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Modelling the effect of phosphorylation on the circadian clock of *Drosophila*

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HIGHLIGHTS

- We model phosphorylations of mutually exclusive domains of a circadian protein in the fruit fly.
- The domains act as a switch. Alterations in their phosphorylation increase or decrease the period of circadian oscillations.
- Using realistic parameters we find period values that agree with observations in mutant flies.

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ABSTRACT

It is by now well known that, at the molecular level, the core of the circadian clock of most living species is a negative feedback loop where some proteins inhibit their own transcription. However, it has recently been shown that post-translational processes, such as phosphorylations, are essential for a correct timing of the clock. Depending on which sites of a circadian protein are phosphorylated, different properties such as degradation, nuclear localization and repressing power can be altered. Furthermore, phosphorylation domains can be related in a positive way, giving rise to consecutive phosphorylations, or in a negative way, hindering phosphorylation at other domains. Here we present a simple mathematical model of a circadian protein having two mutually exclusive domains of phosphorylation. We show that the system has limit cycles that arise from a unique fixed point through a Hopf bifurcation. We find a set of parameters, with realistic values, for which the limit cycle has the same period as the wild type circadian oscillations of the fruit fly. The domains act as a switch, in the sense that alterations in their phosphorylation can alter the period of circadian oscillation in opposite ways, increasing or decreasing the period of the wild type oscillations. In particular, we show that our model is able to reproduce some of the experimental results found for switch-like phosphorylations of the *PER* protein of the circadian clock of the fly *Drosophila melanogaster*.

1. Introduction

The behavior of most organisms living in our planet is organized in cyclic patterns of activity that last approximately 24 h, usually called *circadian rhythms*. Even though these cycles are entrained by light or other environmental cues, it has been found that they are endogenous, in the sense that they can persist for many days without such cues. The seminal experiments of Konopka and Benzer (1971) showed that in the fruit fly (*Drosophila melanogaster*) these behaviors are determined by a cycle that takes place at a molecular level inside many cells. The first protein found was called *PER* (Period), and it was shown that mutations of it produced alterations in the circadian behavior of the fruit fly. Later experiments determined that the cycle could be thought as a negative feedback loop where *PER* represses the transcription of its own *mRNA*. Further research added new elements (and complexity) to this core clock. At the level of structure the most relevant feature is that the clock consists of two interlocked loops: the negative loop, involving proteins *PER* and *TIM* represses the transcription of the proteins *CLK* and *CYC*, and a positive loop where these last proteins are in turn activators of the transcription of *PER* and *TIM*. The structure of the circadian clocks of mammals has been found to be basically the same as for flies, with only small differences: the mammal analogs (in terms of function) of the proteins *PER* and *TIM* are the proteins *PER*1, *PER*2 and *CRY*1, *CRY*2, respectively, whereas the analogs of *CLK* and *CYC* are *CLOCK* and *BMAL*1 (see Edery, 2000 and references therein).

In recent years many other processes were discovered that seem to be very important for a correct timing of the clock, even though they do not change its core structure. Typically these processes happen after the *mRNA* has been translated into a

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circadian protein, and are thus called post-translational processes (Mehra et al., 2009), such as phosphorylation, acetylation, sumoylation, ubiquitination or proteasomal degradation (see Leloup, 2009 and references therein). For some simple organisms, as cyanobacteria, phosphorylation processes are so important that they are able to produce circadian oscillations even in the absence of transcriptional loops (Nakajima et al., 2005). For higher organisms post-translational processes are not believed to be essential (although this is a matter of debate; Lakin-Thomas, 2006; Hastings et al., 2008), but they are very important for the correct timing of the clock (Merrow et al., 2006).

The largest family of post-translational processes is the family of reversible phosphorylations of the clock proteins, consisting on the addition or removal of phosphate groups, which are mediated by enzymes called kinases (such as *CKI*, *SHAGGY*; Leloup, 2009 and *NEMO*; Yu et al., 2011) and phosphatases (such as *PP*1, *PP*2, *PP*5, Leloup, 2009), respectively. One of the most important kinases for the *Drosophila* circadian clock is *doubletime* (DBT), whose homolog for vertebrates is *CKI*ϵ. The effects of phosphorylation on the cellular clock properties, which we call *macroscopic* effects, seem to be highly dependent on the specific protein being phosphorylated (or dephosphorylated). Phosphorylation of *TIM* is related to changes in the entrainment and adjustment of the clock, whereas phosphorylation of *CLK* regulates the stability of the clock (Bae and Edery, 2006). In turn, phosphorylation of *PER* alters mostly the timing of the circadian rhythms. It is well known that mutants for which the phosphorylation of *PER* has been altered may have longer or shorter circadian rhythms than wild-type individuals. In humans, it has recently been shown that the advanced sleep phase syndrome (FASPS) (Jones et al., 1999), a rare hereditary sleep disorder that shortens the circadian rhythm by approximately 4 h, is caused by a mutation that alters the phosphorylation of *PER*2 (Toh et al., 2001).

There is also a large amount of research dedicated to understanding the molecular aspects of phosphorylation, and the changes it produces on the target proteins. We call these changes the *microscopic* effects of phosphorylation. In the case of *Drosophila PER* it has been shown that phosphorylation alters the degradation rate (Price et al., 1998), the timing of nuclear entry (Cyran et al., 2005), and the transcriptional repressing potency (Nawathean and Rosbash, 2004) of the protein.

Recently, new techniques of mass spectrometry have led to the identification of the residues of *PER* that are phosphorylated. In some cases it has been possible to unequivocally associate the phosphorylation of one or more residues to a given phenotype, and *in vitro* experiments have unraveled the molecular effects of the different phosphorylations. For example, in humans it is known that FASPS is caused by a mutation at position 662 of human *PER*2 that replaces a serine by glycine (Toh et al., 2001), thus precluding its phosphorylation by the kinase *CKI*ϵ. *In vitro* experiments in mice (Vanselow et al., 2006) have shown that a mutation of the homolog residue S659 of mouse *PER*2 (which generates the same phenotype in mice) leads to premature nuclear clearance as well as to a faster destabilization of the protein in the cytoplasm. It was also found that *S*659 is probably the priming site for a phosphorylation cascade involving downstream sites. This was confirmed by experiments performed on cultures of cells of transgenic mice (with human *PER*) (Xu et al., 2007). The same group showed that phosphorylation of S662 of human *PER* decreases its ability to repress its own transcription.

