The mammalian circadian clock gene Per2 modulates cell death in response to oxidative stress

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INTRODUCTION

Life on earth is under the continuous influence of a light and dark cycle that is caused by the rotation of the earth around its axis and its orbit around the sun. Many organisms have internalized the cyclic change of light and darkness in the form of the circadian clock. This enables them to predict dusk and dawn giving them an edge in competing for limited resources and avoiding predators (1, 2). The circadian timing system provides a temporal organization within an organism to modulate and synchronize biological functions in order to prevent the activation of biochemical pathways that would counteract each other. During the day, catabolic functions in order to prevent the activation of biochemical pathways are turned on while the organism is able to cope with the damaging effects of molecular oxygen known as reactive oxygen species (ROS). Here, we show that Per2, a molecular component of the mammalian circadian clock, is involved in regulating a cell’s response to oxidative stress. Mouse embryonic fibroblasts (MEFs) containing a mutation in the Per2 gene are more resistant to cytotoxic effects mediated by ROS than wild-type cells, which is paralleled by an altered regulation of bcl2 expression in Per2 mutant MEFs. The elevated survival rate and alteration of NADH/NAD+ ratio in the mutant cells is reversed by introduction of the wild-type Per2 gene. Interestingly, clock synchronized cells display a time dependent sensitivity to paraquat, a ROS inducing agent. Our observations indicate that the circadian clock is involved in regulating the fate of a cell to survive or to die in response to oxidative stress, which could have implications for cancer development and the aging process.

Keywords: apoptosis, adenovirus, bcl-2, paraquat, plumbagin, UV, SIN-1, p53
molecular mass antioxidants (e.g., vitamin C, tocopherol, lipoic acid) and the induction as well as the activation of antioxidant enzymes, such as catalase, glutathione peroxidase, and superoxide dismutases (SODs). A disturbance of the balance between pro-oxidant/antioxidant processes leads to alterations in redox homeostasis and oxidation of DNA and cellular biomolecules. In a first line, the cell may respond by autophagy, in order to remove damaged cellular components and damaged organelles. Alternatively, the cell can undergo either necrotic cell death or programmed cell death (apoptosis). However, the switch between the different possibilities for a cell to respond to oxidative stress is not completely understood [reviewed in Ref. (11)].

Since the circadian clock integrates and regulates metabolism, we started to investigate whether the circadian clock is involved in cellular response to oxidative stress. In particular, we were interested in the role of the Per2 gene, which is known to be one of the clock components responding to external signals such as light (12) and temperature (13). We found that at the cellular level Per2 is involved in the response of a cell to oxidative stress.

**EXPERIMENTAL PROCEDURES**

**ISOLATION OF MOUSE EMBRYONIC FIBROBLASTS**

Mouse embryonic fibroblasts (MEFs) from wild type and Per2ΔE3ΔGlob mutant mice were obtained at E13.5–14. Embryos were dissected under sterile conditions, the heads and the internal organs were discarded, and the rest of the bodies were minced and passed through a 2 ml bent syringe into a 60 ml bottle. Embryos were incubated in 5 ml trypsin/2 mM EDTA at 37°C – 5% CO2 for 15 min, pelleted (4000 g for 5 min), resuspended in 10 ml of fresh DMEM + 10% FCS, and plated in 60 mm dishes (1:5 dilution). The next day, the medium was removed the plates were rinsed twice with TBS and covered with 10 ml of fresh medium. The plates were then incubated for 24 h at 37°C – 5% CO2 and split 1:10. About 90% confluent MEF’s were frozen in FCS and stored in liquid nitrogen until used. At least three cell lines for every genotype were obtained and tested for paraquat sensitivity.

