of WT cells, except for a possible small increase in the percentage of S-phase cells (Figure 2C). The normal G1/G2 phase distribution in *Cry1⁻/⁻|Cry2⁻/⁻* MEFs suggests that both cell cycle phase are equally shortened in the absence of CRY proteins.
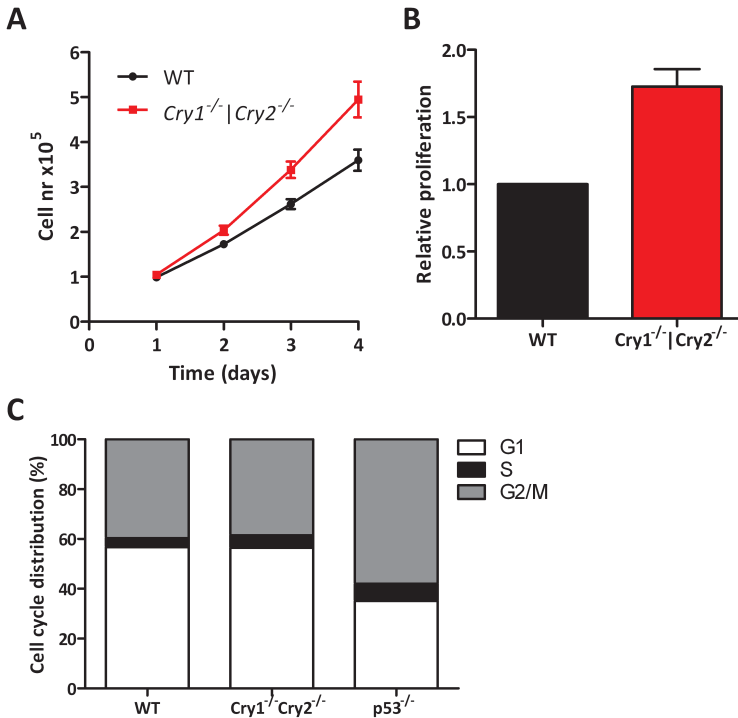

Figure 2: Faster cell cycle progression of *Cry*-deficient primary MEFs (A) Proliferation of WT (n=4) and *Cry1⁻/⁻|Cry2⁻/⁻* (n=3) primary MEFs. 1x10⁵ Cells/well were seeded in triplicate in 6-well plates on day 0 and then counted on the subsequent days. Error bars indicate SEM. (p=0.02 comparison of slopes). **(B)** Quantification of the slopes from four experiments using cell lines from different liters and/or different passage. Due to inter-experimental variability, the slope of the WT cells was set to 1 in each experiment and the *Cry1⁻/⁻|Cry2⁻/⁻* cells are expressed relative to that. Error bars indicate SEM. **(C)** FACS analysis showing normal cell cycle distribution in *Cry1⁻/⁻|Cry2⁻/⁻* cells.

Accelerated cell cycle progression in $Cry1^{-/-}|Cry2^{-/-}$ cells is clock-independent

The faster cell cycle progression of $Cry^{-/-}|Cry2^{-/-}$ MEFs might originate from either the inactivation of the circadian core oscillator or the specific absence of CRY proteins (and accordingly a clock-associated or a yet unknown clock-independent function of these proteins). To distinguish between these two possibilities, we made use of the observation that $Cry1^{+/-}|Cry2^{-/-}$ mice maintain (long period) rhythmicity in constant darkness, while $Cry1^{-/-}|Cry2^{+/-}$ mice become arrhythmic after several days (Van der Horst et al., 1999) and isolated primary MEFs from embryos with the corresponding genotype (genetic background C57BL6/J). We first transduced the primary MEFs with a lentiviral *Per2::luciferase* reporter construct to allow real time imaging of circadian clock performance *in vitro*. In line with recent observations, suggesting that cellular circadian phenotypes can be more extreme as compared to mouse behavior or oscillations in the SCN (Brown et al., 2005; Liu et al., 2007), we observed that $Cry1^{+/-}|Cry2^{-/-}$ MEFs oscillate with a longer period and higher amplitude (as compared to WT MEFs), while $Cry1^{-/-}|Cry2^{+/-}$ MEFs showed severely impaired/absent oscillations (Figure 3A). This latter finding is consistent with the finding that inactivation of *Cry1* alone already severely impairs cellular oscillations (Liu et al., 2007).
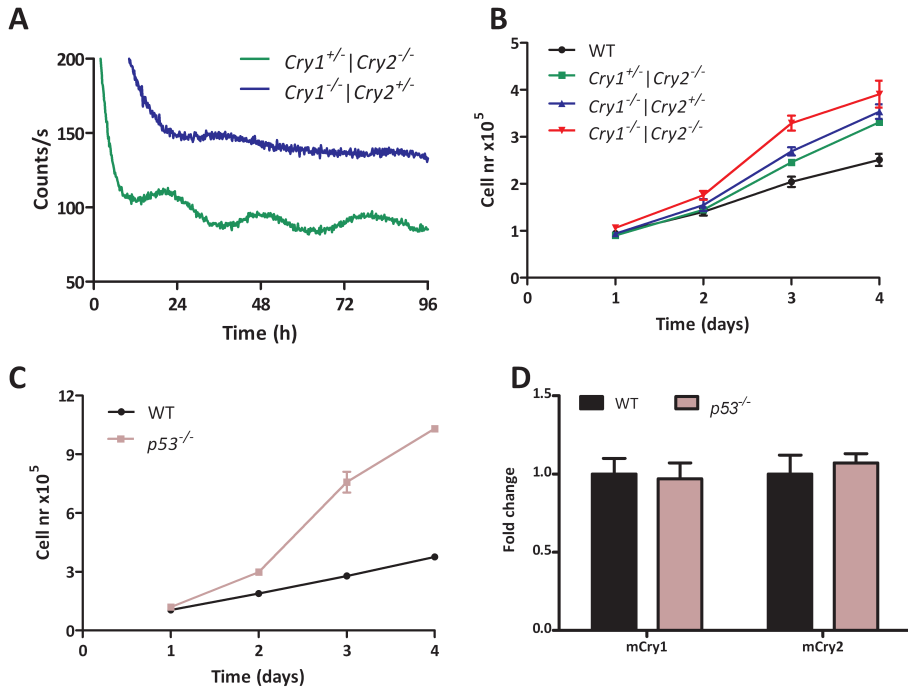

Figure 3: Circadian oscillations do not impede proliferation. (A) Analysis of the circadian clock in $Cry1^{+/-}|Cry2^{-/-}$ and $Cry1^{-/-}|Cry2^{+/-}$ cells transduced with lentivirus containing a luciferase reporter under the control of the *mPer2* promoter.. Confluent dishes were synchronized using Forskolin after which bioluminescence was measured every 10 minutes. **(B)** Proliferation of MEFs with different doses of *Cry* (n=3 for all genotypes). Each line was plated in triplicate. **(C)** Proliferation of WT (n=4) and *p53-/-* (n=1) MEFs. Each line was plated in triplicate. **(D)** Gene expression (qPCR) showing normal levels of *Cry* genes in *p53*-deficient MEFs. For the $p53^{-/-}$ cells the error bars indicate the SEM of 1 line for which we analyzed 3 RNA samples. Error bars for the WT cells indicate the SEM of 4 independent cell lines

Next, we compared the proliferation rate of the various *Cry*-deficient MEF lines and observed that *Cry1^-/-|Cry2^+/-* cells (containing a robust clock with a longer period) and *Cry^+/-|Cry2^-/-* cells (lacking a circadian clock) both proliferate faster than WT MEFs (Figure 3B) which implies that the increased proliferation rate of *Cry1^-/-|Cry2^-/-* MEFs can not be explained by the absence of a circadian clock in those cells. Rather, as one active copy of the *Cry* genes (either *Cry1* or *Cry2*) is sufficient to partially attenuate the accelerated growth associated with *Cry1^-/-|Cry2^-/-* MEFs, our results suggest that the *Cry* gene products impinge on proliferation capacity in a dose-dependent manner, regardless of whether cells are clock-proficient or clock-deficient.

In view of the previous findings, the question arises whether rapidly proliferating primary cells with an intact circadian clock might display reduced expression of the *Cry* genes. In line with the literature (Xu et al., 1998), primary *p53*-deficient MEFs proliferate considerably faster than WT MEFs (Figure 3C). Luciferase imaging experiments using dermal fibroblasts from adult *p53^-/-* mice, which like the MEFs also proliferate very fast, shows that they display robust circadian oscillations (under confluent conditions, not shown). We thus examined the average *Cry1* and *Cry2* mRNA level in proliferating primary *p53^-/-* and WT MEFs by quantitative RT-PCR analysis. As we used non-clock synchronized cells, the detected expression levels of oscillating genes represent the average expression level. As shown in Figure 3D, *p53*-deficient MEFs show wild type *Cry1* and *Cry2* mRNA levels. This suggest that the *Cry* genes do not need to be downregulated in clock-proficient cells to increase proliferation.

Accelerated cell cycle progression in *Cry1^-/-|Cry2^-/-* cells is BMAL1-dependent

The CRY proteins are potent inhibitors of CLOCK/BMAL1-induced transcription activation of core clock and clock-controlled E-box genes (Griffin et al., 1999; Kume et al., 1999). Accordingly, the accelerated proliferation rate of *Cry1^-/-|Cry2^-/-* MEFs might originate from the fact that, in the absence of CRY proteins, such CLOCK/BMAL1-target genes are now constitutively switched on. We therefore asked the question whether inactivation of *Bmal1* (causing constitutive low expression of CLOCK/BMAL1expression of CLOCK/BMAL1-target genes) would slow down proliferation of *Cry1^-/-|Cry2^-/-* MEFs.

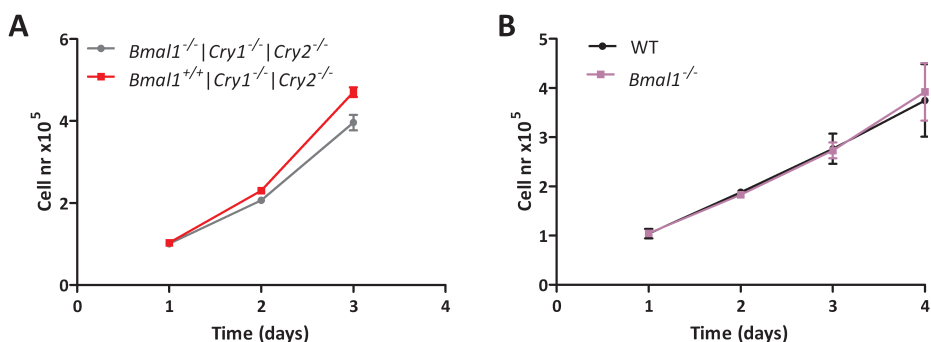**Figure 4: *Cry*-dependent increased proliferation is at least partially dependent on *Bmal1*.** **(A)** Proliferation of *Cry*-deficient MEFs with additional *Bmal1* deletion compared with littermate *Cry*-deficient MEFs without *Bmal1* deletion (n=1, analyzed in triplicate. A replicate experiment with the same cell lines at different passage gave similar results (not shown). **(B)** Proliferation of *Bmal1^-/-* MEFs is indistinguishable from WT littermate MEFs (n=2 for each genotype). MEFs from another litter gave similar results.

To this end, we isolated a panel of primary $Bmal1^{-/-}$, $Bmal1^{+/+}|Cry1^{-/-}|Cry2^{-/-}$, $Bmal1^{-/-}|Cry1^{-/-}|Cry2^{-/-}$ MEFs and determined the proliferative capacity of these cell lines. Although still preliminary, two independent experiments with one cell line indicate that the absence of $Bmal1$ reduces the proliferation rate in a $Cry1^{-/-}|Cry2^{-/-}$ background (Figure 4A). $Bmal1$ deletion alone, causing constitutively low expression of CLOCK/BMAL1-target genes, does not appear to alter the proliferation rate of MEFs (Figure 4B). Thus, as the clock-independent accelerated cell cycle progression phenotype of $Cry1^{-/-}|Cry2^{-/-}$ cells still requires $Bmal1$ expression, it likely originates from constitutive high expression of CLOCK/BMAL1-target genes.

Transcriptome analysis of WT and $Cry1^{-/-}|Cry2^{-/-}$ MEFs

In view of the observed BMAL1-dependency of the accelerated proliferation of $Cry1^{-/-}|Cry2^{-/-}$ cells, and given the finding that various cell-cycle-related genes oscillate *in vivo* and are in part affected by the *Clock* gene (Miller et al., 2007), we aimed at analyzing gene expression in WT and $Cry1^{-/-}|Cry2^{-/-}$ MEFs by transcriptome analysis. In a pilot study, we first determined by quantitative RT-PCR whether core clock genes are differentially expressed in proliferating $Cry1^{-/-}|Cry2^{-/-}$ cells, as expected on the basis of *in vivo* data, obtained with non-proliferating cells and animal tissues. Figure 5A shows a clear up-regulation of the CLOCK/BMAL1 targets *Per2* and *Dbp* in $Cry1^{-/-}|Cry2^{-/-}$ MEFs, while *Bmal1* is down-regulated, which is in full agreement with the literature (Okamura et al., 1999; Shearman et al., 2000; Baggs et al., 2009). For clarity, we wish to mention here that in the absence of CRY-mediated negative feedback, the low expression level of *Bmal1* is still sufficient to allow intermediate to high CLOCK/BMAL1-driven expression of E-box containing clock(-controlled) genes (Shearman et al., 2000). This includes *Rev-erbα*, up-regulation of which in $Cry1^{-/-}|Cry2^{-/-}$ MEFs leads to downregulation of *Bmal1* (Preitner et al., 2002), and other ROR-driven genes. In addition, we measured the expression of a few clock-controlled cell cycle genes, which have been linked to proliferation.

Except for *Wee1* (Matsuo et al., 2003), we found none of the other genes to be differentially expressed (Figure 4A) despite a clear difference for some of these genes (e.g. *p21* is strongly downregulated and *Pten* more mildly) in livers of *Cry*-deficient mice (our unpublished results). *c-Myc* has been found to be upregulated in *Per2*-deficient livers (Fu et al., 2002). Even though the CRY proteins function together with the PER proteins to inhibit CLOCK/BMAL1, we found no difference in *c-Myc* levels in $Cry1^{-/-}|Cry2^{-/-}$ MEFs (Figure 4A) or liver (unpublished), suggesting that PER2 regulates gene expression independently from the clock. Since the WEE1 kinase is an inhibitor of G2/M transition and thus has a negative effect on cell cycle progression, it is unlikely to explain the phenotype of the *Cry*-deficient MEFs.

To gain a comprehensive view of gene expression alterations in proliferating $Cry1^{-/-}|Cry2^{-/-}$ MEFs (as compared to WT MEFs), we performed an mouse full genome microarray study. We found ~3000 out of ~45.000 probe sets to display significantly changed expression levels between WT and MEFs ($p \leq 0.05$ and a ≥ 1.2 fold change up- or down-regulated). The top 50 probe sets up- or downregulated in $Cry1^{-/-}|Cry2^{-/-}$ MEFs are listed in Table 1 and 2, respectively. Metacore pathway analysis of the whole data set revealed that various cell-cycle-related processes were among the top deregulated pathways (Figure 5B). However, most of the genes that belong to these pathways show only mild expression changes (~1.5 to 2-fold), suggesting that altered expression of a larger group of genes is most likely responsible for the increased proliferation in *Cry*-deficient MEFs.
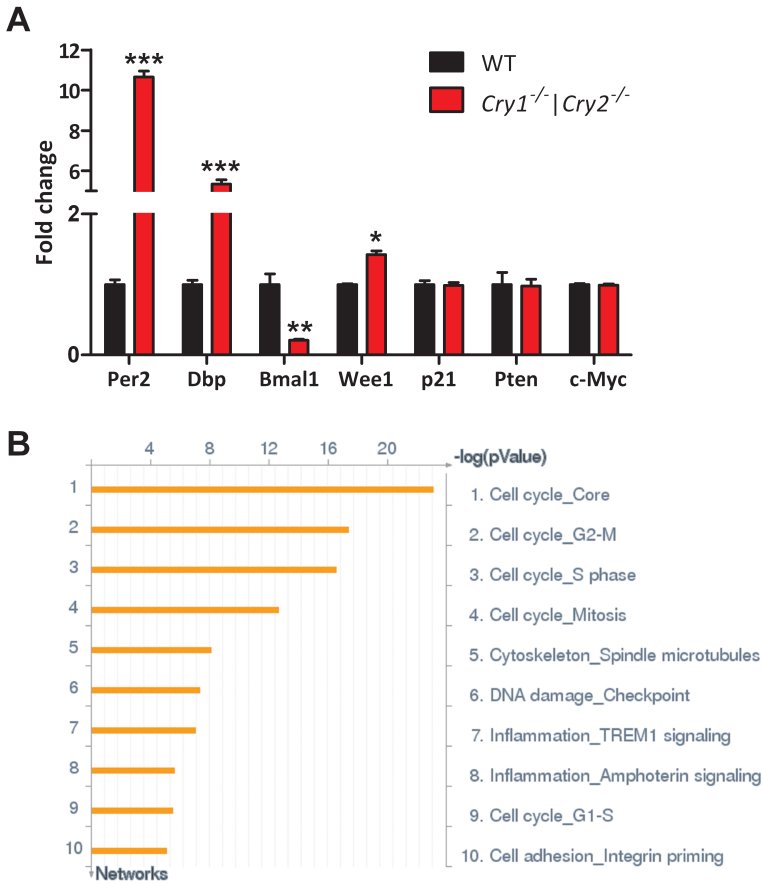
A

B

Figure 5: Gene expression analysis of Cry-deficient MEFs. (A) qPCR analysis of clock genes and cell-cycle-related genes in asynchronous *Cry*-deficient MEFs. WT (n=4) and *Cry1-/-|Cry2-/-* (n=3) were seeded in triplicate for RNA collection and were measured for proliferation in parallel. Student's t-test was used to assess significance (*p<0.05, **p<0.01, ***p<0.001). **(B)** List of top 10 networks based on GeneGo Metacore analysis of all genes that showed at least 1.2 fold up or down regulation (at p<0.05) in *Cry*-deficient MEFs (n=4) compared to WT MEFs (n=4). Cells collected for RNA isolation were analyzed in parallel for proliferation to ensure that the used cell lines behaved as expected

Another interesting aspect revealed by the microarray analysis, is the finding that various genes involved in inflammation and chemokine signalling show altered expression in the *Cry*-deficient MEFs. Some of these genes have recently been implicated in oncogene-induced senescence (OIS, Kuilman and Peeper, 2009). Since *Bmal1* has recently been found to play a role in p53-dependent senescence in human fibroblasts through an as-of-yet unidentified mechanism (Mullenders et al., 2009), these genes may link clock-controlled gene expression to OIS and tumorigenesis.