In *Drosophila*, it is well known that many of the 1224 residues of *PER* can be clustered in domains within which most point mutations give rise to very similar phenotypes (Hamblen et al., 1998). One of them comprises residues 585–601 and is associated to phenotypes with shorter periods ($\approx$19 h) than the wild type (Baylies et al., 1992). It includes *S*589, the residue whose change produces the mutant per^S (Baylies et al., 1987; Yu et al., 1987), discovered by Konopka and Benzer. For this reason the whole domain is known as the per-short domain. But it was not until very recently that the molecular effects on the cell clock of many point mutations could be elucidated (Kim et al., 2007; Kivimäe et al., 2008; Chiu et al., 2008). Several works have shown that the kinase DBT phosphorylates *PER* in many places. Furthermore, as phosphorylation alters several properties of *PER*, the residues can also be clustered in domains within which phosphorylation alters the same properties. For example, Kivimäe et al. (2008) studied the properties of two domains: per-short and a region downstream of it, between *S*604 and *S*629 called domain *perSD*. They found that when phosphorylation of these sites by DBT is hindered (by substituting all serines by alanine), the protein becomes very stable and has almost no repressing power. If only the domain per-short is rendered inactive the protein becomes very unstable but has a large repressing power. On the other hand, only mutating the domain *perSD* results in a protein with intermediate stability and repressor activity. This shows that phosphorylation of each of these domains endows the protein with very different properties. It is worth stressing that it is very difficult to establish the relationship between these properties and the resulting phenotype from shear intuition.

It is well known that DBT mutants also display phenotypes with shorter ($\approx$20 h) and longer ($\approx$27 h) periods than the wild type (Price et al., 1998). One possible explanation for this is that the mutated kinase is impaired in its ability to phosphorylate different sites of *PER* (Preuss et al., 2004), which leads to different effects. If this was the case, DBT mutations could be considered phenotypically equivalent to *PER* mutations. In general, the phosphorylation of *PER* is conceived as a process that takes place progressively. This is confirmed by experimental works which show that the phosphorylation of some domains indeed gates the phosphorylation of other domains or sites (Vanselow et al., 2006; Chiu et al., 2008). However, and most interestingly, Kivimäe et al. (2008) have shown that phosphorylations may act as a switch: the phosphorylation of domain *perSD* seems to be promoted by the *dephosphorylation* of per-short, and vice versa.

The main difference between the experimental studies that determine the molecular effects of point mutations of *PER*, and the studies that determine the phenotype resulting from such mutations is that whereas the former can be performed *in vitro* using cell cultures (and sometimes *in vivo*), the latter can only be performed in living organisms. The problem is that the mutants that would be necessary for these studies are in general not viable (i.e. they die during development). This makes it rather difficult to infer how the observed microscopic effects of phosphorylation may affect the macroscopic properties of the clock. Furthermore, in cyclic systems intuition alone is usually not enough to relate microscopic and macroscopic effects. Consider for example a mutation that increases the repressing potency of the protein *PER*. On the one hand, this implies that smaller amounts of protein need to enter the nucleus to achieve the same levels of repression of transcription, which in turn should lead to a shortening of the clock's period. On the other hand, an increased repressing power leads to less protein being produced and therefore a slower accumulation of it in the cytoplasm, which should increase the period of the oscillator. It is evident that these two arguments cannot be simultaneously valid, but it is difficult to know which one is wrong. Part of the problem lies in the fact that each argument uses only an arbitrary fraction of the information available. On the other hand, the integration of the relevant available information into a mathematical model of the circadian clock can provide a systematic method to asses the relationship between microscopic and macroscopic effects. In fact, this approach has been used recently by Gallego et al. (2006) to give a molecular explanation for the phase-advancement of the

circadian rhythm of a mutant mouse. They found that the model predicted that the macroscopic effect could only be produced through an increase in kinase activity (i.e. larger levels of phosphorylation) even though *in vitro* experiments predicted a decrease in kinase activity. Experiments performed *in vivo* by the same group confirmed the model prediction (Gallego et al., 2006).

Mathematical models of circadian clocks are usually based on the Goodwin oscillator (Goodwin, 1965). The first model that was actually based on the experimental results available for *Drosophila* was the Goldbeter model (Goldbeter, 1995). The model consists of a feedback loop of one protein (*PER*) which must be phosphorylated two consecutive times to be able to enter the nucleus and repress its own transcription. The availability of new experimental results led to more detailed models, incorporating for example a *TIM* loop (Leloup and Goldbeter, 1998) and a positive feedback loop with *CLK–CYC* (Ueda et al., 2001). Recently, some very detailed models have been built that try to integrate most of the available experimental information (Leise and Moin, 2007; Bagheri et al., 2008). Even though these models also include post-translational mechanisms, their complexity sometimes precludes the study of the influence of specific processes. For these reasons, simple models are still used to analyze the effects of phosphorylation (Vanselow et al., 2006; Gallego and Virshup, 2007). More specifically these studies have focused on consecutive (or progressive) phosphorylations that affect the degradation or the nuclear entry of *PER*.

Here we use a simple model to study the effect of *parallel* phosphorylations in the fruit fly. These are defined as switch-like phosphorylations of certain sites that hinder the phosphorylation of other sites, which have been shown to be important in the structure of the circadian clock of *Drosophila* (Kivimäe et al., 2008). Through analytical and numerical analyses of the model we study the influence that phosphorylation of different domains of *PER* may have on properties of the protein, such as degradation, nuclear localization and repressing power. We compare the behavior of the model with available experimental results and discuss its biological implications.

