Acknowledgements We thank M. Rees for comments on statistical analyses. This work was supported by the UK Natural Environment Research Council (NERC).

Competing interests statement The authors declare that they have no competing financial interests.

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Functional consequences of a *CKIδ* mutation causing familial advanced sleep phase syndrome

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Familial advanced sleep phase syndrome (FASPS) is a human behavioural phenotype characterized by early sleep times and early-morning awakening[1]. It was the first human, mendelian circadian rhythm variant to be well-characterized, and was shown to result from a mutation in a phosphorylation site within the casein kinase I (CKI)-binding domain of the human *PER2* gene. To gain a deeper understanding of the mechanisms of circadian rhythm regulation in humans, we set out to identify mutations in human subjects leading to FASPS. We report here the identification of a missense mutation (T44A) in the human *CKIδ* gene, which results in FASPS. This mutant kinase has decreased enzymatic activity *in vitro*. Transgenic *Drosophila* carrying the human *CKIδ-T44A* gene showed a phenotype with lengthened circadian period. In contrast, transgenic mice carrying the same mutation have a shorter circadian period, a phenotype mimicking human FASPS. These results show that CKIδ is a central component in the mammalian clock, and suggest that mammalian and fly clocks might have different regulatory mechanisms despite the highly conserved nature of their individual components.

Phosphorylation has a central role in the regulation of circadian clocks[2–5]. Multiple points of regulation have been proposed, including the nuclear import and export of circadian proteins, and the active/inactive states of these proteins, but the details of this system are not well understood[6–8]. In *Drosophila*, Per and Tim are two core proteins of the circadian clock. Doubletime (Dbt) and casein kinase II (CKII) phosphorylate Per, and Shaggy (Sgg) phosphorylates Tim[9–13]. The Syrian hamster *tau* mutation was identified in the gene for casein kinase Iε (*CKIε*), a mammalian homologue of *Drosophila dbt*[14]. The known mutations in *dbt*, *CKII*, and *CKIε* lead to hypophosphorylation *in vitro*[11,12,14,15]. However, the different mutations can give rise to different phenotypes *in vivo*, with longer or shorter circadian periods (τ).

FASPS is a behavioural phenotype manifest by early sleep times, early-morning awakening, and a short τ (ref. 1). It was shown to result from mutation in a phosphorylation site within the CKI-binding domain of the human (h) PER2 protein. To gain a deeper understanding of the mechanisms of human circadian regulation, we set out to identify mutations in human subjects that lead to FASPS. We report here the identification of a mutation in the human *CKIδ* gene (also known as *CSNK1δ*), which causes FASPS in humans. This mutation results in reduced kinase activity *in vitro* and leads to a shorter circadian period in mice, but a longer period in *Drosophila*.

The proband of this study was noted by one of the authors (R.E.S.) to have FASPS. Further evaluation of this subject's family revealed autosomal-dominant transmission of the behavioural trait. Fifteen family members, spanning three generations, were separately interviewed for their typical work and vacation sleep–wake schedules by two of the authors (C.R.J. and R.E.S.). Five individuals definitely affected with FASPS (age range 20–65 yr, mean 41 yr) were identified (Fig. 1a). In the absence of competing psycho-social demands, both the average sleep onset time (18:12 ± 1.4 h versus 23:24 ± 1.1 h) and final wake time (04:06 ± 0.7 h versus 08:00 ± 1.6 h) of these subjects were significantly earlier ($P < 0.0001$) than the nine unaffected family members. Affected individuals reported the onset of FASPS somewhere between early childhood to the mid-teen years.

Clinical features or a history of depression were found in three of the FASPS subjects, two of who had mildly elevated Beck Depression Inventory scores of 12 (ref. 16). A tendency towards winter depression was reported by a fourth FASPS subject. However, early-morning awakening due to depression was considered an unlikely explanation for FASPS in this family because the affected individuals showed little difficulty initiating or maintaining sleep, and had good energy levels in the morning (subjectively assessed). In addition, the young age of FASPS onset in these individuals was well below the age at which the typical early-morning awakening develops in depression[17].

