Report

NEMO Kinase Contributes to Core Period Determination by Slowing the Pace of the *Drosophila* Circadian Oscillator

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Summary

The *Drosophila* circadian oscillator is comprised of transcriptional feedback loops that are activated by CLOCK (CLK) and CYCLE (CYC) and repressed by PERIOD (PER) and TIMELESS (TIM) [1]. The timing of CLK-CYC activation and PER-TIM repression is regulated posttranslationally, in part through rhythmic phosphorylation of CLK, PER, and TIM [2–4]. Although kinases that control PER and TIM levels and subcellular localization have been identified [5–10], additional kinases are predicted to target PER, TIM, and/or CLK to promote time-specific transcriptional repression. We screened for kinases that alter circadian behavior via clock cell-directed RNA interference (RNAi) and identified the proline-directed kinase *nemo* (*nmo*) as a novel component of the circadian oscillator. Both *nmo* RNAi knockdown and a *nmo* hypomorphic mutant shorten circadian period, whereas *nmo* overexpression lengthens circadian period. CLK levels increase when *nmo* expression is knocked down in clock cells, whereas CLK levels decrease and PER and TIM accumulation are delayed when *nmo* is overexpressed in clock cells. These data suggest that *nmo* slows the pace of the circadian oscillator by altering CLK, PER, and TIM expression, thereby contributing to the generation of an ∼24 hr circadian period.

Results and Discussion

nmo Acts to Lengthen Circadian Period

Because PERIOD (PER), TIMELESS (TIM), and CLOCK (CLK) are maximally phosphorylated when CLK-CYCLE (CYC) transcription is repressed [2–4], we sought to identify kinases that promote transcriptional repression. The loss of such kinases is predicted to increase CLK-CYC transcriptional activity, which is known to shorten circadian period [11]. In contrast, reducing the levels and/or activity of DOUBLETIME (DBT), SHAGGY (SGG), and CASEIN KINASE 2 (CK2) lengthens circadian period by slowing PER-TIM degradation or delaying PER-TIM nuclear localization [5, 8, 9, 12, 13]. To identify kinases that act to repress CLK-CYC transcription, we screened a series of kinase RNA interference (RNAi) strains for short-period rhythms. A *tim*-Gal4 driver was used to express 34 UAS-kinase RNAi lines in all clock cells, and one line (KK104885) targeting *nemo* (*nmo*) displayed a short period of ∼22.0 hr in constant darkness (DD) (Table 1, compare rows 3–5; see also Figure S1 available online). Because the ventrolateral neurons (LN$_v$s) are key pacemaker cells that are sufficient to drive behavioral rhythms [14, 15], we predicted that expressing *nmo* RNAi only in LN$_v$s would also shorten circadian period. Indeed, when the *pdf*-Gal4 driver

was used to drive *nmo* RNAi in LN$_v$s [16], the free-running period of locomotor activity rhythms shortened to ∼22.5 hr (Table 1, compare rows 4, 6, and 7).

To determine whether the short-period phenotype was due to the action of *nmo* RNAi on *nmo* expression and not an off-target effect, we expressed *nmo* RNAi targeting a different portion of the *nmo* transcript (KK101545) in all clock cells or LN$_v$s using the *tim*-Gal4 and *pdf*-Gal4 drivers, respectively. Expression of *nmo* RNAi KK101545 with either driver shortened circadian period to <22.5 hr (Table 1, compare rows 8–12), thus confirming the specificity of *nmo* RNAi. To further demonstrate that loss of *nmo* function shortens circadian period, a severely hypomorphic *nmo* P[lacZ] insertion mutant (*nmo*^{P1}) was tested for behavioral activity rhythms [17–19]. Heterozygous *nmo*^{P1}/*nmo* deficiency (henceforth *nmo*^{P1}/Df) flies shortened the period of circadian activity rhythms to ∼21.5 hr, thus confirming that loss of *nmo* function shortens circadian period (Table 1, compare rows 13–15; Figure S1). It is possible that loss of *nmo* function could shorten period by altering clock cell development. To test this possibility, we expressed *nmo* RNAi in adults only using the Gal80^{ts} TARGET system [20]. At the permissive temperature (18°C), Gal80^{ts} inhibited *tim*-Gal4-driven UAS-*nmo* RNAi and the circadian period was ∼23.4 hr, whereas at restrictive temperature (30°C), *nmo* RNAi was expressed in clock cells and shortened circadian period by ∼1 hr (Table S1, compare rows 5 and 11 and rows 7 and 13). Control flies lacking *nmo* RNAi expression at 30°C had 23.4–24.4 hr behavioral activity rhythms, demonstrating that period shortening does not result from increased temperature (Table S1, compare rows 3, 4, and 6 to rows 5 and 7). These results argue that period shortening is due to *nmo* function in differentiated oscillator cells.