2. Model

We present a simple mathematical model of the circadian oscillations of the *PER* protein. In Fig. 1 we show a scheme of the model that represents a single *Drosophila* cell. It consists of a feedback loop where oscillatory behavior originates from the repression of per *mRNA* (M) synthesis by phosphorylated *PER* proteins. As mentioned in Introduction recent experiments (Kivimäe et al., 2008) have shown that phosphorylation of some sites in *D. melanogaster PER* can have very different effects on the period of the circadian oscillations. Additionally, they have shown that phosphorylations of these sites are mutually exclusive and also that they have very different properties in terms of repressing power and degradation. To model this, we assume that *PER* can be phosphorylated in two different domains, denoted as A and B, and that phosphorylation of one of them precludes phosphorylation of the other. Thus, the following three states of the protein are possible: P_0 is the unphosphorylated *PER*, P_A is the protein phosphorylated in domain A and P_B is the one phosphorylated in domain B. We also assume that the effect of P_A and P_B on repression of transcription is different. This forces us to consider two different variables for the nuclear versions of the phosphorylated proteins: P_{NA} and P_{NB}. It has also been shown that unphosphorylated *PER* has virtually no effect on repression (Kivimäe et al., 2008). To account for this we have simply assumed that P_0 does not enter the nucleus. In the following we show that this simplified approach is enough to reproduce experimental results with realistic values of its parameters.

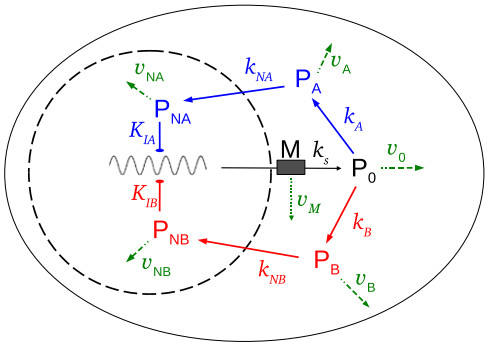

Fig. 1. *Schematic representation of the Drosophila switch model.* The figure presents a schematic representation of a *Drosophila* cell, and the processes considered in the model. The continuous and dashed lines represent the outer limits of the cytosol and the nucleus, respectively. M represents the concentration of *mRNA*, and P_0 the concentration of non-phosphorylated *PER* protein. The concentration of *PER* protein phosphorylated in domain A (B) is denoted by P_A (P_B). The full (dashed) arrows denote the net fluxes (degradation) with their corresponding rates. The concentration of nuclear proteins are P_{NA} and P_{NB}, and their repressing activity is denoted by a chopped arrow.

The equations that define the model are

$$\frac{dM}{dt} = \frac{V_s}{1+\left(\frac{P_{NA}(t)}{K_{IA}}+\frac{P_{NB}(t)}{K_{IB}}\right)^n} - \frac{v_M M(t)}{K_M + M(t)}$$

$$\frac{dP_0}{dt} = k_s M(t) - (k_A + k_B + v_0)P_0(t)$$

$$\frac{dP_A}{dt} = k_A P_0(t) - (k_{NA} + v_A)P_A(t)$$

$$\frac{dP_B}{dt} = k_B P_0(t) - (k_{NB} + v_B)P_B(t)$$

$$\frac{dP_{NA}}{dt} = k_{NA} P_A(t) - v_{NA} P_{NA}(t)$$

$$\frac{dP_{NB}}{dt} = k_{NB} P_B(t) - v_{NB} P_{NB}(t) \quad (1)$$

where the variables denote the abundance of *mRNA* (M) and of the different states of the protein: unphosphorylated *PER* (P_0), *PER* phosphorylated in domain A (P_A), *PER* phosphorylated in domain B (P_B), and the corresponding nuclear variants (P_{NA} and P_{NB}). Note that the equations are symmetrical, in the sense that A and B can be interchanged. The symmetry is broken when parameters are assigned numerical values, thus endowing PER-A and PER-B with different properties.

Repression of the transcription of *mRNA* is represented by a Hill-type term (first term in the right hand of the first equation above) with Hill coefficient n. This coefficient represents the minimum number of binding sites involved in the process (Weiss, 1997) (repression in this case), and thus it is desirable to choose a low value for it. In the original Goodwin oscillator, which has linear degradation terms for all species involved, limit cycle oscillations only appear for large values of n (unless the degradation rates are nearly equal for all species) (Griffith, 1968). In fact in the model developed for FASPS, the Hill coefficient used was $n=12$ (Vanselow et al., 2006), which is probably rather unrealistic. It has been shown that replacing at least one degradation term by a Michaelis–Menten term leads to an important reduction of the threshold value of n (Bliss et al., 1982). Thus, in order to keep the model simple, but with a low value of n, we have chosen a Michaelis–Menten term to model the degradation of *mRNA*.

Table 1
Definitions of the 16 parameters of the model with their corresponding units. *U* is an arbitrary concentration unit.

Parameter	Units	Definition
V_s	U h^{-1}	Maximum rate of *mRNA* synthesis
K_{IA}	U	Inhibition constant for repression by nuclear *PER* P_{NA}
K_{IB}	U	Inhibition constant for repression by nuclear *PER* P_{NB}
n	–	Hill constant
v_M	U h^{-1}	Maximum rate of degradation of *mRNA*
K_M	U	Michaelis constant for degradation of *mRNA*
k_s	h^{-1}	Rate constant for synthesis of unphosphorylated *PER* P_0
k_A	h^{-1}	Rate of A-phosphorylation
k_B	h^{-1}	Rate of B-phosphorylation
v_0	h^{-1}	Rate of degradation for P_0
k_{NA}	h^{-1}	Rate constant for entry of P_A into nucleus
v_A	h^{-1}	Rate of degradation for P_A
k_{NB}	h^{-1}	Rate constant for entry of P_B into nucleus
v_B	h^{-1}	Rate of degradation for P_B
v_{NA}	h^{-1}	Rate of degradation for nuclear *PER* P_{NA}
v_{NB}	h^{-1}	Rate of degradation for nuclear *PER* P_{NB}

In Table 1 we present the definitions of the 16 parameters of the model. Note that since all the constants in the model are positive, if any of the quantities reaches 0, then its derivative will be positive. As a consequence, for positive initial conditions, which represent the initial amount of *mRNA* or *PER* proteins, the system will always remain in the same region of phase space (where the six variables are non-negative).

