A Key Temporal Delay in the Circadian Cycle of *Drosophila* Is Mediated by a Nuclear Localization Signal in the Timeless Protein

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ABSTRACT Regulated nuclear entry of the Period (PER) and Timeless (TIM) proteins, two components of the *Drosophila* circadian clock, is essential for the generation and maintenance of circadian behavior. PER and TIM shift from the cytoplasm to the nucleus daily, and the length of time that PER and TIM reside in the cytoplasm is an important determinant of the period length of the circadian rhythm. Here we identify a TIM nuclear localization signal (NLS) that is required for appropriately timed nuclear accumulation of both TIM and PER. Transgenic flies with a mutated TIM NLS produced circadian rhythms with a period of ~30 hr. In pacemaker cells of the brain, PER and TIM proteins rise to abnormally high levels in the cytoplasm of $tim^{\Delta NLS}$ mutants, but show substantially reduced nuclear accumulation. In cultured S2 cells, the mutant $TIM^{\Delta NLS}$ protein significantly delays nuclear accumulation of both TIM and wild-type PER proteins. These studies confirm that TIM is required for the nuclear localization of PER and point to a key role for the TIM NLS in the regulated nuclear accumulation of both proteins.

DROSOPHILA circadian rhythms are generated and maintained by two interlocked negative and positive feedback loops (reviewed in Allada and Chung 2010). In the primary loop, two transcription factors, Clock (CLK) and Cycle (CYC), activate the transcription of *period* (*per*) and *timeless* (*tim*) in the nucleus. After a period of PER and TIM accumulation and physical association in the cytoplasm, the latter proteins enter the nucleus, repressing their own transcription by inactivating CLK. In the secondary feedback loop, which affects the phase and amplitude of the core oscillator, CLK/CYC promotes the transcription of *Par domain protein 1* (*Pdp1*) and *vrille* (*vri*). In turn, the VRI and PDP1 proteins sequentially repress (in the case of VRI) and then activate (in the case of PDP1) the transcription of *clock*.

In the *Drosophila* brain, there is a network of ~150 neurons that drives circadian behavior (Shafer *et al.* 2006). Anatomically, these clock neurons can be divided into seven different groups (Nitabach and Taghert 2008). The dorsal lateral neurons (LNd), three groups of dorsal neurons (DN1-3), the lateral posterior neurons (LNP), the small ventral lateral neurons (sLNv's), and large ventral lateral neurons (lLNv). The LNv's are the only neurons expressing the neuropeptide PDF, a principle transmitter coordinating circadian rhythms in the fly brain. The sLNv's maintain circadian time in constant darkness and anticipate lights-on in light–dark cycles (Helfrich-Forster 1998; Park *et al.* 2000; Stoleru *et al.* 2005).

Temporal delays between activation and repression are built into the circadian loops that allow the generation of RNA and protein-level oscillations with a 24-hr periodicity. Post-translational modifications are necessary to introduce these temporal delays into the circadian clock. Among the many known modifications, protein phosphorylation and dephosphorylation have been shown to play a critical role in circadian rhythmicity in many organisms (Harms *et al.* 2003; Bae and Edery 2006; Fang *et al.* 2007). For example, PER is phosphorylated by Double-time (DBT, Casein Kinase 1), which increases PER degradation and its activity as a repressor (Kim *et al.* 2007; Kivimae *et al.* 2008), and Casein Kinase 2 (CK2), which appears to promote PER nuclear accumulation (Allada and Meissner 2005). Additionally, *Drosophila* TIM is phosphorylated in a pathway that requires Shaggy/

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GSK3 kinase, and this appears to advance the onset of nuclear accumulation of both PER and TIM (Martinek *et al.* 2001). CLK is phosphorylated by an unknown kinase with the cooperation of PER and DBT (Yu *et al.* 2009).

A key temporal delay in the circadian cycle of *Drosophila* is the timed daily transport of PER and TIM to the nucleus. PER and TIM proteins are retained in the cytoplasm for several hours following their synthesis, and nuclear translocation is highly dependent on the presence of both cytoplasmic PER and TIM (Vosshall and Young 1995; Myers *et al.* 1996; Saez and Young 1996). In a single-cell-based assay involving cultured S2 *Drosophila* cells, we have shown that although PER and TIM expressed in the same cell rapidly associate, they persist in the cytoplasm for ~5.5 hr (Meyer *et al.* 2006; Saez *et al.* 2007). Subsequently, and in a narrow time frame, PER and TIM appear to dissociate and enter the nucleus (Shafer *et al.* 2002; Meyer *et al.* 2006; Saez *et al.* 2007). The relevance of this behavior in S2 cells was supported by parallel studies of the mutation *per-long* (per^L). The PER^L protein differs from wild-type PER by a single amino acid change that has been shown to delay nuclear translocation of PER and TIM in pacemaker cells of adult brains. The per^L mutation causes a long-period (28 hr) circadian behavioral rhythm. per^L was found to similarly delay the nuclear accumulation of PER and TIM in S2 cells without detectably altering the rate of physical association of these proteins. Thus, regulated nuclear entry of PER and TIM seems to play a central role in setting the period length of the *Drosophila* circadian clock. Nevertheless, the interdependence of PER and TIM in regulating this process has been questioned in some studies (*cf.* Shafer *et al.* 2002; Nawathean and Rosbash 2004).

The mechanism by which nuclear accumulation of PER and TIM is triggered is unknown. Macromolecules that move into and out of the nucleus are transported through the nuclear pore complex, and a well-characterized nuclear import process occurs through receptor-based recognition of nuclear localization signals (NLS) on protein cargoes marked for nuclear import (Boulikas 1993). Nuclear import is mediated by specialized import proteins, such as importin β or heterodimers of importin α/β. For example, in importin α/β assemblies, importin α recognizes and binds the NLS in the cargo protein and importin β translocates the trimeric complex through the nuclear pore (for a review see Stewart 2007).

Sequence analysis of PER indicated several stretches of basic amino acids that might provide NLS function, and these segments of PER have been shown to possess some NLS activity *in vivo* and *in vitro* (Vosshall *et al.* 1994; Saez and Young 1996; Chang and Reppert 2003). However, the NLS involved in the temporally regulated nuclear entry of the PER and TIM remains elusive. Unlike PER, the TIM protein contains a single stretch of basic amino acids that matches the bipartite structure of nuclear localization signals found in most well-studied systems (Myers *et al.* 1995). This putative TIM NLS spans 14 amino acids (Figure 1) and is required for the nuclear entry of TIM and TIM^{cld} in S2 cells (Saez and Young 1996).

To ascertain the role of the putative TIM NLS, we mutagenized the sequence and tagged the TIM protein with yellow fluorescent protein (YFP) to facilitate the purification and visualization of the modified protein. We tested the ability of $TIM^{\Delta NLS}$ to enter the nucleus in S2 cells and *in vivo*. In S2 cells, $TIM^{\Delta NLS}$ delayed the nuclear entry of PER and TIM. *In vivo,* tim^o; $tim^{\Delta NLS}$*-yfp* flies suppressed nuclear accumulation of PER and TIM and lengthened the period of the circadian locomotor rhythm. These results confirm the interdependence of PER and TIM in the control of their nuclear entry. We conclude that the NLS of TIM plays a pivotal role in both the timing and the efficiency of PER/TIM nuclear accumulation.

