repair genes, inactivated in hereditary nonpolyposis colon cancer (*23*). The presence of *hCHK2* mutations in a human cancer syndrome highlights the importance of cell cycle checkpoints in tumorigenesis. However, inactivation of the G_2 checkpoint may also have therapeutic implications (*6*), because it may render cancer cells particularly sensitive to DNA damaging agents, triggering a mitotic catastrophe (*24*) as they attempt segregation of damaged chromosomes.

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13. Clinical criteria for diagnosing a family as having LFS are the combination of (i) proband with sarcoma diagnosed under age 45, (ii) first-degree relative with an LFS component tumor (sarcoma, breast cancer, brain tumor, leukemia, or adrenal cancer) diagnosed under age 45, and (iii) first- or second-degree relative with any cancer diagnosed under age 45 or with sarcoma diagnosed at any age. Clinical criteria for LFS-variant are an individual with three separate primary cancers, with the first cancer diagnosed under age 45, or the combination of (i) proband with childhood cancer or LFS component tumor diagnosed under age 45, (ii) first- or second-degree relative with LFS component tumor diagnosed at any age, and (iii) first- or second-degree relative with any cancer diagnosed under age 60 [J. M. Birch *et al.*, *Cancer Res.* **54**, 1298 (1994); C. Eng *et al.*, *Cancer Epidemiol. Biomark. Prev.* **6**, 379 (1997)].
14. RNA was isolated from pelleted peripheral blood mononuclear cells, cultured fibroblasts, or EBV-immortalized lymphoblasts using RNA–Stat 60 (Teltest, Friendswood, TX) and reverse transcribed with random hexanucleotides. Overlapping 250– to 350–base pair RT-PCR fragments of the *hCHK1* and *hCHK2* transcripts were designed to generate homogeneous melting profiles with the Stanford prediction program (available at http://insertion.stanford.edu/melt.html) and were amplified in nested reactions (primers and conditions are available upon request). Uncloned RT-PCR products were analyzed by DHPLC (Transgenomics, Omaha, NE) to detect mismatched sequences resulting from heterozygous mutations [W. Liu, D. Smith, K. J. Rechtzigel, S. N. Thibodeau, C. D. James, *Nucleic Acids Res.* **26**, 1396 (1998)]. Synthetic mutations were tested for all fragments to ensure optimal analytic conditions. To search for homozygous mutations in cancer cell lines, we mixed equal amounts of PCR products derived from different cell lines to generate detectable mismatches. To search for polymorphic variants in the population, we analyzed EBV-immortalized lymphoblasts from 50 healthy individuals for alterations in the *hCHK1* and *hCHK2* transcripts. All samples with abnormal chromatography profiles were analyzed by nucleotide sequencing.
15. For automated nucleotide sequencing analysis, uncloned RT-PCR products were sequenced in both directions, with Energy Transfer Dye Primer Chemistry (Amersham Pharmacia Biotech), and analyzed for the presence of heterozygous mutations. Point mutations and small deletions detected in the *hCHK2* transcript were confirmed by amplification of genomic DNA and nucleotide sequencing. Nucleotides are numbered from the initiator methionine (GenBank accession number AF086904).
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Photic Induction of *mPer1* and *mPer2* in *Cry*-Deficient Mice Lacking a Biological Clock

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Mice lacking *mCry1* and *mCry2* are behaviorally arrhythmic. As shown here, cyclic expression of the clock genes *mPer1* and *mPer2* (mammalian *Period* genes 1 and 2) in the suprachiasmatic nucleus and peripheral tissues is abolished and *mPer1* and *mPer2* mRNA levels are constitutively high. These findings indicate that the biological clock is eliminated in the absence of both mCRY1 and mCRY2 (mammalian cryptochromes 1 and 2) and support the idea that mammalian CRY proteins act in the negative limb of the circadian feedback loop. The *mCry* double-mutant mice retain the ability to have *mPer1* and *mPer2* expression induced by a brief light stimulus known to phase-shift the biological clock in wild-type animals. Thus, mCRY1 and mCRY2 are dispensable for light-induced phase shifting of the biological clock.

