

Article

An inactivation switch enables rhythms in a *Neurospora* clock model

Abhishek Upadhyay^{1*} , Michael Brunner² and Hanspeter Herzel^{1,*} ¹ Institute for Theoretical Biology, Charité – Universitätsmedizin Berlin and Humboldt University of Berlin, Philippstr. 13, 10115, Berlin, Germany; abhishek.upadhyay@charite.de, hans-peter.herzel@charite.de² Biochemistry Center, University of Heidelberg, Im Neuenheimer Feld 328, 69120, Heidelberg, Germany; michael.brunner@bzh.uni-heidelberg.de

* Correspondence: abhishek.upadhyay@hu-berlin.de, h.herzel@biologie.hu-berlin.de; Tel.: +30 2093 98402

Abstract: An autonomous endogenous time-keeping is ubiquitous across many living organisms known as circadian clock when it has a period of about 24 hours. Interestingly, the fundamental design principle with a network of interconnected negative and positive feedback loops is conserved through evolution, although the molecular components differ. Filamentous fungus *Neurospora crassa* is a well established chrono-genetics model organism to investigate the underlying mechanisms. The core negative feedback loop of the clock of *Neurospora* is composed of the transcription activator White Collar Complex (WCC) (heterodimer of WC1 and WC2) and the inhibitory element called FFC complex which is made of FRQ (Frequency protein), FRH (Frequency interacting RNA Helicase) and CK1a (Casein kinase 1a). While exploring their temporal dynamics we investigate how limit cycle oscillations arise and how molecular switches support self-sustained rhythms. We develop a mathematical model of 10 variables with 26 parameters to understand the interactions and feedback among WC1 and FFC elements in nuclear and cytoplasmic compartments. We performed control and bifurcation analysis to show that our novel model produces robust oscillations with a wild-type period of 22.5 hrs. Our model reveals a switch between WC1 induced transcription and FFC assisted inactivation of WC1. Using the new model we also study possible mechanisms of glucose compensation. A fairly simple model with just 3 nonlinearities helps to elucidate clock dynamics revealing a mechanism of rhythms production. The model can further be utilized to study entrainment and temperature compensation.

Keywords: Circadian clock, Mathematical modelling, Molecular switch, *Neurospora crassa*, Glucose compensation

1. Introduction

The earth's rotation around its own axis gives rise to the 24 hours day and night cycles. To anticipate these daily environmental changes in light and temperature, life in all its kingdoms has evolved a time keeping molecular machinery [1–3]. In humans, disruption in the clock functioning due to jet lag, shift work, light at night or other reasons increases the risk of diseases such as cancer, obesity and sleep disorders [4–6].

In order to understand basic clock mechanisms we study a filamentous fungus, *Neurospora crassa* (*N. crassa*) [7]. Their natural habitats are soils, plants, trees and food resources [8]. The ability of *N. crassa* to deconstruct and metabolize plant cell walls is crucial for environmental carbon and other nutrient cycling. There are also various potential biotechnological applications such as the production of biofuels. An example is the production of ethanol from xylose derived from plant dry matter biomass (lignocellulosic substrates). The xylan to ethanol pathway has been found to be clock regulated at each stage [9].

Mathematical modelling is useful to understand the *Neurospora* clock mechanism with multiple known feedback loops [10–15]. There are earlier models which considered only the core clock gene *Frequency* (FRQ) [10–12]. Recently, transcription factor White Collar-1 (WC1) has also been incorporated into the models [13–15]. In order to study glucose compensation, another feedback via Conidial Separation-1 (CSP1) [16] was included.

Figure 1 demonstrates the transcription-translation feedback loop which consists of the inhibitory protein Frequency (FRQ), the activating transcription factor White Collar Complex (WCC), the FRQ stabilizer Frequency-interacting Helicase (FRH), and the Casein Kinase-1a (CK1a). Oscillations are based on delayed negative feedback. In *Neurospora*, clock gene *frequency* inhibits its own transcription after intermediate steps such as transcription, translation, dimerization, phosphorylations and nuclear import. Moreover, stabilization of FRQ is governed by FRH and CK1a binding in forming the FFC complex. Later, FFC is involved in the inhibition of WCC induced transcription [17–19].

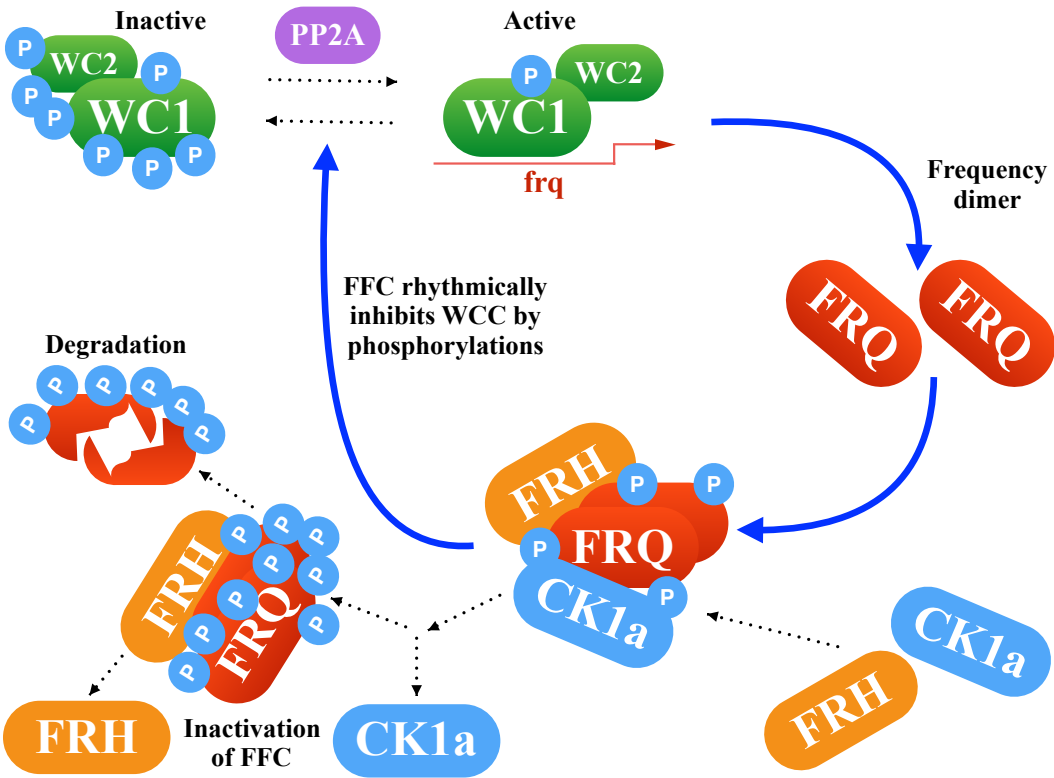


Figure 1. Core network of *Neurospora* clock: The delayed negative feedback via FRQ is controlled by complex formations and phosphorylations.

The detailed mechanisms of the negative feedback are not well understood. For example, the inhibition of WCC via FFC might involve hyperphosphorylation, sequestration and stoichiometric inhibition [20–24]. Moreover, it is not clear how the long delay is realized to obtain daily rhythms. In order to obtain self-sustained oscillations nonlinearities are necessary.

The focus of our study is a deeper understanding of the underlying nonlinearities ("switches") and an analysis of possible intrinsic mechanisms of glucose compensation in the *Neurospora* clock. Thus, our model addresses two general questions: (1) What are the underlying switch mechanisms? (2) How can a steady period be maintained for varying environmental conditions such as glucose availability? A

comprehensive analysis of our mathematical model reveals that an inactivation switch allows self sustained rhythms in the *Neurospora* clock. Our model also suggests in-built glucose compensation mechanisms.

