**Mathematical Analysis of Robustness of Oscillations in Models**

**of the Mammalian Circadian Clock**

**Short title: Robustness of Oscillations in Models of the Mammalian Circadian Clock**

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**ABSTRACT**

Circadian rhythms in a wide range of organisms are mediated by molecular mechanisms based on transcription-translation feedback, which have been extensively studied by mathematical biologists. In this paper, we use bifurcation theory to explore properties of mathematical models based on Kim & Forger’s interpretation of the circadian clock in mammals. Their models are based on a core negative feedback loop whereby a regulatory protein (PER) binds to and inhibits its transcriptional activator (BMAL). For oscillations to occur, the dissociation constant of the PER:BMAL complex, *K*d, must be ≤ 0.03 nM, which is at least 200-fold smaller than a reasonable expectation for this protein complex. We relax this constraint by introducing two modifications to Kim & Forger’s ‘single negative feedback’ (SNF) model: first, by introducing a multistep reaction chain for posttranslational phosphorylations of PER, and second, by replacing the first-order rate law for degradation of PER in the nucleus by a Michaelis-Menten rate law. These modifications increase the maximum allowable *K*d to 300 nM. In a third modification, we consider alternative rate laws for gene transcription to resolve an unrealistically large rate of *PER* transcription at very low levels of BMAL. This modification allows for significant overexpression of PER or BMAL but not both. Additionally, we studied extensions of the SNF model to include a second negative feedback loop (involving REV-ERB) and a supplementary positive feedback loop (involving ROR). Contrary to Kim & Forger’s observations of these extended models, we find that, with our modifications, the robustness of oscillations (compared to the SNF model) is reduced by the additional negative feedback loop and increased by the additional positive feedback loop. Our results provide testable predictions for future experimental studies.

**AUTHOR SUMMARY**

The circadian rhythm aligns bodily functions to the day/night cycle and is important for our health. The rhythm originates from an intracellular, molecular clock mechanism that mediates rhythmic gene expression. It is long understood that transcriptional negative feedback with sufficient time delay is key to generating circadian oscillations. However, some of the most widely cited mathematical models for the circadian clock suffer from problems of parameter ‘fragilities’. That is, sustained oscillations are possible only for physically unrealistic parameter values. A recent model by Kim and Forger nicely incorporates the inhibitory binding of PER, a key clock protein, to its transcription activator BMAL, but oscillations in their model require a binding affinity between PER and BMAL that is orders of magnitude larger than observed binding affinities of protein complexes. To rectify this problem, we make several physiologically credible modifications to the Kim-Forger model, which allow oscillations to occur with more realistic binding affinities. The modified model is further extended to explore the potential roles of supplementary feedback loops in the mammalian clock mechanism. Ultimately, accurate models of the circadian clock will provide better predictive tools for chronotherapy and chrono-pharmacology studies.

**INTRODUCTION**

Most organisms experience perpetual day/night cycles and need to synchronize their physiological functions with this potent external driving rhythm of light and temperature (1). Endogenous circadian rhythms meet this demand. These autonomous clock-like rhythms are driven by molecular mechanisms that generate oscillations of ~24 h period through negative feedback on gene expression (1-3). Although the genes and proteins constituting the circadian clocks in animals, plants and fungi are quite different, their essential interactions are remarkably similar. In all cases, the clock mechanism features a ‘core’ negative feedback loop: *A activates B activates C inhibits A*. In mammals, this loop consists of transcriptional regulation involving six genes: *PER1/2*, *CRY1/2*, *BMAL1*, and *CLOCK* (1-4). For convenience, in this work we drop the distinction between the homologous pairs of proteins PER1/2 and CRY1/2. In this mechanism (Figure 1), the heterodimeric transcription factor BMAL:CLOCK activates *PER* transcription. *PER* mRNA is then translated in the cytoplasm, where PER protein binds with CRY and enters the nucleus. PER:CRY then binds with BMAL:CLOCK to block its activation of *PER* transcription. PER:CRY’s cycle of production, nuclear entry, auto-inhibition, and subsequent degradation is widely acknowledged to be the source of circadian rhythmicity (5).

Over the past 50 years, many people have proposed mathematical models of circadian rhythms (5-11). In 1965, Brian Goodwin proposed a model of periodic enzyme synthesis based on negative feedback on gene expression (12, 13). At the time, Goodwin was not attending to circadian rhythms, because nothing was known then about the negative feedback of PER on its own synthesis. But his model was picked up later by Peter Ruoff (14-17) to explain many characteristic features of circadian rhythms. Recently, the core negative feedback loop of Goodwin’s model was extended with other feedback loops (as in Figure 1) to create more comprehensive and realistic models of circadian rhythms (18-20). One particularly interesting modification to Goodwin’s model was made by Jae Kyoung Kim and Daniel Forger (19), who replaced Goodwin’s view—of negative feedback by cooperative binding of a generic ‘repressor’ to a gene promoter—with their own model of stoichiometric binding of PER:CRY, a repressor, to BMAL:CLOCK, an activator of gene expression. Some characteristic features of the two models have been compared in (21, 22). While all of these models have much to commend, they suffer from some technical problems (parameter ‘fragilities’) that limit their appeal.

In his model of periodic enzyme synthesis, Goodwin assumed that the end-product of a metabolic pathway functioned as an inhibitor of expression of the gene encoding the first enzyme in the pathway. The inhibition was carried out by *p* molecules of end-product binding cooperatively to the transcription factor for the gene. In this scenario the rate of transcription is given by a Hill function, , where *Z* = concentration of end-product, *α*1 = maximum rate of transcription, and *K* = end-product concentration at half-maximal rate of transcription. In the Supplementary Materials, we define Goodwin’s model precisely, discuss its basic problem (for the model to oscillate, *p* must be greater than 8, which is unreasonable), and we describe two changes to Goodwin’s model that permit oscillations for smaller values of *p*.