DISCUSSION

Over the last decade, it has become evident that cell proliferation, as well as the DNA damage response (DDR) that allows the cell cycle to halt in order to clear the genome from DNA lesions, are linked to the circadian system. The circadian clock is thought to gate cell division and to restrict DNA replication to a defined moment of the day and has been show to influence the adverse effects of genotoxic compounds (chronotoxicity). These notions stem mainly from *in vivo* observations and accordingly do not discriminate between a cell-autonomous and/or systemic contribution of the clock to these processes. In the present study, we have used primary, arrhythmic *Cry1^-/-^ Cry2^-/-* MEFs, cultured at physiological O_2-concentration to test whether the cell-autonomous clock contributes to the cell cycle and DDR.

The circadian clock and the DNA damage response

When exposing *Cry1^-/-^ Cry2^-/-* MEFs to several genotoxic challengers (i.e. UV light, ionizing radiation, atmospheric oxygen), cells turned out to be not more sensitive to genotoxic stress than wild type cells. This is consistent with a previous report on the genotoxic sensitivity of *Cry1^-/-^ Cry2^-/-* cells; although the cells in that study spontaneously immortalized (Gauger and Sancar, 2005).

The wild type genotoxic stress sensitivity of primary *Cry1^-/-^ Cry2^-/-* MEFs markedly contrasts a study in which *Cry1^-/-^ Cry2^-/-* mice were shown to be more resistant to the genotoxic agent cyclophosphamide due to increased resistance of B-cells in the bone marrow (representing the main target of cyclophosphamide) (Gorbacheva et al., 2005). Although we currently can not exclude that this discrepancy results from cell type-specific differences, a more attractive hypothesis would be that the circadian clock *in vivo* controls the DDR through a systemic mechanism. One recent example of the circadian clock regulating a physiological process in the periphery is the circadian release of stem cells from the bone marrow (Mendez-Ferrer et al., 2008). To test this hypothesis, one could for instance transplant *Cry1^-/-^ Cry2^-/-* B-cells to wild type mice and expose animals to cyclophosphamide. If systemic effects are involved, *Cry*-deficient B-cells should no longer be cyclophosphamide-sensitive in a clock proficient mouse. Oppositely, wild type B-cells could gain cyclophosphamide sensitivity when transplanted to *Cry* double knock-out mice.

The circadian clock and cell cycle progression

Interestingly, we observed that primary *Cry1^-/-^ Cry2^-/-* MEFs proliferate faster than their wild type counterparts under unstressed conditions, Given the normal cell cycle phase distribution of proliferating *Cry1^-/-^ Cry2^-/-* MEFs cultures, accelerated growth likely involves shortening of both the G_1- and G_2-phase. This finding contrasts a previous report in which dermal fibroblasts from *Cry1^-/-^ Cry2^-/-* mice were shown to proliferate at the same rate as WT cells (Gauger et al., 2005). This discrepancy may originate from the fact that the cells in that study were spontaneously immortalized and grown at 20% oxygen (keeping the cell under chronic oxidative stress) oxidative, whereas we used primary cells at near physiological oxygen levels.

Recent *in vitro* experiments with other circadian clock models also uncovered cell cycle related phenotypes. Primary MEFs isolated from *Clock* mutant mice and primary hepatocytes from *Bmal1^-/-^* mice were both suggested to proliferate at a reduced rate (Miller et al., 2007; Gréchez-Cassiau et al., 2008). Given that CLOCK and BMAL1 activate

transcription of E-box clock (controlled) genes and, oppositely, CRY proteins inhibit CLOCK/BMAL1-mediated transcription, this finding would fit nicely with our observation that $Cry1^{-/-}|Cry2^{-/-}$ cells show accelerated proliferation. However, although we did not analyze $Clock$ mutant MEFs, $Bmal1^{-/-}$ MEFs fail to show a reduced proliferation rate, which again may be attributed to differences in cell types or culture conditions. On the other hand, the data obtained with $Bmal1^{-/-}$ hepatocytes, can also be explained by assuming that it is not the proliferation rate that is altered, but rather the cell cycle re-entry of the hepatocytes, which may have been in a quiescent state in the mouse before isolation. Consistent with this interpretation, the decreased proliferation rate of $Clock$ mutant MEFs is only observed under conditions where cells in a quiescent state are induced to re-enter the cell cycle (Miller et al., 2007).

The accelerated proliferation rate of $Cry1^{-/-}|Cry2^{-/-}$ cells is not originating from the inactivation of the circadian clock in these cells. Rather it appears determined by the level of Cry gene expression, as clock-proficient ($Cry1^{+/-}|Cry2^{-/-}$) and clock-impaired ($Cry1^{-/-}|Cry2^{+/-}$) MEFs, carrying 1 active Cry gene, both proliferate more rapidly than WT cells, though not as fast as the double knockout cells. To confirm whether Cry-genes dose-dependently affect the proliferation rate, future studies should include additional genotypes with different doses of Cry alleles. The immediate consequence of our finding that it is not the absence of circadian oscillations, and by inference circadian gating, that is causing the increase in proliferation. Although our results can not exclude that there is gating in WT cells, a very recent report shows that there is no correlation between cell cycle division and circadian phase in cultured immortalized rat fibroblasts expressing a luciferase reporter for the cell cycle or the circadian clock (Yeom et al., 2010).

By deleting $Bmal1$ in a $Cry1^{-/-}|Cry2^{-/-}$ background, we found that the action of the Cry genes is at least in part dependent on the presence of $Bmal1$. Since we do not find a reduction in the proliferation rate of $Bmal1^{-/-}$ MEFs compared to WT MEFs, this suggests that it is the constitutively high expression of CLOCK/BMAL1-target gens that is driving. Since it seems that gene expression changes in circadian genes are less profound in asynchronous, cultured MEFs than they are $in vivo$ (e.g. in the liver), it seems likely that it is the concerted action of a larger number of genes, each with a small change in expression, that underlie the alteration in proliferation in the $Cry1^{-/-}|Cry2^{-/-}$ MEFs . Consistent with this, we found widespread deregulation of cell cycle related genes in the $Cry1^{-/-}|Cry2^{-/-}$ MEFs. Another interesting aspect to emerge from our microarray study is the deregulation of various inflammatory and chemokine signaling genes. These pathways have recently been linked to senescence (reviewed in Kuilman et al., 2009) and may thus shed some light on how BMAL1 plays a role in $p53$-dependent oncogene-induced senescence in human fibroblasts (Mullenders et al., 2009) and $Bmal1^{-/-}$ MEFs (our unpublished results).

Another player that might explain the phenotype of the $Cry1^{-/-}|Cry2^{-/-}$ MEFs is PER2, which is known to be unstable in the absence of the CRY proteins (Shearman et al., 2000; Yagita et al., 2002) and has been linked to increased proliferation of primary osteoblasts when deleted together with $Per1$ (Fu et al., 2005).

Seminal experiments from the Okamura lab revealed that the regeneration of the liver after partial hepatectomy is gated by the circadian clock and that this gating was absent in $Cry1^{-/-}|Cry2^{-/-}$ mice (Matsuo et al., 2003). Furthermore, they found that the proliferation of $Cry1^{-/-}|Cry2^{-/-}$ hepatocytes was slower (Matsuo et al., 2003). As elegant as these experiments are, they do not distinguish between systemic and cell-

autonomous control. In this context it is interesting to note that findings from several studies demonstrate that liver regeneration is affected by systemic signals (reviewed in Michalopoulos and DeFrances, 1997). Thus, our hypothesis that the circadian system controls cell cycle through systemic cues, could also explain why hepatocytes of *Cry*-deficient mice proliferate slower after partial hepatectomy *in vivo* (Matsuo et al., 2003) instead of proliferating faster like the *Cry*-deficient fibroblasts *in vitro*, as we demonstrate here.

In conclusion, our results provide a new view of how the circadian system is connected to the cell cycle and DDR and provide avenues for further investigation.

METHODS

Mice

Cry1 and *Cry2* knockout mice have been described before (van der Horst et al 1999) and were backcrossed over 6 times with C57BL/6J mice. *Bmal1* knockout mice in a C57BL/6J background were generated in the Bradfield lab (Bunger et al., 2000) and provided by Prof. Franck Delaunay. Mice lacking *p53* (Donehower et al., 1992) were obtained from The Jackson Laboratory. All animal experiments were evaluated and approved by the National Committee for Genetic Identification of Organisms and the Animal Ethics Committee and were conducted according to national and international guidelines.

Cells culture

Primary wildtype (WT), $Cry1^{-/-}|Cry2^{-/-}$, $Cry1^{+/-}|Cry2^{-/-}$, $Cry1^{-/-}|Cry2^{+/-}$, $Bmal1^{-/-}$, $Bmal1^{+/+}|Cry1^{-/-}|Cry2^{-/-}$, and $Bmal1^{-/-}|Cry1^{-/-}|Cry2^{-/-}$, and $p53^{-/-}$ mouse embryonic fibroblasts (MEFs) were isolated from 13.5E embryos and routinely cultured in DMEM/F10 (1:1) medium, containing 10% fetal calf serum and antibiotics, in a mixed gas incubator (5% CO_2 and 3% O_2) at 37°C as described before (van de Ven et al., 2006). Primary $p53^{-/-}$ MEFs have been previously generated in our lab and were kindly provided by Dr. Jay Mitchell.

Genotoxic treatment of cultured MEFs

UV sensitivity was determined as described (Sijbers et al., 1996) except that the (primary) cells were plated at a higher density. Petri dish cultures were exposed to different doses of UV (254 nm, Philips TUV lamp) after removing the medium and washing the cells with PBS. Mock treated control cultures went through the same procedure, except that the UV lamp was not turned on. After 3 days, the number of proliferating cells was estimated from the amount of radioactivity incorporated during a 2 hr pulse with [^{3}H]thymidine. Cell survival was expressed as the percentage of radioactivity in exposed cells in relation to the radioactivity in mock-treated cells.

Ionizing radiation sensitivity was assayed by plating early passage cells (passage 2–5) cells in triplo in six-well plates at a density of 100,000 cells. The next day, cells were either gamma-irradiated (^{137}Cs γ-radiation, doses as indicated in the text) or left untreated. Cells were counted 3 d after treatment using a Coulter Multisizer Z2 (Beckman Coulter) cell counter and plotted as the percentage of the total number of cells from the corresponding mock-treated cultures.

Oxidative stress sensitivity was determined as described for the IR treatment, except that culture dishes were transferred to a 5% CO_2/20% O_2 incubator and mock-treated control cultures were kept in the 5% CO_2/3% O_2 incubator.

Proliferation and cell cycle distribution assays

Proliferation assays were performed by counting cells using a Coulter Multisizer Z2 (Beckman Coulter) cell counter and seeded in duplicate or triplicate in 6-well plates at a density of 100.000 cells/well. Cells were trypsinized and counted each day for 3-4 days (as indicated in the graphs).

Cell cycle phase distribution was determined by propidium iodide/BrdU staining after 70% ethanol fixation and cell sorting (BD Facscan or a BD Facscalibur) as described (Smits et al., 2000).

Real-time bioluminescent imaging

For real-time bioluminescence monitoring of circadian core oscillator function, primary MEFs were infected with a lentivirus expressing firefly luciferase from the *Per2* promoter (Oklejewicz et al., 2008). After synchronization of confluent cultures with forskolin (Sigma; dissolved in 100 % ethanol, added to the culture medium at a final concentration of 10 μM) or horse serum (50 %, 1 hour incubation), bioluminescence was recorded for 7 days (75 sec measurements at 10 min intervals) using a LumiCycle 32-channel automated luminometer (Actimetrics) placed in a dry, temperature-controlled incubator at 37 °C as described before (Oklejewicz et al., 2008).

Quantitative real time PCR analysis

Total RNA was isolated from cells (cultured under the same conditions as used for the proliferation assays) in triplicate for each cell line (n=3-4 per genotype) using Trizol (Invitrogen) following manufacturer's instructions. For qPCR, reverse transcription was done using 1 μg total RNA with oligo(dT) and SuperScript reverse transcriptase (Invitrogen). Real-time qPCR analysis of clock and cell cycle genes was performed using SYBR green and an iCyclerIQ detection system (Bio-Rad) following the manufacturer's protocol. Primers were designed to span at least one intron and to amplify a ~100-250 bp region. Specificity was determined by melt-curve analysis and efficiency was determined by testing the primers with mixed, serially diluted cDNA. Expression levels were normalized using *Hypoxanthine phosphoribosyltransferase 1* (*Hprt1*) mRNA levels and plotted relative to WT cells (set at 1). The generation of specific PCR products was confirmed by melting curve analysis and each primer pair was tested with a serial dilution of a cDNA mix which was used to calculate the primer pair efficiency.

Transcriptome analysis

Total RNA was isolated as described for quantitative RT-PRC analysis and confirmed to be of good quality by Agilent Bioanalyzer analysis and qRT-PCR for selected clock genes. After qPCR analysis, the triplicate samples for each cell (n=4 per genotype) were pooled, further processed (cDNA synthesis and labeling) and hybridized hybridized to full mouse genome oligonucleotidearrays (Affymetrix 430 V2.0) as described in detail before (Niedernhofer et al., 2006). Significantly differentially expressed genes (p<0.05 and fold-change >1.2 up- or down-regulated) were subjected to GeneGo Metacore analysis to identifiy significantly overrepresented processes..

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CHAPTER 5

Phase Resetting of the Mammalian Circadian Clock by DNA Damage

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SUMMARY

To anticipate the momentum of the day, most organisms have developed an internal clock that drives circadian rhythms in metabolism, physiology, and behavior (Ko and Takahashi, 2006). Recent studies indicate that cell-cycle progression and DNA-damage-response pathways are under circadian control (Chen-Goodspeed and Cheng Chi Lee, 2007; Collis and Boulton; Kondratov and Antoch, 2007). Because circadian output processes can feed back into the clock, we investigated whether DNA damage affects the mammalian circadian clock. By using Rat-1 fibroblasts expressing an mPer2 promoter-driven luciferase reporter, we show that ionizing radiation exclusively phase advances circadian rhythms in a dose- and time-dependent manner. Notably, this *in vitro* finding translates to the living animal, because ionizing radiation also phase advanced behavioral rhythms in mice. The underlying mechanism involves ATM-mediated damage signaling as radiation-induced phase shifting was suppressed in fibroblasts from cancer-predisposed ataxia telangiectasia and Nijmegen breakage syndrome patients. Ionizing radiation-induced phase shifting depends on neither upregulation nor downregulation of clock gene expression nor on de novo protein synthesis and, thus, differs mechanistically from dexamethasone- and forskolin-provoked clock resetting (Izumo et al., 2006). Interestingly, ultraviolet light and tert-butyl hydroperoxide also elicited a phase-advancing effect. Taken together, our data provide evidence that the mammalian circadian clock, like that of the lower eukaryote Neurospora (Pregueiro et al., 2006), responds to DNA damage and suggest that clock resetting is a universal property of DNA damage.

RESULTS AND DISCUSSION

Ionizing Radiation Phase Advances the Circadian Clock

To study the effect of DNA damage on the circadian system, we used Rat-1 fibroblasts stably expressing an *mPer2* promoter-driven luciferase reporter gene, (Rat-1 *mPer2:luc* cells (Nishii et al., 2006)). The *mPer2* promoter is activated by the CLOCK/BMAL1 heterodimer and repressed by the activity of the PER/CRY complex and allows real-time monitoring of circadian oscillations (Ko and Takahashi, 2006). Confluent Rat-1 *mPer2:luc* cells (arrested in the G0/G1 phase of the cell cycle; see Figure 1) were first treated with forskolin (30 μM) to synchronize clock gene expression between individual cells.
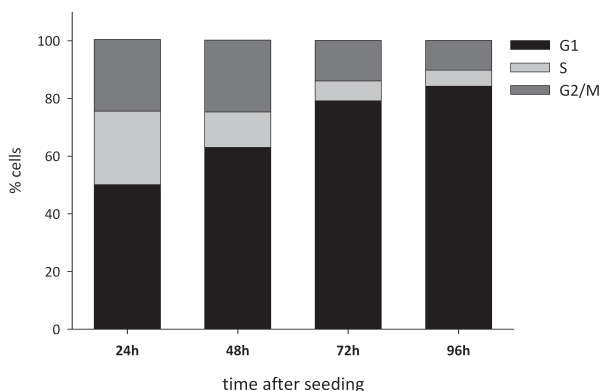

Figure 1. Ionizing radiation phase advances circadian rhythms in fibroblasts

Representative examples of luminescence rhythms in confluent Rat-1 *mPer2:luc* cell cultures, either exposed to a single pulse of γ-radiation (red lines) at a dose of 1 Gy (upper panel) or 10 Gy (middle panel) or mock-treated (black lines) 30 hours after forskolin synchronization. Lower panel presents example of confluent Rat-1 *mPer2:luc* cell cultures exposed to 10 Gy of γ-radiation 40 hours after forskolin synchronization.

