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TIMELESS mutation alters phase responsiveness and causes advanced sleep phase

Short title: TIMELESS is a mammalian circadian gene regulating sleep phase

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33

34**Abstract:** Many components of the circadian molecular clock are conserved 35from flies to mammals; however, the role of mammalian Timeless remains 36ambiguous. Here, we report a novel mutation in the human *TIMELESS* (h*TIM*) 37gene that causes familial advanced sleep phase (FASP). *Tim* CRISPR mutant 38mice exhibit FASP with altered photic entrainment but normal circadian 39period. We demonstrate that the mutation prevents TIM accumulation in the 40nucleus and has altered affinity for CRY2, leading to destabilization of 41PER/CRY complex and a shortened period in non-mature mouse embryonic 42fibroblasts (MEFs). We conclude that TIM, when excluded from the nucleus, 43can destabilize the negative regulators of the circadian clock, alter light 44entrainment, and cause FASP.

45**Keywords:** TIMELESS, Human genetics, Mammalian Circadian Clock 46Regulation, Familial Advanced Sleep Phase

47

48**Significance Statement:** TIMELESS has a clear role in the regulation of 49circadian rhythms in *Drosophila*, but its role in mammalian circadian 50regulation remains unclear. A mutation identified in a small family with 51advanced sleep phase causes cytoplasmic accumulation of TIMELESS. We 52confirm that TIMELESS can bind the critical negative regulators of the 53circadian clock, PER2 and CRY2, and destabilize them when TIMELESS 54remains in the cytoplasm. The mutant mouse model has a phase advance of 55sleep-wake behavior and altered sensitivity to light pulses, but normal period 56length. These data demonstrate that TIMELESS may play a role in regulating 57minor phase changes and therefore contribute to the maintenance of 58circadian rhythmicity in mammals.

59**body**

60

61**Introduction**

62The circadian clock governs the timing of many body functions (1), including
63the onset and offset of sleep, that oscillate with an approximately 24-hour
64period (2–5). The mammalian core clock is comprised of the positive
65transcription activators, BMAL1 and CLOCK, and the key negative regulators,
66PERs and CRYs (3–5). In recent years, studies of human circadian phenotypes
67have contributed significantly to a better understanding of the molecular
68clock. Familial Advanced Sleep Phase (FASP) is an inherited condition where
69affected subjects wake and sleep early (6). We previously showed mutations
70in the genes (*PER2*, *CRY2*, *PER3*) for negative regulators of the clock that
71cause the FASP phenotype (7–9). A common feature of these identified
72mutations is the instability of PER and CRY, causing derepression of
73BMAL1/CLOCK. This leads to a shortened circadian period and advanced
74sleep phase. While we have demonstrated that the shortened period is
75sufficient to cause FASP (7, 8), a mutation that alters entrainment could
76potentially produce the FASP phenotype with preserved period (10).
77
78Although studies have shown the importance of key conserved components
79of the circadian clock across species (4), much remains unknown of the role
80for mammalian homologs of *Drosophila Timeless* (*Tim*). Tim is a core
81component of the *Drosophila (d)* clock, and it functions as the light-sensitive
82partner of dPer to provide the negative regulation necessary for generating
83rhythmicity and photo-entrainment in flies (11–13). Based on phylogenetic
84analysis, mammalian (both mouse [m] and human [h]) *Tim* has higher
85sequence homology to *Drosophila Timeless2* (*Tim2*), which was shown to
86participate in DNA metabolism, maintenance of chromosomal integrity, and
87light entrainment of the adult clock (14). Homozygosity for m*Tim* knockout is
88embryonic lethal (at the preimplantation stage), and analysis of viable
89heterozygous m*Tim* mutant mice showed no change in circadian period (15).
90Previous studies have shown that m*Tim* levels in the SCN oscillate over
91circadian time with a peak of expression at the day-night transition, similar
92to that of m*Per2* (16). Conditional knockdown of *Tim* disrupted the rhythmic

93neuronal firing in *ex vivo* rat suprachiasmatic nucleus (SCN) slices, affected
94the expression of other core clock proteins, and altered phase
95responsiveness in tissues (17). In addition, functional analysis of truncated
96TIM revealed the role of the N-terminal portion in binding to partners like CRY
97and the C-terminal domain for nuclear translocation (18). These data clearly
98implicate a role for TIM in mammalian circadian regulation; however, a
99precise functional mechanism for its contribution remains incomplete.
100
101We report a mutation in the human *TIMELESS* gene that is responsible for the
102FASP phenotype in a family. CRISPR-generated mutant mice recapitulate the
103advanced sleep phase phenotype with normal period and altered light
104entrainment properties. Moreover, shortened period was found in CRISPR-
105generated cells and MEFs. The mutant protein has weakened repressor
106activity, lower stability, exclusive cytoplasmic localization and reduced
107affinity for CRY2. Importantly, we found that wild type (WT) TIM can
108destabilize the PER2-CRY2 complex and the mutation enhances this function
109of TIM. These results indicate that mammalian TIM plays a role in regulating
110circadian clock by fine-tuning levels of the PER2-CRY2 complex.
111

112**Results**

113**Identification of a *TIMELESS* mutation in a family with FASP**

114A nonsense mutation in h*TIM* was identified in one FASP family (Table 1) by
115two independent screening approaches. **In the non-carrier individual in**
116**this kindred, the apparent discordance between sleep/wake times**
117**relative to activity onset and offset times is likely due to movement**
118**captured by actigraphy after the onset of sleep and prior to wake**
119**onset.** We first employed candidate gene screening of 25 circadian clock-
120relevant genes (7). An unbiased whole exome sequencing was subsequently
121applied to confirm the identified mutation (Fig. 1*A*). This mutation changes
122an arginine to a stop codon at amino acid position 1081 (*TIMELESS R1081X,*
123*TIM R1081X*). The mutation causes the loss of 128 amino acids at the C-

124terminal end of the conserved TIMELESS_C domain (Fig. 1*B*) (19–21) which 125contains the fourth and last putative nuclear localization signal (NLS4) 126conserved among mammalian species (18). The *TIM R1081X* mutation and 127the FASP phenotype co-segregate in this family (Fig. 1*A*) (6, 22) although the 128family is too small to prove this locus is linked. Thus, additional *in vitro* and 129*in vivo* experiments were required to prove causation. This mutation 130represents a novel variant in the SNP databases.

