ensures that circadian-controlled processes maintain the appropriate phase relationship to environmental cues. Photoreceptor diversity and redundancy, therefore, appear to be the key features in the photocontrol of the circadian clock in higher plants.

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26. We thank P. H. Quail for the phyB-overexpressing line, A. R. Cashmore for the cry1-overexpressing line, and C. Lin for early release of the *cry2-1* mutant. Supported by competitive research grants NSF MCB 9724120 and NIH GM56006 (S.A.K.). D.E.S. was supported, in part, as an NSF Postdoctoral Fellow in Plant Biology (BIR-9403981). P.F.D. is supported by an European Molecular Biology Organization Long-Term Fellowship (ALTF 720-1997).

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Role of Mouse Cryptochrome Blue-Light Photoreceptor in Circadian Photoresponses

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Cryptochromes are photoactive pigments in the eye that have been proposed to function as circadian photopigments. Mice lacking the cryptochrome 2 blue-light photoreceptor gene (*mCry2*) were tested for circadian clock-related functions. The mutant mice had a lower sensitivity to acute light induction of *mPer1* in the suprachiasmatic nucleus (SCN) but exhibited normal circadian oscillations of *mPer1* and *mCry1* messenger RNA in the SCN. Behaviorally, the mutants had an intrinsic circadian period about 1 hour longer than normal and exhibited high-amplitude phase shifts in response to light pulses administered at circadian time 17. These data are consistent with the hypothesis that CRY2 protein modulates circadian responses in mice and suggest that cryptochromes have a role in circadian photoreception in mammals.

Circadian rhythms are oscillations in the biochemical, physiological, and behavioral functions of organisms with a ~24-hour periodicity (*1*). Circadian rhythms are synchronized with light-dark cycles, but the molecular basis of this "photoentrainment" is not known (*2*). Indeed, there is no consensus on the nature of the circadian photoreceptor. Three classes of pigments have been considered as candidates: opsin/retinal-based photopigments (*3*), tetrapyrrole-based heme pigments (*4*), and pterin/flavin-containing cryptochrome blue-light photoreceptors (*5, 6*). Cryptochromes were first identified in plants as structural homologs of the DNA repair enzyme DNA photolyase (*7*), but they lack DNA repair activity (*8*) and are involved in mediating growth (*9*), flowering time (*10*), and phototropism (*11*) in response to blue light. Recently, two human and mouse homologs of the plant cryptochromes were discovered (*5, 12*). Cryptochromes 1 and 2 (CRY1 and CRY2, respectively) lack DNA repair activity (*5*) and are expressed in the mouse retina (*6*), and *mCry1* exhibits circadian oscillations of expression in the SCN (*6*) wherein the central pacemaker of the body resides. These observations led to the proposal that cryptochromes were likely to be photopigments for circadian photoentrainment (*6*).

To test this hypothesis, we created a mouse strain that lacks the predominant form of cryptochrome found in the mouse retina, CRY2, and analyzed its circadian behavior using biochemical and behavioral tests. We generated $Cry2^{+/-}$ heterozygous mutant mice by established methods (*13*) using the targeting construct shown in Fig. 1A. Interbreeding of heterozygotes yielded progeny of wild type:$Cry2^{+/-}$:$Cry2^{-/-}$ at a ratio of 1:2:1 (Fig. 1B). The mutant mice were phys-

