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Science **316**, 1898 (2007);
DOI: 10.1126/science.1138426

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favored in a small region of southern Italy but less favored farther north or south. We propose that an origin of the derived *ls-tim* allele in southern Europe, followed by its subsequent spread by directional selection, provides—counterintuitively—a more compelling model for understanding the elevated frequencies of *ls-tim* in this geographical region.

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25. Supported by the European Community (EC Biotechnology program ERB-B104-CT960096 and 6th Framework Project EUCLOCK 018741), Ministero dell' Università e della Ricerca Scientifica e Tecnologica (MURST)/British Council (C.P.K. and R.C.); NERC (C.P.K., E.T., and E.R.); a Royal Society Wolfson Research Merit Award (C.P.K.); a Marie Curie postdoctoral fellowship (E.T.); Ministero dell' Università e della Ricerca (MIUR) and Agenzia Spaziale Italiana (ASI, DCMC grant) (R.C.); Università di Padova grant 116 g03 (F.S.) and Assegno di Ricerca CPDR042471 (C.B.); and a Socrates studentship (A.S.). We thank our colleagues throughout Europe who provided many of the natural populations. EMBL sequence accession numbers are AM501534–AM501545, AM502183–AM502218, AM502183–AM502218, AJ748796–AJ748819, and AM503548–AM503569.

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5 December 2006; accepted 1 May 2007
10.1126/science.1138412

A Molecular Basis for Natural Selection at the *timeless* Locus in *Drosophila melanogaster*

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Diapause is a protective response to unfavorable environments that results in a suspension of insect development and is most often associated with the onset of winter. The *ls-tim* mutation in the *Drosophila melanogaster* clock gene *timeless* has spread in Europe over the past 10,000 years, possibly because it enhances diapause. We show that the mutant allele attenuates the photosensitivity of the circadian clock and causes decreased dimerization of the mutant TIMELESS protein isoform to CRYPTOCHROME, the circadian photoreceptor. This interaction results in a more stable TIMELESS product. These findings reveal a molecular link between diapause and circadian photoreception.

Wild European populations of *Drosophila melanogaster* have two major alleles of the *timeless* (*tim*) gene, *ls-tim* and *s-tim* (*1*). These alleles differ in their use of two alternative translational starts to generate longer (L-TIM$_{1421}$) and/or shorter (S-TIM$_{1398}$) isoforms (*2*). The *ls-tim* allele is derived from the *s-tim* allele, and directional selection is thought to have created a latitudinal gradient of *ls-tim* frequency within the past 10,000 years, perhaps due to an enhanced fitness of *ls-tim* individuals in temperate environments (*1*). TIM is a cardinal component of the circadian clock (*3*), and its light sensitivity via its physical interaction with the circadian photoreceptor cryptochrome (CRY) (*4*) mediates the fly's circadian responses to light (*5*). This photoresponse can be quantified at the behavioral level by studying the fly's locomotor response to brief light pulses delivered at zeitgeber time 15 (ZT15), three hours into the night phase of a light/dark [12 hours of light alternating with 12 hours of darkness (LD12:12)] cycle that generates a phase delay of a few hours; the same light stimulus administered late at night (ZT21) generates a phase advance (*6*).

Flies homozygous for each natural *tim* allele (*ls-tim* and *s-tim*) were established from isofemale lines from natural populations in Italy, the Netherlands, and Russia (*1*, *7*). We examined the two natural variants' locomotor phase response to 20-min saturating light pulses delivered at ZT15 and ZT21. Because we were interested in observing whether *tim*-mediated behavioral photoresponsiveness might be relevant to its latitudinal distribution, we initially used two temperatures, 18° and 24°C. For phase delays (ZT15 light pulse), analysis of variance (ANOVA) revealed significant genotype [$F_{(1,164)} = 11.1$, $P = 0.001$], temperature [$F_{(1,164)} = 23.8$, $P < 0.001$], and population [$F_{(2,164)} = 4.47$, $P < 0.002$] effects. For phase advances (ZT21 light pulse), significant genotype [$F_{(1,192)} = 10.5$, $P < 0.0015$], population [$F_{(2,192)} = 3.17$, $P = 0.044$], and temperature × population [$F_{(2,192)} = 8.4$, $P < 0.0005$] interactions were observed. In these tests, the *s-tim* variants clearly showed a larger phase response, as compared with that of *ls-tim* (Fig. 1A).

