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Exploring mechanisms of oscillations in p53 and NF- κ B systems

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Abstract: A number of regulatory networks have the potential to generate sustained oscillations of irregular amplitude, but well conserved period. Single cell experiments revealed that in p53 and NF- κ B systems the oscillation period is homogenous in cell populations, insensitive to the strength of the stimulation, and is not influenced by overexpression of p53 or NF- κ B transcription factors. We propose a novel computational method of validation of molecular pathways models, based on the analysis of sensitivity of the oscillation period to the particular gene(s) copy number and the level of stimulation. Using this method we demonstrate that existing p53 models, in which oscillations are born at a *saddle-node-on-invariant-circle* or *subcritical Hopf* bifurcations (characteristic for systems with interlinked positive and negative feedbacks), are highly sensitive to gene copy number. Hence, these models cannot explain existing experiments. Analyzing NF- κ B system, we demonstrate the importance of saturation in transcription of the NF- κ B inhibitor I κ B α . Models without saturation predict that the oscillation period is a rapidly growing function of total NF- κ B level, which is in disagreement with experiments. Our study supports the hypothesis that oscillations of robust period are based on *supercritical Hopf* bifurcation, characteristic for dynamical systems involving negative feedback and time delay. We hypothesize that in the p53 system the role of positive feedback is not sustaining oscillations, but terminating them in severely damaged cells in which the apoptotic program should be initiated.

1 Introduction

Many molecular regulatory networks exhibit sustained oscillations or have the potential to generate such oscillations in response to external stress or cytokine stimulation (reviewed in [1]). Although a single negative feedback loop is capable of generating sustained oscillations, regulatory systems

like p53, NF- κ B, or circadian rhythm contain both negative and positive feedbacks (reviewed in [2] for NF- κ B in [3] for p53). This property, together with the observation that oscillators based solely on a negative feedback and time delay tend to oscillate over the restricted range of parameters value, drew Zhang et al. [4] and Tsai et al. [5] to conclusion that the positive feedback is needed to generate the sustained robust oscillations.

Here we propose a novel computational method of validation of molecular pathway models, which is based on analysis of sensitivity of the oscillation period to particular gene(s) copy number and the level of stimulation. With the help of this method we will demonstrate that regulatory network models based on *saddle-node-on-invariant-circle* or *subcritical Hopf bifurcations* (characteristic for systems having interlinked negative and positive feedback loops) produce oscillations of period highly sensitive to parameters, and may not properly reproduce existing experimental data. We will concentrate on two systems of transcription factors, p53 and NF- κ B, which are known to produce sustained oscillations of varied amplitude but well conserved period, and for which the single cell data are available [6, 7]. The advantage of single cell experiments is that they show single cell dynamics, which is masked in population studies. In experiments by Nelson et al. [7] and Geva-Zatorsky et al. [6] oscillations were visualized by fluorescently tagged proteins in individual transfected cells. Since NF- κ B and p53 systems are nonlinear, the transfected cells having an elevated number of RelA (a key component of NF- κ B) or p53 gene copies, respectively, could have dynamics dramatically different than the normal diploid cells, especially if the regulatory system is based on positive feedback [9]. However, the experimental evidence is that the length of oscillation period in transfected cells and in normal diploid cells, that are analyzed in population experiments, is similar. In addition, the length of oscillation period is not sensitive to the level of stimulation (irradiation dose - in the case of p53, and TNF α dose - in the case of NF- κ B). These experimental observations pose restrictions on regulatory mechanisms responsible for oscillations - restrictions that were apparently overlooked in previous studies.

The bifurcation type is the key determinant of sensitivity of oscillation period to parameters. There are four main types of codimension one bifurcations (codimension is the number of equality conditions that characterize bifurcation) leading to oscillations e.g. [10]; superH (*supercritical Hopf*), subH (*subcritical Hopf*), SNIC (*saddle-node-on-invariant-circle*) and SL (*saddle-loop*) bifurcation, see Fig. 1.

- 1) In superH bifurcation, oscillations are born with zero amplitude and finite (nonzero) period.
- 2) In subH bifurcation oscillations are born with finite period and finite amplitude. This type of bifurcation is typically associated with the CF (*cyclic fold*) bifurcation in which stable and unstable periodic orbits are annihilated (or born). Such systems exhibit hysteresis; stable oscillations are born as bifurcation parameter increases at λ_1 and vanish at λ_0 as bifurcation parameter decreases,

see Fig. 1. For $\lambda \in (\lambda_0, \lambda_1)$ stable steady state and stable limit cycle coexist.

3) At SNIC bifurcation, a saddle-node collapse and a limit cycle of high period (infinite at the bifurcation point) arises. At the bifurcation point the system has homoclinic orbit.

4) SL (or homoclinic) bifurcation is somehow similar to SNIC; at bifurcation point there exists an orbit homoclinic to saddle, which then gives the origin to the periodic orbit of high period (infinite at the bifurcation point). Note that the “minimal system” exhibiting SL bifurcation has not any stable recurrent state (steady state or limit cycle) for $\lambda < \lambda_0$. It is thus not a good candidate for a biological model.

SuperH bifurcation may arise in regulatory systems having single negative feedback loop. Systems of interlinked negative and positive feedback loops may produce subH or SNIC bifurcations, but also superH bifurcation. In general, systems based on superH bifurcation have oscillations of relatively well conserved period (it changes slowly with the bifurcation parameter), but varied amplitude. In contrast, for the SNIC and SL bifurcations amplitude is rather fix, but period of oscillations may be arbitrarily high (see also [11]).

As already said, we will concentrate on regulatory systems of two transcription factors: p53 and NF- κ B.

Transcription factor p53 is responsible for regulation of DNA repair, cell cycle suppression or apoptosis and functions as a tumor suppressor. In healthy cells the level of p53 remains low under the control of its primary inhibitor, Mdm2, which in turn is p53 responsive. In response to DNA damage p53 becomes activated and its nuclear concentration increases. It binds to its affinity sites in the genome inducing transcription of numerous genes that are involved in cell cycle arrest and DNA repair. If the DNA damage is irreparable or its repair lasts too long, p53 induces transcription of apoptotic factors that initiate programmed cell death. The DNA damage results in a rapid change of p53 and Mdm2 degradation rates (p53 is stabilized by phosphorylation, while Mdm2 is destabilized), which leads to transition from the steady state, characterized by a low level of p53, to the oscillatory state. Existence of these apparently undamped oscillations, of characteristic period of 5-6 hours, but highly varied amplitude, was demonstrated by Geva-Zatorsky et al. in single cell experiments using fluorescently tagged p53 and Mdm2 [6].

Based on western blots analysis Geva-Zatorsky et al. [6] estimated that the concentration of exogenous p53-CFP and Mdm2-YFP protein in studied transfected cells is comparable to the endogenous p53 and Mdm2 protein concentration. This suggests that the total number of p53 or Mdm2 gene copies in analyzed cells is about four. Taking this estimation as a reference, we will focus on dynamics of cells, which have between one and six copies of p53 or Mdm2, which enables us to study the effects of overexpression or loss of an allele.

The cytoplasmic transcription factor NF- κ B is a major mediator of innate immune responses

(reviewed in [8, 12]). In resting cells NF- κ B is sequestered in cytoplasm by dimerization with inhibitory proteins, mainly its primary inhibitor called I κ B α . Binding of extracellular cytokines such as TNF α and IL-1 leads to I κ B α phosphorylation and rapid proteasomal degradation. Liberated NF- κ B is then rapidly translocated into the nucleus, where it binds to high affinity sites in the genome, thereby influencing target gene expression. The two levels of autoregulatory negative feedback control, termed the NF- κ B–I κ B α and NF- κ B–A20–IKK feedback loops, arise because both I κ B α and A20 genes are directly regulated by NF- κ B binding. In the NF- κ B–I κ B α feedback loop, NF- κ B enters the nucleus after I κ B α degradation, NF- κ B induces I κ B α resynthesis to recapture activated nuclear NF- κ B and return it to the cytoplasm. In the NF- κ B–A20–IKK feedback loop, A20, a protein that is not expressed prior to stimulation, is also strongly NF- κ B responsive. A20 is an inhibitor of IKK kinase that mediates I κ B α phosphorylation; without A20 expression, the IKK retains activity which leads to rapid degradation of the newly resynthesized I κ B α , which destroys the NF- κ B–I κ B α feedback loop [13, 14].

