Veela defines a molecular link between Cryptochrome and Timeless in the light-input pathway to *Drosophila*'s circadian clock

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Organisms use the daily cycles of light and darkness to synchronize their internal circadian clocks with the environment. Because they optimize physiological processes and behavior, properly synchronized circadian clocks are thought to be important for the overall fitness. In *Drosophila melanogaster*, the circadian clock is synchronized with the natural environment by light-dependent degradation of the clock protein Timeless, mediated by the blue-light photoreceptor Cryptochrome (Cry). Here we report identification of a genetic variant, *Veela*, which severely disrupts this process, because these genetically altered flies maintain behavioral and molecular rhythmicity under constant-light conditions that usually stop the clock. We show that the *Veela* strain carries a natural *timeless* allele (*ls-tim*), which encodes a less-light-sensitive form of Timeless in combination with a mutant variant of the F-box protein Jetlag. However, neither the *ls-tim* nor the *jetlag* genetic variant alone is sufficient to disrupt light input into the central pacemaker. We show a strong interaction between *Veela* and *cryptochrome* genetic variants, demonstrating that the Jetlag, Timeless, and Cry proteins function in the same pathway. *Veela* also reveals a function for the two natural variants of *timeless*, which differ in their sensitivity to light. In combination with the complex array of retinal and extraretinal photoreceptors known to signal light to the pacemaker, this previously undescribed molecular component of photic sensitivity mediated by the two Timeless proteins reveals that an unexpectedly rich complexity underlies modulation of this process.

F-box | polymorphism | photoreception

Most organisms live throughout the year in light/dark (LD) cycles. This natural fluctuation represents a crucial stimulus to adjust the internal circadian clocks to operate in synchrony with the environment (1). Exposure to constant light dramatically affects biological rhythms and molecules comprising the circadian clock in many organisms. In *Drosophila*, the chronic presence of light (constant light, called LL) usually results in behavioral arrhythmicity and a breakdown of molecular oscillations in the circadian clock (2–4). Although the experimental LL situation is artificial, mutations that abolish this LL effect define essential components of light-signaling pathways that synchronize the central pacemaker to the external world. So far, this has been shown for the Cryptochrome (Cry) mutations cry^b and cry^m (5–7). Crys are related to photolyases, blue-light photoreceptors that use harvested light energy to repair UV-damaged DNA (8). In animals and plants, Cry proteins have been shown to function in the circadian system as photoreceptors, clock factors, or both (8, 9).

Opsin-mediated retinal, extraretinal, and Cry-independent photoreception contributes to light synchronization of the circadian clock in *Drosophila* (5, 10, 11). However, the main entrainment pathway is believed to involve light-dependent Cry and Timeless (Tim) and perhaps Period (Per) interactions within the behavioral pacemaker neurons of the fly brain (7, 12, 13). Upon light activation, Cry is thought to undergo a conformational change that allows it to bind to Tim in a way that irreversibly targets this clock protein for degradation by the proteasome (7, 14, 15). This light-induced degradation of Tim is crucial for molecular and behavioral clock resetting. If Tim is degraded prematurely by light pulses given at the end of the night, as a consequence the molecular feedback loops comprising the circadian clock and regulating rhythmic locomotor behavior are phase-advanced. Vice versa, the clock reacts with phase delays in case Tim is degraded by light exposure in the early night (reviewed, for example, in ref. 16).

By characterizing the genetic variant *Veela*, which, like *cry* mutants, behaves abnormally rhythmically in constant light, we identified a factor involved in the Cry-dependent light-input pathway of *Drosophila*. *Veela* genetically interacts with cry^b and shows decreased light sensitivity of Tim degradation. We demonstrate that these effects are caused by the simultaneous presence of a natural (less light-sensitive) form of Tim and a mutation in the F-box protein Jetlag (Jet). Importantly, the same *jetlag* (*jet*) mutant in combination with another natural and common variant of Tim behaves like wild type (WT). Therefore, previous findings attributing observed light-input defects solely to mutations in the *jet* gene (17) need to be revised.

Results

Isolation and Initial Mapping of *Veela*. During behavioral analysis of potential light-synchronization mutants in *D. melanogaster*, we identified a strain that exhibits robust rhythmicity in LL (Fig. 1 and Table 1). The variant mapped to chromosome 2 and, because of its elusive nature (see *Supporting Text*, which is published as supporting information on the PNAS web site), was named *Veela* (18). The mutant showed a semidominant effect: ≈30% of *Veela*/+ flies exhibited weak rhythmicity in LL (Table 1). Behavior in constant darkness was not abnormal (Table 2, which is published as supporting information on the PNAS web site). Mapping by meiotic recombination placed *Veela* between the *aristaless* (*al*) and *dumpy* (*dp*) markers, close to the clock gene *timeless* (refs. 19 and 20; see also *Materials and Methods*).

***Veela* Genetically Interacts with cry^b and Stabilizes Tim in the Light.** Photic responsiveness of Tim is mediated by its light-induced interaction with Cry, resulting in rapid degradation of Tim, thus representing a crucial mechanism by which *Drosophila*'s clock synchronizes to LD cycles (5, 7, 21). Because a mutation in the

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Abbreviations: LD, light/dark; LL, constant light; Cry, Cryptochrome; Per, Period; Tim, Timeless; LN, lateral neurons; DN, dorsal neurons; l-LNv, large LN; LRR, leucine-rich repeat; ZT, Zeitgeber time.