Figure 2. Ionizing radiation phase advances circadian rhythms in fibroblasts

(A) Representative examples of luminescence rhythms in confluent Rat-1 *mPer2:luc* cell cultures, either exposed to a single pulse of γ-radiation (gray lines) at a dose of 1 Gy (upper panel) or 10 Gy (middle panel) or mock-treated (black lines) 30 hours after forskolin synchronization. Lower panel presents example of confluent Rat-1 *mPer2:luc* cell cultures exposed to 10 Gy of γ-radiation 40 hours after forskolin synchronization.
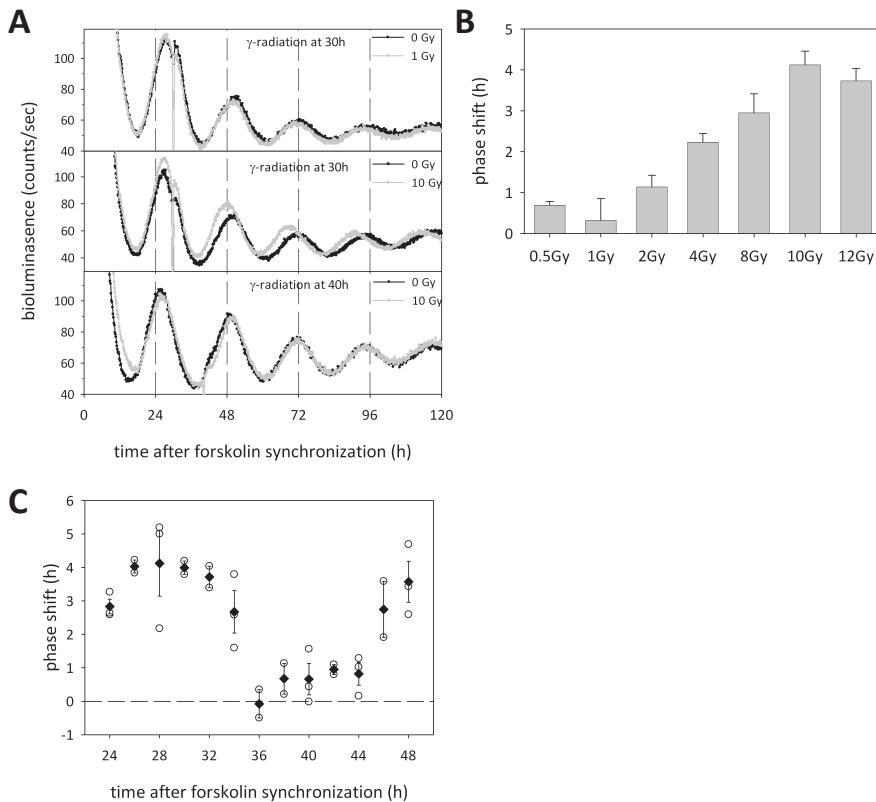
(B) Dose dependency of the γ-radiation induced phase advance of luminescence rhythms in Rat-1 *mPer2:luc* cell cultures. Each bar represents the mean of three independent experiments. Error bars represent the s.e.m.

(C) Phase response curve of ionizing radiation induced phase shifts. Forskolin-synchronized Rat-1 *mPer2:luc* cell cultures were exposed to 10 Gy of γ-radiation at different phases of the second circadian cycle. Individual and averaged data are represented by open and closed symbols respectively. Error bars represent the s.e.m

When applied 30 hr after synchronization, a single dose of gamma (γ)-radiation elicited a clear phase advance of bioluminescence rhythms in a dose-dependent manner (Figure 2A), with a maximum shift of about 4 hr being reached at doses of 10 Gy and higher (Figure 2B). At this dose, we did not observe significant cell death, as further illustrated by the comparable levels of bioluminescence signals in irradiated and mock-treated cells (Figure 2A). Because the phase of circadian oscillations hardly changed when cells were γ-irradiated 40 hr after synchronization (Figure 2A, bottom), we next examined whether the magnitude and direction (i.e., advance versus delay) of ionizing radiation-induced phase shifts were dependent on the phase of the circadian clock. As evident from the phase response curve (PRC), obtained by exposing Rat-1 *mPer2:luc* cells to 10 Gy of γ-radiation at various phases of the circadian cycle (Figure 2C), a maximal phase advance was elicited between 26 and 32 hr after forskolin synchronization. This shift was intermediate at 34 hr and

negligible between 36 and 44 hr after synchronization. At 48 hr, cells again displayed an intermediate phase advance, similar to that observed 24 hr earlier. Interestingly, the PRC for ionizing radiation exclusively shows phase advances. This finding markedly contrasts the reported Rat-1 cell PRCs for forskolin and dexamethasone, which exhibit both phase advances and delays (Izumo et al., 2006). The lack of phase delays in the PRC for ionizing radiation is not due to an unforeseen artifact in our batch of Rat-1 *mPer2:luc* cells because forskolin instigated a phase delay 32 hr after synchronization of cells with horse serum (Figure 3). Interestingly, forskolin and dexamethasone PRCs (Izumo et al., 2006) provoke phase delays at the time when ionizing radiation produced maximum phase advances (Figure 2C).
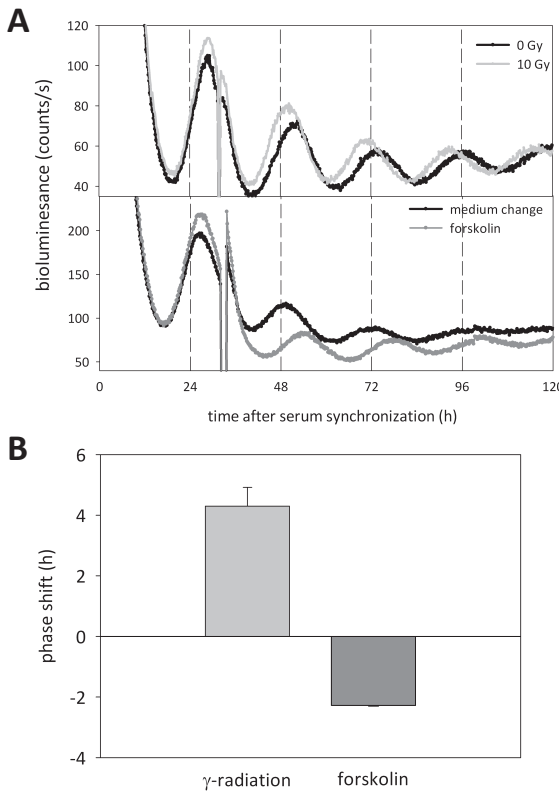

Figure 3. Phase shift responses of Rat-1 *mPer2:luc* cells to γ-radiation and forskolin.

(A) Representative examples of luminescence rhythms in confluent Rat-1 *mPer2:luc* cell cultures, synchronized with 50% horse serum at time point 0 hr, and 32 hours later either exposed to 10 Gy of γ-radiation (upper panel, gray line) or 10 μM forskolin (lower panel, dark gray line). Mock treated cells (black lines) were used as a control. **(B)** Quantative analysis of the direction and magnitude of phase shifts in γ-radiation and forskolin treated cells (as compared to mock-treated cells). Each bar represents the average of two independent experiments. Error bars represent the s.e.m

Having shown that γ-radiation can phase advance peripheral oscillators *in vitro*, we next investigated its impact on the master circadian clock in the suprachiasmatic nuclei (SCN), as visualized by voluntary wheel-running-behavior recordings. Free-running C57BL/6J male mice were subjected to a nonlethal dose of γ-radiation (4 Gy) given at circadian time (CT) 6 or 22. This choice is based on behavioral PRCs for nonphotic stimuli, showing maximum phase advances and delays at CT6 (middle of the subjective day) and CT22 (end of the subjective night), respectively (Daan and Pittendrigh, 1976; Hastings et al., 1998; Rosenwasser and Dwyer, 2001). Exposure to γ-radiation at CT6 significantly phase advanced locomotor activity rhythms by 1.2 ± 0.2 hr (as compared to the −0.6 ± 0.2 hr

shift elicited by sham-treatment; p < 0.001; Figure 4). In contrast, radiation of mice at CT22 did not produce a significant response (0.3 ± 0.3 hr and −0.4 ± 0.3 hr in exposed and sham-treated mice, respectively; p = 0.08).
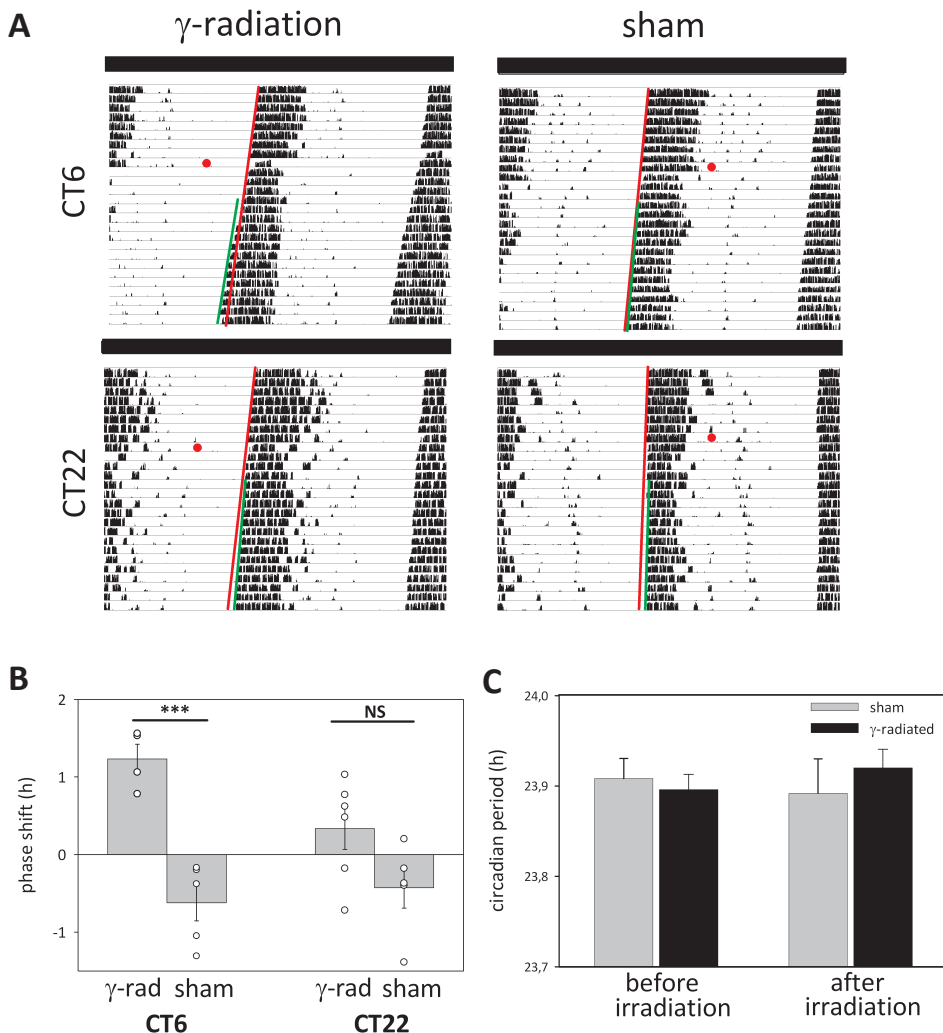

Figure 4. Ionizing radiation can phase advance behavioral rhythms in free-running mice
(A) Representative examples of double-plotted activity records of C57BL/6J male mice, γ-radiated (left panel) or sham-treated (right panel) at CT6 (upper panels) and CT22 (lower panels). Red dots indicate moment of treatment. Lines through the onsets of activity indicate the phase of rhythmicity before (red line) and after treatment (green line).
(B) Quantitative representation of ionizing radiation induced phase shifts in ionizing radiation exposed or sham-treated C57BL/6J mice. Individual data points and mean values are represented by open dots and grey bars, respectively. Error bars represent the s.e.m. ;*** - p<0.001, NS - not significant.
(C) Periodicity of voluntary wheel running behavior was determined before and after treatment. As the time of (sham) treatment (CT6 vs CT22) did not significantly affect the periodicity determined for the four groups (i.e. before sham treatment, after sham treatment, before γ-radiation, after γ-radiation) As the circadian period determined for the four groups (i.e. before sham treatment, after sham treatment, before γ-radiation, after γ-radiation) did not significantly differ between CT6 and CT22 (p values >0.7), data were pooled. Neither ionizing radiation, nor sham-treatment significantly affected circadian period (p=0.9; ANOVA).

Importantly, neither the period of circadian rhythms nor the overall wheel-running activity per day was affected by ionizing radiation (Figure 4C), excluding that the observed effect was influenced by changes in core oscillator performance. This animal study shows that the phase-advancing properties of ionizing radiation are not limited to peripheral oscillators but extend to the master clock in the SCN. In line with the notion that the mechanism and molecular makeup of the circadian clock in cultured cells and the SCN are alike (Balsalobre et al., 1998; Yagita et al., 2001), ionizing radiation exerted its effect at the same circadian phase (i.e., when the CLOCK/BMAL1 complex is engaged in transcription activation of E-boxes containing genes like *Per2*) (Bae et al., 2001).

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

DNA double-strand breaks (DSBs) form the primary type of DNA lesion introduced by ionizing radiation and trigger DNA damage signaling pathways mainly through members of the phosphatidylinositol 3-kinase-related kinase (PIKK) family of protein kinases, notably ATM (ataxia telangiectasia mutated) and ATR (ATM and Rad3 related) (Hurley and Bunz, 2007). These kinases initiate the signaling cascade by phosphorylating a wide spectrum of cell-cycle regulators and DNA-repair proteins (Matsuoka et al., 2007; Kim et al., 1999). Treatment of synchronized Rat-1 *mPer2:luc* cell cultures with the ATM-specific inhibitor Ku-55933 (Hickson et al., 2004) reduced the γ-radiation-induced phase advance in a dose-dependent manner, whereas mock treatment did not have any effect (Figure 5A, right). Similarly, the nonselective ATM/ATR inhibitor caffeine (Hickson et al., 2004; Sarkaria et al., 1999) caused a dose-dependent reduction of the ionizing radiation-induced phase advance (Figure 5A, left). These findings strongly suggest that ATM is a mediator of the clock-resetting properties of ionizing radiation and point to DNA damage as the ultimate trigger. Nonetheless, as caffeine inhibits ATM and ATR at reported IC50 levels (half inhibitory dose) of 0.2 mM and 1.1 mM, respectively (Sarkaria et al., 1999; Blasina et al., 1999), the magnitude of the radiation-induced phase advance at 1.1 mM caffeine (>5 × IC50 for ATM) suggests that other kinases (i.e., ATR) also may contribute to the response.

To further evaluate the involvement of ATM in ionizing radiation-mediated resetting of the circadian clock, we extended our analysis to ATM- and NBS1-deficient human primary dermal fibroblasts (HDFs) obtained from patients with the ionizing radiation-sensitive, cancer-predisposing disorder ataxia telangiectasia (AT) and Nijmegen breakage syndrome (NBS), respectively (reviewed in Chun and Gatti). NBS1 (also termed nibrin) is a component of the MRN complex (MRE11-RAD50-NBS1), which recruits ATM to the proximity of DSBs and activates the latter protein (Zhou et al., 2006). To visualize circadian clock performance, we first stably infected control (n = 4), ATM-deficient (n = 3), and NBS1-deficient (n = 3) HDFs with a lentiviral *mPer2:luc* reporter construct. Interestingly, forskolin-synchronized patient cell lines were oscillating with a circadian period shorter than that of control cell lines (24.4 ± 0.3 hr and 25.8 ± 0.4 hr, respectively, p = 0.01; Figure 5B). Importantly, however, all ATM- and NBS1-deficient cells were moderately to severely impaired in their phase-advancing response upon ionizing radiation exposure, whereas rhythms shifted as expected in control cells (p < 0.001; Figures 5B and 5C). These results provide definite evidence that ATM (directly or indirectly) communicates DNA damage information to the core clock machinery. Given the notion that ATM-mediated damage-signaling pathways are active in neuronal tissues (Dar et al., 2006), which likely includes the SCN, and given the shorter circadian periodicity of the patient

lines (final proof requiring analysis of larger numbers of cell lines), it would be of interest to investigate the chronotypes of AT and NBS patients.
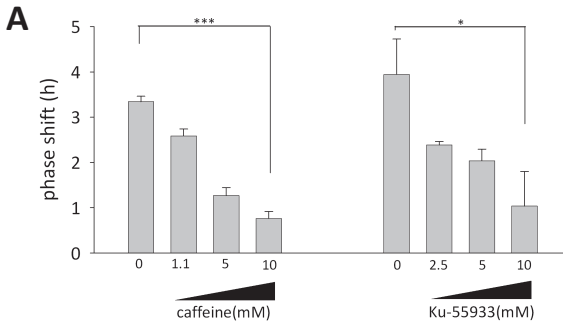

Figure 5. ATM/ATR kinases are involved in ionizing radiation induced phase advances

(A) Caffeine (left panel) and Ku-55933 dose-dependently inhibit ionizing radiation-induced phase advances in forskolin-synchronized confluent Rat-1 *mPer2:luc* cell cultures. Up to one hour prior to γ-radiation (10 Gy) or mock-treatment (administered 28 hour after synchronization), cells were exposed to caffeine or Ku-55933 at the indicated dose. Bars represent the average of three independent experiments. Error bars indicate the s.e.m.
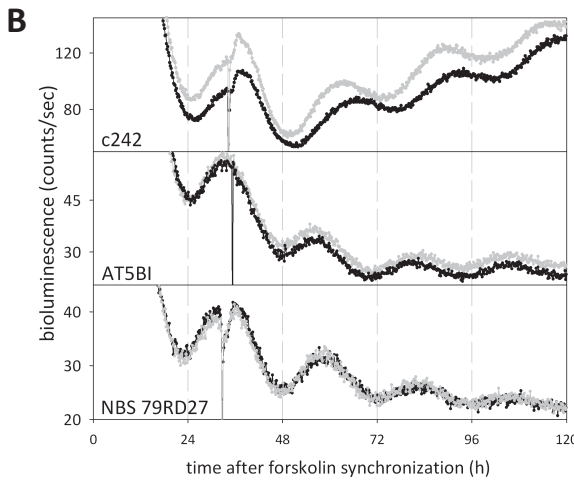
(B) Representative examples of bioluminescence rhythms in human primary dermal fibroblasts from control subjects and AT, and NBS patients exposed to γ-radiation (5 Gy, gray lines) or mock-treated (black lines) approximately 34h after initial synchronization with forskolin.