**ENZYMATIC ASSAYS**

Cells (passage 4–5) were cultured in DMEM/10% FCS until confluency was reached, then trypsinized and collected for the enzymatic assays (ca 6 × 10^6 cells). The aconitase assay was performed according to Gardner et al. (14). The SOD assay was done according to the manufacturers instruction in the Trevigen kit (order # 7500-100-K). Lactic dehydrogenase (LDH) assay was performed according to Gardner et al. (15). Cell homogenates were prepared in ice-cold PBS pH 7.4 as described above. The assay was performed in 200 µl reaction mixture containing 50 mM PBS, pH 7.4, 50 µl of sample, 0.2 mM NADH, and 1 mM sodium pyruvate. The decrease in absorbance at 340 nm was recorded for 3 min at 25°C and correlated to LDH activity using a standard curve. LDH activity was normalized for the protein amount.

Total NADH/NAD\(^+\) ratio in cell extracts was estimated by the lactate/pyruvate ratio (16, 17). Lactate and pyruvate were measured in whole cell extracts (ca 6 × 10^6 cells) of wild type and Per2ΔE3ΔGlob mutant cells synchronized by a 100 nM dexamethasone shock (see last paragraph) and taken 6 h after the shock. The samples were tested for lactate and pyruvate levels by adding to the reaction mixture (glycine buffer pH 10.0, 25 µl of sample and 10 units of lactate dehydrogenase) 0.2 mg of NAD\(^+\) or NADH, respectively. The increase or the decrease in absorbance at 340 nm was recorded after 15 min and correlated to lactate and pyruvate concentration through standard curves. Values were calculated using the formula in Ref. (16) with K\(_{eq}\) = 4.4 × 10^-2. Complex I activity was measured according to Chretien et al. and Klement et al. (18, 19).

**PARAQUAT, PLUMBAGIN, HYDROGEN PEROXIDE, SIN-1, AND UV LIGHT TREATMENTS**

Mouse embryonic fibroblasts were treated with paraquat (Supelco, PS-366), plumbagin (Sigma P7262), SIN-1 (Sigma M-5793), and hydrogen peroxide (Sigma H-1009) as reported in Ref. (20) or treated with ultra-violet (UV) light. Briefly, cells were plated in DMEM-10%FCS until confluent and then seeded in 96-wells plates (3000–5000 cells/well). The day after the medium was replaced and paraquat and H\(_2\)O\(_2\) were added at different concentrations (0, 200, 400, 600, 800, 1000 µM, total volume 150 µl). Plumbagin was added at a final concentration of 1.5 µM, SIN-1 at a concentration of 2 mM, or cells were exposed to UV light at 120 mJ/cm\(^2\). After 24 h, the cytotoxic effect was measured by a commercial kit based on the colorimetric determination of the LDH released upon cell lysis (Promega, G-1780). The cytotoxicity was evaluated by calculating the ratio between the LDH released spontaneously and the total LDH contained in the cells and expressed as percentage of cytotoxicity (dead cells relative to the total number of cells). The rescuing of paraquat cytotoxicity through N-acetyl-cysteine (NAC) was performed according to Macip et al. (21) using 50 µM NAC.

**CRYSTAL VIOLET STAINING**

Cells were seeded into 24 well plates (4 × 10^4 cells/well) and treated with paraquat, NAC, or paraquat + NAC for 24 h (see above). Medium was aspirated; cells were washed once with PBS and incubated with 0.02% crystal violet/2% EtOH for 20 min at room temperature. Plates were thoroughly rinsed with tap water and dried. Crystal violet was solubilized in 1% SDS, and sample absorption was read at 595 nm.