The sustained oscillations in nuclear-cytoplasmic NF- κ B localization (N-C oscillations) in response to tonic TNF α stimulation have been demonstrated in single cell experiments by Nelson et al. [7] on SK-N-AS (human S-type neuroblastoma) cells transfected with plasmids expressing RelA fused to the red fluorescent protein (RelA-DsRed). As reported, 91% of these cells showed prolonged oscillations in RelA N-C oscillations. These oscillations appeared quite synchronous among cells in the first few cycles, but then they became out of phase and thus appeared damped when observed at the population level in earlier experiment by Hoffmann et al. [15]. Recently, Sun et al. [16] demonstrated that more than 4000 mRNAs oscillate in response to TNF α stimulation.

2 Results

2.1 *p53 system*

There are two main classes of models that were proposed to explain oscillations of p53|Mdm2 regulatory module;

1) The group of J. Tyson proposed four models in which oscillations exist due to interlinked positive and negative feedbacks [4, 17]. In these models oscillations are born at subH bifurcation (Tyson group models I and III, [4]) or at SNIC bifurcation (Tyson group models II and IV, [17, 4]).

2) In other models oscillations result from negative feedback(s) and time delay(s), which are due to the presence of some intermediates [18, 19], or are introduced at the level of Mdm2 mRNA transcription or translation [20, 22, 21]. In all these models oscillations are born at superH bifurcation.

Our aim is to verify which models are capable of explaining the observed insensitivity of the oscillation period to changes in p53 and Mdm2 gene copy numbers and the stimulation level. First, we analyze four models proposed by the group of Tyson, then our novel "Toy model", based on the same two feedbacks as the Tyson group model IV, [17], but involving the superH bifurcation instead of SNIC. Finally, we analyze models of Ma et al. [20], Puszynski et al. [19] and Batchelor et al. [21], in which oscillations result from negative feedback and time delay and are born at superH bifurcation.

The proper model of p53|Mdm2 signaling pathway should satisfy at least the following constraints posed by experimental data:

1. Oscillations of p53 and Mdm2 levels should be induced by a decrease of p53 degradation rate or an increase of Mdm2 degradation rate, that follow DNA damage.
2. Period of oscillations of p53 and Mdm2 levels should be robust to the change in p53 and Mdm2 gene copy numbers and the extent of DNA damage.
3. Range of Mdm2 degradation parameter (which is controlled by the extent of DNA damage), in which oscillations exist, should not be very sensitive to the number of p53 and Mdm2 gene copies.

Since the gene copy number is not explicitly present in any of the models we aim to analyze, we introduce it by assuming that the mRNA or protein (if the mRNA transcription step is missing) production rate is proportional to the number of gene copies of p53 or Mdm2. Specifically, we assume that the original synthesis coefficients correspond to diploid cells and then we proportionally modify these coefficients for cells having arbitrary number of gene copies.

The numerical analysis of the models was performed using MATLAB solvers; ode23tb for ordinary differential equations models or time delay differential equations solver for Ma et al. [20] and Batchelor et al. [21] models. The stable fixed points and limit cycles were determined as asymptotic solutions.

2.1.1 Analysis of models based on interlinked positive and negative feedback loops

The common idea for the Tyson group models [17, 4] is that oscillations are induced by DNA damage via destabilization of Mdm2 (upregulation of Mdm2 degradation coefficient). We calculated thus the period of oscillations with respect to Mdm2 degradation coefficient for cells with different p53 and Mdm2 gene copy numbers, see Figs. 2 and 3. Namely, we focus on cells having $n = 2$ copies of Mdm2 and $m \in \{1, 2, 3, 4, 5, 6\}$ copies of p53, and $m = 2$ copies of p53 and $n \in \{1, 2, 3, 4, 5, 6\}$ copies of Mdm2. This way we are able to reproduce p53 and Mdm2 transfected cells.

In the first three models considered by Tyson and colleagues [4] the range of Mdm2 degradation coefficient for which the system possesses stable limit cycle is very sensitive to the number of p53

and Mdm2 gene copies, see Fig. 2. In addition, for a given number of p53 and Mdm2 gene copies the period of oscillations strongly varies with Mdm2 degradation coefficient. Specifically:

- In model I, oscillations are born as Mdm2 degradation increases at subH bifurcation and vanish as Mdm2 degradation decreases at CF bifurcation, with large but finite period. For $m = 2$, $n > 4$ or $n = 2$, $m > 3$ the system does not exhibit any oscillations.

- In model II, oscillations are born at SNIC bifurcation with infinite period. For $n = 2$ and $m = 6$ system oscillates for arbitrarily small k_{d2} (in this model, k_{d2} controls only Mdm2 degradation in nucleus, while Mdm2 degradation in cytoplasm is governed by independent parameter), for $m = 2$, $n = 1$ system does not exhibit any oscillations. The oscillation range for each (m, n) pair is very narrow, and there is only a small overlap between ranges corresponding to different (m, n) pairs.

- In model III, as in model I, oscillations are born at subH bifurcation with nonzero amplitude and vanish as Mdm2 degradation decreases at CF bifurcation with large but finite period. For $n = 2$, $m > 3$ oscillations do not exist; for $n = 2$, $m = 3$ oscillations are born not at subH but superH. As in model II, the oscillation range for each (m, n) pair is very narrow, and there is only a small overlap between ranges corresponding to different (m, n) pairs.

All these three models would imply a very broad distribution of oscillation periods in cell population, which is not observed. In addition, since for each (m, n) pair the system oscillates in a different range of Mdm2 degradation parameter, at a given level of stimulation only cells of the particular (m, n) pair would oscillate.

Apparently, more robust is the Tyson group model IV proposed in [17] and reconsidered in [4], see Fig. 3. In this model a negative feedback arises since p53 induces Mdm2 synthesis, which then translocates to the nucleus, where it catalyses p53 degradation. A positive feedback arises since Mdm2 phosphorylation (necessary for its nuclear entry), is suppressed by p53 (by a long pathway involving PTEN, PIP3 and Akt – not explicitly included in the model). This way, p53 inhibits its own degradation. For diploid cells ($m = n = 2$), the model generates p53 pulses by SNIC bifurcation. For cells with one Mdm2 gene copy missing ($m = 2$, $n = 1$) the system oscillates for arbitrarily small k_{d2} , which controls only Mdm2 degradation in nucleus, while for $m = 2$, $n \in \{3, 4, 5, 6\}$ and $m = 1$, $n = 2$ oscillations are born at superH bifurcation, and only for these (m, n) period is a slowly varying function of Mdm2 degradation coefficient. For $n = 2$, $m \in \{3, 4\}$ the system oscillates for arbitrarily small k_{d2} , while for $n = 2$, $m \in \{5, 6\}$ the system does not exhibit any stable oscillations. Summarizing, the Tyson group model IV [17] produces oscillations with period insensitive to Mdm2 degradation parameter, only in the case of Mdm2 transfection ($m = 2$, $n \in \{3, 4, 5, 6\}$), i.e. when oscillations are born at superH bifurcation. This suggests that the proper model should be based on superH bifurcation rather than on SNIC or subH bifurcations.