Because *nmo* levels are severely reduced in *nmo*^{P1}/Df flies [17–19], we reasoned that expressing *nmo* specifically in clock cells would rescue the short-period rhythm phenotype. When *tim*-Gal4 was used to express UAS-GFP-tagged NMO (UAS-GFP-*nmo*) in *nmo*^{P1}/Df flies [21], short-period behavioral rhythms were reverted to periods of ∼25 hr (Table 1, compare rows 16–18; Figure S1). Because the reverted period was ∼1.5 hr longer than controls carrying only UAS-GFP-*nmo*, the period lengthening beyond a precise (e.g., ∼23.5 hr period) rescue may be due to *nmo* overexpression. To determine whether this was the case, we used *tim*-Gal4-driven UAS-GFP-*nmo* to overexpress NMO in clock cells from wild-type flies, which resulted in long-period (∼25 hr) activity rhythms (Table 1, compare rows 3, 19, and 20). The period shortening of *nmo* RNAi and *nmo*^{P1}/Df mutants and the period lengthening of *nmo* overexpression flies suggested that NMO levels and/or activity are important determinants of circadian period. Though microarray and qPCR analysis demonstrate that *nmo* mRNA levels do not cycle in fly heads [22, 23] (data not shown), cycling of NMO protein levels could not be assessed because NMO antibodies are not available.

nmo Interacts with Core Clock Components and Alters CLK and PER Levels

Drosophila NMO is the founding member of the *nemo*-like kinase (Nlk) family of proline-directed protein kinases, which

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Table 1. Circadian Locomotor Activity of Clock Cell-Specific *nmo* RNAi, *nmo* Mutant, *nmo* Mutant Rescue, and *nmo* Overexpression Flies

Row	Genotype	n (Animals Tested)	% of Rhythmic Animals	Period
1	*per*[01]	47	0	NA
2	*w*[1118]	59	98.3	23.5 ± 0.03
3	*w*[1118];+/*tim*-Gal4	55	90.9	24.0 ± 0.07
4	*w*[1118];+/*nmo* RNAi-1*	41	90.2	23.5 ± 0.05
5	*w*[1118];*tim*-Gal4 /*nmo* RNAi-1	16	75	22.0 ± 0.17[a]
6	*yw*;;+/*pdf*-Gal4	11	100	24.3 ± 0.10
7	*yw*;+/*nmo* RNAi-1;+/*pdf*-Gal4	16	87.5	22.6 ± 0.11[b]
8	*yw*;+/*tim*-Gal4	14	92.9	23.9 ± 0.08
9	*w*[1118];;+/*pdf*-Gal4	15	100.0	24.0 ± 0.10
10	*w*[1118];+/*nmo* RNAi-2****	35	97.1	23.6 ± 0.05
11	*w*[1118];*tim*-Gal4/*nmo* RNAi-2	16	75.0	22.1 ± 0.14[c]
12	*w*[1118];+/*nmo* RNAi-2;+/*pdf*-Gal4	20	75.0	22.4 ± 0.14[d]
13	*yw*;;+/*nmo*[P1]**	35	94.3	23.4 ± 0.03
14	*w*[1118];;+/*nmo*Df***	26	88.5	23.7 ± 0.05
15	*w*[1118];;*nmo*[P1]/*nmo*Df	74	36.5	21.4 ± 0.09[e]
16	*w*[1118];*tim*-Gal4/+;*nmo*[P1]/*nmo*Df	18	27.8	22.0 ± 0.22
17	*w*[1118];UAS-GFP-*nmo*/+;*nmo*[P1]/ *nmo*Df	14	42.9	21.3 ± 0.44
18	*w*[1118];UAS-GFP-*nmo*/tim-Gal4;*nmo*[P1]/*nmo*Df	25	56.0	25.4 ± 0.16[f]
19	*w*[1118];UAS-GFP-*nmo*****/+	20	100	23.7 ± 0.08
20	*w*[1118];UAS-GFP-*nmo*/tim-Gal4	12	100	25.1 ± 0.14[g]

