# Supporting Online Material

## Materials and Methods

### Diagnostic and classification criteria

Subjects signed a consent form approved by the Institutional Review Boards at the University of Utah and University of California San Francisco. Self-reported habitual sleep-wake schedules were obtained during structured interviews by one of the authors (C.R.J.) and some were confirmed by 10-day sleep logs with coincident wrist actigraphy (Ambulatory Monitoring Inc, model "Micromini" recorded in zero-crossing mode with one-minute bins and analyzed by the Cole-Kripke algorithm). The short-sleeper mutation-carriers had strikingly early morning wake-up times but neither of them fit clinical or research criteria for FASPS because they lacked the early evening sleep-onset times, even in the absence of psycho-social obligations or opportunities, that typify FASPS (*1, 2*). One non-mutation carrier did have an early morning wake-up time but had a conventional habitual sleep duration of 7.5 hours in his fifth decade (currently). This is a trait that has been lifelong in the 2 mutation carriers. The early awakening despite a conventional sleep onset time was reported by the 44 year old daughter to have continued from childhood to adulthood, with a short reprieve during adolescence. The 69 year old mother recalled no significant change in her habitually short sleep time since the age of 22. Blood sample collection and DNA preparation were performed as previously described (*2*).

### Identification of mutation in *DEC2*

We have collected human families that show habitual early rising trend in the family. In order to identify the mutations that are responsible for their phenotype, we used candidate gene approach to screen at least one DNA sample from each family. In our screening

procedures, we included all clock related genes including all the core clock components (*CLK, BMAL, PER, CRY, DEC,* and *CKI*). *DEC1 and 2* have been shown to be circadian related genes and were therefore screened. *DEC2 P385R* mutation was found only in this family (out of 60 families). No other mutation was found in mutation carriers in this family. One hundred bases into each intron were included in all the sequencing for exons of candidate genes.

**Generation of *DEC2* transgenic mice**

WT and P385R *DEC2* transgenic mice were generated according to a procedure described previously (*3*). Human BAC: RP 11-288E19, containing the entire *DEC2* gene on a 132-kb genomic insert, was obtained from Children's Hospital Oakland Research Institute. This BAC clone includes 54 kilobases (kb) of sequence upstream from the start codon and is expected to contain all cis-acting regulatory elements needed to accurately reproduce an endogenous expression pattern (*4*). All relevant segments generated by PCR and recombination were sequence confirmed. Detailed mapping was carried out for the modified BAC to ensure that the correct construct was obtained. All mouse work was performed in accordance with the guidelines of University of California San Francisco Institutional Animal Care and Use Committee.

**Locomotor activity analysis**

To assess α and daily activity, 11-18 *DEC2-P385R* mice and their WT littermate controls (3-4 month old) were individually housed in cages with running wheels. Locomotor activity was determined by electronically counting revolutions of the running wheel in 6 min intervals using Clocklab (Colbourn Instruments). The procedure for intrinsic period determination was described previously (*5*). To determine α, the daily onset and offset of locomotor activity was identified and the intervening time calculated. For each animal, daily α was averaged over a 7 day period in LD (12 hours light:12 hours dark). Activity

profiles varied slightly between experiments; the comparisons shown in each figure were thus always from the same experiment. Values were evaluated statistically using Student's t-test with a significance threshold of $p<0.05$.

**Surgery, EEG/EMG monitoring and analysis**

The surgery, EEG/EMG monitoring, and EEG data acquisition were performed for the indicated number of 6-7 month old mice in the Stanford Sleep and Circadian Neurobiology Laboratory and were carried out as described previously (*6*). Wakefulness was determined by low amplitude and mixed frequency (> 4 Hz) EEG with continuous large fluctuation in EMG, slow-wave or non-REM (NREM) sleep was determined by high amplitude and low frequency (0.25–4 Hz) EEG with no fluctuation in EMG, and REM sleep was determined by low amplitude and high frequency EEG (similar to wake stage, but with rhythmic α waves at 8–9 Hz) with no fluctuation in EMG. For power spectral analysis, NREM delta and REM theta EEG power were analyzed with fast Fourier transform (FFT) for band frequencies between 0.25–4 Hz and 4–9 Hz, respectively.

