Casein kinase 1 promotes synchrony of the circadian clock network

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Abstract

Casein kinase 1, known as DOUBLETIME (DBT) in Drosophila, is a critical component of the circadian clock that phosphorylates and promotes degradation of the PERIOD (PER) protein. However, other functions of DBT in circadian regulation are not clear, in part because severe reduction of dbt causes pre-adult lethality. Here we report the molecular and behavioral phenotype of a viable dbt<sup>EY02910</sup> loss-of-function mutant. We find that DBT protein levels are dramatically reduced in adult dbt<sup>EY02910</sup> flies, and the majority of mutant flies display arrhythmic behavior, with a few showing weak, long period (~32h) rhythms. Peak phosphorylation of PER is delayed, and both hyper- and hypo-phosphorylated forms of the PER and CLOCK proteins are present throughout the day. In addition, molecular oscillations of the circadian clock are dampened. In the central brain, PER and TIM expression is heterogeneous and decoupled in the canonical clock neurons of the dbt<sup>EY02910</sup> mutants. We also report an interaction between dbt and the signaling pathway involving Pigment Dispersing Factor (PDF), a synchronizing peptide in the clock network. These data thus demonstrate that overall reduction of DBT causes long and arrhythmic behavior and reveal an unexpected role of DBT in promoting synchrony of the circadian clock network.

Introduction

In Drosophila, daily rhythmic behavior is governed by a network of ~150 brain clock neurons (1, 2), each of which contains a molecular oscillator (3). Within this oscillator, the CLOCK (CLK) and CYCLE (CYC) proteins activate transcription of the repressor genes period (per) and timeless (tim) during the day, and accumulated PER and TIM proteins repress the transcriptional activity of CLK-CYC in the late night/early
morning. Phosphorylation of clock proteins, which is mainly regulated by casein kinases (CK1 and CK2) (4-6), glycogen synthase kinase (GSK3β) (7) and specific protein phosphatases (8, 9), plays a pivotal role in circadian timing, at least in part by regulating the stability and nuclear localization of PER and TIM (3).

Robust circadian behavior relies on the coupling of clock neurons within the network. The neuropeptide Pigment Dispersing Factor (PDF) is required for synchrony of the circadian clock circuit and its output (10-12). A similar framework operates in the mammalian circadian pacemaker, the suprachiasmatic nucleus (SCN), where a peptide, Vasoactive Intestinal Polypeptide (VIP), released by some clock cells synchronizes individual oscillators to generate a coherent output (13, 14). How PDF and VIP synchronize the circadian clock network and its output is unclear. Interestingly, in Ck1εtau hamsters, there is a tissue-specific misalignment of some peripheral clocks with the SCN, suggesting that the tau mutation in CK1 causes a defect in synchronization (15). However, tau appears to be a gain-of-function mutation (16) and the redundancy of CK1 isoforms in mammals makes it difficult to address consequences of CK1 loss.

In Drosophila, Ck1ε is known as double-time (dbt) and mutations in this gene, such as dbtS, dbtL and dbtar, produce different effects on circadian rhythms, specifically short periods, long periods and arrhythmia respectively. However, on a molecular level, they all produce relatively normal levels of protein with reduced overall kinase activity (4, 17-21). It is possible that allele-specific non-catalytic functions of DBT contribute to different circadian phenotypes. Over-expression of a kinase-dead DBT results in cytoplasmic localization PER in some cells but nuclear in others, raising the possibility
that DBT plays a role in synchronizing PER expression (22). A severe hypomorphic
mutation, dbt\textsuperscript{P}, causes pupal lethality (4, 23), thus precluding analysis of adult flies.

Here, we report the adult circadian phenotype of a new dbt allele that dramatically
reduces protein levels, but is viable. In addition to robust behavioral and molecular
phenotypes, dbt hypomorphs show heterogeneous expression of PER expression in the
central brain, among different groups of canonical clock neurons, and also within each
group. TIM expression is also heterogeneous and decoupled from PER. These data
thus uncover an unexpected role of DBT in synchronizing the circadian clock network.

**Materials and Methods**

**Drosophila genetics**

A w\textsuperscript{1118}, or y\textsuperscript{1}, w\textsuperscript{1} genetic background was used in most experiments. The
P[EPgy2] insertion allele of dbt\textsuperscript{EY02910} was obtained from the Bloomington stock center
and outcrossed into an isogenic w\textsuperscript{1118} for 4 generations, and balanced over TM6, Sb to
establish a stock. per\textsuperscript{S}, dbt\textsuperscript{AR}, dbt\textsuperscript{L}, dbt\textsuperscript{R}, dbt\textsuperscript{P}, tim\textsuperscript{01}, Pdfr\textsuperscript{5304}, cry-Gal4 and tim-Gal4 lines
were reported previously (1, 4, 20, 24-27). To generate the UAS-dbtx transgenes, the
corresponding coding sequences of dbt were inserted into a pUAS-V5 vector. We
hypothesized that cytoplasmic DBT would more likely interact with PDF signaling, so we
added a nuclear export sequence at the N terminus of dbt. However, we do not know if
this transgene is exclusively expressed in the cytoplasm. P-element transformation was
performed by Rainbow Transgenic Flies Inc. (Camarillo, CA, USA).