(C) Quantative analysis of the magnitude of ionizing radiation-induced phase advances in human control, AT, and NBS primary dermal fibroblasts. On right the overall mean values are shown. Error bars represent the s.e.m.
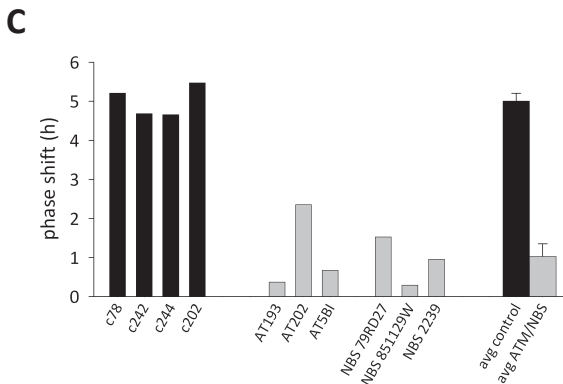
Induction of Clock Gene Expression Is Not Required for Ionizing Radiation-Mediated Resetting

The mechanism of clock resetting in the SCN involves early induction (after photic stimuli) or repression (after nonphotic stimuli) of *Per* gene expression (Miyake et al., 2000; Maywood et al., 1999; Hamada et al., 2004). Because the molecular mechanism of clock resetting in cultured cells by either chemical synchronizers or ionizing radiation is not documented, we next analyzed clock mRNA levels in oscillating Rat-1 *mPer2:luc* cells exposed to either dexamethasone or γ-radiation 30 hr after synchronization. Remarkably, whereas dexamethasone strongly stimulated transcription of *Per1* and *Per2* genes up to 7-fold (peaking 2–4 hr after the pulse), ionizing radiation neither up- nor downregulated transcript levels of these genes (Figure 6). The levels of other clock gene transcripts like *Clock*, *Bmal1* (Figure 7), *Cry1*, and *Cry2* (data not shown) were unaffected by both treatments. The opposite direction of forskolin and dexamethasone versus ionizing radiation-induced phase shifts at the same circadian phase (i.e phase delay and phase advance, respectively (Izumo et al., 2006; this study), as well as the nonresponsiveness of *Per* genes upon γ-radiation, further points to differences in the underlying resetting mechanism.
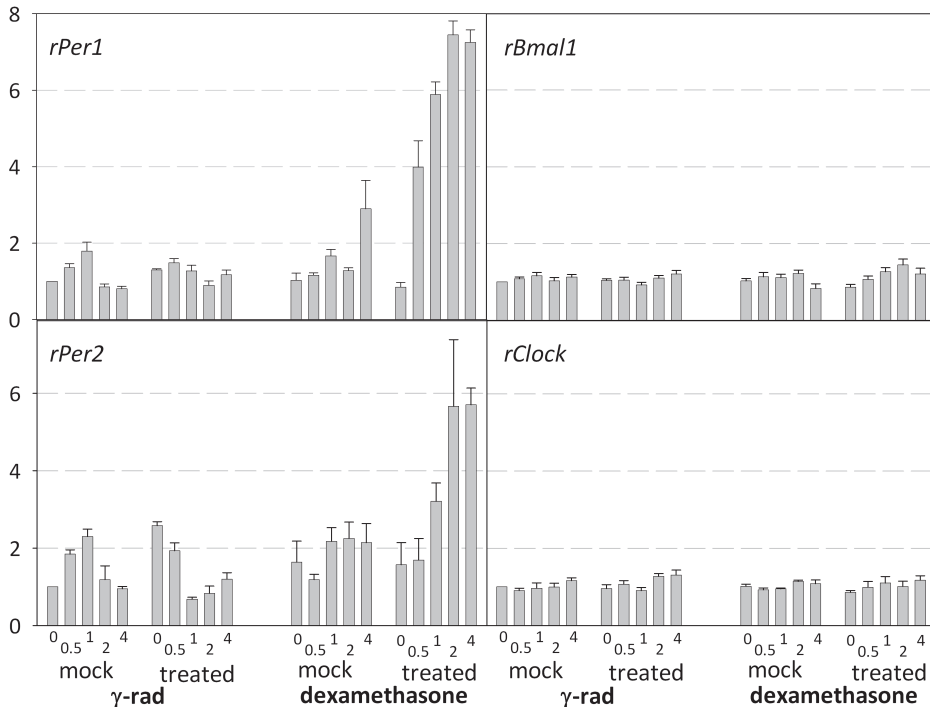

Figure 6. **Clock gene expression in ionizing radiation and dexamethasone exposed Rat-1 *mPer2:luc* cells.**
Quantitative RT-PCR analysis of *Per1, Per2, Bmal1, and Clock* expression 0, 0.5, 1, 2, and 4 hour after γ-radiation (10 Gy) or dexamethasone (100 nM) and mock treatment of forskolin-synchronized Rat-1 *mPer2:luc* cell cultures.

Additionally, to investigate whether *de novo* protein synthesis may be required for clock resetting by ionizing radiation, we treated Rat-1 *mPer2:luc* cells with cycloheximide (CHX) prior to and after γ-radiation. Inhibition of translation by CHX did not significantly prevent phase resetting (3.6 ± 0.6 and 3.0 ± 0.6 hr for solvent and CHX treatment, respectively; p > 0.05, t test), which implies the involvement of a posttranslational regulation mechanism. Noteworthy, PER1 has been identified as one of the many substrates phosphorylated by ATM/ATR after DNA damage (Matsuoka et al., 2007). Moreover, PER1 and TIM proteins are engaged in complex formation with the ATM and ATR kinases, respectively (Gery et al., 2006; Unsal-Kacmaz et al., 2007). Therefore, these clock proteins may be the primary targets for posttranslational modifications to subsequently change their abundance and activity in a fast and controllable manner.

Finally, we assessed whether clock-resetting potential is restricted to ionizing radiation-induced genotoxic stress or whether it extends to other types of DNA-damaging agents. Interestingly, ultraviolet light (crosslinking the base moiety of adjacent pyrimidines) and tert-butyl hydroperoxide (causing oxidative DNA damage), when applied to Rat-2 *mPer2:luc* cells 30 hr after forskolin synchronization, also elicited a dose-dependent, phase-advancing effect on the circadian clock (Figure 7). This suggests that clock resetting could be a universal property of DNA damage.
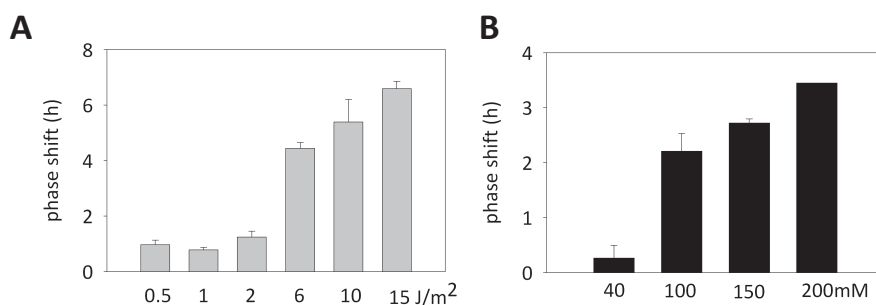

Figure 7. **UV and oxidative stress phase advance circadian rhythm in fibroblasts.**
Treatment of confluent Rat-1 *mPer2:luc* cell cultures with UV-light (panel A) or t-butyl-peroxide (panel B) 30 hours after forskolin synchronization, dose-dependently phase advances bioluminescence rhythms. Each bar represents the average of two independent experiments. Error bars represent the s.e.m.

CONCLUSIONS

In summary, whereas the mammalian circadian clock has been reported to control cell-cycle progression and DNA-damage-response pathways (Gery et al., 2006; Unsal-Kacmaz et al., 2007; Fu et al., 2002; Gorbacheva et al., 2005), the current study provides the first evidence that, conversely, DNA damage can act as a resetting cue for the mammalian circadian clock *in vitro* and *in vivo*. The underlying mechanism differs from that of known resetting agents. Thus far, the only other example of DNA damage impinging on the circadian clock is represented by the lower eukaryote Neurospora crassa, in which the radiomimetic MMS caused phase advances through a mechanism involving Check2-mediated phosphorylation of the clock protein FRQ (Pregueiro et al., 2006). In view of the hypothesis that circadian clockworks might have originated from protective mechanisms to escape from DNA/RNA damage (as imposed by diurnal exposure to ultraviolet light and ultradian respiratory metabolic cycles) that ultimately evolved in a self-sustained

oscillator (Roenneberg and Merrow, 2002; Tauber et al., 2004), the question remains open whether DNA-damage sensitivity of the circadian clock is a remnant of clock evolution or whether it is the immediate consequence (or by-product) of the close coupling between the circadian clock and cell-cycle control. Alternatively, as recently put forward by Chen and McKnight (Chen and McKnight, 2007), circadian and metabolic cycling might have coevolved with cell-cycle gating. Considering DNA-damage avoidance as the underlying unifying evolutionary driver, it was hypothesized that DNA damage might act as Zeitgeber. The present data, showing that physical and chemical genotoxicants can phase shift the clock, well support this idea. Yet, except for radiotherapeutical purposes, organisms are unlikely to be exposed to the ionizing radiation doses used in this study. It would be interesting, therefore, to investigate to what extent daily exposure to low but timed doses of genotoxicants might affect the the circadian system.

MATERIALS AND METHODS

Cells and culture conditions

Rat-1 *mPer2:luc* fibroblasts (a generous gift from Dr. K. Yagita) were grown in Dulbecco's modified Eagle (DMEM)/Ham-F10 medium (1:1) supplemented with 10% fetal calf serum and a mixture of penicillin-streptomycin antibiotics. Primary human dermal fibroblasts (HDFs) from normal subjects and patients with Ataxia telangiectasia (ATM) and Nijmegen breakage syndrome (NBS) were cultured in 100% of Ham-F10 medium supplemented with 15% fetal calf serum and antibiotics. All cells were grown at 37 ^{0}C in a humidified atmosphere with 5-10% CO_2 and 20% O_2. Determination of cell cycle profile by propidium iodide staining and cell sorting (BD Facscan or a BD Facscalibur) was performed as described (Darzynkiewicz and Juan, 2001).

Lentivirus production and cell infection

A detailed description of lentivirus technology used will be reported elsewhere (Tamanini et al., manuscript in prep.). In brief, a *mPer2* promoter fragment (spanning 1.5 Kb upstream of the first ATG) was cloned in front of the luciferase reporter gene (pGL3 Basic, Promega). The pND-Per2-luc plasmid was generated by subsequently cloning a 3.1 Kb *mPer2:luc* fragment in the Lentiviral vector pND. HEK293 cells were used to produce virus stocks (dissolved in phosphate buffered saline and stored at -80 ºC. Transduction of primary HDFs was performed by seeding cells in 12 well plates at 50% confluency and infection with 15 μl retroviral stock. All experiments involving the virus were performed under ML2 laboratory conditions.

Real-time bioluminescence monitoring

For real-time bioluminescence monitoring of circadian core oscillator function, cells were cultured in medium buffered with 25 mM HEPES and containing 0.1 mM luciferin (Sigma). After synchronization of confluent cultures with forskolin (dissolved in 100 % ethanol, added to the culture medium at a final concentration of 30 μM) or horse serum (50 %, 1 hour incubation), bioluminescence was recorded for 7 days (75 sec measurements at 10 min intervals) using a LumiCycle 32-channel automated luminometer (Actimetrics) placed in a dry, temperature-controlled incubator at 37 ^{0}C.

Raw data, collected from 2 to 144 hr after treatment, were analyzed using a curve-fitting program based on a nonlinear sine wave regression procedure where additional linear

and quadratic terms to the trend and dampening variables were added. The general sine function used was:

$y = l + mt + nt^2 + (a + bt + ct^2) \sin [2\pi ((t - \varphi)/\tau)]$, in which symbols represent: y, raw luciferase signal; t, time (hours); l, base line; m, linear baseline trend; n, parabolic baseline trend; a, amplitude; b, linear dampening; c, parabolic dampening; φ phase (hours); τ circadian period (hours).

The fitted curves generated an excellent description of the raw data, yielding highly significant fits and r^2 values >0.9. The zenith time of each oscillation cycle was used as the cycle phase marker. Because circadian period stabilized after two transient cycles after treatments, the phase shift was assessed by calculating the phase difference of the third peak in the control and the stimulated curve

Culture treatments

Ionizing radiation exposure was performed by placing culture dishes in a [137]Cs γ-radiation source at various time points after synchronization (as indicated in the text). Tert-butyl-peroxide (concentrations as indicated in the text, Sigma) was added to DMEM/F10 (1:1) medium and cells were incubated for 1 hour, after which pre-treatment medium was returned. Before UV treatment (254nm, Philips TUV lamp) culture medium was removed and cells were washed with a pre-warmed phosphate-buffered saline. The pre-treatment culture medium was return to culture dish after UV exposure.Cycloheximide (CHX, Sigma; dissolved in 100 % ethanol, used at a final concentration of 100 µg/ml), caffeine (1,3,7-trimethyl xanthine, Sigma; dissolved in phosphate-buffered saline, used at various concentrations), or Ku-55933 (KuDOS; dissolved in DMSO, used at various concentrations) treatment was performed by adding drug containing DMEM/F10 medium (1:1) and antibiotics to forskolin-synchronized cells 40-60 min prior to γ-radiation. After two hours culture dishes were washed with culture medium and pre-treatment medium was returned. In all experiments, mock-treated cells (culture dishes having been subjected to exactly the same procedures, except that γ-radiation was omitted, or in case of chemical compounds, only the solvent was added) served as an internal control.

Quantitative Real time PCR analysis

Total RNA from cultured cells was extracted using Triazol reagent (Invitrogen) as described by manufactures. First-strand cDNA was synthesized from 1 µg of total RNA using oligo (dT) primers and SuperScript reverse transcriptase (Invitrogen), according to the manufacturer's protocol. Real-time quantitative RT-PCR for determination of *Per1, Per2, Cry1, Cry2* and *Bmal1* mRNA levels was performed in duplicate with SYBR Green using an iCyclerIQ system (Bio-Rad) and primer pairs generating intron-spaning products of 150-300 bp. Expression levels were normalized using *Hprt* (hypoxanthine guanine phosphoribosyl transferase) and *ß2m* (beta-2-microglobulin) mRNA levels. The generation of specific PCR products was confirmed by melting curve analysis and each primer pair was tested with a logarithmic dilution of a cDNA mix to generate a linear standard curve, which was used to calculate the primer pair efficiency.

Animal studies

C57BL/6J mice were housed in a controlled environment (food and water ad libitum, 12h light:12 hr dark cycle) at the Animal Resource Center (Erasmus University Medical Center), which operates in compliance with the "Animal Welfare Act" of the Dutch government,

using the "Guide for the Care and Use of Laboratory Animals" as its standard. All animal studies were approved by an independent Animal Ethical Committee (Dutch equivalent of the IACUC).

For the analysis of circadian behavior C57BL/6J male mice (n=24, age 12 weeks) were individually housed in cages equipped with a running wheel (14 cm diameter). Animals were kept under 12 hr light:12 hr dark cycles for 10 days and subsequently released to constant darkness (DD). After 10 days in DD, animals (n=6 per time point) received a total body exposure to a non-lethal dose of 4 Gy of γ-radiation (^{137}Cs-source, exposure time 7.36 min) at CT6 (middle of subjective day) or CT22 (end of subjective night). Sham-treated mice (n=6 per time point) were handled in exactly the same manner, except that the shutter of the ^{137}Cs-source remained closed. All handlings were performed in total darkness, using a binocular night-vision goggles (Rigel 3200 Pro). Activity recordings were analyzed by Clock Lab software (Actimetrix). The free-running period was calculated from 7 days before radiation and 10 days after by using chi-square periodogram. The first four transition days after IR were omitted from analysis. Magnitude of steady-state phase shift was determined on a base of regression line on activity onsets before and after treatment.

Acknowledgments

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CHAPTER 6

MICRORNA-MEDIATED POSTRANSCRIPTIONAL CONTROL OF MAMMALIAN CLOCK GENE EXPRESSION REGULATES CIRCADIAN OSCILLATOR PERFORMANCE

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Chapter 6

ABSTRACT

Self-sustained circadian rhythms in behavior, physiology and metabolism are generated by a molecular oscillator composed of interlocked transcription/translation feedback loops. Added to this, post-translational mechanisms control the stability and localization of the clock proteins, mainly through phoshorylation and ubiquitylation. Recently, microRNAs have been implicated in the post-transcriptional control of circadian behavior in mice. Although in *Drosophila* one microRNA was found to affect circadian behavior through direct post-transcriptional regulation of the *clock* gene, this has not been fully elucidated for the mammalian circadian oscillator. To investigate to what extent microRNAs regulate mammalian circadian rhythms through direct regulation of clock genes, we screened 40 microRNAs for their impact on circadian oscillations in cultured cells. We found that a large fraction of the overexpressed microRNAs altered period and/or amplitude. For some of the microRNAs that we investigated further, we show that they regulate core clock gene expression. We also provide evidence indicating that endogenous microRNAs regulate the circadian oscillator through direct postranscriptional regulation of clock gene expression.

INTRODUCTION

Day-night rhythms in mammalian behavior, physiology, and metabolism are generated by a self-sustained circadian clock with a periodicity of approximately 24 h. The driving force behind circadian rhythms is a molecular oscillator, composed of a set of clock genes that act in transcription/translation feedback loops and subsequently drive rhythmic expression of a large series of clock-controlled output genes (Reppert and Weaver, 2002; Takahashi et al., 2008). The mammalian circadian system consists of a central clock in the neurons of the suprachiasmatic nucleus (SCN) in the brain, that is reset daily by light to keep synchrony with the light-dark cycle (Moore and Eichler, 1972; Stephan and Zucker, 1972). In addition, peripheral clocks are found in virtually any other cell and tissue in the body (Dibner et al., 2010). Clock gene defects (as present in laboratory-made clock mutant/knockout mice and patients with sleep phase syndromes) can accelerate, slow down, or completely abolish the circadian clock (Reppert et al., 2002; Ptáček et al., 2007). While the molecular make up of the central SCN clock is essentially the same as that of the peripheral clock in other cells, the SCN can partially compensate for mutations in clock genes through neuronal coupling of SCN neurons (Liu et al., 2007). This explains why behavioral phenotypes of mutant mice and circadian gene expression in cultured SCNs from these mice are less extreme than tissues or cells from the same mice (Yagita et al., 2001; Brown et al., 2005; Liu et al., 2007).

At its core, this molecular oscillator is generated by a negative feedback loop that comprises CLOCK/BMAL1-mediated transcriptional activation of the *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) clock genes through E-box elements in their promoters. Negative feedback is obtained through inhibition of the CLOCK/BMAL1 heterodimer by the CRY/PER complex, which is formed in the cytoplasm and then translocates to the nucleus. This core loop is interlocked with other transcription/translation feedback loops. In one such loop, ROR-driven transcription of the *Bmal1* gene is circadially repressed by REV-ERBα and REV-ERBβ, expression of which is controlled by the core loop (Preitner et al., 2002; Liu et al., 2008; Sato et al., 2004; Akashi and Takumi, 2005). These additional feedback loops, while not essential for maintaining rhythmicity, are thought to confer robustness and precision to the core negative feedback loop (Preitner et al., 2002; Tsai

et al., 2008).

