Climatically driven changes in the genetic constitution of *D. melanogaster* populations are not entirely unexpected, because climate-associated changes in chromosomal inversions have been documented for the *Drosophila* species *D. robusta* (*23*) and *D. subobscura* (*24*). In addition, previous *Drosophila* field studies suggest that changes in frequencies of inversions and single-gene alleles can occur rapidly (*25*) as well as seasonally (*26*, *27*). On the basis of distribution records, it is thought that *D. melanogaster* first entered Australia from the north about 100 years ago (*28*). Despite the relative recency of this introduction and molecular data indicating a high rate of gene flow (*29*), clinal variation is well established for several traits and several genetic polymophisms along the Australian eastern coast (*3*, *30*). Therefore, the genetic shift documented here is unlikely to be the tail end of adaptation to a temporally stable environmental transect by an introduced species; instead, it may be part of a rapid genetic response of a species to climate change, as has recently been suggested for an adaptive trait in another insect species (*31*).

The *Adh* enzyme polymorphism in *D. melanogaster* and the common cosmopolitan inversions in this species are valuable examples of clinal variation along latitudinal gradients, resulting in genetic constitutions appropriate to local climatic conditions. The shift in polymorphisms with climatic change indicates how *Adh* [and potentially *In(3R)Payne*] can be linked to the dynamics of adaptive processes. We have shown that adaptive polymorphisms could provide monitoring tools for detecting the impact of climate change on populations.

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PERIOD1-Associated Proteins Modulate the Negative Limb of the Mammalian Circadian Oscillator

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The clock proteins PERIOD1 (PER1) and PERIOD2 (PER2) play essential roles in a negative transcriptional feedback loop that generates circadian rhythms in mammalian cells. We identified two PER1-associated factors, NONO and WDR5, that modulate PER activity. The reduction of NONO expression by RNA interference (RNAi) attenuated circadian rhythms in mammalian cells, and fruit flies carrying a hypomorphic allele were nearly arrhythmic. WDR5, a subunit of histone methyltransferase complexes, augmented PER-mediated transcriptional repression, and its reduction by RNAi diminished circadian histone methylations at the promoter of a clock gene.

About 10% of all mammalian transcripts show daily oscillations of abundance (*1*). These rhythmic fluctuations are governed by a molecular circadian clock, whose function relies on two interconnected feedback loops of transcription. In the major negative feedback loop, transcription of the *Period* (*Per1* and *Per2*) and *Cryptochrome* (*Cry1* and *Cry2*) genes, and of *Rev-Erbα*, an orphan nuclear receptor gene, is activated by the transcription factors CLOCK and BMAL1 and repressed by the PERIOD (PER) and CRYPTOCHROME (CRY) proteins themselves (*2*). Although PERs interact with CRYs (*3*), the mechanism by which they repress CLOCK:BMAL1–mediated clock gene transcription remains poorly understood.

To elucidate the sizes of the PER1 and PER2 complexes involved in this process, we fractionated nuclear extracts of mouse livers by gel filtration chromatography. During the night, the two proteins formed similarly large complexes (>1 MD) (Fig. 1A), whose abundance and size distribution changed during the day (fig. S1). Thus, PERs associate with other proteins that may play roles in the function of the circadian oscillator. To identify them, we generated a Rat-1 fibroblast cell line that expresses a PER1 protein containing a 6xHis tag and a V5 epitope tag at its C terminus. The expression of a His-V5-PER1 protein was three times higher than that of endogenous PER1 in unsynchronized cells (Fig. 1B, lanes 1 and 2) and did not interfere with the circadian transcription of other clock or clock-controlled genes (*4*).