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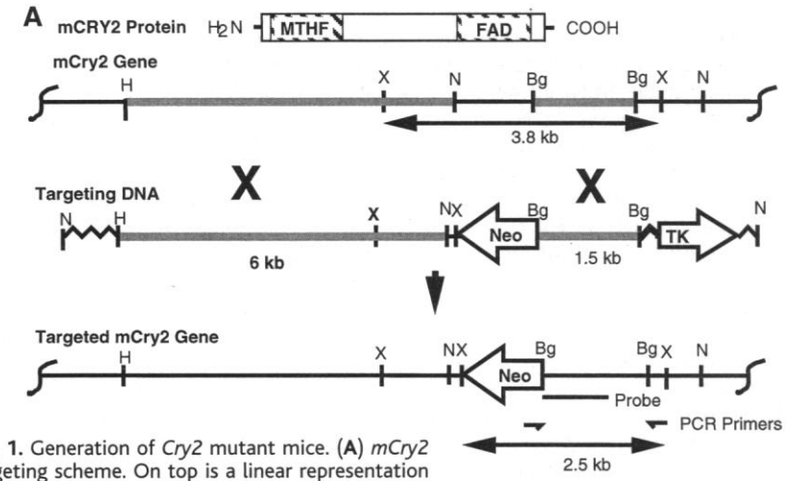

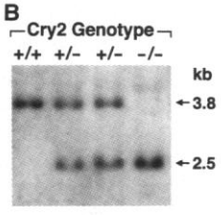

Fig. 1. Generation of *Cry2* mutant mice. (**A**) *mCry2* targeting scheme. On top is a linear representation of the structural map of CRY2 indicating the approximate locations of binding sites for methenyl tetrahydrofolate (MTHF) and flavin adenine dinucleotide (FAD) as represented in the genomic clone. The top line shows a partial restriction map of the gene, indicating the 1.1 kb that was deleted and replaced by the neomycin (Neo) gene. After homologous recombination (targeting DNA, middle) diagnostic Southern (DNA) hybridization of the recombinant allele in ES cells and mice detected the presence of an Xba I site introduced by the targeting vector that leads to a change of fragment length from 3.8 kb in the wild-type allele to 2.5 kb in the recombinant allele (targeted *mCry2* gene, bottom). Primers from the 3′ end of the Neo gene and the 3′ end to the 1.5-kb arm of homology yield a PCR product of 1.6 kb from the recombinant gene. Bg, Bgl I; H, Hind III; N, Not I; X, Xba I. (**B**) Southern blot analysis of mouse genomic DNA. About 10 μg of mouse genomic (tail) DNA was digested with Xba I and subjected to electrophoresis through a 0.8% agarose gel, which was then probed with ^{32}P-labeled DNA made from the *mCry2* genomic clone in (A).

ically and behaviorally normal. We used mice that were at least 6 weeks old and age and sex matched. To determine if CRY2 is a circadian photoreceptor, we tested the light response of the mutant mice using biochemical and behavioral assays. Because the mutant mice still express the CRY1 photoreceptor, we expected a reduction in, rather than elimination of, the circadian photoresponse reactions.

We first quantified induction of *mPer1* mRNA expression in the SCN by acute light pulses. *mPer1* is a candidate circadian clock gene (*14*) that is expressed in the SCN with a robust circadian rhythmicity in 12-hour light: 12-hour dark (LD12:12) cycles, reaching a maximum at zeitgeber time 4 (ZT4) and a minimum at ZT20 (by convention ZT0 is the time at which the light is turned on). Importantly, *mPer1* is one of the immediate-early genes in the SCN that can be induced with acute light pulses at night when mRNA levels are low (*15*). To test whether CRY2 protein functions as a circadian photopigment, we exposed wild-type and *Cry2* null mice to two subsaturating light doses at ZT18 and quantified *mPer1* induction in the SCN by in situ hybridization (Fig. 2A). At both light doses, the induction of *mPer1* in the SCN of $Cry2^{-/-}$ mice was reduced by 50 to 60% relative to the wild type. This reduction was roughly proportional to the reduction of total cryptochrome mRNA levels in the retina of