While screening candidate genes for circadian mutations (Supplementary Information), an A-to-G change was identified in the DNA sequence of *CKIδ*, which causes a threonine-to-alanine alteration at amino acid 44 in the protein (Fig. 1b). This

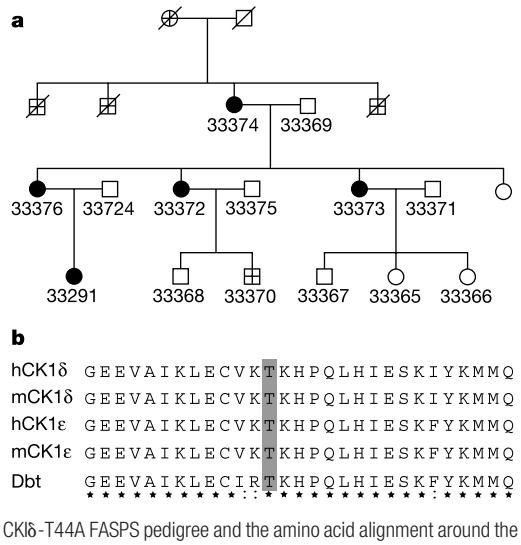

Figure 1 CKIδ-T44A FASPS pedigree and the amino acid alignment around the mutation. **a**, FASPS kindred 5231. Circles represent women, squares denote men, filled circles and squares show affected individuals; empty circles and squares show unaffected individuals. The individual marked with a cross is 'probably affected' but was conservatively classified as unknown. Diagonal lines across symbols indicate deceased individuals. **b**, Alignments for *Drosophila* Dbt and mouse (m) and human (h) CKIδ and CKIε proteins. The T44A mutation is highlighted.