**Fly strains, transgenesis, cultures, and rest/activity measurements**

Transgenic flies were outcrossed to an isogenic wild-type strain for over 10 generations. Flies were cultured and tested at 25°C, 68% humidity, on yeast, dark corn syrup and agar food. UAS- constructs of full length m*Dec2* with or without the human point mutation were subcloned into the pUAST vector for embryo injection to generate transgenic flies. A GFP or FLAG tag was inserted on the N-terminus before the start codon of WT and P385R m*Dec2*..

For rest/activity analysis, 1-2 days old flies were monitored in light-dark conditions at 25°C. Locomotor activity of individual flies was monitored and collected

with the DAM system as previously described (*7*). All analyses were based on the definition of fly 'sleep-like behavior' as 5-6 minutes of immobility. Daily 'sleep-like behavior' was averaged over 4 days (12 hrs of light: 12 hrs of dark). For each genotype, activity was recorded (beam crossings) for three independent transgenic lines with 28-32 female flies from each line.

**Cell Culture, transfections, Co-immunoprecipitation, and luciferase assay**

HEK293 cells were used for all transfections and maintained in Dulbecco's modified medium (DMEM, Hyclone) with 10% fetal bovine serum (FBS, Sigma). Cells were plated for 2-3 days in 96-well plates (luciferase assay) or 10cm petri-dishes (co-immunoprecipitation) and transiently transfected with plasmids using LipofectAMINE 2000 (Invitrogen). For luciferase assays, cells were harvested in passive lysis buffer and the luciferase assays were preformed according to the manufacurer's instructions (Promega). The co-transfected mixtures contained WT or mutant (P385R, R57A and R57K) *mDec2*, *Clock/Bmal1*, m*Per2* promoter (in firefly luciferase reporter plasmid). Empty vector plasmid was used to equalize the amount of plasmid DNA for each transfection. Transfection efficiencies were normalized by co-transfecting 0.2 or 5ng of the HSV-TK generating Renilla luciferase activity. All transfections were repeated at least 5 times. For HDAC inhibitor experiments, TSA was applied to cells 24 hrs following plasmid transfections. Luciferase activity was measured in untransfected cells for controls. Co-immunoprecipitations were performed using an immunoprecipitation kit (Roche). The antibody for protein pull-down was anti-FLAG monoclonal antibody (Sigma). The precipitates were analyzed for the presence of mDec2 and hSIRT1 by western blots with anti-GFP and anti-FLAG (AbCam) antibodies. GFP-*mDec2* and FLAG-*hSIRT* were replaced with empty FLAG-cMV and GFP-N1 vectors as controls for co-immunoprecipitation.

## DNA subcloning and constructs

For luciferase and co-immunoprecipitation assays, mouse *Dec2* was subcloned into the pCMV-Tag2A expression vector (Stratagene). The Pro385 to Arg, Arg57 to Ala or Lys substitutions were generated by Quick-Change Site-directed Mutagenesis (Stratagene) and confirmed by sequencing. A 1Kb *Per2* promoter (containing three tandem copies of E-box) was subcloned into the luciferase reporter plasmid pGL3 (Promega).

## Statistical Analysis

Data were presented as mean ± s.e.m. and data comparison was undertaken using Student's *t* test or one-way ANOVA with Newman-Keuls post hoc test. Significant difference was set at $P<0.05$ unless otherwise stated.