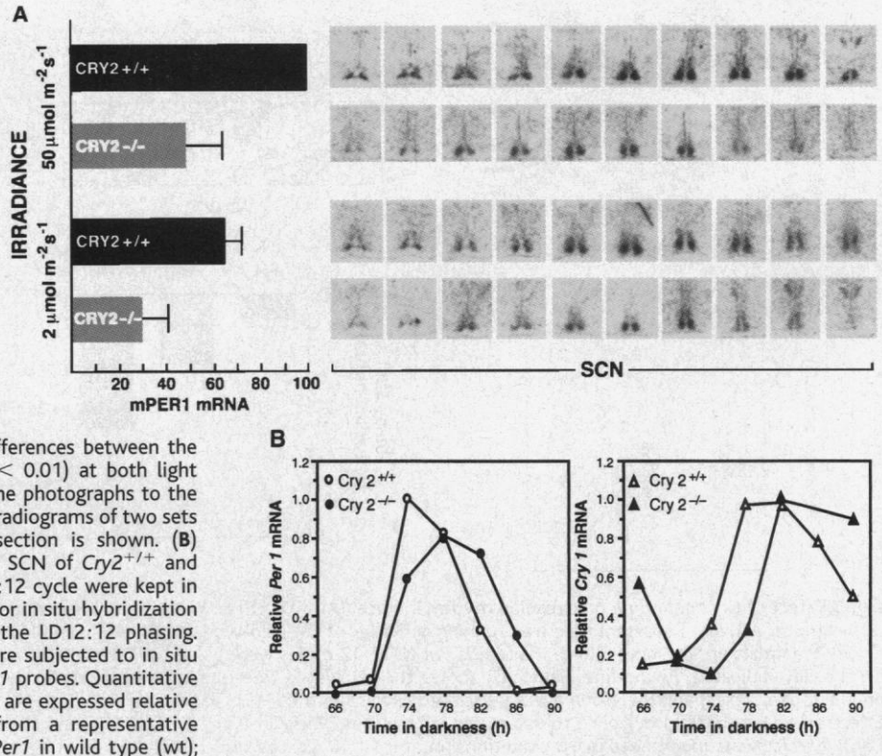

Fig. 2. (**A**) Reduced *mPer1* induction by acute light exposure in the SCN of $Cry2^{-/-}$ mutant mice. $Cry2^{+/+}$ and $Cry2^{-/-}$ mice kept under a LD12:12 cycle were exposed to a light pulse (2 μmol m^{-2} s^{-1} or 50 μmol m^{-2} s^{-1} for 30 min) at ZT18, returned to darkness for 30 min, and then decapitated. Coronal brain sections (18 μm thick) were sampled from the rostral to the caudal end (images left to right) of the SCN. The sections were hybridized to a ^{35}S-labeled probe (*24*) consisting of nucleotides 539 to 1481 of *mPer1* cDNA (GenBank accession number AB002108). Autoradiograms were digitized with a COFU 4815 charge-coupled device camera, and NIH Image 1.6 software was used for quantitation. All the *mPer1* signals from the rostral to the caudal regions of the SCN were summed (18 to 24 sections). The transcript levels are expressed relative to that in $Cry2^{+/+}$ mice after 50-μmol m^{-2} s^{-1} light pulse. The differences between the wild-type and mutant mice were significant ($P < 0.01$) at both light fluences. The bars represent SEM ($n = 4$ to 5). The photographs to the right of each column are from representative autoradiograms of two sets of experiments. For clarity, only every other section is shown. (**B**) Circadian expression of *mPer1* and *mCry1* in the SCN of $Cry2^{+/+}$ and $Cry2^{-/-}$ mice. Animals maintained under a LD12:12 cycle were kept in DD for 54 hours and then time points were taken for in situ hybridization every 4 hours starting at CT18 on the basis of the LD12:12 phasing. Coronal brain sections encompassing the SCN were subjected to in situ hybridization with ^{35}S-labeled *mCry1* (*24*) or *mPer1* probes. Quantitative analyses were performed as in (A), and the values are expressed relative to the highest wild-type figures. Data points from a representative experiment are shown. Symbols: open circles, *mPer1* in wild type (wt); closed circles, *mPer1* in $Cry2^{-/-}$; open triangles, *mCry1* in wt; closed triangles, *mCry1* in $Cry2^{-/-}$. Note the phase delay, but normal amplitude, in the $Cry2^{-/-}$ mice relative to the $Cry2^{+/+}$ animals. The phase difference between the two is consistent with the longer free-running period in the $Cry2^{-/-}$ mice (see Fig. 3).

the mutant mice (*mCry2* mRNA represents about 70% of the total) and suggests that $Cry2^{-/-}$ mice are deficient in photic induction of *mPer1*.