2. Results

2.1. Modelling the core clock elements

The negative feedback loop involves an implicit delay consisting of frq transcription and translation, FRQ dimerization, CK1a binding, nuclear import, formation of the FFC complex, and FFCn interaction with WC1n. A positive feedback involves the nuclear export of FFC supporting WC1c translation [25,26]. It is well known that FRQ and WCC are phosphorylated at many sites. Rhythmic phosphorylation influences complex formation and stability [27,28]. In our current model we take these phosphorylations implicitly into account via appropriate rate constants.

Figure 2A shows the turnover of frq, wcc and associated negative and positive feedback loops. Using primarily linear kinetic terms this scheme can be directly translated into a system of ordinary differential equations (ODEs) [29–32]. These equations are presented in Figure 2B. The 10 time-varying concentrations are denoted with brackets and 26 kinetic parameters are enumerated as a01, a02, a1, a2,, a21, n, K1, K2(Appendix A1).

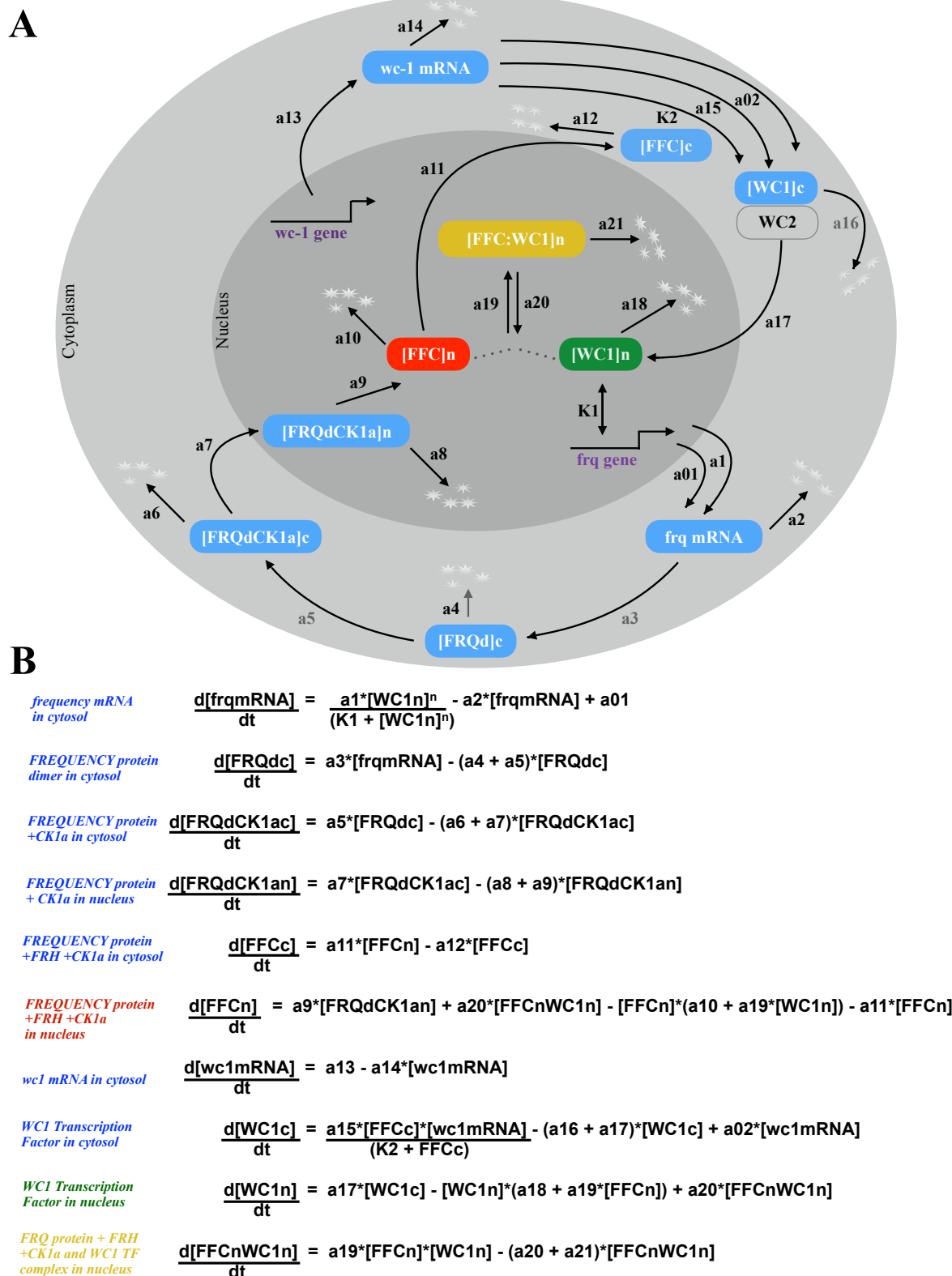


Figure 2. *Neurospora* circadian clock model: Wiring diagram of the model shows compartmentalization into nucleus and cytoplasm, turnover of *frq* and *wc1* coloured in blue, complex formations and nuclear translocation. The core components of the inactivation switch are marked by green, red and yellow throughout the paper (Fig 2A). Ordinary differential equations with 10 variables and 26 parameters (Fig 2B).

2.2. The model reproduces self sustained rhythms

The basic scheme of the model is adapted from a previous study [15]. In order to study the role of FRH and FFC, we introduce 3 additional variables [FRQdCK1a]c, [FRQdCK1a]n and [FFC]c (see Figure 2). The corresponding 5 parameters (a4, a6, a8, a10, a12) were taken from the proteins half-lives in associated experiments [27,33]. In order to adapt expression levels we increased frq transcription rate (a1) and translation rate (a3, a15) (see Appendix A1).

We kept most parameter values from the original model [15] (compare Appendix A1). The remaining 2 parameters corresponding to the FRQdCK1a and FFC complex formation rates (a5 and a9) were adjusted to get a period of 22.5 hours and robust limit cycle oscillations. Moreover, our model includes a stronger positive feedback on cytoplasmic WC1 by FFC. We decreased the activation threshold from $K2 = 1$ in [15] to $A2 = 0.3$ in our model to strengthen the experimentally verified positive feedback [25] (compare Appendix A1).

Our resulting model consists of 10 ordinary differential equations (ODEs) and 26 kinetic parameters. In order to get self-sustained rhythms nonlinearities are necessary. Inspection of the equations in Figure 2B reveals that there are just 3 nonlinear terms: The transcription of the gene frq is modeled via a quadratic Hill function representing multiple WCC binding sites. The complex formation of FFC and WC1 is described by a bilinear term. The third nonlinearity models the positive feedback of the FFCc complex on WC1c translation as in the original model [15].