Period of activity in constant darkness is given in hours ± standard error of the mean. **nmo* RNAi-1, VDRC# KK104885; ***nmo*[P1], P[lacW]*nmo*[P1]; ****nmo*Df, Df(3L)Exel6279; *****nmo* RNAi-2, VDRC# KK101545; ******UAS-GFP-*nmo*, UAS-GFP-*nmoII*. The genotypes in rows 8, 9, 11, and 12 contain +/UAS-*Dcr-2* on chromosome 2 (rows 9 and 12) or chromosome 3 (rows 8 and 11) because a very low number of rhythmic flies were observed when *nmo*RNA-2 was crossed with the Gal4 drivers in rows 3 and 6. See also Table S1 and Figure S1.
[a] Significantly shorter than *w*[1118];+/*tim*-Gal4 and *w*[1118];+/*nmo* RNAi-1 controls (p < 10⁻⁸).
[b] Significantly shorter than *yw*;;+/*pdf*-Gal4 and *w*[1118];+/*nmo* RNAi-1 controls (p < 10⁻⁸).
[c] Significantly shorter than *yw*;+/*tim*-Gal4 and *w*[1118];+/*nmo* RNAi-2 controls (p < 10⁻¹⁰).
[d] Significantly shorter than *w*[1118];;+/*pdf*-Gal4 and *w*[1118];+/*nmo* RNAi-2 controls (p < 10⁻⁹).
[e] Significantly shorter than *yw*;;+/*nmo*[P1] and *w*[1118];;+/*nmo*Df controls (p < 10⁻²⁵).
[f] Significantly longer than *w*[1118];*tim*-Gal4/+;*nmo*[P1]/*nmo*Df and *w*[1118];UAS-GFP-*nmo*/+;*nmo*[P1]/*nmo*Df controls (p < 10⁻⁸).
[g] Significantly longer than *w*[1118];+/*tim*-Gal4 and *w*[1118];UAS-GFP-*nmo* controls (p < 10⁻⁸).

phosphorylate serine or threonine residues immediately preceding a proline residue [24]. During fly development, NMO acts in multiple signaling pathways (e.g., WNT and BMP) to regulate pattern formation in embryos [25], planar cell polarity [17, 19, 21], and apoptosis [18, 19]. NMO and its mammalian NLK counterpart are known to impart regulation by phosphorylating transcription factors such as Mad, TCF-4, and Eve [25–27].

The short-period phenotype produced by clock cell-specific *nmo* RNAi implies that *nmo* is expressed in clock cells. To determine the pattern of *nmo* expression in adults, we employed the *nmo*[P1] P[lacZ] enhancer trap insert, which was previously used to detect *nmo* spatial expression during development [17]. Brains from *nmo*[P1]/+ flies were dissected and coimmunostained with antisera against the oscillator cell marker CLK and the *nmo*[P1] enhancer trap marker β-galactosidase. CLK-positive neurons were assigned to cell groups according to size and position. β-galactosidase colocalized with CLK in a subset of small ventrolateral neurons (sLN$_v$), large ventrolateral neurons (lLN$_v$), dorsolateral neurons (LN$_d$), and dorsal neuron 1s (DN$_1$), but not in dorsal neuron 2s (DN$_2$) or dorsal neuron 3s (DN$_3$) (Figure 1). In addition, cells lacking CLK also expressed β-galactosidase (Figure 1). These results demonstrate that *nmo* is expressed in a subset of oscillator neurons and in nonoscillator cells in the brain. Although we did not detect β-galactosidase expression in all brain oscillator neurons, it is likely that the *nmo*[P1] insert does not trap all enhancers that drive expression in adults within the >70 kb *nmo* gene.