**Behavioral assays**
Flies were maintained on standard *Drosophila* food containing cornmeal, molasses, and yeast. 5-day-old male flies were entrained to a 12 h:12 h light:dark (LD) cycle at 25°C for 3 d, and then loaded into locomotor assay tubes containing 5% sucrose and 2% agar. Activity records were collected in 1-min bins in the DAM system (TriKinetics, Waltham, MA, USA) for more than 7 days and analyzed with Clocklab (Actimetrics, Wilmette, IL, USA). For the *Pdfr* experiments, data from DD4-9 were used for analysis. Circadian periodicity was determined by χ2 periodogram analysis, coupled with visual inspection of the actogram. A fly is considered rhythmic when activity is clearly rhythmic in the actogram and χ2 periodogram analysis reveals a single peak above the 95% confidence line. For those individuals that had a weak rhythmic pattern and/or the periodogram displayed multiple peaks over the 95% confidence line, the strength of the rhythm corresponding to the major peak was determined via a fast Fourier transform algorithm. An FFT value >0.01 was generally considered rhythmic, and an FFT of 0.004-0.01 was scored as weakly rhythmic. Note that these rhythms with very low FFT values are unique to this *dbt* line as typically the period of a rhythmic fly has an FFT of >0.01. Detection of very low strength rhythms may also have been facilitated by our use of smaller bin sizes (1 min) for activity data collection.

**Quantitative real-time PCR**

To maximally synchronize the population of flies, 5-day-old adult flies were maintained in a 12 h:12 h LD cycle at 25°C for 3 days and then collected on dry ice at indicated time points on the third day of LD. Total RNA was isolated from heads by using Trizol (Invitrogen, Carlbad, CA, USA), and cDNAs were synthesized by using a high-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA).
Quantitative real-time PCR was performed in an ABI prism 7000 Sequence Detection System using a SYBR Green kit (Applied Biosystems), following manufacturer’s suggestions. Values for clock gene mRNAs were normalized over those of Actin. The dbt primers were designed downstream of the p[EY02910] insertion site. Primer sequences are: Actin-F, 5’-gcgcgggttacttttcacca-3’; Actin-R, 5’-atgtcacggacgatttacg-3’; per-1803F, 5’-cgtcaatccatggtcccg-3’; per-1861R, 5’-cttgaagagccgatggc-3’; Pdp1 RD1401F, 5’-tttggaacagcttggccc-3’; Pdp1 RD1453R, 5’-gagattttctgcctggatgcg-3’; Clk-1462F, 5’-ttctcgatggttcttcggtg-3’; Clk-1512R, agttcgaaacgcaacg; cry-1365F, 5’-gcagtacgtccagtgagttc-3’; cry-1417R, 5’-agggctcgtgaacaaattcct-3’; dbt-F, 5’-ctctcgggtctccggagttc-3’; dbt-R, 5’-gacgaaagacagtgccagaa-3’.

Western blot analysis
To maximally synchronize the population of flies, 5-day-old flies were entrained to 12 h:12 h LD cycles for 3 day, and collected at indicated time points. Fly heads were separated on dry ice for total protein extraction using a passive lysis buffer (Promega, Madison, WI, USA), with the additions of protease inhibitors (Roche) and phosphatase inhibitor okadaic acid (Sigma, St. Louis, MO, USA). For the larval tissues, 3rd instar larvae were dissected in PBS. SDS loading buffer was added to the whole homogenate and samples were boiled. For the λ phosphatase treatment, okadaic acid was not included in the lysis buffer, and samples were equally divided into untreated and treated groups. λ phosphatase (New England Biolabs, MA, USA) treatment was carried out following manufacturer’s suggestion. A higher volume was loaded into the gel to compensate for sample dilution. Total proteins were separated through SDS–PAGE, and transferred onto nitrocellulose membrane and processed for antibody incubation.
Primary antibodies rabbit anti-PER (PA1139), rat anti-TIM (UPR41) and guinea pig anti-CLK (GP50) (28) were used at 1:1000 dilution. Rat anti-DBT(17) was used at 1:3000 dilution. Mouse anti-Armadillo (Developmental Studies Hybridoma Bank, University of Iowa) was used to control the loading of total proteins. Following enhanced chemiluminescence, blots were exposed to X-ray film, and images were processed in Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA).

**Immunohistochemistry and microscopy**

Brains were dissected in fresh 4% paraformaldehyde (in PBS) and fixed for 20 min at indicated time points, washed for 1 h in PBS buffer, and incubated with primary antibodies (in PBS buffer with 3% normal donkey serum and 0.3% Triton X-100) overnight at 4°C. Following 3 washes of 30 min each at room temperature, brain samples were incubated with Cy3 donkey anti-rat (or guinea pig), FITC donkey anti-guinea pig or mouse, and Cy5 anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1.5 h at room temperature, followed by extensive washes in PBS. Samples were mounted onto slides with Vectashield mounting medium (Vector Laboratories, CA, USA). Primary antibody dilutions were as follows: PER (GP1140), 1:1500; PDF (C7; Developmental Studies Hybridoma Bank), 1:1000; TIM (UPR41), 1:1000. Secondary antibody dilutions were 1:1000. Fluorescent images were taken with a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany). For cell number counting, samples were visually examined under the fluorescent microscope, PER and TIM positive neurons were arbitrary scored (0-5) for fluorescent intensity. For the analysis of light-dependent degradation of TIM, flies were entrained to an LD cycle for 4 days and at ZT20 of the
fourth LD, one group of flies was exposed to a 10-min light pulse (LP, 2000 lux), and then returned to the dark for 50 min. Both light-pulsed (LP) and unpulsed (ctl) groups were collected at ZT21 for immunohistochemistry procedures as described above.

**Results**

**Characterization of a new dbt loss-of-function allele**

We obtained a strain from the Bloomington Stock Center that contains a P[EPgy2] insertion in the 5'UTR of dbt. The insertion dramatically reduces DBT expression (Figure 1). However, DBT protein is detectable when the immunoblots are over-exposed (Figure 1B), indicating that dbt\textsuperscript{EY02910} is a strong hypomorphic allele. We did not find gross defects of the adult brain, in that PER is expressed in canonical clock neurons in the central brain of this new dbt mutant (Figure 1D and see below), and neuronal processes from central clock cells, the ventral lateral neurons, are not altered.