Nuclear extracts from His-V5-PER1 cells were harvested 3 hours after the induction of circadian rhythms by serum treatment. Because clock-controlled genes such as *Dbp* and *Rev-erbα* are repressed during this time period (*5*), PER-mediated transcriptional repression would likely be operative as well. After chromatography of this extract was performed on nickel

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chelate resin, superose 6, and V5 antibody–Sepharose (fig. S2A), the eluted proteins were size-fractionated by gel electrophoresis and stained with colloidal Coomassie blue (Fig. 1B, lane 3). Individual bands were excised, and the peptides they contained were identified by tandem mass spectrometry. In addition to peptides from CRY1 and CRY2, which are already known to be PER1-interacting proteins (*3*), peptides from two other factors, NONO and WDR5, were identified. NONO has been characterized previously as an RNA- and DNA-binding protein that could be involved in splicing, transcriptional repression, and RNA export (*6*); and its *Drosophila* homolog, NonA, has been implicated in visual acuity and courtship behavior (*7*). WDR5 is a member of a histone methyltransferase complex (*8*) and has been implicated in cell differentiation processes (*9*). RNA and proteins for both factors are expressed at constant levels throughout the day in a variety of tissues, including the suprachiasmatic nucleus, which is the site of the central circadian clock in the mammalian hypothalamus (fig. S3). However, the apparent sizes of the NONO protein complexes in liver nuclei varied during the day, and a size difference of these complexes was also observed between wild-type mice and mice lacking functional PER proteins (Fig. 1C).

To verify the association of WDR5 and NONO with PER1, gel filtration chromatography was performed on cellular extracts. A portion of the NONO protein coeluted with PER1, and complete immunodepletion of His-V5-PER1 before fractionation resulted in a decrease in apparent size for some NONO proteins (fig. S2B, fractions 56 to 58). Because the majority of the WDR5 protein was present in complexes of sizes close to those that contain PER1, and because we estimate WDR5 to be 10 times more abundant than PER1 in the cell, it was impossible to estimate the fraction of WDR5 that co-migrates with PER1 by using this assay (*4*). Nevertheless, epitope-tagged PER1 or PER2 coimmunoprecipitated with WDR5 and NONO (Fig. 1D), and both WDR5 and NONO were immunologically de-