Although the most likely explanation for defective induction of *mPer1* in $Cry2^{-/-}$ animals is a reduction in the amount of the photoactive pigment mediating the response, the data are also consistent with an alteration of the circadian clock itself. That is, the reduction could be due to a change in the circadian regulation of *mPer1* in the SCN, rather than simply an alteration at the photoreceptor level. To determine if there were alterations in endogenous rhythmic expression of clock-related genes in the SCN of the $Cry2^{-/-}$ mice, we measured the mRNA levels of *mPer1* and of *mCry1*. Both these genes exhibit robust circadian oscillations in wild-type mice (*6, 15*), and they represent two different rhythmic outputs of the clock in the SCN. There were no differences in the amplitudes of the circadian rhythms of expression of either *mPer1* or *mCry1* in the SCNs of wild-type and *Cry2* mutant mice (Fig. 2B), suggesting that the basic oscillations of the circadian clock in the SCN are normal in $Cry2^{-/-}$ mice. The phases of *mCry1* and *mPer1* mRNA rhythms in the mutant mice, however, were shifted by about 4 hours relative to the wild type on the third cycle of constant darkness, which is consistent with a lengthening of the period by about 1 hour in the mutant.

We used locomotor activity (wheel-running) as a behavioral output to examine the mutant mice for the three basic circadian properties: intrinsic period, persistence of rhythmicity under constant conditions, and ability to synchronize with light/dark cycle (photoentrainability). There were no obvious differences in periods or amplitudes of activity among wild-type, $Cry2^{+/-}$, and $Cry2^{-/-}$ animals in LD12:12 cycles (Fig. 3, A to C). Similarly, there were no significant differences among *Cry2* genotypes in the phase angle of entrainment to LD12:12 as determined from the initial free-run in constant darkness (DD). However, as might be expected from a partial disruption of entrainment function, the $Cry2^{-/-}$ mice had significantly greater variance (wild type, 595 min; $Cry2^{+/-}$, 1150 min; $Cry2^{-/-}$, 2801 min; $F(9,11) = 4.70$; $P < 0.01$) in the free-run phase than did wild-type mice. The variance of heterozygotes was intermediate, but not significantly different from wild type. These data suggest that $Cry2^{-/-}$ mice are capable of normal entrainment to bright-light cycles, but the increased variance suggests that they do so with less precision than wild-type animals (*16*).

In contrast to its modest effect on photoentrainment, the $Cry2^{-/-}$ genotype had a drastic and paradoxical effect on the free-running period in DD (Fig. 3, A to D). The $Cry2^{-/-}$ mice had significantly ($P < 0.05$) longer free-running periods than either $Cry2^{+/-}$ or wild-type mice (Fig. 3D). One homozygous *Cry2* null animal became arrhythmic on transfer to DD but exhibited a persistent rhythm after exposure to a saturating light pulse (*17*). Thus, the *Cry2* null mutation appears not to affect the persistent rhythmic behavior under constant conditions but clearly causes an increase in the length of the intrinsic period (*18*). Lengthened circadian period may result from an alteration in some input process to the circadian pacemaker, such as an alteration mimicking constant