We tested dbt\textsuperscript{EY02910} for circadian rhythms of locomotor activity and found that under free-running conditions of constant darkness, greater than half the mutant flies are arrhythmic, and about 40% display very weak rhythms with a period of 32 h (Figure 2, Table 1). A small proportion of mutant flies (6%) retains relatively strong rhythmicity, with a period of ~32 h. As the orientation of the UAS element in the P[EPgy2] transposon allows it to drive transcription of dbt (Figure 1A), we were able to rescue the behavioral phenotype by tim-Gal4 driven expression of endogenous dbt (Figure 2A).

When heterozygous over other dbt alleles or a wildtype chromosome, this new dbt allele (Table 1) behaves like the dbt\textsuperscript{P} or deficiency alleles (4). Thus it is a bona fide loss-of-function allele. These data demonstrate that a severe reduction of DBT causes long
period and disrupts behavioral rhythms. Importantly, we found that light-dependent degradation of TIM is largely intact in the mutant flies (Figure 2B), suggesting that the primary response of the clock to light is unimpaired.

Phosphorylation of PER and CLK proteins is altered in the \textit{dbt}\textsuperscript{EY02910} mutants

As noted above, DBT is a critical kinase that phosphorylates PER and CLK, but due to lack of viable null mutants most assays of this kinase have relied upon the expression of mutant proteins, or deletion of the DBT binding domain in PER (4, 18, 19, 23, 29-31). This new loss-of-function allele allowed us to directly examine the effect of severe reduction of DBT on the PER phosphorylation profile over a 24 h day. We examined molecular oscillations under LD conditions, so the population of flies is maximally synchronized. In wildtype flies PER is phosphorylated in a cyclic fashion, such that on Western blots of adult head extracts it appears largely in a hyper-phosphorylated, low mobility form at ZT2 (ZT0 is light-on and ZT12 is light-off) and a hypo-phosphorylated, high mobility form at ZT14 (Figure 3). In \textit{dbt}\textsuperscript{EY02910} mutants, the phosphorylation profile of PER is delayed, and PER is never present in an exclusively, or largely, hyper-phosphorylated form. Even when hyper-phosphorylated forms are seen (ZT6 and ZT10), a large amount of the PER protein still appears in a hypo-phosphorylated state. These observations indicate that a severe reduction of DBT mainly affects the synchronized phosphorylation of PER proteins over the course of the day. Levels of PER are also higher in \textit{dbt}\textsuperscript{EY02910} mutants, which is consistent with the role of DBT in promoting the degradation of PER (4). However, overall levels of PER as well as its phosphorylation status still cycle in mutant flies, albeit with a much lower amplitude than they do in wildtype flies.
As a decrease in DBT stabilizes PER, enhancing PER degradation in a dbt mutant background might be expected to rescue the phenotype. Indeed, it was previously reported that short period alleles of per can rescue the arrhythmic behavior of the dbt\(^{AR}\) allele (20). Accelerated degradation of nuclear \(\text{PER}^{T}\) (or \(\text{PER}^{S}\)) proteins (32-34) apparently restores PER turnover in the \(\text{dbt}^{AR}\) background, which would explain the rescue of rhythmicity. Interestingly, the per\(^S\) allele did not rescue the arrhythmicity of \(\text{dbt}^{EY02910}\), even though it shortened the period of a few weakly rhythmic flies (Table 1). These data indicate that severe reduction of DBT does not just stabilize PER, but causes additional defects in the clock network (see below).

In wildtype flies, levels of CLK do not cycle over the course of a day, but its phosphorylation status displays a rhythmic pattern, such that maximal phosphorylation occurs towards the end of the night/beginning of the day (31, 35) (Figure 3A). In the \(\text{dbt}^{EY02910}\) mutants, maximal phosphorylation of CLK is reduced (compare ZT22 between wildtype and mutant in Figure 3A, ZT2 and 24 in 3B). However, considerable hyper-phosphorylation of CLK is still observed at these time points, and at other time points CLK phosphorylation in the \(\text{dbt}^{EY02910}\) mutants is slightly higher or equivalent to that in wildtype flies (Fig 3A). Thus severe reduction of DBT does not significantly reduce total CLK phosphorylation, rather, it dampens and delays the circadian oscillation of CLK phosphorylation. Such a dampening of molecular rhythms may be due to a weaker oscillator, but it may also reflect desynchrony of individual clocks (see below).

The role of DBT in circadian clock gene transcription
The role of DBT in the repression of CLK mediated transcription is still unclear. While some studies support the idea that DBT regulates transcription indirectly through its effect on PER localization (although the effect of DBT on PER localization is also controversial) (21, 29, 36), others propose that DBT directly participates in transcriptional repression (18, 30). To investigate the effect of severe reduction of DBT on clock gene transcription, we assayed mRNA oscillations of clock genes in adult heads of \( \text{dbt}^{\text{EY02910}} \) mutants (Figure 4 A-B). We found that peak expression of \( \text{per} \) and \( \text{Pdp1}\epsilon \) is reduced and delayed, whereas trough levels are increased in \( \text{dbt}^{\text{EY02910}} \) mutants. Daily cycling of \( \text{Clk} \) and \( \text{cry} \) expression is abolished, with intermediate levels of both transcripts throughout the day. As in the case of the Western blot analysis above, these RNA assays of adult heads largely report clock gene expression in the compound eye (37, 38), which constitutes a peripheral oscillator. We infer that the peripheral clock in the eye is dampened in \( \text{dbt}^{\text{EY02910}} \) mutants.