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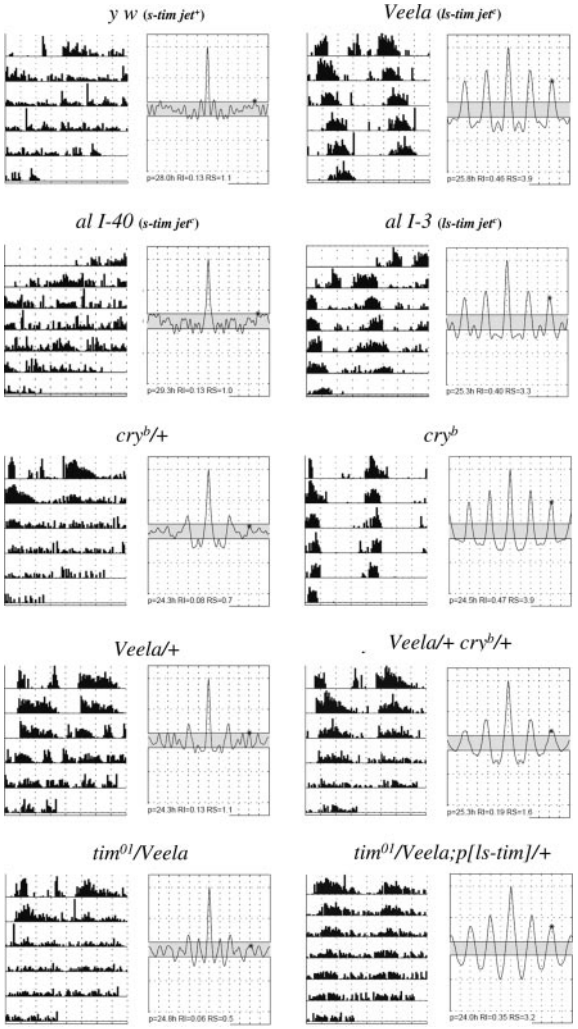

Fig. 1. Locomotor activity of individual flies during constant light. Genotypes are indicated on top of each actogram accompanied by its corresponding autocorrelogram. Actograms show the raw activity of each fly, in which the height of each bar indicates the amount of locomotion during a 30-min interval. Autocorrelograms show period and rhythmicity index values of each fly as an objective way to determine rhythmicity (see *Materials and Methods*). The upper four flies also have their polymorphisms in regard to the *tim* and *jet* variants indicated (see text). Flies to the left are arrhythmic; individuals to the right are rhythmic. *al I-40* and *al I-3* each designate recombinants resulting from meiotic crossing over between two second chromosomes in females heterozygous for *Veela* and the marker combination *al dp b pr*. Both the *I-40* and the *I-3* recombinants carry the *al* marker and the jet^c variant but differ with respect to *tim*. Note that *al I-40* flies behaved arrhythmically in LL (like the controls), although they carry jet^c.

cryptochrome gene (cry^b) also results in anomalous rhythmicity in LL (ref. 6; see also Fig. 1 and Table 1), we reasoned that *Veela* may interfere with the Tim–Cry interaction, perhaps, given our mapping results, because of a mutation in *tim* itself. To examine this possibility, we analyzed the behavior of flies that carried one copy of cry^b in combination with one copy of *Veela*. Strikingly, these double heterozygotes exhibited robust rhythmicity in LL (Fig. 1 and Table 1). Because this phenotype is never observed in heterozygous $cry^b/+$ flies and is much stronger compared with that of *Veela*/+ flies, our results indicate a strong genetic interaction between *Veela* and cry^b (Fig. 1 and Table 1).

These findings suggested that *Veela* impairs light inputs to the clock because of interference with the usual Tim–Cry interaction. If true, light-induced degradation of Tim should be affected in *Veela*

Table 1. Locomotor activity rhythms in constant light

Genotype	N_T	n_{Rhy}	Period	RI	% Rhy
y w	70	2	22.3	0.30	3
wild-type Athens	8	0	—	—	0
cry^b/cry^b	22	21	24.3 ± 0.1	0.33 ± 0.03	96
$cry^b/+$	23	1	27.5	0.30	4
Veela/*Veela*	75	64	25.7 ± 0.1	0.33 ± 0.02	85
Veela/+	35	11	24.4 ± 0.4	0.18 ± 0.01	31
Veela/+ $cry^b/+$	26	20	25.9 ± 0.6	0.24 ± 0.02	77
al I-3/*al I-3* (*ls-tim* jet^c)	10	9	26.2 ± 0.6	0.33 ± 0.04	90
dpp 20/*dpp 20* (*ls-tim* jet^c)	8	6	25.9 ± 0.3	0.20 ± 0.04	75
dp b pr I-28/*dp b pr I-28* (*ls-tim* jet^n)	13	1	25.8	0.27	7
dpp ed 23/*dpp ed 23* (*s-tim* jet^c)	8	0	—	—	0
al I-40/*al I-40* (*s-tim* jet^c)	12	2	24.2	0.16	16
al I-40/*al I-40*	40	2	23.2	0.25	5
$tim^{01}/+$	24	0	AR	—	0
Veela/tim^{01}	42	13	25.9 ± 1.1	0.21 ± 0.02	22
tim^{01}/tim^{01}; *P[ls-tim]*/*P[ls-tim]*	14	1	25	0.20	7
Veela/tim^{01}; *P[ls-tim]*/+	46	29	24.6 ± 0.2	0.25 ± 0.02	63

