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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 post-translationally, 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 knock down 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 is 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 a ~24-hour circadian period.

Results and Discussion

nmo acts to lengthen circadian period

Because PER, TIM and CLK are maximally phosphorylated when CLK-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 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 ~22.0h in constant darkness (DD) (Table 1, compare rows 3–5; Fig. S1). Since the LN_vs are key pacemaker cells that are sufficient to drive behavioral rhythms [14,15], we predicted that expressing *nmo* RNAi only in LN_vs would also shorten circadian period. Indeed, when the *pdf*-Gal4 driver was used to drive

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nmo RNAi in LNvs [16], the free-running period of locomotor activity rhythms shortened to ~22.5h (Table 1, compare rows 4, 6, 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, *nmo* RNAi targeting a different portion of the *nmo* transcript (KK101545) was expressed in all clock cells or LNvs using the *tim*-Gal4 and *pdf*-Gal4 drivers, respectively. Expression of *nmo* RNAi KK101545 with either driver shortened circadian period to ~21.5h (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 ~21.5h, thus confirming that loss of *nmo* function shortens circadian period (Table 1, compare rows 13–15; Fig. 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 only in adults using the Gal80^{ts} TARGET system [20]. At the permissive temperature (18°C) Gal80^{ts} inhibits *tim*-Gal4 driven UAS-*nmo* RNAi and the circadian period is ~23.4h, whereas at restrictive temperature (30°C) *nmo* RNAi is expressed in clock cells and shortens circadian period by ~1h (Table S1, compare rows 5 and 11, and rows 7 and 13). Control flies that lack *nmo* RNAi expression at 30°C have 23.4h–24.4h behavioral activity rhythms, demonstrating that period shortening doesn't 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.

Since *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 ~25h (Table 1, compare rows 16–18; Fig. S1). Since the reverted period was ~1.5h longer than controls carrying only UAS-GFP-*nmo*, the period lengthening beyond a precise (e.g. ~23.5h period) rescue may be due to *nmo* overexpression. To determine if this was the case, GFP tagged *nmo* (GFP-NMO) was used to overexpress NMO in clock cells from wild-type flies, which resulted in long period (~25h) activity rhythms (Table 1, compare rows 3, 19, 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 phosphorylate serine or threonine residues immediately preceding a proline residue [24]. During fly development, NMO acts in multiple signaling pathways (e.g. WNT, 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 co-immunostained 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 was co-localized with CLK in a subset of small ventrolateral neurons (sLNv), large ventrolateral neurons (lLNv), dorsolateral neurons (LNd), and dorsal neuron 1s (DN1), but not in dorsal neuron 2s (DN2) or dorsal neuron 3s (DN3) (Fig. 1). In addition, cells lacking CLK also expressed β -galactosidase (Fig. 1). These results demonstrate that *nmo* is expressed in a subset of oscillator neurons and in non-oscillator cells in the brain. Although we do 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 >70kb *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 if this is the case, GFP antiserum was used to immunoprecipitate GFP-NMO complexes from flies expressing GFP-NMO in all clock cells and controls that lack GFP-NMO expression. During LD cycles, PER, TIM and CLK co-immunoprecipitate with GFP-NMO at ZT22, PER and CLK co-immunoprecipitate with GFP-NMO at ZT2, TIM co-immunoprecipitates with GFP-NMO at ZT14, and CLK co-immunoprecipitates with GFP-NMO at ZT10 and ZT14 (Fig. 2). Since TIM levels are low at ZT2 and ZT10 and PER levels are low at ZT10 and ZT14, we can't 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 co-immunoprecipitation results, GFP-NMO is present in the nucleus of LNvs when transcription is being repressed at ZT21 (Fig. 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 if reducing NMO levels alters PER, TIM or CLK phosphorylation or abundance, antibodies against these proteins were used to probe western blots containing head extracts from *nmo* RNAi (*tim*-Gal4 driven UAS-*nmo* RNAi) and control (UAS-*nmo* RNAi/+) 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 (Fig. 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 (Fig. 3a; Fig. 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 knock-down flies, overexpression of NMO in clock cells lengthens period by ~1.5h (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, antibodies against CLK, PER and TIM were used 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 (Fig. 3b; Fig. 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 (Fig. 3b). TIM also accumulated in a delayed fashion (Fig. 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 *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 due to NMO overexpression wouldn't 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 phosphorylation 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 ~1.5h [31]. Another proline-directed phosphorylation site is situated in the “short period domain” of PER, a region spanning amino acids S585 to 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 ~22h [33]. Replacing S596 with a non-phosphorylatable residue also shortens circadian period, and analysis using an antibody that detects phosphorylated S596 shows that this residue is phosphorylated by NMO (Chiu and Edery, personal communication). 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 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 increased PER and CLK phosphorylation. These results suggest that NMO may directly phosphorylate CLK, which reduces CLK levels to maintain a 24h 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 prolonging PER repression and lengthening period. These changes in CLK abundance likely work in concert with NMO-induced delays in PER accumulation 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_vs. 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 since mPER and CLOCK are also highly phosphorylated when transcription is repressed [37], and a single *nmo* ortholog, Nemo-like kinase (NLK), is present in mice and humans [38,39].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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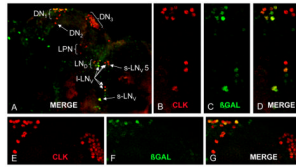


Figure 1.