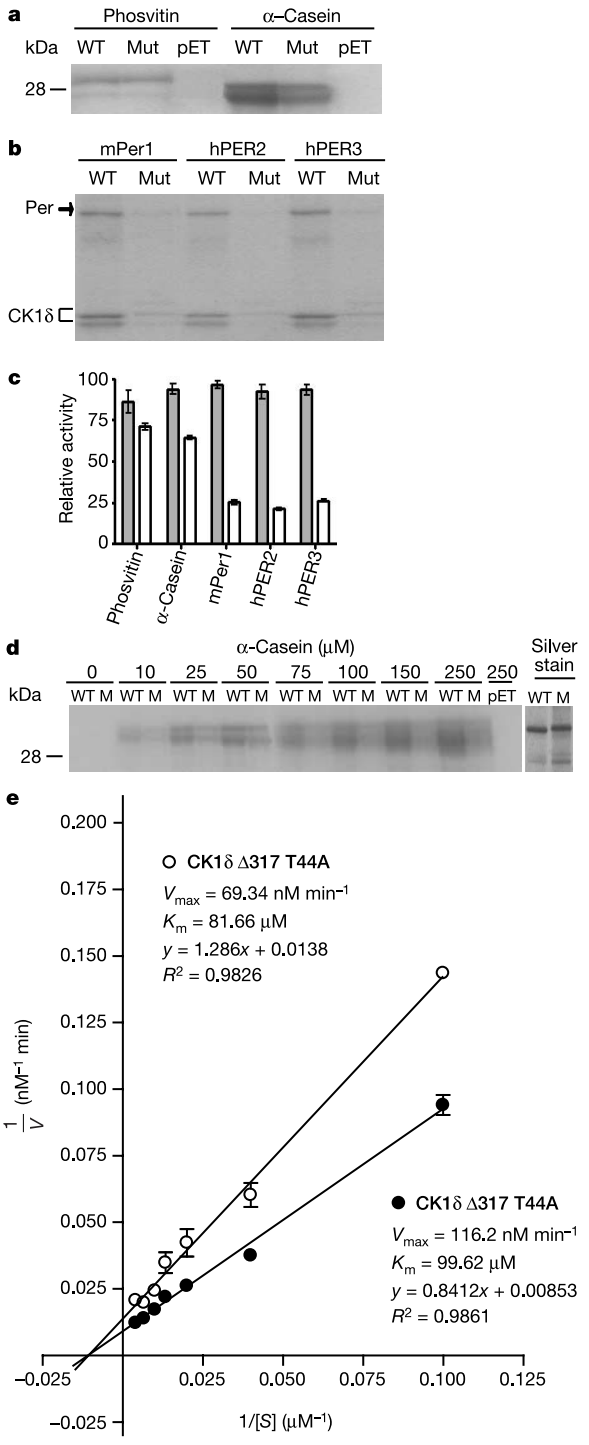

Figure 2 Biochemical characterization of CKIδ-T44A. **a**, **b**, *In vitro* kinase assays of wild-type and mutant CKIδΔ317 using the generic substrates phosvitin (100 μM) or α-casein (100 μM) (**a**), or *in vitro* translated circadian substrates, mPer1, hPER2 and hPER3 (**b**). pET, empty vector control. **c**, Quantitative analysis of kinase assays from **a** and **b**. Relative activity is defined as the percentage of activity relative to the highest measured activity for the same substrate. The experiment was performed in triplicate. Error bars indicate s.d. **d**, Kinetic analysis of wild-type (WT) and mutant (M) CKIδΔ317 using varying concentrations of α-casein. An empty vector control (pET) was included. One autoradiogram representative of nine experiments is shown. **e**, Lineweaver–Burk (double-reciprocal) plot of data derived from **d**. Each point represents the average ± s.e.m. from three experiments.

mutation (CKIδ-T44A) occurs at a residue that is conserved from mammalian CKIs through *Drosophila* CKI (also known as Dbt), and co-segregates with the FASPS phenotype in this family. This particular change was not found in over 250 control DNA samples.

We expressed the wild-type and mutant carboxy-terminal-truncated CKIδΔ317 (ref. 18) in a bacterial expression system (Supplementary Information). *In vitro* kinase assays with these bacterially expressed proteins and exogenous substrates (phosvitin and α-casein) showed that CKIδ-T44A has decreased activity compared with wild-type protein (Fig. 2a). Similar results were obtained with circadian-relevant substrates, PER1–3 (Fig. 2b). Interestingly, the defect in phosphorylation is greater when using PER polypeptides compared with α-casein or phosvitin (Fig. 2c). This observation suggests that CKIδ-T44A may have different effects on different substrates. Michaelis–Menten kinetic analyses were performed using wild-type and CKIδ-T44A enzyme (Fig. 2d). The Michaelis constant (K_m) and maximum reaction velocity (V_{max}) for both wild-type and CKIδ-T44A were calculated from nonlinear regressions of untransformed data. The resulting Lineweaver–Burk plot is shown in Fig. 2e. The V_{max} for CKIδ-T44A was 60% of the wild-type V_{max}, but the difference between K_m values is less dramatic (K_m for CKIδ-T44A is 82% of wild type).

We introduced the human *CKIδ* gene carrying the T44A mutation into flies in order to examine the effect on circadian activity (Supplementary Information). For accurate comparison, transgenic flies were generated containing either the wild-type or the mutant *hCKIδ* genes driven by the UAS–GAL4 system, using *tim*-UAS-*gal4* for expression in the lateral neurons, which are the central pacemaker cells of the *Drosophila* clock[19]. Three independent lines for each wild-type and mutant UAS-*hCKIδ* were crossed to *tim*-UAS-*gal4* flies and their locomotor activity was studied in constant darkness (DD) after entrainment for 3–4 days under 12 h light–12 h dark (LD) conditions (Supplementary Fig. 1a). Transgenic lines expressing the human *CKIδ* gene showed a longer circadian period length than the wild-type lines and undriven lines (which contain the transgene but are not crossed to *gal4* lines, and so do not express *CKIδ*). In all the *tim*-UAS-*gal4*/UAS-*hCKIδ* lines studied, transgenic flies expressing the mutant form of the *hCKIδ* gene had a significantly longer period than the flies with the wild-type *hCKIδ* gene (Table 1).