PER, TIM, and CLK phosphorylation is maximal when they form a complex in the nucleus during the late night and early morning that represses transcription [2–4, 12]. If NMO phosphorylates one or more of these core clock components, we would expect NMO to be present in PER-TIM-CLK complexes when transcription is being repressed. To determine whether this is the case, we used GFP antiserum to immunoprecipitate GFP-tagged NMO (GFP-NMO) complexes from flies expressing GFP-NMO in all clock cells and controls lacking GFP-NMO expression. During 12 hr light/12 hr dark (LD) cycles, PER, TIM, and CLK coimmunoprecipitated with GFP-NMO at ZT22 (zeitgeber time, where ZT0 is lights-on and ZT12 is lights-off); PER and CLK coimmunoprecipitated with GFP-NMO at ZT2; TIM coimmunoprecipitated with GFP-NMO at ZT14 and CLK coimmunoprecipitated with GFP-NMO at ZT10 and ZT14 (Figure 2). Because TIM levels are low at ZT2 and ZT10 and PER levels are low at ZT10 and ZT14, we cannot rule out the possibility that these proteins interact with GFP-NMO at these times. These results demonstrate that NMO is present in circadian transcriptional repression complexes and that NMO remains associated with CLK during times of transcriptional activation. Consistent with these coimmunoprecipitation results, GFP-NMO was present in the nucleus of LN$_v$s when transcription was being repressed at ZT21 (Figure S2).

The presence of NMO in complexes with PER, TIM, and CLK suggests that NMO may lengthen circadian period by phosphorylating one or more of these proteins, thereby altering their levels and/or activity. To determine whether reducing NMO levels alters PER, TIM, or CLK phosphorylation or abundance, we used antibodies against these proteins to probe western blots containing head extracts from *nmo* RNAi (*tim*-Gal4-driven UAS-*nmo* RNAi) and control (UAS-*nmo* RNAi/+)
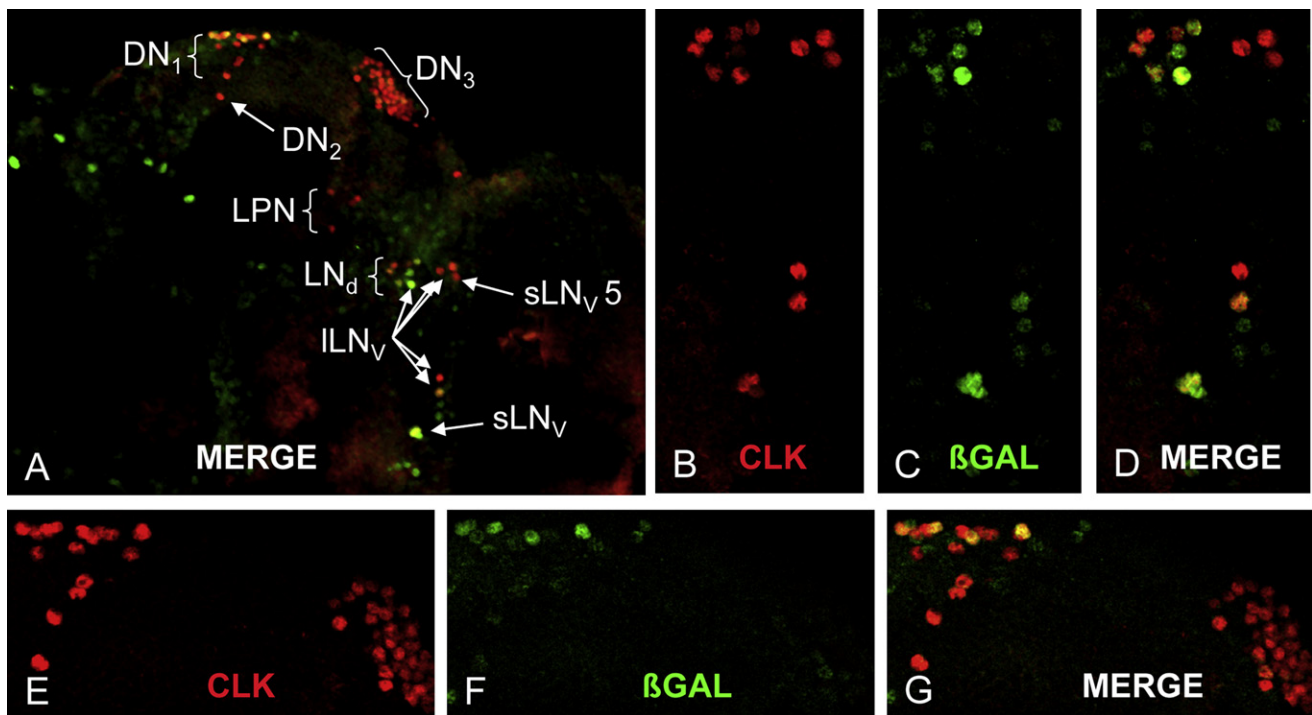
Figure 1. A *nmo*-lacZ Enhancer Trap Is Expressed in a Subset of Clock Brain Neurons