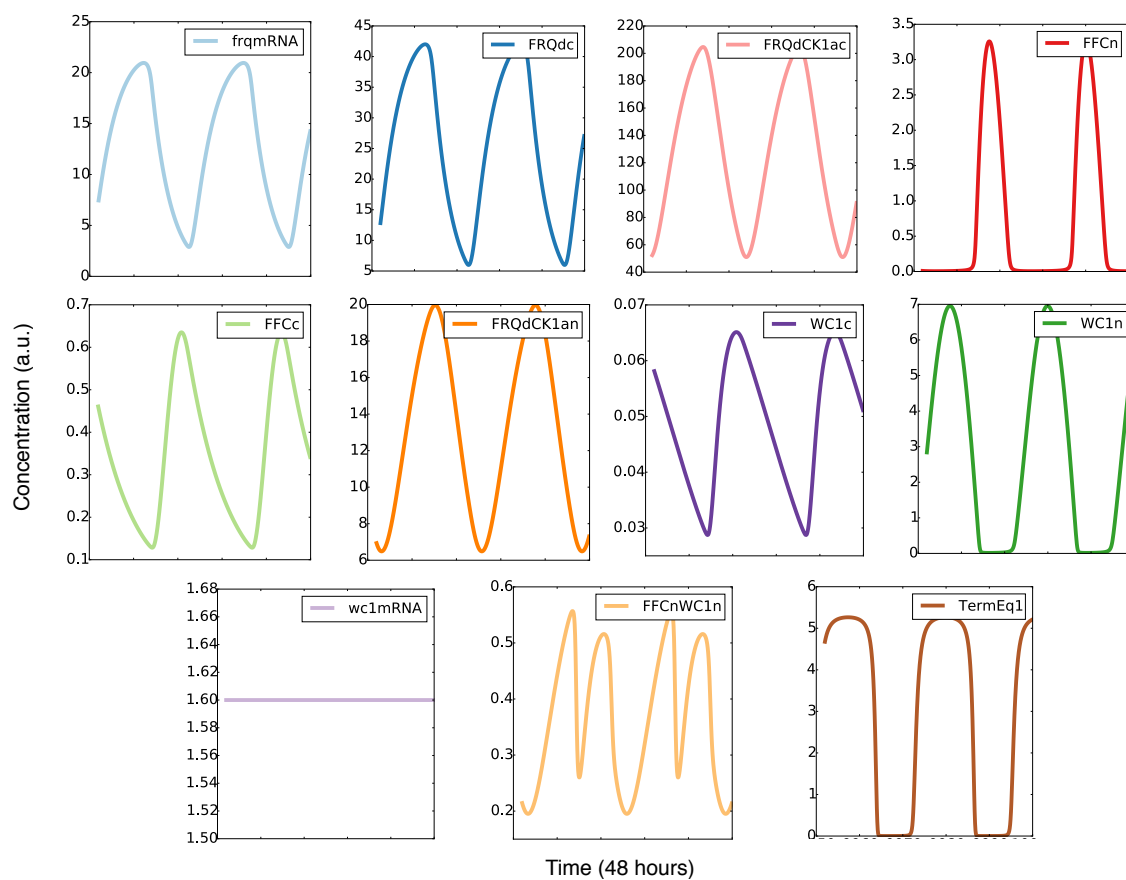


Figure 3. Simulated time series: There are sinusoidal and spike-like waveforms, harmonics and a temporal switch (see text).

Figure 3 shows simulations of our model for the default parameters listed in Appendix A1. The oscillations of Frequency mRNA and protein (blue) are almost sinusoidal whereas the levels of the inhibitory complex FFCn (red) and the transcription factor WC1n (green) display spike-like behaviour. The resulting FFCnWC1n complex (yellow) exhibits even two peaks during one circadian cycle. This observation illustrates that nonlinearities can generate pronounced "harmonics", an example of ultradian rhythms. Harmonics have been described in large-scale transcription profiles in mouse [34,35] and recently also in *Neurospora* [36]. The final graph in Figure 3 represents the transcription of the *frq* gene. It is evident that there is a sharp ON/OFF switch of transcription. In a later section we relate this temporal switch to FFC-assisted inactivation of the transcription factor WC1.

2.3. Bifurcation analysis of our model

The available kinetic data are not sufficient to determine all model parameters precisely. Moreover, external conditions can induce parameter variations. Consequently, we varied all 26 parameters in a comprehensive manner to explore the robustness of the model and to evaluate the role of the different kinetic terms along the lines of previous studies [37,38].

The self-sustained oscillations are obtained in wide ranges of parameters (see Figure 4 and Appendix A2). Amplitudes and periods vary smoothly with most parameters. The onset of oscillations occurs in most cases via a Hopf bifurcation, a transition from damped to self-sustained rhythms. Close to Hopf bifurcations we find large amplitude variations but weaker dependencies of the period on parameters.

Interestingly, for 3 parameters we observe hard onsets of oscillations (compare Appendix A2), i.e. the amplitude jumps abruptly. Such bifurcations are characterized by hysteresis and co-existing attractors [39–41] (see basins of attractors in Appendix A3). Details of these phenomena are presented in Appendices A2 and A3.

Nonlinearities are necessary to obtain limit cycle oscillations. As discussed above our model exhibits just 3 nonlinear terms. In order to check their relevance we replaced the terms by their mean (termed "clamping" in [42,43]) or by a linearized kinetics. Interestingly, for 2 of the nonlinearities the oscillations persisted. This implies that the dimerization and the positive feedback are not essential to obtain self-sustained oscillations. Thus, the only essential nonlinearity in our model is the Hill function describing *frq* transcription. Details of the detection of essential nonlinearities are provided in the supplementary information (Appendix A4).

In summary, the developed model exhibits robust self-sustained oscillations with just a single nonlinearity and a fairly small Hill coefficient $n=2$. In earlier models artificially high Hill coefficients have been used [11,12]. As in [15] we use a biologically plausible small Hill coefficient [44]. Since there are two distinct binding sites for WCC in the *frq* promoter [22] a Hill coefficient of $n=2$ is justified [45].

We can exploit now our model to discuss mutants, to analyze the underlying transcriptional switch, and to explore possible glucose compensation mechanisms.

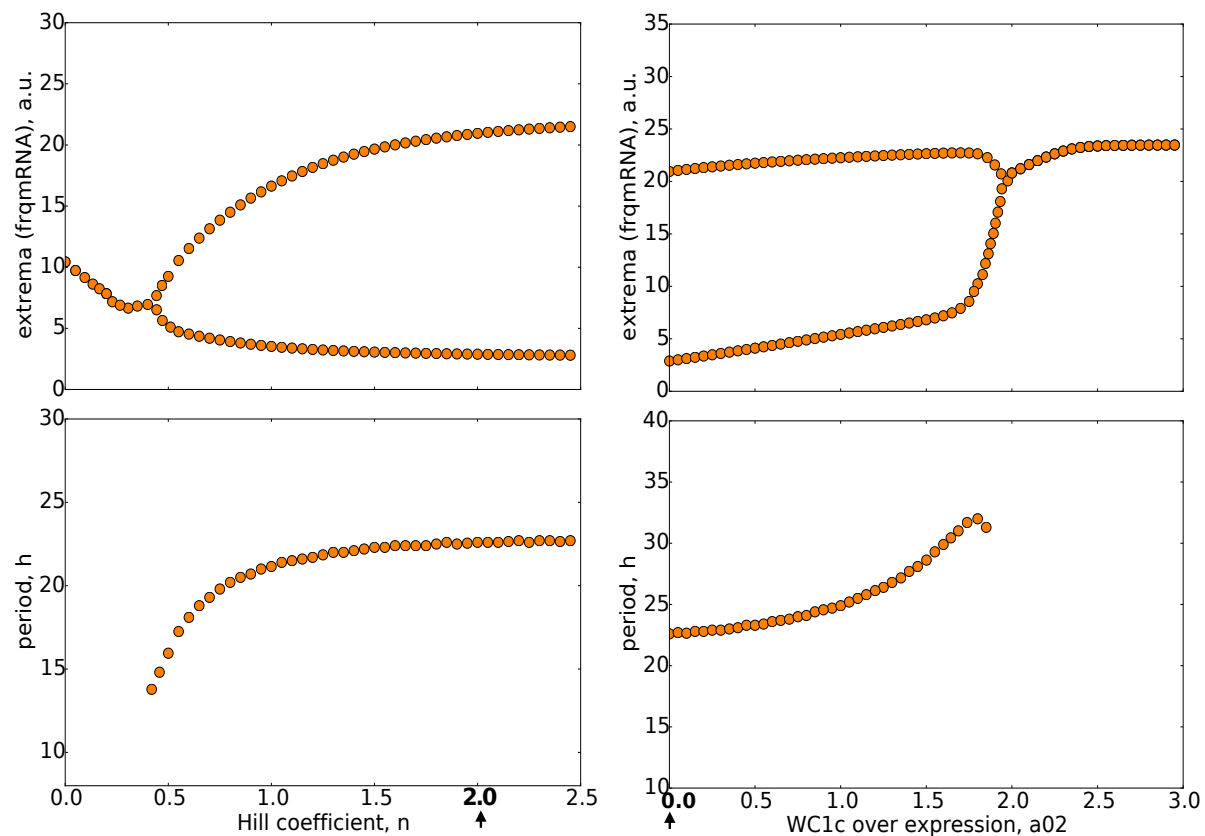


Figure 4. Bifurcation diagrams: The upper graphs show the maxima and minima of oscillations for varying parameters n (Hill coefficient) and a_{02} (WC1c overexpression). The lower graphs depict the corresponding periods. It turns out that oscillations persist for Hill coefficient n between 0.5 to 2.5 whereas overexpression of WC1 (a_{02}) can terminate rhythms. Default parameter values are marked by bold numbers and arrows.