Since we are interested in the stable oscillations of the *mRNA* and *PER* proteins, we will analyze in which regions of phase space limit cycles appear. As a starting point, we consider the fixed point, where all the derivatives are zero, and the system is in a stationary state with no oscillations. The study of the local stability of this fixed point will allow us to determine the regions where oscillations are possible. Since the system has only one non-linear equation, the fixed points of the dynamics amounts to finding the solutions of the polynomial equation

$$f(M)=0 \tag{2}$$

where

$$f(M)=V_s k_M+(V_s-v_M)M-\alpha M^{n+1}$$

$$\alpha=v_M\left(\frac{B_a}{K_{IA}}+\frac{B_b}{K_{IB}}\right)^n$$

$$B_a=\frac{k_s k_{NA} k_A}{v_{NA}(k_{NA}+v_A)(k_A+k_B+v_0)}$$

$$B_b=\frac{k_s k_{NB} k_B}{v_{NB}(k_{NB}+v_B)(k_A+k_B+v_0)} \tag{3}$$

For $M>0$ the function $f(M)$ has either one maximum or is monotonically decreasing. This, together with the fact that $f(0)>0$, implies that for all values of the parameters there is one and only one zero of the polynomial. In other words, the system has only one fixed point for $M>0$. Its linear stability is given by the eigenvalues of the Jacobian matrix at the fixed point (Strogatz, 1994).

It can be shown that the characteristic polynomial of the Jacobian is a sixth order polynomial with real positive coefficients. This implies that the trace of the Jacobian is negative which in turn implies that at least some of the eigenvalues must have negative real parts. Furthermore, the positivity of the coefficients also implies that the system cannot have null eigenvalues. In other words, the fixed point can only become unstable by passing through a Hopf bifurcation, where two conjugate eigenvalues cross the imaginary axes. If this bifurcation is supercritical, it leads to a limit cycle (Strogatz, 1994). In the next section we show that there are regions of parameter space where a limit cycle is indeed present. In Fig. 2 we present a parametric plot of M vs. total *PER* protein, P_T, for a typical limit cycle. The curves correspond to trajectories with exactly the same parameters, but starting from different initial conditions. The figure clearly shows the evolution of the system to a limit cycle.

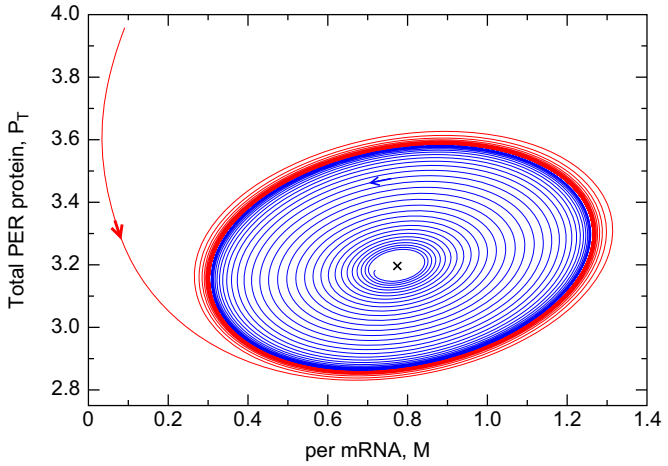

Fig. 2. *Limit cycle in the* (M,P_T) *plane*. Parametric plot of the absolute values of M vs total *PER* protein P_T. The curves correspond to trajectories with the parameters given in the caption of Fig. 3, and different initial conditions. The cross at the center of the limit cycle gives the position of the fixed point.

3. Results

3.1. Transformation between sets of parameters

Even for moderately complex models, searching the parameter space for values that correspond to a given phenotype can be an extremely demanding task. For this reason it is important to find equivalences between the different parameters in the model, or even relationships that constrain their values, in order to reduce the dimensions of parameter space. Furthermore, these equivalences and constraints might shed some light on the relationships between the processes that are being modelled.

In our case, we found that given a set of parameters for which an oscillatory solution of Eqs. (1) exists, there is a transformation that gives a new set of parameters for which the solutions are simply a rescaling of the original ones, and therefore preserving the period of the oscillations. The transformation is defined by the equations

$$v_A^*+k_{NA}^*=v_A+k_{NA},\quad v_B^*+k_{NB}^*=v_B+k_{NB}$$

$$\frac{k_{NA}^*}{K_{IA}^*}=\frac{k_{NA}}{K_{IA}},\quad \frac{k_{NB}^*}{K_{IB}^*}=\frac{k_{NB}}{K_{IB}} \tag{4}$$

The parameters which do not appear in these equations keep their values unchanged under the transformation. In the transformed system (corresponding to the starred parameters) the solutions are the same as for the original one but rescaling the nuclear abundances as follows:

$$\frac{P_{NA}^*(t)}{K_{IA}^*}=\frac{P_{NA}(t)}{K_{IA}}$$

$$\frac{P^*_{NB}(t)}{K^*_{IB}} = \frac{P_{NB}(t)}{K_{IB}} \tag{5}$$

In the transformation given by Eqs. (4) two features stand out. The first is that the parameters related to species A can be transformed independently from those of species B. The second is that, for each species, there are only two equations for three starred parameters. Thus, in principle one could set one of the starred parameters to any value, and use Eqs. (4) to find the value of the remaining two starred parameters that lead to the same solutions as for the set of unstarred parameters (up to a constant rescaling of the nuclear abundance, Eq. (5)). Evidently, this can be done as long as the value obtained for the remaining starred parameters is positive. For example, v^*_A can be set to any value between 0 and $v_A + k_{NA}$.

This transformation implies that, in this model, an increase in the rate of degradation can be compensated by a decrease of the rate of nuclear entry and of the repressing power, which in turn leads to a decrease of the nuclear abundance (Eqs. (5)). It also implies that, given a system with a given relation for two equivalent parameters of species A and B, we can find a system with the same abundances (up to a constant rescaling) for which the relation between those two parameters is reversed. For example, if we have a system with $v_A < v_B$, one can find an equivalent system with $v^*_A > v^*_B$ simply by setting $v^*_A = v_A$ and v^*_B to any value lower than v_A (which is always possible).

3.2. Numerical results

We model two different mutations by restricting the possibility of phosphorylation in one of the domains. By setting $k_B = 0$ we block domain *B*, and as a consequence only phosphorylations in domain *A* take place. We refer to this case as Mutant *A*. Mutant *B* is similarly defined, by setting $k_A = 0$. In order to see whether our simple model can account, at least qualitatively, for the experimental results in the literature, we have searched the parameter space for a set producing a system with sustained oscillations with the following properties: a period of approximately 23.5 h (to model wild type *Drosophila*), sustained oscillations with a shorter period for mutant A (i.e. setting $k_B = 0$), and sustained oscillations with a longer period for mutant B ($k_A = 0$). Biologically, this corresponds to a mutation that has rendered inactive one of the phosphorylation domains of *PER* or, equivalently, to a mutation that has rendered the *DBT* kinase unable to phosphorylate that domain. This behavior has been found both in DBT-mutants (Price et al., 1998) as well as in *PER*-mutants (Kivimäe et al., 2008).