Many physiological and behavioral systems are controlled by an internal self-sustaining molecular oscillating mechanism with a periodicity of approximately 24 hours, known as the biological clock. The core oscillator consists of an autoregulatory transcription-(post) translation–based feedback loop involving a set of clock genes (*1*). In mammals, as in *Drosophila*, three recently identified *mPer* genes (*mPer1*, *mPer2*, and *mPer3*) are thought to be oscillator genes (*2–4*). Transcription of *mPer1* is driven by the CLOCK/BMAL transcriptional activator complex and in turn is repressed by its own gene product (*5*). To maintain synchrony with the solar day/night cycle, the master clock in the suprachiasmatic nucleus (SCN) of the brain needs to be reset by daily light through receptors in the eye (*6*). In mammals, mCRY1 and mCRY2, members of the light-harvesting cryptochrome/photolyase protein family (*7*), have been proposed as candidate photoreceptors required for light-entrainment of the biological clock (*8*). Mouse mutants lacking *mCry1* show an acceleration of the free-run-

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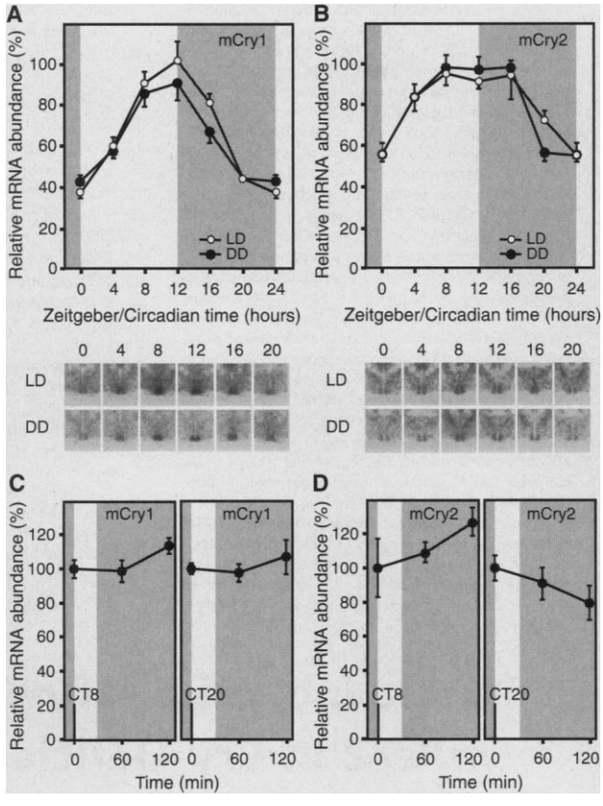

Fig. 1. Expression of *mCry1* and *mCry2* in the suprachiasmatic nucleus (SCN) of wild-type mice. (A and B) Circadian expression of (**A**) *mCry1* and (**B**) *mCry2* mRNA in the SCN in LD 12:12 (open circles) and in DD (closed circles). For each *mCry* gene, the relative RNA abundance was determined by quantitative in situ hybridization, with the value at CT12 adjusted to 100%. Values are expressed as means ± SEM (n = 5). Representative autoradiograms for each time point are shown below the graphs. (C and D) Light-pulse experiments for (**C**) *mCry1* and (**D**) *mCry2*. A 30-min light pulse (600 lux; fluorescent light) delivered at CT8 (left panels) and at CT20 (right panels) does not induce *mCry1* and *mCry2* mRNA. The relative RNA level was measured by quantitative in situ hybridization with the value just before onset of light adjusted to 100%. Values are expressed as means ± SEM (n = 3).

