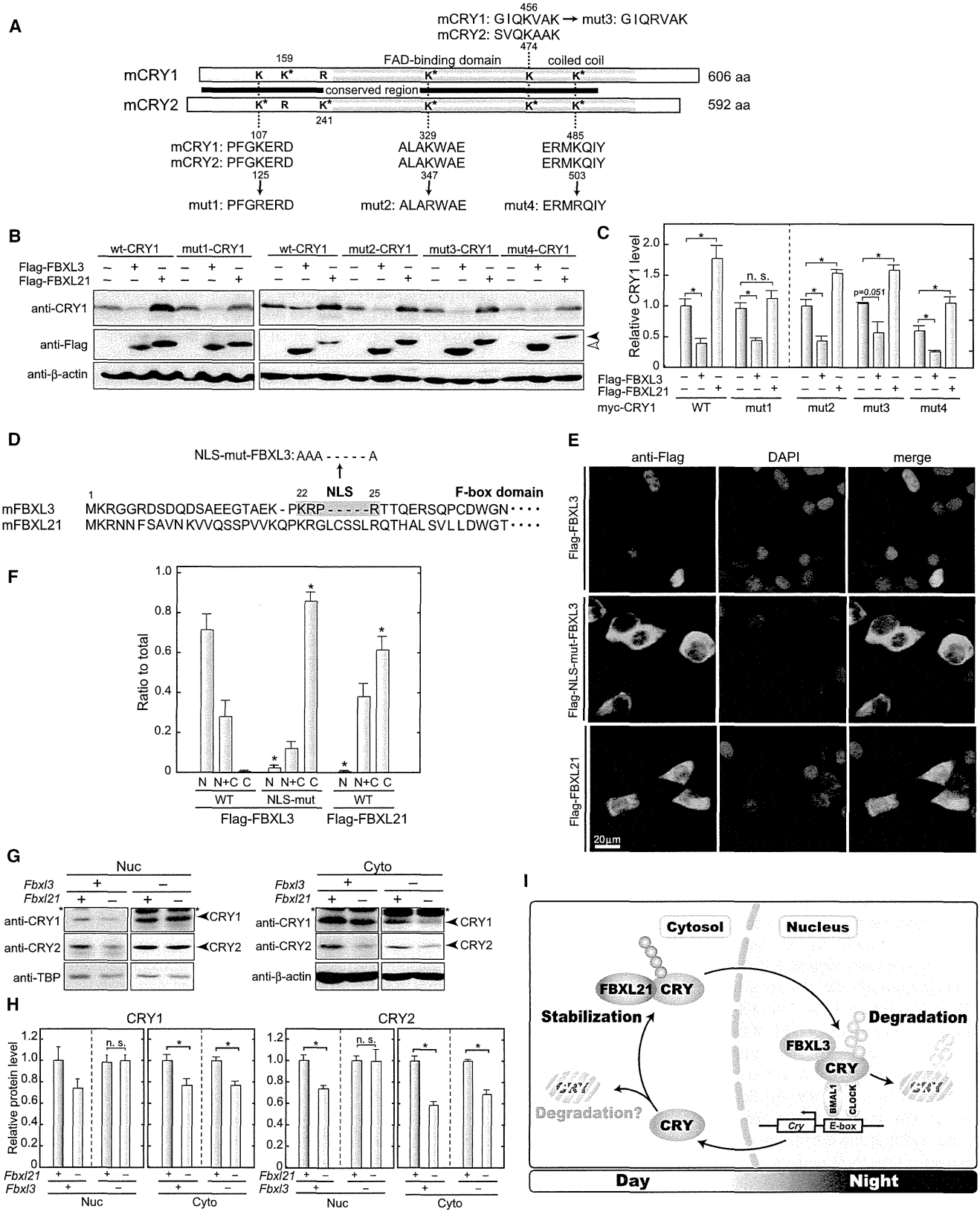

(legend on next page)

directed by posttranslational modifications (Brooks and Gu, 2003; Perkins, 2006) underlies the regulatory processes of many biological events.

EXPERIMENTAL PROCEDURES

Animals

All the mouse experiments were approved by the animal ethics committee of The University of Tokyo and Kyushu University. Mice were housed in cages with free access to commercial chow (CLEA Japan) and tap water. See Extended Experimental Procedures for details on the generation of *Fbxl3*$^{-/-}$ and *Fbxl21*$^{-/-}$ mice.

Wheel-Running Activity

Five- to ten-week-old mice were housed individually in cages equipped with running wheels. The animals were maintained in a light-tight chamber at a constant temperature (23°C ± 1°C) and humidity (65% ± 10%). Mice were entrained to the LD cycle for at least 5 weeks and released into DD for 21 days or longer. Wheel revolutions were recorded in 5 min bins and analyzed with ClockLab analysis software (Actimetrics). The circadian period and periodogram amplitude of the activity rhythms in DD were determined using chi-square periodogram procedure with ClockLab.