(A) Confocal image of brain hemisphere from w^{1118};nmo^{P1}/+ flies coimmunostained with β-galactosidase (βGAL; green) and CLOCK (CLK; red). The following abbreviations are used: DN$_{1-3}$, dorsal neuron groups 1–3; lLN$_V$, large ventral lateral neurons; sLN$_V$, small ventral lateral neurons; LN$_d$, dorsal lateral neurons; LPN, lateral posterior neurons.
(B–D) Magnification of the LN region from (A).
(E–G) Magnification of the DN region from (A).
Images are Z stacks of 50 μm thickness at 2 μm per Z image.

flies collected during LD. PER and TIM phosphorylation (as measured by the higher-mobility forms) and levels were not noticeably different in *tim*-Gal4-driven *nmo* RNAi flies versus *nmo* RNAi/+ controls (Figure 3A). In contrast, CLK phosphorylation and abundance were higher in *tim*-Gal4-driven *nmo* RNAi flies than in *nmo* RNAi/+ controls throughout the diurnal cycle (Figure 3A; Figure S3). This result is consistent with the progressive period shortening as *Clk* copy number

increases [11] and suggests that NMO may act to destabilize CLK. It is also possible that knocking down *nmo* expression may increase CLK levels indirectly by boosting *Clk* transcription, but we favor the possibility that NMO destabilizes CLK because it is direct and there is ample precedent for phosphorylation-dependent degradation of clock proteins [28].

In contrast to the period shortening seen in *nmo* mutant and RNAi knockdown flies, overexpression of NMO in clock cells
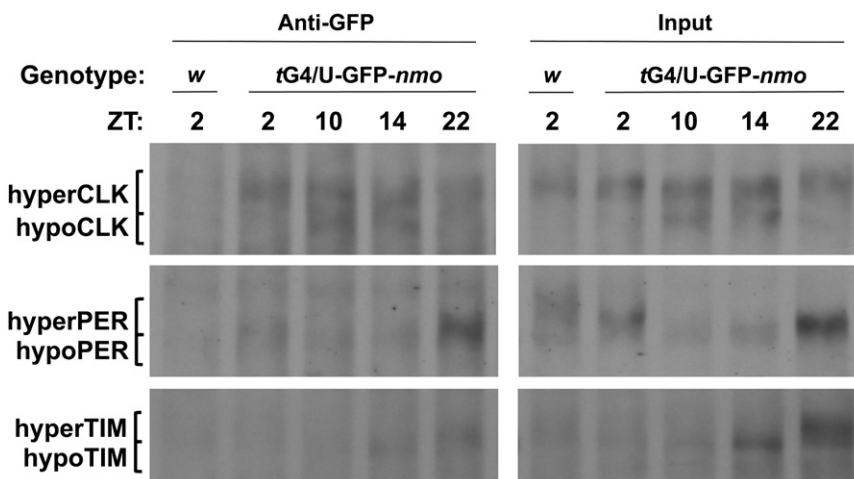