131

132**Measuring circadian period of *Tim R1078X* mice and cells**

133To further examine the circadian phenotype of the human *TIM R1081X* 134mutation *in vivo*, we generated CRISPR-edited mice (*Tim R1078X*) harboring 135the causative mutation at the conserved codon position (R1078 in mouse) 136(Fig. S1 *A-C*). *Tim R1078X* (*RX/+*) heterozygous and *WT* (+/+) littermate mice 137were subjected to circadian behavior testing, first entrained under conditions 138of 12 hr. light and 12 hr. darkness (LD 12:12) before being released into 139constant darkness (DD) to determine the endogenous circadian period. The 140free-running period of *RX/+* mice (23.60±0.043 hr.) was not significantly 141different from that of +/+ controls (23.71±0.022 hr.; Fig. 2*A*, *B*) by line fitting 142analysis.

143To test whether the heterozygous *TIM R1081X* mutation at the endogenous 144locus is sufficient to cause shortened period *in vitro*, a U2OS cell line with a 145heterozygous *TIM R1081X* mutation was generated by CRISPR-mediated 146genome-editing (Fig. 2*C*). Out of 128 edited clones, a single clone (#37) with 147heterozygous *R1081X/+* genotype (green dots, genotyping done in 148duplicates, Fig. 2*D*) was identified and verified by Sanger sequencing (Fig. 1492*E*). The heterozygous *R1081X/+* U2OS cells showed ~29 mins shortening of 150period as compared to +/+ cells (23.29 vs. 23.78 hr., Fig. 2F).

151

152Circadian period was further assessed in peripheral tissues of *RX/+* and +/+

153mice that had been crossed to m*Per2*^{Luc} knock-in mice (23). Similar to what

154was observed for the running period of mice, liver and lung *ex vivo* cells
155showed no period change (Fig. S2*A*). Although *Tim* expression had previously
156been reported to be low in non-proliferative tissues (18), we further
157confirmed TIM expression levels in proliferative and non-proliferative tissues
158and in MEFs (Fig. S2*D,E,F*). We next examined periodicity using MEFs from
159these mice and observed a modest yet significant reduction in period in *RX/*
160+ MEFs compared to +/+ MEFs (24.0 vs 24.36 hr.; Fig. S2*B*). Moreover,
161expression of TIM R1081X in HEK293T cells shortened the period by ~37 min
162compared to HEK293T cells expressing TIM WT (25.92 vs. 26.54 hr., Fig.
163S2*C*), similar to the shortened period phenotype observed in MEFs.
164
165***hTIM R1081X/mTim R1078X* mutation alters light entrainment**
166We next assessed light entrainment in the mutant mice. In LD 12:12 (lights
167on 0600-1800), no change in the activity onset and offset was seen (Fig. 2*A*).
168Light masking is a strong confounding factor when examining locomotor
169behavior to assess phase in LD 12:12 (24, 25). Therefore, we entrained mice
170to a skeleton photoperiod to minimize the masking effect of light with 15-min
171light arms from clock times 06:00-06:15 and again from clock times 17:45-
17218:00, with darkness at all other times. Under these conditions, activity
173onset was significantly advanced in heterozygous *RX/*+ mice compared to +/
174+ mice (-40.32±12.53 minutes vs 9.545±6.756 minutes) (Fig. 3*A,B,C*).
175Activity offset was more heterogenous in the skeleton photoperiod without a
176significant difference of offset of activity in mutant animals (-18.13±15.37
177minutes vs 14.73±7.397 minutes; Fig. 3*A,B,C*). Since rest and activity
178behavior quantification is a surrogate for sleep/wake states and is influenced
179by the effect of light masking, we subjected mice to electroencephalography
180(EEG) assessment in LD 12:12 to objectively score sleep/wake epochs. Phase
181advance of wakefulness and sleep at the transition from light to dark and
182from dark to light was approximately 30 minutes (Fig. 3*D*). Under LD 12:12,
183overall sleep duration, sleep bout length and number, and total non-rapid
184eye movement (NREM) and REM sleep percentages were preserved (Fig.

185S3*A*, *B*), indicating that the advanced phase is gated by circadian parameters
186and does not affect sleep architecture.

187The strength of entrainment was examined using 30-min light pulses at ZT
18814 and ZT 22 and by assessing the magnitude of phase shift in either
189condition as compared to the activity onset in the absence of a light pulse.
190**Heterozygous *RX/+* mice exhibited a significantly increased phase**
191**advance after initial release into DD compared to +/+ mice**
192**(30.22±5.125 minutes vs 4.886±4.324 minutes; Fig. 3*E*, upper**
193**panels & leftmost graph). Heterozygous *RX/+* mice exhibited a**
194**significantly longer phase delay** with a light pulse at ZT 14 as compared
195to +/+ controls (-77.11±12.20 minutes vs. -21.41±13.41 minutes; Fig. 3*E*,
196middle panels & middle graph). The ZT 22 light pulse elicited a diminished
197phase advance from *RX/+* mice (-3.686±5.916 minutes vs 41.20±10.15
198minutes; Fig. 3*E*, lower panels & rightmost graph). These data indicate that
199the *TIM R1081X* mutation changes sensitivity to entraining stimuli, a finding
200that is consistent with previous studies of timed *Tim* knockdown in *ex vivo*
201rat SCN slices (17).
202
203**TIM R1081X is less stable and localized to the cytoplasm, and TIM**
204**R1081X-expressing cells have reduced repressor activity**
205We next examined the molecular alterations underlying the observed
206changes in period and entrainment. Since inhibition of CLOCK-BMAL1
207transactivation by hTIM had been shown previously (20), we tested the
208repressor activity of TIM R1081X. TIM WT expression inhibited the CLOCK-
209BMAL1−mediated luciferase expression in a dose-dependent manner,
210resulting in a more than 80% decrease in CLOCK-BMAL1−induced luciferase
211activity at the highest dosage (9.92 vs 1.84 normalized luciferase activity,
212with or without TIM-WT, respectively, Fig. 4*A*). Overall repressor activity for
213CLOCK-BMAL1−mediated expression is weaker in TIM R1081X-expressing
214cells vs. TIM WT-expressing cells across different dosages with a significant