In the following section, we describe the Kim-Forger model and its basic problems, in order to frame our proposals for more robust and realistic mathematical models of circadian clocks. Then, in the ‘Results and Discussion’ section, we present our results in detail.

**Kim & Forger’s Model**

In 2012, Kim and Forger (19) presented a model of the negative feedback loop controlling mammalian circadian rhythms (Figure 2a). The Kim-Forger (KF) ODEs are:

Kim-Forger SNF Model.

|  |  |  |
| --- | --- | --- |
|  |  | (1) |
|  |  | (2) |
|  |  | (3) |
|  | | (4) |

SNF stands for ‘single negative feedback’ (i.e., the core negative feedback loop involving PER:CRY inhibition of BMAL:CLOCK). As originally written, the KF model has three dynamical variables: = [*PER* mRNA], = [PER protein in the cytoplasm], = [PER protein in the nucleus] (i.e., PER:CRY in the nucleus). The BMAL:CLOCK transcription factor is denoted by A; is the total concentration of BMAL:CLOCK in the nucleus, and is the concentration of ‘free’ BMAL:CLOCK in the nucleus. (The ‘hat’ on each variable indicates a concentration in nanomole/liter; and is time in hours.) The factor is the probability that BMAL:CLOCK is not bound to its repressor, PER:CRY. By expressing the rate of transcription of *PER* mRNA to be proportional to , Kim & Forger are implicitly assuming that the total number of BMAL:CLOCK dimers is large enough to saturate the E-boxes on the *PER* genes, and that PER:CRY binds equally well to BMAL:CLOCK dimers that are either bound or not bound to an E-box (Supplementary Materials, ‘Deriving the rate laws for *PER* transcription’). Equation (4) is derived by solving the condition for equilibrium binding of BMAL:CLOCK (A) and PER:CRY (P) to form an inactive complex (C); namely,

The ’s and ’s are rate constants with appropriate units of concentration and time. It is commonplace in these models to assume that , because this condition is most conducive to oscillations.

First of all, we point out a ‘homogeneity’ property of KF’s SNF model: Eqs. (1)‒(4) are unchanged under the substitutions We take this property into account by casting the equations on the left side of (1)‒(3) into dimensionless form on the right side by defining a dimensionless time, , and dimensionless concentrations, where is a relevant concentration of the binding partners, BMAL and PER (to be defined later), and is the dimensionless rate of synthesis of *PER* mRNA (in a wild-type diploid cell). The other dimensionless parameters in Eqs. (1)‒(4) are = total concentration of BMAL in the nucleus and = dissociation constant of the PER:BMAL complex. Because all the variables and parameters in Eq. (4) are scaled with the same factor , the expression for *A*free/*A*T is true for either the hatted variables (with dimensions) or the dimensionless variables. In Tables S1 and S2 we provide definitions of the variables and kinetic constants in Kim & Forger’s SNF model.

In addition to the SNF model, Kim & Forger proposed two extended models, in which the core negative feedback loop involving PER and BMAL is supplemented with (either) an additional negative feedback from REV-ERB on transcription of the *BMAL1* gene (called the NNF model, Figure 2b) (or) an additional positive feedback from ROR on transcription of the *BMAL1* gene (called the PNF model, Figure 2c). Evidences for these interactions are found in references (23-27). The ODEs of the ‘NNF’ and ‘PNF’ models are presented in the Supplementary Materials, along with definitions of the variables and kinetic constants in Tables S1 and S2.

Notice that, in the SNF model, nonlinearity in the transcription term is due to tight stoichiometric binding between PER and BMAL, not (as in Goodwin’s equations) to cooperative participation of nuclear PER in the regulation of *PER* gene expression. Consequently, the SNF model circumvents the unreasonable cooperativity constraint (*p* > 8) of Goodwin’s model. (Don’t confuse the Hill exponent, *p*, in Goodwin’s model with the concentration of nuclear PER, *P*, in the KF model.)

While the SNF model appears to oscillate robustly and avoid Goodwin’s unrealistic constraint (*p* > 8), the SNF model has some undesirable properties of its own. To elaborate, we derive an equation for oscillations to arise in the SNF model.

*Locus of Hopf bifurcations in the KF SNF model.*

The condition for a Hopf bifurcation to arise in Eqs. (1)‒(4) is

|  |  |
| --- | --- |
|  | (5) |

where *P*ss, the steady-state solution of Eqs. (1)‒(4), satisfies the equation

|  |  |
| --- | --- |
|  | (6) |

Solving Eqs. (5) and (6) simultaneously, we derive

|  |  |
| --- | --- |
|  | (7) |

and from Eq. (6) we derive

|  |  |
| --- | --- |
|  | (8) |

Substituting (8) into (7), we find, after a little algebra, the condition for a Hopf bifurcation (HB):

|  |  |
| --- | --- |
|  | (9) |

where,

|  |  |
| --- | --- |
|  | (10) |

and, for the time being, we introduce the notation and . That the condition for a Hopf bifurcation depends only on the ratios and , is a consequence of the homogeneity property of the SNF model.

Solving the quadratic equation (9), we obtain as a function of , as plotted in Figure 3a. We must locate a wild-type (WT) cell somewhere within the oscillatory domain, far enough from the HB locus so that mutant cells overexpressing or under-expressing BMAL and PER are still rhythmic. We propose that, if the point locates a WT cell on the bifurcation diagram, then the points should also lie within the oscillatory domain. We introduce this constraint because: *Bmal1*+/− and *Clock*+/− cells, i.e., are rhythmic [references?]; *Per1*−/−*Per2*+/+ and *Per1*+/+*Per2*−/− cells, i.e., are rhythmic [Cermakian, 2001; Steinlechner, 2002; Pendergast, 2010]; mouse embryonic fibroblasts (MEFs) retain rhythmicity when co-overexpressing both *Bmal1*and *Clock* up to at least four-fold [Lee, 2011, Fig 3c]; and MEFs carrying extra copies of *Per1* or *Per2*, driven by a *Per2*-promoter, also retain rhythmicity [Lee, 2011, Fig 6]. The smallest values of that satisfy these requirements are ; we choose the smallest values so as to maximize the value of *K*d, the dissociation constant of the PER:BMAL complex. We still have one degree-of-freedom left among the parameters (*K*d, *α*, *A*T); to fix their values, we choose the set (*K*d = 10−4, *α* = 2, *A*T = 0.1) to correspond to a WT cell.