Figure 2. NMO Interacts with PER, TIM, and CLK

w^{1118};UAS-GFP-*nmo*/*tim*-Gal4 (*t*G4/U-GFP-*nmo*) and control w^{1118} (*w*) flies were entrained for 3 days in 12 hr light/12 hr dark (LD) conditions and collected at the indicated times. Western blots of nuclear protein extracts of fly heads alone (input) or after immunoprecipitation with GFP antiserum (anti-GFP) were probed with CLK, PER, and TIM antisera. Bands corresponding to hyperphosphorylated CLK, PER, and TIM (hyperCLK, hyperPER, and hyperTIM, respectively) and hypophosphorylated CLK, PER, and TIM (hypoCLK, hypoPER, and hypoTIM, respectively) are shown. See also Figure S2.
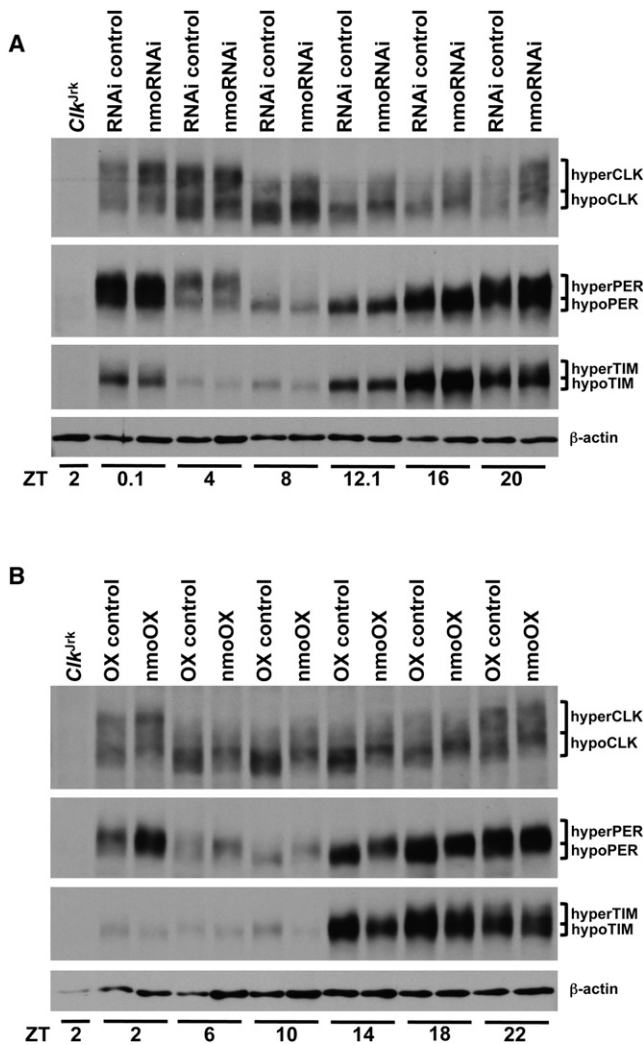
Figure 3. NMO Acts to Stabilize CLK

Flies were entrained for 3 days under LD conditions and collected at the indicated times.

(A) Western blot of head extracts from *Clk*^Jrk^, *w*^1118^;+/UAS-*nmo* RNAi-2 (RNAi control), and *w*^1118^;UAS-*nmo* RNAi-2/*tim*-Gal4;+/UAS-*Dcr-2* (nmoR-NAi) flies probed with CLK, PER, and TIM antisera. Bands corresponding to the different phosphorylation states of CLK, PER, and TIM are marked as in Figure 2. Actin serves as a loading control.

(B) Western blot of head extracts from *Clk*^Jrk^, *w*^1118^;UAS-GFP-*nmo*/+ (OX control), and *w*^1118^;UAS-GFP-*nmo*/*tim*-Gal4 (nmoOX) flies probed with CLK, PER, and TIM antisera. Bands corresponding to the different phosphorylation states of CLK, PER, and TIM are marked as above. Actin serves as a loading control.

See also Figure S3.

lengthens period by ~1.5 hr (Table 1). If NMO functions to destabilize CLK, then we would expect that CLK levels would be lower when NMO is overexpressed. To determine whether NMO alters the levels and/or phosphorylation of CLK and other core clock repression complex components, we used antibodies against CLK, PER, and TIM to probe western blots containing head extracts from NMO overexpression (*tim*-Gal4-driven UAS-GFP-*nmo*) and control (UAS-GFP-*nmo*/+) flies collected during LD. CLK levels were generally lower and CLK phosphorylation was higher in NMO overexpression flies compared to controls (Figure 3B; Figure S3), consistent with a role for NMO in destabilizing CLK. Although *nmo*