215genotype effect (P < 0.01) and dosage effect: (P < 0.001) without a
216genotype-dosage interaction.
217
218Mutations that destabilize transcriptional repressors in the circadian
219transcription-translation feedback loop can speed up the circadian clock and
220shift sleep phase (7, 26, 27). We therefore examined the protein stability of
221TIM WT and TIM R1081X by assessing the decline in protein levels after
222cycloheximide (CHX) treatment and found that TIM R1081X is less stable
223than TIM WT (Fig. 4*B*). With a significant genotype effect (P < 0.01), and time
224effect (P < 0.001) without a genotype-time interaction.
225
226TIM R1081X leads to a truncated protein lacking a putative nuclear
227localization signal (NLS4, see Fig. 1*B*), which has been shown to be
228necessary and sufficient for nuclear localization (18). Indeed, in contrast to
229TIM WT (predominantly localized in the nucleus), TIM R1081X is mostly
230cytoplasmic, as shown by both immunocytochemistry (66.9% vs 23.7%
231nuclear TIM signal, WT vs R1081X; Fig. 4*C*) and Western blotting (94% vs
23234% nuclear TIM signal, WT vs R1081X; Fig. 4*D*). These findings indicate that
233the last 128 amino acids (including the NLS4) of mammalian TIMELESS is
234critical for proper nuclear translocation of TIM.
235
236To determine whether the loss of NLS4 contributes to the molecular deficit
237underlying the TIM-R1081X phenotypes, we generated TIM without NLS4
238(TIM-ΔNLS4). We found that TIM-ΔNLS4 is restricted to cytoplasm, and the
239period of TIM-ΔNLS4-expressing cells is shortened compared to that of the
240TIM WT-expressing cells (Fig. S4*A*, *B*). Taken together, our results suggest
241that the *TIM-R1081X* mutation leads to FASP partly due to the loss of NLS4.
242
243**TIM-R1081X has weakened interaction with CRY2, and leads to**
244**destabilization of CRY1/2 and PER1/2**

245Since TIM was shown to bind CRY proteins (18, 28), we examined whether 246the *TIM R1081X* mutation affects interactions between TIM and CRY2. Upon 247immunoprecipitation of CRY2, the abundance of co-immunoprecipitated TIM 248R1081X was only 2.8% of bound TIM WT (Fig. 5*A*). Consistently, the amount 249of CRY2 co-immunoprecipitated with TIM R1081X was 48.7% less than the 250amount bound to TIM WT (Fig. S5*A*). These results indicate that the 128 251amino acid C-terminal region of TIMELESS protein is required for stabilizing 252its interaction with CRY2 protein.

253

254TIM was shown to bind PER2 in the SCN (17) but not in cell culture systems 255(18). Given the interaction of TIM with CRY2, we sought to re-examine 256whether PER2 and TIM interact and if the TIM R1081X mutation alters any 257putative association with PER2. We found that PER2 co-immunoprecipitated 258with both WT and R1081X TIM, but unlike CRY2, the interaction was not 259affected by the mutation (Fig. 5*B* and Fig. S5*B*).

260

261The stability and levels of major repressors of the molecular clock are critical 262for the regulation of clock speed (7). Because both PER2 and CRY2 can bind 263to TIM R1081X and TIM WT but with different affinities, we investigated 264whether the stability of PER2 or CRY2 is altered by the TIM R1081X mutation. 265HEK 293T cells were transfected with either *TIM R1081X* or *TIM WT* together 266with either *CRY2:luc* or *PER2:luc*, and the stabilities of PER2 and CRY2 were 267assayed after CHX treatment. The half-life of both CRY2 and PER2 were 268significantly reduced in *TIM R1081X*–transfected cells compared to *TIM WT*–269transfected or empty vector control cells (Fig. S5*C*). Similarly, the half-life of 270both CRY1 and PER1 were significantly reduced in *TIM R1081X*–transfected 271cells compared to *TIM WT*–transfected or control cells (Fig. S5*D*). These 272results suggest that PER1/2 and CRY1/2 interacts with TIM and that TIM 273destabilizes these proteins when restricted to cytoplasm. To examine the 274effect of *TIM R1081X* on the stability of the PER2-CRY2 heterodimer, we co-275transfected cells with *TIM WT* or *TIM R1081X* together with *PER2 (or CRY2)*

276and *CRY2:luc* (or *PER2:luc*) constructs and assayed the stability of CRY2:luc
277(or PER2:luc). TIM WT expression results in CRY2 and PER2 having
278intermediate stability compared to vector control or TIM R1081X (Fig. 5*C*).
279The destabilizing effect of TIM WT on the PER2-CRY2 complex is dose
280dependent (Fig. 5*C,* right panels). These results imply a role for TIM WT as a
281destabilizer of the PER–CRY complex, and this destabilization effect of TIM is
282enhanced by the *R1081X* mutation.
283
284**Discussion**
285Although the role of mammalian TIMELESS in circadian regulation has been
286ambiguous, its transcriptional repression activity for CLOCK/BMAL1 and
287interactions with mPER1/mCRY have previously been demonstrated (18, 29,
28830). Here, we report that a mutation in human *TIMELESS R1081X* causes
289FASP leading to reduced nuclear presence of TIM and reduced stability of
290PER1/2 and CRY1/2.

