Supporting Online Material

Materials and Methods

Animals and Circadian Time

LE/BluGill rats (University of Illinois) 6-12 weeks old were used for the in studies of the SCN. A dense genome scan was performed at a 10cM interval between markers on LE/BluGill progenitors. The results of this scan, performed by the Medical College of Wisconsin Human and Molecular Genetics Center as part of the NHLBI Programs for Genomic Applications (PGA) U01 HL66579, showed that the colony is inbred, yielding one allele at each locus tested (http://pga.mcw.edu/pga-bin/strain_desc.cgi). Use of this inbred strain greatly reduced inter-experimental variation, contributing to high statistical significance with small sample sizes. Rats were entrained to a daily cycle of 12-h light:12-h dark (LD) and provided food and water *ad libitum*. Zeitgeber time (ZT) is determined from the animal's LD cycle, with time of lights-on designated ZT 0. For samples obtained *ex vivo*, ZT is reckoned from this point. Because brain slices are maintained in constant conditions where the clock functions with a period of ~24-h without external time cues, the time of lights-on in the donor colony is designated as circadian time 0 (CT 0). Thus, subjective day (CT 0-12) corresponds to the light portion of the donor's former lighting schedule; subjective night (CT 12-24) corresponds to the dark portion of the donor's cycle. All animal protocols were approved by the IACAC and in compliance with NIH and University guidelines.

Brain Slice Preparation

Hypothalamic slices (500 μm) containing the SCN were prepared on a mechanical chopper and immediately placed at the interface of a Hatton-style perfusion chamber containing Earle's Balanced Salt Solution (Gibco BRL/Invitrogen, Carlsbad, CA), pH 7.25 (EBSS, supplemented with 26 mM glucose, 10 mM NaHCO3 and 0.005% gentamicin). For certain experiments, 750 μm slices containing the SCN were prepared and surgically reduced to minimize non-SCN tissue. These reduced slices retain clock properties attributed to the SCN. Reduced slices were immediately frozen for analysis by Western blot or co-immunoprecipitation. All slices were prepared at least 2-h prior to the onset of the dark phase of the light:dark cycle to avoid phase shifts during preparation (S1).

mTim αODN Treatment of Brain Slices

Slices maintained in the brain slice chamber were continuously perfused with medium. For experiments where short-term treatments of ODN were applied, perfusion was stopped during treatment. Antisense (5'acaagtccatacacc3'), missense (5'tacgatccaccacaa3'), 1-mismatch (5'acaagtcgatacacc3'), and 3-mismatch (5'acacgtcgataaacc3') *mTim* ODN corresponding to nucleotides 101–115 of *mTim* (Accession NM_031340); and antisense to AVP (5'tggcgagcataggtg3') corresponding to nucleotide of AVP were synthesized (Operon/Qiagen, Alameda, CA). ODN (10 μM, 2-h) was applied to the bath of the brain slice chamber. Perfusion was resumed after completion of treatment. For experiments requiring long term application of ODN, slices

were incubated continuously using a series of 50-ml aliquots of 10 μM ODN dissolved in EBSS. The 50-ml aliquot of ODN was recirculated through the chamber for 4-h. At 4-h intervals, 50-ml treatment aliquots were replaced with fresh ODN throughout the duration of the experiment. Slices were exposed to ODN for up to 36-h without deleterious effects.

Single Unit Recordings of SCN Neuronal Activity

The effects of ODN applied to SCN slices *in vitro* were assessed using the standard extracellular single unit recording technique (S1). Spontaneous firing rates of individual neurons were grouped into 2-h running averages using 15-min lags. The time-of-peak for each experiment was determined by visual inspection of a plot of 2-h running averages for the symmetrically highest point. A characteristic sinusoidal pattern of change, such that activity was low at night and peaked near CT 7, was observed in vehicle-treated control slices. In certain experiments, data were collected continuously and analyzed for the presence of a rhythm. In other experiments, phase shifts were determined by comparing the mean time-of-peak from treatment groups to vehicle-treated controls. All of the long-term ODN recordings and some short-term pulse recordings were performed with the experimenter blind to the treatment conditions.