We also examined phase responses of flies transformed with the transgenes *P[LS-tim]*, *P[L-tim]*, and *P[S-tim]* (*1*), which are designed to generate both or each TIM length isoforms, respectively, in a tim^{01} mutant background at three temperatures (18°, 24°, and 28°C). ANOVA for delays gave highly significant effects for genotype [$F_{(2,311)} = 28.7$, $P < 0.0001$] and temperature [$F_{(2,311)} = 3.52$, $P = 0.03$], with *P[S-tim]* flies consistently showing larger delays than the other genotypes. Similarly for advances, ANOVA of the data for *P[L-tim]* and *P[S-tim]* transformants at all three temperatures gave only a significant genotype effect [$F_{(1,201)} = 12.28$, $P = 0.0006$]. A similar result was obtained for all three transformants at 18° and 28°C (*P[LS-tim]* data was not collected at 24°C), with a resulting significant genotype effect [$F_{(2,187)} = 4.94$, $P = 0.008$]; as with delays, the advances of *P[LS-tim]* were intermediate between those of *P[S-tim]* and *P[L-tim]*.

We next examined whether the *ls-tim* variants would show the normal arrhythmic behavioral response to constant bright light (LL) (*8*). We placed the natural *tim* variants, as well as the *P[tim]* transformants, in LD12:12 for 3 days and then in LL for 7 days. Locomotor arrhythmicity in LL of all genotypes was high (90 to 100%), and no *tim* allele (natural or transgenic)

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significantly differed in the time it took for the line to reach arrhythmia (table S1). Thus, *P[L-tim]* and *ls-tim* are able to mediate normal behavior in LL and constant darkness (DD), though they are less responsive to short light pulses (*1*).

Our results imply that in natural *ls-tim* and transformant *P[LS-tim]* flies, the longer isoform is translated and has biological activity that leads to a reduction in the circadian response to light. To investigate whether this was indeed the case, Western blot analysis on fly heads was used to study whether *ls-tim* flies did express the longer L-TIM isoform, and if so, whether they also expressed S-TIM. The *ls-tim* allele putatively encodes a protein that is 23 residues longer than the *s-tim* allele (*2*), a relatively small difference between isoforms that are ~1400 residues long. At ZT1 (the first hour of light), when TIM levels were low because of degradation by light, and at ZT13 (the first hour of darkness), when TIM levels began to rise (*9*), we detected two isoforms in *ls-tim* (Fig. 2). *P[L-tim]* transformants generated only the longer isoform, whereas *ls-tim* flies and the *P[LS-tim]* transformants produced both long and short TIM isoforms; *s-tim* flies and *P[S-tim]* transformants produced only the short isoform (Fig. 2). TIM bands from *ls-tim* flies also appeared more intense than did those from *s-tim* in flies from the same or different genetic backgrounds (Fig. 2). The higher–molecular weight band was maintained in *ls-tim* samples that had prior phosphatase treatment, in spite of a change in mobility for both *ls-tim* and *s-tim* genotypes that was consistent with dephosphorylated TIM isoforms (Fig. 2). The same phosphatase treatment had a more dramatic effect on the mobility of PER isoforms, given its more extensive levels of phosphorylation (*10*, *11*). On the basis of these findings, we suggest that the L-TIM isoform mediates an enhanced diapause response (*1*) and is also responsible for the attenuated circadian light sensitivity of *ls-tim* flies.