291With the exception of the *PER2 S662G* FASP phenotype, the other reported
292mutations found in families with FASP (*CRY2, CK1δ, PER3*) show subtle
293phenotypes in mouse models (7, 9, 27). This is expected given that carriers
294of these mutations are normal, healthy humans. The *RX/+* mutant mice have
295advanced phase of activity onset in conditions where light masking is
296minimized while activity offset was not advanced in the skeleton photoperiod
297due to higher variability in offsets. Phase advance of both wake onset and
298offset was confirmed with EEG analysis under standard lighting conditions
299(Fig. 3*B*). EEG data also revealed that the overall quantity of sleep was
300preserved (Fig. S3*A*, *B*).

301Common features of the previously reported circadian mutants include
302altered period of variable magnitude (22 hr. to 23.4 hr.) and changes in
303entrainment. The *TIM R1081X* mutation is the first example with an
304advanced phase phenotype and altered entrainment but no significant
305change in period compared to WT at the organismal level. We previously

306posited that the FASP phenotype can result from either a shortened circadian 307period or altered entrainment. *TIM R1081X* represents a model in which ASP 308is present in the setting of an altered entrainment phenotype, but in the 309absence of period shortening *in vivo*. The period shortening and protein 310instability demonstrated *in vitro* when TIM is expressed at higher levels in 311tissues or cells suggests that the phase phenotype could be a function of low 312TIM expression and the period shortening only happens when TIM is more 313highly expressed. Forward mutagenesis screens in model organisms have 314focused primarily on phenotypes of arrhythmicity and altered period. 315Because our human genetic studies of FASP have focused on a phase 316phenotype (rather than period), we expected to find mutations affecting 317period and/or entrainment. The finding of altered entrainment in the absence 318of period shortening in this *TIM* mutant validates this idea. **The difference 319in the baseline phase after release into DD between *TIM* mutant and 320WT animals may represent the derepression of the phase advance 321(caused by masking) and altered entrainment which may largely 322account for the differences observed between groups in the ZT 14 323and ZT 22 light pulses.** Interestingly, *Drosophila Tim2* knockdown affects 324the phase response to light (31). Further, transient disruption of m*Tim* RNA 325across circadian time alters the phasing of circadian rhythmicity (17). TIM 326protein is predominantly and robustly expressed in proliferative organs 327compared to more differentiated tissues (18). We found that the period of 328mature peripheral tissues (lung and liver) from *RX/+* mice was not altered, 329whereas the period of proliferating MEFs derived from *RX/+* mice was 330moderately yet significantly shorter than that of +/+ mice (Fig. S2*A*). 331Importantly, CRISPR generated *TIM R1081X/+* heterozygous U2OS cells have 332a shortened period compared to +/+ control cells (Fig. 2*F*). Moreover, the 333period observed in HEK293 cells overexpressing *TIM R1081X* (Fig. S2*C*) also 334showed shortening similar to that observed in CRISPR U2OS cells. Further 335studies are needed to unravel this discrepancy in period between mature vs. 336proliferating tissues and how this relates to behavioral phase advance in the

337setting of normal period *in vivo*.

338Consistent with previously reported FASP mutations, *TIM R1081X* affects its 339repressor activity and stability. With the exception of *PER2 S662G*, all the 340FASP mutations render weakened repression of CLOCK/BMAL1 mediated 341transactivation and instability of the respective proteins. The TIM R1081X 342protein localizes predominantly in the cytosol unlike TIM WT which localizes 343to the nucleus (Fig. 4*C*, *D*). NLS4 deleted TIM protein recapitulates the 344shortened period and altered subcellular localization phenotypes. The latter 345finding confirms the previously reported necessary role of C-terminal portion 346of mammalian TIM in its nuclear localization (18). Combining these results, 347we cannot say whether the TIM R1081X protein has reduced repressor 348activity itself, or whether its instability and extranuclear localization 349contributes to observed derepression of CLOCK/BMAL1 mediated 350transactivation. Nevertheless, the *TIM R1081X* mutation causes protein 351instability and overall reduced repression of BMAL1/CLOCK which is 352consistent with other examples of FASP.

353The protein levels of core clock components are known to play critical roles 354in maintaining clock stability. We show here that TIM R1081X can destabilize 355CRY1/2 and PER1/2. The association between TIM R1081X and CRY2 is 356weakened as compared to TIM WT. PER2 also binds TIM, but the R1081X 357mutation does not alter this association. Interestingly, CRY 1 and CRY2 have 358been reported to have opposing roles in the maintenance of period length 359(32, 33). Thus, the finding that TIM R1081X destabilizes both CRY1 and CRY2 360may explain the lack of period change in mutant mice. Because the de-361stabilization of CRY2 has been associated with phase behavior differences 362(7), this instability may continue to exert its effect in the context of phase 363alone and supports the idea that regulation of phase (phase advance of 364behavior) can be independent of period length. While the R1081X mutation 365destabilizes both PERs and CRYs, TIM WT does not increase PERs or CRYs 366stability when expressed alone. TIM WT destabilizes CRY2 and PER2 when

367they are both present, and this destabilization is further enhanced by the 368*R1081X* mutation. These results suggest the existence of a TIM–PER2–CRY2 369complex in which TIM plays the role in fine-tuning the levels of CRY2/PER2. 370Collectively, these data demonstrate a role for mammalian *Timeless* in 371circadian regulation, and we posit that it has a role in destabilizing the PER–372CRY complex in the cytoplasm, creating the conditional ability to adjust to 373minor changes in phase. How TIM nuclear migration is regulated may provide 374insight into its function as a destabilizer of PER and/or CRY proteins, and 375investigation into the precise functions of TIMELESS can further reveal the 376mechanism of phase modulation.

377**Materials and Methods**

378Nomenclature

379For humans—gene (*TIM*), protein (TIM). For mouse—gene (*Tim*), protein 380(TIM). +/+ refers to wild type animals or unaffected human subjects and RX/381+ refers to heterozygous mutant animals or affected human subjects.