An extensive and important additional layer of control over the molecular oscillator is provided through post-translational modifications (e.g. phosphorylation, ubiquitylation, acetylation) of clock proteins (Gallego and Virshup, 2007; Vanselow and Kramer, 2007). These modifications control the stability and localization of the clock proteins and thereby create a delay in the timing of nuclear entry of CRY/PER complexes, which contributes to the ~24-hour periodicity of the circadian oscillator. A recent genome-wide RNAi screen greatly expanded the number of possible clock modifying genes, as knockdown of hundreds of genes was shown to influence the periodicity and/or amplitude of circadian oscillations in cultured cells (Zhang et al., 2009).

In addition to transcriptional regulation and posttranslational modifications, control over gene expression has been shown to also occur at the posttranscriptional level. In recent years a lot of studies have uncovered roles for microRNAs in postranscriptional gene expression regulation. microRNAs are small RNA products that are encoded by introns of protein-coding genes or by their own genes. In both cases, hairpin RNAs are processed in several steps by different proteins leading to the final mature microRNA that controls gene expression post-transcriptionally by guiding the RNA Induced Silencing Complex (RISC) complex to target mRNAs. The RISC complex inhibits gene expression through translational inhibition, degradation of mRNAs or a combination of both (Bartel, 2004; Flynt and Lai, 2008). Computational predictions indicate that individual microRNAs potentially regulate hundreds of genes. This notion was recently strengthened by two reports that described hundreds of genes that were regulated at the protein level by changing the levels of individual microRNAs (Baek et al., 2008; Selbach et al., 2008). microRNAs have been implicated in a very wide range of processes (e.g. immunity, cardiovasculature, cell cycle and differentiation) (O'Connell et al., 2010; Liu and Olson, 2010; Chen et al., 2010; Mallanna and Rizzino, 2010). microRNAs have also been implicated in the circadian system. Recently, inhibition of endogenous miR-219 in the mouse SCN was shown to shorten the period of wheel-running behavior, while inhibition of miR-132 altered light-induced clock resetting (Cheng et al., 2007). Both microRNAs were suggested to alter circadian behavior by, at least in part, modulating neuronal excitability (Cheng et al., 2007). Although there is some data suggesting that microRNAs also regulate mammalian clock genes (Cheng et al., 2007; Nagel et al., 2009), it is still unknown to what extent the molecular circadian oscillator itself is modulated by microRNAs through direct regulation of clock gene expression.

To identify additional clock-modifying microRNAs, we screened 40 microRNAs (including microRNAs with predicted target sites in clock genes) for their ability to alter the circadian clock in cultured cells and found about half of the tested miRNAs to affect period and/or amplitude of the molecular oscillator

RESULTS AND DISCUSSION

Validation

Cultured cells have proven to be an invaluable model in elucidating the molecular mechanism of the circadian oscillator (Yagita et al., 2001; Zhang et al., 2009). In the present study, we set out to use the NIH/3T3 cell line (representing immortalized mouse embryonic fibroblasts) for co-transfection of the Bmal1::luciferase circadian clock reporter construct and microRNA mimics or, as positive controls, siRNAs targeting core

clock genes (*Bmal1* or *Cry2*). As expected on the basis of the circadian phenotype of *Bmal1* and *Cry2* knockout mice and mouse embryonic fibroblasts (Bunger et al., 2000; Van der Horst et al., 1999; Yagita et al., 2001), siRNA mediated silencing of these genes resulted in a strongly impaired rhythmicity and lengthening of the period, respectively (Figure 1A). Thus, transiently transfected NIH3T3 cells can be used as a test platform to determine the role of micorRNAs in circadian clock performance.

To make an initial assessment of whether microRNAs play a prominent role in the circadian oscillator, we globally reduced the expression or activity of all endogenous microRNAs by siRNA-mediated silencing of *Dicer1* or *Ago2*, important for microRNA processing and activity, respectively (Bartel, 2004; Liu et al., 2008). Compared to cells transfected with the control *siGfp* (25.35 h +/- 0.07 h), knockdown of *Dicer1* and *Ago2* significantly lengthened the period with 0.65 h ((26.0 h +/- 0.0 h, p=0.005) and 0.48 h (25.73 h +/- 0.09 h, p=0.05), respectively (Figure 1B). Taken together, these results strongly suggest that endogenous microRNAs regulate the circadian oscillator and that the net effect of inhibiting microRNAs globally is an increase in the period length of oscillations.
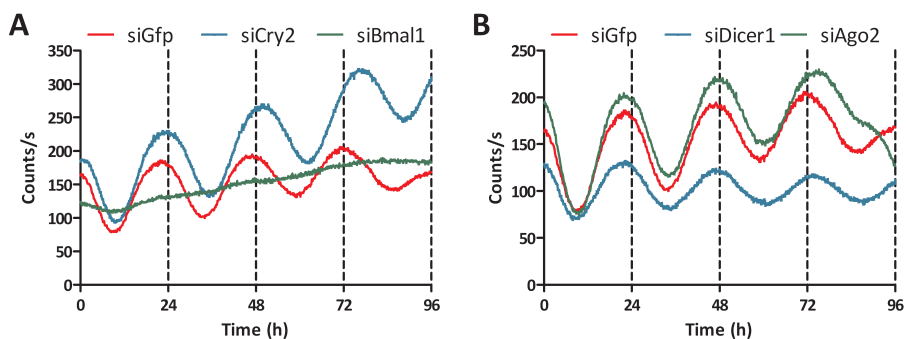

Figure 1: Validation of screen (A) Representative example of a siRNA/luciferase reporter cotransfection experiment in NIH/3T3 cells. Cells were transfected with a Bmal1::luciferase reporter and the indicated siRNAs. An siRNA targeting *gfp* was used as a negative control. Forty-eight hours after cells were synchronized using Forskolin. The first 12h are omitted from the graph for clarity. **(B)** To globally inhibit the microRNA pathway, cells were transfected with siRNAs targeting a key component of the microRNA processing machinery (*Dicer1*) or a gene involved in microRNA-mediated silencing (*Ago2*). Cells were treated as in (A).

Screening

We co-transfected NIH/3T3 cells with 40 individual microRNA mimics (small double-stranded RNA oligos with chemical modifications that mimic endogenous microRNA pre-cursors) and the Bmal1::luciferase reporter to test their effect on circadian oscillations. Biological endpoints determined included the period (Tau) and robustness (as determined by the goodness of fit; GOF) of luciferase oscillations (for a more detailed description of the criteria used, see the *Methods* section). As a control for the effect of the transfection procedure, we also co-transfected the cells with a non-targeting microRNA (miR-con). The raw data of this screen, which was performed 3 times, is shown in Figure 2. At a p-value of <0.01 and a GOF above 80%, we found 13 microRNAs to alter the period of luciferase rhythms (Figures 2 and 3A and table 1). Furthermore, we identified microRNAs that

affected the robustness of luciferase rhythms (Fig. 3B). Ten microRNAs severely or even completely abolished oscillations (GOF below 80 %). Given that microRNAs potentially target hundreds of genes, it may be that in some cases the very poor oscillations elicited by microRNA transfection are due to interference with important pathways and affect the viability/health of the cells. However, as evident from the luciferase expression level in most of the non-oscillating microRNA-transfected cells (comparable to, or sometimes even higher than those detected in control transfected cells), the loss of circadian clocks is not likely due to cell death. Next, we embarked on investigating in more detail the mode of action of some of the identified candidate clock-modifying microRNAs.
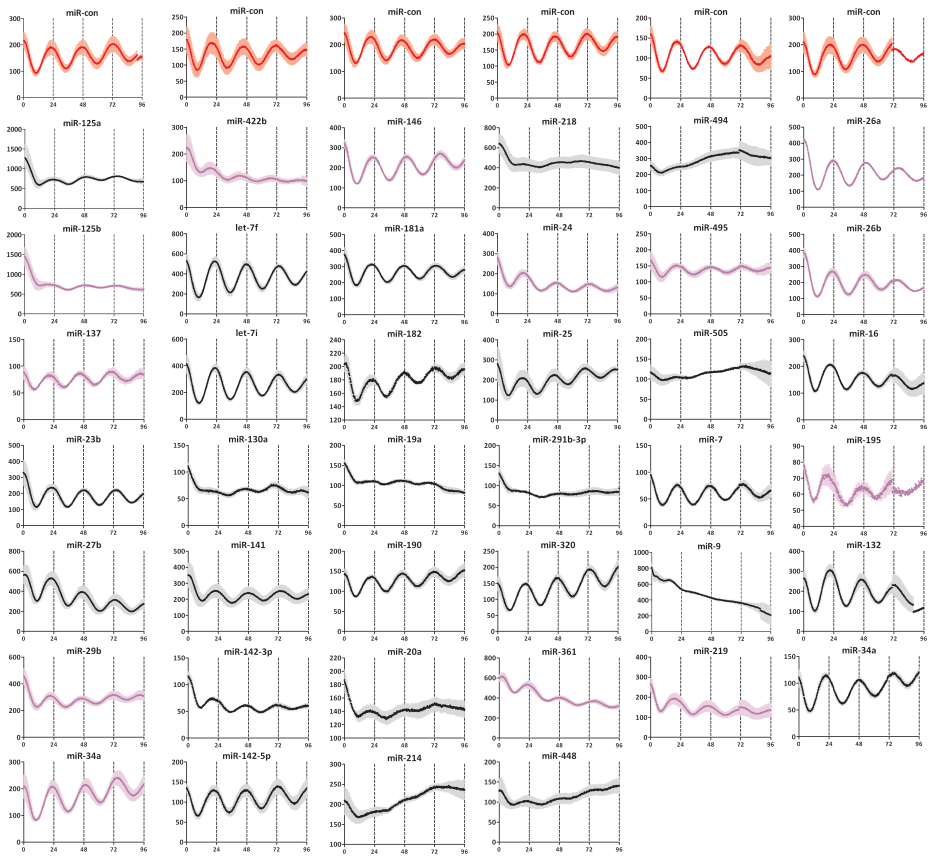
Figure 2: microRNAs alter period and amplitude of circadian oscillations. Graphs represent the raw data from the screen. NIH/3T3 cells were cotransfected with the Bmal1::Luc reporter construct and microRNA mimics. Graphs represent the raw bioluminescent data collected over ~5 days after synchronization of the cells with forskolin. The first ~12h after synchronization, which is the induction period, were left off to emphasize the oscillations. Each LumiCycle channel included a control transfection and up to 7 microRNA transfections (vertically together in the figure). The y-axis shows the counts/s of the luciferase reporter; the x-axis indicates the time in hours. The thick line represents the mean and the shaded area the standard error of the mean (SEM) of 3 independent experiments (for 3 microRNAs only 2 experiments were available).

Figure 3. Period and Goodness-of-fit calculations. (A) Goodness-of-fit (GOF) percentages (as determined by LumiCycle analysis software of the fitted sine-wave) for the Bmal1::Luc oscillations in microRNA mimic transfected cells. The microRNAs are sorted by percentage. Cells with oscillations with a GOF below 80% were considered poorly rhythmic or non-rhythmic. Transfection of cells with *siBmal1* (used as a positive control), which resulted in very poor/absent oscillations, has a GOF of ~50% (indicated in light b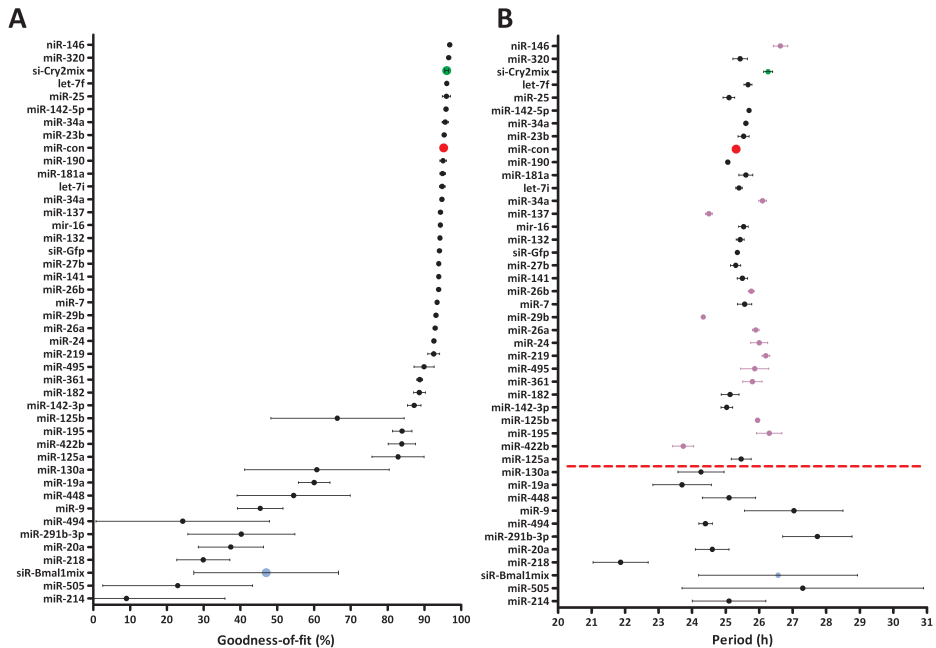lue), while the control transfection (red) has a GOF of 95.2 %. **(B)** The period of *Bmal1::Luc* oscillations in microRNA mimic transfected cells was analyzed using LumiCycle Analysis software. The raw data was de-trended using the 24-h moving average method. The microRNAs are in the same order as in (A). For statistical analysis, data from all control transfections were pooled. Oscillations with a significantly altered period (Student's t-test p<0.01) and a Goodness-of-fit above 80% (indicated by the dashed red line) are indicated in purple. As a positive control we cotransfected *siCry2*, known to lengthen the circadian period (indicated in green).

miR-218 controls PER2 proteins levels

One interesting microRNA to emerge from the screen is miR-218. This microRNA severely impaired the oscillations of *Bmal1::luc* (Figure 4A). Among the predicted target mRNAs for miR-218 are two subunits of the PP1 phosphatase complex: *Ppp1cb* (encoding the beta isoform of the catalytic subunit) and *Ppp1r12c* (encoding one of the many regulatory subunits). The PP1 complex has been implicated in the stabilization of the core clock protein PER2 (Gallego et al., 2006). Data from the genome-wide RNAi screen indicate that knockdown of *Ppp1cb* and *Ppp1r12c* individually impairs oscillations (Zhang et al., 2009), as microRNAs usually inhibit expression of their target genes this finding would fit with the circadian phenotype of NIH/3T3 cells overexpressing miR-218. Similar to PP1 inhibition and miR-218 overexpression, *Per2* reduction in cells leads to an arrhythmic circadian clock (Brown et al., 2005; Liu et al., 2007). If miR-218 targets the *Ppp1cb* and *Ppp1r12c* mRNAs, the reduced expression of PP1 phosphatase complex might lead to a reduction in the levels of PER2 due to enhanced degradation (Gallego et al., 2006).

Table 1: Period and Goodness-of-fit analysis

microRNA	Period (h)	SEM	p-value	GOF	SEM
miR-con	25.31	0.05		95.22	0.19
miR-125a	25.47	0.30	0.36225	82.84	7.08
miR-125b	25.95	0.07	0.00131	84.43	2.33
miR-137	24.50	0.10	0.00001	94.41	0.25
miR-23b	25.53	0.17	0.15116	95.38	0.49
miR-27b	25.30	0.15	0.95206	93.92	0.43
miR-29b	24.33	0.03	0.00000	93.15	0.26
miR-34a	25.60	0.00	0.05153	95.68	0.86
miR-422b	23.73	0.32	0.00000	83.85	3.70
let-7f	25.67	0.12	0.02285	96.07	0.42
let-7i	25.40	0.10	0.53710	94.85	0.79
miR-130a	24.27	0.69	0.00052	60.78	19.63
miR-141	25.50	0.15	0.21453	93.91	0.56
miR-142-3p	25.03	0.18	0.08292	87.22	1.84
miR-142-5p	25.70	0.06	0.01173	95.90	0.52
niR-146	26.63	0.22	0.00000	96.88	0.16
miR-181a	25.60	0.21	0.07507	94.97	0.78
miR-182	25.13	0.27	0.29666	88.66	1.63
miR-19a	23.70	0.87	0.00003	60.05	4.32
miR-190	25.07	0.03	0.10131	95.09	0.91
miR-20a	24.60	0.50	0.00285	37.36	8.84
miR-214	25.10	1.10	0.58030	9.00	26.79
miR-218	21.87	0.82	0.00000	29.90	7.23
miR-24	26.00	0.25	0.00030	92.59	0.58
miR-25	25.10	0.17	0.18149	96.00	1.12
miR-291b-3p	27.73	1.03	0.00000	40.23	14.56
miR-320	25.43	0.22	0.43805	96.58	0.02
miR-361	25.80	0.29	0.00774	88.71	0.81
miR-448	25.10	0.80	0.47610	54.45	15.40
miR-494	24.40	0.28	0.00932	45.28	26.40
miR-495	25.87	0.42	0.00832	89.94	2.73
miR-505	27.30	3.60	0.09421	22.92	20.38
miR-7	25.57	0.22	0.11542	93.46	0.31
miR-9	27.03	1.47	0.00160	45.39	6.17
miR-26a	25.90	0.14	0.00275	92.94	0.61
miR-26b	25.77	0.09	0.00427	93.87	0.37
mir-16	25.53	0.15	0.14548	94.33	0.19
miR-195	26.30	0.38	0.00002	83.94	2.65
miR-132	25.43	0.12	0.40543	94.23	0.48
miR-219	26.20	0.12	0.00000	92.54	1.63
miR-34a	26.10	0.12	0.00002	94.75	0.51
siGfp	25.35	0.07	0.81645	94.11	0.49
siCry2mix	26.27	0.13	0.00000	96.07	0.50
siBmal1mix	24.20	0.28	0.00000	27.28	0.53

SEM = standard error of mean, p-value was calculated using Student's t-test.
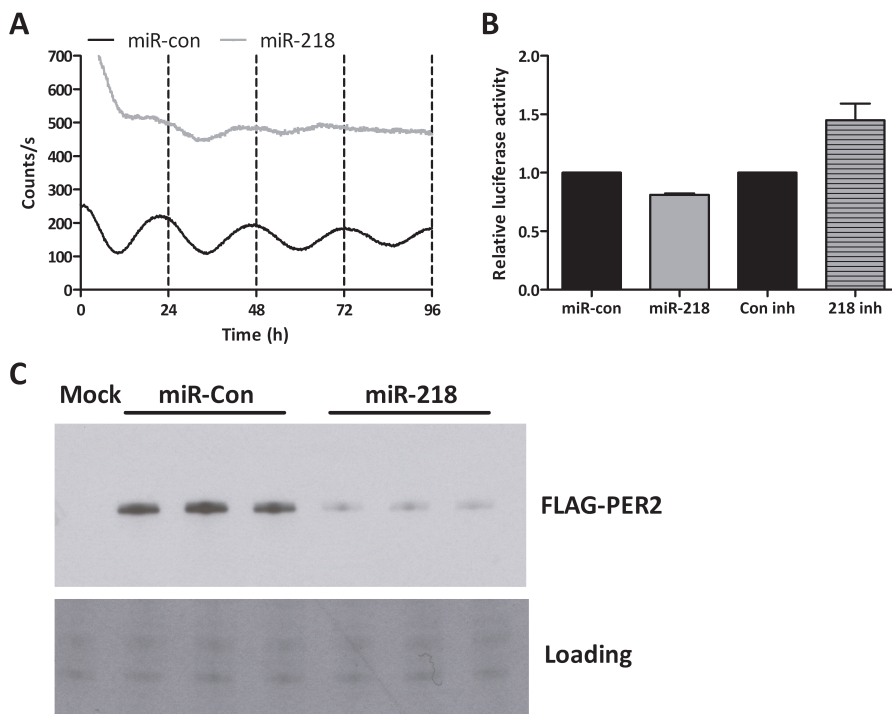
Figure 4: miR-218 controls amplitude, *Ppp1cb* expression and PER2 proteins levels. (A) Representative example of the effect of miR-218 overexpression on oscillations in NIH/3T3 cells co-transfected with the Bmal1::Luc reporter and microRNA mimics. Two days after transfection, cells were clock-synchronized using Forskolin and placed in a LumiCycle device for real-time imaging of luciferase activity. **(B)** miR-218 regulates the 3'UTR of *Ppp1cb*. 293T cells were transfected in quadruple with the Bmal1::Luc reporter and either a miR-218 mimics (overexpresion) or an inhibitor of endogenous miR-218 (silencing). miR-218 tends to reduce the 3'UTR luciferase reporter activity (n=3), while inhibition yields the opposite effect (n=2). **(C)** Overexpression of miR-218 reduces the levels of exogenous Flag-PER2. 293T cells were co-transfected in triplicate with microRNA mimics and a Flag-PER2 expression construct. FLAG-PER2 expression was analyzed using Western blot analysis. A non-specific band was used as loading control. Mock indicates transfection with empty expression vector.