The oscillatory solution of the SNF model for this set of parameter values is illustrated in Figure 3b. The dimensionless period of oscillation is 3.8, which would correspond to a 24 h rhythm if *β* = 0.16 h−1. Nuclear PER, *P*(*t*), executes nearly sinusoidal oscillations around a mean value of 0.1. The oscillations of *PER* mRNA, on the other hand, are slightly non-sinusoidal. This property of the model is not in contradiction to the evidently sinusoidal oscillations exhibited by luciferase ‘reporter’ genes driven by *PER2* promoters [Xu et al 2015] because those observations were made on populations of imperfectly synchronized cells. In Supplementary Figure Sxx we show that the *PER* mRNA oscillations reported in Figure 3b, when averaged over a population of cells with a 10% dispersion of phase, appear perfectly sinusoidal.

|  |  |
| --- | --- |
| Fig. 3a N=3 | Fig. 3b. AT=0.1, alf=2, Kd=0.0001 |
| Fig. 4a N=8 | Fig. 4b. AT=0.4, alf=2, Kd=0.01  Period = 15.5, Ptot = 0.77\*7 = 5.4 |

The oscillations in Figure 3b require *K*d << [BMAL]total ≈ [PER]nuclear, i.e., that the dissociation constant of the PER:BMAL complex is much smaller than the concentrations of the binding partners. To see why this is a problem, we must convert the dimensionless value of *K*dinto a real concentration, , using an estimate of , the scaling factor in the SNF model.

*Estimation of* *from experimental data.*

We can estimate from the fact that there is a maximum of ~30,000 molecules of PER in a mammalian cell (28); hence, . In the SNF model, cytoplasmic PER is transported into the nucleus, so the rate at which PER molecules are lost from the cytoplasm, , must equal the rate at which PER molecules are gained in the nucleus, , assuming that there is not significant degradation of PER in the cytoplasm. (For an order-of-magnitude estimation, this is a reasonable simplifying assumption.) In this case, , and . From the simulation in Figure 3b, we find that at the peak of its oscillation, and from BioNumbers (29), we find that the volume of a typical mammalian cell nucleus is ~500 fL. Hence, 400 nM. In this case, *Â*T ≈ 40 nM, and the total number of BMAL molecules in a nucleus of volume ~500 fL would be ~12,000. The observed number of BMAL molecules in a cell is ~25,000 (28), which is not too far off, considering that some fraction of BMAL molecules may not localize to the nucleus or act as functional transcription factors.

With this estimate of we find our choice of for circadian oscillations in the SNF model corresponds to . We expect the time constant for dissociation of the PER:BMAL complex to be on the order of minutes (i.e., ), because, if dissociation of the complex were much slower, then the negative feedback of PER on BMAL would react sluggishly to changes in nuclear PER concentration, and also ‘sticky’ PER binding to BMAL would disrupt PER degradation. With this estimate of the dissociation rate constant, the binding constant for the complex would have to be However, protein-protein binding rate constants are typically on the order of 106 M−1s−1 (30). So, a physically realistic, minimum value for the dissociation constant of the PER:BMAL complex is , or in dimensionless terms, *K*d ≈ 0.02. Hence, the dissociation constant we used in the SNF model, is unrealistically small by 200-fold.

Fribourgh et al. (31) recently studied the docking of PER2:CRY1/2 to the core PAS domain of BMAL:CLOCK and measured . This estimate of is likely too large because the authors used partial protein sequences. So we might reasonably assume that , or in dimensionless terms, 0.025 < *K*d < 0.25 (assuming = 400 nM).

~~In Figure 3c we illustrate how the domain of oscillations expands if we overexpress the~~ *~~PER~~* ~~and~~ *~~BMAL~~* ~~genes at constant (~~*~~φ~~* ~~= fold-overexpression of~~ *~~PER~~*~~,~~ *~~λ~~* ~~=~~ *~~A~~*~~T~~~~/0.055 = fold-overexpression of~~ *~~BMAL~~*~~). For larger values of~~ *~~λ~~*~~, SNF oscillations become more robust (i.e., they persist over a larger range of~~ *~~φ~~* ~~values), but the waveform of~~ *~~PER~~* ~~mRNA oscillations becomes increasingly non-sinusoidal (Figure 3d).~~

To summarize, we find that circadian oscillations in KF’s original SNF model require a value of the PER:BMAL dissociation constant, ≈ 0.04 nM (or smaller), that is 200-fold less than a physically realistic minimum of = 8 nM, and three orders of magnitude less than a reasonable estimate of = 40 nM. ~~Furthermore, as a consequence of the homogeneity property of the SNF model, its domain of oscillations (Figure 3c) is too robust; as~~ *~~A~~*~~T~~ ~~increases, the range of expression of the~~ *~~PER~~* ~~gene (i.e., the range of~~ *~~φ~~*~~) grows very large indeed. It is unlikely that, for fixed expression of BMAL, PER can be overexpressed several orders of magnitude.~~

In this work we consider some realistic changes to the SNF model that increase the maximum permissible value of *K*d for oscillations and address the overly robust nature of the original model. In the process, we come up with some other surprising reassessments of the KF model and its extensions.