382

383Human data and mutation screening

384All human subjects signed a consent form approved by the Institutional 385Review Boards at the University of Utah and the University of California, San 386Francisco (IRB# 10–03952). The consent form includes all confidentiality and 387ethics guidelines.

388

389Subjects were characterized by a previously published procedure (22). The 390data were interpreted by one of the authors (CRJ, LJP) as possible, probable, 391or definite advanced sleep phase by age 30. Though ancillary features of ASP 392(earlier spontaneous wake time if an earlier bed time is selected) and 393potential confounding or masking influences were considered, participants 394categorized as ‘definite ASP’ reported spontaneous vacation sleep onset and 395offset time no later than 21:30 and 05:30, respectively and had H-O score of 396at least 70. Sleep logs, actigraphy and Zeo (Zeo Incorporated, Boston, MA)

397EEG recordings were obtained to characterize consecutive nights of sleep at
398home. Activity onset and offset were defined by actigraphy and sleep onset
399and offset were defined by Zeo recordings. DNAs purified from blood
400samples were used to screen for mutations.
401
402DNA was extracted from blood samples taken from members of kindred
4035602. Specific primers covering the coding sequence of the circadian
404candidate genes were used to amplify fragments for sequencing. The
405candidate genes screened included CLOCK, BMAL1, PER1-3, CRY1-2, DEC1-2,
406CSNK1D, CSNK1E, PRKAA2, NPAS2, CSNK2A2, CSNK2B, FBXL3, GSK3B, PKCA,
407PRKAA1, PRKAA2, RAB3A, RORA, TIMELESS, NR1D1, and PRKCG. TIMELESS
408R1081X was identified as a novel genetic variant specific to this family, and
409it is not found in any public genome database as of June 2018.
410
411Exome sequencing was performed on 2 individuals, one ASP and one
412control. Omicia Opal 0.10.0 software was used to annotate the variants for
413potential disease-causing mutations in the exomes of each of the affected
414individuals (after filtering out common variants, dbSNP MAF > 1%) using the
415HGMD and OMIM databases. Variant prioritization (using Ingenuity Variant
416Analysis) included generating a list of all variants, filtered for 1) minor allele
417frequency < 0.01% according to 1000 Genomes Project, Complete Genomics
418public genomes, and NHLBI ESP exomes; 2) nonsynonymous, near a splice
419site/promoter, or a structural variant; 3) occur in affected and not in the
420control sample.
421
422<u>Wheel Running and Circadian Analysis</u>
423*RX/+* and +/+ mice were maintained on a C57BL/6J background.
424Experimental mice were male and ~8 weeks-old at the initiation of
425behavioral assays. Mice were individually housed in wheel-running cages
426with *ad lib* access to food and water. Mice were initially entrained to LD
42712:12 at 250 lux for at least 14 days. Onset times, offset times, and

428acrophase were analyzed using data from stably entrained mice to generate
429activity profiles using ClockLab software (Actimetrics, Wilmette, IL; RRID
430SCR_014309). After entraining mice for a minimum of 14 days, mice were
431released into constant darkness (DD) to assess free-running period. Period
432determination was made with line fitting of activity onsets from day 1 to
433day14 in DD using ClockLab software. To examine whether masking
434confounded observed locomotor behavior in LD conditions, a skeleton
435photoperiod was employed with two entrainment light pulses of 250 lux from
436clock times 6:00 to 6:15 to designate the “morning” light cue and again from
437clock times 17:45 to 18 to designate the “evening” light cue. Mice were kept
438on this schedule for 10 days after stable entrainment to LD 12:12 conditions.
439To determine phase shifting behavior mice were stably entrained to LD 12:12
440conditions and then subjected to a 30-minute light pulse (250 lux) from ZT
44114 to ZT 14:30 or, separately from ZT 22 to ZT 22:30 and then the mice were
442released into DD for at least 10 days. Phase-shifts were determined by line
443fitting of activity onsets from day 1 to day 10 in DD and compared to line
444fitting of activity onsets at baseline in DD conditions without a light pulse. All
445data collection and analysis was done using ClockLab software (Actimetrics,
446Wilmette, IL; RRID SCR_014309). Activity onset and offset were defined using
447the ClockLab software algorithm.
448
449EEG and Sleep Wake Analysis
450For electroencephalogram (EEG) analysis, mice were surgically fitted with
451EEG electrodes. The mice were anesthetized using isoflurane anesthesia,
452placed on the surgical stage and the skull was immobilized. The hair was
453shaved and betadine was applied to the scalp and allowed to dry. A vertical
454incision was made and the skull was exposed with a sterile cotton swab. A
45523-gauge sterile surgical needle was used to make 4 epidural guide holes
456through the skull over the frontal cortical area and over the parietal area (1
457mm anterior to bregma, 1 mm lateral to the midline and 3 mm posterior to
458bregma, 2.5 mm lateral to midline respectively). One ground and three lead