To determine whether the catalytic PP1 subunit is a direct target of miR-218, we cloned the 3'UTR of *Ppp1cb* behind the cDNA of firefly luciferase in the pmirGLO vector. This vector also encodes a *Renilla* luciferase, expression of which allows normalization of the firefly luciferase measurements. Overexpression of miR-218 slightly, but reprodicbly, reduced luciferase activity originating from the *Ppp1cb* 3'UTR reporter Figure 4B). Silencing of endogenous miR-218 (which does not have other family members) in NIH/3T3 cells by transfection of a small RNA oligo, which binds to, and accordingly inhibits its function, showed the opposite effect (Figure 4B). These results together suggest that *Ppp1cb* is a direct target of miR-218. To further strengthen this connection, we addressed the effect of overexpression of miR-218 on PER2 protein levels. If miR-218 indeed targets *Ppp1cb,* the reduced PP1 complex level is expected to result in enhanced degradation of PER2 (Gallego et al., 2006). We therefore co-transfected 293T cells (which were also used by Gallego et al.) with a plasmid allowing constitutive expression of FLAG-tagged PER2, and either miR-218 or a control microRNA. Western blot analysis revealed that

PER2 protein levels were markedly reduced in miR-218 overexpressing cells (Figure 4C). Taken together, these results suggest that miR-218 controls the amplitude of circadian oscillations by targeting mRNAs encoding subunits of the PP1 complex, which in turn regulates the levels of PER2 protein.

let-7 controls period by regulating CRY2

Furthermore, we found that overexpression of let-7f (which belongs to the well-studied let-7 microRNA family) slightly lengthened the period and increased the amplitude of Bmal1::luc oscillations (Figure 5A and table 1). Although let-7f did not reach the cut off of $p<0.01$, it did reach the $p<0.05$ cut off and was studied further due to Cry2 being one of the predicted targets.
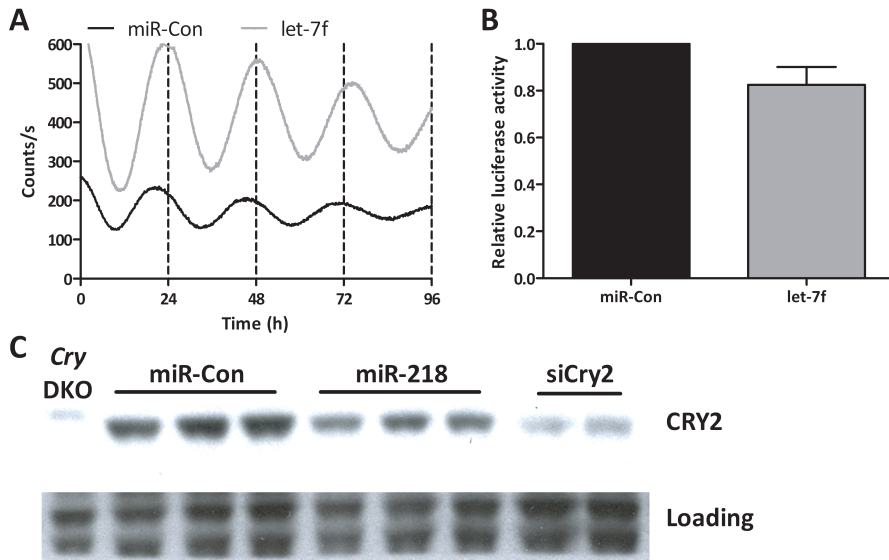

Figure 5: let-7 regulates period and CRY2 levels. (A) Representative example of the effect of let-7f on the period amplitude and level of circadian oscillations in NIH/3T3 cells co-transfected with the Bmal1::Luc reporter and microRNA mimics. Two days after transfection, cells were synchronized with Forskolin and placed in a LumiCycle device for real-time imaging of luciferase activity. **(B)** Effect of let-7f on the 3'UTR of *Cry2* in 293T cells. Cells were co-transfected with a 3'UTR luciferase reporter and a control or let-7f microRNA mimic. Error bars indicate the SEM (n=3). **(C)** Wsetern blot analysis of endogenous CRY2. NIH/3T3 cells were transfected in triplicate with a control or let-7f microRNA mimic. As a control for the specificity of the CRY2 antibody, we used *Cry1/Cry2*-deficient cells (Yagita et al., 2001). Knockdown of *Cry2* (siCry2) was used as a positive control for the transfection and as a comparison. A non-aspecific band was used as a loading control. A second experiment gave qualitatively similar results.

Cry2 inactivation in the mouse and in cultured cells increases period and enhances the amplitude of oscillations (Van der Horst et al., 1999; Brown et al., 2005; Liu et al., 2007). This is very similar to the effect of let-7f overexpression. To determine whether the effect of let-7f on the clock might be caused by direct downregulation of *Cry2*, we tested the effect of let-7f overexpression on the *Cry2* 3'UTR in 293T cells. Although there was a trend towards slight downregulation (Figure 5B), this did not reach significance (p=0.15, 1-column t-test). To determine the effect of let-7 on CRY2 protein levels, we examined endogenous CRY2 protein levels in NIH/3T3 cells after overexpression of either let-7f or

a control microRNA. As shown in Figure 5C, let-7f overexpression resulted in a reduction of the endogenous CRY2 protein level, which, however, is not as pronounced as that observed after siRNA-mediated silencing of *Cry2*. This finding is consistent with the less pronounced effect of let-7f overexpression (as compared to siRNA-mediated silencing of *Cry2*) on circadian period (Table 1). Although at present it is unclear why there is no clear effect in the luciferase assay, the Western blot data strongly suggest that let-7 regulates CRY2. Consistent with this notion, a preliminary experiment indicates that let-7 does not lengthen the period in *Cry2*$^{-/-}$ cells (data not shown), suggesting that let-7 requires *Cry2* to lengthen the period.

miR-29 controls period and targets the *Per1* 3'UTR
Another interesting microRNA to emerge from the screen is miR-29b. Overexpression of this microRNA led to strong (~ 1 hr) shortening of the period and a slight decrease in the amplitude of Bmal1::luc oscillations (Figure 6A and Table 1). This phenotype is similar to, although less extreme than the phenotype of cells with an inactive *Per1* gene, which is one of the predicted targets of miR-29. To determine whether miR-29 directly targets the 3'UTR of *Per1* we generated a 3'UTR reporter construct. As shown in Figure 6B, miR-29b overexpression resulted in a small reduction of luciferase activity, indicating that miR-29 might directly target the *Per1* mRNA. To determine whether endogenous miR-29 also acts upon the molecular clock, we inhibited two out of three miR-29 family members and found that both inhibitors slightly, but sigificantly increased the period ((n=4, p<0.05), Figure 6C). Furthermore, inhibition of these microRNAs also led to a decrease in the level of Bmal::luc expression, while oppositely overexpression increased the levels (compare Figures 6A and C). Together, these results show that miR-29 controls the clock through regulation of *Per1*.

miR-9 overexpression abolishes oscillations and targets *Fbxl3*
Like miR-218, miR-9 overexpression strongly impaired oscillations (Figure 7A and table 1). One of the predicted targets of miR-9 is *Fbxl3*. This gene encodes an ubiquitin ligase that was recently found to target the CRY proteins for proteaseome-mediated degradation (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007). Whereas *Fbxl3* mutant mice maintain behavioral rhythmicity, albeit with a longer period (Godinho et al., 2007; Siepka et al., 2007), explanted peripheral tissues from these mice and cells with RNAi-mediated knockdown of *Fbxl3* show strongly impaired oscillations (Busino et al., 2007; Godinho et al., 2007; Siepka et al., 2007). This makes *Fbxl3* a good candidate to explain the phenotype of miR-9 expression. Figure 7B shows that miR-9 overexpression clearly reduced luciferase expression from the *Fbxl3* 3'UTR reporter construct (p<0.001, 1-column t-test), showing that miR-9 affects oscillations through direct regulation of FBXL3 expression.

CONCLUDING REMARKS
Our results provide evidence that endogenous microRNAs regulate the mammalian circadian oscillator through direct post-transcriptional regulation of core clock genes. One reason for their involvement in the circadian clock might be to keep clock protein levels within a certain range. Since microRNAs can be regulated by a variety of processes that are known to affect the circadian clock, e.g. the DNA damage response, they may also serve as new links between these processes and the circadian clock.
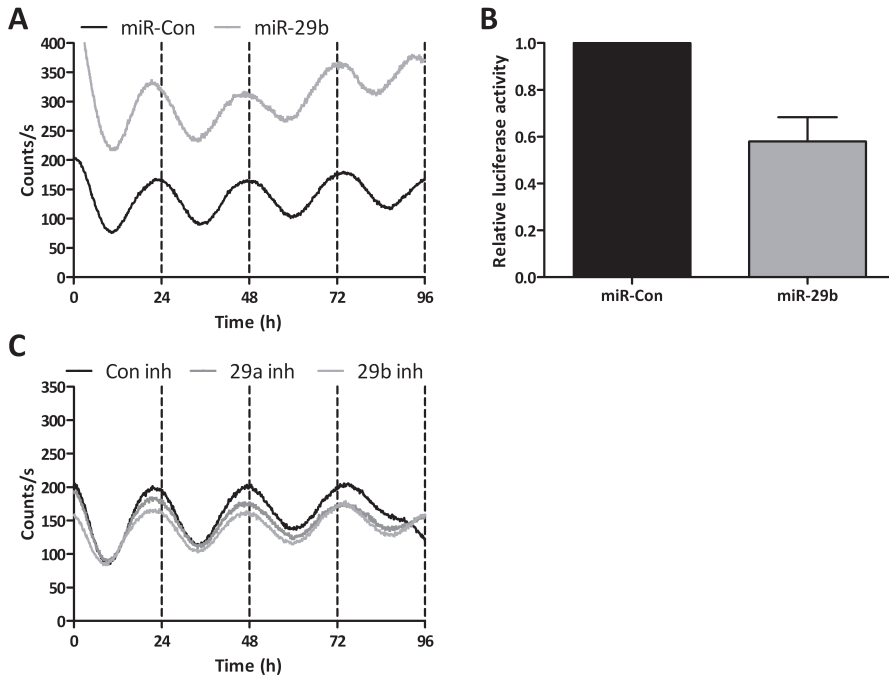
Figure 6: miR-29 controls period and targets the Per1 3'UTR. (A) Representative example of the effect of miR-29b on the period and level of circadian oscillations in NIH3T3 cells co-transfected with the Bmal1::luc reporter and microRNA mimics. Two days cells were synchronized using Forskolin and placed in a LumiCycle device for real-time imaging of luciferase activity. **(B)** miR-29b reduces the level of bioluminescence originating from the *Per1* 3'UTR luciferase reporter construct. 293T cells were transfected in triplicate with a 3' UTR luciferase reporter and a control or miR-29b mimic. Error bars indicate the SEM (n=2). **(C)** Inhibition of endogenous miR-29a or b lengthens period and reduces Bmal1::Luc expression levels. NIH/3T3 cells were transfected as described for panel A. Both inhibitors caused a lengthening of the period by about 0.3h (+/- 0.1h, n=4, p<0.05) and have an opposite effect on Bmal1::Luc levels as compared to that provoked by miR-29b overexpression.
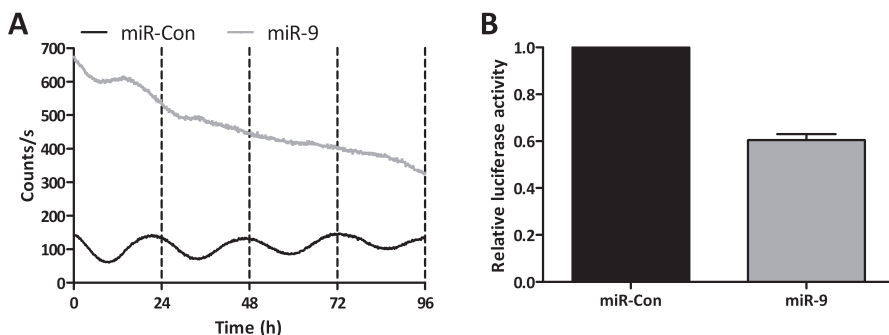
Figure 7: miR-9 overexpression abolishes oscillations and targets *Fbxl3*. (A) Representative example of the effect of miR-218 on clock performance in NIH/3T3 cells co-transfected with the Bmal1::Luc reporter and microRNA mimics. Two days after transfection cells were synchronized with Forskolin and placed in a LumiCycle device for real-time imaging of luciferase activity. **(B)** miR-9 overexpression reduces the expression level of an *Fbxl3* 3'UTR luciferase reporter in 293T cells co-transfected in triplicate with the reporter and a control or miR-9 mimic. Error bars indicate the SEM (n=4).

Although microRNAs can be very tissue-specific (e.g. miR-9) others have more ubiquitous expression patterns (e.g. let-7 and miR-29) (Landgraf et al., 2007), opening up the possibility that these microRNAs may also play a role in regulating the circadian oscillator *in vivo*. The microRNAs with tissue-specific expression may be a contributing factor to the tissue-specific differences in period that have been observed for tissues explanted from PER2::LUCIFERASE reporter mice (Yoo et al., 2004). Furthermore, a recent report demonstrated that ES cells do not have a circadian clock, even though all the core clock genes are expressed (at the mRNA level) (Yagita et al., 2010). The ES-cell specific miR-290-295 locus comprises ~70% of the total microRNA fraction in ES cells (Marson et al., 2008). One of these microRNAs, miR-291b-3p, severely reduced oscillations when overexpressed in NIH/3T3 cells. Given the high expression in ES cells, this indicates that microRNAs could be the explanation of why ES cells do not have a circadian clock and is subject of further study.

METHODS
Cell culture and siRNA/microRNA transfections
NIH3T3 immortalized mouse embryonic fibroblasts and 293T human renal epithelial cells were grown in Dulbecco's modified Eagle medium (DMEM)/Ham-F10 medium (1:1) supplemented with 10% fetal calf serum and a mixture of penicillin-streptomycin antibiotics. Small interfering RNAs (siRNAs) against *Bmal1*, *Cry2*, *Dicer1*, *Ago2* (4 oligos were pooled for each gene) and *Gfp* (control) were obtained from Ambion. microRNAs mimics (including the mini-library) were also obtained from Ambion. microRNAs were dissolved in RNAse-free water, aliquoted and stored at -20 °C and -80 °C for short- and long-term storage, respectively. microRNA inhibitors, dissolved in RNAse-free water, were obtained from Exiqon. NIH3T3 cell cultures (35-mm dishes containing ~5×10^5 cells in 2 ml medium) were transfected with 50 pmole microRNAs or siRNAs (25 µM final concentration) using the Lullaby transfection system (OZ Bioscience). To visualize the effect of siRNA and microRNAs on circadian clock performance, cultures were co-transfected with 200 ng Bmal1::luciferase reporter (kindly provided by Dr. K. Yagita, Osaka University).

Real-time bioluminescence imaging
Two days after transfection, when dishes were fully confluent, cells were synchronized by replacing the old medium with 2 ml fresh medium containing Forskolin (10 µM), luciferin (100 µM), HEPES (25 nM), 10 % fetal calf serum and antibiotics. Dishes were sealed with a glass coverslip and parafilm and placed in a LumiCycle 32-channel automated luminometer (Actimetrics) in a dry, temperature-controlled incubator at 37 °C. Real time bioluminescence recording (75 sec measurements at 10 min intervals) and data processing have been described before (Oklejewicz et al., 2008).
In screening assays, the following biological endpoints were determined: a) periodicity of oscillations (Tau) and b) robustness of oscillations (GOF; goodness of fit). The period was calculated from a sine-wave fit to the 24-h moving average subtracted data using LumiCycle Analysis software (ActiMetrics). For microRNAs that had a GOF above 80%, the period was compared to the control transfection using Student's t-test. For the screen, $p < 0.01$ was considered significant, in all other experiments we considered $p < 0.05$ significant.

3'UTR reporters.