In particular, some of the recent experimental results of Kivimäe et al. (2008) can be reproduced by our model. Using for the parameters the values given in Fig. 3, we obtain circadian oscillations with a period of 19.5 h for mutant A. Interestingly, the parameter values are of the same order of magnitude as those found in experiments with mammals (Vanselow et al., 2006) and *Drosophila* (Syed et al., 2011). For example the half lives of PER2 for mice found in Vanselow et al. (2006) are ≈ 2.5 h for the wild type and ≈ 1 h for the FASPS mutant. In the case of *Drosophila* the half lives of wild type are ≈ 1 h and ≈ 6 for the mutant DBT^{ar} and ≈ 7 for the mutant DBT^{K38R} (Syed et al., 2011). In our model the half lives (given by the inverse of the degradation rates) present values between 1 and 20.

In Fig. 3 we plot the oscillations of *mRNA* and total *PER* protein. In order to allow for a clear comparison between cases we always started from arbitrary initial conditions, then let the system evolve for 600 h (in order to reach the limit cycle), and finally set the time to $t = 0$ when the first maximum of *mRNA* was obtained. We have checked that, in general, the limit cycles of this model are rather close to Hopf bifurcations, which implies that the mean value of the oscillations of the variables is very close to the fixed point. As the figure shows only normalized values for the abundance of the proteins, in Table 2 we give the absolute values of the protein abundances at the fixed points for the wild type and two mutants, for the set of parameters used.

For the wild type, the shift between the peaks of total *PER* and *mRNA* is approximately 5 h, which is in agreement with experimental results (So and Rosbash, 1997). In the parameter set found, *PER* with domain A phosphorylated has the same properties as *PER* with domain perSD phosphorylated (Kivimäe et al., 2008): it has high repressor activity (K_{IA} is small), it is relatively unstable ($v_0 < v_B < v_A$) and is hyperphosphorylated (its rate of phosphorylation, k_A is low, as should be expected of a protein that

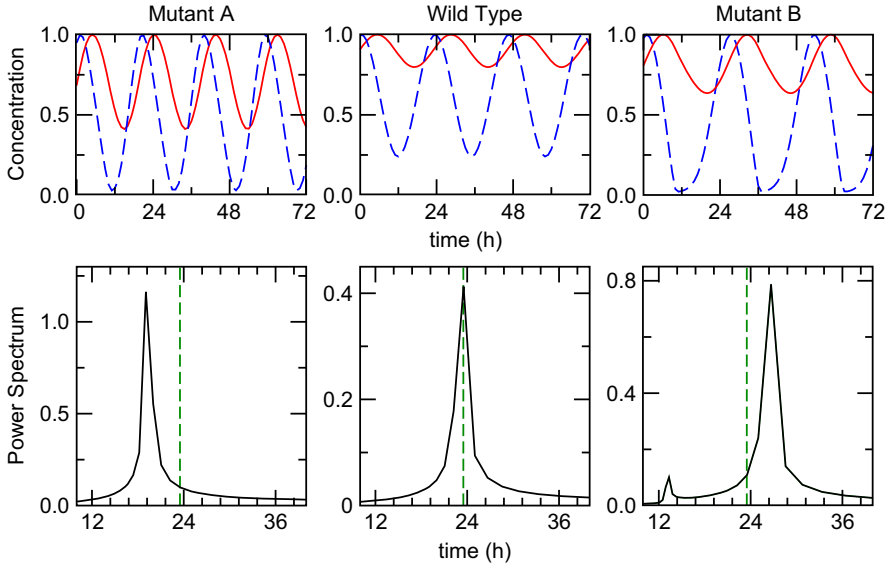

Fig. 3. *Oscillations of mRNA and total PER protein.* The top row shows the oscillations in normalized concentration of *mRNA* (dashed line) and total *PER* (continuous line) for the wild type (center figure) and mutants *A* (left) and *B* (right). The bottom row shows the power spectrum of the oscillations. The vertical dashed lines indicate the period of the wild type oscillations (23.5 h). The values of the parameters are $V_s = 1.3\ \mathrm{U\,h^{-1}}$, $K_{IA} = 0.09$ U, $K_{IB} = 2.8$ U, $n = 4$, $v_M = 0.6\ \mathrm{U\,h^{-1}}$, $K_M = 0.03$ U, $k_s = 0.22\ \mathrm{h^{-1}}$, $k_A = 0.16\ \mathrm{h^{-1}}$, $k_B = 1.6\ \mathrm{h^{-1}}$, $v_0 = 0.02\ \mathrm{h^{-1}}$, $k_{NA} = k_{NB} = 0.5\ \mathrm{h^{-1}}$, $v_A = v_{NA} = 0.95\ \mathrm{h^{-1}}$, $v_B = v_{NB} = 0.05\ \mathrm{h^{-1}}$.

Table 2
Abundances of the variables of the system at the fixed point for the wild type and mutants A and B.

Variable	Mutant A	Wild type	Mutant B
M	1.35	0.77	0.75
P_T	2.135	3.195	3.37
P_0	1.86	0.10	0.11
P_A	0.18	0.01	0.0
P_B	0.0	0.28	0.30
P_{NA}	0.095	0.005	0.0
P_{NB}	0.0	2.80	2.96

has to be phosphorylated in many sites). *PER* with domain B phosphorylated corresponds to *PER* with domain per-S phosphorylated (or maybe only with site 589 phosphorylated) and, again in agreement with experimental results (Kivimäe et al., 2008), its properties are opposite to those of *PER* with domain A phosphorylated: it has low repressor activity (i.e. K_{IB} is large), it is relatively stable, and it is hypophosphorylated (i.e. k_B is large). In turn, unphosphorylated *PER* is the most stable protein and has no repressor activity (in fact, for the sake of simplicity, we have assumed that it does not even enter the nucleus). One of the predictions of our model is that a mutation that deletes (or inactivates) domain *perSD* should increase the period. In fact, for the set of parameters shown in Fig. 3 we have obtained a period of 26.5 h for mutant B. At this point, we should stress that mutations of the kinase DBT also produce mutants with shorter and larger periods than the wild type. Interestingly, we find that the values of the periods of the mutants obtained with our model are similar to those experimentally observed, which are 18 and 27 h (Price et al., 1998). As a matter of fact, only changing the phosphorylation rates to k_A=0.16 and k_B=1.6 we obtain exactly those values.