**CONSTRUCTION OF A RECOMBINANT ADENOVIRUS EXPRESSING THE FULL LENGTH Per2 ORF (Ad-Per2)**

The Per2 gene, cloned into a Tet-repressible expression vector (pSCOT), was inserted into the E1 region of a cloned ∆E1∆E3 adenoviral backbone (vmRL-CMV1) through homologous recombination in E. coli BJ5183. Bacteria were transformed by electroporation (2.5 kV in 0.2 cm gap cuvettes, 25 µF, 200 Ω) with the vector carrying the Per2 gene and the virus mid. Both constructs were carrying the CMV promoter and a region spanning the rabbit β-globin intron 2 and the β-globin exon 3, where the homologous recombination occurred. Positive clones were screened by PCR and the virus mids were isolated from the bacteria and purified by a standard equilibrium centrifugation in CsCl-ethidium bromide gradient (22). Virus mids were transfected with the calcium–phosphate method in a packaging cell line (HER911 Tet) to get the recombinant adenovirus (FeG generation). The transfected cells were harvested, lysed by repeated freezing-thawing and centrifuged. The supernatant, containing...
the viral particles, was used to infect HER911 Tet cells for a larger scale preparation (F1 generation). The titer (TCID\textsubscript{50}) of the F1 viral generation was finally determined with the Reed–Muench method (23).

**INFECTION OF MEFS**

Mouse embryonic fibroblasts were seeded in 96-wells plates (3000 cells/well) and infected with the Ad-Per2 at 600 or 60 m.o.i (100 μl volume of infection, 48 h incubation). Expression of the PER2 protein in cells was confirmed by Western blotting. As a control, MEFS were infected with an adenovirus containing the green fluorescent protein (GFP) gene in order to follow infection by microscopic inspection and to check the effects of the adenovirus on cell viability. After incubation, cells were treated with 600 μM paraquat and incubated for 24 h at 37°C – 5% CO\textsubscript{2}. Cell death was evaluated with the cytotoxicity test mentioned above (Promega). All the experiments were performed in duplicate with populations of wild type or Per2\textsuperscript{bdomi} mutant cells composed of three different cell lines equally represented. Data were compared by one-way ANOVA and Bonferroni’s post hoc test.

**CIRCADIAN PROFILE OF SENSITIVITY TO PARAQUAT**

Cells were grown in 20% FCS to confluency and 100 nM dexamethasone was added for clock synchronization (24). Dexamethasone was carefully washed away after 15 min and replaced with fresh DMEM with 20% FCS. Six, 12, 18, and 24 h after dexamethasone shock 600 μM paraquat was added to the wells and after 24 h incubation the cytotoxicity was evaluated as described above.

**H\textsubscript{2}DCFDA STAINING**

Cells were grown in 96-well plates to 80–90% confluency. After washes 25 μM H\textsubscript{2}DCFDA (2′,7′-dichlorodihydrofluorescein diacetate) diluted in phenol-red free medium without FCS were added, and cells were incubated for 30 min at 37°C. Subsequently, cells were washed once with PBS and fresh PBS was added. Fluorescence of DCF (2′,7′-dichlorofluorescein) was measured at 485 nm excitation and 528 nm emission wavelength in a Synergy HT multi-mode plate reader (BioTek). Values for cells treated with phenol-red free medium was subtracted from all other values.

**MITOCHONDRIAL STAINING**

Wild type and Per2\textsuperscript{bdomi} mutant MEFS were cultured in 8-well slide chambers at a density of 10\textsuperscript{4} cells/well. Culturing medium was replaced with fresh medium containing 100 nM MitoTracker Green FM (Molecular Probes, M7514). After 45 min at 37°C, the medium was removed and the slides inspected under the microscope and photographed.

**SEMIQUANTITATIVE PCR**

PCR amplification of a 2.3 kb fragment of mitochondrial DNA (mtDNA) was performed by using the primers described in Ref. (25). The PCR was performed as follows: denaturation step of 3 min at 94°C, then 15–35 cycles of denaturation–annealing–extension (denaturation 94°C 1 min, annealing 55°C 2 min, extension 72°C 2 min). After a final extension step of 10 min at 72°C, the tubes were chilled on ice and 8 μl of PCR products were loaded onto a 1% agarose gel.