Materials and Methods

Plasmids

The *hs-per, hs-per-cfp, hs-tim*, and *hs-tim-yfp* plasmid were previously described (Saez and Young 1996; Meyer *et al.* 2006). The *hs-tim*$^{\Delta NLS}$*-yfp* construct was generated by site-directed mutagenesis using a QuickChange site-directed mutagenesis kit (Stratagene) and verified by DNA sequencing.

Transgenic flies

To generate transgenic flies, we used the Casper4 transformation vector containing the 4.3-kb sequence upstream of the transcription initiation site of the *Drosophila melanogaster tim* gene (Ousley *et al.* 1998) fused to *tim-yfp* and *tim*$^{\Delta NLS}$*-yfp* cDNA. Transgenic flies were generated by BestGene (Chino Hills, CA) using w^{1118} embryos as hosts. Independent germline transformants containing the *tim*$^{\Delta NLS}$*-yfp* construct were obtained and crossed to *yw,* tim^o flies to yield *yw,* tim^o*;* *tim*$^{\Delta NLS}$*-yfp*. The wild-type control transgenic flies *yw,* tim^o*;* *tim-yfp* were generated by similar crosses.

Behavioral analysis

Individual flies were monitored and their locomotor activity was analyzed with the *Drosophila* Activity Monitoring System IV (TriKinetics). The flies were raised at 25° until the pupal stage and were then entrained to a light:dark cycle (LD) until eclosion. For LD experiments and for experiments in constant darkness (DD), the flies were entrained for 3 days in LD and monitored at 25° in subsequent days of LD and/or DD as specified. Locomotor activity was analyzed during free-run (DD), and period length was calculated using ClockLab Software (ActiMetrics).

Western blot analysis

To prepare head extracts, 100 μl of adult heads per time point were homogenized in 100 μl of head extraction buffer [100 mM KCl, 20 mM HEPES (pH 7.5), 10% glycerol, 10 mM EDTA (pH 8), 0.1% Triton X-10, 50 mM NaF, 1 mM DTT] with 1× protease and phosphatase inhibitors (Roche) using a handheld homogenizer (Kontes). Samples were centrifuged at 14,000 × *g* for 15 min at 4°. The supernatant was transferred to a new tube and centrifuged as above for an

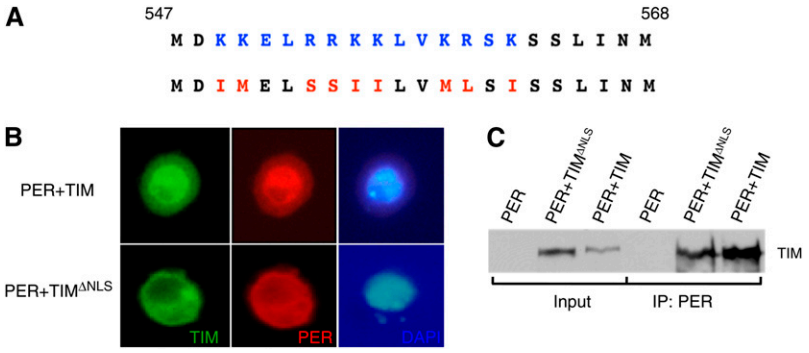

Figure 1 Mutations in the NLS of *timeless* impair nuclear entry of PER and TIM in S2 cells. (A) Amino acid sequence of the NLS of TIM (blue) depicting the mutated amino acids in red. (B) S2 cells were transiently cotransfected with hs-*per*/hs-*tim* or hs-*per*/hs-*tim*$^{\Delta NLS}$ and subjected to heat shock 48 hr post transfection. Cells were fixed 8 hr after heat induction as described (see Materials and Methods) and processed for immunohistochemistry. TIM protein was visualized with anti-TIM antibody, and the complex was detected with FITC-conjugated anti-rat IgG. PER was detected using anti-PER antibody label with rhodamine-conjugated anti-rabbit IgG. (C) S2 cells were transiently cotransfected with hs-*per-myc* and with hs-*tim* or hs-*tim*$^{\Delta NLS}$ (indicated at the top of C). After 48 hr of transfection, cells were heat-shocked, and after 6 hr of induction, the cells were harvested. Samples were immunoprecipitated with anti-myc antibody and the immune complex was visualized with anti-TIM antibody. Input represents 2% of the total sample.

additional 10 min. A total of 10–30 μg of total protein was loaded on a NuPAGE Novex 3–8% Tris–acetate gel (Invitrogen) and run following the manufacturer's instructions. Samples in these gels were transferred to a nitrocellulose membrane (Schleicher & Schuell). Membranes were blocked for at least 1 hr at room temperature with 5% nonfat dry milk in 1× TBST. Primary antibodies were diluted in blocking solution [1:10,000 for α-PER (rabbit); 1:2,000 for α-TIM (rat)] and incubated with the membranes at 4° overnight. The membranes were washed four times for 10 min each in 1× TBST and incubated with secondary antibodies (1:10,000) (Jackson ImmunoResearch) for 1 hr at room temperature. The membranes were washed as before, and detection was carried out using ECL (Amersham Pharmacia Biotech).

Northern blot analysis

Total RNA was extracted from ~100 μl of adult heads per time point using RNA-STAT60 (Tel-Test). Ten micrograms of total RNA was denatured for 5 min at 65° and resolved on a 1% formaldehyde–agarose gel [20 mM MOPS (pH 7), 5 mM NaOAc, 1 mM EDTA]. The resolved RNA was transferred to Nytran membrane (Schleicher & Schuell) in 10× SSC overnight. Probe templates were radiolabeled as specified for the DECAprime II kit (Ambion). Hybridizations were carried out at 50° in UltraHyb solution (Ambion) supplemented with denatured fish sperm DNA. Radioactive signals on the blots were visualized and quantitated with a Typhoon Phosphorimager (Molecular Dynamics), and the results were plotted in Microsoft Excel.

S2 cell culture

S2 cells (Invitrogen) were grown in Schneider *Drosophila* media (Gibco) with 15% serum, and transient transfection was performed using efectene (Qiagen) according to the manufacturer's instructions. Briefly, for PER and TIM co-expression, equal amounts of *hs-per-cfp* and *hs-tim*$^{\Delta NLS}$*-yfp* DNA were cotransfected overnight, the media was replaced the next day, and the cells were allowed to recover for another 24 hr. Induction of *per-cfp* and *tim*$^{\Delta NLS}$*-yfp* expression was initiated by heat-shocking the cells for 30 min at 37°, and *per-cfp* and *tim*$^{\Delta NLS}$*-yfp* expressing cells were set up for imaging as previously described (Meyer *et al.* 2006) or collected for analysis after 8 hr.