N_T, total number of flies tested; n_{Rhy}, number of individuals behaving rhythmically in LL. Period, free-running cycle durations (hr ± SEM), determined by autocorrelation; RI, Rhythmicity Index, indicating the significance level associated with a given period (see *Materials and Methods*); % Rhy, percentage of rhythmic flies of a given genotype. Male flies of the indicated genotypes were analyzed. As controls, flies with the *X*-chromosomal body- and eye-color markers *y* and *w* were used (*Materials and Methods*). The *y w* strain used here carries the *s-tim* allele (Fig. 4). The wild-type Athens strain carries the *ls-tim* allele. Genotypes containing a digit in conjunction with a recessive marker designation (e.g., *al I-40*) specify recombinants obtained from crossing homozygous *Veela* flies to multimarker chromosomes (*Materials and Methods*). The *P[ls-tim]* transgene is inserted on chromosome 3 and contains the *ls-tim* version of *timeless* including *tim* promoter sequences (25).

flies, and we therefore analyzed Tim abundance during the light portion of the LD cycle [during Zeitgeber times (ZT)0–12; Fig. 2], during which Tim levels are normally very low (5). As expected, Tim abundance in head protein extracts was increased in *Veela* flies (compared with controls monitored between ZT1 and ZT9), whereas we did not see any significant differences during the night (ZT13; Fig. 2). Next, we analyzed Tim levels in *Veela*/+ $cry^b/+$ flies, which also showed robust behavioral rhythms in LL (Fig. 1 and Table 1). Here, too, the doubly heterozygous flies showed increased Tim levels at ZT2 and ZT9 compared with both kinds of singly heterozygous controls (Fig. 2). Importantly, Tim signals in the *Veela*/+ $cry^b/+$ extracts were stronger compared with those of homozygous *Veela* flies and more similar to those of homozygous cry^b flies. Thus, at the molecular level as well as behaviorally, *Veela*

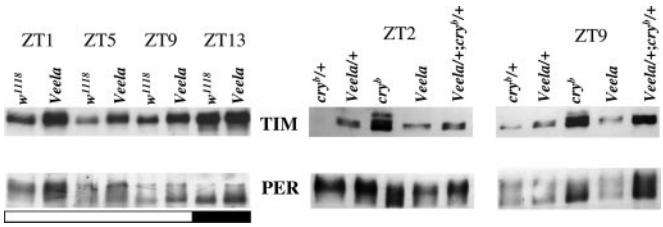

Fig. 2. *Veela* stabilizes Tim during the light portion of LD cycles. Western blots of head extracts from flies collected during LD cycles. Genotypes and time points of collection (ZT) are indicated above the blots. (*Upper*) Anti-Tim. (*Lower*) Anti-Per. Below the left blots, white and black bars indicate when the lights were on and off, respectively. *Veela* and cry^b have similar effects on stabilization of Tim as on Per. Heterodimerization of both proteins has been proposed to stabilize Per (16) and is probably the reason for increased levels of Per in *Veela*, cry^b and *Veela*/+ $cry^b/+$ flies.