**RESULTS and DISCUSSION**

**Longer Feedback Loop and Saturating PER Degradation Increase the Oscillatory Robustness of the Kim-Forger SNF Model.**

Our primary goal in modifying KF models is to alleviate the unreasonable constraint on *K*d, the dissociation constant of the PER:BMAL complex. To this end, we consider two changes to the SNF model: first, increasing the number of dynamical species in the PER-BMAL negative feedback loop, and second, introducing a Michaelis-Menten rate law for the degradation of nuclear PER. These same changes are known to increase the robustness Goodwin’s model (as explained in the Supplementary Materials).

Longer feedback loop. In the SNF model, there is only one intermediate (Pc) between *PER* mRNA (M) and nuclear PER protein (P). However, the primary gene transcript must be processed and exported to the cytoplasm, where it is translated into nascent PER protein, which must be phosphorylated multiple times (PER has 10-20 phosphorylation sites (32, 33)) and bound to CRY before it is transported into the nucleus. These steps insert a considerable time lag between *PER* gene transcription and the negative feedback on BMAL activity. To account for this time delay, we replace Pc in the SNF model by a sequence of species, P0 , …, P*J* (some combination of mRNA and protein intermediates), to obtain the modified ODEs:

|  |  |  |
| --- | --- | --- |
|  |  | (11) |
|  |  | (12) |
|  |  | (13) |
|  |  | (14) |

where *N* = *J*+3 is the total length of the negative feedback loop, and *A*free is still given by Eq. (4). This change lengthens the time between *PER* mRNA transcription and the negative feedback signal generated by nuclear PER and consequently increases the oscillatory potential of the negative feedback loop (34).

The longer feedback loop changes the condition for a Hopf bifurcation to arise in ODEs (11)-(14): the number ‘8’ on the left-hand-side of Eq. (5) is replaced by the number . Following the same derivation as before, we find that Eq. (9) determines *α*/*K*d as a function of *A*T/*K*d at the Hopf bifurcation, provided that

|  |  |
| --- | --- |
|  | (15) |

In Figure 4(a) we plot log(*α*/*K*d) vs log(*A*T/*K*d) for *N* = 8. Using the same argument as for *N* = 3, we place the WT cell at *α*/*K*d = 200, *A*T/*K*d = 40; or, *K*d = 0.01, *α* = 2, *A*T = 0.4. For this choice of parameter values, the oscillation is illustrated in Fig. 4b: period = 15.5, and the maximum value of *P*tot = 5.4. So, in this case we might estimate that = 100 nM/5.4 = 18 nM. However, ‘*P*tot’ includes *PER* mRNA species as well as PER protein species. So a better estimate of *P*tot might be ‘4’, in which case = 25 nM. With this estimate of , we estimate = 10 nM (3000 molecules in a nucleus of volume 500 fL; perhaps too small compared to the observed number of ~25,000 BMAL molecules) and = 0.25 nM, which is still two orders of magnitude smaller than our estimate of 35 nM for the binding of PER to BMAL.

Saturating degradation of nuclear PER. PER is degraded by proteasomes after it is poly-ubiquitinated by the E3 ligase β-Trcp (35). Because the rate of this enzyme-mediated reaction likely saturates at large substrate concentration, it is reasonable to replace the linear kinetics for degradation of nuclear PER in Eq. (3) by a Michaelis-Menten rate law (35),

|  |  |  |
| --- | --- | --- |
|  |  | (8’) |

This change also has the potential to increase the oscillatory robustness of the model. Intuitively, the upper limit to the rate of PER degradation introduced by the Michaelis-Menten rate law causes nuclear PER concentration to react sluggishly to changes in the rate of *PER* mRNA production, which is another sort of ‘lag’ in the negative feedback loop.

The introduction of ‘saturating degradation’ disrupts the ‘homogeneity’ property of KF’s original SNF model. The locus of Hopf bifurcations, for fixed *V*max and *K*m, now depends on all three parameters, *α*, *A*T and *K*d, not just on the ratios *α*/*K*d and *A*T/*K*d. For example, in Figure 5a we show that (for *N* = 8, *V*max = 0.4, *K*m = 0.5) the oscillatory domain in the *α*, *A*T plane is bounded and shrinking for increasing values of *K*d. If *K*d > 0.05, the domain of oscillations is too small to be consistent with our requirements for over- and under-expression of PER and BMAL. If we assign a WT cell to the parameter set *K*d = 0.05, *α* = 3, *A*T = 3, then the ODEs give oscillations in Figure 5b, with period = 29 and Ptot = 8 (≈ 5 when corrected for mRNA species). Hence, we estimate that = 100 nM/5 = 20 nM and = 1 nM, which is still considerably smaller than our estimate of 35 nM for the binding of PER to BMAL.

|  |  |
| --- | --- |
| Kd = 0.01 | Kd = 0.02 |
| Kd = 0.05 | Kd = 0.07 |
| Figure 5 a. These curves can be merged onto one panel, by plotting log(AT) vs log(alpha) | |

