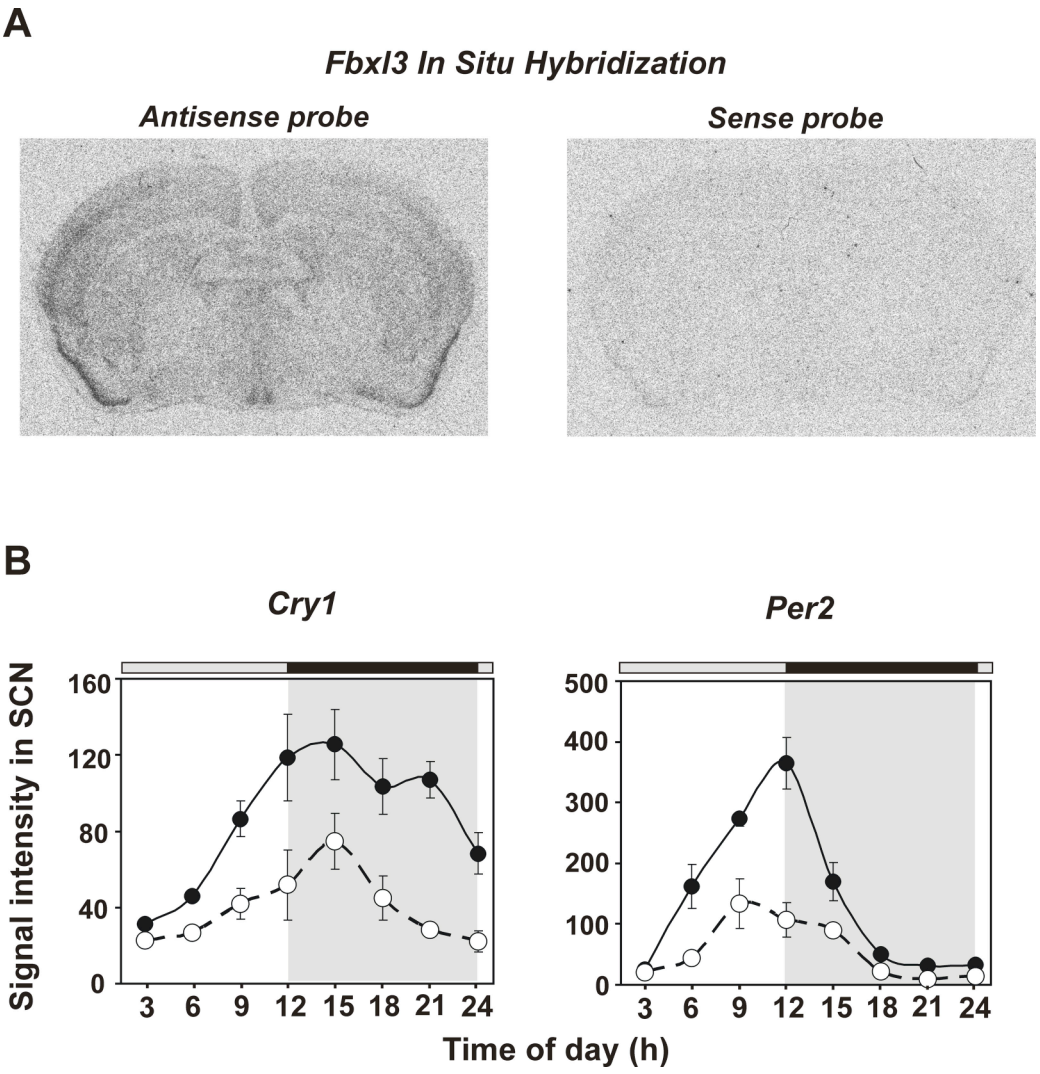
Cell, Volume *129*

Supplemental Data

Circadian Mutant *Overtime* Reveals F-box Protein FBXL3 Regulation of *Cryptochrome* and *Period* Gene Expression

Sandra M. Siepka, Seung-Hee Yoo, Junghea Park, Weimin Song, Vivek Kumar, Yinin Hu, Choogon Lee, and Joseph S. Takahashi



Supplemental Figure 1. In situ hybridization of *Fbxl3, Cry1* and *Per2* in the SCN

(A) In situ hybridization was performed with *Fbxl3* probe on brains from WT mice killed at ZT 12. Left panel: antisense probe. Right panel: sense probe.