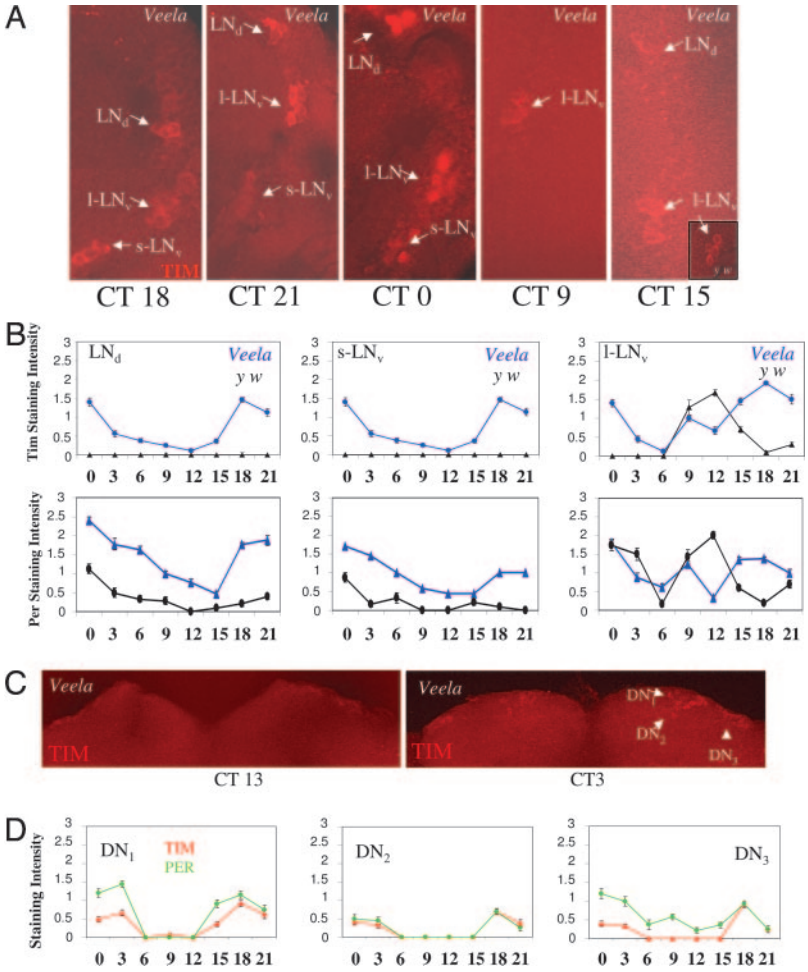

Fig. 3. Rhythmic Tim and Per expression in constant light in clock neurons of *Veela* flies. Control and *Veela* flies were synchronized to 12-h:12-h LD cycles and subsequently released into LL. After 2 days, males were killed at the indicated circadian times (CTs; below the images or *x* axis), and whole-mounted brains were stained with anti-Tim and -Per. (*A*) Rhythmic Tim immunoreactivity during LL in all LN of *Veela* flies and in the cytoplasm of the l-LNvs of *y w* controls (see *Inset*, CT15). (*B*) Quantifications of anti-Tim (*A*) and anti-PER stainings, including *y w* control flies. Note that controls show rhythmic Tim and Per accumulation in the l-LNv. Error bars indicate SEM. (*C*) Rhythmic Tim accumulation in the DNs of *Veela* flies during the second day of constant light. (*D*) Quantification of Per and Tim immunoreactivity in DNs of *Veela* flies during LL. No staining was observed in *y w* control flies under these conditions. Between 12 and 20 (*y w*) or 16 and 25 (*Veela*) brain halves for each time point were analyzed. Error bars indicate SEM.

and Cry interact robustly (Figs. 1 and 2). We have no explanation for why Tim signals in the heterozygous *Veela*/+ flies were stronger compared with those in homozygous *Veela* flies. Similar effects of *Veela* and of the combination of *Veela* with *cry^b* were observed for the Per protein (Fig. 2 *Lower*), which is thought to be stabilized by heterodimerization with Tim (16).

Tim and Per Are Rhythmically Expressed in the Pacemaker Neurons and Glia Cells of *Veela* Flies in Constant Light. Rhythmic locomotor activity is driven by clock gene expression within certain neurons of the fly brain (22). Based on their location within the lateral and dorsal brain, respectively, these neurons are historically divided into lateral neurons (LNs) and dorsal neurons (DNs): three groups of both LNs [small (s-LNvs), large (l-LNvs), and dorsal LNs] and DNs (cell groups 1–3; ref. 22). Because *Veela* individuals behave rhythmically in LL, we asked which subset of the clock neurons would drive this behavior or whether all neurons would be affected equally by this genetic variant. To answer this question, we stained whole-mounted brains of *Veela* adults during the second day in LL with anti-Tim and -Per. We observed robust rhythmic expression of Tim and Per in all clock-neuronal cell types, indicating that *Veela* disrupts light inputs into all six groups (Fig. 3). In all of these LNs and DNs, peak expression of both Tim and Per were observed at the end of the subjective night (a term for the second half of L in an LL "cycle") through the beginning of the subjective day, demonstrating synchronized expression of clock genes among these cells. As expected, in the LNs of control flies that are behaviorally arrhythmic after 2 days in LL (Fig. 1), no rhythmic expression or accumulation of the two clock proteins was observed, indicating light-induced Cry- and *Veela*$^+$-mediated constitutive degradation of Tim (Fig. 3*B*). A noticeable exception in the controls involved the l-LNv cells, which showed peaks of Tim and Per signals at the end of the subjective day and an additional Per peak early in the subjective day (Fig. 3*B*). Interestingly, Tim and Per signals in the l-LNs of *y w* controls flies were always cytoplasmic, perhaps explaining why this coordinated clock protein expression in the l-LNvs is not able to drive rhythmic behavior (see Fig. 3*A Inset* and *B*; see also Fig. 1).

Rhythmic and prominent Tim and especially Per expression in LL was also observed in glia cells of the medulla optic lobe in *Veela* flies (Fig. 6, which is published as supporting information on the PNAS web site). *cry^b* mutant flies also behave rhythmically in LL, and consequently rhythmic accumulation of Per and Tim occurred in the LNs of this mutant in LL (Fig. 7, which is published as supporting information on the PNAS web site). We did not observe rhythmic or significant Tim accumulation within glia cells of *cry^b* flies, although Per levels did cycle (Fig. 6).

***Veela* Flies Express a Less-Light-Sensitive Form of Tim and Carry a Mutation in the *jet* Gene.** Because *Veela* mapped to the same genetic interval as *tim* and genetically interacts with *cry* (Figs. 1 and 2, Table 1, and *Materials and Methods*), we sequenced the ORF of *tim* in the *Veela* strain but did not find any changes compared with several published WT *tim* sequences (data not shown). We did notice, though, that the *tim* form (*ls-tim*) in the *Veela* strain encodes both the "long" and "short" forms of Tim, in which the longer form contains 23 additional amino acids at its N terminus by use of alternative translation-start codons (ref. 23; Fig. 4). This *ls-tim*