It was previously reported that PER is a strong repressor of CLK mediated transcriptional activity in the \( \text{dbt}^{\text{AR}} \) mutants in the absence of TIM (21). Those experiments could not address the role of DBT in PER-mediated transcriptional repression as \( \text{DBT}^{\text{AR}} \) is expressed at similar levels as wildtype DBT and so may have contributed to this repression. To evaluate the role of DBT in repression by PER, we conducted experiments similar to those reported by Cyran et al (2005)(21), but using the \( \text{dbt}^{\text{EY02910}} \) mutant. Thus, we crossed \( \text{dbt}^{\text{EY02910}} \) into a \( \text{tim}^{01} \) null (25, 39) background. Because PER levels are very low in \( \text{tim}^{01} \) flies, there is little repression of CLK transcriptional activity. Thus, the CLK target \( \text{Pdp1}\epsilon \) is expressed at close to peak levels in \( \text{tim}^{01} \) flies (Figure 4D). We found that \( \text{Pdp1}\epsilon \) and \( \text{vri} \) mRNA levels are only slightly
reduced in the \textit{tim}\textsuperscript{01};\textit{dbt}\textsuperscript{EY02910} double mutants. Thus, stabilization of PER in the double mutant is not sufficient to repress \textit{Pdp1\epsilon} and \textit{vri} expression, which suggests a role for DBT. Yet another CLK target, \textit{per}, is even slightly increased in \textit{tim}\textsuperscript{01};\textit{dbt}\textsuperscript{EY02910} double mutants relative to \textit{tim}\textsuperscript{01} single mutants (Figure 4D), again supporting the idea that DBT participates in the feedback repression of \textit{per} transcription. As discussed later (see Discussion), we believe the effects on \textit{Pdp1\epsilon} and \textit{per} are compatible with a role of DBT in repression of CLK-mediated transcription.

\textbf{Heterogeneous expression of PER in clock neurons of \textit{dbt}\textsuperscript{EY02910} mutants}

In a normal daily cycle, hyper-phosphorylation of PER is correlated with maximal nuclear localization in the late night and early morning (39-42). Although phosphorylation of PER at specific sites may promote its nuclear localization, the accumulation of hyper-phosphorylated, low mobility forms of PER appears to be secondary to its nuclear localization (43). The presence of hypo- and hyper-phosphorylated PER at many time points in \textit{dbt}\textsuperscript{EY02910} mutants raised the possibility that nuclear expression of PER is desynchronized in these mutants. To test this idea, we examined PER expression in the central clock neurons at various time points in a daily cycle. In wildtype flies, PER expression peaks and is nuclear in all cell types at the end of the night/beginning of the day, and it is low at the end of the day. In \textit{dbt}\textsuperscript{EY02910} mutants PER expression is variable among clock neurons at most time points (Figure 5A). Within each group of the canonical clock neurons, namely, ventral lateral neurons (LN\textsubscript{v}s), dorsolateral neurons (LN\textsubscript{d}s), and dorsal neuron group1 (DN1s), at almost all time points we noticed high levels of PER in some cells, but low in others. Because of the high variability in expression across cells, we quantified the number of cells that...
strongly express PER at each time point rather than the intensity of expression. Even
with this measure, wildtype neurons show robust daily rhythms of PER expression. In
the \textit{dbt}^{EY02910} mutant, the large ventral lateral neurons (I-LN\textsubscript{s}) and LN\textsubscript{s}s show a trough
at ZT18, but mixed expression at other time points. The subcellular localization of PER
is likewise variable; indeed high levels of PER usually correspond to nuclear
expression. Strong nuclear PER is present in some s-LN\textsubscript{s}s and DN1s throughout the
day (Fig 5B). Heterogeneous expression of PER in the s-LN\textsubscript{s}s is more obvious at ZT16-
17 than at other time points (Fig 6B). Such prolonged nuclear PER and heterogeneity
among clock neurons may account for the weak and long period behavioral rhythms of
some \textit{dbt}^{EY02910} flies.

\textbf{PER and TIM expression is decoupled in clock neurons of the \textit{dbt}^{EY02910} mutants}

To evaluate the role of DBT in promoting synchrony of the clock network in
further detail, we examined the temporal expression of PER and TIM in the adult lateral
neurons. In general, TIM is low during the daytime and it undergoes cytoplasm-to-
nucleus translocation at night in \textit{dbt}^{EY02910} mutants, as it does in wildtype flies. As noted
above, PER does not show this cycle in the \textit{dbt}^{EY02910} mutants, so the expression of PER
and TIM appears to be decoupled in the \textit{dbt}^{EY02910} mutant. Interestingly, in contrast to
wildtype where TIM accumulates shortly after lights-off (ZT14) in all clock neurons, we
found fewer TIM positive cells in the mutants, suggesting that the underlying phase of
TIM accumulation is altered among these cells. In addition, although the general cycling
of TIM is maintained in LN\textsubscript{s}s, we found that at ZT22 (Figure 6A) and ZT18 (Figure
6B, D), TIM is expressed in the nucleus in some cells, but cytoplasm in others.
Decoupling between TIM and PER is evident at times when PER is stable and nuclear

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and TIM is absent (e.g. ZT8) and also after TIM accumulates. Figure 6 shows that at ZT14, PER is expressed strongly in the nucleus of 4 l-LNₙs, whereas TIM is expressed weakly in the cytoplasm; in 2 s-LNₙs, PER is expressed in the nucleus, whereas in the other 2 s-LNₙs, TIM is cytoplasmic, but there is no detectable PER (Figure 6A).

Although PER is nuclear for the most part in \textit{dbt}^{EY02910} mutants, there is also some variability in its localization: it is in the nucleus of some LNₙs, but cytoplasmic in other LNₙs at ZT16-17 (Figure 6B, D). These data suggest that PER and TIM expression in the central clock cells is desynchronized when DBT is severely reduced.