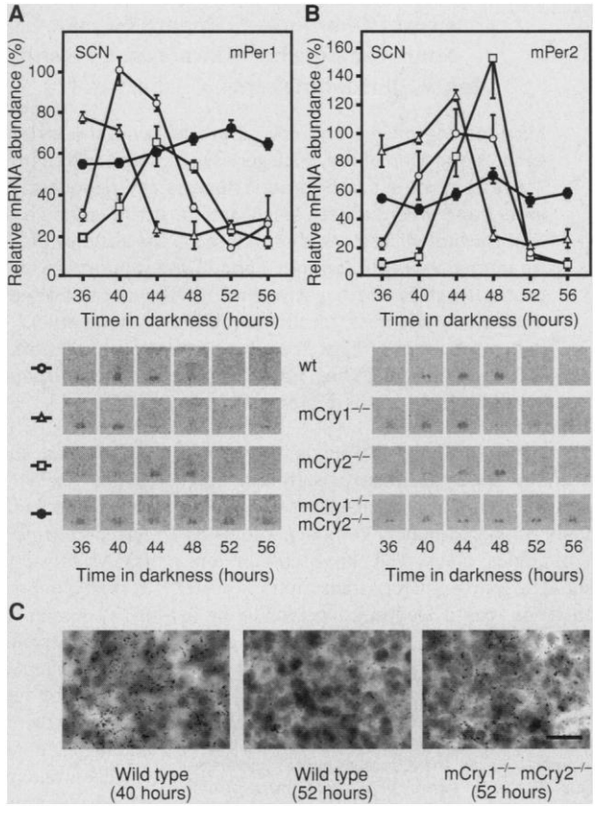

Fig. 2. Expression of *mPer1* and *mPer2* in the SCN of wild-type and *mCry* mutant mice in constant darkness. (A and B) The relative RNA abundance of *mPer1* (**A**) and *mPer2* (**B**) in the SCN of wild-type (open circles), *mCry1*$^{-/-}$ (open triangles), *mCry2*$^{-/-}$ (open squares), and *mCry1*$^{-/-}$*mCry2*$^{-/-}$ (closed circles) mice in constant darkness was determined by quantitative in situ hybridization. The peak values observed in wild-type animals were adjusted to 100%. Values are expressed as means ± SEM (n = 4). Representative autoradiograms are shown below the graphs. Because *mCry1*/*mCry2* double-mutant mice are arrhythmic, we used an environmental time scale (hours in DD) rather than a circadian time (CT) scale. (**C**) Representative examples of *mPer1* mRNA signals (dotted grains) in emulsion-coated sections of the SCN in wild-type and *mCry1*$^{-/-}$ *mCry2*$^{-/-}$ mice. (Left panel) wild-type SCN, 40 hours in darkness (≈CT4; 68 to 80% cells with 10 to 25 grains per cell); (middle panel) wild-type SCN, 52 hours in darkness (≈CT16; <10% cells with 10 to 25 grains per cell); (right panel) *mCry1*$^{-/-}$*mCry2*$^{-/-}$ SCN, 52 hours in darkness (62 to 74% cells with 10 to 25 grains per cell). Bar, 10 μm.

ning clock, as measured by wheel-running activity in constant darkness (*9*), whereas loss of *mCry2* slowed the clock (*9, 10*). Unexpectedly, mice lacking both *mCry* genes completely lack free-running rhythmicity (*9*). Although these results point to an antagonistic clock-adjusting function as well as to an absolute requirement of mCRY proteins for maintenance of circadian rhythmicity, they failed to resolve a possible function of the proteins as circadian photoreceptors and did not elucidate the consequences of mCRY dysfunction at the molecular level. To investigate how mCRY proteins act in the circadian core oscillation mechanism, we examined temporal and light-induced expression profiles of *mCry1*, *mCry2*, *mPer1*, and *mPer2* in the SCN and peripheral tissues of wild-type and *mCry* mutant mice by quantitative in situ hybridization or quantitative polymerase chain reaction (PCR) using real-time TaqMan technology.

Consistent with a possible function as clock gene, mRNA levels of *mCry1* oscillate in a circadian manner in the SCN and skeletal muscle of wild-type mice (*8, 11*). Similarly, *mCry2* expression exhibits a daily rhythm in the skeletal muscle (*11*). In contrast to previous reports (*8, 11*), using a highly sensitive method (*12, 13*), we observed that *mCry2* is rhythmically expressed in the SCN in a manner similar to *mCry1* (Fig. 1, A and B) (*14*). Levels of *mCry2* mRNA peak at the (subjective) day/night transition whether mice were kept under 12-hour light and dark cycles (LD 12:12) or under constant darkness (DD). Expression of the *mCry* genes is not significantly up-regulated by a brief light pulse given during the subjective day (CT8) or night (CT20) (Fig. 1, C and D). This indicates that, unlike *mPer1* and *mPer2* (*3, 15*), *mCry* expression is not directly regulated by light.