To ensure that the elongation of period seen in the *hCKIδ-T44A* transgenic flies was not due to increased expression of the transgene, we carried out semi-quantative polymerase chain reaction with reverse transcription (RT–PCR) analysis using messenger RNA from fly heads (Supplementary Fig. 1b). The wild-type fly line (E25), which had a higher expression of the *hCKIδ* gene than mutant lines (H3 and H10), had a shorter period than all the mutant lines. This suggests that the mutation has functional consequences, as the mutant transgene leads to greater period elongation than the wild-type transgene. We then introduced the mutation at the conserved T44 residue of *dbt*, the *Drosophila* homologue of *hCKIδ*. Flies expressing the wild-type *dbt* gene driven by *tim*-UAS-*gal4* showed a period of 24.97 h. Flies expressing *dbt* with the *hCKIδ-T44A* mutation showed period elongation to 25.56 h (Supplementary Table 1). This period elongation is similar to observations when the human *CKIδ-T44A* mutant gene was expressed in flies. As an additional control, we also expressed the dbt^h allele, which is caused by a Thr–Ile mutation at the same residue as the FASPS *hCKIδ* mutation. These flies showed an extreme elongation of circadian period to 29.45 h, similar to the period seen in the dbt^h homozygotes[20] (Supplementary Table 1). This shows that the period lengthening we observed in the fly system is indeed a consequence of the mutation and not an artefact caused by overexpressing the *hCKIδ* or *dbt* genes.

We also created transgenic mice with a human BAC clone (RP11-1376P16) containing the entire wild-type *CKIδ* gene. This

Table 1 **Period variation in control and transgenic flies**

Genotype	Line	Period length (h)	s.d. (h)	Number of rhythmic flies	Total number tested
tim-UAS-*gal4*/UAS-*hCK1δ*	Line 1 (E25)	25.54	0.31	38	39
	Line 2 (E27)	25.39	0.28	27	27
	Line 3 (F5)	25.14	0.32	38	38
tim-UAS-*gal4*/UAS-*hCK1δ-T44A*	Line 1 (H3)	25.96	0.39	36	36
	Line 2 (H8)	26.00	0.32	35	36
	Line 3 (H10)	25.86	0.26	24	26
UAS-*hCK1δ*/+	Line 1 (E25)	23.56	0.29	9	10
	Line 2 (E27)	23.88	0.39	13	13
	Line 3 (F5)	23.80	0.26	9	10
UAS-*hCK1δ-T44A*/+	Line 1 (H3)	23.68	0.36	10	10
	Line 2 (H8)	23.60	0.46	10	10
	Line 3 (H10)	23.67	0.34	10	10
tim-UAS-*gal4*/+		24.13	0.28	10	10
*w**		23.57	0.30	18	21

Period length (τ) was significantly longer in *tim*-UAS-*gal4*/UAS-*hCKIδ-T44A* flies compared with *tim*-UAS-*gal4*/UAS-*hCKIδ* flies ($P < 0.0001$, ANOVA).
**w* is a recessive allele (*white*) affecting eye pigmentation. It serves as a control because all the transgenic lines were created using this background.

BAC clone was modified to introduce the T44A variant (Supplementary Fig. 2a). The resulting BAC construct contains the promoter as well as an additional 32 kilobases (kb) upstream of the gene, and is expected to contain all *cis*-acting regulatory elements needed to accurately reproduce the endogenous pattern of expression in humans[21]. Transgenic mice had no gross phenotypic or behavioural abnormalities. Analysis of transgene copy number showed that two lines contained 2–3 copies (H lines) and four lines had one copy each (L lines) (Supplementary Fig. 2b). RT–PCR with human- and murine-specific primers was used to compare the transcription levels from human *CKIδ* and murine *Csnk1δ* gene expression (Supplementary Fig. 2c). Only transgenic mice gave PCR products when human-specific primers were used, confirming human *CKIδ* expression in these mice. Semi-quantitative PCR analysis showed that different transgenic lines had endogenous *Csnk1δ* expression levels similar to wild-type mice, and that the expression level of human *CKIδ* is higher in H lines than in L lines. These results imply that BAC transgene expression levels are related to copy number and independent of the site of integration.