(B) Circadian rhythm of *Cry1* and *Per2* expression in WT and *Ovtm* mice in the SCN. The expression of *Cry1* and *Per2* are both significantly reduced in the SCN. Mice were maintained in constant darkness for two weeks before being killed, and three animals were collected at each CT point. Filled circles with solid line represent wildtype, and open circles with dotted line represent values from *Ovtm* mice.

EXPERIMENTAL PROCEDURES

Heritability testing.

Putative mutants (or putants) were mated with wild-type *BTBR* T^+ tf/J mice to produce G4 offspring. These G4 mice were intercrossed to produce G5 mice for phenotyping. All G3 mice that produced G5 mice with the identical or with a more extreme phenotype were considered bona fide mutants.

Genetic mapping

The following SSLP markers were used to map *Ovtm* to mouse chromosome 14: *D1Mit440*, *D2Mit395*, *D3Mit256*, *D4Mit17*, *D5Mit277*, *D6Mit116*, *D6Mit209*, *D6Mit373*, *D7Mit267*, *D8Mit281*, *D9Mit336*, *D10Mit230*, *D11Mit4*, *D12Mit91 D14Mit39*, *D14Mit60*, *D15Mit107*, *D16Mit139*, *D17Mit51*, *D18Mit222* and *D19Mit28 (Dietrich et al., 1992; Dietrich et al., 1996)*. The location of the *Ovtm* mutation was narrowed to a smaller region of mouse chromosome 14 using the following markers: *D14Mit39*, *D14Mit7*, *D14Mit197*, *D14Mit170*, *D14Mit200*, *D14Mit97*, *D14Mit157*, *D14Mit265* and *D14Mit266*. PCR conditions were as follows: 1x Amplitaq buffer + Mg, 1 mM forward primer, 1 mM reverse primer, 100 mM dNTP mix, 1M betaine, 50 – 100 ng template DNA and 1 unit Amplitaq. PCR cycling conditions were as follows: 95^{o}C for 2 minutes followed by 35 cycles of 95^{o}C for 15 seconds, 52^{o}C for 30 seconds and 72^{o}C for 15 seconds, followed by a final extension time of 72^{o}C for 3 minutes. PCR products were separated on a 3% agarose/1% NuSieve gel in 1x TBE buffer and visualized by ethidium bromide staining. Linkage analysis was performed using MapManager QTXb18 software (Manly et al., 2001).

Gene trap mouse production

Mouse embryonic stem cell gene trap line S13-12G1 was obtained from the Soriano Lab Gene Trap Resource at the Fred Hutchinson Cancer Research Center, Seattle WA (http://www.fhcrc.org/science/labs/soriano/trap.html). The cell line contains a ROSAFARY gene trap vector (Chen et al., 2004) that disrupts expression of *Fbxl3* to create a null allele of *Fbxl3*. The cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) with high glucose, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 0.1 mM b-mercaptoethanol, 15% fetal bovine serum (FBS), penicillin and streptomycin (50 mg/ml each) and 1000 units/ml Leukemia Inhibitory Factor (LIF) in an incubator at 37^{o}C with 10% CO_2.

Three-week-old C57BL/6J female mice were superovulated by intraperitoneal injection of 5 units of pregnant mare serum gonadotropin (Sigma # G-4877, St. Louis, MO) at noon on day 1 and 5 units of human chorionic gonadotrophin (hCG) (Sigma # CG-5) at noon on day 3. After the hCG injection, the females were immediately mated to C57BL/6J males. The following morning, the females were checked for vaginal plugs. At 3.5 days post coitum, blastocysts were collected from the female mice and placed in M2 medium. The blastocysts were incubated in M16 medium in an incubator at 37^{o}C, 5% CO_2.