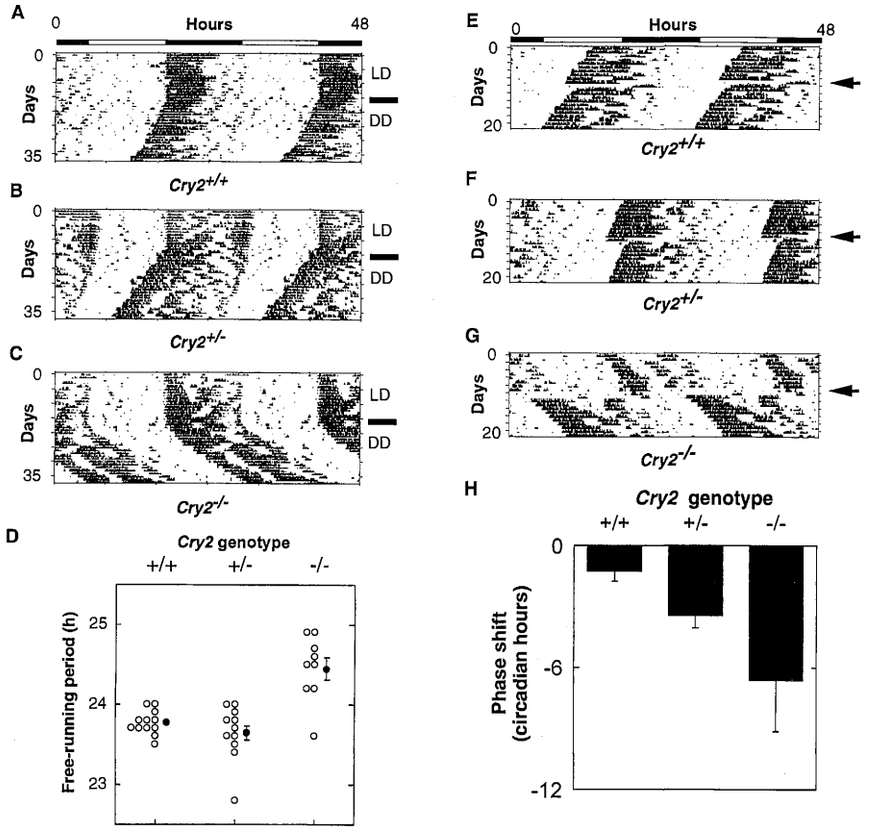

Fig. 3. Effect of *Cry2* genotype on circadian rhythm in mice. (**A** to **D**) Effect on free-running period of locomoter activity. Representative free-running activities of (A) wild-type, (B) $Cry2^{+/-}$, and (C) $Cry2^{-/-}$ animals are presented. Mice maintained in LD12:12 cycles were placed in DD conditions on the day indicated by the line. Panel (D) shows the calculated free-running periods for each animal (open circles) and the group averages (closed circles) with the SEM for each group. (**E** to **H**) Effect on phase shifts in response to saturating light pulses. (E) Wild-type, (F) $Cry2^{+/-}$, and (G) $Cry2^{-/-}$ mice were maintained in DD conditions for at least 10 days before and after a 6-hour light pulse was given at CT17 on the day indicated by an arrow. The average value for the phase shifts between CT16.5 and CT18.5 in each group of mice (n = 10, 10, and 6 for $Cry2^{+/+}$, $Cry2^{+/-}$, and $Cry2^{-/-}$ animals, respectively) is shown in (H) along with the SEM.