Wheel-running activity of wild-type and *hCKIδ-T44A* transgenic mice was examined by entraining the animals to a schedule of 12 h light–12 h dark (LD) for 7 days before transferring them to constant darkness (DD). Under LD and DD conditions, wild-type and *hCKIδ* transgenic lines showed similar and robust circadian rhythms of activity (Fig. 3a–f). Free-running periods were significantly shorter in transgenic animals compared with wild-type mice (analysis of variance (ANOVA), $P < 0.001$; Fig. 3g). To address the possibility of

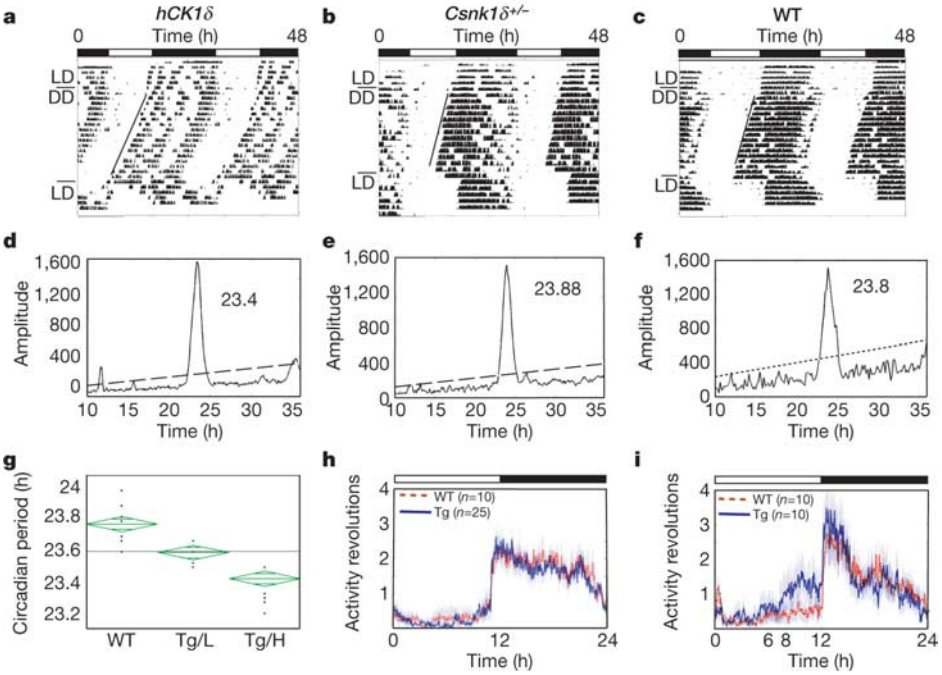

Figure 3 Circadian locomotor activity of *hCKIδ* transgenic mice. **a**–**c**, Voluntary locomotor activity was recorded as wheel-running activity for *hCKIδ* transgenic mice, *Csnk1δ* $^{+/-}$ heterozygous knockout mice and wild-type mice under LD and DD conditions. **d**–**f**, Periodograms of locomotor activity for **a**–**c**, showing periods of 23.4 h (**d**), 23.88 h (**e**) and 23.8 h (**f**). **g**, The distribution of period length determined for days 8–21 in wild-type, low copy number transgenic mice (Tg/L), and high copy number transgenic mice (Tg/H). Average lengths of the free-running periods (±s.d.) were: wild type, 23.78 ± 0.12 h ($n = 17$); Tg/L, 23.62 ± 0.07 h ($n = 19$); Tg/H, 23.42 ± 0.12 h ($n = 17$). $P < 0.001$, ANOVA. **h**, **i**, Onset activity analysis of wild-type (red) and transgenic (blue) mice during initial LD entrainment (**h**) and re-entrainment after the free-running wheel test (**i**). During entrainment, the light was switched on at T = 0 h and switched off at T = 12 h.

dosage effect, we generated knockout mice for the human *CKIδ* homologue, *Csnk1δ* (Supplementary Fig. 3). The *Csnk1δ*$^{-/-}$ homozygous mice died within days of birth. We therefore obtained *Csnk1δ*$^{+/-}$ heterozygotes (containing one copy of the wild-type gene), and measured their activity rhythms. These mice showed no change in τ (23.88 ± 0.2 h, $n = 7$) when compared to wild-type mice (23.80 ± 0.1 h, $n = 17$). These results provide strong evidence that the circadian period change in *hCKIδ-T44A* transgenic mice is due to the T44A mutation. These results represent the first direct evidence that *CKIδ* is a core clock component, and that a *CKIδ* mutation gives rise to similar circadian phenotypes in humans and mice.