Confocal imaging

Larval and adult fly brains were dissected in cold PBS, and a minimum of 10 brains were collected for each time point. For each time point, wild-type and *tim*$^{\Delta NLS}$ flies were entrained; brains were collected and processed simultaneously for comparison. Larval brains were fixed in cold 4% paraformaldehyde for 30 min in a nutator. Adult fly heads were fixed in cold 4% paraformaldehyde for at least 3 hr (in the dark when required) and dissected in PBS and 0.5% Triton. Brains were washed several times with PBS, with PBS containing 1% Triton for 20 min and incubated with blocking solution (PBS, 0.5% Triton, 5% goat serum) for at least 2 hr. Primary antibody was added overnight in blocking solution. The final dilutions of the antibodies used were the following: anti-PDF antibody 1:500 (C7-DSHB), anti-TIM antibody 1:2000 (Myers *et al.* 1996), and anti-PER antibody 1:1000 (from J. Hall, Brandeis University, Walthman, MA). Brains were washed several times with PBS–0.5% triton and incubated with the secondary antibody in blocking solutions for 3 hr in the dark. After extensive washing, the brains were mounted onto slides using Fluoromount-G (SouthernBiotech) and analyzed in a LSM 510 laser scanning confocal microscope (Zeiss) at The Rockefeller University Bio-Imaging resource Center. The confocal images obtained were processed with ImageJ program (National Institutes of Health) and Photoshop (Adobe System).

Results

Mutation of a timeless *NLS impairs nuclear entry of PER and TIM in S2 cells*

To learn more about the potential role that the NLS of *tim* might have in the nuclear entry of the PER-TIM complex, we mutagenized sequences encoding the *tim* NLS, such that all the basic amino acids in the bipartite NLS region would be changed to methionine, isoleucine, or serine (see Figure 1A). We then asked if the expression of *tim*$^{\Delta NLS}$ in S2 cells

Table 1 Locomotor activity rhythms of *tim* mutants and control flies

Phenotype	τ	SEM	Power	Total flies[a]	Rhythmicity (%)
yw	23.5	0.113	80.0 ± 0.7	10	100
tim^o	ar			10	0
+; +; *tim-yfp*	23.6	0.150	101.5 ± 0.5	8	100
+; +; $tim^{\Delta NLS}$-*yfp*	24.7	0.250	110.0 ± 10	24	80
yw; tim^o; *tim-yfp*	23.0	0.317	74.5 ± 15.3	10	90
tim-yfp; tim^o	23.4	0.327	81.7 ± 1.9	10	90
yw; tim^o; $tim^{\Delta NLS}$-*yfp*	30.3	0.257	90.0 ± 0.9	30	70
$tim^{\Delta NLS}$-*yfp*; tim^o	31.8	0.375	82.7 ± 1.5	10	90
$tim^{\Delta NLS}$-*yfp*; tim^o; $tim^{\Delta NLS}$-*yfp*	31.0	0.243	75.3 ± 10.5	10	100

Flies were entrained at 25° for 4 days in LD cycle, followed by 7 days in DD.
[a] Total numbers of flies that survived until the end of the experiment.

impaired the nuclear accumulation of PER and TIM. We had shown previously that when wild-type PER and TIM are co-expressed in S2 cells, both proteins remain in the cytoplasm for 5–6 hr, followed by their rapid transfer to the nucleus (Saez and Young 1996; Meyer *et al.* 2006). In this study, we found that co-expression of PER and $TIM^{\Delta NLS}$ in S2 cells prolonged the cytoplasmic retention of both proteins so that nuclear accumulation was still not observed 8 hr after their production (Figure 1B). Since the NLS of *tim* interacts with the PAS domain of PER (Saez and Young 1996), we asked whether the impaired nuclear entry of PER and $TIM^{\Delta NLS}$ was due to the inability of PER and $TIM^{\Delta NLS}$ to physically associate in S2 cells. We performed immunoprecipitation studies using S2 cells that co-expressed either PER-myc and TIM or PER-myc and $TIM^{\Delta NLS}$. Protein complexes were isolated with anti-myc antibodies and subsequently tested for the presence of TIM (Figure 1C). TIM was detected in both PER/TIM and PER/$TIM^{\Delta NLS}$ immunoprecipitates, but not in control cells expressing PER alone (Figure 1C). These data indicate that $TIM^{\Delta NLS}$ can still bind to PER and that PER and $TIM^{\Delta NLS}$ accumulate as PER/TIM complexes in the cytoplasm of S2 cells. These conclusions were further corroborated by single-cell assays that tracked physical associations and movements of fluorescent PER and TIM proteins in living S2 cells (see below).

$tim^{\Delta NLS}$ flies produce long-period behavioral rhythms

To ascertain the role of $tim^{\Delta NLS}$ in the regulation of nuclear entry of PER and TIM *in vivo*, we generated transgenic flies that expressed either TIM or $TIM^{\Delta NLS}$ under the control of the wild-type *tim* promoter. Both transgenes included a sequence encoding the YFP fused to the carboxyl end of the *timeless* coding region. Several independent *tim* and $tim^{\Delta NLS}$ lines were obtained and tested as homozygotes in a wild-type and tim^o (null) background (Table 1). Under conditions of constant darkness (DD), wild-type locomotor activity was restored in tim^0; *tim-yfp* flies, indicating that fusion with YFP does not substantially affect the activity of TIM. However, the locomotor activities of all tim^0; $tim^{\Delta NLS}$-*yfp* flies were aberrant. Most of these flies produced long-period (~30.5 hr) rhythms while the remainder (~30%) were arrhythmic (Table 1; see also supporting information, Figure S1). We also tested the locomotor activity of flies carrying four, rather than two, $tim^{\Delta NLS}$-*yfp* transgenes ($tim^{\Delta NLS}$-*yfp*; tim^0; $tim^{\Delta NLS}$-*yfp*; Table 1). These flies produced ~31-hr rhythms. In a wild-type background, $tim^{\Delta NLS}$ behaves as a dominant negative allele, increasing the period length of locomotor activity rhythms by ~1 hr (Table 1). Thus, the long-period locomotor activity of $tim^{\Delta NLS}$ flies appears to reflect a qualitative change in TIM function.

Period length is temperature compensated in $tim^{\Delta NLS}$ flies

An important general feature of circadian rhythms is their temperature compensation: The period of the circadian rhythm remains relatively constant when measured at different physiological temperatures (Pittendrigh and Cosbey 1974). Nevertheless, several clock mutants have previously been shown to alter temperature compensation in *Drosophila* and in Neurospora (Gardner and Feldman 1981; Price 1997). To determine whether the $tim^{\Delta NLS}$ mutation affects temperature compensation, circadian rhythms of wild type, per^L, and tim^o; $tim^{\Delta NLS}$-*yfp* mutants were compared at several temperatures (Figure 2). We tested per^L mutants in this study because their periods are not temperature compensated and lengthen with higher temperatures (Rutila *et al.* 1996; Bao *et al.* 2001). Neither wild-type flies nor tim^o; $tim^{\Delta NLS}$-*yfp* mutants showed significant changes in period at the different temperatures tested. Thus, tim^o; $tim^{\Delta NLS}$-*yfp* flies retain their ability to be temperature-compensated.