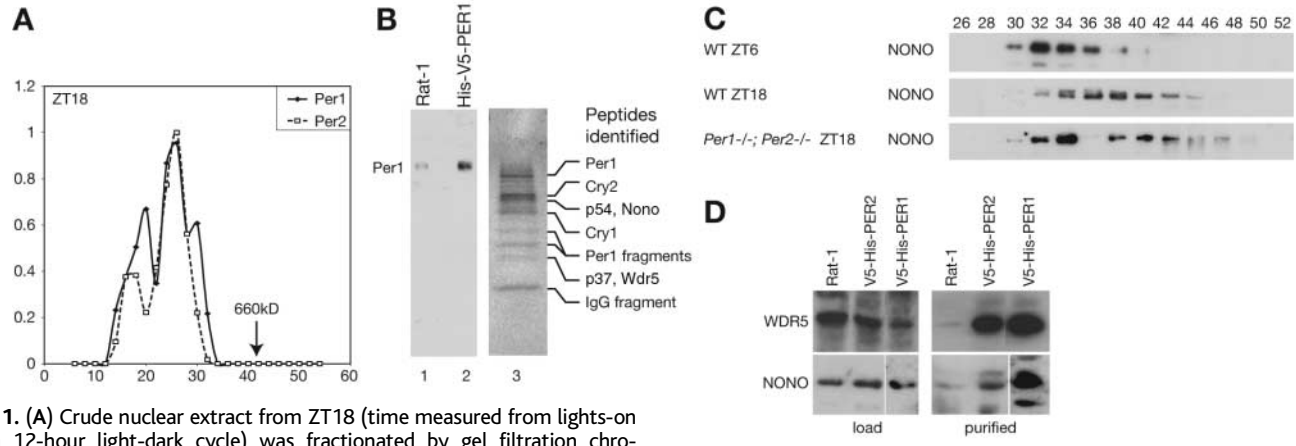

Fig. 1. (**A**) Crude nuclear extract from ZT18 (time measured from lights-on in a 12-hour light-dark cycle) was fractionated by gel filtration chromatography (Biogel A1.5m, Bio-Rad, Hercules, CA), and fractions were analyzed by Western blot probed with anti-PER1 and anti-PER2. *x* axis, fraction number (smaller numbers are larger complexes); *y* axis, signal intensity in arbitrary units. The elution peak of thyroglobulin (660 kD) is shown for reference (see also fig. S1). (**B**) Lanes 1 and 2 show whole-cell extracts from RAT1 cells and His-V5-PER1 cells that were analyzed on a Western blot probed with anti-PER1. Lane 3 shows colloidal Coomassie-stained gel of purified material; peptides identified by mass spectrometry are shown at right. (**C**) Nuclear extract from wild-type mice killed at ZT6 (top) or ZT18 (middle), or from *Per1*$^{-/-}$-*Per2*$^{-/-}$ double-knockout mice (bottom) was purified by nickel-nitriloacetic acid (NiNTA) chromatography and then analyzed on a Western blot probed with anti-NONO. (**D**) Extracts from serum-treated RAT1 cells, V5-His-PER1 cells, or V5-His-PER2 cells were purified by NiNTA chromatography and then analyzed by Western blot with anti-WDR5 (top) or with anti-NONO (bottom). (Left) One-fiftieth of the crude extract used; (right) purified.