Activity patterns in wild-type and *hCKIδ-T44A* transgenic mice were indistinguishable under regular LD conditions (Fig. 3h). However, after free running, the *hCKIδ-T44A* transgenic mice required a significantly longer time to re-entrain to a LD regimen compared with wild-type mice (Fig. 3i). This observation is different from the *tau* hamster, for which clear phase-shift under LD conditions was found[22]. Whether this results from an altered phase angle or from decreased sensitivity to masking by light remains to be elucidated.

Precise control of the mammalian clock requires careful regulation of clock protein production, post-translational modifications and protein–protein interactions[6,23,24]. Phosphorylation affects the stability and nuclear translocation of clock proteins[6,11,14,25]. Understanding how CKI mutations alter circadian period length requires an understanding of how the mutations affect the enzymatic properties of CKI and its interactions with clock-relevant substrates. Here, we showed that the human CKIδ-T44A mutation has attenuated enzyme activity *in vitro*. This mutation co-segregates with the FASPS phenotype in the affected family but was not found in controls. Whether CKIδ-T44A also causes a delay in the appearance of hyper-phosphorylated substrates (as in the *tau* hamster) remains to be seen. Interestingly, the activity pattern of *CKIδ-T44A* mice is different from that of the *tau* hamster even though the mutations are in homologous genes, suggesting that these mutations influence different downstream effects. The results presented here suggest a direct role for CKIδ in circadian clock regulation.

We have previously noted a tendency of FASPS-affected individuals to have higher scores on the Beck Depression Inventory[16]. It is intriguing that four of the five affected individuals in the K5231 kindred have clinical features or a history of depression. This might be a simple coincidence, or the depression might be situational, resulting from being 'out of phase' with the rest of the world. A more provocative possibility is that circadian rhythm variants contribute to affective behaviour. Sensitivity to light is a shared characteristic of both the circadian system (the process of entrainment) and affective disorders (such as seasonal affective disorder). *shaggy*, a circadian mutant in flies[11], results from mutations in GSK3β, the protein which is a principal target of lithium, a drug used to treat severe depression[26]. It is also noteworthy that sleep deprivation is therapeutic in some patients with severe depression that is resistant to pharmacological therapy[27]. These *CKIδ* transgenic mice will allow testing of this and other possibilities prompted by observations in human FASPS subjects.

It is interesting that the same mutation in the *hCKIδ* gene produces opposite effects on the circadian period when expressed in flies as opposed to mice. This suggests that despite the highly conserved nature of individual components of the circadian clock in mammals and flies, the interactions of these components as part of the circadian regulatory network may be different. The differences in the activity pattern and period length change between transgenic mice carrying the *hCKIδ* mutation and the *tau* hamster indicate that different mutations in the similar protein may have different downstream effects, and that separate regulatory mechanisms are responsible for period length and entrainment. These models provide unique opportunities to dissect further the molecular mechanisms of circadian clocks, and to perform extensive characterization of a gene variant relevant to human circadian function. New insights into CKI functions could have implications for the development of novel compounds for the treatment of sleep disorders. □

Methods

Diagnostic and classification criteria

Subjects signed a consent form approved by the Institutional Review Board at the University of Utah. Self-reported habitual sleep-wake schedule was obtained during structured interviews by two of the authors (C.R.J., R.E.S.). Self-report was supplemented by parental report for several of the children still at home. Individuals were considered to have FASPS if (1) they habitually preferred to fall asleep before 20:30 and to wake up spontaneously by 05:30 local time throughout the year, in the absence of self-imposed early morning bright lights, stimulant or sedative drugs, early morning work demands, or psychosocial preferences that compete with sleep; (2) the onset of FASPS was before 30 years of age, and not within three months after traumatic brain injury; and (3) there was only one major sleep period per 24-h day[1]. Individuals were considered unaffected if they preferred to go to sleep after 21:30 and wake after 06:30 in the absence of psychosocial demands or drug use, and if this preference was stable before the age of 30. Individuals not meeting either 'affected' or 'unaffected' criteria were considered to be of 'unknown' phenotype. Blood sample collection and DNA preparation were performed as previously described[28].