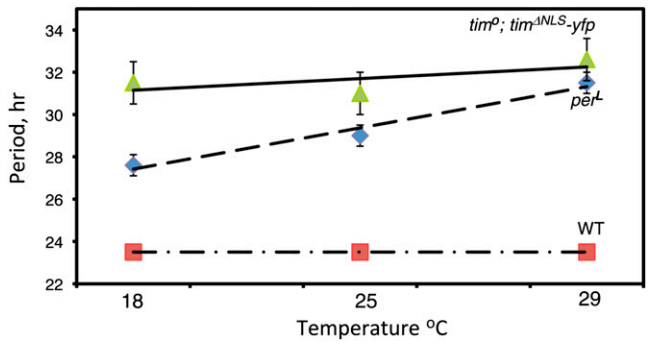

Figure 2 The $tim^{\Delta NLS}$ mutation retains its temperature compensation. Flies were entrained for 4 days at 25° and tested for locomotor activity at the temperatures indicated. Periods, in hours, were calculated for *yw*, per^L and tim^0; $tim^{\Delta NLS}$-*yfp*.

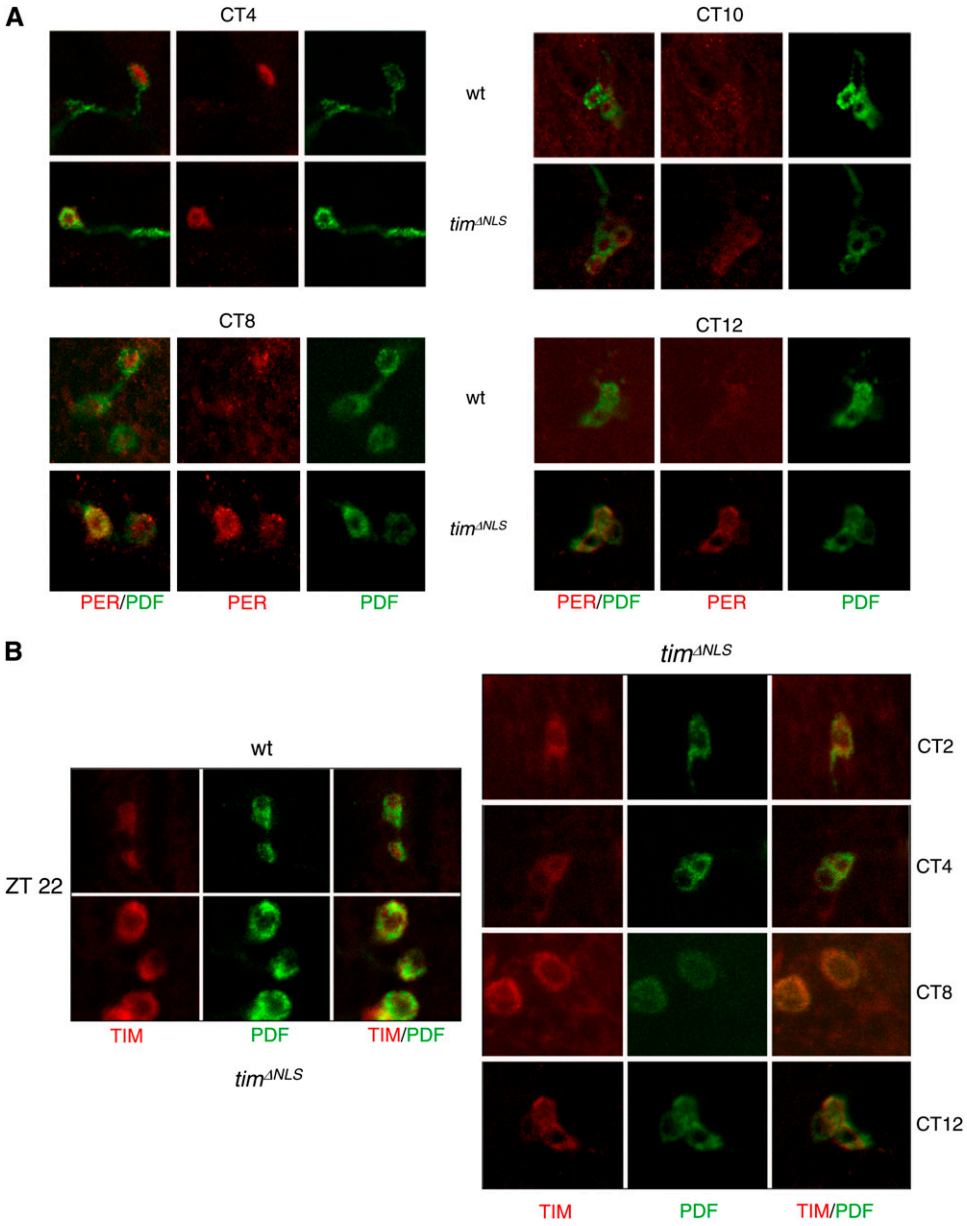

Figure 3 Subcellular localization of PER and TIM proteins in the LNs of larval brains. (A) Wild-type and tim^{o}; $tim^{\Delta NLS}$-*yfp* (indicated $tim^{\Delta NLS}$) larval brains were collected at CT4, CT8, CT10, and CT12 on the first day in DD and stained with antibodies to PER (red) and PDF (green). (B) Wild-type and tim^{o}; $tim^{\Delta NLS}$-*yfp* larval brains stained with antibodies to TIM (red) and PDF (green). Brains were collected at ZT22, CT2, CT4, CT8, and CT12. Three experiments were performed for each time point and representative images are shown.

Cytoplasmic accumulation of PER and TIM is prolonged in $\text{tim}^{\Delta NLS}$ *larvae and flies*

To determine the effect of $tim^{\Delta NLS}$ on the nuclear entry of PER and TIM *in vivo*, we followed subcellular movements of the TIM and PER proteins in pacemaker cells of the brains of wild-type and mutant larvae and in adult flies. Initially, we looked for YFP fluorescence emanating from the TIM–YFP fusion. However, we could not resolve this emission in our tissue samples, although we could detect the presence of YFP by Western blots (data not shown). We therefore decided to use antibodies to resolve the TIM and PER proteins and to localize the PDF neuropeptide, which labels the lateral neurons (LNs). Following entrainment to 12-hr:12-hr light:dark cycles (LD12:12), we collected larval brains and adult heads at several time points in subsequent constant darkness.

For wild-type larvae collected in DD, PER was localized to the nucleus at CT4 and CT8. Low levels of cytoplasmic accumulation were observed at CT10 and CT12 (Figure 3A). A very different pattern of PER accumulation was seen for tim^{o}; $tim^{\Delta NLS}$-*yfp* larvae: PER was predominantly cytoplasmic at CT4 and CT8, when PER is nuclear in wild-type larvae, and cytoplasmic levels of the protein remained high through CT12 (Figure 3A).