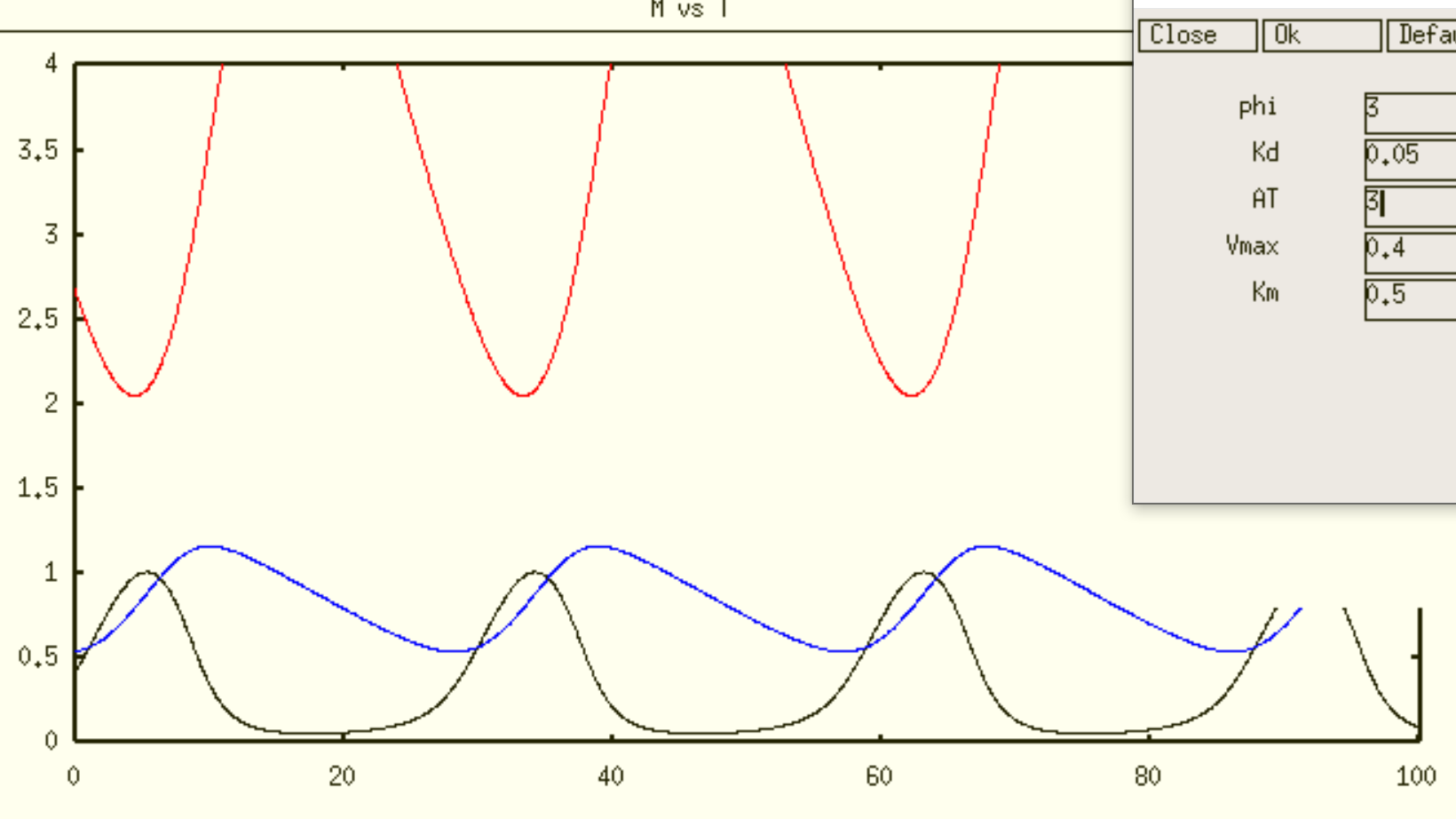


Figure 5 b. Oscillations for WT cell.

|  |  |
| --- | --- |
| Kd = 0.04; Vmax = 0.4, Km = 0.1; AT -> zero… | Kd = 0.01; Vmax = 0.4, Km = 0.2; AT -> zero… |
| Figure 5 c. Use just one of these. | |

~~and our ‘~~*~~N~~*~~-component feedback loop’ , then the maximum value of~~ *~~K~~*~~d~~ ~~permissible for oscillations increases considerably; for example, for~~ *~~φ~~* ~~= 1,~~ *~~β~~* ~~= 4,~~ *~~K~~*~~m~~ ~~= 0.1,~~ *~~K~~*~~d~~ ~~may be as large as 0.6 for~~ *~~N~~* ~~= 8 (Figure 3b). However, given our standard requirement that heterozygous diploid and homozygous tetraploid cells be rhythmic, we estimate that a ‘WT cell’ might be associated with the parameter set:~~ *~~N~~* ~~= 8,~~ *~~K~~*~~d~~ ~~= 0.04,~~ *~~A~~*~~T~~ ~~= 0.4,~~ *~~φ~~* ~~= 1,~~ *~~β~~* ~~= 4,~~ *~~K~~*~~m~~ ~~= 0.1. In this case, the WT cell oscillates with a period of 21 and~~ *~~P~~*~~tot~~ ~~= 4.7 (= 3.3 when corrected for mRNA species), from which we estimate~~ *~~P~~*~~\* ≈ 30 nM, and = 1.2 nM, which is a five-fold improvement over the~~ *~~N~~* ~~= 8 model with linear degradation of PER.~~

A curious feature of the doubly-modified SNF model is illustrated in Figure 5c: for certain parameter sets, the model exhibits oscillations even as *A*T → 0, which is clearly impossible because there can be no expression of the *PER* gene when BMAL concentration is zero. The problem, of course, is that the rate law for *PER* transcription (rate *A*free/*A*T) is valid only if BMAL saturates *PER* E-boxes, which clearly cannot be true as *A*T → 0. To get around this problem, we consider some other potential rate laws for *PER* gene transcription (see Supplementary Materials, ‘Deriving the rate laws for *PER* transcription’).

STOPPED HERE. Do we like this approach???

**Modifying the *PER* Transcription Rate Law Increases the Robustness of the SNF Model Still Further.**

We consider two slightly different rate laws to replace the KF expression for the rate of *PER* gene transcription:

|  |  |  |
| --- | --- | --- |
| Rate Law 0: |  | (9-0) |
| Rate Law 1: |  | (9-1) |
| Rate Law 2: |  | (9-2) |