Next, we examined the expression profiles of *mPer1* and *mPer2* in entrained wild-type and *Cry* mutant mice (LD 12:12; 2 weeks) starting 36 hours after animals were placed in constant darkness (DD). Both *mPer1* and *mPer2* show a high-amplitude oscillation in the SCN of wild-type animals (Fig. 2, A and B). As expected on the basis of animals' circadian wheel-running behavior (*9*), robust oscillation of *mPer1* and *mPer2* mRNA is maintained in animals mutant for *Cry1* or *Cry2*. On the absolute time scale, consistent with the differences in free-running period length (*9*), *mPer1* and *mPer2* expression peaks earlier in *mCry1* mutant mice (tau = 22.5 hours) and later in *mCry2* mutant mice (tau = 24.6 hours), compared to wild-type animals (tau = 23.8 hours). In marked contrast, *mCry* double-mutant mice fail to show significant *mPer1* and *mPer2* mRNA cycling in the SCN (Fig. 2, A and B); instead, *mPer1* and *mPer2* transcript levels are intermediate to high at all times examined. In addition, we observed a homoge-

neous pattern of *mPer1* labeling in SCN tissue sections of mice deficient in *mCry1* and *mCry2* (Fig. 2C), indicating that transcript levels of *mPer1* are similar in all cells. This suggests that the absence of *mPer1* cycling in double mutants (Fig. 2A) is not due to a synchronization defect.

Because non-SCN tissues also rhythmically express clock genes (*4, 16*), we examined whether oscillation of these putative peripheral clocks was also affected in the absence of CRY. In contrast to wild-type mice, in the retina (Fig. 3A) (*17*) and liver (Fig. 3B) (*18*) of *mCry1/mCry2* double-mutant mice, *mPer1* mRNA no longer cycles and expression is constantly high. Similarly, *mPer2* mRNA did not show a prominent rhythm in retina and liver of *mCry1/mCry2* double-mutant mice (*19*). From the total absence of *mPer1* and *mPer2* oscillation in the SCN and in peripheral tissues, we conclude that the behavioral arrhythmicity observed in *mCry1/mCry2*-deficient mice is the direct consequence of a complete impairment of the molecular clock and that mCRY proteins are indispensable components of the core oscillator. Furthermore, the presence of accelerated and retarded clock oscillation in *mCry1* and *mCry2* single mutants indicates at the molecular level that mCRY1 and mCRY2 have overlapping functions in running the clock and an antagonistic role in determining its pace. The high mRNA levels of *mPer1* and *mPer2* in *mCry1/mCry2*-deficient mice suggest that mCRY proteins negatively affect *mPer* expression. Reppert and co-workers (*11*), as well as other groups (*20, 21*), have shown that whereas mPER and mTIM proteins only have moderate inhibitory effects on CLOCK/BMAL transactivating activity, mCRY1 and mCRY2 can almost completely block transcription from the *mPer1* promoter, which puts the mCRY proteins directly in the negative limb of the circadian feedback loop (*11*). Our findings that, in the absence of mCRY, *mPer1* and *mPer2* appear constitutively expressed at high levels are in agreement with this conclusion.