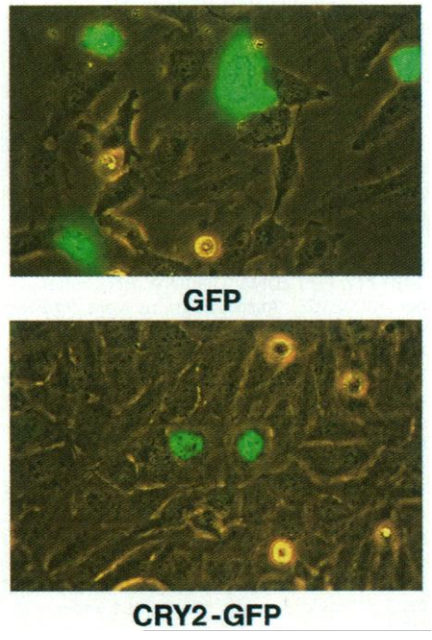

Fig. 4. Nuclear accumulation of CRY2-GFP fusion protein after transient transfection. HeLa cells were seeded at a density of 2×10^6 cells per 100-mm dish and transfected with 5 μg of either *pEGFP* (vector control) or *pHCRY2-GFP* construct by using LiipofectAMINE (Life Technologies). The cells were analyzed 4 hours after transfection with an Olympus fluorescence microscope. Magnification about ×400.

light (Aschoff's rule) (*19*), or from an alteration in the pacemaker mechanism itself. To examine these two possibilities, we determined phase shifts of the activity rhythms in response to light pulses as a measure of the circadian clock's responsiveness to light. The phases of the light pulses were determined according to the phase and period of the activity rhythm for each individual mouse, so that pulses were given at a predetermined circadian time (CT12 being defined as the onset of activity). The animals were exposed to saturating light pulses of 6-hour duration (*17*) at CT 17.5 + 1 with at least 10 days between pulses to determine the responses to light in the phase delay and near the "breakpoint" (or maximum) region of the mouse phase response curve to light (*20*).

The resulting data show that the response to saturating light pulses near the breakpoint (~CT18) was significantly different among the three genotypes (Fig. 3, E to H). The magnitude of phase shifting was highest in the *Cry2*$^{-/-}$ mice and lowest in the wild-type animals (*21*). A high-amplitude phase shift with light pulses given near the breakpoint is indicative of "type 0" phase resetting (*22*) and can occur when the strength of the stimulus is enhanced or amplified, or when the amplitude of the pacemaker is reduced. Because the *mPer1* and *mCry1* circadian mRNA rhythms in the mutant animals are normal (Fig. 2B), we suggest that the lack of CRY2 enhances the strength of the inducing signal.

The combination of reduced photic sensitivity (Fig. 2A) and increased phase shifts in response to light pulses (Fig. 3, E to H) is not without precedent. Increased phase shifts combined with reduced light sensitivities have been reported in old golden hamsters (*23*), and old mice show a lengthening of the circadian period in constant darkness and a reduced light sensitivity (*24*). Whether these phenomena are caused by alterations in the circadian photoreceptor remains to be determined. The marine dinoflagellate *Gonylaux polyhedra* contains two circadian photosensors, and complex interactions occur between these two photoreceptive pathways (*25*). In *Arabidopsis*, *Cry2* mutants antagonize the phytochrome B pathway that regulates flowering time (*10*). The increased phase shifts in the null animals implies that, in mice, CRY2 may antagonize phase-shifting responses mediated by CRY1 or another photoreceptor.

A second unexpected effect of the *Cry2* mutation was the lengthening of the free-running period. If cryptochromes function solely as photoreceptors, then they would not be expected to affect circadian rhythms in constant darkness. However, in *Arabidopsis*, photoreceptor mutants also have lengthened circadian periods (*26*). Although the "dark effect" of CRY2 seems counterintuitive, it is not surprising. The prototype of this class of proteins, DNA photolyase, in addition to repairing DNA by phototransduction, performs a dark function by interacting with the excision repair system when light is absent (*27*). Conceivably, CRY2 may directly interact with components of the pacemaker mechanism.

Recent evidence from mouse (*28*) and *Drosophila* (*29*) indicates that the molecular components of the clock are localized in the nucleus. If CRY2 functions at the interface of signal input and the clock mechanism, it should also be nuclear. Nuclear localization of cryptochromes is inferred by the presence of a bipartite nuclear localization sequence Pro-Lys-Arg-Lys-X13-Lys-Arg-Ala-Arg (where X13 represents 13 nonconserved amino acids) in mouse and human hCRY2 (*5*) and by the finding that hCRY2 interacts with the nuclear serine-threonine phosphatase 5 (*30*). To examine the subcellular localization of CRY2, we constructed a hCRY2-GFP (green fluorescent protein) fusion gene, transfected HeLa cells, and determined the localization of the fusion protein by fluorescence microscopy. CRY2 was found almost exclusively in the nucleus (Fig. 4), consistent with a possible role in circadian photoreception.