Rate law 0 is, of course, the original KF expression. When *A*free = *A*T, the maximum rate of transcription is *φ*. (In our scaling of the differential equations, the maximum rate of transcription of *PER* mRNA = *φ* = 1 for WT homozygous diploid cells, and the rate increases or decreases with *φ* for cells over- or under-expressing *PER* mRNA, either by manipulating *PER* gene dosage or the strength of the *PER* gene promoter.) For rate laws 1 and 2, the maximum rate of transcription is , i.e., the maximum rate depends on how strongly BMAL:CLOCK binds to the E-box, as characterized by the dissociation constant *K*A. Rate laws 1 and 2 also imply that the transcription rate is proportional to *A*free (not *A*free/*A*T) when *A*T becomes small. These three rate laws represent different limiting cases of BMAL:CLOCK binding to E-boxes, as explained in detail in the Supplementary Materials (‘Deriving the rate laws for *PER* transcription’). Recall that the KF rate law 0 applies to the case in which binding between PER:CRY and BMAL:CLOCK is independent of the binding between BMAL:CLOCK and E-box, and BMAL:CLOCK complexes saturate *PER* E-boxes. Rate law 1 is a slight modification of the KF rate law 0, relaxing the saturation of *PER* E-boxes by BMAL:CLOCK. Finally, rate law 2 is based on a different model of the regulation of *PER* transcription, where PER:CRY binds tightly to BMAL:CLOCK, and only the free form of BMAL:CLOCK binds to the E-box. Electron microscopy studies of Aryal et al. (36) indicate that PER:CRY::BMAL:CLOCK complexes are bound to E-boxes, suggesting that rate law 2 may be less appropriate than rate laws 0 and 1. Nonetheless, we study all three possibilities.

**Modified Kim-Forger SNF equations.** Taking all of the aforementioned changes into account, we have:

|  |  |  |
| --- | --- | --- |
|  |  | (10) |
|  |  | (11) |
|  |  | (12) |
|  |  | (13) |
|  |  | (14) |

where , and . (15)

To keep track of all these different versions, we introduce the notation SNF(TDN), where T denotes the *PER* transcription rate law (0, 1 or 2), D denotes the PER degradation rate law (L for linear or M for Michaelian), and N denotes the number of species in the negative feedback loop. For example, the original KF model is denoted SNF(0L3).

We have already shown the two-parameter bifurcation diagram (log *K*d versus log *A*T) for SNF(0LN) and SNF(0MN) in Figures 3a and b. In the remaining panels of Figure 3 we plot the corresponding bifurcation diagrams for SNF(1LN), SNF(1MN), SNF(2LN) and SNF(2MN). These diagrams show that our SNF(1MN) models are more robust than the original SNF(0LN) model in that oscillations are now possible for *K*d as large as 0.6. Applying again our requirement that heterozygous diploid and homozygous tetraploid cells be rhythmic, we estimate that a ‘WT’ SNF(1M8) model might be associated with the parameter set: *N* = 8, *K*d = 0.05, *A*T = 1, *φ* = 1, *β* = 2, *K*m = 0.1, *K*A = 0.1. In this case (see Supplementary Figure S1), the maximum value *P*tot is ~3.5 (corrected value ~2.5 if P0 and P1 are mRNA species), so 40 nM, and 2 nM, which is close to the range of dissociation constants that we have estimated for PER:BMAL binding. Moreover, 40 nM ≈ 13,000 BMAL molecules in a nucleus of volume 500 fL. If the remaining 12,000 molecules of BMAL are dispersed through a cytoplasm of volume 5000 fL, the cytoplasmic concentration of BMAL would be 4 nM, which is believable for a ‘nuclear’ protein like BMAL.

From Figure 3, it appears that saturating degradation of nuclear PER is crucial for permitting oscillations for *K*d > 0.01. Figure 3f indicates that transcription rate law 2 is only marginally oscillatory for *K*d = 0.1 and which is another reason to prefer rate law 1 over rate law 2.

**An Additional Positive Feedback Loop Involving ROR Increases the Domain of Oscillations.**

Next, we explore Kim & Forger’s NNF and PNF models (see Supplementary Material), with modified rate laws for gene transcription. For the rates of transcription of *PER*, *rev-erb* and *ror* genes governed by BMAL:CLOCK binding to E-boxes, we consider all three rate laws 0, 1, 2. For the transcriptional activation and repression of the *BMAL* gene by ROR (variable *R*) and by REV-ERB (variable *V*), respectively, we replace the functions *γR* and *γ/V* (as originally proposed by Kim & Forger) by *A*MAX∙*R*/(*R*+*K*R) and *A*MAX/(*V*+*K*V), where *K*R and *K*V are the dissociation constants for ROR and REV-ERB binding to the promoter (RORE) of the *BMAL* gene. These new rate laws remedy an issue in KF’s original PNF and NNF models, for which the rate of BMAL synthesis does not saturate as *R*→∞ or *V*→0.

**Modified Kim-Forger NNF model.** Equations (10)-(14) plus

|  |  |  |
| --- | --- | --- |
|  |  | (16) |
|  |  | (17) |

**Modified Kim-Forger PNF model.** Equations (10)-(14) plus

|  |  |  |
| --- | --- | --- |
|  |  | (18) |
|  |  | (19) |

In Eqs. (16) and (18), the parameter *λ* is the gene dosage of *BMAL1*, relative to homozygous diploid, *λ* = 1.

In Figure 4a we compare the ‘robustness’ of models SNF(0L8), NNF(0L8), and PNF(0L8) by plotting the regions where each model oscillates with respect to fold changes in *BMAL* (*λ*) and *PER* (*φ*) transcription rates. In panel (a) we set *K*d = 10−3, so that all three models exhibit oscillations for N = 8. Figure 4a is comparable to Figure 5a in the Kim-Forger paper (19), and the major conclusions are the same: oscillatory robustness of the model is increased by the additional negative feedback loop and decreased by the additional positive feedback loop. Notice that these ‘0L8’ models oscillate for any values of *φ* > 0.1 and *λ* > 0.1, provided (e.g., for SNF(0L8), *a*min ≈ 0.015 and ≈ 1.8). This condition of ‘stoichiometric balance’ for oscillations in KF models is consistent with the evidence of some experimental observations (37), but it seems unlikely that oscillations would persist for arbitrarily large levels of overexpression of BMAL and PER.