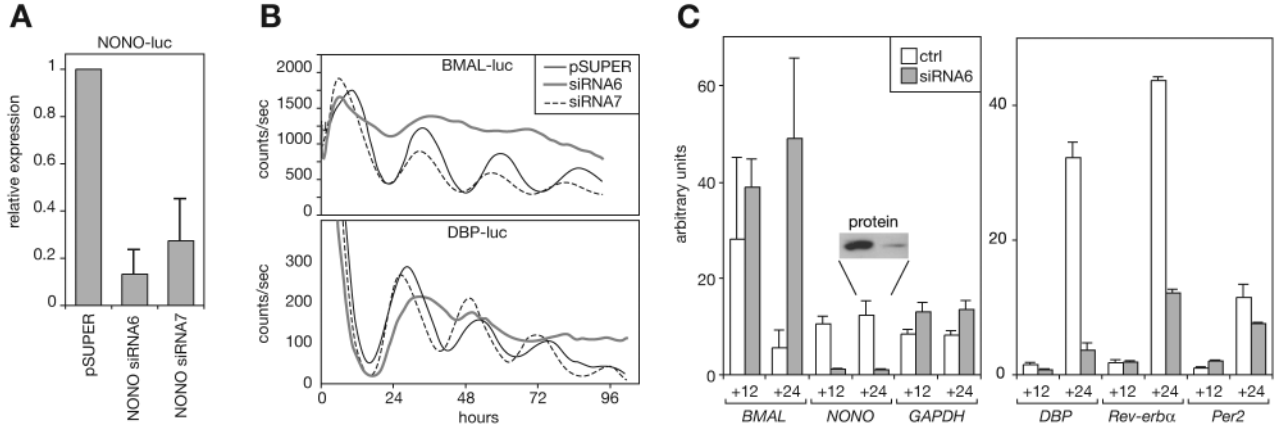

Fig. 2. (**A**) 3T3 cells were transfected with *NONO*-luciferase fusions, as well as the pSUPER empty vector or the *NONO*-specific siRNA hairpin expression vectors pSUPER-NONO-siRNA6 or pSUPER-NONO-siRNA7. NONO-luciferase levels were measured by luminometry of whole-cell extracts. (**B**) 3T3 cells were transfected with *NONO*-luciferase fusions, as well as with the pSUPER blank siRNA hairpin expression vector or the *NONO*-specific hairpin vectors pSUPER-NONO-siRNA6 or pSUPER-NONO-siRNA7. After synchronization of circadian rhythms with dexamethasone, *BMAL11-luciferase* (top) and *DBP-luciferase* (bottom) expression levels were measured by continuous live-cell luminometry. (**C**) Cells cotransfected with pSUPER or pSUPER-NONO-siRNA6 and the pCS2 YFP-Venus expression vector were treated with dexamethasone and then FACS-sorted for the presence of YFP 12 or 24 hours later. RNA from YFP-positive cells was analyzed by real-time quantitative polymerase chain reaction (PCR) by using indicated probes and normalized as described in (*20*). In addition, NONO protein in sorted whole-cell extract from duplicate plates was measured by Western blot (inset). GAPDH, glyceraldehyde phosphate dehydrogenase; ctrl, control.

tectable after chromatography as per the initial purification (fig. S2C).

To determine whether WDR5 and NONO control circadian gene expression, RNA interference (RNAi) experiments were conducted to reduce target protein levels. For NONO, 12 short RNAi hairpin sequences (*10*) were screened for their ability to reduce luciferase expression in mouse 3T3 fibroblasts that were transiently transfected with a plasmid that expresses a luciferase-NONO hybrid transcript. Two of these hairpins, which elicited 4- or 10-fold reductions in luciferase levels (Fig. 2A), were expressed in 3T3 cells with the circadian reporter plasmids pBmal1-luc or pDbp-luc that express luciferase in opposite circadian phases. The circadian rhythms of transfected cells were synchronized (*11*), and circadian cycles of *Bmal-luc* and *Dbp-luc* expression were measured by real-time luminometry (*12*). The RNAi hairpin vector that reduced *NONO-luc* transcription fourfold shortened the circadian period of transcription by approximately 2 hours. The other hairpin, which reduced *NONO-luc* transcription 10-fold, attenuated circadian transcription of both reporter genes by at least 20 times and increased the expression of the *Bmal1-luc* fusion construct (Fig. 2B).

To measure the effect of NONO depletion on endogenous gene expression, 3T3 cells were cotransfected with a *NONO*–small interfering RNA (siRNA) vector and a yellow fluorescent protein (YFP) vector, synchronized, and then sorted with a fluorescence-activated cell sorter (FACS) to purify YFP-expressing cells at two different times of day. Endogenous NONO transcript and protein levels were reduced in these cells. *Bmal1* transcript levels exhibited no temporal variation and were constantly high. However, transcript levels of *Rev-erbα* and *Dbp* were constantly low (Fig. 2C), suggesting that the wild-type NONO protein does not aid in the repression of these genes but rather antagonizes it.

NonA, the *Drosophila* homolog of NONO, plays an important physiological role, because flies harboring a P element–mediated deletion of this gene are extremely sick (*13*). However, because flies homozygous for the strongly hypomorphic *nonA* allele *P14* are viable and fertile (*7*), we were able to examine whether NonA also plays a role in the generation of circadian rhythms in *Drosophila*. *nonA*$^{P14/P14}$ flies were behaviorally nearly arrhythmic and hyperactive (Fig. 3A and fig. S4). In addition, the rhythmicity of mRNA from the clock gene *timeless* was greatly attenuated, indicating that the *nonA*^{P14} mutation interferes with the basic function of the circadian oscillator (Fig. 3C).

The WDR5 protein has been identified as a member of a histone methyltransferase complex (*8*). Consistent with this function, histone methyltransferase activity could be precipitated from His-V5-PER1 fibroblast extracts with antibodies to V5 (anti-V5), anti-PER2, and anti-WDR5. It could also be enriched from evening but not morning nuclear extracts of mouse livers with anti-PER2 (Fig. 4A). Because PER-regulated gene expression is low at night, it is likely that this methyltranferase activity has a repressive function. Such a repressive effect would be characteristic of histone H3 lysine 9 (H3K9) methylation, among other types (*14*). Nevertheless, WDR5 has been associated with a histone H3K4 methyltransferase containing a SET1 domain (*8*), which is mostly thought to be involved in the activation of transcription (*15*). Hence, both histone modifications were examined at a PER1-regulated circadian promoter in the presence and absence of WDR5.