Circadian photoreceptors in mammals reside in the eye: loss of eyes abolishes photoentraining of the biological clock (*22*). However, rod- and cone-less mice show intact entrainment, indicating that rhodopsins are not involved (*23*). mCRY proteins are likely candidates for the circadian photoreceptors involved in light-entrainment because (i) they belong to the family of blue-light receptors (*7*), (ii) their expression is high in the ganglion cells and inner nuclear layers of the retina (*8*), and (iii) they can exert a clock-adjusting function (*9*) which would be required in the context of effecting a light input into the clock. However, direct proof for the presumed light input function is lacking because, ironically, the absence of a functional clock in *mCry* double-mutant mice precludes analysis of a resetting function by classical phase-shifting experiments. Nevertheless, to investigate a potential mCRY-mediated photoreceptor role, we examined the effect of light pulses on *mPer1* and *mPer2* expression: when light is given early during the subjective night concomitant with phase shifting of the clock, wild-type mice respond with an acute induction of *mPer1* and *mPer2* mRNA (Fig. 4) (*3, 14, 24*). Surprisingly, *mCry* double-mutant mice retained the ability to respond to the brief light pulse with acute induction of *mPer1* and *mPer2* mRNA (Fig. 4). This indicates that the rapid induction of *mPer1* and *mPer2* mRNA by light is still intact in complete absence of mCRY proteins and can occur without a functional clock or core oscillator.

In conclusion, our results show that a complete inactivation of the molecular oscillator driving the biological clock underlies the arrhythmicity observed in *mCry1/mCry2* double-mutant mice. Thus, mCRY1 and mCRY2 are indispensable components of the core oscillator, and *mCry1/mCry2*-deficient mice are, to our knowledge, the first mammals completely lacking a circadian pacemaker. In *mCry1/mCry2* double-mutant mice, the mechanism of light-induced phase shifting by up-regulation of *mPer1* and *mPer2* expression is still intact, which points to the involvement of different transcription regulatory processes for core oscillation and light-mediated phase-shifting. Our data suggest that photoreceptors other than mCRY proteins and rod/cone opsins [for example, mammalian homologs of the recently discovered fish VA-opsin (*25*) or *Xenopus laevis* melanopsin (*26*)] may be responsible for photic entrainment. Although *mPer* transcription repression by mCRY proteins (and thus their function in core oscillation) is light-independent (*20*), we do not completely rule out that mCRY proteins may act as photoreceptor proteins. First, it is possible that phase-shifting is mediated via more than one photoreceptor system, implying functional redundancy. Second, mCRY proteins may be involved in transmitting light inputs to the clock other than those required for phase shifting, such as changes in the length of the day and night, and information on dusk and dawn. Future experiments should shed light on the myste-

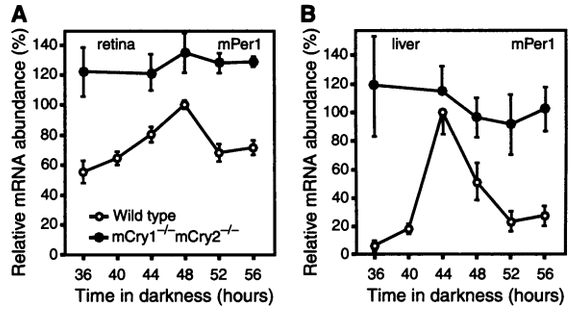

Fig. 3. Expression of *mPer1* in the retina and liver of wild-type and *mCry1*$^{-/-}$*mCry2*$^{-/-}$ mutant mice in constant darkness. (**A**) The relative abundance of *mPer1* mRNA in the retina was determined by quantitative in situ hybridization (five sections per animal; three independent experiments). Values (CT48 was determined as 100%) are expressed as the mean ± SEM. (**B**) The relative abundance of *mPer1* mRNA in the liver was determined by the reverse transcription PCR method with TaqMan technology as described. The calculated expression level of *mPer1* of wild-type at 44 hours divided by that of GAPDH was normalized to 100%. Values are expressed as means ± SEM (n = 3).