In Figure 4b,c,d, we compare the robustness of SNF, NNF and PNF for the ‘0M8’, ‘1M8’ and ‘2M8’ models to get an idea of how our modifications change the robustness properties of feedback loops. For these models, we choose *K*d = *K*m = *K*A = 0.1, so that the dissociation constants, , are biophysically reasonable values. In all three model types, ‘fold change in PER transcription’ is just a descriptive name for parameter *φ*. For SNF models, ‘fold change in BMAL transcription’ = *λ* = *A*T/0.3 (which is to say *A*T = 0.3 for a homozygous diploid cell). For NNF and PNF models, *λ* is defined in Eqs. (16) and (18), and *A*MAX is adjusted so that the average value of *A*T(*t*) over the course of an oscillation = 0.3 (the fixed value of *A*T for the SNF model).

Comparing panels (a) and (b), we see that our ‘0M8’ models are less robust than the ‘0L8’ models with respect to overexpression of the *PER* gene. ‘0M8’ models, unlike ‘0L8’ models, no longer mandate a stoichiometric balance between *BMAL* and *PER* expression. On the contrary, ‘0M8’ models oscillate over a restricted range of *PER* transcription rates (0.5 < *φ* < 5, approximately) and a wide range of *BMAL* transcription rates (0 < *λ* < 10 or more). Oscillations in both ‘0L8’ and ‘0M8’ models depend on the accumulation of enough nuclear PER to turn off *PER* transcription followed by clearance of enough PER to revive *PER* transcription. For a given level *λ* of BMAL transcription in the system, a *PER* transcription level *φ* too low never generates enough PER to inhibit BMAL and turn off *PER* transcription. Even though *PER* transcription rate is maximal, PER abundance is low because *PER* gene dosage is small. On the other hand, if *PER* transcription level *φ* is too high, PER cannot be cleared from the nucleus fast enough to revive *PER* transcription. Although *PER* transcription rate is small, PER abundance is high because *PER* gene dosage is large.

In ‘0L8’ models, with linear degradation of nuclear PER, the concentrations of all PER species scale linearly with *φ*, the rate of *PER* transcription. As the level of BMAL scales up or down, the level of nuclear PER must scale up or down as well, with *φ* proportional to *λ*. For this reason, both the upper and lower boundaries of the oscillatory regions of ‘0L8’ models are straight lines in the *φ*‒*λ* plane (except in the lower-left corner). In this manner, ‘stoichiometric balance’ is built into ‘0L8’ models. (Similar reasoning applies to all ‘0LN’ models.)

In ‘0M8’ models, however, with Michaelis-Menten degradation, nuclear PER concentration no longer scales linearly with *φ*, the rate of *PER* transcription. Furthermore, the oscillations we observe in Figure 4(b) must often be operating in the region of limiting rate of PER degradation (*P* > *K*m) because otherwise ‘0M8’ models become ‘0L8’ models, which do not oscillate for *K*d = 0.1. Consider a simplified model, . If then the maximum rate of PER accumulation can never exceed the saturating rate of PER degradation, so PER settles into a stable steady state of low PER abundance. If then the leaky transcription of *PER*, , is apparently strong enough to balance the saturating degradation rate, , and the system stabilizes with a high PER level, . Between these limits, 0.4 < *φ* < 5 (approximately), the ‘0M8’ model is able to adjust PER synthesis and degradation to oscillate over a broad range of *A*T values.

~~Eq. (4) implies that when , approaches , which does not depend on . Hence, the balance between PER synthesis and degradation has little dependence on , and mandates roughly the same~~ *~~PER~~* ~~transcription level over a broad range of BMAL level. Taken together, the Michaelis-Menten degradation of PER in ‘0M8’ models significantly changes the characteristics of oscillation robustness.~~

In addition, we note that, in contrast to ‘0L8’ models, oscillatory robustness of ‘0M8’ models is decreased by the additional negative feedback loop and increased by the additional positive feedback loop. Finally, we observe that oscillations in all three ‘0M8’ models persist as *A*T → 0, for 0.7 < *φ* < 4.4, which is an annoying property of ‘0MN’ models for N sufficiently large.

This problem is fixed in ‘1M8’ models (Figure 4c). Furthermore, in this case, oscillations are now possible for large values of *φ* (multiple copies of the *PER* gene) as long as *λ* (multiple copies of the *BMAL* gene) is small enough, because the rate of *PER* gene transcription, , scales (with *λ*) like , as . Hence, oscillations are maintained as provided constant. Notice that, although oscillations are possible for either *φ* large or *λ* large, ‘1M8’ models to not support oscillations when both *φ* and *λ* are large, which contradicts the principle of ‘stoichiometric balance’ in ‘0LN’ models.

Lastly, Figures 3f and 4d show that ‘2M8’ models (Figure 4d), for *K*d = *K*m = *K*A = 0.1, are not robust with respect to overexpression of BMAL. Rate law 2 posits that BMAL cannot bind simultaneously to PER and E-box. Because neither binding reaction is super-strong, there will always be some fraction of active BMAL transcription factor bound to the E-box of the *PER* gene. Hence, if BMAL protein is overexpressed, then *PER* gene transcription cannot be shut down, and the system stabilizes at a steady state with abundant levels of both PER and active BMAL.

Although the PNF(1M8) model appears to be the most ‘robust’ of all the models in Figure 4 (we disregard the ‘0L8’ models because they do not oscillate at all for *K*d = 0.1), we show in the next section that the PNF(1M8) model is not robust with respect to 24 h oscillations.