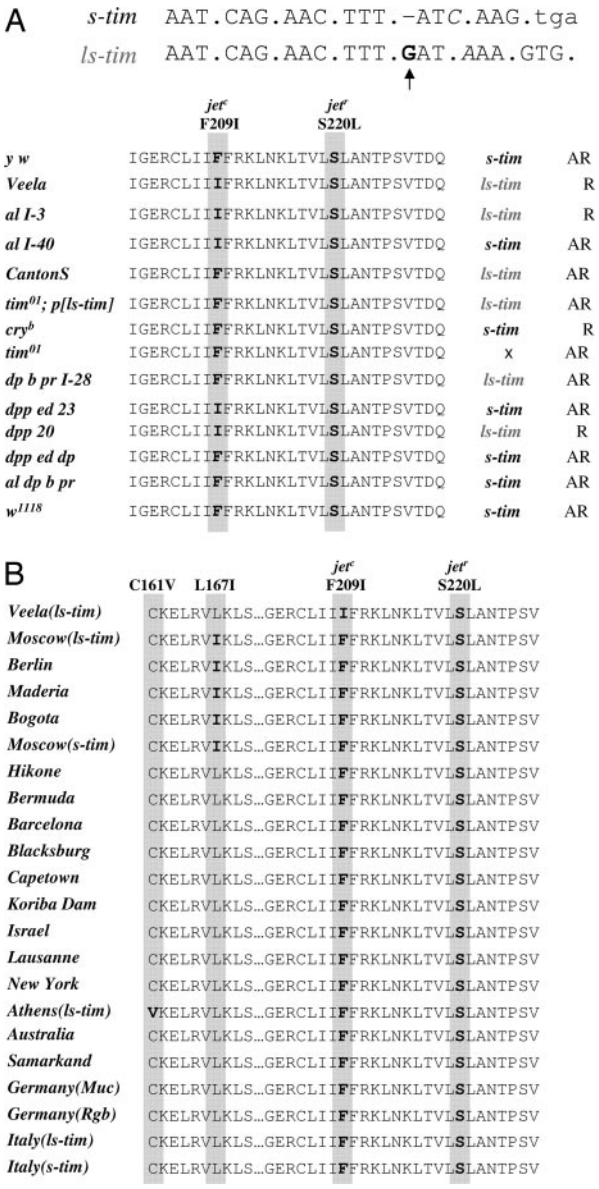

Fig. 4. *tim* and *jet* polymorphisms in Veela and other fly strains. (*A*) (*Upper*) Nucleotide sequence of the two *tim* polymorphisms. A deletion of the G nucleotide at position 294 of the *tim* cDNA (19) results in the generation of a stop codon immediately 5′ of the translational start of *s-tim* (23). Note the additional polymorphism three nucleotides downstream of the G deletion (indicated in italics). Fly lines carrying the single base-pair G deletion produce only the short (more light-sensitive) form of Tim. (*Lower*) Amino acid residues of the fourth and fifth LRR domains of the Jet protein. Highlighted in gray and bold are positions 209 and 220, respectively, which carry the common (F209I) or rare (S220L) polymorphisms (17). Genotypes expressing the respective forms are indicated to the left; their associated *tim* polymorphism along with the LL-behavioral phenotype is indicated to the right (R, rhythmic; AR, arrhythmic in LL). Meiotic recombinants are listed with their second chromosomal markers and a numerical indicator (e.g., *al I-3*). (*B*) Jet polymorphisms in WT fly strains. Displayed are the amino acid residues of the fourth and fifth LRR domains of the Jet protein from different WT fly strains. The fly strains' origin is shown on the left along with the nature of the *tim* allele, if determined (see *Materials and Methods* for details). Natural polymorphisms occurring at positions 161 and 167 are highlighted in gray and bold (Cys to Val and Leu to Ile, respectively). None of the WT strains carried the jet^c or jet^r variant.

variant is common to many strains of *D. melanogaster*, as is exclusive production of the short form (*s-tim*) for other naturally occurring WTs (ref. 23; F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta,

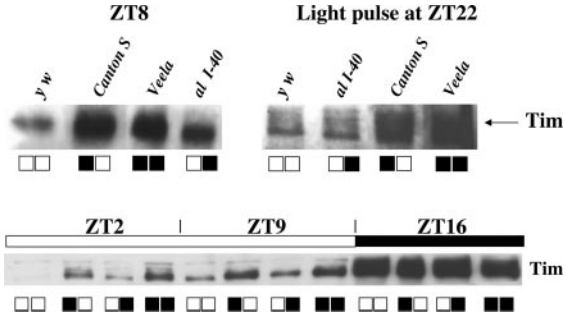

Fig. 5. *ls-tim* flies express a less-light-sensitive form of Tim, which is independent of *jet*. Flies of the indicated genotypes (*Upper*) were raised in 12-h:12-h LD cycles and either collected at the indicated ZT or subjected to a 2-min light pulse at ZT22 and collected at ZT23. Below each lane, the respective allele of *tim* (left square) and *jet* (right square) is indicated for each genotype. Open squares indicate *s-tim* (*Left*) and jet^+ (*Right*), black squares indicate *ls-tim* and jet^c. (*Lower*) Four genotypes, from left to right: *Italy (s-tim)*; *Canton S*; *al I-40*; *b I-27*), all flies had WT eye color. Note that the presence of *ls-tim* leads to a drastic increase in Tim levels, irrespective of *jet* or eye color.