459screws were surgically placed into the skull through the guide holes. The
460three lead screws were soldered onto a 6-pin connector EEG/EMG headstage
461(Pinnacle Technologies, Lawrence, Kansas). EMG leads from the headstage
462were surgically placed into the neck muscle and the base of the headstage
463was covered with black dental cement to form a solid cap on the head. The
464incision was closed with VetBond (3M, Santa Cruz Biotech) and animals were
465given a subcutaneous injection of Marcaine (0.05 mg/kg) adjacent to the
466vertical incisions prior to recovery on a heating pad. Mice were allowed to
467recover for 3 weeks prior to behavioral analysis. During EEG/EMG recording,
468mice were singly-housed and a tethered pre-amplifier (to record EEG signals)
469was attached to the headstage. The mice habituated to the recording cable
470for 7 days in LD 12:12 conditions, allowing for freedom of movement in the
471base of the cage. EEG data was then collected in LD 12:12 conditions. Data
472was acquired with Sirenia software (Pinnacle Technologies, Lawrence, KS).
473EEG signals were sampled at 500 Hz. A 24-hour epoch of EEG data was pre-
474scored semi-automatically by Sirenia Sleep Pro software, and then
475subsequently hand-scored by researchers blinded to genotype.
476
477Cell culture and constructs
478HEK293 cells (ATCC CRL-3216; RRID: CVCL_0063) were purchased from
479ATTC. Authentication of the cell lines was performed using STR profiling by
480ATCC. A stable U2OS-B6 cell line that expresses a destabilized firefly
481luciferase gene under the control of the m*Bmal1* promoter was obtained
482from Dr Satchidananda Panda (34). All cell lines were confirmed as
483Mycoplasma-free every 6 months. Cells were cultured in DMEM (Millipore
484Sigma) containing 10% FBS and maintained by standard methods. DNA
485constructs were introduced into the cells by using X-tremeGene 9 DNA
486Transfection Reagent (Roche). DNA constructs used were as follows:
487pCMV4a-CRY2-FLAG, pCMV2a-HA-CRY2, pCMV10-3xFLAG-CRY2, pCMV-GFP-
488TIM, pCMV-GFP-TIM R1081X, pCMV4a-TIM-FLAG, pCMV4a-TIM R1081X-FLAG,
489pCMV10-3xFLAG-TIM, pCMV10-3xFLAG-TIM R1081X, pCMV10-3xFLAG-

490hCRY2-WT-LUC, pCMV10-3xFLAG-hCRY1-WT-LUC, pCMV10-3xFLAG-hPER2-
491WT-LUC, pCMV10-3xFLAG-hPER1-WT-LUC, pCS2-MT-hPER2-WT, pCMV10-
4923xFLAG-TIM dNLS3, pCMV10-3xFLAG-TIM dNLS4, pSQT1313-TIM G1,
493pSQT1313-TIM G2, pSQT1313-TIM G5, pSQT1313-TIM G6, pSQT1601
494(Addgene #53369), pX458-TIM G1, pX458-TIM G5. For guide sequence, see
495gene editing section. For protein stability assays, cycloheximide (100 μg/ml,
496Santa Cruz Biotechnology Inc.) was added to the culture for intended length
497of treatment (0-8 hours). All cells were harvested 40-hours post-transfection,
498and lysates were assayed by Western blots.
499
500<u>Western blot assays</u>
501For whole-cell extracts, HEK293 cells were lysed in NP-40 buffer (150 mM
502NaCl, 1.0% NP-40, 50 mM Tris pH 8.0) plus complete Mini protease inhibitor
503cocktail (Roche). Preparation of the cytosolic and the enriched nuclear
504fractions was prepared with the Nuclear Extract Kit (Active Motif).
505Immunoprecipitation was performed with Ezview Red Anti-FLAG M2 affinity
506gels (Millipore Sigma). Proteins were separated by SDS-PAGE and transferred
507to Immobilon-FL PVDF membranes (Millipore Sigma), which were then
508blocked by SuperBlock (PBS) Blocking Solution (Thermo Fisher Scientific).
509Primary antibodies were diluted in PBST buffer (8mM Na_2HPO_4, 150mM NaCl,
5102mM KH_2PO_4, 3mM KCl, 0.05% Tween 20, pH 7.4) and interacted with blots at
5114°C overnight. The blots were then probed with IRDye secondary donkey anti
512rabbit/mouse/goat antibodies (LI-COR) at RT for 1 hr. The blots were imaged
513using the LI-COR Odyssey system (LI-COR). Protein intensities were
514measured using Image J software. β-actin was used as a loading control.
515LaminB and GAPDH were used as nuclear and cytoplasmic markers,
516respectively. Relative protein levels or protein abundance were normalized
517to levels in the control group. Proteins were detected with the following
518antibodies: anti-HA (Abcam, ab9110), anti-FLAG M2 (Sigma Aldrich, F1804),
519anti-GFP (Abcam, ab290), anti-β-actin (Abcam, AC-15), anti-GAPDH (Millipore
520Sigma, MAB374), anti-LaminB1 (Abcam, ab16048 and Santa Cruz, C20), anti-

521mPER2 (Alpha Diagnostic International, PER-21A), anti-hCRY2 (Santa Cruz,
522sc-130731) and anti-hTIM (Abcam, ab50943).
523
524Luciferase Assay
525Dual-Luciferase Reporter Assay (Promega) was performed in HEK293T cells
526transfected with a *Per2::luc* reporter and other expression constructs 24 hrs.
527post-transfection. For transfection, 100 ng per construct per well of a 24-well
528plate was used, except for TIM titration, when 100ng, 20ng, or 4ng of *TIM-WT*
529or *TIM-R1081X* constructs were transfected. A constitutively expressed
530*Renilla* Luciferase construct was used as an endogenous control. Luciferase
531activity was measured by Synergy H4 Hybrid Microplate Reader (BioTek).
532Results are expressed as mean ± SEM. All experiments were performed at
533least 2 times and all data presented are the average of 3 technical
534replicates.
535
536Immunocytochemistry
537293T cells were plated on 2-well chamber slides (Nunc Lab-Tek II) and
538transfected with TIM expression constructs. Cells were then fixed,
539permeabilized, and exposed to primary and secondary antibodies. Images
540were taken under a fluorescence microscope (Olympus). The LSM images
541were projected and quantified using ImageJ. Cell nucleus boundary was
542defined by DAPI-stained area. TIM immunocytochemical signal from cell body
543and nucleus of individual cells was calculated as total integrated density
544minus background of the cell body area.
545
546Generation of *Tim-R1078X* Mice
547sgRNAs were generated by MEGAshortscript T7 Transcription Kit (Thermo
548Fisher Scientific) and quantitated by NanoDrop (Thermo Fisher Scientific),
549according to Ran et al. (35). One cell stage C57BL/6 embryos were injected
550with Cas9 mRNA, sgRNAs, and 200-nt single-stranded oligodeoxynucleotides
551(ssODN, purchased from IDT) repair template and later transplanted to