2.1.2 The "Toy" model involving interlinked positive and negative feedbacks, based on superH bifurcation

We constructed a novel "Toy" model of p53|Mdm2 regulatory core that involves the same positive and negative feedback loops as the original Tyson group model IV [17], but has essentially different dynamics. In contrast to [17], where the oscillations arise at two different types of bifurcation, in the Toy model oscillations arise at superH bifurcation for all pairs $m, n \in \{1, 2, 3, 4, 5, 6\}$.

The model consists of three components only: total p53, cytoplasmic Mdm2 and nuclear Mdm2, see Fig. 4. The negative feedback arises since p53 induces production of Mdm2, while Mdm2, when in nucleus, enhances p53 degradation. The positive feedback arises since p53 blocks Mdm2 nuclear import. The regulatory module is described by the system of three ordinary differential equations for amounts of total p53, cytoplasmic Mdm2 and nuclear Mdm2,

$$\frac{d}{dt}p53 = ms_1 - k_{d1}p53(Mdm2_{nuc})^2, \quad (1)$$

$$\frac{d}{dt}Mdm2_{cyt} = n \left(s_2 + s_3 \frac{(p53)^3}{s_4 + (p53)^3} \right) - k_1 \frac{k_2}{k_2 + p53} Mdm2_{cyt}, \quad (2)$$

$$\frac{d}{dt}Mdm2_{nuc} = k_1 \frac{k_2}{k_2 + p53} Mdm2_{cyt} - k_{d2}Mdm2_{nuc}, \quad (3)$$

where m and n are the numbers of p53 and Mdm2 gene copies, respectively. Rates s_1 , s_2 and s_3 are the production rates per gene copy, see Table I for parameters. Mdm2 transcription is regulated mostly by p53 tetramers, therefore we assumed, following [17], that p53 induces Mdm2 transcription according to Hill function with exponent 3. The nonlinearity in p53 degradation results from the fact that Mdm2 must attach several ubiquitines to p53 to initiate its degradation. As in [17], transport of Mdm2 from the cytoplasm to the nucleus is inhibited by action of p53.

The DNA damage is not explicitly present in our model but can be introduced by increase of Mdm2 degradation rate or decrease of p53 degradation rate. The model has the required property, that the transition from a stable point to a stable limit cycle is a consequence of either increased Mdm2 degradation or decreased p53 degradation, see Fig. 5. Although, superH bifurcation is rather characteristic for systems with negative feedback only, in this model the presence of positive feedback is needed for oscillations, i.e. oscillations vanish after assuming that the nuclear import of Mdm2 is p53 independent.

As for Tyson group models [17, 4], we investigated the dependence of oscillation period on Mdm2 degradation rate and p53 and Mdm2 gene copy number, see Fig. 6. As shown in Fig. 6, the period of oscillations weakly depends on Mdm2 degradation parameter and gene copy number.

However, the range of oscillations in Mdm2 domain depends on p53 and Mdm2 gene copy number, see Figs. 7 and 8. As shown in Fig. 7, Mdm2 transfection ($m = 2, n = 4$) results in a decrease of the parameter range for which system oscillates, while p53 transfection ($m = 4, n = 2$) increases the oscillatory range of parameters. In cotransfected cells having duplicated number of p53 and Mdm2 copies ($m = n = 4$) the oscillatory region is smaller than in normal diploid cells, but larger than in cells with duplicated number Mdm2 gene copies only. As shown in Fig. 8, the lack of one of two p53 gene copies causes the oscillatory region to shrink, while the lack of one of Mdm2 gene copies causes the oscillatory region to grow.

The Toy model implies that oscillations may be triggered solely due to p53 transfection, or may be suppressed (in DNA damage cells) in response to Mdm2 transfection. Similarly, lack of Mdm2 gene copy may promote oscillations even when DNA is intact, and the lack of p53 copy may inhibit oscillations even when DNA is damaged. These model predictions are in agreement with some experimental findings. Since oscillations of p53 and Mdm2 are needed to initiate transcription of p53 dependent genes involved in cell cycle arrest, DNA repair or apoptosis, the model may explain p53-haploinsufficiency resulting in cancer development. In contrast, lack of one of Mdm2 gene copies makes cells more sensitive to DNA damage and suppress cancer, as observed by Alt et al. [23], who showed that Mdm2-haploinsufficiency profoundly inhibits lymphomagenesis, while p53-haploinsufficiency induces lymphomagenesis development. Deleting p53 blocks Mdm2 haploinsufficient B cells from undergoing spontaneous and Myc-induced apoptosis. Mice engineered to express low Mdm2 levels are more sensitive to gamma irradiation induced apoptosis, which is rescued by loss of p53 [24]. This confirms opposite action of loss of p53 and Mdm2 gene copies, as shown in Fig. 8.

With respect to Tyson group model IV [17] our Toy model produces oscillations of period less sensitive to p53 and Mdm2 genes copy number and Mdm2 degradation parameter. This demonstrates that the bifurcation type is a more important determinant of system dynamics than existence of positive feedback.

Tyson group model IV [17] as well as our Toy model should be considered with caution; both are based on the positive feedback mediated by PTEN, which is hardly inducible in MCF-7 cell line studied by Geva-Zatorsky et al. [6]. In addition, even in cell lines having p53 responsive PTEN, the presence of intermediates in the positive feedback loop (i.e. PTEN, PIP3 and Akt) introduces the long time delay (omitted in both models), that may substantially change system dynamics.

2.1.3 *Models based on negative feedback and time delay*

Here we analyze two models in which oscillations are driven by negative feedback coupling p53 with its inhibitor Mdm2 and time delay. In Ma et al. model [20] time delay is introduced at the level of Mdm2 transcription and translation. In this model, oscillations are born at superH bifurcation. As shown in Fig. 9, period of oscillations very weakly depends on Mdm2 degradation rate (which is controlled by DNA damage) and on gene copy number.

Puszynski et al. model [19] is based on two feedbacks: negative and positive. However, the oscillations are solely driven by negative feedback and presence of intermediates (which introduce time delay) in the negative feedback loop, which involves p53, Mdm2 transcript, cytoplasmic Mdm2, cytoplasmic phosphorylated Mdm2 and nuclear phosphorylated Mdm2 that controls p53 degradation.

The positive feedback follows even longer loop of interaction involving phosphatase PTEN, PIP3 and Akt. First, p53 triggers synthesis of PTEN, which hydrolyzes PIP3 to inactive PIP2. PIP3 is needed to phosphorylate kinase Akt, which in turn phosphorylates Mdm2 enabling its nuclear entry and catalysis of p53 degradation. The positive feedback works thus via “double negation”: $p53 \rightarrow PTEN \dashv PIP3 \rightarrow Akt \rightarrow Mdm2 \dashv p53$, switching of the negative coupling of p53 and Mdm2.

DNA damage leads to Mdm2 destabilization and p53 phosphorylation, triggering oscillations of p53 and Mdm2 levels during which DNA may be repaired. If DNA repair is not completed in about 15 hours, the signal passing through positive feedback blocks the negative feedback loop and triggers p53 on even higher level at which apoptosis may be initiated.

With a disconnected positive feedback loop and DNA repair suppressed, the system, in response to DNA damage, exhibits sustained oscillations. For current analysis of Puszynski et al. model [19], we introduce DNA damage by change of Mdm2 degradation rate (leaving p53 phosphorylation rate unchanged). As shown in Fig. 9, similarly to Ma et al. model [20], oscillations are born at superH bifurcation and their period is neither sensitive to Mdm2 degradation nor to the number of p53 and Mdm2 gene copies. As in Ma et al. model [20], p53 transfection promotes oscillations, while Mdm2 transfection inhibits oscillations.