Development of mTIM antisera

Polyclonal antiserum was raised in rabbit to synthetic peptides chosen from both the N- and C-terminal ends of mTIM. The N-terminal peptide (5'SSDLGKQPRRVPKR3') corresponding to residues 308-321 of mTIM (Accession AF098161) was synthesized at the Protein Sciences Facility of the University of Illinois Biotechnology Center. Conjugation of synthetic peptide, immunization of the rabbit, and collection of serum were performed using standard protocol at the Immunological Resources Center at the University of Illinois Biotechnology Center. Preabsorption of antisera was performed by incubation of the synthetic peptide bound to Protein G Sepharose (Pharmacia/Amersham, Piscataway, NJ) with mTIM antiserum. C-terminal polyclonal antisera were raised in chicken using a synthetic peptide (5'DEEEDDEGRRQAVSGTPRVHRKKRFQIEDEDD3') corresponding to residues 1161-1197 of *mTim* (Accession AF089161). The peptide was synthesized by Quality Controlled Biochemicals (QCB, Hopkinton, MA). Conjugation of synthetic peptide, immunization of the chicken, and collection of the eggs were performed at the Immunological Resources Center of the University of Illinois Biotechnology Center. Purification of the chicken mTIM antiserum was performed using the Eggstract kit (Promega, Madison, WI). Preabsorption of the C-terminal antisera was performed by incubation of the synthetic peptide bound to Protein G Sepharose (Pharmacia/Amersham, Piscataway, NJ) with mTIM antiserum.

Western Blotting

One frozen reduced SCN slice per timepoint or treatment was homogenized by repeat pipetting in 75 μl ice-cold T-PER (Pierce, Rockford IL) with 1x Complete Protease Inhibitor Cocktail (PI, Roche, Indianapolis, IN). Samples were centrifuged at 10,000 RPM for 5 min at 4°C, and the protein supernatant transferred to a new chilled tube. Protein content of each sample was determined by the Micro BCA Protein Assay (Pierce, Rockford, IL). Total protein (25 μg) was resolved by 8% or 4-15% SDS-PAGE and

transferred to nitrocellulose. Each blot was probed with N-terminal mTIM antiserum (1:1000), C-terminal chicken mTIM antiserum (1:1000), mPER1, mPER2, mPER3 antiserum (1:1000, Alpha Diagnostics Intl., San Antonio, TX), mCRY1 and mCRY2 antiserum (1:250, Santa Cruz Biotechnology, Santa Cruz, CA), β–tubulin antiserum (1:10.000, Sigma, St. Louis, MO), or TAP antiserum (1:500, S2). Goat anti-rabbit, donkey anti-chicken, or donkey anti-goat HRP-linked secondary antibodies (Chemicon, Temecula, CA) were used at dilutions of 1:5000. Blots were developed using Supersignal chemiluminescent substrate (Pierce, Rockford, IL) and the Biochemi Imaging system (UVP, Upland, CA) was used to digitally quantitate blots.

Co-immunoprecipitation (Co-IP)

Confluent 60 mm^2 plates of SCN 2.2 cells were rinsed twice with PBS. Ice-cold T-PER with PI (300 µl) was added to each plate, and the plate was set on ice for 2 min. Cells were scraped from the plate and the cell solution transferred to a chilled 1.5 ml tube. Cells and slices were lysed by repeat pipetting and samples centrifuged at 10,000 RPM for 5 min at 4°C. The protein supernatant was then transferred to a new chilled tube and protein concentration derived as described above. Total protein (150 µg) was brought to a total volume of 500 µl in T-PER with PI. Protein G Sepharose (20 µl of 50% slurry, Pharmacia/Amersham, Piscataway, NJ) in T-PER with PI was added to each Co-IP sample. Antiserum (1 µl, approximately 1 µg) was added to each sample. Each Co-IP experiment was performed with the antibody of interest and an isotype control antibody to evaluate nonspecific interactions. Co-IP samples were gently rotated overnight at 4° C. The samples were then washed three times with T-PER with PI. Laemmli's Buffer (30 µl) was added to each sample, the samples were heated at 100° C for 5 min and centrifuged at 14,000 RPM for 10 min. Each Co-IP sample was then loaded and resolved on an 8% or 4-15% SDS-PAGE gel and blotted as outlined above for the antibody of interest.