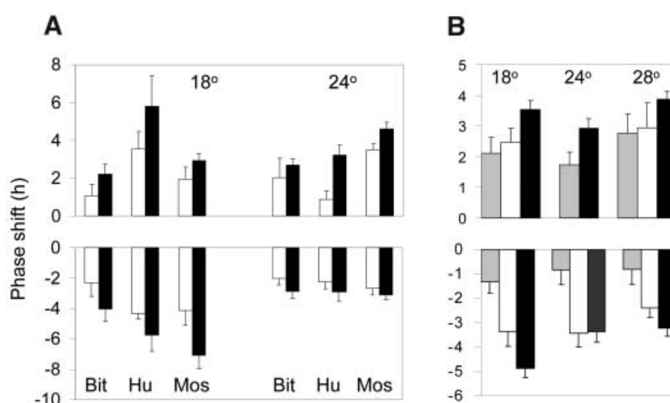

Fig. 1. Phase response to light pulses of *tim* genotypes. Mean phase responses [hours (h) ±SEM] to 20-min light pulses delivered at ZT15, giving phase delays (–, below), and ZT21, giving phase advances (+, above), are shown. (**A**) Natural lines: Bitetto (Bit), southern Italy; Houten (Hu), Netherlands; and Moscow (Mos), Russia at 18° and 24°C. Black bars, *s-tim*; white bars, *ls-tim*; h, hours. (**B**) Transformants at 18°, 24°, and 28°C. Black bars, *P[S-tim]*; gray bars, *P[L-tim]*; white bars, *P[LS-tim]*.

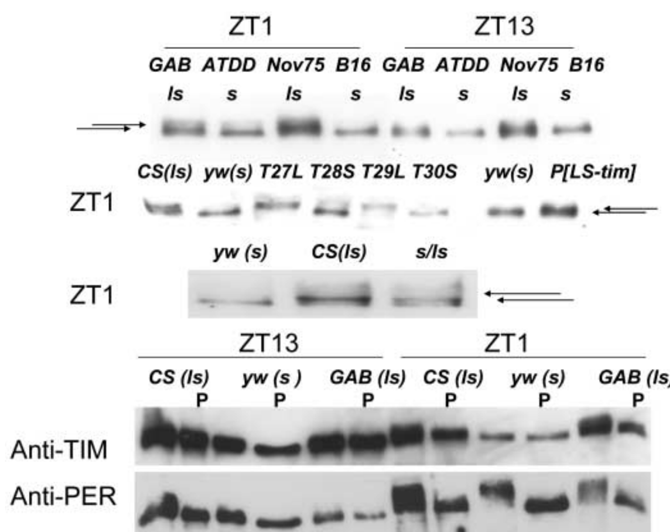

Fig. 2. TIM Western blots reveal different TIM isoforms. Fly heads harvested at ZT1 or ZT 13 are shown. The upper row shows natural lines: columns 1 and 5, *GAB* (*ls-tim*); 2 and 6, *ATDD* (*s-tim*); 3 and 7, *Nov75* (*ls-tim*); and 4 and 8, B16 (*s-tim*). The broader "doublet" TIM band in *ls-tim* genotypes is shown with arrows. The upper middle row shows CS, Canton-S (*ls-tim*); y w(s), *y w* (*s-tim*); *T27L* and *T29L* (*P[L-TIM]*); *T28S* and *T30S* (*P[S-TIM]*); and (*P[LS-TIM]*) from fly heads harvested at ZT1. There are doublets in CS and *P[LS-tim]* transformants, with single bands in (*P[S-TIM]*) and (*P[L-TIM]*) transformants. The lower middle row shows larger-scale figures of *y w* (*s-tim*), Canton-S (*ls-tim*) and their heterozygote s/ls [(*s/ls-tim*)] at ZT1. The bottom row shows the results of a phosphatase (P) treatment applied to *ls-tim* and *s-tim* samples at ZT1 and ZT13. Blots were performed with anti-TIM and anti-PER. *ls-tim* genotypes maintain the higher–molecular weight isoform after phosphatase treatment.

