

# A Role for the Segment Polarity Gene *shaggy*/GSK-3 in the *Drosophila* Circadian Clock

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## Summary

Tissue-specific overexpression of the glycogen synthase kinase-3 (GSK-3) ortholog *shaggy* (*sgg*) shortens the period of the *Drosophila* circadian locomotor activity cycle. The short period phenotype was attributed to premature nuclear translocation of the PERIOD/TIMELESS heterodimer. Reducing SGG/GSK-3 activity lengthens period, demonstrating an intrinsic role for the kinase in circadian rhythmicity. Lowered *sgg* activity decreased TIMELESS phosphorylation, and it was found that GSK-3 $\beta$  specifically phosphorylates TIMELESS in vitro. Overexpression of *sgg* in vivo converts hypophosphorylated TIMELESS to a hyperphosphorylated protein whose electrophoretic mobility, and light and phosphatase sensitivity, are indistinguishable from the rhythmically produced hyperphosphorylated TIMELESS of wild-type flies. Our results indicate a role for SGG/GSK-3 in TIMELESS phosphorylation and in the regulated nuclear translocation of the PERIOD/TIMELESS heterodimer.

## Introduction

Genetic and biochemical studies of circadian behavioral rhythms in *Drosophila* and rodents have revealed a cell-autonomous mechanism that is substantially conserved throughout the animal kingdom (Dunlap, 1999; Edery, 2000; Wager-Smith and Kay, 2000; Reppert and Weaver, 2000; Cermakian and Sassone-Corsi, 2000; King and Takahashi, 2000; Young, 2000). In *Drosophila* locomotor activity, cycles are regulated by a group of cells in the central brain, the lateral neurons or LNs (Ewer et al., 1992; Frisch, et al., 1994; Renn et al., 1999). Within the LNs, circadian rhythmicity can be seen at a molecular level. At the beginning of a cycle, two transcription factors, dCLOCK (dCLK) and CYCLE (CYC), coordinately activate transcription of the *period* (*per*) and *timeless* (*tim*) genes. Toward the end of the cycle, nuclear PERIOD (PER) proteins interact with dCLK and CYC to fully suppress their activity (Hall, 2000; Hardin and Glossop, 2000; Edery, 2000; Giebultowicz, 2000; Scully and Kay, 2000; Young, 2000).

Formation of nuclear PER requires a series of closely

regulated steps that generate molecular oscillations. Cytoplasmic accumulation of PER is delayed by the activity of a kinase, DOUBLE-TIME (DBT), which promotes phosphorylation and degradation of PER (Edery, 2000; Giebultowicz, 2000; Young, 2000). PER is eventually stabilized in the cytoplasm by heterodimerization with the TIMELESS protein (TIM). This physical association also permits nuclear translocation of both proteins (Edery, 2000; Young, 2000). In the nucleus, PER/TIM complexes appear to initiate downregulation of *per* and *tim* expression while upregulating expression of *dClk* (Edery, 2000), but loss of TIM is needed for full suppression of dCLOCK/CYCLE activity by a TIM-free nuclear PER protein (Rothenfluh et al., 2000a). DBT-dependent phosphorylation and degradation of TIM-free nuclear PER allows reinitiation of the molecular cycle (Suri et al., 2000; Young, 2000; Rothenfluh et al., 2000a; Kloss et al., 2001).

Environmental light:dark cycles can adjust the phase of this molecular clock through effects on TIM protein that are mediated by the photoreceptor CRYPTOCHROME (CRY; Ceriani et al., 1999; Emery et al., 2000a, 2000b; Hall, 2000; Hardin and Glossop, 2000; Edery, 2000; Scully and Kay, 2000; Young, 2000). Two additional proteins expressed in the LNs, the transcription factor VRILLE (VRI) and the neuropeptide PDF, help to couple molecular oscillations to rhythmic locomotor behavior (Blau and Young, 1999; Renn et al., 1999; Helfrich-Förster et al., 2000; Park et al., 2000).

Unlike the *per*, *tim*, *dClk*, and *cyc* genes, whose sole function may be the regulation of circadian rhythms, the clock genes *dbt* and *vri* are required for viability (Kloss et al., 1998; Price et al., 1998; Blau and Young, 1999). Certain genes organizing rhythmic behavior in response to the clock have also been found to perform vital functions (e.g., *lark*; Newby and Jackson, 1993; McNeil et al., 1998). Because classical genetic screening might fail to identify all clock-associated genes with vital functions, we began a series of alternative studies to extend the search for genes influencing circadian behavior in *Drosophila*. Prior work has shown that constitutive overexpression of either *per* or *vri* is sufficient to stall the molecular oscillator (Zeng et al., 1994; Blau and Young, 1999). Therefore, we initiated a screen involving pacemaker-cell-specific overexpression of individual genes throughout much of the *Drosophila* genome. In this report, we describe the characterization of *shaggy* (*sgg*), which encodes the *Drosophila* ortholog of glycogen synthase kinase-3 (Bourouis et al., 1990; Siegfried et al., 1990), as a component of the *Drosophila* clock.

## Results

### Gain of *sgg* Function Shortens the Period of the Circadian Oscillator

Using *timeless*-(UAS)-GAL4 as driver (Blau and Young, 1999), screening a collection of 2300 EP lines (Rørth, 1996; Berkeley *Drosophila* Genome Project) for effects on locomotor activity rhythms yielded two arrhythmic,

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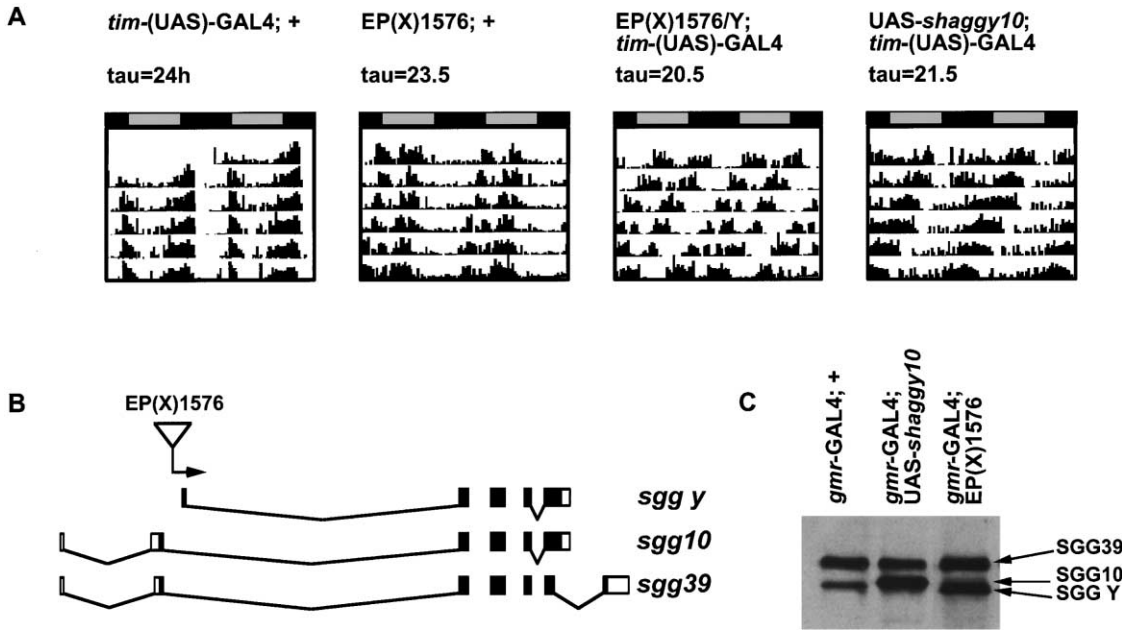


Figure 1. Overexpression of *sgg* Shortens the Period of the Locomotor Activity Rhythm in *Drosophila*

(A) Each panel represents the locomotor activity of an individual fly over time. For visual continuity, 2 days are plotted per row. On top of the panels, the subjective light and dark phases, genotypes, and average period lengths are indicated. Flies were entrained for 3 days in a 12 hr:12 hr light:dark (LD 12:12) cycle and subsequently maintained in constant darkness (DD) for the locomotor analysis.