To independently confirm that a severe reduction of DBT is the underlying cause of the phenotype, we used the \textit{dbt}^{P} mutation. This causes severe reduction of \textit{dbt} mRNA (23) and results in pupal lethality, but allows for clock gene expression analysis in larvae. In wildtype larvae, TIM starts accumulating before PER, and is visible at ZT14 in all clock neurons, and both PER and TIM are present in all clock neurons from ZT16-23. However, as in the adult lateral neurons of \textit{dbt}^{EY02910} mutants, we found that PER and TIM expression is heterogeneous and decoupled in the clock neurons of \textit{dbt}^{P} larval brains (Figure 7). At most time points, some cells show high expression of PER and low of TIM while other cells express low PER and high TIM. Importantly, PER is present in the nucleus in some cells but in the cytoplasm in others from ZT16-23, and we also found similar coexistence of neurons with cytoplasmic and nuclear expression TIM from ZT7-23. Thus overall reduction of DBT results in heterogeneous expression of PER, and decoupling of PER and TIM, in the central pacemaker cells.
To further investigate the temporal dynamics of PER and TIM in individual clock neurons, we examined the larval dorsal neurons. We found that PER is constantly present in the nucleus in DN1 cells of dbt larvae in LD cycles (Figure 8). On the other hand, TIM expression undergoes normal cycling in DN1 cells of mutant larvae (Figure 8). These data further support the idea that PER and TIM are decoupled in mutants that have a severe reduction of DBT.

**DBT interacts with the PDF signaling pathway**

The strong effect of dbt mutants on the synchrony of the circadian clock network is reminiscent of the phenotype produced by loss of pigment dispersing factor (PDF), the only known factor that is released from the s-LN_s to synchronize the circadian clock network. In Pdf^{01} null flies (44), oscillation of clock genes gradually loses synchrony in the s-LN_s, and is also affected in some other clock neurons (10-12, 45). To test if DBT interacts with the PDF signaling pathway, we expressed wildtype and mutant forms of DBT in the Pdfr^{5304} mutant background. Consistent with previous reports, we found that most Pdfr^{5304} mutants are arrhythmic or have weak rhythms (26). Interestingly, when UAS-dbta expression was driven by cry-Gal4 in most clock cells, it partially restored rhythmicity in the mutant background (Table 1). We note that this genetic interaction does not necessary indicate a direct interaction between DBT and PDF signaling. Nonetheless, these data suggest that DBT and PDF signaling act on some common pathways to regulate the circadian clock network.

**Discussion**
Despite the well-known function of CK1 in the phosphorylation and subsequent degradation of PER, the molecular mechanism by which CK1 phosphorylates a specific target site is just beginning to be unraveled. In *Drosophila*, substrate recognition of DBT seems to be temporally and spatially regulated (46, 47). A reduced rate of PER degradation can explain the behavioral phenotypes of *dbt^AR* and *dbt^L* (48), but not of *dbt^S*, although all produce DBT proteins that have overall reduced kinase activity (4, 17, 19, 22, 23, 48). In addition, DBT may play a non-catalytic role in influencing CLK-dependent transcription (18). Thus studies of known *dbt* mutants have uncovered multiple allele-specific effects on the circadian clock (4, 18, 20, 21, 49). Developmental lethality associated with the *dbt^P* strain, perhaps due to the *dbt* mutation itself, limited its use in investigating the effect of reduction of DBT on the circadian clock (4, 23). The bona fide loss-of-function allele, *dbt^EY02910*, we report here provides a valuable reagent to address basic questions regarding the circadian function of DBT.

**Severe reduction of DBT causes long period and disrupts circadian rhythms**

We found that more than half the *dbt^EY02910* flies are arrhythmic, and about 40% display very weak rhythms with a period of 32 h (Table 1). As other allelic combinations of *dbt* show dissociations of period length and arrhythmicity (4, 20, 23) and some ultra-long period mutants (*tim^UL*, 33 h; *per^L;tim^UL*, 41 h) are largely rhythmic (50), long period itself is not necessarily the cause of disrupted rhythms. We suggest that DBT has separate roles in regulating period length and rhythmicity. In *dbt^EY02910* flies, both effects are probably caused by general reduction of catalytic activity, because overexpression of a kinase-dead DBT protein (K38R mutation) produces similar phenotypes (22).
The expression of DBT protein is reduced by ~95% in the \textit{dbt}^{EY02910} mutant flies, reminiscent of the \textit{dbt}^{P} mutant larvae that show similar reduction of \textit{dbt} mRNA levels (23). We were not able to reliably compare DBT protein levels between \textit{dbt}^{EY02910} and \textit{dbt}^{P} mutant larvae due to high background noise. Behaviorally, \textit{dbt}^{P} seems to be a more severe mutation (20) than \textit{dbt}^{EY02910} (Table 1) when tested in-trans over the \textit{dbt}^{AR} allele. Interestingly, the \textit{per}^{S} mutation does not rescue the arrhythmicity of \textit{dbt}^{EY02910} mutants (Table 1), although it rescued the arrhythmicity of \textit{dbt}^{AR} mutants (20). Perhaps DBT^{AR} prevents phosphorylation at specific degradation-promoting sites on \textit{PER}, and this can be offset by the destabilizing effect of the \textit{per}^{S} mutation (20, 33). Lack of rescue by \textit{per}^{S} supports the idea that severe reduction of DBT in \textit{dbt}^{EY02910} mutants has some different effects from other \textit{dbt} alleles, e.g. desynchrony of the clock network (see below).