We then systematically performed Western blot analyses for a full day in LD12:12 (Fig. 3) and in the second cycle of DD (fig. S1). TIM levels were significantly elevated in the *ls-tim* genotype in LD12:12 at all points of the cycle, as compared with *s-tim* [ANOVA for genotype: $F_{(1,79)} = 22.7$, $P < 0.0001$; for time: $F_{(7,79)} = 8.3$, $P < 0.0001$] (Fig. 3A), suggesting that *ls-tim* generates a higher combined level of the two isoforms or has more stable products. This same pattern was also observed in DD, where ANOVA revealed a significant genotype effect [$F_{(1,73)} = 10.2$, $P = 0.002$], but the time (the oscillation began to damp by the second cycle) and genotype × time interactions were both insignificant (fig. S1). The expression of TIM in the four independent *P[L-tim]* and *P[S-tim]* transformant lines was also studied in LD12:12. A nested ANOVA revealed significant time [$F_{(7,42)} = 5.21$, $P = 0.0003$] and time × genotype [$F_{(7,42)} = 4.53$, $P = 0.0008$] interactions, reflecting the observation that P[L-TIM] levels were similar between day and night, as compared with P[S-TIM] (Fig. 3B). These results suggest a stability difference between the two TIM isoforms, rather than a difference in translational efficiency (*12*), and they are further supported by the mRNA profiles for the two natural variants, which are very similar [time: $F_{(5,36)} = 91.02$, $P < 0.0001$; genotype: $F_{(3,36)} = 1.52$, NS (not significant)] (fig. S2).

The enhanced stability of L-TIM might therefore be expected to contribute to the higher levels of TIM observed in natural *ls-tim* flies and to reduced circadian photoresponsiveness. Circadian light responses in *Drosophila* are mediated both by the canonical visual pathway, which uses rhodopsins, and by CRY (*13*). After stimulation by light, CRY can physically interact with TIM and/or PERIOD in yeast, in *Drosophila* S2 cells, and in vivo (*4*, *14*–*16*). These PER/TIM/CRY interactions lead to TIM degradation (*5*, *15*) and subsequent PER instability, which releases the negative autoregulation of PER on the *per* and *tim* genes (*17*). We therefore studied the physical interaction of the L-TIM and S-TIM isoforms with CRY in the yeast two-hybrid system (*16*). No interactions between TIM and CRY occurred in the dark, and the level of interaction between CRY and L-TIM in light was weaker than that between CRY and S-TIM in both plate and liquid assays (Fig. 4, A and B). As a control, we also examined the interaction of L-TIM and S-TIM with the large fragment of PER (residues 233 to 685) that is stable in yeast (*16*), but these PER/TIM interactions were not significantly different (Fig. 4, C and D). These results indicate that the differences in interaction between the two TIM isoforms and CRY are a specific effect due to the additional N-terminal 23 residues in L-TIM, which interfere with the light-dependent dimerization of CRY.

A reduced L-TIM/CRY interaction may explain the differences in the fly's circadian

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photoresponsiveness and the enhanced L-TIM stability. The observation that *ls-tim* females are more prone to diapause at any day length (*1*) is also consistent with the results presented here. As in the corresponding diapause profiles (*1*), the transformants conclusively reveal that the circadian photoresponsive phenotypes of natural *tim* variants are not due to linkage disequilibrium between *tim* and a nearby locus, but they are attributable to *tim* itself. Furthermore, the similarity in behavior of natural *s-tim* variants and *P[S-TIM]* transformants suggests that the residual putative truncated N-terminal 19-residue TIM product from the *s-tim* allele does not play any major role in the phenotypes we have studied (*2*).

It has been argued that the light sensitivity of the circadian clock needs to be abated in temperate zones because of the dramatic increase in summer day lengths in northern latitudes (*18*, *19*). One mechanism for this process involves a reduced sensitivity to light-induced disturbance by having a higher pacemaker amplitude (*18*, *19*). However, the amplitude of TIM cycling in DD was not significantly different between the two variants (fig. S1), nor were there any significant differences in amplitude or phase of the *tim* mRNA cycle between the *s-tim* and *ls-tim* genotypes (fig. S2). Another way to attenuate circadian photoresponsiveness in temperate zones may be by filtering light input into the clock. The molecular changes to the L-TIM protein may buffer the circadian response to light in *ls-tim* individuals, even in the presence of S-TIM, and may contribute to the positive Darwinian selection observed for *ls-tim* in the European seasonal environment (*1*).