(B) Genomic organization of the *sgg* locus. Exons of transcripts are represented by boxes, filled areas represent translated regions. Shared sequences between transcripts are drawn on top of each other. The position of EP(X)1576 is represented as a triangle with an arrow pointing in the direction of GAL4-induced transcription. Nomenclature for the transcripts was taken from Ruel et al. (1993). However, not all known transcripts were included in the diagram. The position for the first exon of *sggy* was determined by sequence alignment of the unique N terminus encoded by *zw3-C* (Siegfried et al., 1992) with genomic sequence.

(C) SGGY overexpression in EP(X)1576. Western blot analysis of extracts from indicated genotypes with an antibody against SGG. Arrows on the side of the gel point out the different SGG isoforms. For comparison, overexpression of SGG10 is shown, *gmr-GAL4/+* serves as wild-type control.

four long period, and one short period strain. Here, we report a molecular and phenotypic characterization of the short period strain.

The X-chromosomal genetic element EP(X)1576 in combination with *tim*-(UAS)-GAL4 as a driver decreased the locomotor activity period by approximately 3 hr. Similar results were obtained when *tim*-GAL4 (Emery et al., 1998) was employed as a driver (Figure 1A, Table

1). Excision of EP(X)1576 reverted this short period phenotype to wild-type, indicating that activation of EP(X)1576 was responsible for the observed phenotype (data not shown).

Isolation of genomic DNA and sequence comparison revealed that EP(X)1576 is an insertion in the *shaggy* (*sgg*) locus, which encodes the *Drosophila* ortholog of glycogen synthase kinase-3 (GSK-3; Bourouis et al.,

Table 1. Period Shortening of Locomotor Activity Rhythms by *sgg* Overexpression

Genotype	tau [Hours]	SD [Hours]	Number Rhythmic	Total Number
EP(X)1576/Y; <i>tim</i> (UAS)-GAL4/+	20.3	0.4	20	20
EP(X)1576/+; <i>tim</i> (UAS)-GAL4/+	21	0.4	13	13
EP(X)1576/+; <i>tim</i> -GAL4	21.1	0.3	12	14
UAS- <i>sgg</i> / <i>tim</i> (UAS)-GAL4 <sup>a</sup>	20.9	0.4	5	10 <sup>b</sup>
UAS- <i>sgg</i> / <i>tim</i> (UAS)-GAL4 <sup>a</sup>	20.8	0.3	5	10 <sup>b</sup>
UAS- <i>sgg</i> / <i>tim</i> -GAL4 <sup>a</sup>	22.2	0.3	16	16
UAS- <i>sgg</i> / <i>tim</i> -GAL4 <sup>a</sup>	21.8	0.6	10	10
<i>tim</i> (UAS)-GAL4/+	23.6	0.4	20	20
<i>tim</i> -GAL4/+	23.9	0.5	14	16
EP(X)1576/+	23.5	0.4	17	17
UAS- <i>sgg</i> /+	23.3	0.6	12	16

Flies of indicated genotypes were entrained for three 12 hr:12 hr light:dark cycles and subsequently subjected to a locomotor activity analysis in constant darkness. tau: period length in hours; SD: standard deviation.

<sup>a</sup> Separate entries are data from different crosses of the same fly lines.

<sup>b</sup> Nonrhythmic flies turned arrhythmic during the analysis. However, this arrhythmicity coincided with reduced locomotor activity, indicating that the decreased longevity of those flies causes this phenotype.

1990; Siegfried et al., 1990). The *sgg* gene is essential for development of the fly and is probably best known for its role in *wingless* signaling where it regulates the subcellular distribution of ARMADILLO (Peifer et al., 1994a, 1994b; Siegfried et al., 1992, 1994).

The *sgg* locus produces multiple transcripts that encode proteins with identical kinase domains but different N and C termini (Ruel et al., 1993a, 1993b; Siegfried et al., 1990, 1992). Since lethality associated with mutations of *sgg* can be rescued by expressing the SHAGGY10 isoform alone, the different forms of SHAGGY (SGG) must have redundant functions (Ruel et al., 1993a, 1993b). Closer investigation of genomic DNA surrounding EP(X)1576 revealed that the ORF for *sgg10* begins 693 bp upstream of the EP-element insertion site (Figure 1B). However, translation start sites of other forms of SGG are located downstream of the insertion site of EP(X)1576. To examine whether a form(s) of SGG is affected by EP(X)1576, total protein from heads of flies activating EP(X)1576 by means of the *glass*-responsive element (*gmr*-GAL4) were subjected to Western blot analysis. As shown in Figure 1C, only the SGGY isoform was more abundant than in wild-type. It has been suggested that SGGY is translated from a transcript termed *zw3-C* (Ruel et al., 1993b; Siegfried et al., 1990, 1992). Consistent with activation of *zw3-C* by EP(X)1576, the translation start of SGGY is located 1022 bp downstream of EP(X)1576.

To confirm that overexpression of *sgg* is responsible for the short period phenotype, a *sgg10* cDNA was expressed under control of the *tim* promoter. As shown in Figure 1 and Table 1, these flies have a period length of 21–22 hr depending on the driver, demonstrating that overexpression of *sgg* can shorten the circadian cycle of locomotor activity rhythm and that a proper level of *sgg* activity is required for wild-type circadian behavior. Overexpression of *sgg10* by means of *tim*(UAS)-GAL4 appeared to also affect longevity, preventing the analysis of data obtained from approximately 50% of these flies (Table 1). The slightly weaker phenotype of *sgg10* compared to activated EP(X)1576 could be attributed to the difference in the N terminus. Only SGG10 contains a serine at a position homologous to serine 9 in GSK-3 $\beta$ . This residue is known to negatively regulate the kinase activity of GSK-3 $\beta$  upon phosphorylation (Stambolic and Woodgett, 1994; Sutherland et al., 1993). As SGGY lacks this serine, and therefore a potential negative regulator, its potentially higher kinase activity could explain the stronger phenotype.

#### Overexpression of *sgg* Alters TIMELESS Oscillation

To determine whether *sgg* overexpression affects molecular oscillations in pacemaker cells, the pattern of TIM accumulation in third instar larval LNs was followed immunohistochemically. Prior studies have demonstrated that period-altering mutations of *per*, *tim*, and *double-time* (*dbt*) produce corresponding effects on these molecular cycles and on behavioral rhythms (Kaneko et al., 1997; Price et al., 1998). It has also been shown that the molecular oscillations can be entrained with light:dark (LD) cycles by the beginning of the first larval instar (Sehgal et al., 1992). Because *Drosophila*'s circadian rhythms are temperature compensated (Ko-

nopka et al., 1989), wild-type and *sgg*-overexpressing larvae for this experiment were reared at 20°C instead of 25°C. This lengthened developmental time and allowed an extended analysis encompassing several days of constant darkness (DD).