For analysis of the circadian expression profile of Cry1 in synchronized cultures, cells were synchronized with dexamethasone as described above and samples were prepared 0, 3, 6, 12, 18, and 24 h after synchronization. For the assessment of bcl-2 expression following paraquat treatment, cells were treated with paraquat for 24 h. Total RNA was prepared using RNAzolB (WAK Chemie Steinbach, Germany; WAK-CS-1005) according to manufacturer’s instructions. Contaminating genomic DNA was removed using DNA-free (Ambion, 1906) according to manufacturer’s instructions followed by phenol:chloroform extraction. RNA integrity was checked on a 0.8% agarose gel and concentration was determined spectrophotometrically. Two micrograms of total RNA were reverse transcribed using SuperScriptII reverse transcriptase (Invitrogen, 18064-014).

For amplification of histone2Az, bcl-2, and Cry1, the following primers were used:

- **H2Az forward** 5′-CTGTAATCATCGACACCTGAAA-3′
- **H2Az reverse** 5′-CTGGTTGCTTCTTCTCCGAT-3′
- **bcl-2 forward** 5′-CCCCACCGAATCTCAAAAGAG-3′
- **bcl-2 reverse** 5′-CGGGAGAACAGGGTATGATA-3′
- **Cry1 forward** 5′-CTCTGGACAGATCATAGAATCA-3′
- **Cry1 reverse** 5′-CCAAAGCGGAGATAAGCGTGAG-3′

PCR conditions were 30 s denaturation at 94°C, 30 s primer annealing (H2Az: 50°C, bcl-2: 56°C, Cry1: 54°C), and 1 min elongation for 30–37 cycles. PCR products were run on 1.5% agarose gels, bands were quantified (QuantityOne 3.0, Biorad), and bcl-2 and Cry1 expression was normalized to H2Az. Subsequently, the data were analyzed by a t-test.

**ANNEXIN V AND PROPIDIUM IOIDE STAINING**

Annexin V and propidium iodide staining were performed using a BD Biosciences kit (no. 550911 and 556463). Cells were plated in an eight chambered slide (5–10 x 10\textsuperscript{4} cells/well) and treated with 600 μM paraquat. Ten and 16 h after the beginning of the treatment the medium was removed and cells were washed once with PBS and annexin V binding buffer (250 μl/well). Cells were then incubated with annexin-V-FITC antibody (diluted 1:10 in annexin V binding buffer, 250 μl/well) and propidium iodide (0.5 μg/250 μl) at 37°C in darkness for 30 min.

**GEArray**

Apoptosis GEArray Q series membranes (SuperArray Biosciences, Bethesda, MD, USA, No. MM-002) were hybridized with labeled cDNA obtained from mRNA extracted from MEFS of the two genotypes according to the manufacturer’s instructions. The dried array membranes were then scanned using an Odyssey infrared imaging system (LI-COR Biosciences). Images were analyzed using the web-based GEArray expression analysis suite (Super Array Biosciences, Bethesda, MD, USA).

**WESTERN BLOTS**

Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were incubated with anti-PER2 antibody (Beckton-Dickinson, GP81620-050) 1:1000, anti-p53 antibody (Oncogene, OP03) 1:1000, or anti-actin antibody.
As a next step, we addressed the question whether the sensitivity of the cells to paraquat is of circadian nature. Therefore, we expected that the observed cytotoxicity is due to radicals. We find that the NADH/NAD⁺ ratio is significantly higher in clock synchronized Per2²brdm1 mutant cells compared to wild type (wild type: 0.14 ± 0.02, Per2²brdm1: 0.33 ± 0.06, NADH/NAD⁺ ± SEM, p < 0.05). This ratio can be normalized to wild-type levels when the complete Per2 gene is introduced via adenovirus into Per2²brdm1 mutant MEFs (0.1 ± 0.04), but not when GFP is introduced (0.41 ± 0.03, Figure 2C). Over expressing the Per2 gene in Per2²brdm1 mutant MEFs probably has a strong influence on the cellular clock. Therefore, it is not clear whether the rescue of oxidative state is a direct consequence of Per2 or whether other clock components such as cryptochromes are the mediators of this process. These results, however, indicate that Per2 is involved in regulating the oxidative state of a cell and influences the cellular response to oxidative stress.