2.4. Our model reproduces clock mutants

N. crassa became a model organism in chronobiology since a band mutation in the *ras-1* pathway leads to an overt periodic conidiation phenotype. A growth front of rhythmic conidiation and mycelium in so-called race tubes (solid culture) and gene expressions from bioluminescence signals using a *frq*-promoter luciferase reporter assay help visualizing a period of about 22.5 hours [46,47]. These techniques also allowed the identification of point mutations in the *frq* gene with altered period lengths. For example, the *frq7* mutation stabilizes FRQ leading to a longer period of about 29 hours [46].

Table 1 lists several mutant phenotypes and associated experimental findings regarding protein stability. If we adapt our model rate constants accordingly we can simulate the phenotypes of wildtype (*frq+*), shorter and longer period mutants (*frq1* and *frq7*) as shown in Appendix A5. Moreover, our model also reproduces the behaviour of a FWD-1 knockout experiment demonstrating that complete turnover of FRQ is not required for the clock to run [48] (see Appendix A5). This implies that the model is faithful to the experimental results, which is a good indication for any mathematical model.

2.5. An inactivation-switch allows self sustained oscillation

It is widely known that molecular switches support limit cycle oscillations [50–53]. Moreover, the switch-like waveforms in the time series of our model discussed above indicate a switch. However, the biological mechanism of such a switch is not immediately evident.

Table 1. Modeling *Neurospora* clock mutants: A list of selected mutants and their properties (simulations in Appendix A5).

Geno- -type (name)	Geno- -type (mutation)	Period (racetube)	Pheno- -type (racetube)	Core clock genes (luciferase reporter)	Temperature compensation	Glucose compensation	Proposed effects experimentally	Model representation of effects	Refer- -ences
<i>frq</i> ⁺ wild type	ras1 band	22.5 hours	rhythmic	oscillate	normal	normal	[FRQ]n < [WC1]n	default parameters (a7 = 0.05, a8 = 0.34, a10 = 0.10)	[46]
<i>frq</i> ¹	Gly-Ser G482S	16.5 hours	rhythmic	oscillate	normal	normal	[FRQ]n of <i>frq</i> ¹ > of <i>frq</i> ⁺ and decreased FRQ stability and [FRQ]n > [WC1]n	faster nuclear import rate a7 = 2.5 and increased FRQ degradation rate a8 = 0.68	[46]
<i>frq</i> ⁷	Gly-Asp G433D	29 hours	rhythmic	oscillate	partially lost		increased FRQ amplitude and increased FRQ stability and approx. [FRQ]n = [WC1]n	decreased FRQ degradation rate a8 = 0.17	[46,49]
<i> fwd1</i>	<i> fwd1</i> ^{RIP} COP9- signalosome	undefined	arrhythmic	oscillate			incomplete turnover of FRQ	no FFCn degradation rate a10 = 0	[48]

Multiple mechanisms have been discussed that allow switch-like behaviour. For example, cooperativity [54,55], zero-order ultrasensitivity [56] and multiple phosphorylations [57,58] can lead to quite steep input-output relationships [59]. Furthermore, sequestration [60] and positive feedback [61] can generate bistable switches [62–65].

Our model shows that expression of FFCn is mostly smaller than WC1n. Only a small fraction of WC1n is bound in the complex FFCWC1n (yellow line in Figure 5A). Therefore, a pure sequestration of WC1n by FFCn is unlikely. However, an inspection of the rate constants (Appendix A1) reveals the underlying switch mechanism: The degradation rate a_{18} of the active WC1n is quite low. If the levels of FFCn grow around time point 10 in Figure 5A, fast binding of FFCn to WC1n ($a_{19} > a_{20}$) allows the sudden deactivation of WC1n ($a_{21} > a_{18}$). A switch between active transcription to FFC assisted fast inactivation serves as the basic mechanism. Note, that these steps require neither high Hill coefficients nor positive feedback as in many other oscillator models [12,66].

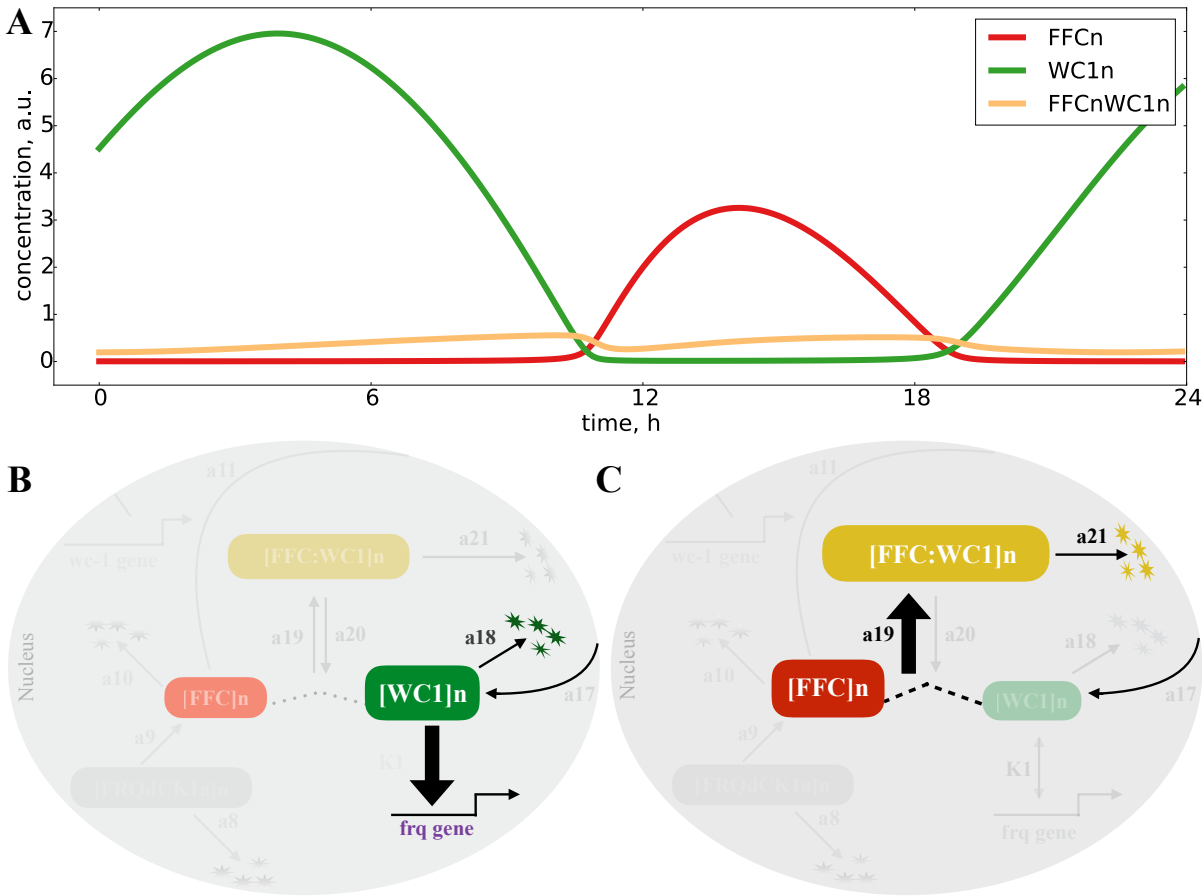


Figure 5. Inactivation switch in *Neurospora* clock model: The FFCn levels (red line) are most of the time lower than the WC1n levels (green line) and only a small fraction of WC1n is bound to FFCn (yellow line) (see Fig 5A). Below we distinguish 'on' and 'off' phases. Fig 5B points to an active WC1 (thick line indicates strong binding to *frq* promoter) with small degradation rate a_{18} (thin line). Fig 5C illustrates the 'off' state with FFC assisted inactivation of WC1. Here we have a strong binding of FFC to WC1 ($a_{19} > a_{20}$) and fast degradation ($a_{21} > a_{18}$).