**DBT kinase activity regulates nuclear localization of \textit{PER} in vivo**

We found that nuclear localization of \textit{PER} and \textit{TIM} in the late night is fairly well coupled in wildtype flies. Although we did not find clear sequential nuclear localization of \textit{PER} and \textit{TIM} as previously reported (51), \textit{PER} was more nuclear than \textit{TIM} in some cells from ZT16-18 (Figure 6). It should be noted that although Shafer et al (51) reported a higher abundance of \textit{TIM} in the cytoplasm at ZT16-20, they also detected \textit{TIM} in the nucleus at this time (51). The absolute amount of \textit{TIM} in the nucleus is difficult to quantify, because different antibodies are used to label \textit{PER} and \textit{TIM}.

In contrast to the situation in wildtype, nuclear localization of \textit{PER} and \textit{TIM} is decoupled in the \textit{dbt}^{EY02910} and \textit{dbt}^{P} mutants. These data suggest that DBT plays an
important role in regulating the nuclear localization of PER and TIM. Severe reduction of DBT in \textit{dbt}$^{\text{EY02910}}$ results in predominantly nuclear expression of PER, which is consistent with previous reports showing that PER is constitutively nuclear in clock neurons when its phosphorylation is blocked by phosphatase expression (9), or by reduction of DBT activity (4, 21, 22). In addition, the PERΔ protein, which lacks the DBT binding domain, is mainly nuclear in clock cells (30). All these data suggest that reduction of DBT activity increases nuclear expression of PER in flies. However, in S2 cells, PER is expressed predominantly in the cytoplasm, even when endogenous \textit{dbt} expression is knocked down by RNAi (29). It is likely that the lack of circadian oscillation and dynamic interaction of PER-TIM-DBT proteins (52) in S2 cells accounts for some of the differences. For instance, DBT displays PER-associated rhythmic redistribution between the nucleus and the cytoplasm in wildtype clock neurons, while it is mostly nuclear in \textit{per}$^{\text{01}}$ (no PER) and \textit{tim}$^{\text{01}}$ (very low PER) mutants (52).

**Role of DBT in the transcriptional repression of CLK target genes**

The relationship between DBT mediated hyper-phosphorylation and repressor potency of PER is not clear, although one model posits that PER brings DBT to phosphorylate and repress CLK activity (18, 31, 35). Most data for the DBT effect in this model are based upon experiments using S2 cells. As a viable mutant with very little DBT expression, \textit{dbt}$^{\text{EY02910}}$ provided us with a valuable tool to address the role of DBT in the transcriptional repression of CLK by PER \textit{in vivo}.

In the DBT bridge model (18), DBT recruits some unknown kinase to phosphorylate CLK and suppress its activity. We predicted a dramatic reduction of CLK
hyper-phosphorylation in the \textit{dbt}^{EY02910} mutants. Surprisingly, we found that CLK is still abundantly phosphorylated in the \textit{dbt}^{EY02910} mutants (Figure 3), which suggests that a 1:1 stoichiometric ratio of DBT:CLK is not necessary for CLK hyper-phosphorylation. Alternatively, DBT may not be the primary kinase that phosphorylates CLK. Importantly, CLK in the mutant is always expressed as hypo- and hyper-phosphorylated forms, and it still has significant transcriptional activity (see below).

We found that rhythms of mRNA levels of CLK target genes \textit{per} and \textit{Pdp1ε} are dampened, such that peak levels are reduced, whereas trough levels are increased in the \textit{dbt}^{EY02910} mutants. This dampening of clock gene transcription can be simply explained by dampened oscillation of PER and CLK protein levels and phosphorylation states. In order to examine the steady state of clock gene transcription, we analyzed CLK-mediated transcription in the \textit{tim}^{01} mutant background. We observed a small increase of \textit{per} mRNA levels in clock-less \textit{tim}^{01}; \textit{dbt}^{EY02910} (compared to \textit{tim}^{01} single mutants (Figure 4D). Although we did not detect an increase in other CLK target gene, \textit{Pdp1ε} and \textit{vri}, between \textit{tim}^{01}; \textit{dbt}^{EY02910} double and \textit{tim}^{01} single mutants (Figure 4D), these data do not negate a role for DBT in repression (see below). A previous report showed that \textit{Pdp1ε} and \textit{vri} are actually dramatically reduced in \textit{tim}^{01}; \textit{dbt}^{AR} (compared to \textit{tim}^{01} single mutants) (21). Presumably the very stable and nuclear PER in the \textit{tim}^{01}; \textit{dbt}^{AR} double mutant (in \textit{tim}^{01} flies PER is very unstable) effectively represses transcription of \textit{Pdp1ε}. However, even though PER is stabilized in the \textit{tim}^{01}; \textit{dbt}^{EY02910} mutant, it does not efficiently repress transcription of \textit{per}, \textit{Pdp1ε}, and \textit{vri}, most likely because the DBT protein itself is required for this effect. Thus, a non-catalytic function of DBT(18), present in DBT^{AR}, is sufficient to repress CLK activity. The role for DBT
indicated by the \( dbt^{EY02910} \) mutant is consistent with a previous finding that RNAi knockdown of \( dbt \) decreases PER repressor activity (29). The mechanism by which DBT modulates repression is unclear. Given that considerable phosphorylation of CLK still occurs in \( dbt^{EY02910} \) flies, it is possible that phosphorylation is not the trigger and DBT recruits other factors that repress CLK. On the other hand, phosphorylation events critical for repression may not occur in the mutant.