In vitro kinase assays

Kinase assays were performed as described elsewhere[14] using 150 ng recombinant CKIδΔ317. Phosvitin, α-casein, or 8 μl of an in vitro translation reaction (Promega) was used as the substrate. For substrates consisting of circadian proteins, kinase was first incubated with translation reaction for 1 h at room temperature, followed by five washes using 1 × Bind/Wash Buffer (Novagen). Bound beads were then subjected to the kinase assay. The reaction was terminated by the addition of 4 × LDS sample buffer and 10 × reducing agent (Invitrogen) followed by a 10 min incubation at 70 °C. Products were resolved on a 12% Bis-Tris NuPAGE gel (Invitrogen). Gels were fixed, dried and exposed to X-OMAT AR film (Kodak). Bands were quantified using NIH Image and substrate–velocity plots were determined using nonlinear regression (GraphPad) to determine V_{max} and K_m.

Fly locomotor activity analysis

Young, male, adult flies of the specified genotype were entrained for 3–4 days in 12 h light–12 h dark (LD) conditions. Locomotor activity was subsequently recorded in constant darkness (DD) for 7–8 days using Trikinetics locomotor activity monitors at 25 °C. The circadian period of the flies was calculated using χ^2 periodogram analysis ($\alpha = 0.01$) on the ClockLab software package. Only flies for which the χ^2 statistic exceeded the significance line by 5 were judged to be rhythmic and used for period calculations.

Generation of mice transgenic for human *CKIδ*

Transgenic mice were generated using standard procedures. The transgenic founders were on a C57BL/6 × SJL F_1 background and were backcrossed to C57BL/6 mice in successive generations. Founders were determined by PCR analysis using 6 primer pairs every 15 kb around this gene. Subsequent to the first generation, genotyping was performed using PCR analysis of tail biopsy DNA with primers (5′-AAGAGGGGAGTACGTGTGTAGAAC-3′ and 5′-AATATAAATGTGGGGACTGAGCAT-3′). The PCR protocol consisted of 3 min at 94 °C, 35 cycles of amplification (30 s at 94 °C, 30 s at 57 °C, and 90 s at 72 °C), and a final extension phase (10 min at 72 °C), and yielded a ~500 bp band. Transgene copy number was estimated by Southern blot. Tail DNA or 0×, 1×, 3×, 5× and 10× BAC DNA-spiked tail DNA (10 μg per lane) was digested with *Bgl*II, electrophoresed on 0.8% agarose, and transferred to a Hybond N+ membrane. The blot was hybridized with a random prime-^{32}P-dCTP-labelled probe amplified from the BAC clone. Inclusion of the copy standards allowed us to calculate the approximate number of transgenes integrated for each line. For RT–PCR, total RNA was extracted from mouse brain with TRIzol (Invitrogen) according to the manufacturer's instructions, then subjected to DNase treatment (Ambion). RNA (10 μg) was reverse-transcribed using Superscript III (Invitrogen). The primer sequences for human *CKIδ* were 5′-CTGCTCCAGGAAATTCA GCC-3′ and 5′-AGGTCGGACGAGGAGATGTT-3′. The primer sequences for mouse *Csnk1δ* were 5′-TCTGTTCAAGGAAGTTTAGT-3′ and 5′-AGATCAGATGAGGAGAC GTT-3′. The primer sequences for the actin gene (used as an endogenous control reference) were 5′-CTCTTTGATGTCACGCACGATTTC-3′ and 5′-GTGGGCCGCTC TAGGCACCAA-3′. The following conditions were used for PCR reactions: 3 min at 94 °C, 30 cycles of amplification (30 s at 94 °C, 30 s at 60 °C, and 90 s at 72 °C), followed by 7 min final extension at 72 °C.

Mouse behavioural analysis

Three- to four-month-old animals were housed individually in cages equipped with running wheels, and exposed to a 12 h light–12 h dark cycle for one week before being released into constant darkness (wild type, $n = 17$; *CKIδ* transgenic mice, $n = 37$). Dim red light was present in each compartment at all times, including those designated as 'dark'. Activity data were collected[29,30]. Using ClockLab (Actimetrics) software, data

analysis included a χ^2 periodogram, fast Fourier transformation (FFT), least squares fit of activity onset, and total activity analyses. The χ^2 periodogram was performed at 1-min resolution for days 1–14 of constant darkness, and saved in digital format.