As shown in Figure 2, with the exception of a single time point (CT10 of the third day), TIM was not detectable in wild-type lateral neurons at either CT4 or CT10 during any of 4 consecutive days in constant darkness (CT0 is subjective dawn and CT12 is subjective dusk in DD). Conversely, at CT16, TIM was largely cytoplasmic and at CT22, predominantly nuclear in wild-type LNs during all 4 days of the analysis. This confirmed that molecular oscillations in wild-type lateral neurons had been entrained by a prior LD cycle (Figure 2, legend) and persisted with a period length of ~24 hr in constant darkness. In contrast, overexpression of *sgg* caused a drift in the appearance of TIM immunoreactivity and nuclear subcellular localization toward earlier time points with each consecutive day (Figure 2). A total shift of ~12 hr was observed over 4 days, showing that the period-shortening effect of *sgg* overexpression on molecular cycling was correlated with the new period of the circadian locomotor rhythm.

#### Reduction of *sgg* Function Lengthens Period

A gain of function phenotype alone would not be sufficient to demonstrate a role for *sgg* in the organization of circadian rhythmicity in wild-type *Drosophila*. We therefore tested the effects of reducing *sgg* gene expression on circadian behavior. Since it had previously been shown that expression of *sgg10* under control of a heat shock promoter can rescue the lethality of *sgg* mutants (Ruel et al., 1993a), we attempted to rescue the development of otherwise inviable *sgg* mutants by supplying heat shock-controlled *sgg10* during the larval and pupal stages. Two strains of *Drosophila*, each carrying a different mutant allele of *sgg* and a heat shock-controlled *sgg10* transgene (HS-*sgg*), were heat shocked twice a day for 1 hr at 37°C beginning after hatching of the first larval instar. Heat shocks were discontinued at the mid-pupal stage, and flies were subsequently maintained at a temperature (24°C) well below that necessary to activate the heat shock promoter to determine the contribution of each defective *sgg* allele to circadian rhythmicity. Adults were entrained for 3 days in 12 hr:12 hr LD cycles (LD 12:12) and then subjected to locomotor activity analysis in constant darkness. As shown in Table 2 and Figure 3A, *Drosophila* bearing mutant alleles of *sgg* produce uniform, long period phenotypes when rescued with the *sgg10* transgene. For example, an approximately 2 hr lengthening of the period to 26 hr was observed for the strong allele, *sgg*<sup>D127</sup> (*sgg*<sup>D127</sup>/Y; HS-*sgg10*).

Our observation that a reduction of *sgg* activity substantially lengthens period, while *sgg* overexpression produces a contrasting short period phenotype, demonstrates that SGG is an intrinsic regulator of the period of the circadian clock in *Drosophila*. However, interference with *sgg* function failed to block rhythmicity, which could indicate that SGG sets the frequency of cycling, but is not required to produce molecular oscillations. Alternatively, residual SGG activity in the rescued mutants may