2.1.4 *Batchelor et al. model*

As a last p53 model, we analyze the recent model proposed by the group of Galit Lahav, which is based on two negative feedback loops [21]. As in all other models one loop couples p53 with its primary inhibitor Mdm2. The second loop involves p53 upstream kinases ATM and Chk2, which activate p53, and their inhibitor, phosphatase Wip1, which is p53 responsive. As a result pulses of p53 level and activity are tightly coupled with pulses of active ATM. The existence of this

novel feedback loop is well documented by the series of experiments performed on MCF-7 breast cancers cell line. The model involves two explicit delays associated with p53-controlled synthesis of Mdm2 and Wip1. Since in the considered model Mdm2 degradation is controlled by the *Signal* (representing active ATM and Chk2), which in turn is regulated by Mdm2 via p53, we choose the *Signal* production rate β_s , instead of Mdm2 degradation coefficient, as a bifurcation parameter for our analysis. Parameter β_s controls the strength of the input signal in DNA damaged cells.

In the model, the limit cycle oscillations are born and vanish at SuperH bifurcations and exist for a finite range of signal production rate β_s , see Fig. 10. The period of oscillations weakly depends on the strength of the signal, and for $m, n \in \{2, 3, 4\}$ the range of signal production rate β_s is almost independent on m and n . Only for $m = 1$ and $n = 2$ the oscillation range is much smaller than that for non-transfected cells ($m = n = 2$). The limit cycle oscillations do not exist for $m = 2$ and $n > 5$, which imply that threefold Mdm2 overexpression may inhibit any oscillations. However, this effect may be compensated by p53 transfection; cells having $m = n \in \{2, 3, 4, 5, 6\}$ oscillate in about the same parameter range as the non-transfected cells, data not shown.

2.2 *NF- κ B system*

In last years several models of the NF- κ B regulatory module have been developed, reviewed in [25] and [26]. The first one was a one-feedback loop model that concentrated on the interplay between the three I κ B isoforms [15]. This original model has numerous descendants developed by groups of Hoffmann and Levchenko including Werner et al. model [27]. The next was our two feedback loop model [14], incorporating effects of both NF- κ B inhibitors, I κ B α and A20, which was further developed to include stochasticity in I κ B α and A20 genes activation/inactivation [28]. In all of the three deterministic models [4, 14, 27], oscillations arising in response to tonic TNF α stimulation are damped (there is no limit cycle) for the assumed (fitted) sets of parameters. Only in the stochastic models [28, 31], oscillations are persistent due to the stochastic resonance.

The mathematical structures of these models have a number of important differences, however, with respect to our analysis the main difference is in the form of transcription regulation of I κ B α ; The original Hoffmann-Levchenko model [15] assumed that transcription rate is proportional to the square of nuclear NF- κ B concentration, while in Werner et al. model [27], and the models following it, the cubic transcription rate is assumed. In contrast, in the Lipniacki et al. model the transcription rate is linear [14]. In the latter stochastic model [28], the activity of I κ B α and A20 genes is regulated by binding and dissociation of NF- κ B dimers, and it is assumed that the active gene is transcribed with the constant rate of 4.5 mRNA/second/gene copy, close to the physiological maximum.

As shown by Barken et al. [29], Hoffmann-Levchenko model [15] produces oscillations of period very sensitive to the amount of total NF- κ B. This led to the conclusion that oscillations recorded in the overexpressed feedback system by Nelson et al. [7] do not allow one to conclude that oscillations of the same persistence, amplitude and period occur in morphogenetically unaltered cells [29]. In fact, according to the Hoffmann-Levchenko [15] model the fourfold NF- κ B overexpression causes the second peak in nuclear NF- κ B, to appear not at 2 h (as normally), but at 5.5 h of persistent TNF α stimulation ([29], Fig. 1A). However, as shown by Nelson et al. [30], the oscillation period is almost insensitive to NF- κ B expression and it is enough to replace quadratic transcription rate by the linear one to substantially minimize sensitivity of period length to the total level of NF- κ B ([30], Fig. 2). Latter, we showed the assumption that the transcription rate is bounded leads to oscillations of period almost insensitive to the total NF- κ B level ([28], Fig. 6).

The models in which transcription rate is proportional to the transcription factor concentration (especially in higher power), without any saturation, lead to transcriptional efficiencies exceeding physiological rates and thus may not properly reproduce cell dynamics. In this particular case, the improper choice of quadratic transcription rate leads to high sensitivity of predicted period to the total level of NF- κ B. At high level of transcription factor the transcription rate of NF- κ B inhibitor I κ B α exceeds many times the maximum physiological rate (see [31] for discussion), and causes the I κ B α to accumulate at a very high level inhibiting NF- κ B reentry for many hours which elongates the oscillation period. In addition, the elongated degradation time of accumulated I κ B α introduces time delay and leads to appearance of a stable limit cycle.

3 Discussion

We proposed a novel computational method, which is helpful in validation of molecular pathways models, in which dynamics of regulatory systems are studied with respect to the particular gene(s) copy number. Based on this approach we demonstrated that four p53 system models that are based on superH bifurcation, produce oscillations of period insensitive to variation of Mdm2 degradation parameter and gene copy number, in contrast to the other four models that are based on SNIC or subH bifurcation. The later models would imply a broad spectra of oscillation periods in cell population, which is not observed.

Considering the four p53 models, in which oscillations are born at superH bifurcation, we found that the oscillatory range (in parameter domain) is a growing function of p53 gene copy number and decreasing function of Mdm2 gene copy number. The dependence of oscillation range to gene copy number is stronger in our Toy model, which is based on the interlinked positive and negative feedback loops, than in the models of Ma et al. [20] and Puszynski et al. [19], in which oscillations

are driven solely by the negative feedback and time delay. The robustness of Batchelor et al. model [21] is somewhere in the middle of models [20] or [19] and the Toy model; The oscillation range (in parameter domain) and oscillation period are well conserved for Mdm2 and p53 gene copy numbers $m, n \in \{2, 3, 4\}$, but the oscillations may not arise for $m = 2, n > 5$, also the oscillation range is much smaller for $m = 1, n = 2$ and $m = 2, n = 5$ than for $m = n = 2$. However, in contrast to the Toy model in the Batchelor et al. model [21] the effect of Mdm2 transfection may be compensated by p53 transfection; i.e. cells having $m = n \in \{2, 3, 4, 5, 6\}$ oscillate in about the same parameter range and with the approximately the same period as the non-transfected cells ($m = n = 2$).

According to the Toy model, Mdm2 transfection may inhibit oscillations even in DNA damaged cells, while p53 transfection may promote oscillations even when DNA is intact. Since oscillations in p53-Mdm2 system can be potentially visualized in p53- or Mdm2-transfected cells, this prediction can be used for experimental verification of the Toy model concept. If the idea proves to be correct, it would suggest that the persistent oscillations observed by Geva-Zatorsky et al. in p53-CFP and Mdm2-YFP co-transfected MCF-7 cells are partially an artifact of the experimental setup [6]. As found in the experiment 19 out of 48 p53-Mdm2 co-transfected cells exhibited oscillations without any gamma irradiation, while strong 10 Gy irradiation caused that 37 out of 54 cells oscillated [6]. This may suggest that p53-Mdm2 co-transfection and gamma irradiation are both responsible for oscillations. According to the Toy model cells with the doubled number of p53 and Mdm2 copies have larger oscillatory range (in parameter domain) than the non-transfected diploid cells, see Fig. 7.