Table 2 shows that the concentration of PER-A is much smaller than that of PER-B. The influence of PER-A, however, is far from negligible: mutant *B*, where PER-A is not present, has almost the same levels of PER-B (and therefore almost the same levels of total *PER*), but the period of the clock is almost 3 h larger. This is an effect not only of the larger power of repression of PER-A, but also of its higher rate of phosphorylation. For the same reason, the levels of nuclear PER-A (P_{NA}) and nuclear PER-B (P_{NB}) peak at very different times, as Fig. 4 shows.

Table 2 also shows that the localization of *PER* is predominantly nuclear all the time, both in wild type as in mutant *B*. On the other hand, in mutant *A* most of the protein is located in the cytoplasm. Interestingly, this difference in localization is not due to slower nuclear entry of protein *A*, as compared to protein *B*, since both of them have the same entry rate ($k_{NA}=k_{NB}$). Instead it is due to a difference in the rates of phosphorylation: protein *B* is phosphorylated much faster than protein *A* and therefore the mean time between translation and entry to the nucleus is much shorter. It must also be mentioned that the levels of PER-A in this mutant are much larger than in the wild type where, as mentioned above, its levels are very low. In principle, the differences in the localization of PER-A and PER-B could be changed by using the transformations mentioned in the previous section. There we have shown that the transformation leaves invariant the temporal properties of the model, but rescales the concentrations of nuclear PER-A and PER-B. For example changing the parameter of Fig. 3 to $k^*_{NB}=0.05$, $v^*_B=0.5$, $k^*_{NA}=1.0$, $v^*_A=0.45$, $K^*_{IA}=0.18$, $K^*_{IB}=0.28$ gives the same relation between nuclear and cytoplasmic abundances for PER-A and PER-B.

As mentioned above for *Drosophila*, it has been found (Kivimäe et al., 2008) that their analogs of phosphorylated PER-A and PER-B have very different degradation properties, a feature that our model can reproduce (i.e. we find the same phenotype using the same properties for the proteins). However, as explained in the previous subsection, a system with the same phenotype (i.e. the same abundances for the wild type and both mutants, up to a constant rescaling of the nuclear abundances) can be obtained using degradation rates that do not satisfy $v_A > v_B$. To be specific, the same phenotype is obtained by using, for example, $v^*_A=0.05$, $v^*_B=0.4$, $k_{NA}=1.4$, $k_{NB}=0.15$, $K_{IA}=0.252$, $K_{IB}=0.84$. In other words, it is not essential that $v_A > v_B$ to obtain the phenotype described above. However, it can be argued that even though the transformed system has $v^*_A < v^*_B$, the fact that $k_{NA} > k_{NB}$ compensates for this (the transformation does not change the degradation rates of the nuclear proteins).

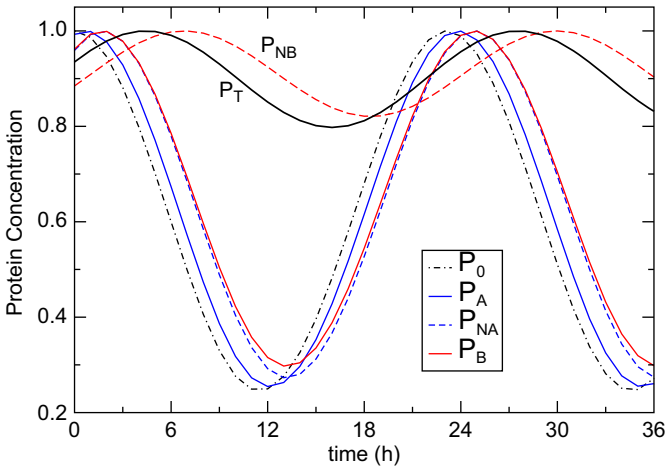

Fig. 4. *Temporal oscillations in protein concentration*. The normalized concentration of phosphorylated (P_A and P_B) and nuclear (P_{NA} and P_{NB}) protein are compared to the oscillations of total *PER* protein P_T. Parameters values are the same as in Fig. 2. Note that only P_{NB} presents an advanced phase of approximately 2 h with respect to P_T, while the others present delays up to 5 h.

3.2.1. *Identical degradation rates*

To test the hypothesis that differences in the degradation rates are not essential to produce shorter and longer period mutants we have searched the region of parameter space where all degradation rates are equal everywhere (i.e. $v_0=v_A=v_B=v_{NA}=v_{NB}$). In this case, differences in phosphorylation or nuclear entry rates do not change the degradation properties of the proteins. We have found a parameter set for which the wild type has oscillations with a period of 23.5, and to mutants with periods of 19.5 and 33 (see Fig. 5 for the values of all parameters). Interestingly, it has recently been found that for the mutant dbt^S the half life of *PER* is the same as in the wild type (Syed et al., 2011). Thus, the model of parallel phosphorylations with identical degradation rates could be used as a minimal model in this case.

There are other features of the experimental system studied by Kivimäe et al. (2008) that our model is able to reproduce. They compared the abundance of *PER* in wild-type flies and the transgenic *Drosophila* strain *per*ΔS, where the entire per-short domain has been deleted. Unexpectedly, they found that even when the abundance of *PER* in this mutant did not differ significantly from that seen in the wild-type, a higher level of *mRNA* was found. In order to test if this feature was present in our model we compared the absolute values of total *PER* protein and *mRNA* both for the wild type and mutant *A*. The left panel of Fig. 6 shows that, for the wild type, the total protein levels are much larger than the *mRNA* levels. For the mutant A (right panel of Fig. 6) there is a noticeable increase in the *mRNA* level, whereas