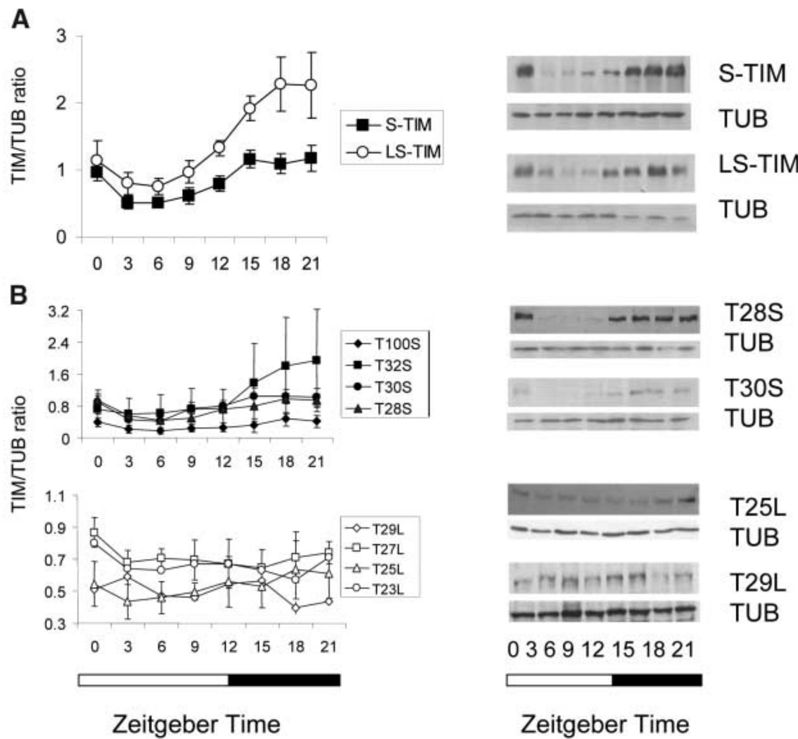

Fig. 3. Circadian TIM profiles in natural lines and transformants. (**A**) Natural variants. Left panels show mean ± SEM TIM/TUB ratios from Western blots of the Moscow line (23°C in LD12:12, $n = 6$ blots for each variant); right panels show examples of corresponding Western blots. (**B**) Transformants. Left panels show mean ± SEM TIM/TUB ratios for Western blots (right) of each of the *P[S-tim]* ($n = 11$) and *P[L-tim]* ($n = 10$) transformants.

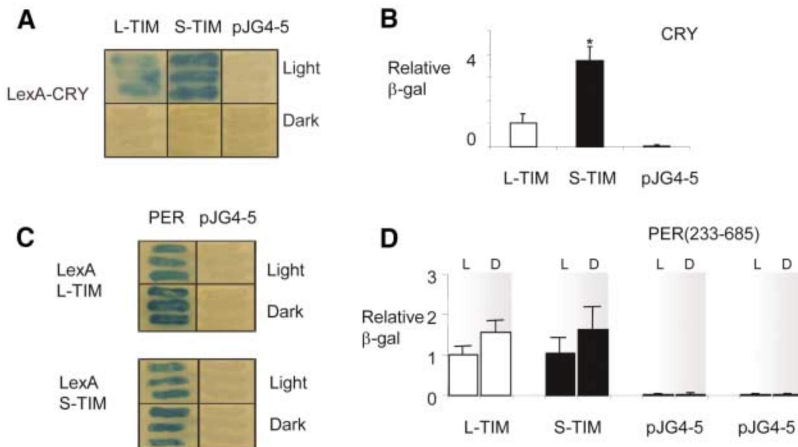

Fig. 4. TIM interactions with CRY and PER in the yeast two-hybrid system. In light, L-TIM shows a diminished interaction with CRY in (**A**) plate assays (pJG4-5, empty vector control) and (**B**) liquid assays (mean ± SD), as compared with S-TIM [$F_{(1,16)} = 141.4$, $P < 0.001$] for at least nine cultures derived from at least eight independent clones are shown. (**C** and **D**) L-TIM and S-TIM show equally robust interactions with the $PER_{(233\text{-}685)}$ fragment in light or darkness in both plate and liquid assays [$F_{(1,\ 20)} = 0.04$, NS].

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