The other possibility is that positive feedback does not play any role in maintaining p53 oscillations, which result from the negative feedback(s) and time delay(s) as in the models by Ma et al. [20], Puszynski et al. [19] or Batchelor et al. [21]. Period of oscillations in these models is almost insensitive to the numbers (m, n) of Mdm2 and p53 gene copies at least for $m, n \in \{2, 3, 4\}$. Instead, as proposed by Puszynski et al. [19], the positive feedback, associated with long time delay, may be responsible for termination of oscillations by triggering p53 to a very high level, that leads to apoptosis in severely damaged cells. The new experimental data are needed to verify this hypothesis, however the Puszynski et al. [19] model appears to be better justified than the Toy model, since the latter neglects the long time delay associated with the positive feedback loop.

With respect to NF- κ B system, in which all deterministic models predict damped oscillations only, we showed that the saturation in the $I\kappa B\alpha$ transcription rate is necessary to produce oscillatory responses of the robust period. The models in which the transcription rate is proportional to the concentration of NF- κ B in the second or third power, without any saturation, lead to transcriptional efficiencies exceeding physiological rates and predict that period of oscillations rapidly grows with the level of transfection which is not observed.

All but one models analyzed in the current study describe p53 dynamics only in the deterministic way. The intrinsic noise, which arises due to small number of reacting molecules, may substantially influence the system dynamics. Our observation from p53 (Puszynski et al. [19]) and NF- κ B (Lipniacki et al. [28]-stochastic, [14]-deterministic) systems, for which we build stochastic models accompanied with their deterministic approximations, is that the characteristic frequency of oscillations is almost identical for stochastic and deterministic version. Although the intrinsic noise can cause the system (originally exhibiting the damped oscillations only) to oscillate, it may not severely change the characteristic period of oscillations. This suggests that introduction of noise to the considered deterministic models, which exhibit oscillations sensitive to parameters would not make the oscillations more robust. Unfortunately, the robustness of the model to parameters or extrinsic noise does not necessary imply its robustness to the intrinsic noise. For example, studying T cell receptor signalling, we found that in the bistable system, deterministic trajectories are qualitatively different from stochastic ones; in a broad range of parameters the deterministic simulations show persistent T cell activity, meanwhile the stochastic simulations show that the cell activity is transient, and for most of the time cell remains inactive [33]. We can thus expect that after taking noise into account bad models will remain bad, while good models not necessarily remain good.

The fluorescent reporter proteins are becoming the leading visualization method of individual cells behavior, which otherwise, is obscured by population averaging [32]. These experiments are performed on cells with elevated gene copy number(s) and thus must be analyzed with caution, but potentially can give a deeper insight to the regulatory pathways than experiments on the wild type cells only. As demonstrated by Nelson et al. [7, 30] for NF- κ B pathway and by Geva-Zatorsky et al. [6] for p53 system, oscillations that are produced in the response to stress have period insensitive to transcription factor gene copy number and the level of stress. These observations pose restrictions on the structure of regulatory systems that are responsible for the visualized oscillations. We found that bifurcation type is the most important determinant of system dynamics; oscillations of the most robust period arise in models based on superH bifurcation, while SNIC or subH bifurcations, characteristic for systems based on interlinked negative and positive feedbacks, lead to oscillations sensitive to the gene copy number and the level of stimulation. Particularly, in the case of SNIC (or SL) bifurcation the oscillations are born with the infinite period. In systems with SubH bifurcation oscillations are born with finite period, however the period may become long close to CF bifurcation, which is typically associated with SubH bifurcation, see Tyson group models I and III. One can thus expect that positive feedback plays no or minor role in maintaining oscillations in p53 system.

Our final conclusion differs from that of Tsai et al. [5], who recently proposed that the positive feedback is needed to produce robust oscillations of "tunable" frequency. As demonstrated by Tsai et al., systems involving interlinked positive and negative feedbacks may oscillate in thousand-fold

range of frequencies, with frequency controlled by the strength of positive feedback. However, we showed in our analysis, that the oscillation frequency in such systems would be also sensitive to other parameters. Since cells in population are not identical, the "tunable" frequency, would thus imply a broad range of oscillation periods in cell populations. However, in most of regulatory systems including p53, NF- κ B, circadian rhythm, HSF1/(hsp70, hsp90) oscillation period is insensitive to the strength of stimulation and there is a little variability of periods in cell populations. The later fact allows for observation of these oscillations also at the population level. This suggests that persistent oscillations in these systems are born at superH bifurcation or exist due to stochastic resonance (as in NF- κ B system) and that the positive feedbacks (even if present in particular regulatory pathways) do not play major role in generation of these oscillations.

4 Acknowledgments

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5 Figure captions

Figure 1 Four codimension-one bifurcations leading to oscillations; SuperH, SubH, SNIC and SL. First column: bifurcation diagrams, next columns: phase portraits for a given bifurcation parameter λ .

Figure 2 Analysis of Tyson group models I, II and III [4]. Period of stable limit cycle oscillations as a function of Mdm2 degradation parameter (k_{d2}). Colors: blue, red, black, green, orange and violet correspond to cells with $n = 1, 2, 3, 4, 5, 6$ (number of Mdm2 gene copies) and fixed number of p53 gene copies $m = 2$ (left column) or to cells with $m = 1, 2, 3, 4, 5, 6$ (number of p53 gene copies) and fixed number of Mdm2 gene copies $n = 2$ (right column).

Figure 3 Analysis of Tyson group model IV [17]. Period of stable limit cycle oscillations as a function of Mdm2 degradation parameter (k_{d2}). Colors: blue, red, black, green, orange and violet correspond to cells with $n = 1, 2, 3, 4, 5, 6$ (number of Mdm2 gene copies) and fixed number of p53 gene copies $m = 2$ or to cells with $m = 1, 2, 3, 4, 5, 6$ (number of p53 gene copies) and fixed number of Mdm2 gene copies $n = 2$. In the right column we show the enlarged fragments of the left column panels.

Figure 4 Diagram of p53|Mdm2 regulatory core: the Toy model. Dotted lines with arrowheads represent induction; dotted line with hammerhead represents inhibition.

Figure 5 Domains of stable limit cycles and stable steady states in (p53deg, Mdm2 deg) plane for diploid cells ($m = n = 2$) for the Toy model.

Figure 6 Analysis of the Toy model. Period of stable limit cycle oscillations as a function of Mdm2 degradation parameter (k_{d2}). Colors: blue, red, black, green, orange and violet correspond to cells with $n = 1, 2, 3, 4, 5, 6$ (number of Mdm2 gene copies) and fixed number of p53 gene copies $m = 2$ (left Panel) or to cells with $m = 1, 2, 3, 4, 5, 6$ (number of p53 gene copies) and fixed number of Mdm2 gene copies $n = 2$ (right Panel).

Figure 7 Bifurcation diagrams for normal and transfected cells for the Toy model. Recurrent states (steady states and limit cycles) are plotted as a function of p53 (left Panel) and Mdm2 (right Panel) degradation rates; red: normal cells ($m = n = 2$), blue: Mdm2 transfected cells ($m = 2, n = 4$) orange: p53-transfected cells ($m = 4, n = 2$) and green: Mdm2 and p53-cotransfected cells ($m = 4, n = 4$). Dotted lines represent upper and lower limits of stable limit cycles, the continuous lines represent stable steady states.