P. Cisotto, *et al.*, unpublished results). We performed Western blot analysis to see whether there are any differences in regard to Tim expression between the *ls-tim* and *s-tim* variants. Tim protein levels in *ls-tim* flies collected during the day were substantially higher compared with *s-tim* (Fig. 5). This was also the case when flies with WT eye color were compared (Fig. 5 *Lower*). Therefore, missing pigments in the white-eyed *y w* flies are not the cause of increased light exposure and degradation of Tim. The same was true when both genotypes where exposed to a light pulse late at night (Fig. 5). Because Tim levels were similar in both genetic variants during the night portion of flies kept in LD cycles (Fig. 5 *Lower*), we conclude that the *ls-tim* allele produces a less-light-sensitive form of Tim. In a recent study, we showed that this difference is caused by a reduced affinity of "long-Tim" to CRY (F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta, P. Cisotto, *et al.*, unpublished results). However, because flies of the *ls-tim* type do become arrhythmic in LL (Figs. 1 and 4 and Table 1; ref. 2), there must be an additional light-input defect in *Veela* that further stabilizes Tim.

During our mapping experiments, we learned that a genetic variant with a similar LL phenotype to that of *Veela* is located close to our mutant but mapped to the right of *dp* instead (17). Koh *et al.* (17) mapped the phenotype to a gene (*CG8873*, now called *jetlag*) that encodes an F-box protein with leucine-rich repeats (LRR). Although *Veela* mapped to the other side (leftward) of *dp*, we decided to sequence the *jetlag* (*jet*) gene in *Veela* mutant flies because of the similar phenotype of the two mutant strains. Koh *et al.* (17) found four different variants of *jet* in various fly stocks that exist in unknown frequencies in various fly strains of *D. melanogaster*. The variants that correlated with the LL-rhythmic behavioral phenotype were reported to involve a phenylalanine-to-isoleucine substitution in one LRR domain or a serine-to-leucine replacement in the neighboring LRR (ref. 17; Fig. 4). Because they found the former substitution in six of seven behaviorally mutant stocks, the Ile variant was named the *common* (*c*) mutation, and the Leu isoform was named the *rare* (*r*) mutation. The WT variants would apparently encode Phe and Ser at these positions within Jet; however, the strain frequency for these alleles of *jet* was not determined (17). Our sequence analysis of this region in *Veela* revealed that its *jet* variant belongs to the *c* variant type. No other coding changes were found in the *jet* gene of *Veela* flies compared with the published WT sequence, suggesting that the LL-rhythmic phenotype of *Veela* is solely caused by this particular jet^c variant.

jet^c Flies Behave Normally in Constant Light When They also Carry the *s-tim* Allele. Our meiotic mapping placed *Veela* to the left of *dp* (and *jet*). Crucially, two of our genetic recombinants (generated with two

different marker chromosomes) carried the *c* form of the *jet* gene but did not show any phenotype in the LL assay; their behavior was indistinguishable from WT controls (Figs. 1 and 4, Table 1, and *Materials and Methods*). These behavioral-genetic results unequivocally demonstrate that the *c* variant alone is not sufficient to block light input into the circadian clock. Instead, careful inspection of all our genetic recombinants ($n = 26$) revealed that the presence of both the *ls-tim* form and the jet^c variant was correlated one to one with abnormally rhythmic behavior in LL ($n = 11$; Fig. 4 and see *Materials and Methods*). Because our two mapping stocks carry the *s-tim* form (Fig. 4 and see *Materials and Methods*), this explains why we were not able to map *Veela* to the *jet* locus *per se*; the relevant meiotic crossovers would have linked (and did link, in two cases) *s-tim* with jet^c, and this combination is phenotypically normal (Figs. 1 and 4, Table 1, and *Materials and Methods*).

In theory, it would be possible that another factor instead of *ls-tim* is responsible for the light-response defect in combination with jet^c. To firmly establish that the presence of the less-light-sensitive form of Tim is required, we compared the phenotypes of heterozygous jet^c flies, expressing either one copy of *ls-tim* and one copy of *s-tim* (*Veela*/+) or two copies of *ls-tim*, whereby one is carried on a transgene ($Veela/tim^{01}$;*P[ls-tim]*; ref. 24, Fig. 1, and Table 1). Strikingly, the presence of two copies of *ls-tim* resulted in doubling the proportion of LL-rhythmic individuals (31–63%; Table 1), further demonstrating that both the form of Tim associated with reduced light sensitivity (Fig. 5; F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta, P. Cisotto, *et al.*, unpublished results) and jet^c must be present to block light input into the circadian clock.