Quantitative Real-Time RT-PCR (qPCR)

Single SCN slices were collected after 2-h treatment with *mTim* αODN or missense ODN and immediately placed in TRIzol reagent (Gibco BRL/Invitrogen, Carlsbad, CA). Isolation of RNA was performed according to the manufacturer's protocol. The concentration and purity of the RNA samples was determined by UV spectrophotometry, with all samples displaying an Abs 260/Abs 280 ratio ≥1.8. For the reverse transcription, 1 µg of total RNA was brought up to a volume of 10 µl, and 200 ng of random hexamers was added. Samples were then heat-denatured at 85º C for 10 min and quick chilled on ice to minimize secondary structure present in the RNA. Invitrogen SuperScript II (200 U) was added along with buffer and 500 µM dNTP mix (final reaction concentration) to bring the total reaction volume to 20 µl. Reactions were incubated for 1 h at 45º C followed by a 10 min deactivation at 92º C. Samples were then diluted to 100 µl with Millipore water and stored at -80º C until time of use.

Primers were chosen using Primer Express 2.0 software (Applied Biosystems, Foster City, CA). A BLASTN search was then performed against GenBank to ensure that all primers were unique to the gene of interest. To avoid amplification from genomic DNA contamination, primers sets were designed to span an intron. For *mTim,* only the 5' sequence was used during primer generation in order to ensure that amplification from

the *mTim-s* splice variant was excluded. Sequences are as follows: *mTim-fl* (Forward: gccttcagaggactcatggatagt, start site, 68; Reverse: tggtaagtgcctccttccaagta, start site, 182, spans intron 1); ribosomal protein *Arbp* (Forward: cgtgatgcccagggaaga, start site, 26; Reverse: tcccacaatgaagcattttgg, start site, 122, spans intron 1). The *Arbp* primers also recognize the acidic ribosomal protein P0.

All reactions were carried out on the ABI 8700 prism in triplicate with 12.5 µl SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 300 nM forward and reverse primer final concentrations, 5 µl of diluted RT reaction, and Millipore water to final reaction volume of 25 µl. Initial denature was carried out at 95º C for 10 min, followed by 50 cycles of 15 sec at 95º C and 15 sec at 60º C. A heat disassociation was performed at the end of every run to avoid non-specific signals from SYBR, and to ensure that all signal was coming from an amplicon with the correct disassociation temperature.

The qPCR results were represented as Ct values, or the cycle at which an arbitrary fluorescence threshold was reached. This threshold was placed at a value that was in the exponential portion of the amplification plot for all of the products being generated and was held constant for all runs. Data were normalized against values obtained from amplification of the ribosomal protein, whose Ct value did not vary across the treatment groups. The Ct value of this gene was considered to be a general indicator of transcript abundance and was thus subtracted from the Ct value of the gene of interest to give ΔCt. ΔCts from different treatment groups could then be compared by subtracting one from the other to give ΔΔCt. $2^{\Delta\Delta Ct}$ gives the fold change in gene expression between these two groups. For the purposes of this experiment, SCN slices treated with *mTim* αODN or missense ODN were compared with the SCN slices receiving no treatment.

Transient transfection and RNA interference

A pGEM1 plasmid (Promega, Madison, WI) containing the human U6 locus (S28) was used as the template for a PCR reaction. This vector contains ~500bp of upstream U6 promoter sequence. Since an SP6 sequence flanks the upstream portion of the U6 promoter, we used an SP6 oligonucleotide (5'GATTTAGGTGACACTATAG3') as the sense primer and a rat *timeless* antisense primer (5'AAAAAAGGCAGGAGACCACTCCGCAGGATCCGCACCAAGCTTCGCGCAGA TCCTGCAGAGTGATCTCCTGCCGGTGTTTCGTCCTTTCCACAA3') as the antisense oligonucleotide for PCR amplification. This created a construct that would generate a shRNA directed at nucleotides 276 to 305 of rat *timeless* (Accession NM_031340). The PCR was performed using Taq polymerase with 4% DMSO and 50pm of each primer. PCR conditions: 95º C for 3 min; 30 cycles of 95º C for 30 sec, 55º C for 30 sec, and 72º C for 1min followed by one elongation step of 72º C for 10 min. The PCR product (~600bp in length) was cloned into the pCR2.1 TOPO vector (Invitrogen, Carlsbad, CA) following the product manual instructions. HEK293 cells (CRL-1573, ATCC, Manassas, VA) were maintained in EMEM supplemented with 10% FBS. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to product manual instructions, and incubated for 72 h before harvesting for Western blot, as described above.