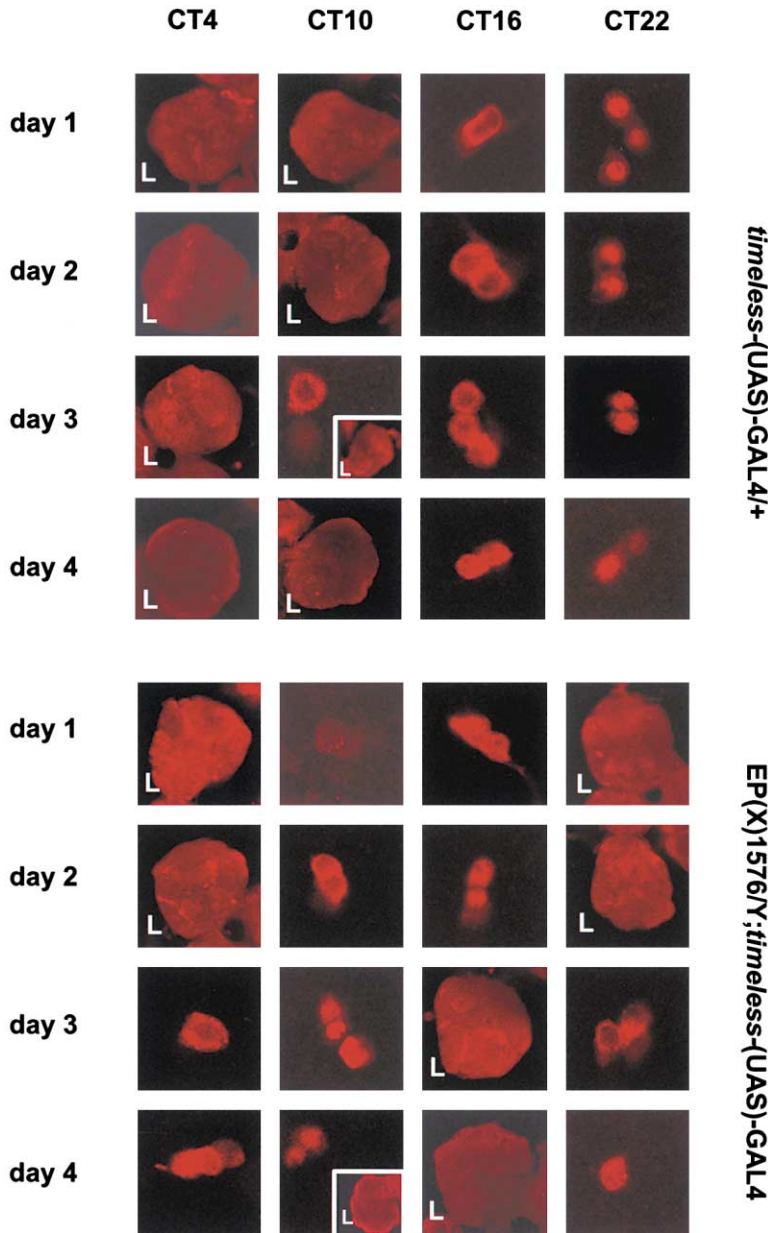


Figure 2. Overexpression of *sgg* Alters the Pattern of TIM Oscillation

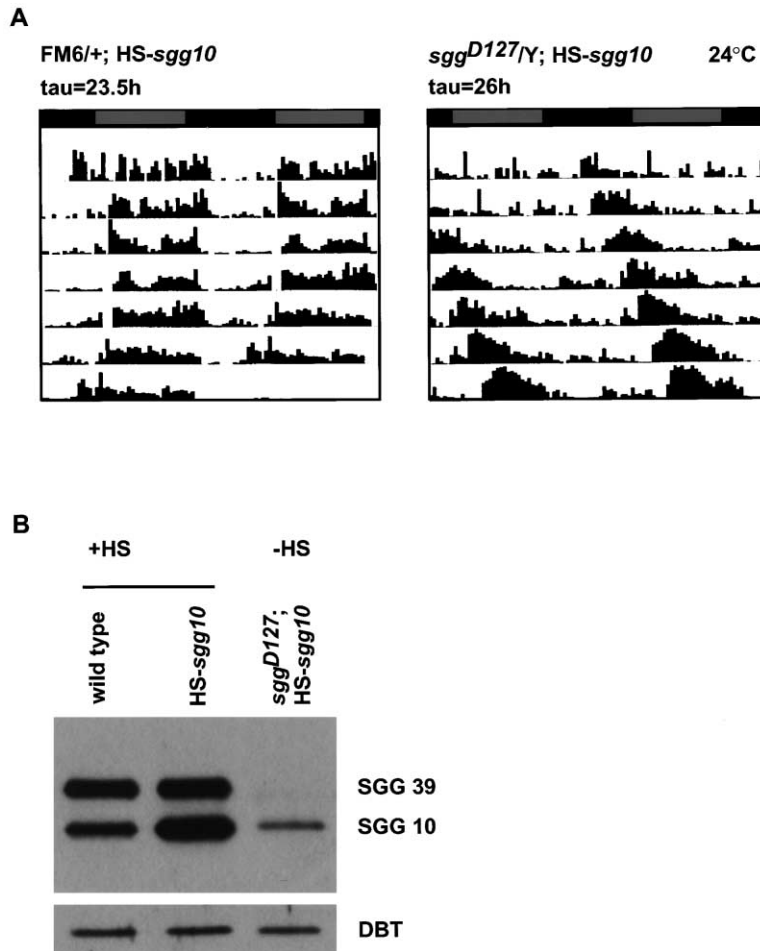
Eggs of indicated genotypes were collected at 20°C in consecutive 1 day bins and entrained for at least three LD 12:12 cycles. Subsequently, larvae of different developmental ages were transferred into constant darkness (CT0 is onset of subjective day). Third instar larvae were subjected to immunohistochemistry every 6 hr using TIM-specific antibodies. Three larval brain hemispheres were analyzed for each time point. Time in DD is indicated on top of the diagram, the day of the collection in DD is indicated on the left. The (L) in some panels denotes lower magnification views of the larval brain hemisphere and absence of pacemaker cell (lateral neuron) staining. Note that wild-type (*tim(UAS)-GAL4/+*) TIM oscillations have a period of ~24 hr, whereas the period is substantially shorter (~3 hr/day) in *sgg*-overexpressing lateral neurons.

prevent us from determining the complete loss of *sgg* phenotype. In order to test this possibility, total head protein was isolated from rescued adult *sgg<sup>D127</sup>* mutant flies (*sgg<sup>D127</sup>/Y*; *HS-sgg10*) approximately one week after the last heat shock, and the extract was subjected to a Western blot analysis using SGG-specific antibodies. As shown in Figure 3B, SGG10, which presumably is derived exclusively from the *HS-sgg* transgene, is still detectable in flies carrying the strong mutant allele *sgg<sup>D127</sup>*. Our results indicate that *sgg<sup>D127</sup>* is a null mutant, and that the residual SGG10 supplied by the transgene is likely required for viability since rearing at lower temperatures eliminated recovery of adult flies. We also found that if the last heat pulse was given more than 4 days prior to eclosion, no rescued flies were produced (S.M., unpublished observation), again suggesting that the residual level of SGG10 protein observed in Figure

3B is required for viability. Due to the persisting SGG10 protein, the ~26 hr, long period behavioral phenotype of the rescued *sgg<sup>D127</sup>* mutant cannot reflect a complete loss of *sgg* function.

**Advanced Nuclear Entry of PER/TIM by *sgg* Overexpression**

In order to investigate the basis for *sgg*-mediated changes in the clock, behavioral responses to photic stimuli were measured to construct a phase response curve (PRC; Aschoff, 1965; Figure 4A). A light pulse administered in the early subjective night, when PER and TIM are cytoplasmic, produces a phase delay in the molecular oscillator, whereas a similar pulse administered late at night eliminates TIM from nuclear PER/TIM complexes to advance the molecular mechanism. Thus, the behavioral response to a photic stimulus reflects the



**Figure 3. Reduced *sgg* Function Lengthens the Period of the Locomotor Activity Rhythm**  
(A) Locomotor activity rhythms of representative, individual flies in DD of the indicated genotypes are shown. Data for all flies tested are presented in Table 2. Flies homozygous for the heatshock-*sgg10* construct (HS-*sgg10*) were crossed with *sgg*<sup>D127</sup> heterozygous flies. Eggs were collected for 1 day at 24°C. After hatching, all developing *Drosophila* were reared together at 24°C and exposed to 1 hr, 37°C heat pulses twice a day until the mid-pupal stage. After eclosion, the rescued *sgg*<sup>D127</sup> hemizygous flies (*sgg*<sup>D127/Y</sup>; HS-*sgg10*) and their siblings (FM6/+; HS-*sgg10*) were entrained by exposure to three LD 12:12 cycles and then subjected to a behavioral analysis in DD for one week at 24°C.

(B) Protein extracts from heads of flies of the indicated genotypes were subjected to a Western blot analysis using SGG-specific antibodies. Heat shocks, where indicated, were administered 1 hr before protein purification. The rescued *sgg*<sup>D127</sup> hemizygotes were subjected to a regimen of 37°C heat pulses through mid-pupal development that was identical to that administered to *Drosophila* tested for locomotor behavior (see [A]). *sgg*<sup>D127/Y</sup>; HS-*sgg10* flies were subsequently maintained in LD 12:12 at 24°C until protein extraction. Proteins were extracted at least one week after the last heat pulse to conform to the midpoint of the behavioral records shown in (A). Note that residual SGG10 persists in the rescued *sgg* mutants despite the absence of continued heat shocks. Antibodies to DOUBLE-TIME (DBT) provided a control for equal loading of protein extracts (Kloss et al., 2001).

subcellular localization of TIM (Young, 1998; Rothenfluh et al., 2000a).

As shown in Figure 4A, overexpression of *sgg* alters the PRC mostly if not exclusively during the delay portion. The delay portion of the PRC of flies overexpressing *sgg* is shortened by 4 hr (compare the two-headed arrows in Figure 4A). This is coextensive with the period-shortening effect of *sgg* overexpression, suggesting that SGG affects a cytoplasmic event of the molecular oscillator.

To test this possibility, subcellular localizations of PER were followed at time points that would reveal the timing of nuclear translocation in larval LNs. Figure 4B and Table 3 show that in wild-type larvae, PER begins to

enter nuclei at ~ZT20 (ZT0 is lights on and ZT12 is lights off in an LD 12:12 cycle) and is predominantly nuclear at ZT21. In contrast, larval lateral neurons overexpressing *sgg* begin to show nuclear PER immunoreactivity at ZT17.5, and by ~ZT19, PER is mostly nuclear. The premature nuclear entry of PER proteins in *sgg*-overproducing larvae should thus account for most, if not all, of the period-shortening phenotype.