To evaluate potential miRNA target sites, the 3'UTR of genes of interest were cloned into the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) using a PCR-based approach. 3'UTRs were amplified using primers with restriction sites from cDNA made from total NIH/3T3 RNA and inserted into the pmirGLO multiple cloning site, localized behind the firefly luciferase cDNA, which is expressed under control of the PGK promoter. The same construct also contains a *Renilla* luciferase reporter under control of the CMV promoter, which is used as an internal control for normalization. 3'UTR sequences were obtained from Ensembl and the Santa Cruz Genome Browser. In case of sequence length differences between the databases, we chose the longest sequence for cloning. To determine the effect of microRNAs on the 3'UTR luciferase reporter, we co-transfected 293T or NIH3T3 cells as above except that cells were grown in 24-well format and transfected in triplicate or quadruplicate. One day after transfection, cells were lysed and the activity of the firefly and *Renilla* luciferase reporters was determined using a Dual Luciferase kit, as described by the manufacturer (Promega).

Western blotting

To test the effect of microRNAs on exogenously expressed tagged PER2, we co-transfected 293T cells in triplicate in 6-well plates with microRNAs and FLAG-PER2 or GFP-PER2 expression constructs as described above. After 3 days culturing, cells were lysed directly in sample buffer. Proteins were separated on 4-12% gradient polyacryl amide gels (Invitrogen) and transferred to nitrocellulose membranes. α-FLAG (Sigma, 1:1000) and α-GFP (Roche, 1:1000) antibodies were used to detect FLAG-PER2 and GFP-PER2, respectively. As a secondary antibody, we used rabbit polyclonal anti-Mouse immunoglobins (Dako, 1:10000), conjugated to horseradish peroxidase, which were visualized using an ECL kit (Amersham) following the manufacturer's protocol. α-Actin antibodies (Millipore, 1:1000) or an aspecific band were used as a loading control..

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Chapter 7

CHAPTER 7

Discussion

DISCUSSION

The circadian clock regulates processes such as detoxification, drug metabolism, DNA repair, cell cycle and apoptosis, which has implications for chronotoxicity and chronotherapy (Lévi et al., 2010). Because of this, we wanted to determine whether exposure to a mutagenic compound would result in different mutation frequencies and/or spectra depending on what time during a day the compound would be given **(Chapter 2)**. To do so, we injected LacZ transgenic mice with the short-lived alkylating agent ethyl nitrosurea (ENU). Although an initial pilot experiment suggested that time-of-day was a factor in determining mutation frequency in spleen, this could not be confirmed in a subsequent experiment with a larger number of mice per time point. One simple possible explanation could be that time-of-day and thus the circadian clock do not play a role in determining mutation frequency. However, recent results from the Sancar lab suggest that the nucleotide excision repair (NER) pathway is under circadian control in brain and liver (Kang et al., 2009, 2010). Direct removal by MGMT and NER are the main pathways by which alkylated DNA is repaired. An earlier report has shown that the activity oscillated in liver with a peak of activity around ZT19 (Martineau-Pivoteau et al., 1996). This peak of activity is approximately 12 h earlier than the peak of NER activity in liver (Kang et al., 2010). Although it is not clear yet whether these results also apply to other peripheral tissues like spleen, it is a possible explanation for the lack of a diurnal variation of mutation frequency. Further work would be needed to resolve this. One could, for example, repeat the ENU exposure experiment in mice that lack either NER activity or MGMT. If both processes are involved in the circadian removal of alkylated DNA, one would expect that in mice that are impaired in of the two pathways there would be a variation in the mutation frequency after ENU exposure at different times during a day.

One of the important questions within the circadian clock field is the importance of circadian oscillations for organismal survival/life span (Schibler, 2009). Studies in Cyanobacteria and plants using short and long period mutants have demonstrated that organisms whose clock most closely matches the period of the environment are able to do better than organisms whose clock is out of phase with the environment (Dodd et al., 2005; Ouyang et al., 1998). Mutants with a short or long period do worse compared to WT strains in a 24-h environment, while the same short or long period mutants outperform the WT strain in a short or long period environment, respectively. This shows that it is not the mutation per se that alters survival and/or fitness, but whether the endogenous period matches that of the environment. This is referred to as resonance. While this has been firmly established for Cyanobacteria and *Arabidopsis* plants, it is not clear yet whether this also applies to mammals. **In Chapter 3** we show that mice lacking *Cry1* or *Cry2*, which have short and long periods under constant conditions, respectively (Horst et al., 1999), have circadian gene expression in liver and kidney that is out of phase with their environment in LD12:12 conditions, despite having normal running-wheel behavior under the same conditions (Horst et al., 1999). Even though our conditions do not exactly mimic those used for the Cyanobacteria and *Arabidopsis* experiments, they could still offer insight into whether internal resonance is important mammals. To examine this, we monitored the survival of WT, *Cry1*^{-/-} and *Cry2*^{-/-} mice. Somewhat surprisingly, we found no differences in the survival of either *Cry* mouse strain, despite the fact that these mice have an internal clock in peripheral tissues that is out of phase with the environment

every day of their life. Given the many links between the circadian clock and tumor imitation and progression in mouse models and humans, we wanted to determine whether there is a survival difference when the mice are predisposed to tumorigenesis by exposing them to a single dose of ionizing radiation. Also under these conditions there were no long-term survival differences. These results are in striking contrast to those obtained when mice are placed under conditions of repeated jetlag, which, in different experimental models, has a strong effect on survival (Filipski et al., 2004; Fu and Lee, 2003). Also epidemiologic studies have found a link between prolonged shift work and cancer incidence and metabolic disorders (Schernhammer et al., 2001, 2003). Under jetlag or shift work conditions, the subjects, like the *Cry1*$^{-/-}$ and *Cry2*$^{-/-}$ mice, are out of phase with their environment. However, an important difference between these two situations is that jetlag/shift work subjects have a normal clock but are out of phase with their environment due to working at abnormal hours in the case of humans or being exposed to a regular change in the light regime. Instead, our knock-out mice live under normal conditions and behave normally (presumably due to entrainment of the SCN to LD cycle), but are internally out of phase with their environment due to the fact that circadian gene expression in the periphery follows the genetic makeup of the clock. Our mouse models may mimic humans with very early or late chronotypes (referred as larks and owls) (Roenneberg et al., 2004). These people often suffer from "social jetlag" due to for example work or study obligations (Wittmann et al., 2006). Our results suggest that these people are not exposed to the same long-term risks as people who often work irregular hours. However, our IR exposure experiment did reveal a difference in the acute survival of *Cry2*$^{-/-}$ mice. This acute survival difference is not observed in *Cry1*$^{-/-}$ or arrhythmic *Cry1*$^{-/-}$|*Cry2*$^{-/-}$ mice (ongoing work),. This suggests that it is not the absence of *Cry2* per se, but rather the altered timing in those mice that is causing the reduced survival after IR exposure. It will be interesting and important to repeat the IR exposure at different times throughout the day to determine whether at other time points the *Cry1*$^{-/-}$ would be more sensitive. These experiments might shed more light on whether individual chronotype should be taken into consideration when treating (cancer) patients that have extreme chronotypes with genotoxic agents.

The circadian clock exerts control over a wide range of physiological processes. One these output processes is the cell cycle and the DDR that is connected to cell cycle. In **Chapter 4** we investigated if and how the cell autonomous circadian clock controls cell cycle progression under normal conditions and after exposure to genotoxic stress. Using primary fibroblasts isolated from embryos with different circadian phenotypes, we found that in the absence of a circadian clock due to a lack of both *Cry* genes (Van der Horst et al., 1999; Yagita et al., 2001), cells had a normal DDR. However, we did find that *Cry*-deficient cells had accelerated cell cycle progression under normal conditions. This would be consistent with the notion that the clock gates cell cycle division: removing the gate would allow cells to divide faster. However, cells that still had a robust clock, despite having a reduced amount of *Cry* (*Cry1*$^{+/-}$|*Cry2*$^{-/-}$), still proliferated faster. Cells with a reduced amount of *Cry* but having severely impaired oscillations (*Cry1*$^{-/-}$|*Cry2*$^{+/-}$) proliferated with a similar speed. This suggests that it is not the absence of the clock per se that is allowing *Cry*-deficient cells to proliferate faster. Although these results do not necessarily rule that gating does take place in WT cells, very recent results from wild type immortalized fibroblasts expressing luciferase reporters for the clock or the cell cycle that found no correlation between cell cycle division time and the phase of the

circadian clock (Yeom et al., 2010). So far, the CRY proteins are only known to inhibit CLOCK/BMAL1-mediated transcription. Using *Cry*-deficient cells that were additionally deficient in *Bmal1*, we found that this, at least in part, decreased the proliferation, while *Bmal1* deficiency alone did not alter proliferation. This suggests that the increased proliferation in *Cry*-deficient cells is due to alterations in the expression levels of *Bmal1*-dependent genes. Indeed, using unbiased genome-wide expression analysis we found that a great number of cell cycle related genes had altered expression in *Cry*-deficient fibroblasts. Furthermore, the microarray experiments also suggest a possible explanation for the finding that knockdown of *Bmal1* in humans cells resulted in escape from p53-dependent senescence (Mullenders et al., 2009). Our preliminary results suggest that primary *Bmal1* knockout MEFs escape from senescence during prolonged culture at 20% O_2. From the microarray analysis performed on WT and *Cry*-deficient MEFs, we found various inflammatory genes with altered expression in *Cry*-deficient cells. This pathway has recently been linked to control of senescence (Kuilman and Peeper, 2009). It would be interesting to determine the amounts of various cytokines in the medium of *Bmal1*- or *Cry*-deficient MEFs to see whether these are altered and may therefore play a role in BMAL1-dependent control of senescence. The fact that there is clear circadian connection between the cell cycle and DDR *in vivo*, but not *in vitro* and together with results described above leads to the hypothesis that the circadian clock, in part, controls these processes through a non-cell-autonomous mechanism. This could be tested by looking at circadian regulation of cell division or DDR in a specific tissue without a clock that resides in a mouse that whose clock is normal in the rest of its body. This can be done using the recently described conditional *Bmal1* knock out mouse model (Storch et al., 2007) or using transplantation experiments between wt and mutant mice using e.g. skin, liver or bone marrow, tissues with known circadian control for cell cycle and DDR(Bjarnason and Jordan, 2000; Matsuo et al., 2003; Gorbacheva et al., 2005; Fu et al., 2005).

Although we did not find an effect of the clock on the DDR in cultured cells, **Chapter 5** shows that the converse does happen: DNA damage exposure phase shifted the circadian clock *in vitro* in an ATM-dependent manner. Also mice exposed to ionizing radiation phase shifted their clock as evidenced by their running-wheel behavior in constant darkness. Somewhat surprisingly, we found that both *in vitro* and *in vivo* DNA damage exposure only led to phase advances and no phase delays of the clock and only when the exposure occurred during a specific part of the circadian cycle. These results are strikingly similar to those obtained in the slime mold *Neurospora crassa* (Pregueiro et al., 2006). This is even more intriguing in light of the independent origins of the clock in these two species (reviewed in Bell-Pedersen et al., 2005). At present it us unclear what the physiological significance is of these observations. One of the hypotheses for the evolution of circadian clock poses that they first occurred in response to daily UV radiation exposure from the sun (Pittendrigh, 1993), which could be an explanation for the observed DNA-damaged induced phase shifts. Another possibility comes from observations that the PER1 protein, one of the core clock proteins, may be directly involved in the DDR by interacting with ATM and CHK2 and influences their activation (Gery et al., 2006). Another report found PER1 to be one of many targets for ATM-mediated phosphorylation after exposure to IR (Matsuoka et al., 2007). It is conceivable that after exposure to DNA damage, part of the PER1 pool becomes phosphorylated by ATM after which it is recruited by the DDR to perform a function outside of the circadian clock. This (temporary) reduction

in PER1 protein available for the circadian clock could result in a phase shift making the DNA-damage induced phase shifts a by-product of non-clock functions of PER1 (or possibly other clock proteins). A further understanding of how the DDR is connected to the circadian clock will be required to gain more insight the significance of DNA-damage induced resetting of the circadian clock and its implications for the initial evolution of circadian rhythms.

To understand how the circadian clock affects clock-controlled processes it is important to know how circadian oscillations are generated. Transcriptional and posttranslational mechanisms are very important and well established mechanism in generating and modifying the circadian oscillator. **Chapter 6** of this thesis shows that a posttranscriptional mechanism, mediated by microRNAs, also plays an important role. Using a small, focussed overexpression screen using ~40 microRNAs, we found that about half of the microRNAs affected the period and/or amplitude of Bmal1::luciferase oscillations. For a few of these microRNAs we provide evidence that they regulate circadian oscillations by postranscriptionally regulating core clock genes. Although we have not tested for all these microRNA whether inhibiting their endogenous activity also leads to a phenotype, we did find that at least a few also affected the clock when they were inhibited. Consistent with this, we found that inhibition of the microRNA machinery through RNAi-mediated knockdown of *Dicer1* or *Ago2* (which reside in distinct complexes) resulted in a lengthening of the period. microRNAs can be induced by a variety of pathways/stimulations (e.g. the DNA damage response, cellular differentiation, metabolism) and may in that way affect the circadian clock under specific conditions, even if the microRNA might not play a physiological role under steady-state conditions. As shown in Chapter 5, activation of the DNA damage response phase shifts oscillations of luciferase reporter. Given the fact that numerous microRNAs respond to DNA damage (e.g. Pothof et al., 2009) it is possible that microRNAs that target clock genes may affect the clock after exposure to DNA damage. This may also occur after other stimuli that result in expression/activity changes of microRNAs that target clock genes. Additionally, one of the microRNAs that we found to blunt circadian rhythms is part of an ES cell-specific microRNA family (miR-290-295) that together comprises 70% of all expressed microRNAs in ES cells (Marson et al., 2008). Given that ES cells recently were found to have no circadian clock (Yagita et al., 2010), we postulate that the high expression of the miR-290-295 cluster in blocking the rhtyhms, as observed after overexpression of one individual member in NIH/3T3 cells (which normally do not express microRNAs from this cluster). This will be tested by imaging ES cells in which the miR-290-295 locus is deleted. If our hypothesis is corrent, we would expect these cells to regain a circadian clock. Similarly, other tissue-specific microRNAs may contribute to other tissue-specific differences observed in clock properties, such as the period differences seen among tissues *ex vivo* (Yoo et al., 2004).

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SUMMARY

Most organisms have evolved an internal timekeeper to anticipate and coordinate internal processes with the external 24-h environment imposed upon all living creatures due to rotation of the Earth around its axis. In the absence of any external cues, this timekeeper has a period of about 24-h (it can be slightly longer or shorter depending on the species) and hence the name circadian clock (from Latin: circa = about, diem = day). The mammalian clock is organized in hierarchical manner with the central clock in the suprachiasmatic nucleus (SCN), a small part of the brain consisting of two paired groups of ~10000 neurons each, coordinating the clock in peripheral tissues. The SCN receives light information from the environment through the retina and transmits this information to peripheral tissues and other brain regions. These peripheral tissues, such liver and kidney and virtually all other organs, have their own cell-autonomous clock that, in addition to signals from the SCN, can also receive other signals that can entrain them. On well-studied example is food, which when only given at times at which animals normally don not eat, can shift the phase of the clock of some peripheral tissues. At the cellular level, the circadian clock is generated by a genetic program in which genes and their protein products generate a molecular oscillator. In mammal cells, this oscillator consists of one essential negative transcription/translation feedback loop and several other positive and negative feedback loops that function mainly to confer robustness and precision to the core negative feedback loop. Posttranslational modifications of the clock proteins impose another layer of control by regulating the stability and localization (nuclear vs. cytoplasmic) of the clock proteins and thereby contribute significantly to the period and strength of the circadian clock. The same genetic program that generates the circadian clock also generates oscillations in gene expression of so-called clock-controlled genes (CCGs). These CCGs can constitute ~10% of all genes in a given tissue and vary from tissue to tissue, presumably reflecting the needs of each specific organ. Not all these genes are directly driven by the circadian clock transcription factors. Among the CCGs are other transcription factors that confer rhythmicity to their target genes. Given the fact that CCGs also include enzymes that, for example, can control the activity or stability of a protein or that can generate a metabolite in a rhythmic manner, the molecular impact of the circadian clock extends beyond the rhythmic expression of genes (i.e. mRNAs). So far, this additional level of circadian output control has remained largely unexplored but has gained more attention in the past couple of years after the demonstration of oscillating proteins of which the mRNA does not oscillate and the finding that production of the metabolite NAD^+ (which is implicated in many processes) is controlled by the circadian clock.

Processes that are under circadian control include detoxification, DNA repair, cell cycle progression and apoptosis (programmed cell death). The fact that processes oscillate throughout the day has important implications for toxicity and/or efficacy of genotoxic (chemotherapeutic) agents and has led to clinical trials in which cancer patients were treated with drugs at a specific time during the day at which the drug was most effective and/or displayed the least side-effects. This is referred to as chronotherapy. Given the genotoxic nature of many chemotherapeutic compounds, we wished to determine whether the mutation frequency would depend on the time-of-day of when a genotoxic mutagenic compound was given to mice that were transgenic for a marker that allows *in vivo* determination of mutation frequency in any tissue. **In Chapter 2**, using ethylnitrosurea

(ENU), which is a potent mutagen with a short half-life and is directly active, we found no difference in the mutation frequency in spleen after single exposures of ENU at different times of the day. Seminal experiments in Cyanobacteria and plants (*Arabidopsis thaliana*) have demonstrated that the circadian clock confers a survival advantage to these organisms, especially under competitive conditions. Cyanobacteria or plants whose endogenous clock most closely matched the period of the environmental light-dark cycle outcompeted Cyanobacteria or plants with a more deviating period. These experiments show that being in phase with environmental light-dark cycle (resonance) is beneficial for those organisms. Conversely, there is evidence from epidemiological studies and rodent models indicating that being out of phase with environment has a negative impact on health. Specifically, frequent shift work correlates with increased cancer incidence in humans and repeated jetlag simulation in mice accelerates tumor growth and increased tumor incidence. Furthermore, genetic disruption of circadian rhythms in mice has also been linked to pathologies such as cancer and metabolic disorders. In **Chapter 3**, we show that mice with a fast or a slow clock due to disruption of the core clock components *Cry1* or *Cry2*, respectively, have gene expression patterns that are out of phase with environment, while the mice still entrain normally with their behavior to the light-dark cycle. Despite the fact that these mice are internally out of phase with their environment every day, we found no significant impact on their survival under normal conditions. Predisposing the mice to cancer by a single ionizing radiation exposure also did not lead to long-term survival differences between the single knock out mice and their wild type counterparts. However, we did find that mice lacking *Cry2* were more prone to die from the acute effects of ionizing radiation. Since mice lacking both *Cry1* and *Cry2* had normal acute survival, this suggests that the increased mortality is due to the altered peripheral phase of the clock in *Cry2* mice and not the absence of *Cry2* itself.