552surrogate mothers. 15 animals were born and genotyped by TaqMan
553genotyping to identify founders (Fig. S1*B*). The *Tim R1078X* allele was
554verified by Sanger sequencing in founder #1L. The founder was backcrossed
555once to generate N1 mice. N1 littermates were then crossed to expand the
556colony. Sequences of guide, ssODN repair template, primer, and probe are
557listed in Table S1. All mouse work was performed in accordance with the
558guidelines of Institutional Animal Care and Use Committee at the University
559of California, San Francisco.
560
561<u>Generation of *TIM-R1081X* U2OS Cell Line</u>
562Guide RNAs were picked using MIT CRISPR Design (http://crispr.mit.edu/) and
563cloned into pX458 plasmids (Addgene #62988), according to Ran, et al (33).
564Cells were cultured for 24 hours before guide plasmid and 200-nt ssODN
565repair template (IDT) were delivered by nucleofection (Nucleofector 2b,
566Lonza). After 36-48 hours, GFP-positive single-cell clones were isolated in 96-
567well plates (33). Surviving and expanded clones were genotyped by TaqMan
568genotyping to identify founders (Fig. 2*D*). The *TIM-R1081X* allele was verified
569by Sanger sequencing in U2OS clone #37 (Fig. 2*E*). Guide, primer and probe
570sequences are listed in Table S1.
571
572<u>Bioluminescence rhythms in tissue culture</u>
573*Tim-RX* mutant mice were crossed with m*Per2*^{Luc} knock-in mice ((23); RRID
574IMSR_ JAX006852). Mice were sacrificed between ZT 2-3. Liver and lung were
575harvested. Liver tissues were thinly sliced under sterile conditions and
576cultured on Millicell culture membrane (PICMORG50, EMD Millpore, Billerica,
577MA) in 35 mm dishes with 1.4 ml of recording medium (phenol-red free
578DMEM (Sigma Aldrich) containing 10 mM HEPES- pH7.0, 3.5 g/L D-glucose,
5790.2 mM luciferin potassium salt, 0.35 g/L sodium bicarbonate, 2% B-27
580supplement (Thermo Fisher Scientific), 50 U/ml penicillin-streptomycin
581(Thermo Fisher Scientific)). Lung tissues were removed in small cubes and

582placed directly on 35 mm dishes which were allowed to adhere for 10
583minutes prior to the addition of recording medium. Bioluminescence was
584recorded continuously in a Lumicycle 32 instrument (Actimetrics, Wilmette,
585IL). Bioluminescence was detrended by subtracting 24 hr. average of
586bioluminescence using the Lumi- Cycle analysis software. The period length
587of each sample was determined by dampened sine-curve fitting using Lumi-
588Cycle analysis.
589
590Luciferase-based degradation assay
591HEK293T cells were co-transfected using Lipofectamine 3000 (Thermo Fisher
592Scientific) with 50 ng hCRY2-LUC or hPER2-LUC vectors and 1000ng TIM-WT,
593or TIM-RX, or empty CMV-10 vectors and cultured for 24 hr. The culture
594medium was replaced with recording medium [phenol-red free DMEM (Sigma
595Aldrich) supplemented with 10% fetal bovine serum, 3.5 mg/ml glucose, 50
596U/ml penicillin-streptomycin (Thermo Fisher Scientific), 0.05 mM luciferin,
597and 10 mM HEPES-NaOH; pH 7.0] containing 100 mg/ml cycloheximide (CHX;
598Santa Cruz Bio- technology Inc., Santa Cruz, CA). Luciferase activity of
599hCRY2-LUC of hPER2-LUC was recorded at 10-min intervals at 37∘C with a
600LumiCycle 32 instrument (Actimetrics). The luminescence signals were fitted
601to an exponential function to quantify the half-life of CRY2-LUC or PER2-LUC.
602
603
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612experiments. A.L. and L.A.P. carried out sequencing and data analysis. C.R.J.

613characterized human subjects. P.K., P-K.H., L.J.P. and Y-H.F. wrote the 614manuscript. **Competing interests:** The authors declare no competing 615interests.

616

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714

715**Figure Legends**
716
717**Table 1. Clinical variables of human subjects in a FASP family**
718
719**Fig. 1. *TIMELESS R1081X* mutation found in a FASP family.** (*A*) In
720kindred 5602, a *TIMELESS R1081X* nonsense mutation co-segregates with
721FASP. Filled and open shapes represent affected and unaffected individuals,
722respectively. Circles and squares represent women and men, respectively.
723Individuals with the *R1081X* mutation are heterozygous. (*B*) The *TIMELESS*
724*R1081X* mutation is located in the conserved TIMELESS_C domain. On the
725lower right, the amino acid alignment around the *R1081X* mutation (marked
726by a red asterisk) is shown. A blue line marks the last putative nuclear
727localization signal in the TIMELESS protein (NLS4). The mutation causes a
728truncated protein lacking NLS4.
729
730**Fig. 2. A Heterozygous h*TIM R1081X*/m*Tim R1078X* mutation was**
731**generated by genome editing and results in shortened period in**
732**cells but not in mice.** (*A*) Representative actograms of wheel-running
733activity for *Tim* +/+ (left) and *RX/*+ (right) mice. The yellow marks the times
734when the lights were on. Blue marks wheel-running activity. Activity onset
735was fitted to the red lines using ClockLab analysis software. (*B*) Period for
736*RX/*+ and *Tim* +/+ mice was determined by line-fitting of activity onset from
737day 1 to day 14 in DD (constant darkness) (n=21 for +/+, n=26 for *RX/+)* p
738> 0.05 (Student's t test). (*C*) The upper panel shows the *TIM* exon 27
739genomic locus (hg38, Chr12: 56418324-56418363, negative strand
740orientation) with the R1081 codon marked and highlighted in green. There is
741an EcoRI site overlapping the R1081 codon. Lower view shows the edited *TIM*
742*R1081X* allele in that genomic locus. The mutated nucleotides are in red, and
743the R1081X codon is marked and highlighted in green. A FLAG sequence was
744also inserted before the stop codon. The codon next to R1081X was mutated