#### Advanced Nuclear Entry Is Not Associated with Increased RNA Accumulation

Because advanced nuclear translocation of PER/TIM could reflect higher levels of *per* and/or *tim* expression, the time course of *per* RNA accumulation in constant

Table 2. Locomotor Activity Rhythms of *sgg* Hypomorphic Alleles

Genotype	tau [Hours]	SD [Hours]	Number Rhythmic	Total Number
<i>sgg</i> <sup>M11/Y</sup> ; HS- <i>sgg10</i>	25.4	0.9	14	15
<i>sgg</i> <sup>D127/Y</sup> ; HS- <i>sgg10</i>	26.2	1.0	12	18
<i>sgg</i> <sup>M11/sgg</sup> <sup>D127</sup> ; HS- <i>sgg10</i>	25.2	1.0	10	10
FM6/+; HS- <i>sgg10</i>	24.0	0.5	26	30
<i>sgg</i> <sup>D127/Dp(1:Y)2D1-2;3D3-4;Y</sup>	23.7	0.5	20	30

Females carrying the respective *sgg* allele were crossed to males carrying a heat shock-*sgg* transgene (HS-*sgg10*). The progeny were heat shocked twice a day for 1 hr at 37°C beginning after hatching until approximately the mid-pupal stage. After eclosion, flies were entrained for three 12 hr:12 hr light:dark cycles and locomotor activity was assayed in constant darkness. tau: period length; SD: standard deviation. Statistically significant differences separated the period lengths of all mutants from the wild-type controls with  $p < 0.0005$  (1-tailed t test).

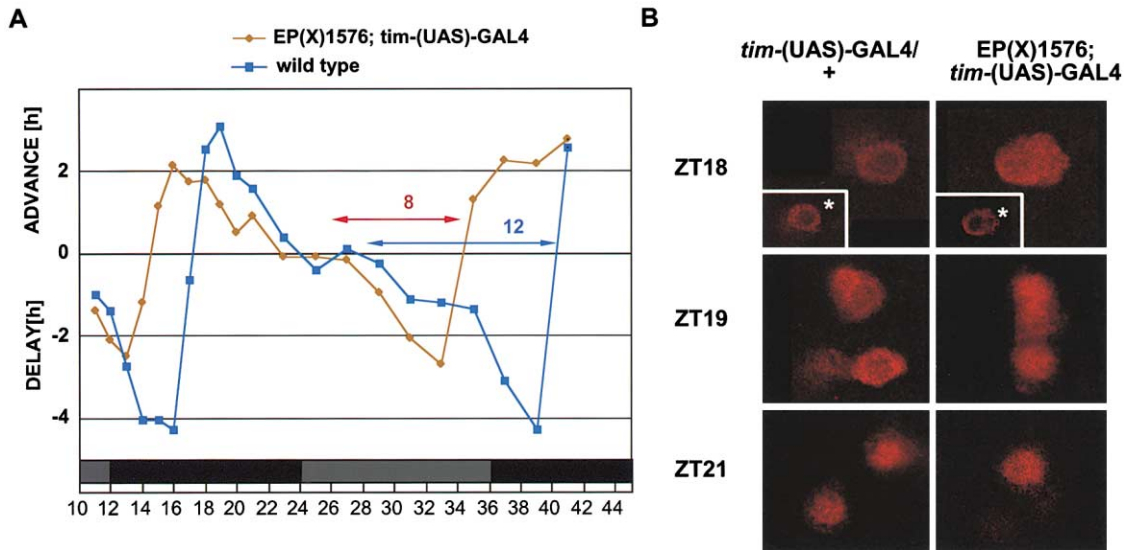


Figure 4. Advanced Nuclear Entry of the PER/TIM Heterodimer Is Associated with a Gain of *sgg* Function

(A) Circadian gating of phase responses to photic stimuli is altered in flies overexpressing *sgg*. Flies were entrained to LD 12:12 for 3 days and released into constant darkness at ZT10 of the fourth day (ZT0 is defined as the onset of light in an LD cycle). Ten minute light pulses of ~2000 lux were given at the indicated times (x axis, in hr). The phase of the subsequent locomotor activity rhythm was measured and compared to the phase of flies that were not subjected to a light pulse. An advanced phase is represented as a positive time and a delayed phase is represented as a negative time on the y axis. Phase responses of flies overexpressing *sgg* are indicated by diamonds (brown). *timeless(UAS)-GAL4/+* (blue squares) serves as a wild-type control. Each time point represents the average of at least two independent experiments. Approximately 10 flies were analyzed for each time point in all experiments. Two-headed arrows indicate the extent of the delay portion of the PRC.

(B) Advanced nuclear entry of PER in lateral neurons overexpressing *sgg*. *Drosophila* were reared in LD 12:12. Third instar larvae were subjected to immunohistochemistry at the indicated time points using anti-PER antibodies to visualize the subcellular localization of PER proteins. For ZT18, the inserts (asterisk) show anti-PDF staining of the same neuron to visualize the cytoplasm.

darkness was determined for *Drosophila* overexpressing *sgg*. Although the phase of *per* RNA accumulation was slightly advanced in these flies, there was no detectable change in the amplitude of the RNA accumulation cycle (Figure 5). Identical results were obtained in studies of the *tim* RNA cycle (data not shown). While the slightly altered phases of *per* and *tim* RNA accumulation could contribute to the short period phenotype, such shifts have been observed previously for period-altering alleles of *per*, *tim*, and *dbt* whose primary defects involved changes in the encoded proteins. Those phase differences were viewed as secondary responses to a fast- or slow-running clock (Price et al., 1998; Rothenfluh et al., 2000b).

### Hypomorphic Mutations of *sgg* Reduce TIM Phosphorylation and Increase Levels of Both the TIM and PER Proteins

A comparison of rescued *sgg<sup>D127</sup>* mutants and wild-type flies also failed to indicate a difference in *per* and *tim*

RNA levels (data not shown), further suggesting that altered *sgg* function might affect nuclear entry of PER/TIM through a posttranscriptional mechanism. To test this possibility, PER abundance was investigated in our rescued *sgg<sup>D127</sup>* flies. As shown in Figure 6A, levels of PER protein were significantly elevated by hypomorphic *sgg* function.

TIM accumulation, like that of PER, was substantially increased in the mutant flies (data not shown). However, these studies also revealed a difference in the pattern of TIM electrophoretic mobility (Figure 6B). During the late subjective night and the early subjective day, TIM is increasingly phosphorylated in wild-type flies (Myers et al., 1996; Zeng et al., 1996). This is associated with the conversion of half or more of the accumulated TIM protein to a slowly migrating species near the end of each molecular cycle. In rescued *sgg<sup>D127</sup>* flies, TIM is predominantly found as a rapidly migrating protein at all times (compare wild-type versus *sgg<sup>D127</sup>* CT2 and CT4 of Figure 6B). Such a difference was not observed for

Table 3. Quantification of Data Obtained in Figure 4B

	timeless(UAS)-GAL4; +			EP(X)1576; timeless(UAS)-GAL4		
	C	C/N	N	C	C/N	N
ZT17.5	6	—	—	—	10	1
ZT18	9	—	—	—	12	2
ZT19	10	—	—	—	1	8
ZT20	3	3	—	—	—	4
ZT21	—	7	5	—	—	10

The number of brain hemispheres found with the indicated subcellular distribution of PERIOD at a given time point is listed. C, cytoplasmic; C/N, cytoplasmic and nuclear; N, nuclear.