RNAi knockdown had little effect on PER levels and phosphorylation, NMO overexpression delayed PER accumulation and degradation and increased PER phosphorylation (Figure 3B). TIM also accumulated in a delayed fashion (Figure 3B) but is rapidly degraded upon exposure to light [29]. The delayed accumulation of PER and TIM and the lower levels of CLK in NMO overexpression flies are consistent with a lengthened circadian period. The delay in PER and TIM accumulation could be due to lower levels of *per* and *tim* transcription, consistent with lower CLK levels in NMO overexpression flies. Alternatively, PER accumulates as a more highly phosphorylated form in NMO overexpression flies, suggesting that NMO may phosphorylate PER. If this phosphorylated form of PER is less stable, it would delay PER accumulation. PER is not required for TIM stability [4, 30]; thus, a delay in PER accumulation as a result of NMO overexpression would not cause a delay in the accumulation of TIM. These results suggest that NMO phosphorylates more than one core clock component to slow the pace of the circadian oscillator.

PER, TIM, and CLK each have a small number of consensus proline-directed phosphorylation sites and could thus serve as NMO substrates. Although the identity of TIM and CLK phosphorylation sites have not been determined, at least six consensus proline-directed sites are phosphorylated when PER is expressed in S2 cells [12]. Eliminating a proline-directed phosphorylation site at S661 in PER delays nuclear localization and lengthens period by ~1.5 hr [31]. Another proline-directed phosphorylation site is situated in the "short-period domain" of PER, a region spanning amino acids S585–Y601 that contains multiple mutations that shorten circadian period including *per*^S^ and *per*^T^ [32–36]. When the only proline-directed phosphorylation site within the short-period domain is mutated by replacing P597 with alanine, the period shortens to ~22 hr [33]. Replacing S596 with a nonphosphorylatable residue also shortens circadian period, and analysis using an antibody that detects phosphorylated S596 shows that this residue is phosphorylated by NMO [37]. It is possible that loss of NMO phosphorylation only at PER S596 could account for the *nmo* mutant and *nmo* RNAi short-period phenotype. For instance, loss of a phosphorylation site that destabilizes PER after dawn would advance feedback repression by PER complexes and shorten circadian period. Alternatively, NMO may phosphorylate multiple components of the PER repression complex to shorten circadian period. Knocking down *nmo* expression in clock cells via RNAi increased CLK levels but did not detectably alter PER and TIM phosphorylation or abundance. In contrast, NMO overexpression decreased CLK levels, delayed PER and TIM accumulation and PER degradation, and increased PER and CLK phosphorylation. These results suggest that NMO may directly phosphorylate CLK, which reduces CLK levels to maintain a 24 hr period. In this scenario, reduced *nmo* expression would increase CLK levels, thereby prematurely terminating PER repression and shortening period, whereas increased *nmo* expression would reduce CLK levels, thereby delaying PER accumulation at night, prolonging PER repression in the morning, and lengthening period. These changes in CLK abundance likely work in concert with NMO-induced delays in PER degradation in the morning to set circadian period.

We have identified *nmo* as a new component of the *Drosophila* circadian oscillator. The short-period behavioral rhythms of *nmo*^P1^/Df and *nmo* RNAi flies indicates that NMO acts to slow the pace of the circadian oscillator, consistent with the lengthening of circadian period when *nmo* is overexpressed in clock cells. A *nmo* P[lacZ] enhancer trap line reveals

nmo expression in the sLN$_v$ and other brain clock cells, consistent with the short-period behavioral rhythms that result from expressing *nmo* RNAi in LN$_v$s. NMO is present in complexes with PER, TIM, and CLK when CLK-CYC transcription is repressed and alters PER, TIM, and CLK levels and/or phosphorylation state. Our results suggest that NMO acts within PER-TIM-CLK regulatory complexes to lengthen circadian period. These results are likely relevant to the mammalian circadian oscillator because mPER and CLOCK are also highly phosphorylated when transcription is repressed [38] and a single *nmo* ortholog, Nemo-like kinase (NLK), is present in mice and humans [39, 40].

Supplemental Information

Supplemental Information includes one table, three figures, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.02.037.

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