Supporting Online Text

1. Assessing rhythmicity in the absence of mTIM-fl has been difficult. Genetic deletion of *mTim* is embryonically lethal, most likely because mTIM-fl is required for embryonic tubule formation. Given the high level of expression of *mTim* RNA in embryonic epithelial tissues that develop through branching morphogenesis (including the kidney, lung, and liver) (S3), disruption of the *mTim* gene should, indeed, have catastrophic consequences to the developing embryo. The assertion that *mTim* does not have a major role in the mammalian circadian system (S4-6) has been based on observations that animals heterozygotic for *mTim* deletion fail to show arrhythmicity or alterations in their circadian period. In *Drosophila*, a single copy of the *dTim* gene is sufficient to produce normal rhythmicity, and knockout of both *dTim* alleles is required to observe circadian alterations (S7). The homozygous *Dbt* mutant fly also proved lethal (S8), yet DBT plays an essential role in the regulation of dPER (S9). Thus, there is ample precedent that putative clock genes can be dosage insensitive and play key roles in both developmental and circadian processes.

2. Long-term treatment with *mTim* αODN knocks down mTIM-fl protein and results in a loss of SCN electrical activity rhythms. The only other report where a molecular alteration of putative clock elements led to abolishment of electrical rhythmicity found that SCN neurons from *mCry1/mCry2* double knock-out mice housed in DD show no electrical rhythmicity as measured by multi-unit recordings (S10). SCN arrhythmicity due to mTIM-fl knockdown places *mTim-fl* within the core or as output of the mammalian clockwork.

3. Electrophysiological recordings from SCN slices treated with *mTim* αODN containing 1- or 3-mismatched bases had no effect on the rhythm of electrical activity (S11).

4. Several studies have analyzed the expression pattern of *mTim* RNA, and the results have shown both a circadian oscillation and lack of oscillation of this transcript (S12-S17). Our finding of constitutive expression in the SCN of mTIM-s, which shares C-terminal sequences with mTIM-fl, offers a basis for resolving differing results in tissue studies of RNA as well as protein. Studies that employed In Situ Hybridization would again be confounded by *mTim-s* if the probe was designed against a C-terminal sequence. Our study utilized an N-terminal probe and detected a statistically significant oscillation of *mTim-fl* RNA. This result was verified by RNAse Protection Assay with an N-terminal probe (S15).

5. The N-terminal mTIM antiserum was further characterized by ectopic expression of mTIM-fl in a stable cell line. An expression vector carrying the *mTim* ORF and 3' UTR (S18) was transiently transfected in HEK293 cells (Fig. S2).

6. Our data extend the differential expression of the two *mTim* isoforms reported for peripheral tissues and brain (S3) to the rat SCN. The differences in expression of the RNA and protein in the SCN and other tissues suggest that *mTim-fl* and *mTim-s* may play different roles. Notably, mTIM-fl, but not mTIM-s, oscillates in the SCN over the

circadian cycle. Structural differences emphasize the potential for distinct clock functions; as shown in Fig. S1A, mTIM-s contains a truncated PER-binding domain. This is the first study to report constitutive expression of mTIM-s and circadian expression of mTIM-fl in the SCN.

7. Post-translational modifications have been shown to play a critical role in the clockwork mechanism in peripheral tissue (S19). However, to minimize the impact of multiple bands on the quantitation of the circadian profile of mTIM-fl and mPER2 in the SCN, we excluded phosphatase inhibitors. Thus, mTIM-fl resolved to a single immunoreactive band in our experiments. Additionally, the timing of our mPER2 oscillation is consistent with previously published profiles for mPER2 in the mouse SCN, but different from timing of expression profiles in peripheral tissue (S20). The SCN oscillator, which synchronizes peripheral clocks, exhibits molecular oscillations that precede those in the periphery by several hours (S19, S21).

8. Reagents recognizing sequences shared by mTIM-fl and mTIM-s would confound analysis of mTIM-fl circadian expression by techniques unable to discern the size of the products detected. Techniques that would be most likely to incur this confound include immunocytochemistry (ICC), which was used in previously published studies examining the expression of mTIM-fl (S22, S23), and its degradation in response to light (S22). These studies showed no oscillation in mTIM-fl in their tissue immunostaining. Their antiserum was directed to the C-terminal region shared by both mTIM-fl and mTIM-s. Because mTIM-s is so much more abundant and does not oscillate, no change would have been detected in the staining level. The Western blot data of SCN extracts using C-terminal antiserum (S22) was limited to a small region surrounding 140 Kd. Based on the described peptide sequence used to generate this antiserum, the antibody would have picked up both isoforms, much like our blot in Fig. 3B. The Co-IPs likely suffer from a similar problem. Because mTIM-s is so abundant, most of the antibody in their Co-IP is likely attached to mTIM-s. mTIM-s has a truncated mPER-binding domain and likely does not bind mPER. The study did show interaction with mCRY, which suggests that mCRY may bind to the C-terminal end of the mTIM protein. A study by Rosato et al (S24) deduced that dCRY binds to dTIM in an area external to the residues 377-915, which raises the possibility that dCRY may bind to residues 915-1388 of dTIM. Thus, it is possible that mCRY binds to mTIM-fl in an area in the extreme C-terminal sequence of mTIM-fl.