**DBT promotes coherence of the circadian clock network**

We uncovered an unexpected role of DBT in synchronizing the circadian clock network in the central brain. \( dbt^{EY02910} \) mutants show variable numbers of neurons expressing high levels of PER from time point to time point, indicating a loss of coherence in timing. PER is mostly nuclear, perhaps because DBT activity or levels set the gate for nuclear translocation of PER, so in cells with severely reduced DBT, the default state of PER is nuclear. However, we do find co-existence of neurons that express cytoplasmic and nuclear PER at certain time points, particularly at ZT16-18, the time of PER nuclear translocation in wildtype flies. These data, together with the finding that about 40% of the \( dbt^{EY02910} \) flies display weak behavioral rhythms, indicate that the residual DBT is capable of maintaining a weak molecular clock in some cells. However, the coherence of the network is lost. We note that heterogeneous expression of PER in the early night has also been reported for flies overexpressing the kinase dead \( DBTK^{38R} \) (22).

While prolonged nuclear expression of PER likely accounts for the long period, it probably does not account for the weak behavioral rhythms of \( dbt^{EY02910} \). As mentioned...
above, some extremely long-period mutants maintain stable rhythms without arrhythmicity (20, 50). Furthermore, the dosage effect of DBT on period length and rhythmicity does not seem to be correlated (23) (Table 1). We suggest that the long period of \( \text{dbt}^{\text{EY02910}} \) mutants derives from effects on clock proteins, while the arrhythmia is attributable, at least in part, to disruption of the circadian circuit. A role of CK1 in maintaining coherence of the circadian timing system has also been proposed in mammals. In \( \text{Ck1}^\epsilon_{\tau\mu} \) mutants, some peripheral clocks are misaligned with the SCN, suggesting that this gain-of-function mutation affects synchrony of the circadian timing system (15). In addition, loss of the VIP receptor in the SCN desynchronizes clock cells and abolishes oscillations of \( \text{Per2-luciferase} \), which can be restored by transiently inhibiting CK1\( \delta \) (53).

As noted earlier, the \( \text{Drosophila} \) equivalent of VIP is PDF, which has also been associated with synchrony of the clock network. Indeed, we show that over-expression of \( \text{dbt}^{\text{AR}} \) by the \( \text{cry-Gal4} \) driver can partially restore rhythmicity to the \( \text{Pdfr}^{5304} \) mutants. We hypothesize that \( \text{dbt} \) is epistatic to \( \text{Pdfr} \), such that a severe reduction of DBT levels causes a strong phenotype even in LD, while the effect of \( \text{Pdfr} \) mutation manifests a weak phenotype over time in DD. We hypothesize that DBT and PDF share some common mechanisms that synchronize the circadian clock network. On the other hand, given that the \( \text{dbt} \) desynchrony phenotype is manifest in the presence of light:dark cycles, it is possible that \( \text{dbt} \) mutants affect entrainment of the clock network to light. TIM degradation by light is normal in these mutants, but we cannot exclude the possibility that events downstream of the TIM response are affected and prevent light-mediated synchrony of the circuit. Regardless, the data indicate that changes in CK1
activity, perhaps in response to peptidergic signals including PDF or VIP, mediate communication across the circadian network. Intriguingly, a recent study using the unicellular marine algae Ostreococcus tauri indicated that CK1 activity is circadian gated (54), which could also be the case in higher organisms.

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References


**Figure legends**

**Figure 1.** Identification of a new dbt mutant allele. (A) A P[EPgy2] element insertion in the 5’ UTR of all isoforms (RA, RB, RC, RD) of dbt disrupts their expression. A pair of blue triangles denotes the location of qPCR primers. (B) DBT is dramatically reduced in the dbt<sup>EY02910</sup> mutants. Levels of DBT protein in adult heads are quantified based on 3 Western blots (6 samples in each blot) (right panel). The bottom panel is over-exposed to visualize the weak band of DBT in the mutants. Armadillo (ARM) protein is shown as a loading control. wt: wildtype; EY: dbt<sup>EY02910</sup>. (C) Levels of DBT protein are barely detectable in the larval tissues of dbt<sup>EY02910</sup> and dbt<sup>2</sup> mutants. (D) No gross defect of the circadian clock network is observed in the central brain of dbt<sup>EY02910</sup> mutants. PDF projections from the s-LN<sub>s</sub> and l-LN<sub>s</sub> are intact and dorsal neuron groups are present in dbt<sup>EY02910</sup> mutants. Green: PDF, Red, TIM (ZT21).

**Figure 2.** Behavioral rhythms are disrupted in dbt<sup>EY02910</sup> mutants. (A) 5-day-old adult males were entrained to 3 light:dark (LD) cycles and released into constant darkness (DD) for 7 days. The majority of dbt<sup>EY02910</sup> mutants (lower panel) display arrhythmic...
behavior in DD; about 40% of the mutants exhibit weak rhythms with a period of ~32 h. This behavioral phenotype can be rescued by expression of the endogenous \textit{dbt} (trapped by the EY insertion) driven by \textit{tim-Gal4} (TG); R: rhythmic; WR: weakly rhythmic; AR: arrhythmic. (B) Light-dependent degradation of TIM protein is intact in \textit{dbt}^{EY02910} mutants. Left panels: compared to unpulsed controls (ctl), TIM levels are dramatically reduced by a 10-min light-pulse (LP). Right panels: TIM is dramatically reduced in both wildtypes and mutants at ZT02, compared to CT02.

\textbf{Figure 3.} Cyclic phosphorylation of circadian clock proteins PER and CLK is altered in \textit{dbt} mutants. (A) Hyper-phosphorylation of PER, which occurs in the late night and early morning in wildtype flies, is delayed in \textit{dbt}^{EY02910} mutants. Hypo-phosphorylated forms of both PER and CLK proteins are present at most times of day, even at times of peak phosphorylation, thus resulting in dampened oscillation of phosphorylation levels. Asterisks point to nonspecific bands also present in \textit{Clk}^{Jrk} mutants (note that the top non-specific band is variable from blot to blot). Similar results were obtained from more than 3 experiments. (B) \textit{\lambda }-phosphatase treatment eliminates the slow moving bands, demonstrating that the majority of CLK proteins are phosphorylated in both wildtype and \textit{dbt}^{EY02910} mutants. Similar results were obtained in two independent experiments.