In wild-type larval brains collected in LD at ZT22, TIM was enriched in the nucleus. In contrast, TIM was predominantly cytoplasmic at ZT22 in tim^{o}; $tim^{\Delta NLS}$-*yfp* larvae (Figure 3B). Subsequently, in constant darkness, TIM continued to accumulate in the cytoplasm from CT2 to CT12 in tim^{o}; $tim^{\Delta NLS}$-*yfp* animals (Figure 3B). Thus, both PER and TIM showed a predominantly cytoplasmic localization

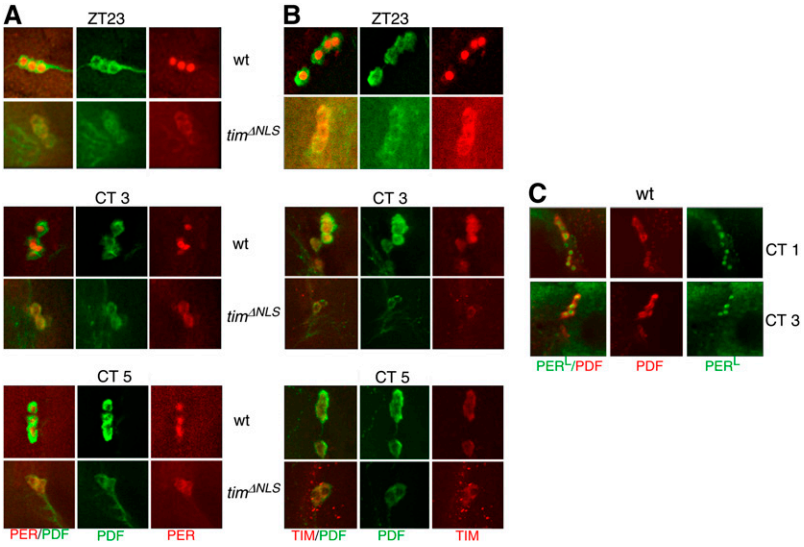

Figure 4 Cytoplasmic accumulation of PER and TIM in the sLNV's of *wt, tim^{o}; tim$^{\Delta NLS}$-yfp* (indicated *tim$^{\Delta NLS}$*) and *per^{L}* adult brains. Immunohistochemistry of whole-mount brains forming a time series of wild-type and *tim$^{\Delta NLS}$* tissues. sLNV's are shown after staining for PER (A, red), TIM (B, red), and PDF (green). (C) *per^{L}* brains collected at CT1 and CT3 stained for PER (green) and PDF (red). Each experiment was performed three times with identical results.

throughout the circadian cycle in the pacemaker neurons of *tim^{o}; tim$^{\Delta NLS}$-yfp* larvae.

In wild-type adults, PER cytoplasmic accumulation, nuclear entry, and degradation in the sLNv's were similar in LD and DD. For example, PER was cytoplasmic at ZT or CT16, and nuclear and cytoplasmic at ZT or CT22 (data not shown). By ZT23, PER appeared to be exclusively nuclear in wild-type sLNv's, and the protein remained nuclear until it disappeared from these cells after CT5 (Figure 4A). A similar pattern of subcellular localization was observed for TIM in wild-type adults (Figure 4B). PER and TIM accumulation followed a different pattern in *tim^{o}; tim$^{\Delta NLS}$-yfp* adult brains. Both proteins remained predominantly cytoplasmic at ZT23, CT3, and CT5 and for the remainder of the subjective day (Figure 4, A and B; data not shown). From these studies we conclude that in *tim^{o}; tim$^{\Delta NLS}$-yfp* flies, most TIM and PER remains in the cytoplasm of the sLNv's for the duration of the circadian cycle. This pattern of PER and TIM subcellular localization also differs from that observed in another long period mutant, *per^{L}* (Konopka and Benzer 1971; Curtin *et al.* 1995). In *per^{L}* flies, which have a 29-hr rhythm, PER was predominantly nuclear at CT1 and CT3 (Figure 4C). Possibly, the ~31-hr circadian rhythms observed in the *tim^{o}; tim$^{\Delta NLS}$-yfp* flies reflect the movement of much lower levels of PER and TIM to the nucleus.

High levels of PER and TIM are found throughout the circadian cycle in tim$^{\Delta NLS}$ *flies*

Near the end of each circadian cycle, PER and TIM enter the nucleus and repress their own transcription. If levels of nuclear PER and TIM in *tim^{o}; tim$^{\Delta NLS}$-yfp* flies are significantly lower than those of wild-type flies, we would expect reduced repression, correspondingly higher levels of total cellular PER and TIM, and a weak molecular oscillation.

To determine whether the impaired nuclear entry of PER and TIM in *tim^{o}; tim$^{\Delta NLS}$-yfp* flies affects the levels of these clock proteins, we assessed the abundance of PER and TIM proteins in wild-type and *tim^{o}; tim$^{\Delta NLS}$-yfp* flies by Western blot analysis. We entrained flies to LD cycles and collected heads during the first day in LD and the two subsequent days in constant darkness. Figure 5, A and B, shows the pattern of TIM and PER expression that was observed in LD. Wild-type flies showed a strong oscillation of TIM and PER over the course of the analysis. In contrast, *tim^{0}*; *tim$^{\Delta NLS}$-yfp* flies produced substantially higher levels of TIM and PER protein at most time points. A delay in PER disappearance after lights-on was also observed in the *tim^{0}*; *tim$^{\Delta NLS}$-yfp* flies, with PER at ZT2 and ZT6 showing persistence of a hyper-phoshorylated form of this protein (Figure 5B). In constant conditions, TIM in wild-type flies showed a strong oscillation in the first and second day in DD by maintaining its 23.5-hr rhythm (Figure 5C). A higher level of TIM protein at all DD time points, a low amplitude, and a long-period molecular cycle that matched the period of the behavioral rhythm were observed in *tim^{0}*; *tim$^{\Delta NLS}$-yfp* flies (Figure 5, C and D). As shown in Figure S2, a similarly elevated pattern of PER accumulation was found in *tim^{0}*; *tim$^{\Delta NLS}$-yfp* flies in DD.