A lentivirally mediated system (*16*) was used to generate a cell line that represses endogenous *Wdr5* RNA and protein levels about five times in a doxycycline-inducible fashion (fig. S5). Circadian cycling in these cells was synchronized (*11*), and chromatin was harvested from cells grown in the presence or absence of doxycycline at the time of maximum and minimum *Rev-erbα* transcription. Methylation of histone H3K4 and histone H3K9 at the *Rev-erbα* promoter was examined by chromatin immunoprecipitation (ChIP). In uninduced cells expressing normal levels of WDR5, circadian methylation was observed in phase with *Rev-erbα* transcription at H3K4, and antiphase to it at H3K9. In doxycycline-treated cells, however, both rhythms were nearly abolished (Fig. 4B). Down-regulation of WDR5 expression produced only moderate changes in clock gene expression. PER-regulated genes such as *Per2* and *Rev-erbα* were somewhat derepressed initially, and mRNA accumulation was then phase-delayed by about 2 hours (Fig. 4B and fig. S6). Expression of other circadian transcripts such as *Cry1* mRNA, *Clock* mRNA, and *Bmal1* mRNA were unaffected (*4*). Similarly, when the same cells were analyzed with circadian luciferase reporter genes and real-time luminometry, the period length of cyclic gene ex-

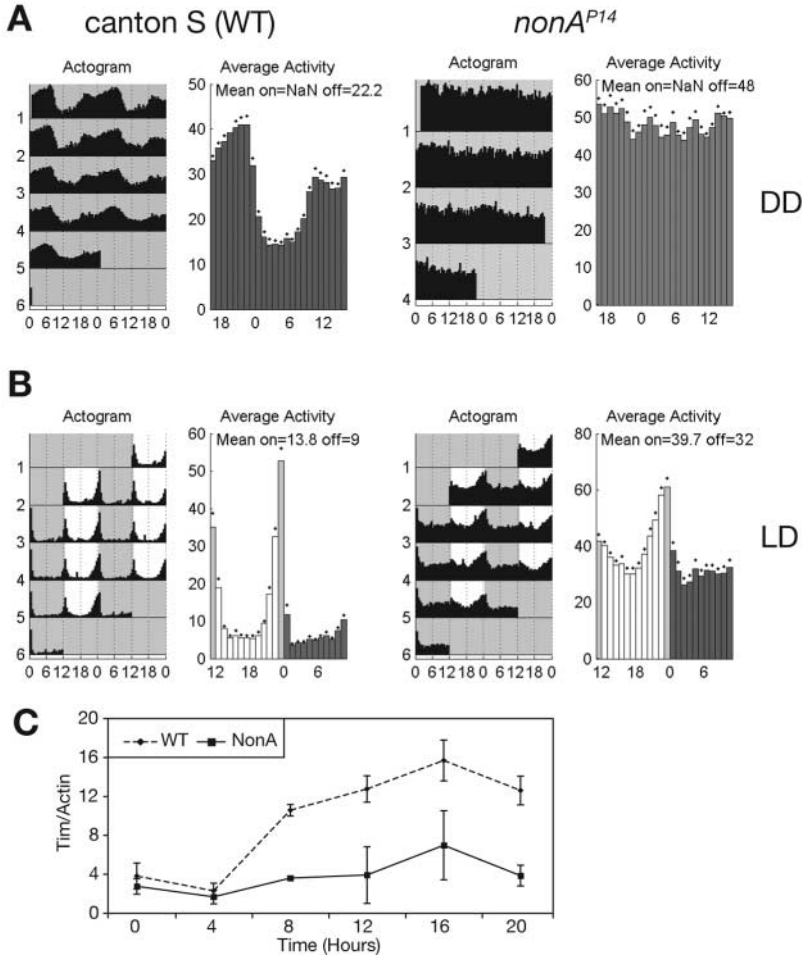

Fig. 3. (**A**) Locomotor activities of male Canton S or *nonA*$^{P14/P14}$ flies were analyzed by infrared beam-break studies in continuously dark (DD) conditions. Sample data from a single fly are shown at left, and data from 10 flies are plotted at right (fig. S3). (**B**) Identical experiment to (A) but measured in flies exposed to 12 hours of light and 12 hours of darkness per day (LD). (**C**) RNA from wild-type and mutant flies was prepared from four separate pools of 10 fly heads harvested at the indicated time of day, and the quantity of *Tim* RNA was determined by Taqman real-time PCR.