A *nmo-lacZ* enhancer trap is expressed in a subset of clock brain neurons. (A) Confocal image of brain hemisphere from *w¹¹¹⁸; nmo^{P1/+}* flies coimmunostained with β -GAL (green) and CLK (red). DN₁₋₃, dorsal neuron groups 1–3; 1-LN_V, large ventral lateral neurons; s-LN_V, small ventral lateral neurons; LN_D, dorsal lateral neurons; LPN, lateral posterior neurons. (B–D) Magnification of the LN region from panel A. (E–G) Magnification of the DN region from panel A. Images are Z-stacks of 50 μ m thickness at 2 μ m per Z-image.

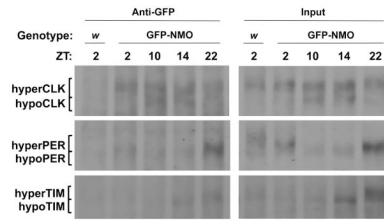


Figure 2.

NMO interacts with PER, TIM and CLK. *w¹¹¹⁸;UAS-GFP-*nmo/tim*-Gal4* (GFP-NMO) and control *w¹¹¹⁸* (*w*) flies were entrained for three days in 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, hypoTIM, respectively) are shown. See also Figure S2.

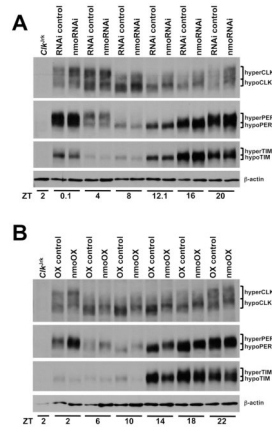


Figure 3.

NMO acts to stabilize CLK. Flies were entrained for three days under LD conditions and collected at the indicated times. (A) Western blot of head extracts from *Clk^{Jrk}, w¹¹¹⁸;+/UAS-nmoRNAi-2* (RNAi control) and *w¹¹¹⁸;UAS-nmoRNAi-2/tim-Gal4;+/UAS-Dcr-2* (nmoRNAi) flies probed with CLK, PER and TIM antisera. Bands corresponding to the different phosphorylation states of CLK, PER and TIM are marked as in Fig. 2. Actin serves as a loading control. (B) Western blot of head extracts from *Clk^{Jrk}, w¹¹¹⁸;UAS-GFP-nmo/+* (OX control) and *w¹¹¹⁸;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 Fig. S3.

Table 1

Circadian locomotor activity of clock cell-specific *nmo* RNAi, *nmo* mutant, *nmo* mutant rescue and *nmo* overexpression flies.

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

^aNumber of animals tested;

^bPercentage of rhythmic animals.

^cPeriod of activity in constant darkness, given in hours ± sem.

* *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) due to a very low number of rhythmic flies observed when *nmo*RNA-2 was crossed with the Gal4 drivers in rows 3 and 6.

^dSignificantly shorter than *w*¹¹¹⁸;+/tim-Gal4 and *w*¹¹¹⁸;+/nmoRNAi-1 controls ($p < 10^{-8}$).

^eSignificantly shorter than *yw*;+/pdf-Gal4 and *w*¹¹¹⁸;+/nmoRNAi-1 controls ($p < 10^{-8}$).

^fSignificantly shorter than *yw*;+/tim-Gal4 and *w*¹¹¹⁸;+/nmoRNAi-2 controls ($p < 10^{-10}$).

^gSignificantly shorter than $w^{1118};+/pdf-Gal4$ and $w^{1118};+/nmoRNAi-2$ controls ($p < 10^{-9}$).

^hSignificantly shorter than $yw;+/nmo^{P1}$ and $w^{1118};+/nmoDf$ controls ($p < 10^{-25}$).

ⁱSignificantly longer than $w^{1118};tim-Gal4/+;nmo^{P1}/nmoDf$ and $w^{1118};UAS-GFP-nmo/+;nmo^{P1}/nmoDf$ controls ($p < 10^{-8}$).

^jSignificantly longer than $w^{1118};+/tim-Gal4$ and $w^{1118};UAS-GFP-nmo$ controls ($p < 10^{-8}$).

See also Table S1 and Figure S1.