2.6. Sensitivity analysis points to inherent glucose compensation mechanisms

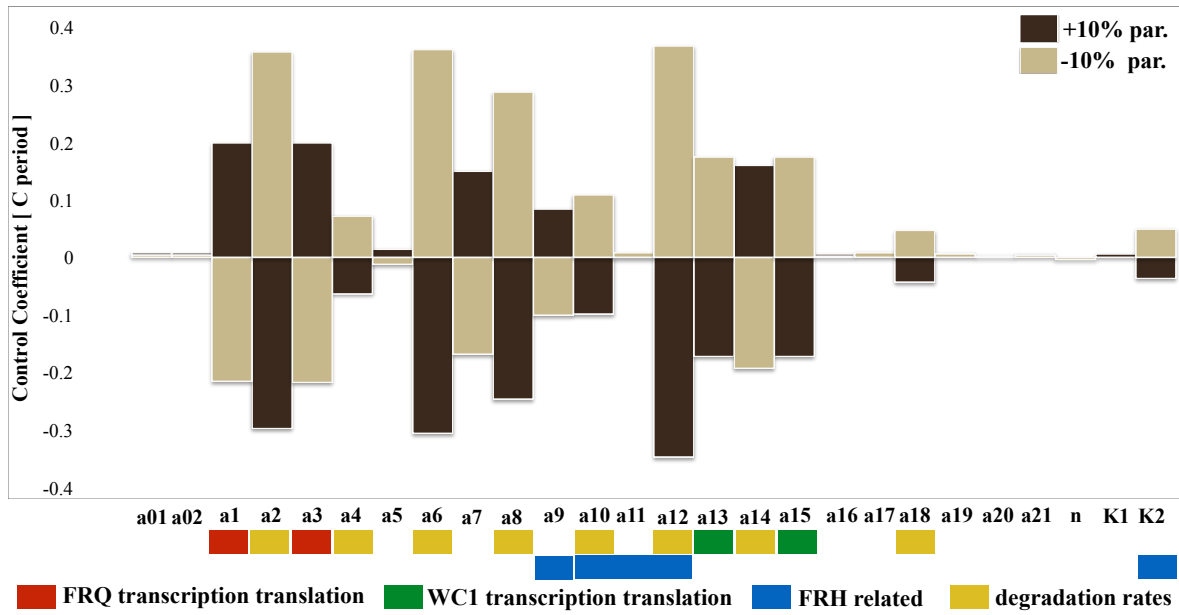
Circadian clocks, in general, have to be robust against environmental fluctuations. Therefore, they utilize compensation mechanisms against the changes in temperature and nutrients (glucose), where changes

in these environmental signals do not affect the overall clock period. There are suggested temperature compensation mechanisms [67–69] and glucose compensation via CSP1 [16].

Here we study the possibilities for glucose compensation without an extra feedback CSP1 [16] using our model. We assume that high glucose facilitates transcription and translation of FRQ and WC1. Simulations show that faster transcription and translation rates (a_1, a_3) of Frequency increase the oscillatory period (compare Figure 6). This is consistent with the data from the mammalian circadian clock [70,71]. With a systematic sensitivity analysis (Figure 6) we search for putative compensation mechanisms. A promising candidate is the helicase FRH since it has a dual role in the network: degradation of nascent mRNAs and formation of FFC complex [27,72].

To quantify period changes for varying the parameters, we calculate the control coefficients quantifying the sensitivity of the system. In Figure 6 we show a comprehensive control analysis of our model along the lines of [37,45,73]. All parameters were changed by ± 10 percent and the corresponding period changes were extracted from simulations. A positive period control coefficient of 0.5 implies that a 10 percent parameter increase induces a period lengthening by 5 percent, i.e. about 1 hour. Our analysis confirms the well-known feature that faster degradation of Frequency leads to shorter periods [74] as discussed above for the *frq¹* mutant (see the yellow bars associated with increasing parameters a_2 , a_4 , a_6 , a_8 and a_{10}).

Now we discuss possible mechanisms to get compensation of high glucose levels. The period increase via faster Frequency production (a_1 , a_3) could be compensated by faster WC1 production (a_{13} , a_{15}) since the corresponding control coefficients have opposite signs. In order to study the dual role of FRH we marked in Figure 6 the FRH related parameters blue (a_9 , a_{10} , a_{11} , a_{12} and K_2). It turns out that the dual role of FRH can indeed serve as a compensation mechanism as follows: enhanced transcription due to high glucose sequesters the helicase FRH. This leads to a slower assembly of FFC (smaller a_9 value) and less stabilization of FFC (larger a_{10} value). According to Figure 6 these effects can compensate the period increase due to an increased Frequency production. Interestingly, the glucose compensation through the dual role of FRH is lost if we cancel the positive feedback of FFCc on WC1c translation via clamping (Appendix A6).



High Glucose:

increase a1, a3 → longer period
increase a13, a15 → shorter period
decrease a9 → shorter period
increase a10 → shorter period

Low Glucose:

decrease a1, a3 → shorter period
decrease a13, a15 → longer period
increase a9 → longer period
decrease a10 → longer period

Figure 6. Sensitivity analysis: All parameters were changed by +/- 10 percent and the resulting periods were calculated. Positive and negative period control coefficients indicate period lengthening and shortening respectively.

3. Discussion

Understanding circadian clocks quantitatively helps to explore its functional significance in different organisms [75]. In mammals, a functional clock can help to prevent diseases such as cancer, obesity, and depression [4]. To reveal the basic of mechanisms of the underlying gene-regulatory network (see Figure 1) *Neurospora crassa* has been established as a useful model organism [3].

Mathematical modeling complements experimental studies and can help to uncover the underlying design principles [12,32]. A transcriptional-translational feedback loop (TTFL) serves as the generator of self-sustained oscillations with a period of about a day but quantitative details of these delayed negative feedback [76] and supporting molecular switches [77] are debated.

The required delay of at least 6 hours [78] is associated with gene expression, phosphorylation, nuclear translocation, complex formations and epigenetic regulations [79,80]. In addition to appropriate delays limit cycle oscillations require nonlinearities such as molecular switches [37] as studied extensively in cell proliferation and differentiation [50,62].

To explore the temporal dynamics of the *Neurospora* clock, we developed a mathematical model (Figure 2) that reproduces basic features of wild type rhythms and selected mutants (Table 1). Simulations reveal spike-like waveforms and a temporal transcriptional switch (Figure 3). Systematic clamping and linearization reveals that there is just one essential nonlinearity with a Hill coefficient of $n=2$. This raises the question how the ON/OFF switch of the Frequency gene is generated without explicit ultrasensitivity or

bistability.

As illustrated in Figure 5 we find sharp transitions between active transcription with slow turnover of WC1 and fast FFC assisted inactivation of WC1. Related protein inactivation switches have been discussed in recent studies [81–84].

Our model can also be exploited to study compensations of environmental fluctuations. It has been found experimentally that the *Neurospora* clock keeps its period almost constant at varying temperatures and glucose concentrations [67,68]. In a previous study an auxiliary transcriptional feedback loop via CSP1 has been postulated for glucose compensation [16].