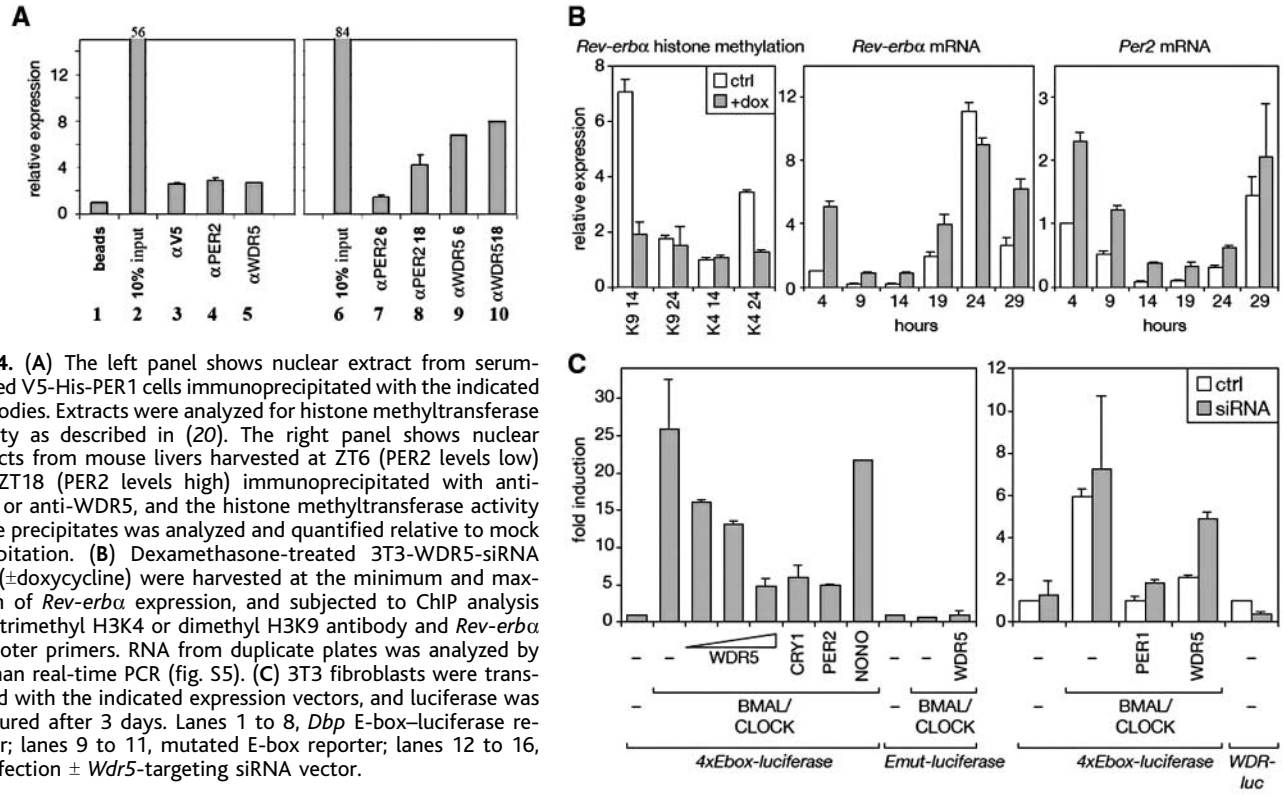

Fig. 4. (**A**) The left panel shows nuclear extract from serum-treated V5-His-PER1 cells immunoprecipitated with the indicated antibodies. Extracts were analyzed for histone methyltransferase activity as described in (*20*). The right panel shows nuclear extracts from mouse livers harvested at ZT6 (PER2 levels low) and ZT18 (PER2 levels high) immunoprecipitated with anti-PER2 or anti-WDR5, and the histone methyltransferase activity of the precipitates was analyzed and quantified relative to mock precipitation. (**B**) Dexamethasone-treated 3T3-WDR5-siRNA cells (±doxycycline) were harvested at the minimum and maximum of *Rev-erbα* expression, and subjected to ChIP analysis with trimethyl H3K4 or dimethyl H3K9 antibody and *Rev-erbα* promoter primers. RNA from duplicate plates was analyzed by Taqman real-time PCR (fig. S5). (**C**) 3T3 fibroblasts were transfected with the indicated expression vectors, and luciferase was measured after 3 days. Lanes 1 to 8, *Dbp* E-box–luciferase reporter; lanes 9 to 11, mutated E-box reporter; lanes 12 to 16, transfection ± *Wdr5*-targeting siRNA vector.

pression was unchanged (fig S6), suggesting that these two histone modifications are either compensatory or irrelevant for circadian clock function. Because overall bioluminescence from multiple reporters was also reduced, we conclude that WDR5 reduction has a pleiotropic effect on cellular processes unrelated to circadian clock function. Therefore, although circadian histone methylation was drastically affected, we could not verify a direct role for WDR5 in the circadian clock via our loss-of-function experiments.

PER proteins repress the activity of the circadian activators CLOCK and BMAL in transient transfection assays (*17*). The transient expression of WDR5 repressed CLOCK:BMAL1–mediated transcription to the same extent as the transient expression of the PER2 or CRY1 proteins, and inhibition of transcription by