Twelve to fifteen ES cells were injected into a single blastocyst. Six to twelve blastocysts were implanted into a single pseudo-pregnant CD1 female mouse. (CD-1 females were mated to vasectomized male mice to produce pseudo-pregnant CD-1 female mice.) Chimeric mice were identified based on coat color. We obtained 19/19 chimeric mice using this protocol. Germline transmission was test in 4 chimeras and 3/4 successfully transmitted the *Fbxl3* gene trap (the fourth mouse was infertile).

High percentage (>95%) ES cell chimeric male mice were mated directly to 6-8 week old *Ovtm/Ovtm* female mice to produce the pups for the complementation assay.

The mice produced from this mating were assayed for altered circadian behavior at 6 weeks of age.

Mice were genotyped for the *Fbxl3* gene trap by PCR to detect the presence of the reporter gene b-*geo*. PCR conditions were as follows: 1x Amplitaq buffer + Mg, 1 mM forward primer (5'-AGTGACAACGTCGAGCACAG-3'), 1 mM reverse primer (5'-CGGTCGCTACCATTACCAGT-3'), 100 mM dNTP mix, 1M betaine, 50-100 ng template DNA and 1 unit Amplitaq. PCR cycling conditions were as follows: 95^{o}C for 2 minutes followed by 35 cycles of 95^{o}C for 30 seconds, 55^{o}C for 30 seconds and 72^{o}C for 45 seconds, followed by a final extension time of 72^{o}C for 10 minutes. The PCR products were separated on a 1.5% agarose gel in 1x TBE buffer and visualized by ethidium bromide staining. This primer set produces a single 300 bp PCR product. In addition, progeny from the complementation cross were also genotyped for the *Ovtm* mutation as described in Experimental Procedures.

Tissue Collection

For tissue collection, wildtype and *Ovtm* mice were entrained to LD12:12 on running wheels. After 5 days in LD, the mice were released into constant darkness and allowed to free run for 10-14 days. Period was calculated as described above. Circadian time (CT) was calculated for each animal before tissue collection began. Three male mice and two female mice for both *Ovtm* and wild-type mice were killed and optic nerves severed under infrared light by cervical dislocation at CT= 3, 6, 9, 12, 15, 18, 21 and 24. Tissues were harvested was under normal room lights, immediately frozen on dry ice and stored at -80^{o}C.

Primer sequences used for real-time PCR:

Cry1 F 5' -TGA GGC AAG CAG ACT GAA TAT TG- 3'
Cry1 R 5' -CCT CTG TAC CGG GAA AGC TG- 3'
Cry2 F 5' -CTG GCG AGA AGG TAG AGT GG - 3'
Cry2 R 5' -GAC GCA GAA TTA GCC TTT GC - 3'
Per1 F 5' -CCC AGC TTT ACC TGC AGA AG- 3'
Per1 R 5' -ATG GTC GAA AGG AAG CCT CT- 3'
Per2 F 5' –TGT GCG ATG ATG ATT CGT GA- 3'
Per2 R 5' –GGT GAA GGT ACG TTT GGT TTG C- 3'
DBP F 5' -CGA AGA ACG TCA TGA TGC AG- 3'
DBP R 5' -GGT TCC CCA ACA TGC TAA GA- 3'
Per2-pre mRNA primers for liver
595 F 5' -CAGATGGACTGGGAGTATGTCA- 3'
686 R 5' -AGCCAAGGTATGTCTCAAAGGA- 3'
Per2-pre mRNA primers for cerebellum
3540 F 5' -CTT TGC TCG AGT CTG TTC TCC T- 3'
3620 R 5' -AAG GGA CAA GGA TGG TAT GAA G- 3'
Cry1-pre-mRNA
1028 F 5' – TCT GGG GGA AAG TAC TAG GTG A- 3'
1130 R 5' –ACT CTC CTG GGG TAA AGT CAC A- 3'
mGAPDH F 5' -CAAGGAGTAAGAAACCCTGGACC- 3'
mGAPDH R 5' -CGAGTTGGGATAGGGCCTCT- 3'