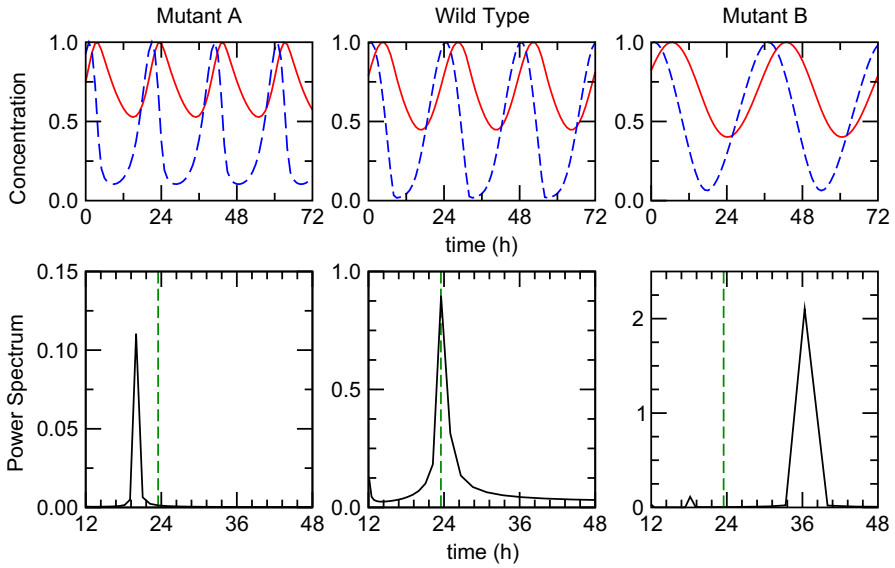

Fig. 5. *Identical degradation rates.* The top row shows the oscillations in normalized concentration of *mRNA* (dashed line) and total *PER* (continuous line) for the wild type (center figure) and mutants *A* (left) and *B* (right). The bottom row shows the power spectrum of the oscillations. The vertical dashed lines indicate the period of the wild type oscillations (23.5 h). Mutant A clearly has a shorter period (19.5 h), while mutant B has a longer period (26.5 h). The values of the parameters are $V_s = 1.3$ U h^{-1}, $K_{IA} = 0.08$ U, $K_{IB} = 3$ U, $n = 4$, $v_M = 0.9$ U h^{-1}, $K_M = 0.03$ U, $k_s = 0.16$ h^{-1}, $k_A = 0.23$ h^{-1}, $k_B = 4.0$ h^{-1}, $k_{NA} = 0.22$ h^{-1}, $k_{NB} = 0.18$ h^{-1}, $v_0 = v_A = v_B = v_{NA} = v_{NB} = 0.1$ h^{-1}.

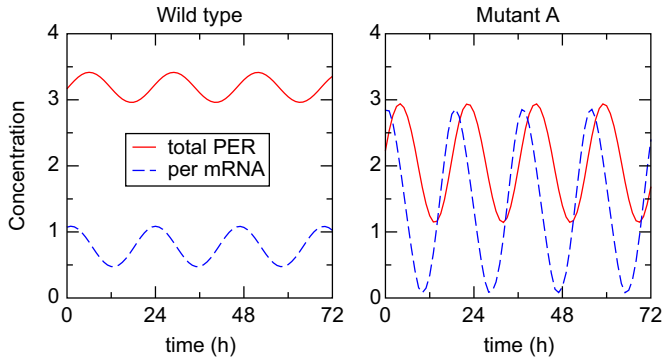

Fig. 6. Absolute values of total *PER* protein and *mRNA* for the wild-type (left) and mutant *A* (right) when the parameters of the model are set to the values of Fig. 3. A clear increase in the level of *mRNA* is observed.

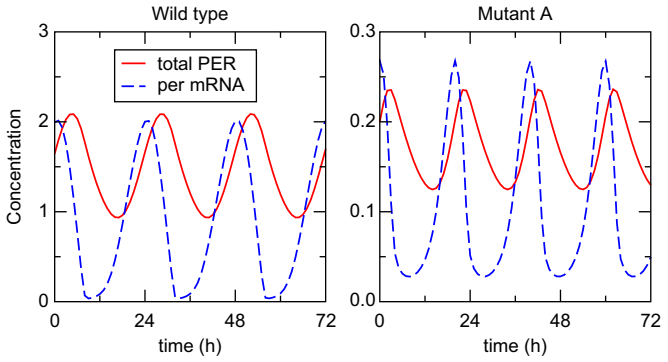

Fig. 7. Absolute values of total *PER* protein and *mRNA* for the wild-type (left) and mutant *A* (right) when all the degradation rates are identical as in Fig. 5. In both cases the ratios of the mean values of *mRNA* and total *PER* are of the same order.

the total protein displays only a slight decrease. This is qualitatively very similar to what was observed by Kivimäe et al. (2008).

It is interesting to compare these results to the case where all the degradation rates are identical, as in Fig. 5. The left panel of Fig. 7 shows that the levels of total *PER* and *mRNA* are of the same order for the wild-type. For the mutant *A* (right panel of Fig. 7) there is a marked decrease in the absolute levels of both *PER* and *mRNA* (notice the change of scale between the figures). However, the ratio between the mean values of *mRNA* and total *PER* is of the same order in both cases.

In our model the absence of the kinase DBT (or its inactivation) corresponds to setting $k_A = 0$ and $k_B = 0$. In this case, the oscillations disappear because unphosphorylated *PER* is assumed to have no repressing power (Kivimäe et al., 2008) (which is equivalent to not being able to enter the nucleus). A less trivial prediction involves the behavior of the system as the concentration of the kinase is uniformly reduced. The experimental prediction of this is that the period lengthens as the proportion of enzymatically active DBT is decreased (Muskus et al., 2007). To model this, we assume that both phosphorylation rates depend linearly on the same parameter α: $k_A = 0.16(1-\alpha)$ and $k_B = 1.6(1-\alpha)$. Fig. 8 shows that the experimental result is qualitatively recovered in our model because the period is an increasing function of α. Remarkably, the system displays oscillations for values of α very close to 1, even though by definition there are no such oscillations when $\alpha = 1$.

We have not been able to find a phenotype having oscillations of 23.5 h and mutants with shorter and longer periods, in a parameter search keeping the phosphorylation constants almost equal. Even though this does not necessarily mean that such a region does not exist, this seems to indicate that whereas a difference in degradation rates is not necessary to generate a phenotype with long and short period mutants, this might not be the case for a difference in phosphorylation rates.