ENDOGENOUS ROS, SENSITIVITY TO PEROXIDE AND MITOCHONDRIAL FUNCTION

To find whether the Per2 gene influences the endogenous amount of ROS in a cell, we evaluated the endogenous amount of ROS present in wild type and Per2²brdm1 mutant MEFs via aconitase activity, an enzyme of the Krebs cycle that is sensitive to ROS (14). Aconitase activity in both wild type and Per2²brdm1 MEFs is comparable and suggests the presence of similar amounts of endogenous ROS in both genotypes (Figure 3A). Because ROS, particularly superoxide anions, can be eliminated in a cell by SOD (27), we measured the amount of total SOD activity (cytoplasmic and mitochondrial) in both genotypes. No differences were observed (Figure 3A), which is consistent with the aconitase assay. There were also no significant differences between the two genotypes in the activity of the control enzyme lactate dehydrogenase (LDH) (Figure 3A). Additionally, LDH did not show a circadian activity pattern in wild type and Per2²brdm1 mutant brain tissue (see Figure S2 in Supplementary Material). These observations indicate that the levels of total endogenous ROS in wild type...
FIGURE 1 | Mutation in the clock gene Per2 leads to altered response of asynchronous cells to oxidative stress. (A) Mouse embryonic fibroblasts (MEFs) of wild type (wt, solid line) and Per2 mutant mice (Per2\textsuperscript{Brdm1}, hatched line) display differences in cytotoxicity in response to various amounts of paraquat. LDH released into the medium versus LDH in living cells was measured after 24 h of paraquat treatment to determine cytotoxicity (n = 4–5 MEF preparations per genotype, *p < 0.05 two-way-ANOVA with subsequent Bonferroni test). Cell death due to plating was deducted (wt = 7 ± 0.8%, Per2\textsuperscript{Brdm1} = 7.4 ± 1%, n = 3).

(B) N-acetyl cystein (NAC), a radical scavenger, reverses paraquat mediated cytotoxicity. About 100 mM NAC was added to MEFs and subsequently the cells were treated with 600 µM paraquat. Top: photomicrographs of cells. Scale bar = 200 µm. Bottom: quantification of viability using the crystal violet method.

(C) Wild type (wt, white bar) and Per2\textsuperscript{Brdm1} (black bar) MEFs display differences in cytotoxicity in response to ultra-violet (UV) treatment (120 mJ/cm\textsuperscript{2}), in response to plumbagin (1.5 µM), and in response to SIN-1 (2 mM) (*p < 0.05).

and Per2\textsuperscript{Brdm1} mutant MEFs are not significantly different under unstressed conditions. However, sub-cellular differences in ROS production cannot be excluded.

To decipher whether the higher resistance of Per2\textsuperscript{Brdm1} mutant MEFs toward oxidative stress was due to the intracellular defense system or to a higher strength of cell membranes toward lipid peroxidation, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) was applied to the cells. Exposure to high H\textsubscript{2}O\textsubscript{2} concentrations for long periods induces necrotic rather than apoptotic cell death by direct damage of cell membranes (28). After 24 h exposure to H\textsubscript{2}O\textsubscript{2} at different concentrations no significant difference between Per2\textsuperscript{Brdm1} mutant and wild-type MEFs could be observed (Figure 3B) indicating that the resistance of Per2\textsuperscript{Brdm1} mutant cells toward paraquat, plumbagin, SIN-1, and UV treatment is not the result of ROS scavenging processes at the cell membrane. Therefore, we tested enzymatic activities in mitochondria that contribute to radical production. We measured the activity of complex I (NADH ubiquinone oxidoreductase) of the oxidative chain in the mitochondria, which transports electrons from NADH to ubiquinone and thereby influences the oxidative state of a cell (Figure 3C). We find that its activity under saturating conditions is significantly lower in Per2\textsuperscript{Brdm1} mutant cells (wild type: 144.4 ± 4.5 nmol/min/mg, Per2\textsuperscript{Brdm1}...
**Table 1 | Per2 influences sensitivity to reactive oxygen species (ROS).**