\textbf{Figure 4.} Role of DBT in transcriptional regulation. (A) Dampened oscillations of circadian clock gene mRNAs in \textit{dbt}^{EY02910} mutants. Peak levels of \textit{per} and \textit{Pdp1} ε mRNA levels are reduced and delayed, whereas trough levels are increased. \textit{Clk} and \textit{cry} mRNAs are expressed at intermediate levels throughout the day. Data are shown as mean±SEM (n=5). The area under the curve (but above the trough point) is significantly
smaller in the mutants as shown in (B) (*p<0.05; **p<0.01, Student's t-test, n=5). (C) While PER is very low in \textit{tim}^{01} mutants, it is present in the nucleus of lateral neurons (upper panel) and fat body cells (lower panel) in the \textit{tim}^{01}; \textit{dbt}^{\text{EY02910}} double mutants (ZT21). PDF staining (green) labels LN's. Nuclei are counterstained with DAPI (blue). (D) Compared to the \textit{tim}^{01} single mutant, the \textit{per} mRNA level is slightly increased in \textit{tim}^{01}; \textit{dbt}^{\text{EY02910}} double mutants; on the other hand, \textit{Pdp1ε} and \textit{vri} mRNA levels are slightly reduced in the double mutants. For the clock-less mutants in which mRNA levels do not cycle, data from ZT2 and ZT14 were pooled. Asterisks indicate significant differences (p<0.05) by Student's t-test (n>4).

**Figure 5.** Heterogeneous expression of PER in adult clock neurons of \textit{dbt}^{\text{EY02910}} mutants. (A) In contrast to the robust rhythm of PER expression in all three groups of clock neurons in wildtype flies, PER is present in some clock neurons most times of the day in \textit{dbt} mutants, resulting in an apparent lack of cycling of PER in the central brain. However, at any given time point PER is strong in some clock neurons but weak in others. This heterogeneity of PER expression is summarized in (B), which depicts the number of cells in each clock cell group that strongly expresses PER at different times of the day. PER positive clock neurons were arbitrarily scored (0-5) for fluorescence intensity under the microscope, and cells with a score higher than 2.5 were counted as strongly PER positive. About 8 brains were examined for each time point.

**Figure 6.** Expression of PER and TIM is decoupled in the adult LN's of \textit{dbt}^{\text{EY02910}} mutants. (A) In contrast to the synchronized expression of PER and TIM in the lateral neurons of wildtype flies, PER and TIM expression is heterogeneous in the lateral
neurons of \textit{dbt}^{EY02910} mutants. TIM is low during the light phase, and it still undergoes cytoplasm to nucleus translocation in the \textit{dbt}^{EY02910} mutants, while PER is largely nuclear at all time points. At ZT14, strong PER and TIM localize to different clock neurons. (B) Similar decoupling is observed at ZT16. Note that PER in the majority of mutant brains at ZT17 is similar to that in ZT16. In a few mutant brains (~20%), PER is strongly nuclear in some cells, but cytoplasmic in others at ZT17 as shown. More than 8 brains were examined for each time point (A and B), and similar results were obtained from two experiments. This heterogeneity is summarized in (C), which depicts the number of clock neurons (mean±SEM, \textit{n}>7) with high PER and high TIM (Hper Htim), high PER but low TIM (Hper Ltim), low PER but high TIM (Lper Htim), low PER and low TIM (Lper Ltim), based on relative levels of intensity. (D) Cellular localization of PER and TIM is less synchronized in \textit{dbt}^{EY02910} mutants. Compared to uniform cytoplasmic (ZT16) and nuclear (ZT24) localization of PER and TIM in wildtypes, PER and TIM are present in the nucleus in some cells, but cytoplasmic in others in \textit{dbt}^{EY02910} mutants.

\textbf{Figure 7.} Expression of PER and TIM is decoupled in the larval lateral neurons of \textit{dbt}^{P} mutants. In the wildtype larval brain, cytoplasmic TIM expression is visible in all LNvs from ZT14 to ZT20. At ZT23, TIM and PER localize to the nucleus in all lateral neurons, and PER expression persists in nuclei to ~ZT7. In \textit{dbt}^{P} mutants PER is visible in the nucleus of most clock neurons at all time points. However, expression levels are more variable, and cytoplasmic PER is detected in some clock neurons at ZT16-23. TIM levels are generally lower and also variable in the \textit{dbt}^{P} mutants. In most cells (ZT16-23), intensities of PER and TIM are generally decoupled from each other (summarized in B and C). Note that TIM is mostly undetectable at ZT01 and ZT07. More than 8 brains
were examined for each time point, and similar results were obtained from two independent experiments.

**Figure 8.** TIM cycling persists in DN1 neurons of *dbt* larvae. (A) In the *dbt* mutants, PER is constantly present in the nucleus, whereas TIM expression is similar to that in wildtype larval brains. This heterogeneity is summarized in (B) and (C). More than 8 brains were examined for each time point, and similar results were obtained from two independent experiments.
Table 1. Behavioral analysis for *dbt* mutants

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<th>Genotype</th>
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<th>FFT1</th>
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*n*: total number of male flies tested (excluding the ones that did not survive for more than 7 days in DD). 1Percentage of rhythmic flies (R) that have a single significant peak (p<0.05) based on the χ2 periodogram analysis; their respective period and FFT (fast Fourier transformation) values are shown in the adjacent columns. 2Percentage of weakly rhythmic flies (WR) (see Materials and Methods); their respective period and FFT values are also indicated. ** indicates significant difference (p<0.001) by χ2 test, when compared to the *Pdfr*^5304^ mutants.