It is thought that the circadian clock gates cell cycle division and influences the DNA damage response. *In vivo*, these connections are well-established, although it is not clear whether these connections are direct (cell-autonomous) or mediated through a systemic mechanism. **Chapter 4** investigates this connection using primary cells cultured *in vitro* lacking core clock components. Cell lacking both *Cry* genes are arrhythmic. Exposure of these cells to several different genotoxic agents did not reveal any differences in their survival, cell cycle arrest and p53 activation. However, we did find that *Cry*-deficient cells proliferated faster than WT cells. Analysis of rhythmic cells lacking all *Cry* alleles except one *Cry1* allele also proliferated faster; arrhythmic cells lacking all *Cry* alleles except one *Cry2* allele behaved the same. Taken together these results suggest that the increased proliferation in *Cry*-deficient cells is not due to the absence of oscillations. Genome-wide gene expression analysis of *Cry*-deficient cells and other experiments suggest that the increased proliferation is due to alterations in the average level of many cell cycle related genes.

Although we found no cell-autonomous effect of the clock on the DDR, the experiments described in **Chapter 5** show that the activation of the DDR phase shifts the circadian clock, both *in vitro* in cultured cells and *in vivo* in mice. In contrast to many other stimuli that can, depending on the phase of the clock at which their applied, both phase advance and delay the clock, the observed phase shifts after DNA damage exposure were always phase advances. Experiments using inhibitors and cell lines derived from patients with mutations in ATM, which is one of the main respondents after DNA damage or NBS,

which is required for ATM activation after DNA damage, showed that the phase shifting of the clock is dependent on the activity of the ATM kinase.

In **Chapter 6** we tried to gain more insight into how circadian rhythms are generated at the molecular level. microRNAs are small endogenous RNA molecules that regulate a wide range of processes in cells by posttranscriptionally regulating gene expression. To do so, we screened a small library of microRNAs for their effect on circadian luciferase oscillations in cultured cells. The results described in **Chapter 6** indicate that many microRNAs, when overexpressed, can change clock properties, including changing the period and amplitude. Importantly, experiments in which specific endogenous microRNAs were inhibited and global inhibition of microRNA biogenesis or activity also resulted in altered clock. For several tested microRNAs we found that they changed the clock by posttranscriptionally regulating core clock gene expression.

SAMENVATTING

De meeste organismes hebben een interne klok ontwikkeld die ervoor zorgt dat ze voorspelbare, dagelijkse veranderingen, opgelegd aan alle levende organismes door de rotatie van de aarde om haar as, kunnen anticiperen en interne processen coördineren ten opzichte van de tijd van de dag. In de afwezigheid van alle externe signalen heeft deze klok een periode van ongeveer 24 uur, vandaar de naam circadiane klok (Latijn: circa = ongeveer, diem = dag). In zoogdieren is het circadiane systeem georganiseerd op een hiërarchische wijze waarin de centrale klok in de suprachiasmatische nucleus (SCN), een klein hersengebiedje dat uit twee gepaarde groepen neuronen van elk ~10000 neuronen bestaat, de klok coördineert van organen in de periferie. De SCN ontvangt lichtinformatie uit de omgeving door de retina en verstuurt deze informatie naar andere hersengebieden en de perifere organen. Deze perifere organen, zoals lever and nier en zo goed als alle andere organen, hebben een eigen celautonome klok, die naast informatie van de SCN, ook andere signalen kunnen ontvangen die de klok kunnen sturen. Een goedbestudeerd voorbeeld is voedsel, welke, wanneer het aan dieren gegeven wordt op een tijd dat ze normaal niet eten, de klok van sommige organen kan wijzigen. Op een cellulair niveau wordt de klok gegenereerd door een genetisch programma waarin genen en de daardoor gecodeerde eiwitten een moleculaire oscillator vormen. In zoogdiercellen bestaat deze oscillator uit een essentiële negatieve feedback loop en een aantal andere positieve en negatieve feedback loops die als voornaamste functie hebben om de kern feedbackloop robuust en precies te houden. Posttranslationele modificaties van de klokeiwitten vormen een additionele laag van controle door de stabiliteit en lokalisatie (celkern of cytoplasma) van de klokeiwitten te reguleren. Deze modificaties vormen een essentieel mechanisme dat bijdraagt aan de 24-uurs periode en amplitude van de klok. Hetzelfde genetische mechanisme dat de circadiane klok genereert, genereert ook circadiane oscillaties van zogenoemde klokgereguleerde genen (KGG). Deze KGG's kunnen ~10% van alle genen in een orgaan vormen en variëren tussen de verschillende organen, waarschijnlijk afhankelijk van de benodigdheden van elk specifiek orgaan. Niet al deze genen worden door de kloktranscriptiefqctoren zelf gereguleerd. Tussen de KGG's zitten andere transcriptiefactoren die oscillaties genereren in hun eigen targetgenen. Doordat er ook enzymen tussen de KGG's zitten, die, bijvoorbeeld, de activiteit of stabiliteit van een eiwit reguleren of metabolieten genereren, is de moleculaire impact van de circadiane klok groter dan alleen het ritmisch genereren van genexpressie (mRNA's). Tot nu toe heeft deze vorm circadiane ouput regulatie weinig aandacht gekregen. In de laatste paar jaren is hier echter verandering in gekomen door de demonstratie dat er eiwitten zijn die oscilleren zonder dat hun mRNA oscilleert en de circadiane regulatie van NAD^+ productie (NAD^+ is een belangrijk metaboliet dat verscheidene cellulaire processen beïnvloedt).

Een aantal van de processen die onder circadiane controle staan zijn detoxificatie, DNA repair, celcyclusprogressie en apoptose (geprogrammeerde celdood). Het feit dat deze processen oscilleren heeft belangrijke consequenties voor de toxiciteit and effectiviteit van (chemotherapeutische) stoffen en heeft geleid tot klinische proeven waarin patiënten werden behandeld met medicijnen op tijdstippen waarop die medicijnen het meest effectief waren en/of het minst bijwerkingen vertoonden. Dit wordt chronotherapie genoemd. Omdat veel chemotherapeutica genotoxisch zijn, wilden we bepalen of de mutatiefrequentie afhankelijk was van het tijdstip van de behandeling. Hiertoe hebben

we in **Hoofdstuk 2** muizen, die transgeen waren voor een marker die het mogelijk maakt om *in vivo* de mutatiefrequentie te bepalen in elk orgaan, behandelt met de mutagene stof ethylnitrosureum (ENU). Uit ons onderzoek blijkt dat het tijdstip van behandeling geen gevolgen heeft voor de mutatiefrequentie en –spectrum in milt (het orgaan waar ENU het grottste effect heeft).

Belangrijke experimenten gedaan met Cyanobacterien en planten (*Arabidopsis thaliana*) hebben aangetoond dat de circadiane klok een overlevingvoordeel verleent aan deze organismes, in het bijzonder onder competitieve omstandigheden. Cyanobacteriën of planten waarvan de endogene klok het dichts bij de periode zat van de licht-donkercyclus (LD-cyclus) uit omgeving deden het beter dan Cyanobacteriën of planten met een klok die meer verschilde met de omgeving. Deze experimenten laten zien dat in fase zijn met de LD-cyclus van de omgeving (resonantie) gunstig is voor deze organismes. Ook voor zoogdieren zijn er bewijzen die suggereren dat resonantie gunstig is. Als eerste is data van epidemiologische studies in mensen en van experimenten met knaagdieren zoals muizen en ratten die indiceren dat uit fase zijn met de omgeving negatieve consequenties heeft voor de gezondheid. Het frequent draaien van ploegendiensten correleert met een verhoogde kans op kanker bij mensen en in knaagdiermodellen is laten zien dat het frequent verschuiven van de LD-cyclus de kans op tumoren verhoogt en de tumorgroei versneld. Verder is ook laten zien dat genetische ontwrichting van de circadiane klok in muizen kan leiden tot kanker en metabolische stoornissen. In **Hoofdstuk 3** laten we zien dat muizen met een snellere of langzamere klok door uitschakeling van de essentiële klokgenen *Cry1* en *Cry2*, respectievelijk, genexpressie patronen hebben in perifere organen die uit fase zijn met de omgeving, ondanks het feit dat de muizen qua gedrag wel gewoon de LD-cylus blijven volgen. Ondanks het feit dat deze muizen elke dag intern uit fase zij met de omgeving, hebben we geen verschillen gevonden in hun overlevingskansen. Onder condities waarbij de muizen een verhoogde kans hadden op kanker door een eenmalige blootstelling aan gammabestraling, vonden we ook geen langetermijn verschillen in de overleving van de muizen. Echter, we vonden wel dat de muizen zonder *Cry2* een hogere kans om dood te gaan door de acute effecten (vermindering van beenmerg en gastrolintestinale problemen) van gammabestraling. Het feit dat muizen waarin zowel *Cry1* als *Cry2* ontbreekt, en daardoor volledig aritmisch zijn, geen verschil in acute overleving hebben na gammastraling blootstelling suggereert dat het niet de afwezigheid van *Cry2* zelf is die de problemen veroorzaakt, maar de veranderde fase van genexpressie in de organen van de muizen zonder *Cry2*.

Volgens het huidige denken zorgt de circadiane klok ervoor dat celdeling alleen plaatsvindt tijdens een specifieke periode. Verder zijn er ook data die laten zien dat de reactie op DNA schade afhangt van de tijd van blootstelling en gereguleerd wordt door de klok. *In vivo* zijn deze connecties vrij goed ondebouwd, alhoewel het nog niet echt duidelijk is of deze connceties direct zijn (cel-autononoom) of dat ze door een systemisch mechanisme gereguleerd worden. **Hoofdstuk 4** onderzoekt deze connectie met behulp van *in vitro* gekweekte primaire fibroblasten zonder klok, door uitschakeling van beide *Cry* genen. Na blootstelling van deze cellen aan verschillende genotoxische behandeling kon geen verschil in overlevering, celcyclusarrest of p53-activatie worden waargenomen. Dit suggereert dat de verschillen die *in vivo* gevonden zijn in de overleving van B-cellen na blootstelling van muizen zonder *Cry* genen wellicht veroorzaakt worden door een systemisch mechanisme. We vonden wel een verschil in de deelsnelheid van de cellen zonder *Cry* genen onder normale condities. Experimenten met cellen die verschillende

hoeveelheden van *Cry* genen hebben en die wel of niet een goede klok bezitten, laten zien dat niet de afwezigheid van de klok, maar iets anders de verhoogde deelsnelheid veroorzaakt. Analyse van genoomweide genexpressie en andere experimenten suggereren dat inhibitie door de CRY eiwitten van BMAL1-afhankelijke genexpressie ten grondslag ligt aan de verhoogde deelsnelheid van cellen zonder *Cry* genen.

Hoewel we geen cel-autonoom effect van de klok op de respons na DNA-schade hebben kunnen vinden, laten de experimenten in **Hoofdstuk 5** zien dat activatie van de DNA-schade response de fase van de klok verschuift in zowel gekweekte cellen als muizen. In tegenstelling tot veel andere stimuli die, afhankelijk van het moment van de klok waarop de stimulering plaatsvindt, zowel een vervroeging als vertraging van de fase van klok tot gevolg kunnen hebben, vonden we na blootstelling aan DNA schade alleen vervroegingen van de klok. Experimenten met remmers van ATM en cellijnen afgeleid van patiënten met mutaties in ATM, een van de belangrijkste eiwitten die de response na DNA schade reguleert door andere eiwitten te fosforyleren, of NBS1, een eiwit dat essentieel is voor activiteit van ATM, laten zien dat de faseverschuiving van de klok na DNA schade afhankelijk is van de kinase activiteit van ATM.

In **hoofdstuk 6** wilden we meer inzicht krijgen in hoe circadiane oscillaties worden gegenereerd op een moleculair niveau. microRNA's zijn kleine RNA moleculen die in cellen voorkomen en daar allerlei processen beïnvloeden door de expressie van genen posttranscriptioneel te reguleren. Om te bepalen of microRNA's ook een effect hebben op de moleculaire klok, hebben we van een kleine verzameling microRNA's getest of ze circadiane oscillaties (gemeten met behulp van een lichtgevende reporter) in gekweekte cellen konden veranderen. Van verscheiden microRNA's vonden we dat de klok konden veranderen na overexpressie in cellen. Verder hebben we ook aan kunnen tonen dat de in cellen zelf voorkomende microRNA's ook de klok kunnen reguleren en dat dit gebeurt door directe regulatie van bepaalde klokgenen.

CURRICULUM VITAE

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Research Experience

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2002-2003:   Research technician, Leiden University Medical Centre, dept. of Molecular Cell Biology (Prof. Peter Verrijzer).

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Oklejewicz M, Destici E, Tamanini F, Hut RA, Janssens R, van der Horst GT. Phase resetting of the mammalian circadian clock by DNA damage. **Curr Biol. 2008** Feb 26;18(4):286-91.

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2007 Cold Spring Harbor Symposium on Clocks & Rhythms, USA: "microRNA-mediated regulation fo the circadian clock." (Poster)

2008 Society for Research on Biological Rhythms conference conference, USA: "Oscillator-independent control of cell cycle progression by circadian clock proteins." (Oral)

2010 Society for Research on Biological Rhythms conference, USA: "microRNA-mediated posttranscriptional control of circadian clock gene expression regulates mammalian oscillator performance." (Oral, awarded with Excelence Award)

Dankwoord

Eindelijk is het dan zover: m'n promotie! Het heeft allemaal wat langer geduurd dan gepland, maar waarschijnlijk zijn de meeste mensen dat wel van me gewend. Ondanks de moeilijkheden soms, heb ik erg genoten van m'n aio-periode door de nieuwe mensen die ik heb leren kennen. Verder heb ik in deze periode erg veel geleerd en ben zowel professioneel als persoonlijk erg gegroeid. Ik ben hier veel mensen dank voor verschuldigd.

Als eerste wil ik m'n promotor, Bert, bedanken voor de kans die ik gekregen heb om als HLO analist een aio-project te krijgen; ik ben je hier zeer dankbaar voor. Ik heb met erg veel plezier in je lab gewerkt en stel het vertrouwen, de vrijheid en de steun die je me hebt gegeven zeer op prijs. Jan, m'n andere promoter, ik heb niet zo heel veel met je kunnen werken, maar de weinige gesprekken die we gehad zijn vaak inspirerend geweest door je enthousiasme aan de ene kant en scherpe kritieken aan de andere. Ik wil ook alle andere (ex-leden van het kloklab bedanken voor alle hulp, wetenschappelijke discussies en plezier: Annelieke, Antonio (thanks for the hip parties), Edwin, Filippo (thanks for organizing the Italian poker holiday), Gosia, Ines, Karen, Karin (bedankt voor je immer vrolijke aanwezigheid), Karl, Monika (I've also had fun with you in cell culture and very much enjoyed seeing you get your PhD despite everything), Roel, Romana, Shoko, Xavier. Magda, the "adopted" clock lab member, I'm very happy you keep coming back to our lab; it's been a lot of fun in and more importantly outside of the lab.

Gedurende al de jaren in het MC heb ik veel mensen leren kennen van andere labs waar ik hulp van of gewoon plezier mee heb gehad: Wendy, de sociale "hub" van Cluster 15, je wordt erg gemist, bedankt voor alle uitjes en feestjes. Debbie, je kwam in Leiden werken in de tijd dat ik er weg ging, maar uiteindelijk ben je alsnog op dezelfde afdeling terechtgekomen als ik en gaan we bijna tegelijk promoveren. Ik heb het hier, in het bijzonder de laatste tijd, erg leuk met je gehad en ben ook erg blij dat je, net als ik, veel tijd in de celweek doorbrengt. Veel succes met je eigen promotie, ik kijk er erg naar uit. I would like to thank Christiano, Enrico, Filippo and Sandrino for two of the most memorable moments during my PhD period: the first poker weekend somewhere in a bungalow in Holland and the even more elaborate poker holiday in the mountains of Italy. I had an amazing time with you guys. Akiko (good luck with your defence), Alex, Alireza, Audrey (another cell culture girl I had a lot of fun with; good luck with your defence), Conny, Ekim, Farzin, George, Harald, Harry, Jaan-Olle, Jay, Joey, Joris, Laura, Lieneke, Marieke (bedankt voor de kick-box lessen), Michael, Mitja, Petros, Renata, Rik, Sanja, Steven, Tiago (good luck with your defence), Willeke (bedankt voor het lenen van je flat) en Yanto: bedankt allemaal! Jasperina en Marike, ik ben jullie veel dank verschuldigd voor de hulp bij een heleboel dingen.

M'n eerste kleine stapjes in de wetenschap nam ik in het LUMC onder begeleiding van Erik Kalkhoven. Erik, het vertrouwen dat jij in mij had, in een tijd dat ik die niet zelf had, heeft me erg geholpen en gemotiveerd. I would also like to thank Peter Verrijzer and the members of his lab in Leiden, especially Adone, Jan, Juan, Prashanth, Rob and Tokameh, for the nice time I had there working as a technician for almost 2 years.

Ik wil m'n mede-Hanenberglanders Dennis, Jürgen en Patrick bedanken voor de leuke herinneringen aan de mooiste buurt in de leukste stad van Nederland en het plezier dat we nog steeds samen hebben na al die jaren.

Finally, I would like to thank my paranimfs Gosia and Karl for their help in preparing for my defence and the stuff around around it. But even more so, I would like to thank them for their friendship and support during the past years. Karl, we haven't gone out much together, but I've spend a lot of nice time with you in the lab talking or, as my other paranimf likes to put it, blabbering about movies, music and other non-scientific topics (and sometimes even a bit of science). I very much appreciate your support and it helps a lot to know I can count on you when it's needed. I wish you, Nicole, Sam and the soon to be New One all the best. Gosia, we've had ups and downs, but in the end I am very happy that you'll be standing next to me during my defence, it means a lot to me. We've worked well together in the lab and I've had many wonderful moments with you that I will always cherish.

Eugin
23 augustus 2010, Rotterdam