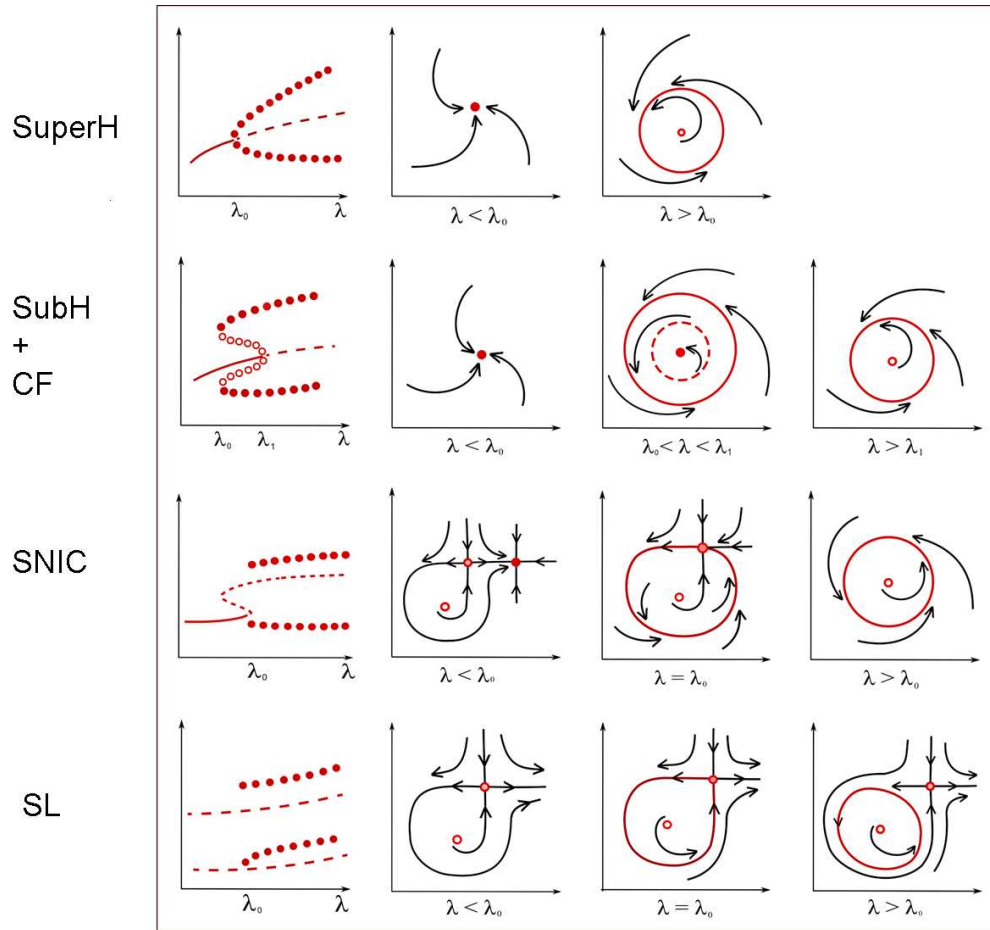
Figure 8 Bifurcation diagrams for normal and haploid cells for the Toy model. Recurrent states (steady states and limit cycles) are plotted as a function of p53 (left Panel) and Mdm2 (right Panel) degradation rates; red: normal cells ($m = n = 2$), blue: Mdm2 haploid cells, ($m = 2, n = 1$) and green: p53 haploid cells ($m = 1, n = 2$). Dotted lines represent upper and lower limits of stable limit cycles, the continuous lines represent stable steady states.

Figure 9 Analysis of Ma et al. [20] and Puszynski et al. [19] models. Period of stable limit cycle oscillations as a function of Mdm2 degradation parameter (k_{d2}). Colors blue, red, black, green, orange and violet correspond to cells with varied number of Mdm2 gene copies $n = 1, 2, 3, 4, 5, 6$ (left column) or with varied number of p53 gene copies $m = 1, 2, 3, 4, 5, 6$ (right column).

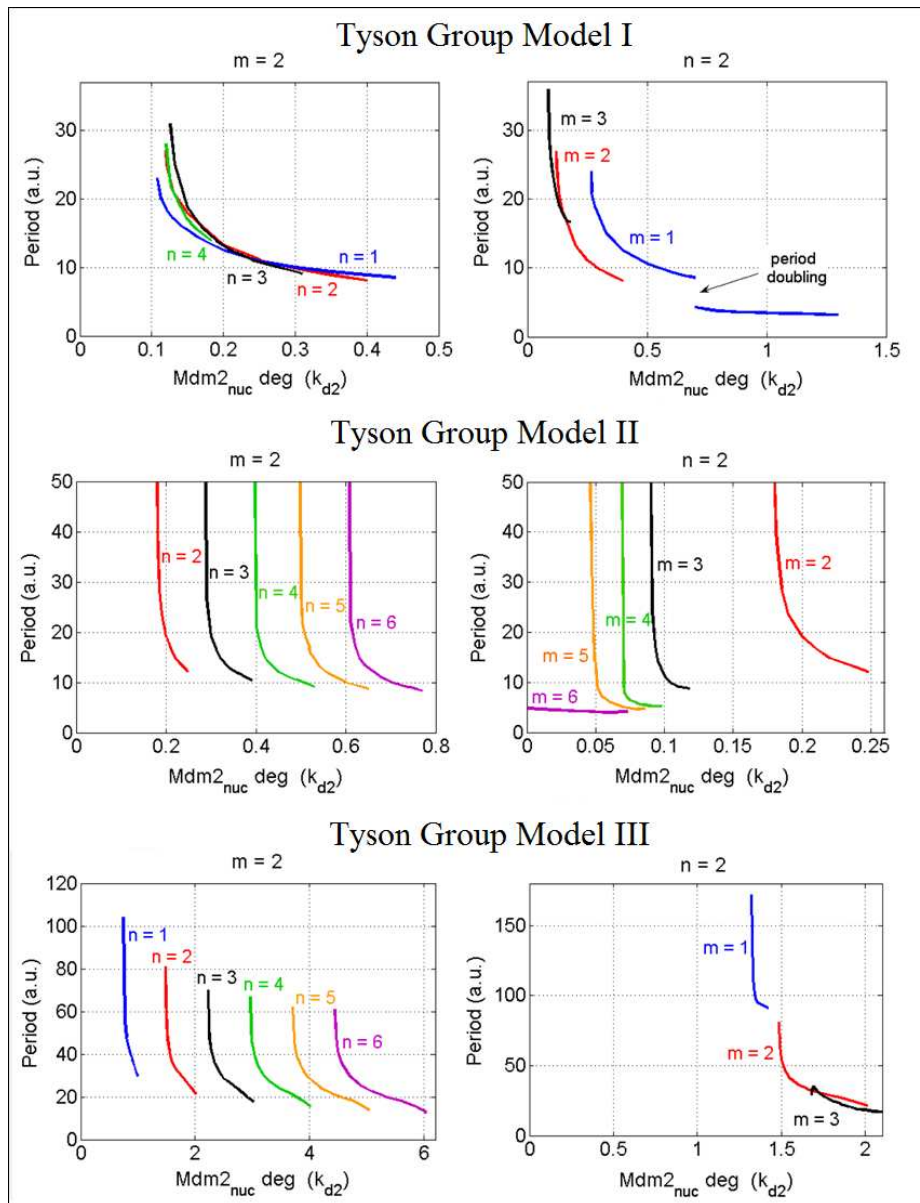
Figure 10 Analysis of Batchelor et al. model [21]. Upper Panel: Recurrent states (steady states and limit cycles) are plotted as a function of signal production rate β_s ; as β_s grows the limit cycle oscillations arise and vanish at superH bifurcations. Period of stable limit cycle oscillations as a function of β_s . Colors: blue, red, black, green, orange and violet correspond to cells with $n = 1, 2, 3, 4, 5, 6$ (number of Mdm2 gene copies) and fixed number of p53 gene copies $m = 2$ (left Panel) or to cells with $m = 1, 2, 3, 4, 5, 6$ (number of p53 gene copies) and fixed number of Mdm2 gene copies $n = 2$ (right Panel).