Cell Culture and Plasmids for Transfection

MEFs were prepared from E12.5 embryos. After the head, paws, and internal organs were removed, embryos were chopped and incubated in 0.25% trypsin in PBS for 24 hr at 4°C. After incubation for 30 min at 37°C in 0.25% trypsin in PBS, cells were dissociated by pipetting. MEFs, NIH 3T3 (Riken Cell Bank), and HEK293T17 cells were cultured and passaged under 5% CO_2 in DMEM (Nissui) containing 1.8 mg/ml $NaHCO_3$, 4.5 mg/ml glucose, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Equitech Bio). NIH 3T3 and HEK293T17 cells were transiently transfected using Lipofectamine Plus reagent (Invitrogen) and Lipofectamine 2000 reagent (Invitrogen), respectively, according to the manufacturer's protocols. Plasmids used for transfection are described in the Extended Experimental Procedures.

Immunoblotting

Proteins separated by SDS-PAGE were transferred to polyvinylidene difluoride membrane (Millipore). The blots were blocked in a blocking solution (1% [w/v] skim milk in TBS [50 mM Tris-HCl, 140 mM NaCl, 1mM $MgCl_2$ (pH 7.4)]) for 1 hr at 37°C and then incubated overnight at 4°C with a primary antibody in the blocking solution. The signals were visualized by an enhanced chemiluminescence detection system (PerkinElmer Life Science). The blot membrane was subjected to densitometric scanning and the band intensities were quantified using Image Gauge Ver.4.0 software (Fujifilm Science Lab). Antibodies were described in the Extended Experimental Procedures.

Degradation Assay

Cells were transfected with FHM- or myc-CRY1 and Flag-FBXLs expression vectors and cultured for 48 hr. The transfected cells were then treated with 100 μg/ml cycloheximide (Nakalai tesque) for time periods specified in the figures and harvested, followed by immunoblotting. Degradation assay in cultured MEFs was performed in essentially the same way except that DNA was not transfected.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, one table, and Extended Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.01.054.

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Figure 6. Identification of Ubiquitination Sites by Mass Spectrometry Analysis

(A) Ubiquitination sites in CRY1 and CRY2 identified by mass spectrometry analysis. Overexpressed FHM-CRY1 or FHM-CRY2 in NIH 3T3 cells was purified by Flag antibody and subjected to mass spectrometry analysis. Ubiquitinated Lys residues (three residues in CRY1 and five in CRY2) are highlighted with asterisks. The gray horizontal bar indicates a region highly conserved between mouse CRY1 and CRY2.

(B) Effect of coexpression of FBXL3 or FBXL21 on mutant CRY1 protein levels. Mut(1-4)-CRY1 and Flag-FBXLs were expressed in HEK293T17 cells. Steady-state levels of CRY1 protein were determined by immunoblotting analysis with CRY1 antibody. The solid and open arrowheads indicate Flag-FBXL21 and Flag-FBXL3, respectively.

(C) Each mut-CRY1 protein levels were quantified and shown with error bars of SEM (n = 3, *p < 0.05 by Tukey's test).

(D) Mouse FBXL3 has an NLS sequence, which is not conserved in FBXL21.

(E) Intracellular distribution of FBXL3, NLS-mut-FBXL3, and FBXL21. Flag-FBXL3, Flag-NLS-mut-FBXL3, or Flag-FBXL21 was expressed in HEK293T17 cells and immunostained with anti-Flag antibody. Cell nuclei were stained with DAPI.

(F) Quantitative analysis of the subcellular distribution of FBXL3, NLS-mut-FBXL3, and FBXL21. The data were obtained from three independent experiments as in (E), and in each experiment at least 100 cells were visually examined and counted. Data are means + SEM. *p < 0.05 by Tukey's test.

(G) CRY1 and CRY2 protein levels in the mouse cerebrum. The mouse cerebrum lysate prepared at ZT18 were fractionated into the nuclear and cytosolic fraction. The same amounts of proteins (50 μg cytosolic proteins or 20 μg nuclear proteins) were loaded.

(H) The band intensities of CRY1 and CRY2 in (G) were quantified and the highest value was set at 1.0. Data are means + SEM (n = 4, *p < 0.05 by Student's t test).

(I) In the cytosol, FBXL21 stabilizes CRY proteins, probably by counterbalancing a degradation mechanism (in gray). This controls appropriate rate of accumulation of translated CRYs in their increasing phase during the daytime. In the nucleus, FBXL3 leads CRYs to proteasomal degradation, which is also competed by FBXL21-mediated stabilization, in the declining phase of CRYs during the nighttime. Dual regulation of CRY protein stabilities by a combination of FBXL21 and FBXL3 plays a critical role for robust oscillation of the circadian clock.

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