| Conditions | Cytotoxicity (%) |
|------------|------------------|
| Vehicle   | Ad-Per2 | Ad-GFP | wt | Per2<sup>Brdm1</sup> |
| + – – – – | 0.2 ± 0.6 | 0 ± 1.0 | |
| + + – – – | 43.9 ± 1.3 | 37.7 ± 2.0* | |
| – – + + – | 4.3 ± 1.3 | 4.4 ± 0.6 | |
| – – – + + | 1.4 ± 0.4 | 0.1 ± 0.9 | |
| + + + – – | 46.6 ± 1.2 | 45.5 ± 3.1 | |
| + + – + + | 44.5 ± 1.6 | 35.2 ± 4.4* | |

MEFs were treated with 600 µm paraquat and infected with 600 moi of adenovirus containing either the wild-type Per2 gene (Ad-Per2) or green fluorescent protein (Ad-GFP). After 24 h, the supernatants of the cultures were assayed for LDH enzyme activity. Cytotoxicity was determined by the ratio of released LDH over maximal LDH. Cell death due to plating was deducted (wt = 7.8 ± 0.4%, Per2<sup>Brdm1</sup> = 7.8 ± 0.5%, n = 3). The data are presented as mean ± SEM of three experiments. *p < 0.05.

**AMOUNT OF MITOCHONDRIA AND bcl-2 EXPRESSION**

Changes in oxidative state have been described for cells depleted of mtDNA (25). Therefore, we tested whether Per2<sup>Brdm1</sup> mutant MEFs contain less mitochondria (Figure 4A). Staining with...
FIGURE 3 | Endogenous reactive oxygen species (ROS) and sensitivity to H$_2$O$_2$ are similar in wild type and Per2$^{Brdm1}$ mutant MEFs but mitochondrial function is different between the two genotypes. (A) Enzymatic activities of aconitase, total superoxide dismutase (SOD), and lactate dehydrogenase (LDH) in wild type and Per2$^{Brdm1}$ mutant MEFs reveal no differences in the amounts of endogenous ROS between the two genotypes ($n=3$). (B) The sensitivity to H$_2$O$_2$ is comparable between wild type and Per2$^{Brdm1}$ mutant MEFs pointing to similar cell membrane sensitivity of the two genotypes to this compound ($n=3$). Cell death due to plating was deducted (wt = 9.2 ± 2%, Per2$^{Brdm1} = 7.4 ± 2.4%$, $n=3$). (C) Activity of complex I (NADH ubiquinone oxidoreductase) of the oxidative chain is significantly reduced in Per2$^{Brdm1}$ mutant compared to wild-type MEFs suggesting altered mitochondrial function in Per2$^{Brdm1}$ mutant MEFs (*$p<0.05$, $n=5$). (D) Assessment of intracellular ROS levels after 6 h of paraquat treatment using H$_2$DCFDA. ROS mediated conversion to DCF was measured and values were normalized to untreated wild-type MEFs (*$p<0.05$, $n=4$, unpaired t-test).