Received 9 November 2004; accepted 4 February 2005; doi:10.1038/nature03453.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements The authors thank the FASPS subjects and their families for participating in this research. We thank U. Heberlein for advice and generous use of fly laboratory facilities, R. Threlkeld for *Drosophila* injections, and A. Rothenfluh for discussions and support with *Drosophila* transgenic lines. We also thank M. W. Young and L. Saez for the *tim*-UAS-*gal4* stock and helpful discussions, and S. Reppert for the mouse *Per1* clone. We acknowledge J. Cheung, E. Stryker and C. Whitney for technical assistance and members of the Fu and Ptáček laboratories for discussions. R.E.S. is supported by an NIH GCRC grant and the FAHC/UVM Office of Patient Oriented Research. This work was supported by an NIH grant to Y.-H.F. and L.J.P., and a Sandler Neurogenetics grant to Y.-H.F. L.J.P. is an investigator of the Howard Hughes Medical Institute.

Competing interests statement The authors declare that they have no competing financial interests.

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Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis

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Human osteoarthritis is a progressive disease of the joints characterized by degradation of articular cartilage. Although disease initiation may be multifactorial, the cartilage destruction appears to be a result of uncontrolled proteolytic extracellular matrix destruction. A major component of the cartilage extracellular matrix is aggrecan, a proteoglycan that imparts compressive resistance to the tissue. Aggrecan is cleaved at a specific 'aggrecanase' site in human osteoarthritic cartilage[1–2]; this cleavage can be performed by several members of ADAMTS family of metalloproteases[3–9]. The relative contribution of individual ADAMTS proteases to cartilage destruction during osteoarthritis has not been resolved. Here we describe experiments with a genetically modified mouse in which the catalytic domain of ADAMTS5 (aggrecanase-2) was deleted. After surgically induced joint instability, there was significant reduction in the severity of cartilage destruction in the ADAMTS5 knockout mice compared with wild-type mice. This is the first report of a single gene deletion capable of abrogating the course of cartilage destruction in an animal model of osteoarthritis. These results demonstrate that ADAMTS5 is the primary 'aggrecanase' responsible for aggrecan degradation in a murine model of osteoarthritis, and suggest rational strategies for therapeutic intervention in osteoarthritis.

The two major structural components of cartilage extracellular matrix are the proteoglycan aggrecan and type II collagen. Pathologic cleavage of aggrecan at Glu 373/Ala 374 (the 'aggrecanase' site) was identified as the major site of aggrecan degradation in human joint disease by analysis of synovial fluid samples from a range of human joint pathologies including osteoarthritis[1,2]. This is also the primary site of aggrecan cleavage in response to inflammatory stimuli[10]. Several members of the ADAMTS (a disintegrin and metalloprotease with thrombospondin-like repeat) family of enzymes (ADAMTS1, 4, 5, 8, 9 and 15) are known to be capable of cleaving aggrecan at the Glu 373/Ala 374 site[3–9], but ADAMTS4 and ADAMTS5 (aggrecanase-1 and aggrecanase-2, respectively) seem to be the most active aggrecanases[8,9]. Which aggrecanase is responsible for aggrecan degradation during human articular cartilage destruction, however, remains unclear. We addressed this question using gene-targeted deletion of the catalytic domains of ADAMTS4 or ADAMTS5 in mice, followed by induction of cartilage degradation by surgically induced joint instability. The functions of ADAMTS4 and 5 activity in normal development and physiology were also investigated by analysis of the histological appearance of organs in adult animals.

We have previously reported the generation of ADAMTS4 knockout ($ADAMTS4^{-/-}$) mice[11]. The ADAMTS5 ($ADAMTS5^{-/-}$) knockout was created by cre/lox mediated recombination, resulting in the deletion of 56 amino acids encoded by exon 3, including disruption of the zinc-binding site and deletion of the conserved 'Met-turn' (Fig. 1a). The deletion was confirmed by polymerase chain reaction (PCR) from tail DNA (Fig. 1b). PCR with reverse transcription (RT–PCR) from spleen total RNA gave PCR products