These patterns of altered TIM and PER accumulation suggested that suppressed nuclear accumulation might lead to weakened repression in *tim$^{\Delta NLS}$* mutants. To study this further, we measured the levels of *tim* RNA in heads from wild-type and *tim^{o}; tim$^{\Delta NLS}$-yfp* flies (Figure 6). In LD, the overall levels of *tim* RNA in both genotypes were similar, with peak RNA levels found between ZT10 and ZT16 in wild type and at ZT22 in *tim^{o}; tim$^{\Delta NLS}$-yfp* mutants. The *tim* RNA levels measured in constant conditions also showed similar overall levels in wild-type and *tim^{o}; tim$^{\Delta NLS}$-yfp* flies, with the latter showing a clear phase delay in the timing of peak *tim* RNA accumulation (Figure 6). These results indicate that the increased levels of PER and TIM in *tim$^{\Delta NLS}$* flies are not simply the result of reduced transcriptional feedback. Instead, there appears to be increased stability of both proteins in the mutant, as well as weakened transcriptional repression. That is, higher levels of PER and TIM$^{\Delta NLS}$ in the

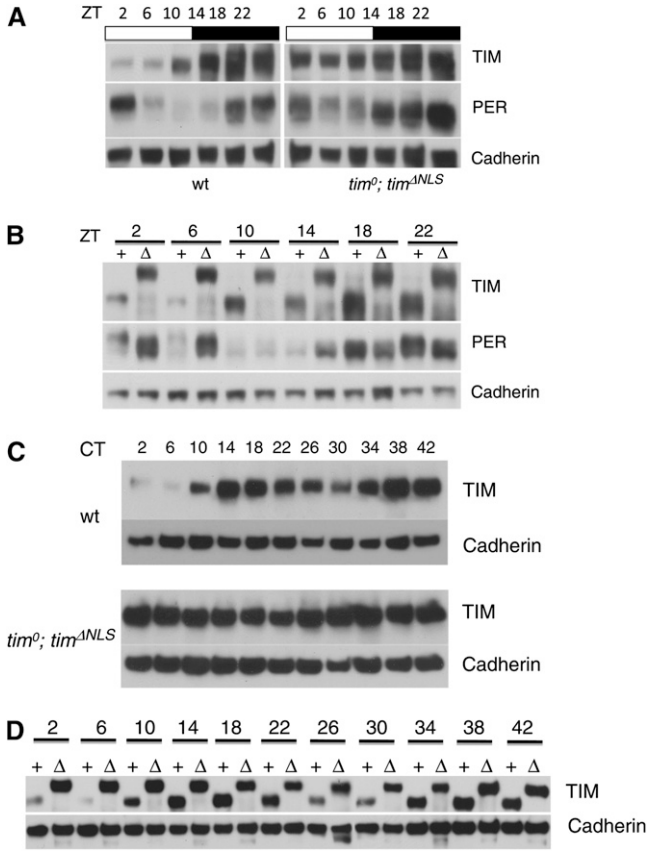

Figure 5 *tim*^{0}; *tim*$^{\Delta NLS}$*-yfp* flies show increased levels of PER and TIM proteins. Adult *yw* (wt or +) and *tim*^{0}; *tim*$^{\Delta NLS}$*-yfp* flies were collected at the indicated circadian times during an LD cycle or in DD after 5 days of entrainment. Head extracts were prepared and analyzed by direct immunobloting. (A) Expression of PER and TIM protein in wild-type and *tim*^{0}; *tim*$^{\Delta NLS}$*-yfp* mutants in LD. (B) Side-by-side comparison of the levels of PER and TIM expression in LD. Note the slower mobility of TIM$^{\Delta NLS}$ (Δ) due to the YFP tag. (C) Timeless expression in wild-type and *tim*^{0}; *tim*$^{\Delta NLS}$*-yfp* flies collected for the first and second day in DD. (D) Side-by-side comparison of the levels of TIM in heads from wild-type and *tim*^{0}; *tim*$^{\Delta NLS}$*-yfp* (Δ) mutant flies. Cadherin levels are shown as a loading control.

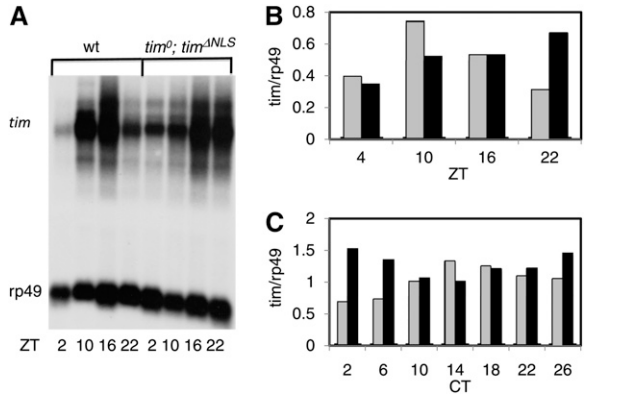

Figure 6 *tim* RNA levels are similar in wild-type and *tim*^{0}; *tim*$^{\Delta NLS}$*-yfp* flies. Adult *yw* and *tim*^{0}; *tim*$^{\Delta NLS}$*-yfp* flies were collected at the indicated circadian times during an LD cycle (A and B) or in DD after 5 days of entrainment (C). A Northern blot of RNA from adult heads (A) was probed for *tim* and rp49 (loading control). Data in A are quantitated in B. Quantitation of a parallel experiment using flies collected in DD is shown in C. Shaded bars represent wild-type (*yw*) RNA levels, and solid bars represent the *tim*^{0}; *tim*$^{\Delta NLS}$*-yfp* RNA levels.

mutant are associated with levels of repression that are typically seen in response to much lower levels of total accumulated PER and TIM in wild-type flies. These observations, coupled with our finding that *tim* RNA accumulation is delayed (peaks at ZT22) in the mutant (Figure 5), also suggest a basis for the persistence of TIM$^{\Delta NLS}$ in the light phase of the circadian cycle: In contrast to wild type, a more stable TIM$^{\Delta NLS}$ protein is synthesized from a delayed pool of RNA just prior to lights on in *tim*^{o}*; tim*$^{\Delta NLS}$*-yfp* flies.

Imaging studies in living S2 cells indicate that TIM$^{\Delta NLS}$ significantly delays the onset of PER and TIM nuclear entry

In our initial *in vitro* studies, we observed only cytoplasmic accumulation of PER and TIM$^{\Delta NLS}$ 8 hr after their induction in S2 cells. We also failed to detect clear nuclear accumulation in transgenic flies expressing *tim*$^{\Delta NLS}$*-yfp*. Nevertheless, most *tim*$^{\Delta NLS}$*-yfp* flies produce long-period circadian rhythms, suggesting that some PER and TIM is transported to the nucleus during each circadian cycle. This prompted us to follow the effects of *tim*$^{\Delta NLS}$ in a time-course study in living cultured cells. We used a previously described, single-cell, fluorescence imaging assay in *Drosophila* S2 cells (Meyer *et al.* 2006). C-terminal fusions of PER and TIM$^{\Delta NLS}$ were constructed with cyan fluorescent protein (CFP) and YFP, respectively, and the subcellular locations of these proteins were followed by time-lapse imaging of their fluorescent tags. Additionally, fluorescence resonance energy transfer (FRET) was used to detect the presence and timing of the physical associations of PER and TIM (Figure 7, A–C).