745to create an EcoRV site for genotyping. (*D*) The upper panel shows a 746schematic of TaqMan-based genotyping design with specific fluorescence 747probes to either *WT* or *R1081X* alleles. The lower panel is the allelic 748discrimination plot of the genotype screening assays on CRISPR-edited U2OS 749cell clones. Fluorescence of probes to *WT* and *R1081X* alleles are shown on 750the X and Y axis, respectively. Red and green dots represent homozygous +/ 751+ and heterozygous *R1081X/+* clones. The genotyping assay was done in 752duplicate. (*E*) Representative Sanger sequencing trace of TA clones of U2OS 753Clone #37 confirming the *R1081X* allele and the EcoRV site. (*F*) On the left, 754are representative rhythms of *Bmal1-luc* reporter in U2OS-B6 cell lines with 755+/+ and *R1081X/+* genotype at endogenous *TIM* genomic locus. Data were 756detrended, normalized to the peak bioluminescence, and aligned to the first 757peak. Periods of the bioluminescence rhythm in the *TIM* +/+ and *R1081X/+* 758cells are quantified on the right. Results are expressed as mean ± SEM 759(n=8). ***$p < 0.001$ (Student's t test).

760

761**Fig. 3. Heterozygous *TimRX* Mutant mice have advanced phase of 762sleep-wake behavior and altered phase responsiveness to timed 763light pulses.** Representative actograms (*A*) and activity profiles (*B*) of 764wheel-running activity for +/+ and *RX/+* mice under skeleton photoperiod 765with fifteen-minute entraining arms for an eleven-day period. Yellow and 766grey bars in (*A*) (right) represent LD 12:12 entrainment and skeleton 767photoperiod paradigms, respectively. Black and yellow bars in (*B*) (below) 768represent periods of darkness and light, respectively. The Y-axis represents 769wheel-running amplitude. (*C*) Activity onset/offset from the onset of the light 770pulse. Results are expressed as mean ± SEM (n=11 for +/+, n=9 for *RX/+*). 771*$p < 0.05$, **$p < 0.01$ NS = not significant (Student's t test). (*D*) Wake 772percentage from EEG recordings in LD 12:12 at the time of light transition. 773Time epochs representing the change from lights-on to lights-off (left), and 774lights-off to lights-on (right) are shown. Yellow blocks indicate lights-on. 775Wake percentage is provided in the Y-axis (n = 6 for +/+, n = 9 for *RX/+*). *p

776< 0.05 (Two-way ANOVA and post-hoc multiple comparison with Fisher's 777test). (*E*) Top: Representative double plotted actograms showing baseline 778behavior (BL) in LD 12:12 and in DD, and phase-shifts (below) in response to 779a 30-minute light exposure at ZT 14 and ZT 22 indicated by red arrows. 780**Bottom: Average phase shifts by minutes for BL, ZT 14, and ZT 22 781(n=15 for +/+, n=20 for *RX*/+). **p < 0.01, ***p < 0.001 (Student's t 782test).**

783

784**Fig. 4. Altered molecular function of TIM by the R1081X mutation.**
785(*A*) Repressor activity of WT or R1081X TIM on CLOCK-BMAL1–mediated
786expression was examined with *Per2* promoter-driven luciferase activity in
787HEK293T cells. Luciferase activity was normalized to activity levels in cells
788without CLOCK/BMAL1 (white column). PER2 from two different constructs
789were positive controls for repressor activity. Genotype effect: **P < 0.01,
790(Two-way ANOVA) (*B*) Western blot and time course of TIM-WT and TIM-
791R1081X abundance after cycloheximide (CHX) was supplied to transfected
792293T cells. Results are expressed as mean ± SEM (n=3). Genotype effect:
793**P < 0.01, ***P < 0.001, (Two-way ANOVA). (*C*) Immunocytochemistry
794demonstrates the subcellular localization of TIM WT and TIM R1081X in 293T
795cells 24 hours post-transfection. Boundaries of nuclei were determined, and
796the proportion of TIMELESS signal within the nucleus vs. whole cell (nuclear
797fraction) was quantified (n=20-25 each group). ***p < 0.001 (Student's t
798test). (*D*) Western blot of TIM WT and TIM R1081X protein in enriched nuclear
799and cytoplasmic fractions. Lamin B and GAPDH were nuclear and
800cytoplasmic markers, respectively. Relative intensities of TIM were quantified
801and are shown on the right (n=5). ****p < 0.0001 (Student's t test).

802

803**Fig. 5. TIM modulates PER2-CRY2 complex stability and this 804modulation is altered by the R1081X mutation.** (*A*) Co-805immunoprecipitation of TIM WT or TIM R1081X by CRY2 in 293T lysates. 806CRY2-bound TIM was normalized to amount of bound TIM WT. Results are

807expressed as mean ± SEM (n=5). ***$p < 0.001$ (Student's t test). (*B*) Co-
808immunoprecipitation of PER2 by TIM WT and TIM R1081X in 293T lysates.
809TIM-bound PER2 was normalized to the amount of input PER2. Results are
810expressed as mean ± SEM (n=6), $p > 0.05$ (Student's t test). (*C*) Left upper
811and left lower views show half-life and decay plots of CRY2:luc after HEK
812293T cells were transfected with TIM WT, TIM R1081X or CMV (vector control)
813and PER2 followed by CHX administration. Right upper and lower panels
814show a dose dependent reduction in CRY2:luc (top) or PER2:luc (below)
815stability when co-transfected with increasing amounts of TIM WT. Results are
816expressed as mean ± SEM (n=10). ****$p < 0.0001$, NS = not significant
817(One-way ANOVA with Tukeys' multiple comparisons test).
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