**Supplementary Table 1**. Period lengths in hours of *Dec2 KO* (*Dec2 -/-*), *DEC2* heterozygous KO (*DEC2* $^{-/+}$), wild-type C57B6, *DEC2-P385R* Tg and *DEC2-P385R* Tg in *Dec2* heterozygous KO background (*DEC2-P385R Tg / DEC2* $^{-/+)}$ under 12 hours of light: 12 hours of dark (LD) and constant darkness (DD).

| | **Period – LD** | **Period - DD** |
|---|---|---|
| *DEC2* $^{-/-}$, n=19 | 23.98 ± 0.02 | 23.81 ± 0.06 |
| *DEC2* $^{-/+}$, n=10 | 24.03 ± 0.01 | 23.78 ± 0.06 |
| WT, n=10 | 24.02 ± 0.02 | 23.68 ± 0.06 |
| *DEC2-P385R* Tg, n=12 | 24.04 ± 0.02 | 23.78 ± 0.09 |
| *DEC2-P385R Tg / DEC2* $^{-/+}$, n=11 | 24.03 ± 0.01 | 23.78 ± 0.06 |

**Supplemental Table 2**. Baseline and SD Mean percentage sleep analysis of wakefulness, NREM, and REM time of *DEC2-P385R* mice and WT littermates in baseline (Day1) and sleep recovery day after SD (Day2).

| | | Day 1 | | | | Day 2 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L1 | L2 | D1 | D2 | SD | L2 | ΔL2 | D1 | ΔD1 | D2 | ΔD2 |
| % Wake | WT | 34.9±1.0 | 34.3±2.6 | 76.1±3.6 | 65.7±3.6 | --- | 32.2±1.6 | -6.1 | 57.3±4.1 | -24.7 | 54.1±4.3 | -17.6 |
| | TG | 42.5±1.9 | 43.3±3.1 | 76.5±5.3 | 67.5±3.2 | --- | 38.5±2.4 | -11.1 | 71.5±3.2 | -6.5 | 65.1±3.0 | -3.5 |
| | P<0.05 | * | * | | | | * | * | * | * | * | * |
| % NREM | WT | 57.6±1.3 | 57.7±2.1 | 22.4±3.4 | 31.9±3.5 | --- | 60.1±1.3 | 4.0 | 38.5±3.9 | 71.8 | 40.5±3.9 | 27.2 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NREM | TG | 51.9±1.8 | 50.6±3.0 | 21.9±4.7 | 30.3±2.8 | --- | 55.5±2.0 | 9.5 | 25.7±3.0 | 17.1 | 30.1±2.6 | 2.0 |
| | P<0.05 | * | * | | | | * | * | * | * | * | * |
| % REM | WT | 7.5±0.7 | 8.0±1.0 | 1.5±0.4 | 2.4±0.3 | --- | 7.7±0.7 | -3.1 | 4.2±0.3 | 175.2 | 5.3±0.8 | 122.4 |
| | TG | 5.6±0.5 | 6.1±0.6 | 1.6±0.7 | 2.2±0.4 | --- | 6.1±0.8 | 0.1 | 2.8±0.4 | 74.4 | 4.0±0.7 | 82.3 |
| | P<0.05 | * | | | | | | | * | * | | |

**Supplemental Table 3.** Baseline episodes of *DEC2-P385R* mice and WT littermates.

| | | Mean duration (sec) | | | Episode number | | |
|---|---|---|---|---|---|---|---|
| | | WT | TG | p<0.05 | WT | TG | p<0.05 |
| 12 hrs | Light | | | | | | |
| | Wake | 116 ± 12 | 97 ± 10 | * | 133 ± 10 | 193 ± 12 | * |
| | NREM | 184 ± 5 | 118 ± 3 | * | 139 ± 9 | 190 ± 10 | * |
| | REM | 64 ± 3 | 63 ± 3 | | 53 ± 6 | 41 ± 5 | |
| | Dark | | | | | | |
| | Wake | 473 ± 79 | 314 ± 48 | | 80 ± 10 | 114 ± 19 | |
| | NREM | 156 ± 6 | 112 ± 5 | * | 80 ± 10 | 112 ± 18 | |
| | REM | 65 ± 5 | 62 ± 6 | | 14 ± 2 | 15 ± 3 | |
| 24 hrs | Wake | 231 ± 23 | 167 ± 17 | * | 212 ± 19 | 306 ± 28 | * |
| | NREM | 174 ± 4 | 114 ± 3 | * | 219 ± 18 | 301 ± 25 | * |
| | REM | 63 ± 2 | 61 ± 3 | | 67 ± 7 | 56 ± 8 | |