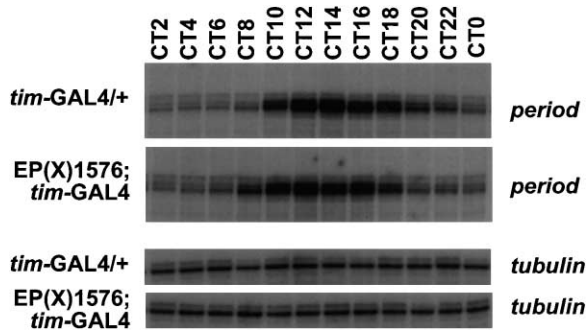


Figure 5. *period* RNA Levels Are Not Elevated by Overexpression of *sgg*

Flies were entrained for 3 days in LD 12:12 and subsequently released into DD (CT0 is defined as subjective dawn in DD). Time points were taken every 2 hr during the first day in constant darkness. Total head RNA was subjected to an RNase protection assay using a *per*-specific probe. Genotypes are indicated on the left of the gel and the time point for each lane is shown on top of the gel. A *tubulin*-specific probe was used as loading control.

PER (Figure 6A), suggesting that *sgg* function specifically influences the phosphorylation and therefore mobility of TIM.

Since the long period rhythms produced by *sgg*<sup>D127</sup> could elicit rather than result from the novel pattern of TIM migration, we examined cycles of TIM mobility in another long period mutant, *double-time*<sup>l</sup> (*dbt*<sup>l</sup>; Price et al., 1998). We focused our analysis on time points corresponding to early subjective day when highest levels of phosphorylated TIM are observed in wild-type flies. Figure 6C shows that even with respect to *dbt*<sup>l</sup>, TIM phosphorylation in *sgg*<sup>D127</sup> is not only delayed, but at all times, TIM migrates predominantly as the hypophosphorylated protein. These observations suggest that *sgg* function is required for proper phosphorylation of TIM.

#### GSK-3 $\beta$ Phosphorylates TIM in an In Vitro Assay

SGG's influence on the phosphorylation state of TIM in vivo prompted us to investigate TIM phosphorylation in vitro. Previous studies have shown that SGG can be replaced by mammalian GSK-3 $\beta$  in a number of *Drosophila* assays. For example, GSK-3 $\beta$  has been shown to rescue the cuticle phenotype and viability of *sgg* mutants (Siegfried et al., 1992; Ruel et al., 1993a). Li and Manley demonstrated that GSK-3 $\beta$  phosphorylates and thereby inactivates the *Drosophila* protein EVEN-SKIPPED in vitro (Li and Manley, 1999). Indeed, the degree of amino acid identity between SGG and GSK-3 $\beta$  within the kinase domain is 85%. We therefore decided to use the mammalian ortholog, whose enzymatic activity is well characterized, for our kinase assay. Different fragments of the TIM protein were synthesized as GST fusion proteins (Myers et al., 1996; Saez and Young, 1996; Lino Saez, unpublished data) and subsequently incubated with GSK-3 $\beta$ . As shown in Figure 7A, the fragments TIM 1–1159 and TIM 222–577 were readily phosphorylated under our conditions. However, neither the carboxy-terminal fragment of TIM (504–1121) nor the clock protein *dbMAL* (full-length *dbMAL* fused to GST) were detectably phosphorylated by GSK-3 $\beta$  in these

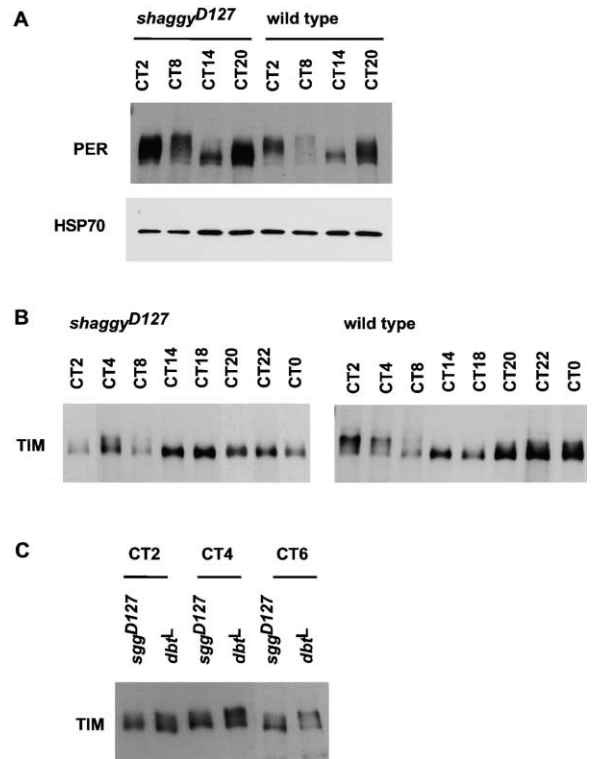


Figure 6. Changes in PER and TIM Proteins due to Reduced *sgg* Function

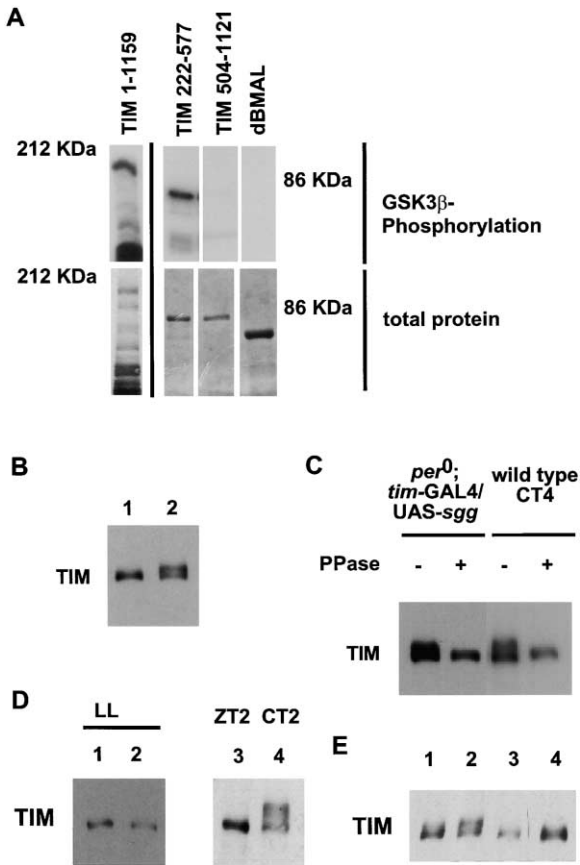
(A) Western blot analysis showing increased levels of PER during the subjective night in *sgg* mutant flies. Flies of the respective genotypes (*sgg*<sup>D127</sup> = *sgg*<sup>D127</sup>/*Y*; HS-*sgg*10, and wild-type = *sgg*<sup>D127</sup>/*Dp* (1:Y)2D1–2;3D3–4;Y) were entrained to three consecutive LD 12:12 cycles and subsequently released into DD. Flies were collected at the indicated time points, head protein was extracted, and subsequently subjected to Western blot analysis.

(B–C) Loss of *sgg* function causes reduced TIM phosphorylation. (B) Blot as in (A) was probed with TIM-specific antibodies. For better comparison of the phosphorylation pattern, exposure time and protein loaded were adjusted to result in similar signal intensities. Consequently, the quantitative difference is not apparent. Note that at CT2 and CT4, TIM in the mutant is less phosphorylated than TIM in wild-type (*sgg*<sup>D127</sup>/*Dp*(1:Y)2D1–2;3D3–4;Y). (C) Flies homozygous for *double-time*<sup>l</sup> (*dbt*<sup>l</sup>) or hemizygous for *sgg*<sup>D127</sup> (*sgg*<sup>D127</sup>/*Y*; HS-*sgg*10) were collected at indicated time points. Head extracts were subjected to a Western blot analysis using antibodies specific for TIM. Note that the low mobility form(s) of TIM are always less abundant in the *sgg* mutant.

assays (Figure 7A). Using the Motif Scanner protein phosphorylation prediction software, two potential GSK-3 sites were found in the sequence encompassed by fragment TIM 222–577 (see Experimental Procedures), and no sites were found in the sequence encompassed by fragment 504–1121; both predictions are in agreement with our in vitro phosphorylation data.