either PER2 or WDR5 could be partially blocked by cotransfecting a WDR5 siRNA vector. This hairpin reduced expression levels of a *WDR5-luc* fusion transcript threefold in parallel experiments (Fig. 4C). Thus, WDR5 can aid the PER-CRY complex in repressing CLOCK:BMAL1–mediated transcription, although this effect might be indirect. Because NONO was 50 times less efficiently expressed than WDR5, PER2, or CRY1 in these experiments, it was not possible to demonstrate its supposed antagonistic effect by this method.

We have identified two proteins, NONO and WDR5, that can associate with the mammalian PER1 protein. Our data suggest that NONO probably operates antagonistically to PER proteins in mammalian cells, and that it is essential to normal circadian rhythmicity in mammals and in *Drosophila*. Mutations in the *Drosophila* homologs of PER1 and NONO were previously coidentified in a screen for courtship behavior defects (*18*). Overexpression experiments suggest that WDR5 assists PER function in mammalian cells. Its reduction affected two different antiphasic circadian histone modifications, H3K4 and H3K9, which are thought to have opposite effects (*14*, *15*), but WDR5 had only moderate consequences for circadian clock function. Hence, further studies of WDR5 and of histone posttranslational modifications at circadian promoters will be essential for understanding its contribution to clock function. We suggest that the antagonistic activities of these proteins might help to render the circadian clock more resilient to noise—caused by changes in temperature and nutrients, or by stochastic fluctuations in transcription rates—that could affect the concentrations of critical clock components (*19*).

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21. Molecular interaction data have been deposited in the Biomolecular Interaction Network Database (BIND) with accession codes 258456 and 258459.

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