As previously observed with wild-type PER and TIM, we detected high levels of FRET in S2 cells expressing PER and TIM$^{\Delta NLS}$ shortly after their induction, and FRET persisted for several hours (Figure 7D). This sustained signal was found to correspond to residence in the cytoplasm. Figure 7E also shows that PER and TIM$^{\Delta NLS}$ nuclear accumulation was observed in these S2 cells, but with a substantial delay compared to that seen for the wild-type proteins in Figure 7B. For several cells tested in this manner, the mean onset of nuclear accumulation for PER and TIM$^{\Delta NLS}$ was ~9.5 hr [compare to a mean onset of ~5.5 hr for wild-type PER and TIM in S2 cells (Meyer *et al.* 2006; Saez *et al.* 2007)]. The timing of nuclear accumulation was also independent of the absolute levels of PER-CFP and TIM$^{\Delta NLS}$-YFP expression in S2 cells (Figure 7F). Our S2 cell studies point to a delay in nuclear translocation of PER and TIM$^{\Delta NLS}$ that may underlie the longer period observed in *tim*$^{\Delta NLS}$ mutant flies.

Discussion

In this study we identified a functional nuclear localization signal in the TIM protein. We also demonstrated a role for this NLS in specifying the timing of nuclear accumulation for

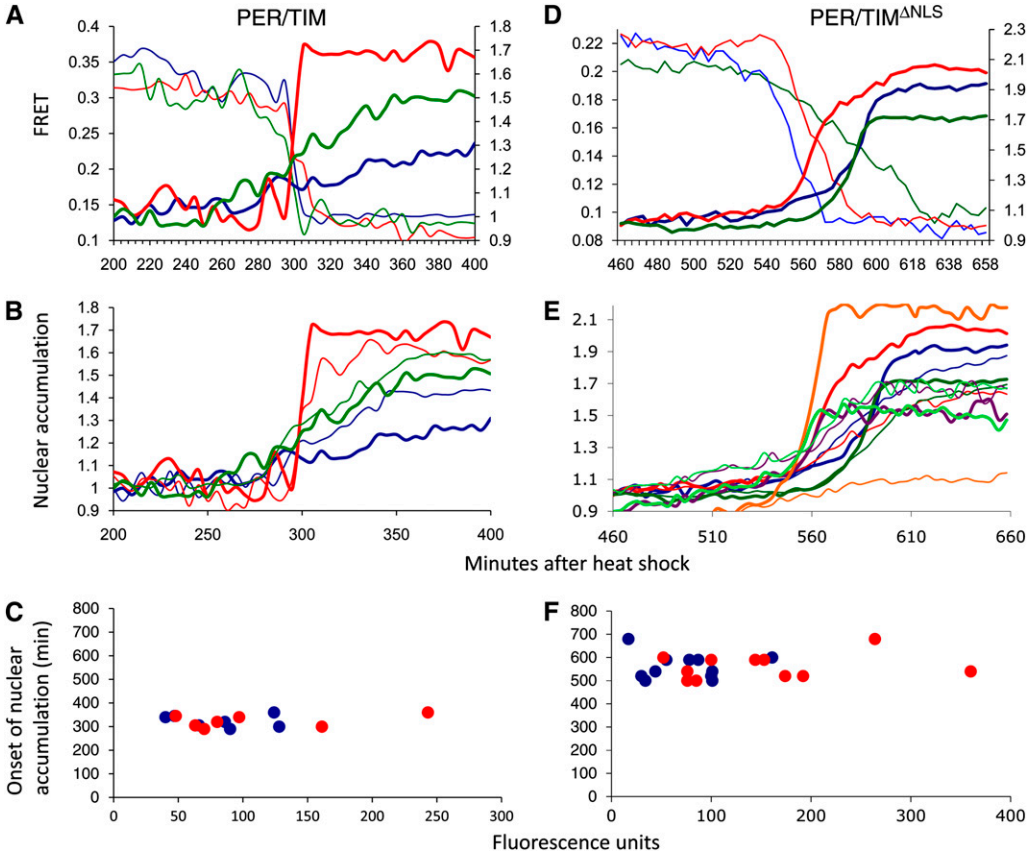

Figure 7 TIM$^{\Delta NLS}$ delays nuclear entry of PER and TIM in S2 cells. Profiles of FRET and nuclear translocation of PER/TIM (A–C) and PER/TIM$^{\Delta NLS}$ (D–F). (A) FRET levels (thin lines) and nuclear translocation profiles for PER (thick lines) in three representative PER/TIM cells shown in different colors. (D) FRET levels (thin lines) and nuclear translocation profiles for PER (thick lines) in three representative PER/TIM$^{\Delta NLS}$ cells shown in different colors. (B and E) Nuclear accumulation profiles for PER-CFP (thick lines) and TIM-YFP (thin lines) for three PER/TIM (B) and five PER/TIM$^{\Delta NLS}$ (E) cells. (C) Onset of nuclear translocation for PER (blue) and TIM (red) relative to PER/TIM protein levels in fluorescence units. (F) Onset of nuclear translocation for PER (blue) and TIM$^{\Delta NLS}$ (red) relative to PER/TIM$^{\Delta NLS}$ protein levels in fluorescence units.

both TIM and its partner protein, PER. Modification of the TIM NLS impaired the nuclear entry of PER and TIM *in vivo* and produced an aberrant, but specific, delay in the onset of nuclear accumulation in living cultured cells. The cytoplasmic residence of PER and TIM$^{\Delta NLS}$ exceeds that of wild-type PER and TIM proteins by ~4 hr in S2 cells. A similar delay *in vivo* might be expected to extend period length to ~28 hr, which is close to the change in behavioral rhythmicity seen in *tim^{0}; tim$^{\Delta NLS}$* mutant flies. Although levels of nuclear accumulation were too low to be assessed directly in neural pacemaker cells (sLNV's) of *tim^{0}; tim$^{\Delta NLS}$* flies, the rhythmic behavior of these flies makes it likely that periodic PER/TIM $^{\Delta NLS}$ nuclear accumulation also occurs *in vivo*. Alternatively, the long-period rhythms of *tim^{0}; tim$^{\Delta NLS}$* flies could be the result of PER and TIM activity in clock cells other than the sLNV's.

The altered profiles of PER and TIM$^{\Delta NLS}$ nuclear accumulation do not appear to be due to a loss of physical association between the two proteins. As shown by inmmunoprecipitation and FRET analyses, PER and TIM$^{\Delta NLS}$ are capable of binding to each other and do so in cultured cells with kinetics that are similar to wild-type PER and TIM. Surprisingly, mutating the identified TIM NLS only delays the time of nuclear translocation in S2 cells. These results suggest that a sequence that poorly resembles a nuclear localization signal can promote nuclear entry in the absence of the known TIM NLS.