#### Overexpression of *sgg* Elevates Phosphorylation of TIM In Vivo

The reduced phosphorylation of TIM in rescued *sgg*<sup>D127</sup> flies and the phosphorylation of TIM by GSK-3 $\beta$  in vitro prompted us to investigate the pattern of TIM phosphorylation in *Drosophila* overexpressing *sgg*. As the cycling accumulation of TIM would complicate our analysis, TIM phosphorylation was studied in an arrhythmic (*per*<sup>o</sup>) ge-



**Figure 7. SGG/GSK-3 $\beta$  Promotes the Phosphorylation of TIM**  
**(A)** Indicated fragments of TIM or dBMAL (CYC) fused to GST were subjected to a kinase assay with GSK-3 $\beta$  and ( $\gamma$ -<sup>32</sup>P) ATP (top). Because of considerable size differences, the fusion proteins were run on different gels (separated by vertical line). As a loading control, the same amount of protein was loaded on a second set of gels and stained with coomassie blue (bottom).  
**(B)** Western blot analysis of protein extracts from fly heads overexpressing *sgg* with antibodies against TIM. Flies were entrained in three consecutive LD 12:12 cycles and subsequently transferred to DD. After 3 subsequent days in constant darkness, total head protein was extracted. Genotypes are as follows: lane 1, *per<sup>0</sup>*; lane 2, *per<sup>0</sup>; timeless-GAL4/UAS-sgg*.  
**(C)** Phosphatase (PPase) treatment abolishes the low-mobility form of TIM. Head extracts of the indicated genotypes were treated with Alkaline Phosphatase and then subjected to gel electrophoresis and Western blot analysis using TIM-specific antibodies. For comparison, untreated samples were run on the same gel.  
**(D)** The low mobility form of TIM in wild-type is light sensitive. Protein extracts from flies raised either in LD 12:12 (ZT), in constant light (LL) or in constant darkness (CT), were subjected to Western blot analysis. Genotypes are as follows: lane 1, *per<sup>0</sup>*; lane 2, *per<sup>0</sup>; timeless-GAL4/UAS-sgg*; lanes 3 and 4, wild-type. Amounts of protein loaded in lanes 3 and 4 were adjusted so that signal intensities would be similar.  
**(E)** Head extracts of indicated genotypes were subjected to a Western blot analysis. The *per-SG* transgene encodes a PER- $\beta$ Gal fusion protein (PER-SG, see text). For *per<sup>0</sup>; timeless-GAL4/UAS-timeless*, only 1  $\mu$ g of total protein was loaded to compensate for the increase in TIM protein abundance. Genotypes are as follows: lane 1, *per<sup>0</sup>; timeless-GAL4/+*; lane 2, *per<sup>0</sup>; timeless-GAL4/UAS-sgg*; lane 3, *per<sup>0</sup>; timeless-GAL4/UAS-timeless*; lane 4, *per<sup>0</sup>; per-SG*.

netic background (Konopka and Benzer, 1971). Although TIM is largely hypophosphorylated in *per<sup>0</sup>* flies, overexpression of *sgg* in *per<sup>0</sup>* converted much of this protein to a slowly migrating form of TIM (Figure 7B); instead of the single immunoreactive band that is readily seen in *per<sup>0</sup>* flies, two equally represented forms of TIM were observed in extracts from *sgg*-overproducing *per<sup>0</sup>* flies.

To test whether the slowly migrating TIM protein observed in flies overexpressing *sgg* is indeed caused by phosphorylation, protein extracts were subjected to treatment with alkaline phosphatase. As shown in Figure 7C, the slower migrating forms of TIM from wild-type at CT4 and from *per<sup>0</sup>* flies overexpressing *sgg* are equally sensitive to dephosphorylation. This demonstrates that the slowly migrating form of TIM is generated mostly or entirely through phosphorylation.

The similar electrophoretic migrations and phosphatase sensitivities of the low-mobility forms of TIM in wild-type and *sgg*-overexpressing flies suggested that these phosphorylated TIM proteins may be identical, and that overproduction of SGG accelerates production of a wild-type, hyperphosphorylated form of TIM. This conclusion was also supported by an examination of the differential light sensitivity of low- versus high-mobility forms of TIM in wild-type and *sgg*-overexpressing flies. Earlier studies of TIM accumulation in wild-type flies have shown that hyperphosphorylated forms of TIM are preferentially lost with the onset of light in LD cycles (Zeng et al., 1996; Rothenfluh et al., 2000a). As shown in Figure 7D, this acute light sensitivity of hyperphosphorylated TIM can be seen by comparing proteins isolated from wild-type flies collected at CT2 (2 hr after subjective dawn in constant darkness) to those from wild-type flies collected at ZT2 (2 hr after lights on in LD). The lower mobility form of TIM is abundant at CT2, but is absent in extracts from flies collected at ZT2. Figure 7D also demonstrates that when *sgg*-overexpressing *per<sup>0</sup>* flies are exposed to constant light, only the hypophosphorylated form of TIM is detected. Thus, the specific pattern of TIM phosphorylation produced by *sgg* overexpression fosters selective degradation of TIM in response to light, as in wild-type *Drosophila*.

In order to test whether a subtle increase in the abundance of TIM in *sgg*-overexpressing flies might be responsible for the change in phosphorylation pattern, *tim* (rather than *sgg*) was overexpressed in *per<sup>0</sup>* flies. As shown in Figure 7E (protein loading has been adjusted, see legend), most if not all of the TIM in those flies is hypophosphorylated, indicating that SGG activity directly influences the phosphorylation pattern of TIM.

In wild-type flies, phosphorylation of TIM can be detected around the middle of the subjective night whereas in *per<sup>0</sup>*, the lower mobility form is weakly detected at all times. This raised the possibility that phosphorylation of TIM is influenced by physical association with PER, and that overexpression of *sgg* might bypass this regulation. To test this, we investigated the phosphorylation pattern of TIM in flies that express the PER- $\beta$ Gal fusion protein PER-SG. PER-SG consists of the amino-terminal half of PER (amino acids 1 to 636) fused to  $\beta$ -Galactosidase (Liu et al., 1988; Vosshall et al., 1994; Stanewsky et al., 1997). Although these fusion proteins physically associate with TIM, they constitutively translocate TIM to the nucleus at an unknown rate, and fail to restore

molecular or behavioral rhythmicity in a *per<sup>0</sup>* background (Vosshall et al., 1994; Stanewsky et al., 1997). As shown in Figure 7E, TIM proteins in *per<sup>0</sup>*; *per-SG* flies appear to be mostly hyperphosphorylated. This result suggests that heterodimerization is not sufficient to induce hyperphosphorylation of TIM.