Sensitivity analysis of our model (Figure 6) suggests in-built mechanisms without additional feedbacks. High levels of glucose enhance gene expression. Our model indicates that higher levels of core clock genes increase the period as found experimentally in *Neurospora* [17,18] and in mammals [70]. Interestingly, enhanced WC1 levels decrease the period (compare Figure 6). This counter-regulation constitutes a first putative compensation mechanism.

Another glucose compensation mechanism is based on the dual role of FRH. This protein serves as an RNA helicase and as a stabilizer of the FFC complex [27,72]. Since at high glucose conditions many more mRNAs are transcribed FRH is partially sequestered. This implies that the FFC complex is destabilized leading to a shortening of the period. This effect can compensate the increased period induced by enhanced transcription. Interestingly this compensation is diminished if we turn off the positive feedback via WC1 translation [25]. This finding points to a possible role of this additional feedback regulation. Note, that this feedback was quite weak in the original model [15]. We decreased the activation threshold K2 for WC1 translation to strengthen the experimentally verified positive feedback [18,25].

In summary, we modified a model by extending the negative loop via FRH complexes and by strengthening the positive feedback. Both modifications contribute to the robustness of the model (Figure 4), to the inactivation switch (Figure 5) and intrinsic glucose compensation (Figure 6). Our proposed mathematical model with 10 dynamic variables and 26 kinetic parameters reproduces many experimentally known features. A comprehensive analysis of the model reveals some unexpected results. It turns out that there is just a single nonlinearity essential for self-sustained oscillations. The switch-like behavior of the model arises from a novel inactivation switch based of WC1 inactivation assisted by the FFC complex. This finding suggests that forthcoming experiments should be oriented towards the detailed mechanisms of WC1-FFC interactions.

Another result concerns the compensation of environmental perturbations. Sensitivity analysis of our model suggests putative in-built mechanisms to achieve glucose compensation. First expression of the WC1 gene could counteract the effects of Frequency expression on the period. Another mechanism points to possible roles of FRH and the positive feedback. Sequestration of FRH via increasing mRNA transcription leads to a destabilization of the FFC complex which can compensate period changes in the presence of the positive feedback.

Even though our model is not quantitative in any detail the comparison of simulations with experimental data can stimulate future investigations. The combination of a delayed negative feedback with an inactivation switch is a robust design principle that could be relevant also in other oscillatory systems such as cell cycle dynamics [85], redox oscillations [86] or somite formation [87,88].

4. Materials and Methods

All the simulations have been performed using codes on a Spyder Python 3.4 platform. Ordinary differential equations have been written into codes using SciPy odeint library. Matplotlib library has been used to generate figures. XPP-Auto has been used to reproduce the bifurcation diagrams generated by Python. Codes are available on request.

Author Contributions: conceptualization by M.B. and H.P.; methodology by A.U. and H.P.; software by A.U.; validation by A.U. and H.P.; formal analysis by A.U.; investigation, A.U. and H.P.; resources by H.P.; data curation by M.B.; writing—original draft preparation by A.U. and H.P.; writing—review and editing, A.U., H.P. and M.B.; visualization by A.U. and H.P.; supervision by M.B. and H.P.; project administration by M.B. and H.P.; funding acquisition by M.B. and H.P.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

<i>N. crassa</i>	<i>Neurospora crassa</i>
frq	<i>Frequency</i> gene
FRQ	Frequency protein
WCC	White Collar Complex Protein
WC1	White Collar-1 Protein
WC2	White Collar-2 Protein
wc1	<i>White Collar-1</i> gene
CK1a	Casein Kinase 1a
FRH	Frequency-interacting Helicase
FFC	Frequency FRH CK1a complex
FFCWC1	FFC and WC1 complex
FWD1	F-box/WD-40 repeat-containing protein-1
CSP1	Conidial separation-1
a.u.	arbitrary units
ODEs	Ordinary Differential Equations
N.A.	Not Applicable
Fig	Figure

289 **Appendix A**

Our model parameters (value in a.u.)	representation into biological processes	Hong model parameters (a.u.) [15]
a01 = 0	frqmRNA over expression	k01 = 0
a02 = 0	WC1c over expression	k02 = 0
a1 = 5.4	frq transcription rate	k1 = 1.8
a2 = 0.23	frqmRNA degradation rate	k4 = 0.23
a3 = 5.4	FRQdc translation rate	k2 = 1.8
a4 = 0.69	FRQdc degradation rate	k5 = 0.27
a5 = 2	FRQdCK1ac transition rate	N.A.
a6 = 0.34	FRQdCK1ac degradation rate	N.A.
a7 = 0.05	FRQdCK1an nucleus import rate	k3 = 0.05
a8 = 0.34	FRQdCK1an degradation rate	N.A.
a9 = 0.15	FFCn transition rate	N.A.
a10 = 0.11	FFCn degradation rate	k6 = 0.07
a11 = 0.05	FFCc cyto import rate	N.A.
a12 = 0.11	FFCc degradation rate	N.A.
a13 = 0.16	wc1 transcription rate	k7 = 0.16
a14 = 0.1	wc1mRNA degradation rate	k10 = 0.1
a15 = 2.4	WC1c translation rate	k8 = 0.8
a16 = 0.05	WC1c degradation rate	k11 = 0.05
a17 = 40	WC1n nucleus import rate	k9 = 40
a18 = 0.02	WC1n degradation rate	k12 = 0.02
a19 = 50	FFCnWC1n complex association rate	k13 = 50
a20 = 1	FFCnWC1n complex dissociation rate	k14 = 1
a21 = 5	FFCnWC1n complex degradation rate	k15 = 5
n = 2	Hill coefficient	n = 2
K1 = 1.25	WCC dissociation constant to frq promoter	K = 1.25
K2 = 0.3	WC1c accumulation by FFCc	K2 = 1

Figure A1. Kinetic parameters: A list of rate constants and their associated biological processes.

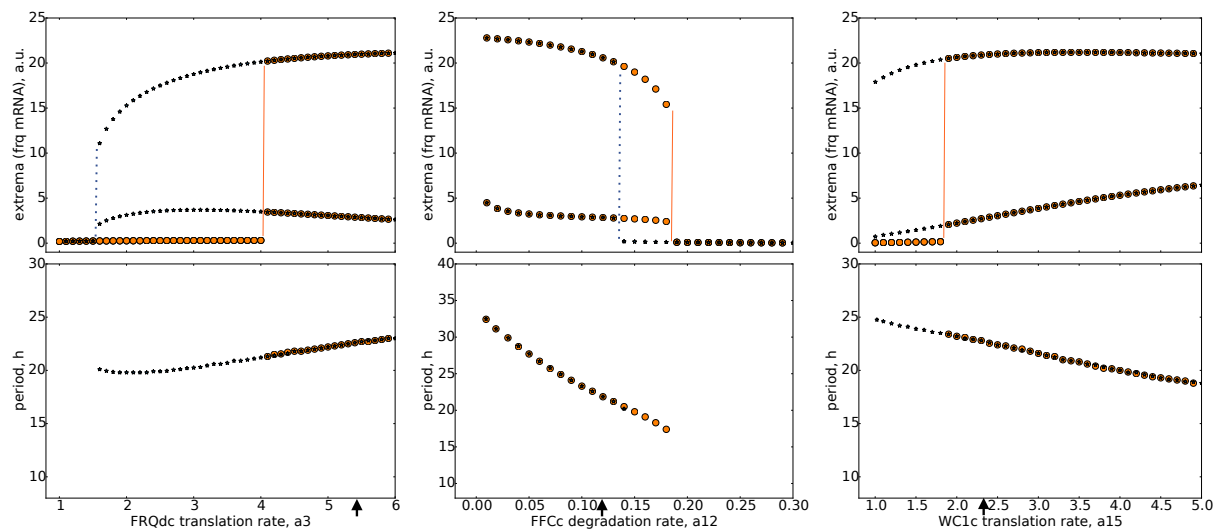


Figure A2. Co-existence of attractors: Bifurcation analysis of three parameters shows co-existence of limit cycle and steady state while parameter is slowly varied. The values for increasing parameters are marked by orange circles. Decreasing parameters correspond to blue stars. Red lines mark jumps between steady states (low frq values) to limit cycle oscillations. The associated periods are given in the lower graphs. Default parameters values are marked by arrows.