The contemporaneous movement of TIM and PER (or of TIM$^{\Delta NLS}$ and PER) to the nucleus in S2 cells (see Figure 7) suggests that the TIM NLS has an important role in determining the time of nuclear entry, but not the co-dependent nuclear accumulation of PER and TIM. That is, in the absence of the identified TIM NLS, although nuclear transfer is substantially delayed, the onset of nuclear accumulation is shifted for both the TIM$^{\Delta NLS}$protein and for wild-type PER expressed in the same cell. That wild-type PER does not accumulate in the nucleus with kinetics that are independent of TIM$^{\Delta NLS}$ indicates that other features of the TIM (and PER) proteins coordinate their timed subcellular movements. Prior studies have repeatedly shown that the presence of TIM significantly enhances the nuclear accumulation of PER (Vosshall and Young 1995; Saez and Young 1996; Saez *et al.* 2007). This tight coupling is most likely to reflect the physical association of PER and TIM as they accumulate in the cytoplasm and immediately prior to the time of their nuclear translocation. The NLS of TIM may participate in a mechanism that facilitates nuclear entry of both proteins. One possibility is that a specific importin recognizes the TIM NLS in cytoplasmic PER/TIM complexes and promotes an association of these complexes with the nuclear pore. As earlier studies have indicated that PER and TIM dissociate at the time of nuclear transfer (Shafer *et al.* 2002, 2006; Ashmore *et al.* 2003; Meyer *et al.* 2006; Saez *et al.* 2007), this model would require recognition of the TIM NLS prior

to separation of the proteins, but could account for the similar timing of PER and TIM nuclear accumulation that has been observed in S2 cells. Possibly a PER NLS or alternate segment of the TIM protein can promote a delayed association with the nuclear pore in $tim^{\Delta NLS}$ mutants.

In summary, our studies have confirmed a role for a specific TIM protein sequence in the regulated nuclear accumulation of TIM and PER. Mutation of the TIM NLS suppressed nuclear accumulation of PER and TIM in neural pacemaker cells of *Drosophila* and lengthened the period of molecular and behavioral rhythms in these flies to ~30 hr. The same $tim^{\Delta NLS}$ mutation also affected nuclear accumulation of PER and TIM when expressed in cultured *Drosophila* cells and caused delays in the onset of PER/TIM nuclear accumulation that are well correlated with the long-period circadian rhythms of $tim^{\Delta NLS}$ flies.

Acknowledgments

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A Key Temporal Delay in the Circadian Cycle of *Drosophila* Is Mediated by a Nuclear Localization Signal in the Timeless Protein

Lino Saez, Mary Derasmo, Pablo Meyer, J. Stieglitz, and Michael W. Young

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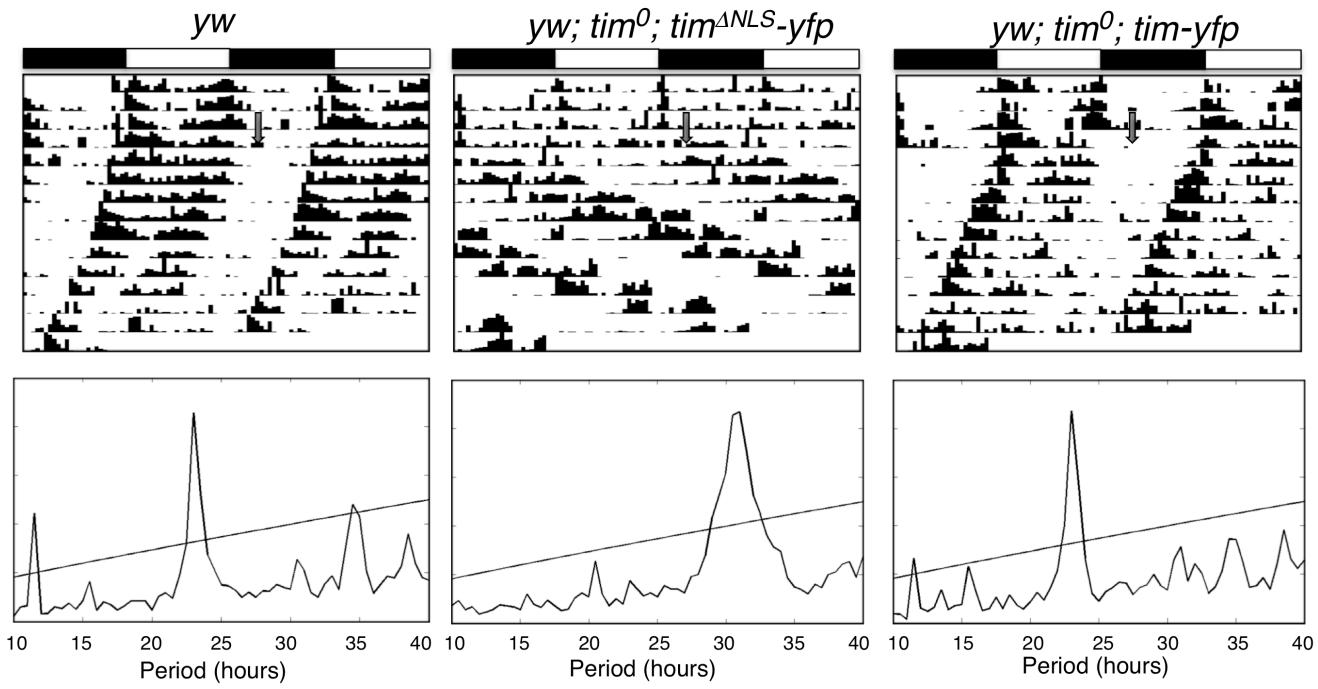

Figure S1 Expression of *tim*$^{\Delta NLS}$ protein lengthens circadian behavioral rhythms. Representative double-plotted actograms (top) and periodograms (bottom) are shown for each of the indicated genotypes. Entrained flies were monitored at 25^0 C for four days in LD and transferred to DD (vertical arrows in actograms) for the duration of each experiment. Periods for which the amplitude lies above the sloping line are statistically significant with a *P* value of <0.01.

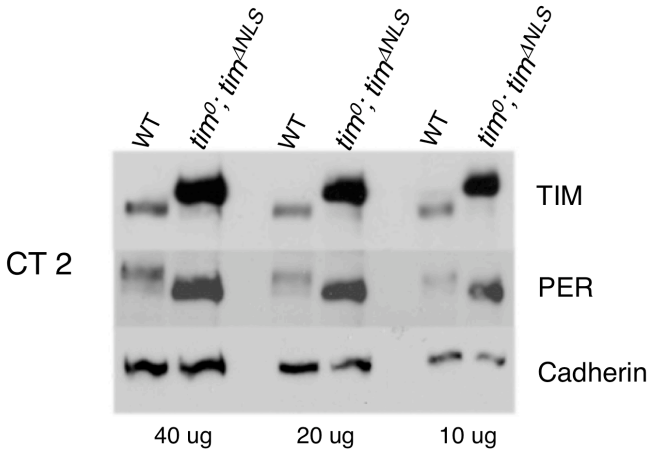

Figure S2 Higher levels of PER and TIM protein expression were found in *tim*^{0}; *tim*$^{\Delta NLS}$*-yfp* flies. Indicated amounts of head protein extract collected at CT2 were loaded in each lane and probed with PER and TIM antibodies. Higher concentrations of TIM and hypo-phosphorylated PER were detected in the TIM$^{\Delta NLS}$ extracts as compared to the wild type extracts. Cadherin was used as a loading control.