Table 1: Parameters and definitions for the Toy model

Symbol	Values	Units	Description	Comments
s_1	16	s^{-1}	p53 synthesis rate	
s_2	8	s^{-1}	Mdm2 constitutive synthesis rate	
s_3	80	s^{-1}	Mdm2 synthesis rate (p53-dependent)	
s_4	10^5		saturation constant in Mdm2 synthesis	
k_{d1}	10^{-13}	s^{-1}	p53 degradation (induced by Mdm2)	value at bifurcation for diploid cells
k_{d2}	2.2×10^{-4}	s^{-1}	Mdm2 degradation rate	value at bifurcation for diploid cells
k_1	3.5×10^{-3}	s^{-1}	Mdm2 nuclear import (controlled by p53)	
k_2	2300		saturation constant in Mdm2 nuclear import	
m			number of p53 gene copies	various m are considered
n			number of Mdm2 gene copies	various n are considered

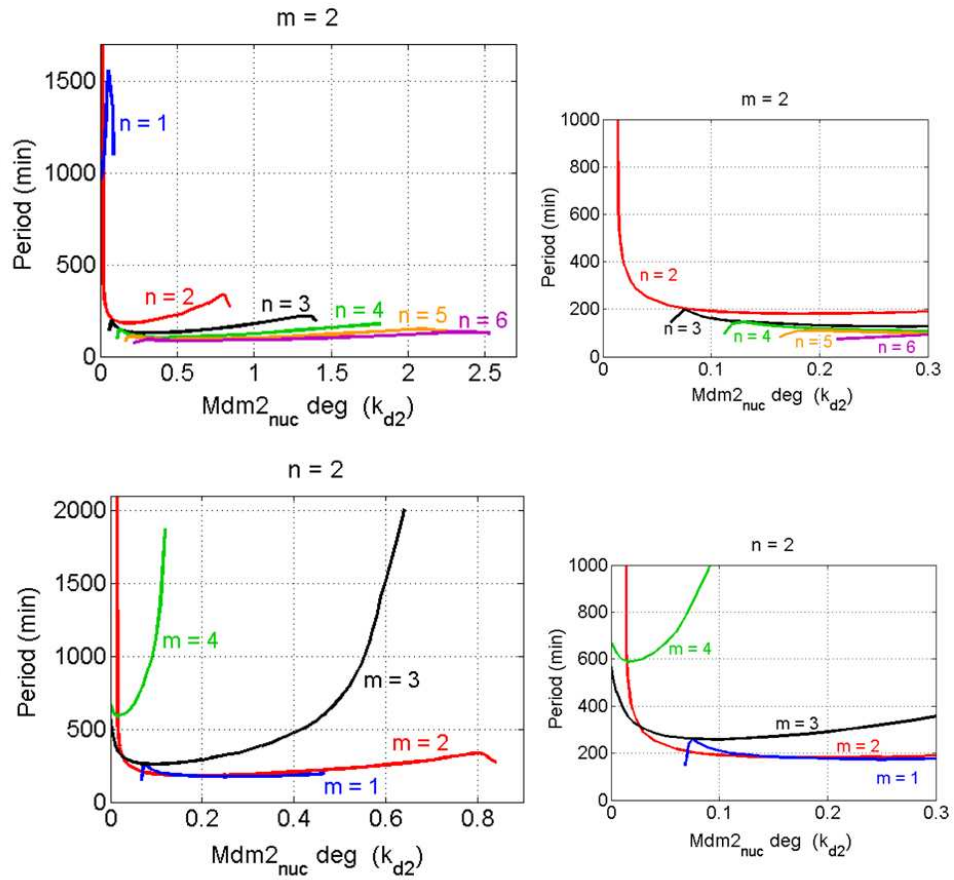


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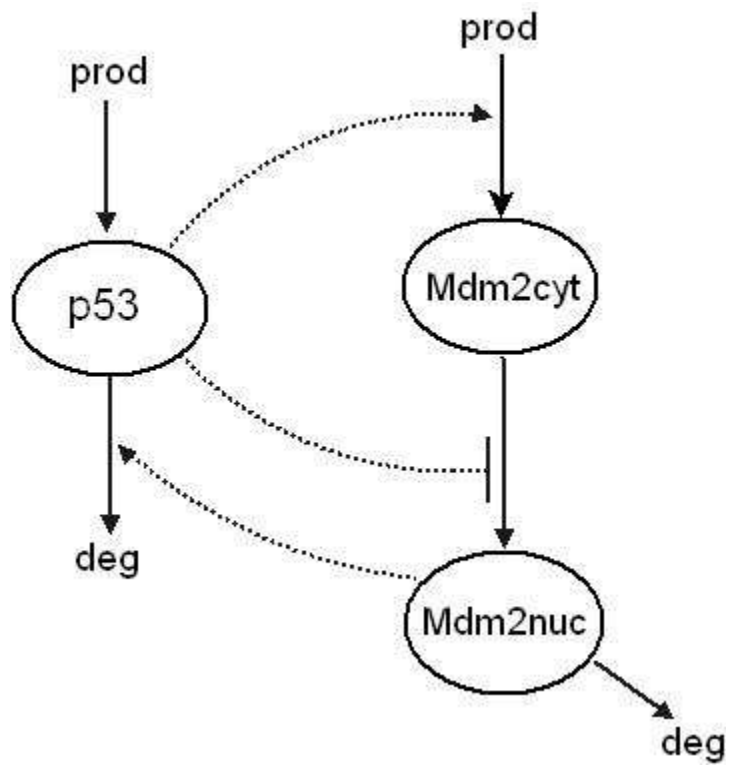


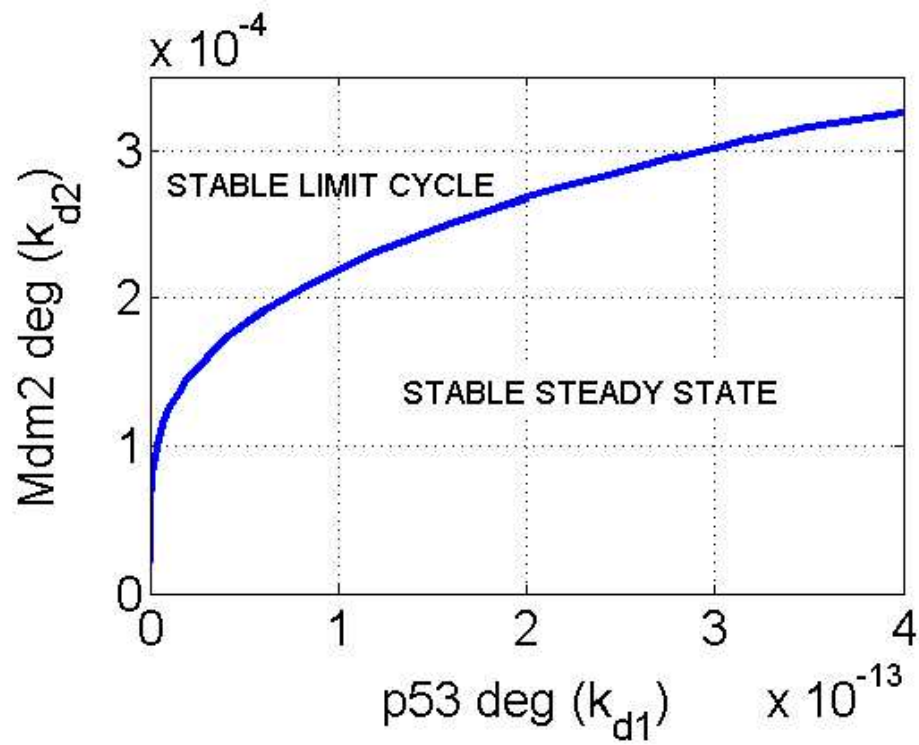
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Tyson Group Model IV