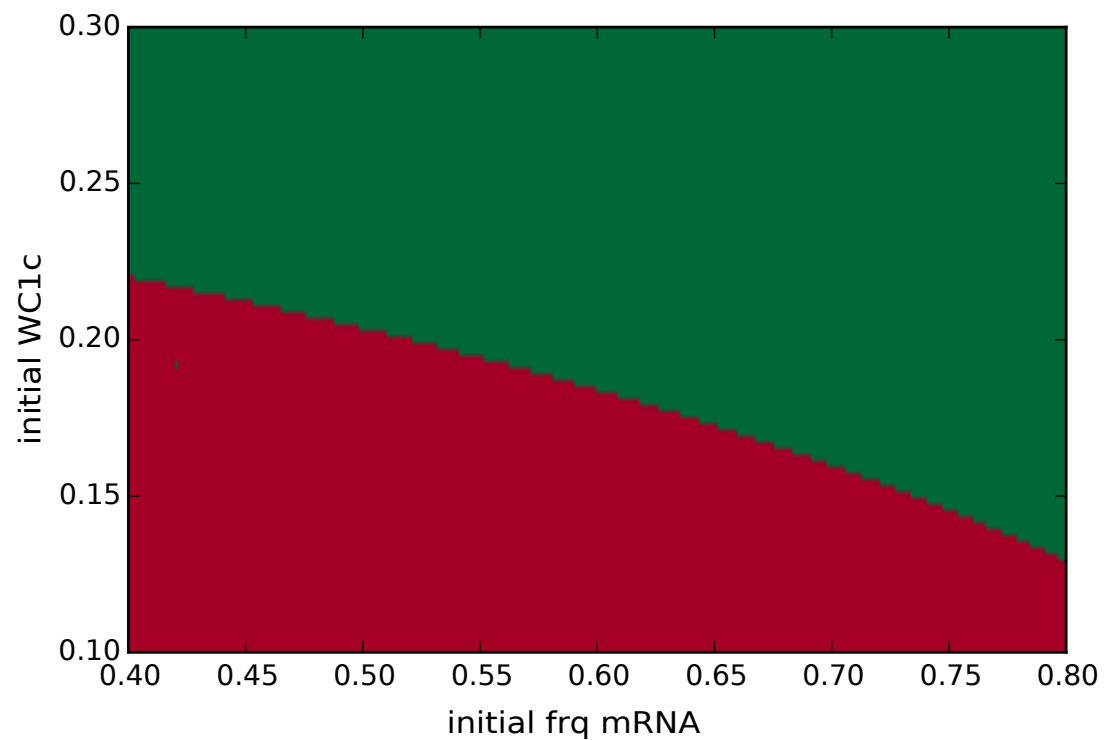


Figure A3. Basins of attractors: Coexisting attractors shown in 'Appendix A2' imply distinct basis of attraction (here $a_3 = 3$). Small initial values of frq and wc1 (red area) approach the steady state whereas initial conditions in green approach the limit cycle. The initial conditions of the other variables were zero.

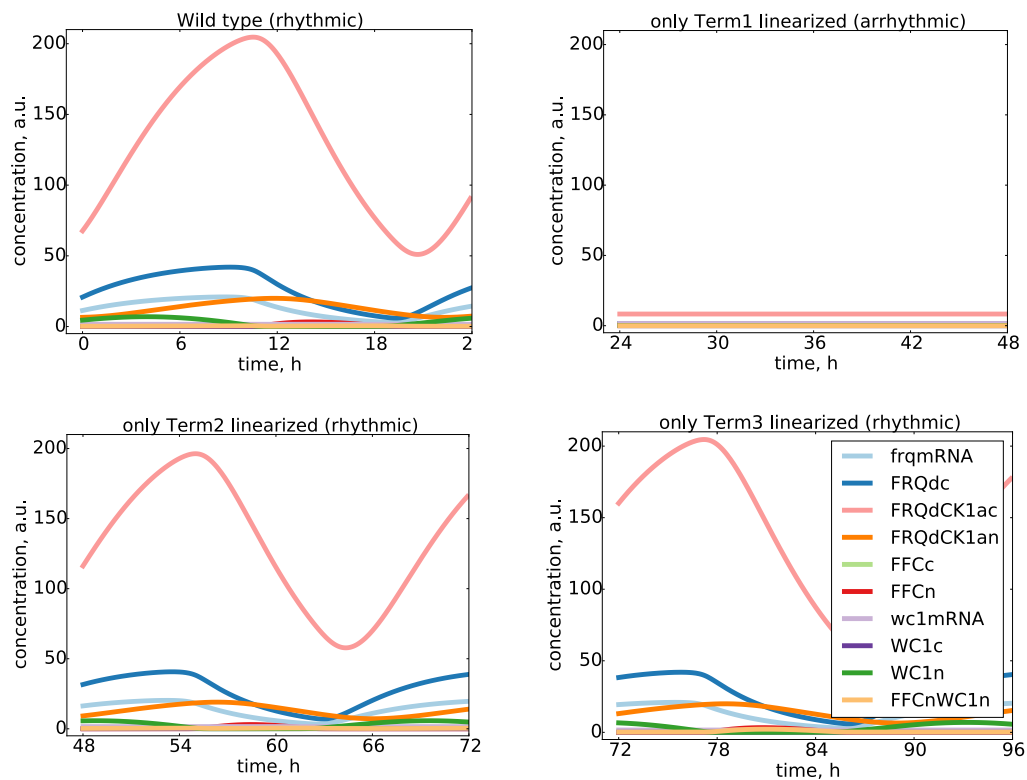


Figure A4. Essential and non-essential nonlinearities in the model: We linearized all the 3 nonlinear terms and find that the Hill function of frq production (Term1) is essential whereas the positive feedback nonlinearity (Term2) and the bilinear dimerization (Term3) are not essential since linearizations kept the rhythmicity.