In conjunction with data from other organisms, our data suggest that cryptochromes are likely to be circadian photoreceptors. First, a *Neurospora* strain defective in riboflavin biosynthesis has a severely compromised circadian photoentrainment potential, suggesting that the *Neurospora* circadian photoreceptor, like cryptochrome, is a flavoprotein (*31*). Second, in *Drosophila*, the action spectrum for phase shifts reveals a peak in the 420- to 480-nm range (*32*), which is consistent with the absorption maximum (420 to 430 nm) of *Drosophila* cryptochrome (*33*). Furthermore, *Drosophila* defective in the visual photoreception-phototransduction system because of either β-carotene deficiency (*34*), mutations in rhodopsin encoding genes (*33*), or mutations in genes involved in visual signal transduction (*35, 36*) are nearly normal in their photoentrainment, suggesting that the pigment for circadian entrainment in *Drosophila* may be cryptochrome and not an opsin. Third, *rd/rd* (retinal degeneration) mice lose all their rods and virtually all their cones upon aging (*2, 3*); although there is no detectable opsin in the retina of 5-month-old animals (*37*) and their electroretinograms are flat (*2*), these blind mice exhibit normal circadian photoresponses (*37, 38*). The action spectrum for phase shifts in aged *rd/rd* mice has a peak at 480 nm (*39*), which is consistent with a role for cryptochromes, and significantly, the retinal ganglion cell and inner nuclear layers (where *mCry1* and *mCry2* genes are expressed) (*6*) are intact (*38*).

Finally, our results are consistent with a dual circadian photoreceptor system in mice, with CRY2 functioning as one of the photopigments. The role of CRY1 or other non-cryptochrome molecules as circadian photopigments remains to be determined.

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13. The mouse *Cry2* gene (12 kbp) was isolated from a λ Charon 35 library of genomic DNA from mouse strain 129/Ola and used to clone arms of homology that were inserted into pOSdupdel targeting vector. Homologous recombination with this construct deletes the region of the gene encoding the FAD-binding domain of the protein [H.-W. Park, S.-T. Kim, A. Sancar, J. Deisenhofer, *Science* **268**, 1866 (1995); T. Tamada *et al.*, *Nature Struct. Biol.* **4**, 887 (1997)] and replaces it with the neomycin gene, which is used for positive selection in recombinant cells. The targeting plasmid was linearized with Not I, and 2×10^7 mouse embryonic stem (ES) cells derived from a subclone (BK4) of E14TG2a of mouse strain 129 [M. Hooper *et al.*, *Nature* **326**, 292 (1987)] were electroporated with 25 μg of DNA. After positive-negative selection with ganciclovir and G418 [S. L. Mansour, K. R. Thomas, M. R. Copecchi, *ibid.* **336**, 348 (1988)], 200 surviving colonies were picked and screened by polymerase chain reaction with one primer from within the Neo gene and one from the region just 3′ to the 3′ arm of homology (Fig. 1A). Seven clones giving a positive PCR signal were identified, and the correctness of the targeting was verified by DNA blotting and hybridization. The ES cells from clone C2A were injected into C57BL/6 blastocysts, which were then implanted into pseudopregnant CD1 females. Male chimeras were bred to C57BL/6 females, and the agouti coat color was used as an indicator of transmission of the 129 genome. Transmitting agouti-coated progeny (F_1) were backcrossed to C57BL/6 animals to obtain progeny that were interbred to produce the experimental animals used in this study. Genotypic analysis of these progeny was by DNA hybridization. Subsequent screening was by PCR alone as there was complete agreement. Analyses of homozygotes by Northern (RNA) hybridization and in situ hybridization revealed a complete lack of *Cry2* mRNA in the mutants.
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Induction and Evasion of Host Defenses by Type 1–Piliated Uropathogenic *Escherichia coli*