DNA Constructs

The *Fbxl3* coding region was amplified from EST BC067203 and cloned into pDONR221. The *Ovtm* mutation was created using Quickchange Mutagenesis kit

(Stratagene). After the mutagenesis, the entire coding region was sequenced to confirm the presence of the desired mutation and the absence of additional mutations. The primers used for mutagenesis are fbxl2OVRT S 5' - GCA AAA ATT TGT CAG CTA CTG GGC TGG GGG AAT- 3' 376 to create the *Ovtm* mutation in *Fbxl3*, GCTATT to GCTACT, Ile to Thr; fbxl2OVRT AS 5' - ATT CCC CCA GCC CAG TAG CTG ACA AAT TTT TGC - 3' 377 to create the OVRT mutation in *Fbxl3*, GCTATT to GCTACT, Ile to Thr. A FLAG tag was added by LR recombination (Invitrogen).

In situ hybridization

Brains were collected at the indicated times, and immediately frozen on dry ice. *In situ* hybridization was performed as described previously (Wilsbacher et al., 2002). Templates for the antisense and sense *Per2* and *Cry1* probes were PCR-generated by using the following primers: Per2-insitu-f 5' - ACG AGA ACT GCT CCA CGG GAC - 3', Per2-insitu-r 5' - ACA GCC ACA GCA AAC ATA TCC GC - 3', Cry1-insitu1f(1890) 5' - TAA GAG GCT TCC CTG CAA AA - 3', Cry1-insitu1r(2220) 5' - CTA CAG CTC GGG ACG TTC TC - 3'. PCR products were cloned into TA cloning vector (Invitrogen). T7 promoter from the TA cloning vector was used to generate both antisense and sense probes. SCN signal density was quantified using ImageJ 1.34s software (NIH, Bethesda, MD) and normalized to radioactive standards as described previously (Sangoram et al., 1998) .

REFERENCES

Chen, W.V., Delrow, J., Corrin, P.D., Frazier, J.P., and Soriano, P. (2004). Identification and validation of PDGF transcriptional targets by microarray-coupled gene-trap mutagenesis. Nat Genet *36*, 304-312.

Dietrich, W., Katz, H., Lincoln, S.E., Shin, H.-S., Friedman, J., Dracopoli, N.C., and Lander, E.S. (1992). A genetic map of the mouse suitable for typing intraspecific crosses. Genetics *131*, 423-447.

Dietrich, W.F., Miller, J., Steen, R., Merchant, M.A., Damron-Boles, D., Husain, Z., Dredge, R., Daly, M.J., Ingalls, K.A., O'Connor, T.J.*, et al.* (1996). A comprehensive genetic map of the mouse genome. Nature *380*, 149-152.

Manly, K.F., Cudmore, R.H., Jr., and Meer, J.M. (2001). Map Manager QTX, cross-platform software for genetic mapping. Mamm Genome *12*, 930-932.

Sangoram, A.M., Saez, L., Antoch, M.P., Gekakis, N., Staknis, D., Whiteley, A., Fruechte, E.M., Vitaterna, M.H., Shimomura, K., King, D.P.*, et al.* (1998). Mammalian circadian autoregulatory loop: a timeless ortholog and mPer1 interact and negatively regulate CLOCK-BMAL1-induced transcription. Neuron *21*, 1101-1113.

Wilsbacher, L.D., Yamazaki, S., Herzog, E.D., Song, E.J., Radcliffe, L.A., Abe, M., Block, G., Spitznagel, E., Menaker, M., and Takahashi, J.S. (2002). Photic and circadian expression of luciferase in mPeriod1-luc transgenic mice in vivo. Proc Natl Acad Sci USA *99*, 489-494.