jet^c and jet^r Variants Are Most Likely Not Natural Polymorphisms. We investigated the possibility that the *jet* variants described here and in a previous study (17) represent a natural polymorphism as described for the *s-tim* and *ls-tim* alleles (ref. 23; F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta, P. Cisotto, *et al.*, unpublished results). To this end, we sequenced genomic DNA from 15 WT stocks available from the stock centers as well as from flies collected at five locations in central Europe (F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta, P. Cisotto, *et al.*, unpublished results; Fig. 4). No strains carrying the jet^c or jet^r variants were identified, but in one case (WT Athens), a base-pair change leading to a single amino change (Cys to Val) at position 161 was identified (Fig. 4). Moreover, several strains harbored a conservative Leu to Ile change at position 167. We analyzed the locomotor behavior of these variants in LL conditions, but their behavior was indistinguishable from other WT controls (Table 1 and data not shown). Although we cannot rule out that the substitutions have subtle consequences for the light sensitivity of the circadian clock, which are not detected in our LL assay, our results indicate they do not grossly alter the function of the Jet protein. Given that we did not identify any WT flies carrying either the jet^c or jet^r alleles, we conclude that both mutations occurred independently and spontaneously in laboratory stocks or were coinduced along with other mutations by chemical mutagenesis. Nevertheless, because we found two new variants in the region harboring the jet^c and jet^r polymorphisms, we cannot rule out that, in other natural strains, additional base-pair changes have occurred in different parts of the *jet* gene, which might more drastically affect the Jet protein and ultimately the light sensitivity of Tim.

Discussion

We identified a genetic variant, *Veela*, that is abnormally rhythmic in constant light, similarly as is shown for mutations affecting the blue-light photoreceptor Cry (6, 7, 10). *Veela*'s phenotype is due to the simultaneous presence of the *ls-tim* allele (encoding a less-sensitive form of Tim) and the jet^c variant encoding a mutant form of the F-box protein Jet (17). We show that *Veela* genetically and molecularly interacts with cry^b, indicating that Tim, Jet, and Cry function in the same circadian light-synchronization pathway. Our findings show that additional factors are necessary to elicit the phenotypes previously associated with *jet* variants (17). In particular, we show that only when jet^c is linked to the *ls-tim* allele, which encodes a less-light-sensitive form of Tim, can abnormal behavioral rhythmicity in LL be observed. The importance of the Jet protein *per se* in the light-entrainment process remains unclear, also when considering certain aspects of the original *jet* study in conjunction with the findings presented here. All control flies used by Koh *et al.* (17) came from a *y w* genetic background (see *Supporting Text*), which we show here carries the *s-tim* allele (Fig. 4). Contrarily, all jet^c or jet^r mutant flies carried the *ls-tim* allele (necessarily; otherwise, they would have behaved like WT). It follows that behavioral and molecular differences between control and mutant flies reported by Koh *et al.* (17) in fact reflect the combined effects of *ls-tim* (vs. *s-tim*) and jet^c (vs. jet^+). In conjunction with our Western blot data showing an increased *jet*-independent stability of the larger Tim form compared with the smaller one (Fig. 5), it seems that the effects on Tim degradation previously attributed to *jet* variants are mainly a reflection of the different features of the two Tim proteins. This may also explain why Koh *et al.* (17) saw only very subtle effects of their mutant Jet proteins on Tim degradation *in vitro*.

Nevertheless, it is clear that *jet* influences the light-input pathway of the circadian clock; WT flies behave arrhythmically in LL, even though they carry *ls-tim*. Moreover, *Veela* strongly interacts with Cry, a crucial protein for circadian light input in flies. Importantly, our findings reveal that, with the current knowledge, an *in vivo* function for *jet*'s F-box protein can be demonstrated only when the available *jet* variants are combined with *ls-tim*. To ultimately resolve the specific function of the Jet protein in the light-input pathway, loss-of-function *jet* mutants (25) or specific RNAi transgenics need to be generated and analyzed chronobiologically (26).

Characterization of *Veela* also led to the assignment of a biological function for the two natural *tim* variants that were identified many years ago (23). We show that Tim encoded by the *ls-tim* allele is more stable after light exposure, and that this increased stability has behavioral consequences when flies are exposed to constant light; if the *ls-tim* allele is linked to jet^c, these flies behave abnormally rhythmically in LL. If jet^c is linked to *s-tim*, the flies behave like WT and become arrhythmic in LL. Therefore, the less-light-sensitive Tim form encoded by *ls-tim* is necessary and sufficient to block light input into the circadian clock of jet^c flies. In nature, the natural polymorphism at the *tim* (and perhaps *jet*) locus might be used to fine-tune the light sensitivity of *Drosophila*'s circadian clock on a purely molecular level (F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta, P. Cisotto, *et al.*, unpublished results). In conjunction with various anatomical light-input routes that are known to send light to *Drosophila*'s circadian pacemaker (10, 27), our findings reveal a glimpse of the potential complexity of this process. The frequent and random occurrence of *tim* and *jet* variants in currently used laboratory strains also speaks to a more cautious strain selection and genotyping in all studies concerning light-input pathways to the circadian clock.

Materials and Methods

Fly Strains. Stocks of *D. melanogaster* and chromosomal markers were as described (5, 20, 28). *y Df (1)w* (*y w*) flies have yellow body color and white eyes and were initially used as control flies. Because *y w* carries the *s-tim* allele, the WT strain *Canton S* (carrying *ls-tim*, like *Veela* flies) was also used as control during this study. The *Veela* variant was isolated from a stock containing the $ninaB^{360d}$ mutation (29) on chromosome 3 (which was replaced by a WT third chromosome in all *Veela* flies analyzed here). The *ls-tim*-encoding transgene contains the full-length *tim* cDNA (generated by PCR from WT *Canton S* flies) and 6 kb of genomic 5′-flanking material (24). The various WT strains that were used to identify potential natural polymorphisms in the *jet* gene were obtained from the Bloomington stock center. The four WT strains from Moscow and two locations in northern Italy are isofemale lines, which were

generated from individuals collected in 1997 and 2004, respectively. From each of the three locations, a *s-tim* and *ls-tim* line was generated (F. Sandrelli, E. Tauber, M. Pergorano, G. Mazotta, P. Cisotto, *et al.*, unpublished results). The two WT variants from Germany stem from individuals collected in 2006 in Regensburg and near Munich.