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Virtually all uropathogenic strains of *Escherichia coli* encode filamentous surface adhesive organelles called type 1 pili. High-resolution electron microscopy of infected mouse bladders revealed that type 1 pilus tips interacted directly with the lumenal surface of the bladder, which is embedded with hexagonal arrays of integral membrane glycoproteins known as uroplakins. Attached pili were shortened and facilitated intimate contact of the bacteria with the uroplakin-coated host cells. Bacterial attachment resulted in exfoliation of host bladder epithelial cells as part of an innate host defense system. Exfoliation occurred through a rapid apoptosis-like mechanism involving caspase activation and host DNA fragmentation. Bacteria resisted clearance in the face of host defenses within the bladder by invading into the epithelium.

Urinary tract infections (UTIs) are among the most common infectious diseases acquired by humans, affecting over 7 million people annually in the United States alone and accounting for substantial morbidity (*1*). *Escherichia coli*, the primary causative agent of UTIs, including cystitis, encodes surface adhesive organelles called type 1 pili (*1*, *2*). These fibrous extensions consist of a 7-nm-thick helical rod composed of repeating FimA subunits joined to a 3-nm-wide distal tip structure containing the adhesin FimH (*3*). Binding of the FimH adhesin to mannosylated host receptors present on the bladder epithelium (urothelium) is critical to the ability of *E. coli* to colonize the bladder and cause cystitis (*2*, *4*, *5*). The lumenal surface of the bladder is lined by a layer of superficial umbrella cells that deposit on their apical surfaces a quasi-crystalline array of hexagonal complexes made up of four integral membrane glycoproteins known as uroplakins (*6*). In vitro binding assays have shown that two of the uroplakins, UPIa and UPIb, can specifically bind to *E. coli* expressing type 1 pili (*7*).

Scanning and high-resolution transmission electron microscopy (EM) techniques were used in a murine cystitis model to investigate the structural basis and consequences of the in vivo interactions between type 1–piliated *E. coli* and the uroplakin-coated host superficial bladder cells. C57BL/6 mice were infected by transurethral inoculation with the *E. coli* cystitis isolate NU14 grown in static broth (*8*), a growth condition that specifically induces the expression of type 1 pili by this strain. The type 1 pili produced by NU14 have been well characterized (*2*, *5*, *9*). Two hours after infection, mouse bladders were removed and processed for EM (*10*). As determined by scanning EM (SEM), numerous bacteria adhered randomly across the bladder lumenal surface, both singly and in large, biofilmlike microcolonies, some of which contained several hundred bacteria (Fig. 1A). The bacteria were often situated in grooves and niches formed by the apical membrane of the superficial cells (Fig. 1B). This uroplakin-embedded membrane has been termed the asymmetric unit membrane (AUM) because of its appearance in cross section (*6*). In contrast to NU14-infected bladders, almost no bacteria were found attached to bladders taken from mice infected with the isogenic *fimH*$^-$ mutant NU14-1 (*2*, *11*), confirming the role of the FimH adhesin in colonization of the bladder.

Examination of infected mouse bladders with high-resolution, freeze-fracture, deep-etch EM revealed the tips of type 1 pili making direct contact with the AUM (Fig. 1, C to H) (*12*). Type 1 pili spanned the distance between the bacterial outer membrane and the AUM directly, with seemingly little slack along their lengths (Fig. 1H). The molecular architecture of these pili was identical to the type 1 pili present on the inoculated NU14 bacteria (*3*, *9*), except that they were shorter. Of 70 pili measured from 23 individual bacteria, the average pilus length from the bacterial outer surface to the host cell membrane was 0.12 ± 0.07 μm. In contrast, type 1 pili present on bacteria in broth culture are typi-

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