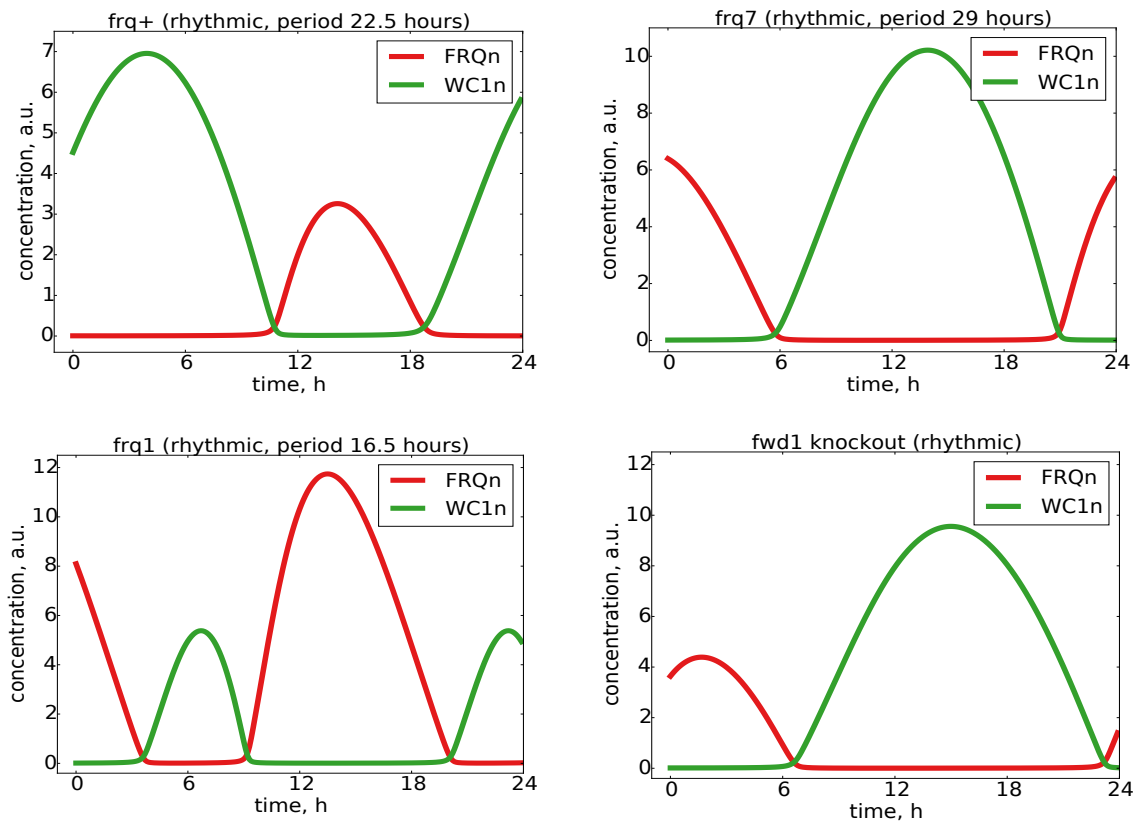


Figure A5. Mutants simulations: Several experimentally observed mutants are also reproduced by our model. The corresponding modified parameters are given in Table 1.

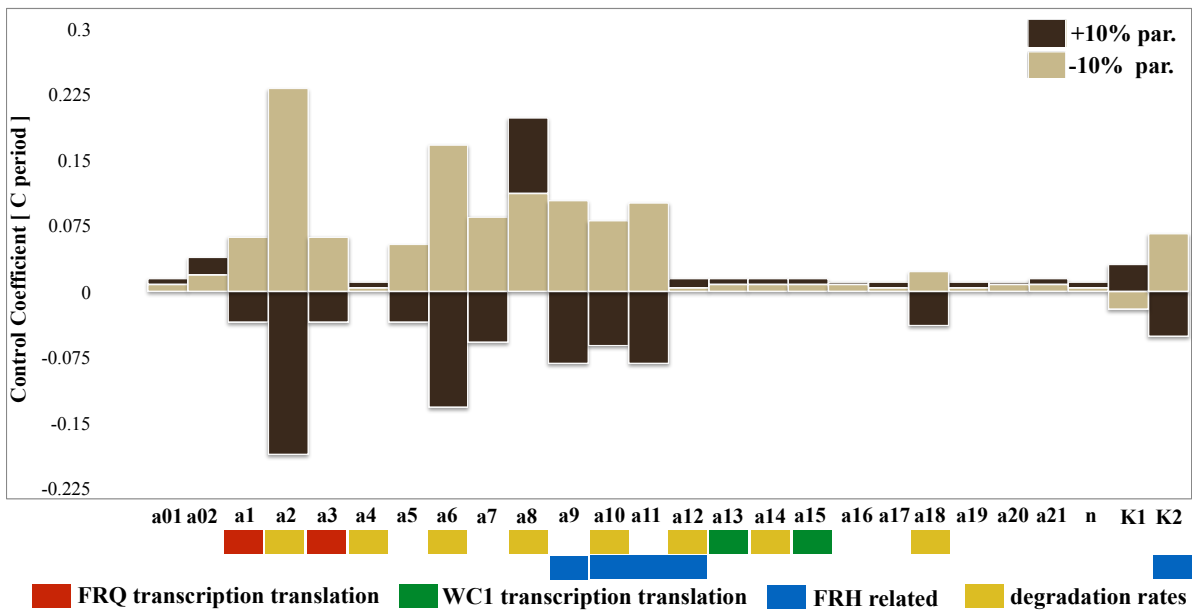


Figure A6. Sensitivity analysis after clamping the positive feedback: All parameters were changed by +/- 10 percent and the resulting periods were calculated. Positive and negative period control coefficients indicate period lengthening and shortening respectively (compare Figure 6).

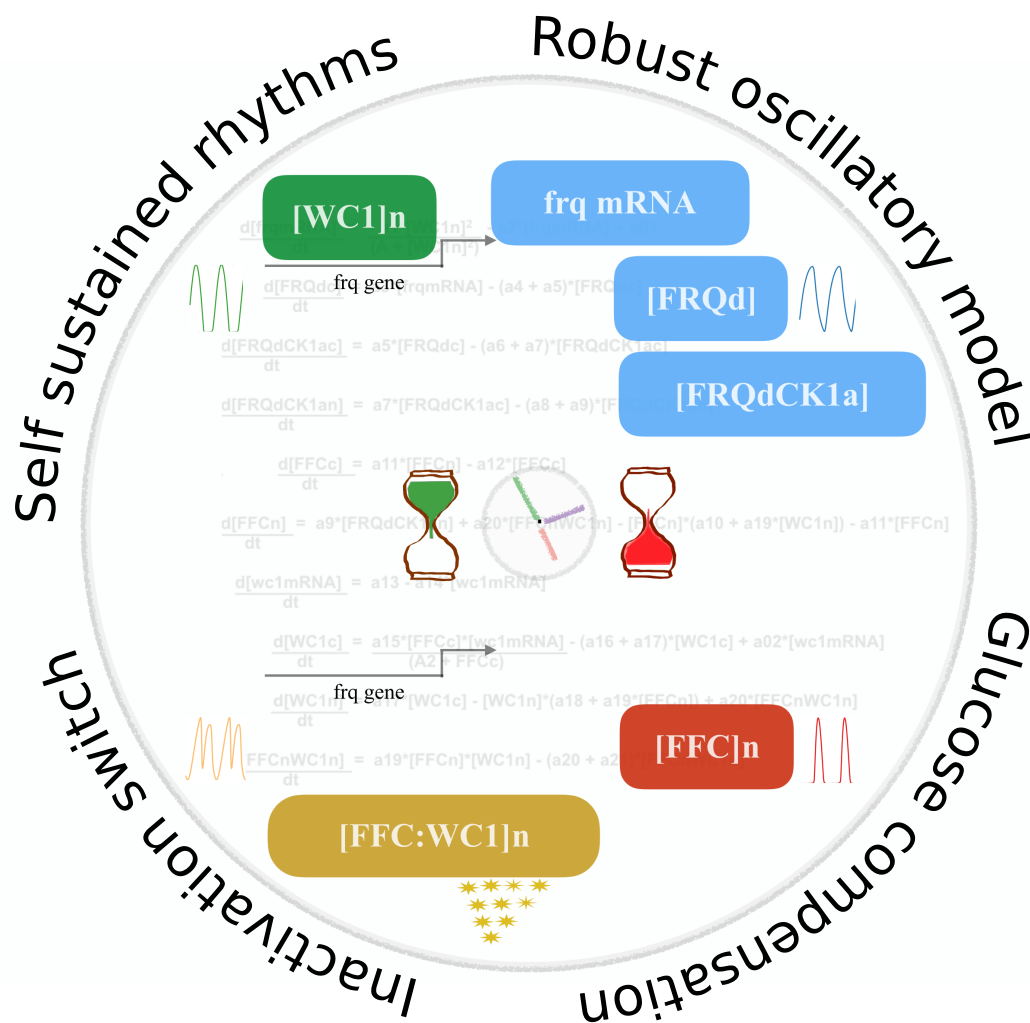


Figure A7. Graphical Abstract

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