Behavioral Analysis. Locomotor rhythms of individual male flies were recorded as described (5). Flies were kept for at least 3 days in 12 h/12 h LD cycles before being transferred to either constant light (300–400 lux LL) or to constant-dark conditions, in which they remained for at least 5 days. Rhythmicity was determined by using autocorrelation and Matlab software as described (30). Flies with period values in the circadian range and with a rhythmicity index value >0.15 were considered rhythmic (see ref. 30).

Chromosomal Mapping of *Veela*. Rhythmic or arrhythmic behavior in LL conditions (indicating the presence or absence of *Veela*, respectively) was used to map the position of *Veela* on chromosome 2. Initially, homozygous *Veela* flies were crossed to the marker stock *al* (map position 0.4) *dp* (13.0) *b* (48.5) *pr* (54.5), all on the left arm of chromosome 2 (20). A total of 22 recombinants was obtained, and all *b pr* ($n = 7$), or single marker *b* ($n = 1$) and *pr* ($n = 1$) recombinants exhibited the *Veela* phenotype. All *al dp* ($n = 4$) *dp b pr* ($n = 4$) *al dp b* ($n = 3$) recombinants were WT, suggesting that *Veela* maps close to *dp*. The two *al* recombinants were WT (I-40) and *Veela* (I-3), respectively (Fig. 1 and Table 1), placing *Veela* between *al* and *dp*, the region that includes the *tim* locus (8.0; ref. 19). Sequence analysis revealed that *Veela* carries the *ls-tim* variant but no other changes in its ORF, which do not occur in other WT or laboratory stocks (Fig. 4 and data not shown). Because other stocks carrying *ls-tim* do not show the *Veela* phenotype, we continued our mapping experiments with a *dpp* (4.0) *ed* (11.0) *dp* (13.0) marker stock in an attempt to separate *Veela* from *tim*. This was not accomplished; of the four total recombinants obtained from *Veela*/*dpp ed dp* females, the one *dpp* recombinant showed the mutant phenotype (Table 1), so that *Veela* must map to the right of *dpp*. All *dpp ed* ($n = 1$) and *ed dp* ($n = 2$) flies were WT (Table 1 and data not shown), placing *Veela* between *dpp* and *dp*, again the region containing *tim*.

Sequence analysis of *jet* in *Veela* flies and several recombinants revealed a correlation between the jet^c variant, *ls-tim*, and the mutant phenotype in all cases ($n = 11$; Fig. 4). Because our two mapping stocks express the *s-tim* variant along with jet^+, our recombinants separated *ls-tim* from jet^c resulting in LL-arrhythmic flies (Figs. 1 and 4; Table 1). Correct meiotic mapping of jet^c would be possible only with both the marker stock and the jet^c strain expressing *ls-tim*.

Western Blot Analysis. Flies of the indicated genotypes were first kept in LD cycles for at least 3 days and collected on dry ice during the indicated ZT in LD. For the light-pulse experiment in Fig. 5, flies were raised identically but exposed to a 2-min light pulse (300–400 lux) at ZT22, allowed to recover for 1 h, and collected on dry ice at ZT23. Preparations of head extracts and protein blots were performed by using anti-PER and -TIM as well as dilutions of these antibodies, as described (5), except that for the blots shown in Fig. 5, a different anti-TIM antibody was applied (31).

Immunohistochemistry. Flies were raised under the same conditions as described above and collected at the indicated circadian times (CTs) during the second day in LL (CTs refer to hours corresponding to ZT in the preceding LD cycles). Whole-mounted brains were prepared and incubated with anti-TIM and -PER, as described (32). Preparations were viewed by using Leica TCS NT (Leica, Deerfield, IL) and Zeiss Meta 510 (Zeiss, Oberkochen, Germany) confocal microscopes. Quantification of stainings was performed (observer blind with regard to genotype) by calculating a staining index, which reflects the number of immunoreactive cells and the staining intensity (on an arbitrary scale from 0 to 4), as described (32).

DNA Sequencing. The *tim* gene of *Veela* flies was sequenced by using genomic DNA and reverse-transcribed RNA fragments, generated by PCR using the methods and oligonucleotides described to sequence the tim^{blind} mutant allele (33). To distinguish between *s-tim* and *ls-tim* in the various fly stocks, the following oligonucleotides were applied to amplify genomic DNA by PCR: 5′-GTGGTTGCGTAATGCCCTGG-3′ (sense) and 5′-GCACCGTCAGATTGACGA-3′ (antisense). Sequencing of *jet* genomic DNA was performed by using oligonucleotides 5′-TGGGATAGAAGTCGTTCAAGT-3 (sense) and 5′-TGCCGATGGCTAACAGAT-3′ (antisense) to determine the variants at the common and rare sites within two LRR-encoding domains. The remaining *jet* DNA sequence was determined by using genomic DNA from *Veela* flies and standard sequencing protocols.

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