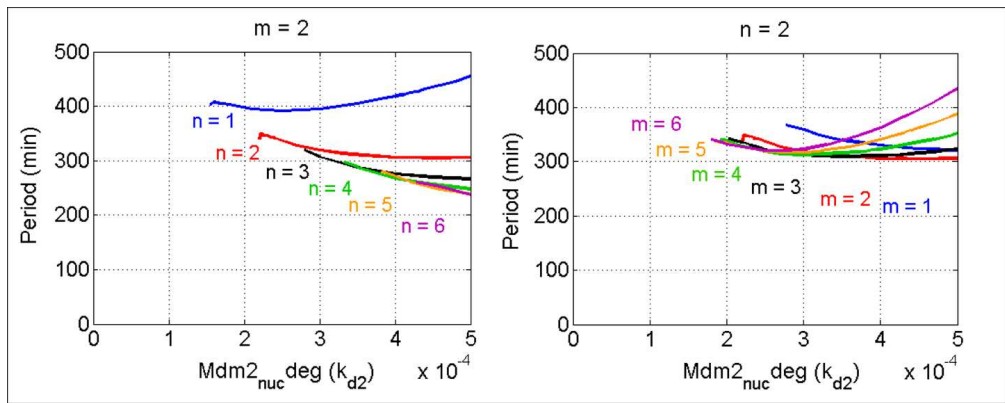


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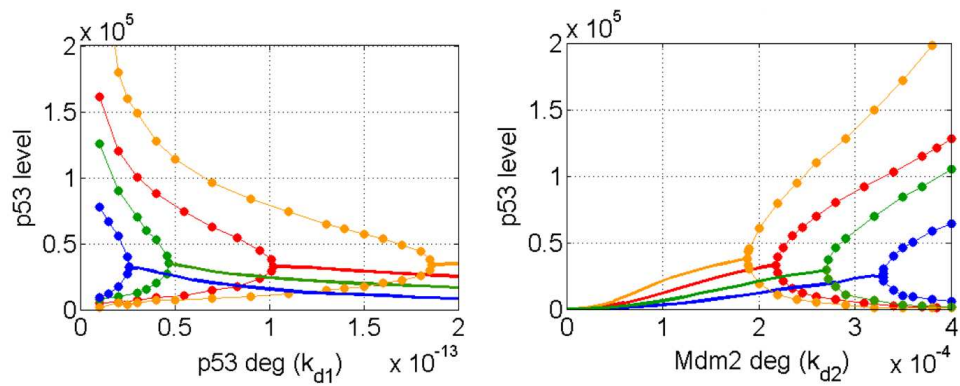




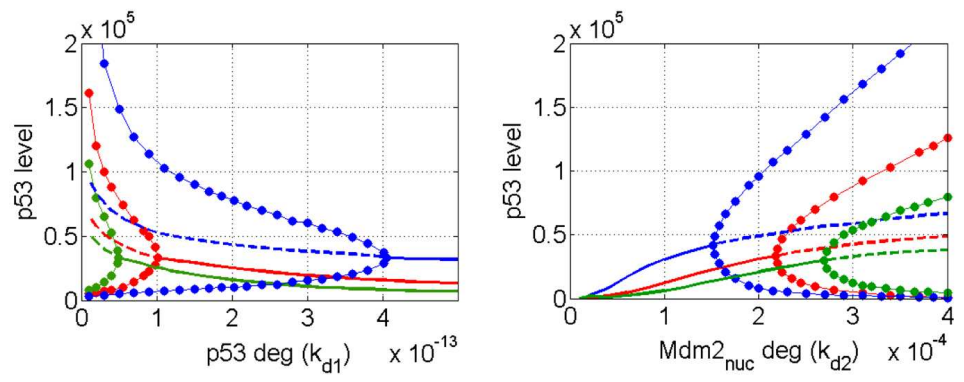
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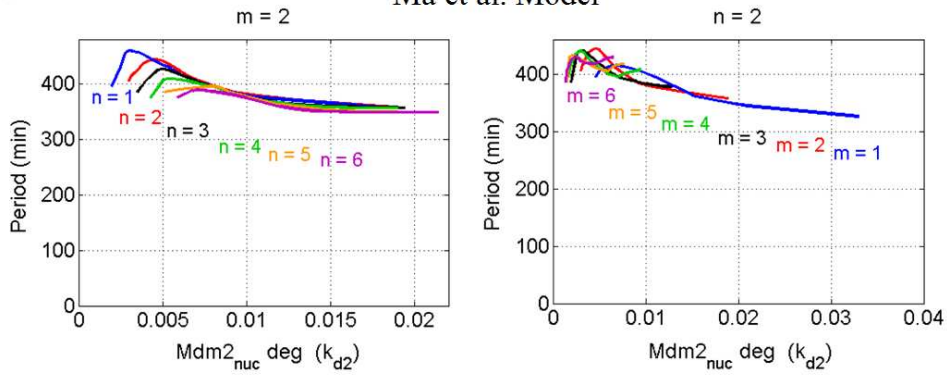


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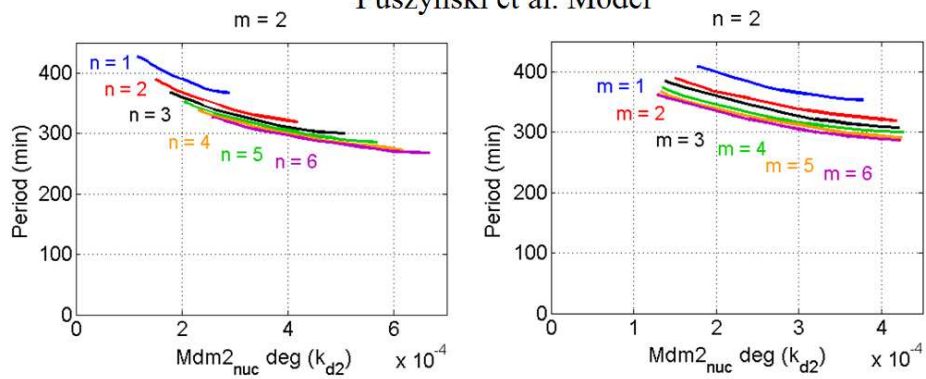


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Ma et al. Model

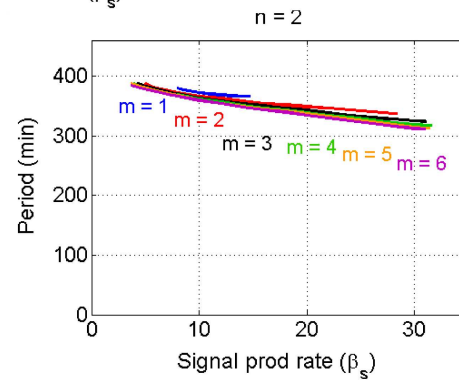
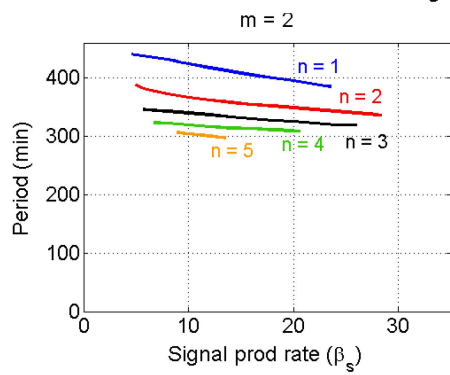
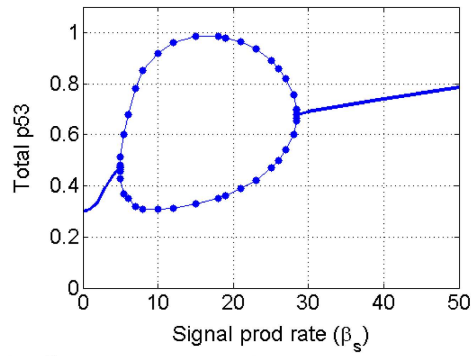


Puszynski et al. Model



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Batchelor et al. Model



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