## Discussion

In this study, it was shown that the segment polarity gene *sgg* contributes to *Drosophila*'s circadian clock. Increased *sgg* function results in period shortening whereas decreased function causes substantial period lengthening of the molecular oscillator. Because we were unable to generate viable adult *Drosophila* that are devoid of SGG protein, we cannot address the consequences of a complete loss of *sgg* function on circadian rhythmicity. However, we note that even very low levels of a well-characterized clock gene, *period*, are sufficient to sustain rhythmicity (Baylies et al., 1987). Under such conditions of *per* depletion, long period rhythms like those associated with *sgg* depletion were observed. In fact, only the complete absence of *per* function has been clearly associated with arrhythmicity in *Drosophila*.

Two independent lines of evidence suggest that *sgg* regulates the period of molecular cycling primarily through effects on nuclear translocation of the PER/TIM heterodimer. First, the transition point between delays and advances of the phase response curve, an indicator for nuclear entry of PER/TIM complexes (Rothenfluh et al., 2000a), is advanced by 3 hr in flies overexpressing *sgg*. Second, nuclear PER was detected ~2 hr earlier in the lateral neurons of larvae overexpressing *sgg* than in wild-type LNs.

*sgg*-induced shifts in the timing of nuclear translocation are likely to reflect changes in TIM phosphorylation that are in turn connected to altered levels of PER and TIM. Because PER and TIM are overproduced when *sgg* activity is low, we suggest that *sgg*-dependent TIM phosphorylation accelerates PER/TIM heterodimerization or directly promotes nuclear translocation of PER/TIM complexes in wild-type flies. In this view, decreased TIM phosphorylation in *sgg* mutants would tend to retard nuclear transfer, and so require higher concentrations of the PER and TIM proteins at times of nuclear entry.

We have shown that TIM can be directly phosphorylated by GSK-3 $\beta$  in vitro. Such experiments suggest a mechanism involving direct interaction of SGG/GSK-3 and TIM in vivo, but do not exclude indirect regulation of TIM phosphorylation by this enzyme in the fly. Nor do our results rule out the involvement of additional protein kinases. For example, a tyrosine-linked phosphorylation of TIM has been implicated in the degradation of TIM by the proteasome (Naidoo et al., 1999). Because SGG would not be expected to promote tyrosine phosphorylation, this kinase should not regulate all aspects of TIM function.

SGG/GSK-3 is well known for its central role in WINGLESS/Wnt signaling. Surprisingly, recent work has indicated that the vertebrate ortholog of DOUBLE-TIME, casein kinase I $\epsilon$ , may also participate in this developmental pathway. For example, in *Xenopus*, inhibition of casein kinase I $\epsilon$  produced developmental abnormalities

closely corresponding to a loss of Wnt function. Casein kinase I $\epsilon$  was also found to stabilize  $\beta$ -catenin and to bind and phosphorylate DISHEVELLED, both established components of the Wnt signal transduction pathway (Peters et al., 1999; Sakanaka et al., 1999). It is remarkable that two kinases that function together to provide specific developmental regulation may both act as controlling elements in a patently unrelated behavioral process. This could reflect an underlying synergism between SGG/GSK-3 and casein kinase I $\epsilon$ . Certainly the activities of both kinases must be integrated at some level for coherent transduction of Wnt signals. Because DBT and SGG appear to produce opposing effects on PER/TIM nuclear transfer, with DBT retarding transfer (Kloss et al., 1998; Price et al., 1998) and SGG accelerating the process (this study), the relative activities of these kinases could establish an important focus for stabilizing the period of *Drosophila*'s circadian rhythms. For example, a control point composed of offsetting kinase activities might contribute to such homeostatic mechanisms as temperature compensation of the clock. In preliminary work, we have briefly explored the effects on circadian rhythmicity of two other elements of the *wg* signal transduction pathway. A temperature-sensitive allele of *wg* failed to show any effect on rhythmic locomotor activity, and a heat shock-*dishevelled*-rescued *dsh* mutant produced no circadian abnormalities (L. Saez, unpublished observation). Thus, *sgg*'s participation in the circadian oscillator may be unrelated to its function in *wg* signaling.

In conclusion, a novel genetic screen has uncovered a role for the segment polarity gene *sgg* in the organization of the *Drosophila* circadian oscillator. The strong evolutionary conservation of SGG/GSK-3 proteins will surely facilitate a search for related function in vertebrate behavioral rhythms.

## Experimental Procedures

### Immunohistochemistry and Western Blot Analysis

Third instar larval brains were prepared for immunocytochemistry as previously described (Kloss et al., 2001). For Western blotting, proteins were extracted from ~50  $\mu$ l heads (~200 heads) in 100  $\mu$ l head extraction buffer (100 mM KCl, 20 mM HEPES, 5% glycerol, 10 mM EDTA, 0.1% TritonX100, 1 mM DTT, [pH 7.5]) with a handheld homogenizer (Kontes). Samples were subsequently centrifuged at 15 K for 10 min at 4°C in an Eppendorf microcentrifuge. 10  $\mu$ g of total protein were loaded per lane on a 5% SDS polyacrylamide gel. Gels were run and blotted on nitrocellulose membrane (Schleicher & Schuell) using the Mini-Vertical Electrophoresis System from Bio-Rad. Membranes were blocked (PBS, 0.5% Tween20, 5% nonfat dry milk) at room temperature and subsequently incubated with the primary antibodies (1:2,000 in blocking solution for anti-TIMELESS) at 4°C overnight. Membranes were washed three times for 10 min in PBT (PBS, 0.5% Tween20), incubated with secondary antibodies (1:1,000 in PBT, Jackson ImmunoResearch), and washed again three times for 10 min. Immunocomplexes were detected using the ECL immunoassay signal reagent (Amersham Pharmacia Biotech).

### RNase Protection Assay

Total RNA was isolated from heads as described in the manufacturer's manual (TEL-TEST, INC.). The RNase protection assay was performed as described in the manufacturer's manual for the RPAII kit (Ambion). Ten micrograms of total RNA were used for each sample. The riboprobes for *period* (Sehgal et al., 1994), *timeless*, and *tubulin* (Sehgal et al., 1995) have been described previously.

### Kinase Assay

The fusion proteins were expressed in the bacterial strain BL21 and purified using Glutathione Sepharose®4B according to manufacturer's instructions (Pharmacia Biotech). After the last wash step, 10  $\mu$ l beads with the attached fusion proteins were incubated with 2 units of GSK-3 $\beta$  (NEB) and approximately 1  $\mu$ Ci ( $\gamma$ -<sup>32</sup>P) ATP (NEN) in phosphorylation buffer for 30 min at 30°C. Following the kinase reaction, the beads were washed to remove free radio-nucleotide and boiled in 2 $\times$  SDS gel loading buffer. The beads were precipitated by centrifugation and the samples were subjected to SDS PAGE. After the electrophoresis, the proteins were either transferred to a nitrocellulose membrane (Protran®, Schleicher and Schuell) for autoradiography or stained with coomassie blue.

### Phosphorylation Site Prediction

The TIMELESS sequence was submitted to the on-line protein substrate motif searching program Motif Scanner at [http://cansite.bidmc.harvard.edu/motifscanner/motifscan1.phtml?database=\\_SWS\\_#](http://cansite.bidmc.harvard.edu/motifscanner/motifscan1.phtml?database=_SWS_#). The analysis was conducted at medium stringency.

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