9. Association of mTIM-fl with mPER1, mPER2, and mPER3 was also detected when SCN 2.2 cell lysate was IP with the respective mPER antiserum and probed with the N-terminal mTIM antiserum (S25).

10. mTIM-fl co-immunoprecipitation (Co-IP) analysis showed no immunoreactivity for β-tubulin, or TAP, which are expressed in the lysate of SCN 2.2 cells. To test the specificity of Co-IP, we probed an N-TIM Co-IP with an anti-TAP antibody, which recognizes another soluble nuclear protein made by the SCN 2.2 cells. TAP is a 619-aa protein that is a mammalian ortholog of Mex67p, a yeast mRNA nuclear export factor (S26). TAP is known to shuttle between the nucleus and cytoplasm (S2), can bind to the Nuclear Pore Complex and is soluble in the nucleus of cells that express it (S27). These

controls demonstrate that our mTIM Co-IP procedure is specific for antigens associating with mTIM-fl.

Supporting Online Figures

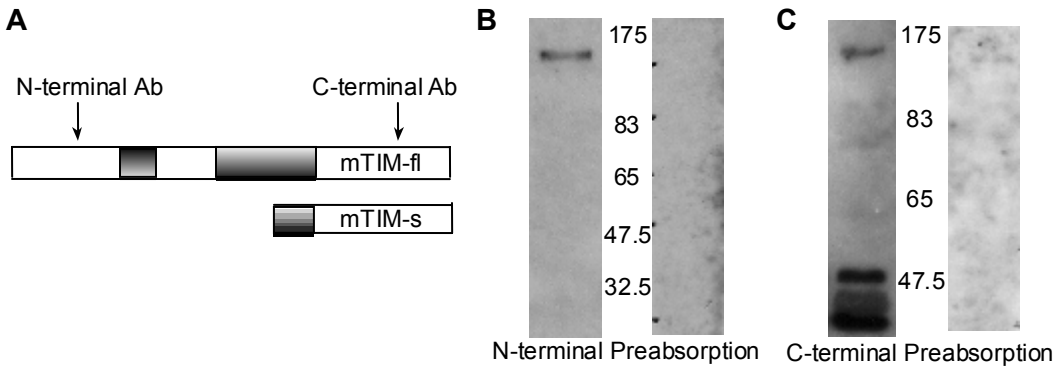

Fig. S1. (**A**) Schematic comparison of mTIM-fl and mTIM-s. The full length mTIM protein contains 1197 residues and retains two regions predicted to contain the PER-binding domains (S14), noted as shaded regions. mTIM-s comprises 465 residues and contains only a portion of one PER-binding domain present in mTIM-fl. (**B**) Western blot of SCN *ex vivo* extracts with N-terminal antiserum identified a single band migrating at ~140 kD, consistent with a predicted molecular weight of 137 kD (S16, S22). Preabsorption of the antiserum with the corresponding peptide abolished immunoreactivity. (**C**) SCN extracts probed with C-terminal antiserum exhibited a single band at ~140 kD and a broad band, which resolved as a triplet, at ~50 kD. These bands were abolished by preabsorption of the antiserum with the corresponding peptide.

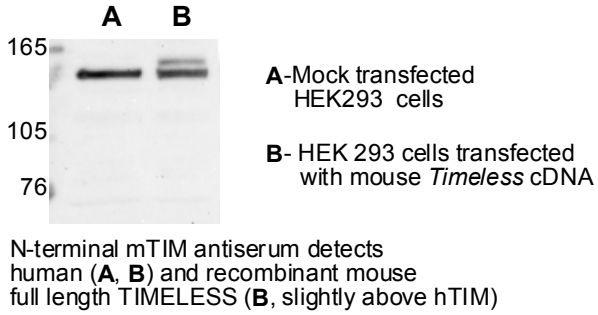

Fig. S2. N-terminal mTIM antiserum recognizes both human and mouse TIMELESS. HEK293 cells were transfected with a vector containing mouse *Timeless* cDNA (S18). An immunoreactive band corresponding to human TIMELESS (hTIM) was present in both samples. An immunoreactive band corresponding to mouse TIMELESS was found only in *mTim* transfected cells.