**Supplemental Table 4.** Baseline and SD percentage sleep analysis of *Dec2* KO mice

| | | Day 1 | | | | Day 2 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | L1 | L2 | D1 | D2 | SD | L2 | ΔL2 | D1 | ΔD1 | D2 | ΔD2 |
| % Wake | WT | 36.0±2.6 | 39.2±3.9 | 80.8±1.2 | 64.5±3.0 | --- | 30.8±1.8 | -21.5 | 65.3±2.9 | -19.2 | 59.6±3.4 | -7.5 |
| | KO | 31.2±1.4 | 34.3±3.0 | 65.3±4.1 | 62.7±2.7 | --- | 32.9±1.6 | -4.4 | 55.0±1.4 | -15.8 | 61.3±4.2 | -2.1 |
| | P<0.05 | | | * | | | | * | * | | | |
| % NREM | WT | 56.5±1.9 | 54.6±3.4 | 18.1±1.1 | 33.0±2.7 | --- | 60.7±1.4 | 11.2 | 31.2±2.6 | 72.0 | 36.2±2.6 | 9.6 |
| | KO | 61.4±1.5 | 58.8±2.4 | 32.5±3.8 | 35.1±2.7 | --- | 58.8±1.4 | -0.1 | 39.7±1.5 | 22.2 | 34.2±3.9 | -2.5 |
| | P<0.05 | * | | * | | | | * | * | * | | * |
| % REM | WT | 7.6±0.9 | 6.2±0.6 | 1.0±0.2 | 2.5±0.4 | --- | 8.5±0.6 | 37.9 | 3.5±0.5 | 245.0 | 4.2±0.8 | 66.2 |
| | KO | 7.5±0.4 | 6.8±0.8 | 2.2±0.4 | 2.3±0.2 | --- | 8.4±0.2 | 23 | 5.3±0.7 | 139.2 | 4.5±0.7 | 97.8 |
| | P<0.05 | | | * | | | | | * | * | | |