**The NNF(1M8) Model Is Most Robust with Respect to Circadian Oscillations.**

Figure 5 redraws the bifurcation plots for the ‘1M8’ models in Figure 4c with colors to indicate oscillatory periods. To convert from dimensionless period *τ* to period in hours, we set of *β*1 = 5/6h, a value that yields a period of ~24 h for the models with their default parameter values (Table S4), in particular, *λ* = *φ* = 1 (Figure S1). For each model the oscillatory period varies over a characteristic range. For SNF(1M8) the period varies from 19 to 36 h, but the range of circadian rhythms (say, 22‒26 h) is quite limited. The NNF(1M8) model is more robust with respect to circadian periodicity. The PNF(1M8) model is much less robust, exhibiting very slow oscillations (up to 45 h) for modest overexpression of BMAL and PER. The broad distribution of oscillatory periods in the PNF model is a common feature of models that combine positive and negative feedback loops (34, 38). The NNF model has the nice property that oscillatory period is relatively insensitive to fold changes in BMAL expression, as observed in Supplementary Figure 1e of Xu et al. (39). All models predict that oscillatory period should be much more sensitive to fold-changes in PER expression than BMAL expression.

**CONCLUSION**

The Kim-Forger (KF) models of mammalian circadian rhythms (called SNF, NNF and PNF) are appealing in many respects; but they rely on an unrealistic requirement for robust oscillations, namely that the (dimensionless) equilibrium dissociation constant of the PER:CRY::BMAL:CLOCK complex must be *K*d < 10−4. To understand why this value is ‘unrealistic’, we must convert *K*d into dimensioned terms, , where *P*\* is the ‘characteristic’ concentration for PER in KF’s SNF model, i.e., *P*\* = 500 nM, which we estimated from experimental data. Hence, < 0.05 nM, which is 200-fold smaller than a reasonable value for the PER:CRY::BMAL:CLOCK complex, > 10 nM. This difficulty can be ameliorated (1) by lengthening the core negative feedback loop between *PER* mRNA transcription and PER:CRY inactivation of BMAL:CLOCK (the transcription factor driving *PER* expression), and/or (2) by implementing a Michaelis-Menten rate law for the degradation of nuclear PER. The KF models were further modified by introducing alternative rate laws for BMAL:CLOCK-mediated transcription of clock genes (*PER*, *REV-ERB* and *ROR*) to correct a problem at low expression of the *BMAL* gene, and to provide more accurate rate laws for the effects of REV-ERB and ROR on *BMAL* expression.

With these modifications, we find (Figure 3d) that the SNF(1M8) model can exhibit oscillations for *K*d ≥ 0.1; e.g., for *K*d = 0.3 the model oscillates over a six-fold range of total BMAL, 0.2 < *A*T < 1.2. To convert these dimensionless parameters into concentrations in nM, we must re-compute *P*\* = 30 nM for the SNF(1M8) model. In this case, then, can be as large as 9 nM, for 6 nM < *Â*T < 36 nM. These order-of-magnitude estimates accord reasonably well with our estimated value of the dissociation constant of the PER:CRY::BMAL:CLOCK complex, 10 nM < < 100 nM (31). For *Â*T = 30 nM, the corresponding number of BMAL proteins in a nucleus of volume 500 fL would be , which is about half the observed number (~24,000) of BMAL molecules in a mammalian cell (28). If the remaining BMAL molecules are dispersed through the cytoplasm of volume 5000 fL, the cytoplasmic concentration of BMAL would be about one-tenth the nuclear concentration, which is not unreasonable for a ‘nuclear’ protein such as BMAL. Furthermore, the model focusses on BMAL:CLOCK complexes that bind E-boxes to regulate gene expression. BMAL in this form may account for only a fraction of total BMAL, if BMAL, like PER, undergoes multi-step post-translational modifications. Indeed, both BMAL and CLOCK are known to be phosphorylated at multiple sites, which affects their stability, nuclear accumulation, and activity of the BMAL:CLOCK complex (40-42).

Replacing the linear rate law for nuclear PER degradation by a Michaelis-Menten rate law causes a dramatic change in the sensitivity of oscillation to the expression levels of PER and BMAL (Figure 4). Models with linear PER degradation predict that oscillations are possible over a very broad range of ‘stoichiometric balance’ between the rates of PER and BMAL expression (denoted *φ* and *λ*, respectively); e.g., in Figure 4a, 0.02 < *λ*/*φ* < 2 (approximately). On the contrary, models with Michaelis-Menten degradation predict that oscillations are favored by a divergent trend between *λ* and *φ*; e.g., Figure 4c. These contrasting results provide a testable prediction for future experimental exploration. By overexpressing *PER/CRY* genes and/or *BMAL/CLOCK* genes under control of their normal (regulated) promoters (i.e., by manipulating *φ* and *λ*), one could test whether nuclear PER degradation operates in a saturated (Michaelis-Menten) or unsaturated (linear) manner, which would be difficult to measure directly *in vivo*. In the same experiment, by measuring the dependence of oscillation period on *φ* and *λ*, one could test a second robust prediction of our models (Figure 5) that period length is much more sensitive to *φ* than to *λ*.

The single negative feedback loop (SNF), whereby PER inhibits its own synthesis, can be supplemented with an auxiliary positive feedback from ROR (PNF) or a negative feedback from REV-ERB (NNF) on the synthesis of BMAL. For their versions of these three models, Kim & Forger observed a ‘robustness trend’ NNF > SNF > PNF, in terms of the size of the oscillatory domain in parameter space. For our versions of these models, we observed the opposite trend. If we define ‘robustness’ in terms of the size of the domains of circadian oscillations (22-26 h) in parameter space, then the trend is NNF > SNF ≈ PNF.

Our models could be employed in the future to explore other features of the mammalian circadian clock. For instance, following the lead of Kim and colleagues (43, 44), we could address our models to the circadian clock’s temperature-compensation and/or phase-shifting properties. Adding these key features may answer some remaining questions about the behaviors of these models. Another question that could be addressed with these models is the function of an anti-sense transcript of the PER2 gene (45). Furthermore, these models could be applied in chronopharmacology and chronotherapy studies (46). One such application would be modeling PER2’s interaction with the tumor suppressor protein p53 in stressed (e.g., DNA damage) cells compared to un-stressed cells (47, 48).

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