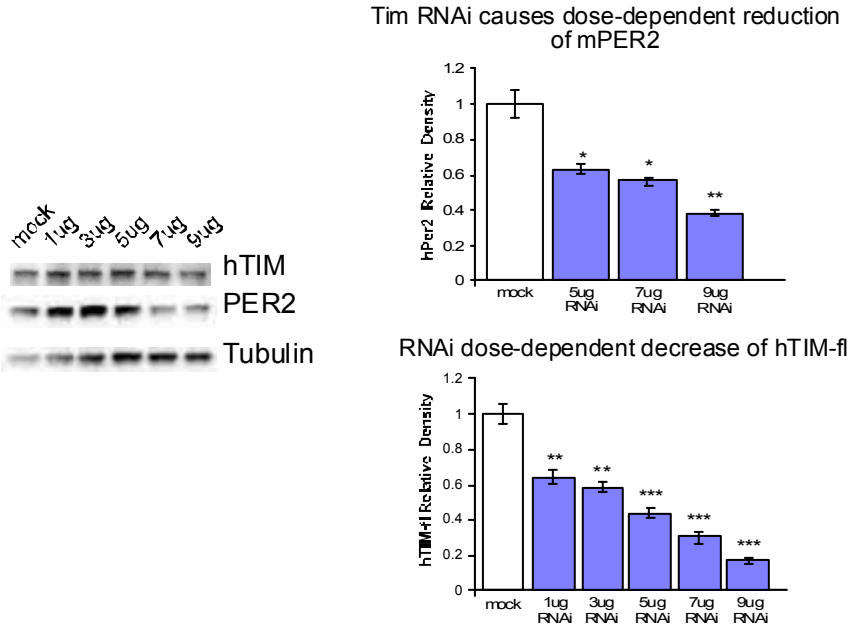

Fig. S3. Double stranded RNA interference (S28) directed at *mTim-fl* induces a dose-dependent decrease of both hTIM-fl and hPER2 in HEK293 cells. This additional method of producing hTIM-fl knockdown caused the same response of hPER2 levels as in *mTim* antisense-treated SCN brain slices. Density of each band (hPER2 and hTIM-fl) was divided by the density of tubulin of the same lane. The hPER2/tubulin or hTIM-fl/tubulin ratio was normalized to control and plotted as the mean +/- s.d. Statistical significance was determined by Student's *t*-test for mock *vs*. each individual treatment (* indicates $p<0.05$, ** indicates $p<0.01$, and *** indicates $p<0.001$, n=3).

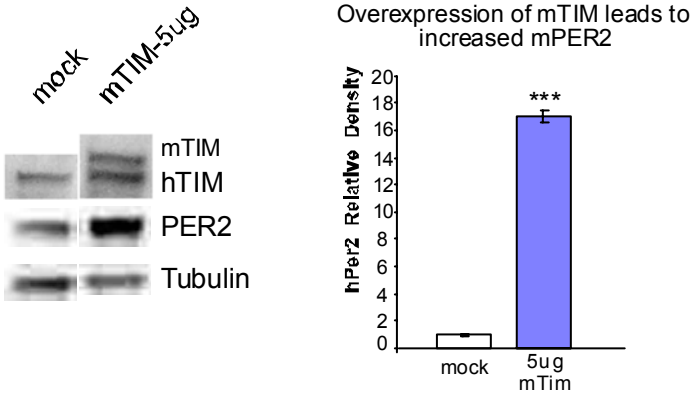

Fig. S4. Overexpression of mTIM-fl leads to increased hPER2 levels. HEK293 cells were transfected with 5 μg of vector containing mouse *Timeless* cDNA (S18). Density of the hPER2 band was divided by the density of tubulin of the same lane. The

hPER2/tubulin ratio was normalized to control and plotted as the mean +/- s.d. *** indicates statistical significance as determined by Student's *t*-test ($p<0.001$, $n=3$).

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S29. Materials and methods are available as a previous section of Supporting online material.