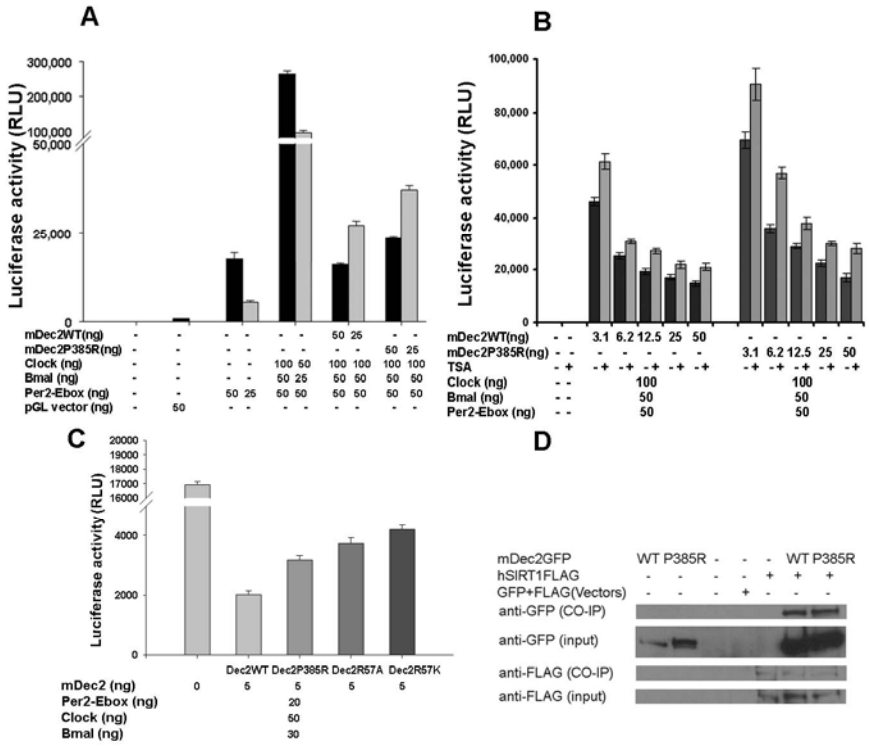


**fig. S1.** *In vitro* analyses of Dec2P385R. (**A**) Luciferase activity of reporter construct containing the *Per2* E-box. Results from two different concentrations of mDec2WT or mDec2P385R expression vectors are shown. Luciferase activities (means ± s.e.m; $n = 8$) were measured after a 24-h incubation. (**B**) Luciferase activity of *Per2* E-Box reporter construct co-transfecting with various amounts of mDec2WT or mDec2P385R expression vectors in the presence (lighter bars) or absence (darker bars) of a HDAC inhibitor (TSA). Luciferase activities (means ± s.e.m.; $n = 8$) were measured after a 24-h incubation with TSA. (C) Luciferase activity of *Per2* E-Box reporter construct co-transfecting with 5ng m*Dec2*WT, m*Dec2*P385R, m*Dec2*R57A or m*Dec2*R57K expression vectors. Luciferase activities (means ± s.e.m.; $n = 6$-8) were measured after a

24-h. (**D**) Interactions of WT or P385R mDec2 with hSIRT1 in mammalian cells by co-immunoprecipitation.

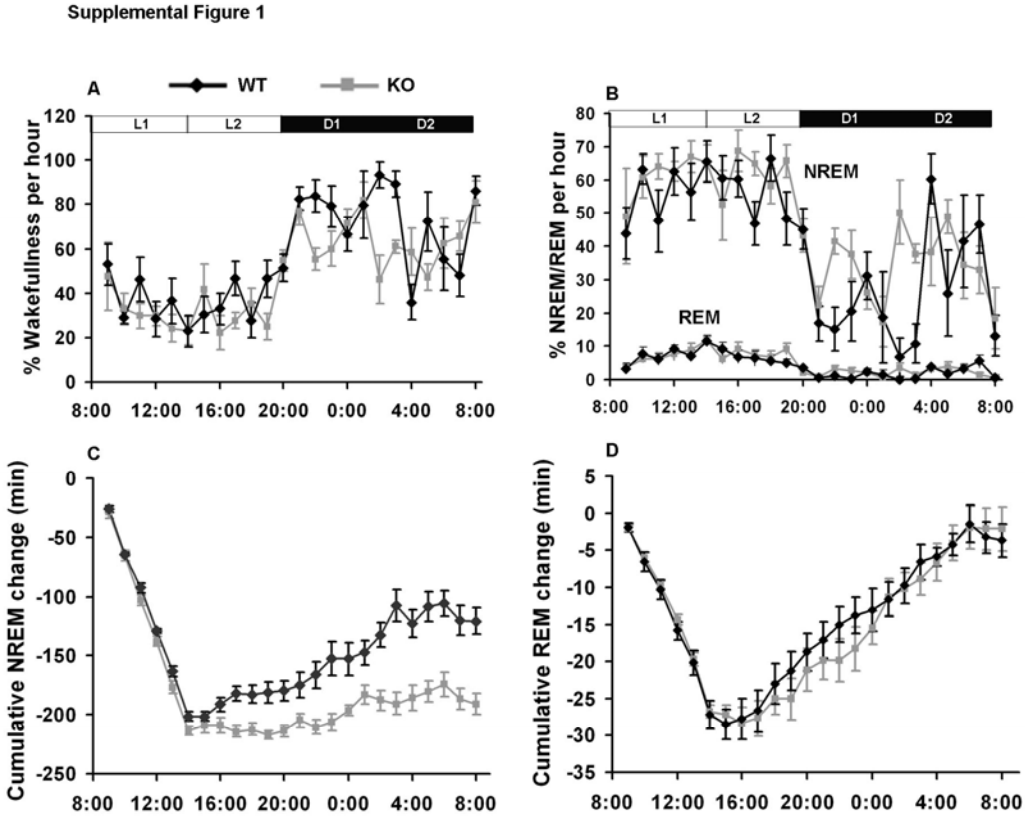


**fig. S2.** EEG and EMG characterization for *Dec2* KO mice and their WT littermate controls on sleep quality and quantity analysis.

**Reference for Supporting Online Material:**


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