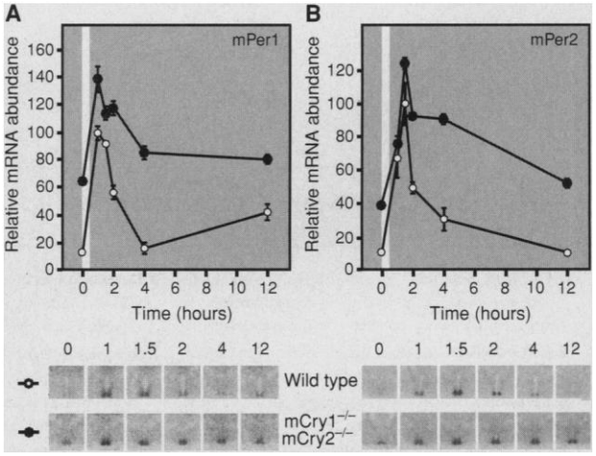

Fig. 4. Light-induced *mPer1* and *mPer2* expression in the SCN of wild-type and *mCry1*$^{-/-}$ *mCry2*$^{-/-}$ mutant mice. A 30-min light pulse (600 lux; fluorescent light) delivered 52 hours after the dark transfer induced (**A**) *mPer1* and (**B**) *mPer2* mRNA in the SCN of wild-type and *mCry1*$^{-/-}$ *mCry2*$^{-/-}$ mutant mice. The relative abundance of *mPer1* mRNA was determined by quantitative in situ hybridization, with the peak induced values of wild-type (1 hour for *mPer1*, 1.5 hour for *mPer2*) adjusted to 100%. Values are expressed as means ± SEM (n = 4). Representative autoradiograms are shown in the lower panels.

rious blue-light receptor properties of mCRY proteins.

Note added in proof: Recently, Vitaterna and colleagues have determined mRNA levels of *mPer1* and *mPer2* in *mCry1/mCry2* double-mutant mice at two time points. In addition, photic induction (measured 30 min after a 1-hour light pulse) was observed for *mPer2* but not for *mPer1* (*27*).

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12. Wild-type, $Cry1^{-/-}$, $Cry2^{-/-}$, and $Cry1^{-/-}Cry2^{-/-}$ animals (C57B16:Ola 129 hybrid, 8 to 12 weeks old) were individually housed under 12-hour light, 12-hour dark cycles (LD 12:12) and constant temperature. In DD (constant darkness) and light-pulse experiments, animals were kept under LD 12:12 conditions for at least 2 weeks and were used the second day of DD. Animals were exposed to fluorescent light (600 lux for 30 min) and killed at the time points indicated. Because $Cry1^{-/-}Cry2^{-/-}$ animals are arrhythmic, in some of the experiments, we used an absolute time scale rather than a Circadian (CT) or Zeitgeber (ZT) time scale. Circadian expression of *mCry1* and *mCry2* was studied in Balb-c mice (Japan Animal Care, 8 to 10 weeks old).
13. Quantitative in situ hybridizations ("free-floating" method) on serial coronal cryostat sections of the mouse brain (40 μm thick) were performed as described (*15*). Radiolabeled cRNA probes for *mCry1* (1074–1768), *mCry2* (1016–1652), *mPer1* (538–1752), and *mPer2* (1–638) were made with [^{33}P]–uridine triphosphate (New England Nuclear) and a standard protocol for cRNA synthesis. The radioactivity in the SCN of each section on BioMax films (Kodak) or Ilford K5 nuclear track emulsions (Ilford) was analyzed with a microcomputer interfaced to an image analyzer system (MCID, Imaging Research Inc., Ontario, Canada) after conversion into the relative optical densities produced by the [^{14}C]-autoradiographic microscale (Amersham). Data from the SCN were normalized with respect to the signal intensities in an equal area of the corpus callosum. The intensities of the optical density of the sections from the rostral-most to the caudal-most of the SCN (10 sections per brain) were then summed; the sum was considered a measure for the amount of mRNA in this region. Values are expressed as means ± SEM (*n* as indicated in the text). "Relative mRNA abundance" values are calculated assuming a wild-type peak value as 100%.
14. Using the highly sensitive and quantitative "free-floating" method (*13*), we found a significant difference among six time points in both LD 12:12 [$F(5,48) = 5.20$, $P = 0.023$] and DD [$F(5,48) = 5.20$, $P < 0.001$] conditions (method: one-way ANOVA).
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17. Quantitative in situ hybridizations on eye sections were performed with the thaw-mounted method. Signals were measured in the external granular layer of the retina (five sections per animal; three independent experiments). Values are expressed as the mean ± SEM.
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