3.2.2. *Influence of the Hill term*

In models of transcription of a gene it is usual to model the repression of transcription with a Hill-type term. In most cases, however, it is assumed that only one species of protein is responsible for repression. When more than one protein is involved in the repression of the same gene, the generalization of the Hill equation is not straightforward. The generalization we

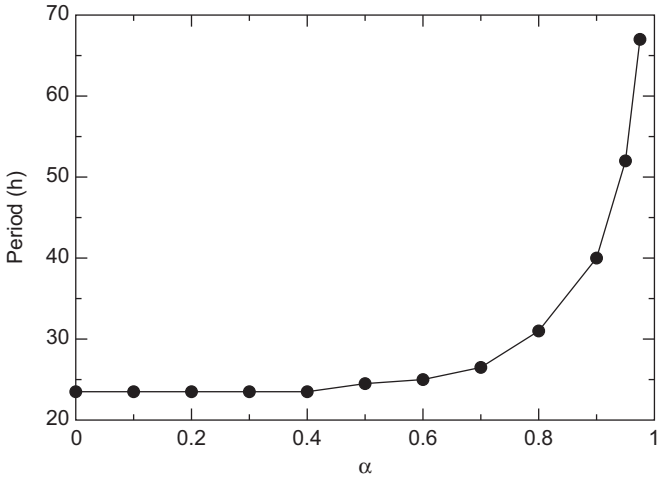

Fig. 8. *Period as a function of the simultaneous decrease of both phosphorylation rates*. The curve shows the period of the oscillations that are obtained when the phosphorylation rates are given by $k_A = 0.16(1-\alpha)$ and $k_B = 1.6(1-\alpha)$. The other parameters have fixed values and are the same as in Fig. 3.

have chosen is given by the first of Eqs. (1), but many others are possible. For instance, the evolution equation for M could be replaced by

$$\frac{dM}{dt} = \frac{V_s}{1+\left(\frac{P_{NA}(t)}{K_{IA}}\right)^n + \left(\frac{P_{NB}(t)}{K_{IB}}\right)^n} - \frac{\nu_M M(t)}{K_M + M(t)} \tag{6}$$

In terms of the usual interpretation of the Hill equation this would represent a different process. In this case the binding of the repressors PER-A and PER-B would happen in different sites, whereas in the case represented by the first of Eqs. (1) both types of *PER* would bind to the same sites. We have replaced the evolution equation for M in Eqs. (1) by Eq. (6) and we have performed a search in parameter space. Even though we have found regions where circadian oscillations are present, we have not been able to find a mutant with larger period, even allowing for different values of the exponents. Mathematically, this happens because the repression term corresponding to the influence of PER-A is much smaller than the one corresponding to PER-B in the new functional form. Thus, its contribution is larger in the functional form assumed in Eqs. (1). The biological interpretation of the fact that only the model given by Eqs. (1) agrees with the experiments is complicated by the fact that it is well known that *PER* does not repress its own transcription by directly acting on the DNA but by altering the function of the activator (*CLK–CYC*) (Lee et al., 1999). However, the difference between the phenotypes given by Eqs. (1) and (6) seems to suggest at least that the mechanisms by which PER-A and PER-B are able to cause repression in transcription are not very different.

4. Discussion

The importance of post-translational processes in the circadian clock has only recently begun to be acknowledged. The most intensively studied of these processes is phosphorylation. Until very recently, it was thought that the only effect of phosphorylation was to prime the protein for degradation. However, experiments both in mammals and in flies have made clear that, even for the same protein, phosphorylation can alter the circadian clock in very different ways. In this paper we have presented a simple mathematical model of a circadian protein that can be phosphorylated in two different domains, generically called A and B, giving rise to two variants of the protein, PER-A and PER-B respectively that have very different properties. At variance to what has been done in other models, we have assumed that phosphorylation of any of these domains precludes the phosphorylation of the other. Two different mutants are obtained by blocking each phosphorylation path. Biologically this corresponds to mutants having either mutations that render certain domains of PER impossible to phosphorylate or mutations that render the kinase DBT selectively unable to phosphorylate such domains. The model allows us to predict how changes in phosphorylations alter the macroscopic properties of the circadian clock.

The simplicity of the model allows us to show that there is always a single fixed point of the dynamics, that can only become unstable at a Hopf bifurcation which, in turn, gives rise to a limit cycle (i.e. all the variables of the system display sustained oscillations). Furthermore, we have shown that by adequately choosing the parameters our model can display a behavior that agrees with that of some recent experimental results (Kivimäe et al., 2008). In particular, there is a region in parameter space where the system displays circadian oscillations where the peaks of *mRNA* are advanced approximately by 5 h respect to the peak of total *PER*. Furthermore, a switch-like behavior of different mutations of *PER* can be obtained by changing only two parameters: by inhibiting phosphorylation of domain A the period decreases to 19.5 h, whereas the inhibition of phosphorylation of domain B leads to a period of more than 26.5 h. Thus, our model shows how a difference in the microscopic effects of phosphorylation (such as found in Kivimäe et al., 2008) can give rise to mutants with very different clock properties (as happens with mutations that affect kinases, such as *DBT*; Price et al., 1998, or phosphorylation sites of *PER*; Kivimäe et al., 2008; Chiu et al., 2008; Preuss et al., 2004).

Our model can also reproduce some other features that have been observed in the experiments. We find that in the short period mutant the increase in the level of mRNA is not matched by a proportional increase of *PER* (Kivimäe et al., 2008). We have also found that decreasing the kinase concentration leads to a progressive increase in the period of the wild type, a fact that has been observed in experiments with mutants with varying concentrations of enzymatically inactive *DBT* (Muskus et al., 2007).

We were able to find a transformation that shows how, given a set of parameters for phenotype nuclear entry, cytoplasmic degradation and nuclear repression, these parameters can be changed to obtain the same phenotype, up to a constant rescaling of the nuclear abundances of proteins. In particular, it is possible to find the same behavior for sets of parameters where $\nu_A > \nu_B$ and also for sets where $\nu_B > \nu_A$ holds. This does not show that degradation rates are not essential because the transformation does not change nuclear degradation rates. However, it has very recently (Syed et al., 2011) been found that in the short period mutant dbt^S the protein *PER* has the same half life as in the wild type. Interestingly, we have found numerically, sets of parameters where all the degradation rates are equal, that produce a wild type with a 23.5 h period and mutants with shorter and larger periods.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at http://dx.doi.org.10.1016/j.jtbi.2012.05.003.

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