MitoTracker®Green, a dye specifically labeling mitochondria regardless of mitochondrial membrane potential, did not reveal gross differences between wild type and Per2$^{Brdm1}$ mutant cells. In addition, semiquantitative PCR did not uncover differences in mtDNA contents relative to nuclear DNA (Figure 4B). Therefore, we conclude that our findings are not due to less mitochondria in Per2$^{Brdm1}$ mutant MEFs. It appears that the described observations are the consequence of transcriptional and/or posttranscriptional events regulated by the Per2 gene. To evaluate whether the MEFs used in the described experiments undergo apoptosis, we took advantage of the fact that apoptotic cells lose plasma membrane asymmetry. We measured the presence of phosphatidylserine (PS), an aminophospholipid that is normally present in the inner leaflet of the plasma membrane, but translocates in early apoptosis to the outer leaflet of the membrane. We measured this process via annexin, a protein with high affinity for PS to indirectly monitor PS translocation. We find that staining for annexin V is already seen 10 h after paraquat treatment (Figure 4C, green color) and only in a few cells DNA was visualized by propidium iodide (Figure 4C, orange color). This indicates that 10 h after paraquat treatment cells are in early apoptosis and the cell membranes are still intact preventing penetration of propidium iodide into the cells. Sixteen hours after paraquat treatment labeled DNA is observed in most cells (Figure 4C, orange color) indicating that cell membranes are damaged typical for late apoptosis. In the cytotoxicity experiments described above assessment was made 24 h after paraquat treatment and hence our evaluation includes cells with defective membranes. To obtain a quantifiable measure for apoptosis, we investigated the expression levels of bcl-2, a well-characterized regulator of apoptosis (31). Using an apoptosis pathway cDNA-array, we found that expression of the anti-apoptotic gene bcl-2 is increased in Per2$^{Brdm1}$ mutant cells compared to wild-type...
FIGURE 4 | Amount of mitochondrial DNA in MEFs, apoptosis after paraquat treatment and *bcl-2* expression.

(Continued)
A change in redox state as manifested by elevated levels of NADH was down-regulated in Per2 mutant tissue (40). The expression of bcl-2 might increase the radical scavenging properties of a cell (40). Apoptosis in a clock component, as shown here for the circadian clock for this purpose (see Figure 2A) (39). A mutation in a clock component, as shown here for Per2, will therefore alter the cellular response to oxidative stress in mammalian cells.

A change in redox state as manifested by elevated levels of NADH might increase the radical scavenging properties of a cell (40), which leads to better survival in response to oxidative stress. This puts an increased pressure on the DNA repair mechanism to avoid accumulation of mutations. Interestingly, however, p53 protein is reduced in its expression in Per2 mutant MEFs [Figure S3 in Supplementary Material, see also Ref. (10)]. This is probably due to the reduced activity of NADH ubiquinone oxidoreductase (Figure 3C), which has been implicated in the regulation of p53 stability and p53-dependent apoptosis (41). Additionally, Per2 mutant MEFs do not reduce expression of the apoptosis gene bcl-2 as strong as wild-type cells in response to the ROS inducing agent paraquat. Therefore, Per2 mutant MEFs undergo less apoptosis and survive better under oxidative stress in vitro. However, if DNA damage occurs it will lead to tumor formation in an organism in vivo as described previously (10, 42). This indicates that the Per2 gene is not only involved in regulating redox state and response to oxidative stress in a cell but also affects apoptosis via p53 regulation. Per2 was identified as a component of the p53 pathway in a large-scale RNAi screen in human cells (43) indicating that the relationship between Per2 and p53 is bidirectional (44). Our results based on lack of Per2 are in agreement with the observation that overexpression of Per2 in various carcinoma cell lines reduced cellular proliferation with up-regulation of p53 and increased apoptosis that was accompanied by down-regulation of bcl-2 (45–47).

Conversely, flutamide, an anti-prostate cancer drug and apoptosis inducer, up-regulated Per2 gene expression in prostate mesenchymal cells (48). Interestingly, two cytotoxicity response regulators Ly49C and Nkg2d are down-regulated in Per2 mutant mice (49) supporting a role of Per2 in the regulation of cytotoxicity responses.

The above discussion illustrates that although a mutation in the Per2 gene makes cells more resistant to oxidative stress, Per2 mutant cells contribute to cancer formation in the organism (10). Hence, the Per2 gene seems to enhance biological fitness by establishing an optimal balance between cell survival and programmed cell death. This does not necessarily lead to maximal longevity under ideal circumstances but optimizes survival of an organism in real life experiencing a variety of different types of environmental stress. In sum, this report provides evidence that the circadian clock gene Per2 influences cellular response to oxidative stress and modulates cell death, which in turn affects cancer development and the aging process.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at [http://www.frontiersin.org/Journal/10.3389/fneur.2014.00289/abstract](http://www.frontiersin.org/Journal/10.3389/fneur.2014.00289/abstract)

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