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Stochastic microtubule-kinetochore attachment drives the metaphase to anaphase transition in mitotic arrested cells

STIER, Alma Beatrix

Molecular Bionics Engineering BSc

PPCU, Faculty of Information Technology and Bionics

Supervisor: CILIBERTO, Andrea

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Abstract

Cells are arrested during the cell cycle by drugs that disrupt essential functions and/or structures such as the mitotic spindle. When this happens, cells arrest cell cycle progression to minimize the risks of undergoing faulty divisions. The arrest is driven by specialized pathways known as checkpoints. In particular, the spindle assembly checkpoint (or mitotic checkpoint) oversees chromosome segregation and thus the integrity of the mitotic spindle. By destabilizing the attachment between microtubules and chromosomes, microtubule targeting drugs activate the mitotic checkpoint which arrests cell cycle progression and favors cell death. Such drugs (e.g., taxanes or vinca alkaloids) are commonly used to treat cancer patients.

Checkpoint activation arrests cells in mitosis. However, some cells manage to escape from the mitotic arrest (they adapt) and resume proliferation. The process of adaptation is stochastic, with some cells escaping the arrest very soon after entry into mitosis and others hours later. In the context of cancer treatments, all escaping cells are dangerous since they do not respond to drugs and may become resistant to treatment. Hence, understanding how cells escape from the arrest is important to improve the efficacy of drugs widely used in cancer treatment.

The overarching objective of my work was to build a model of adaptation to the mitotic checkpoint. I proposed to understand how cells escape the arrest induced by microtubule targeting drugs, testing the hypothesis that the transition from arrest to mitotic exit is driven by noise in protein numbers and microtubule-kinetochore attachment. First, I developed a deterministic model of the molecular network describing the mitotic checkpoint. Secondly, I translated it into a stochastic framework. Finally, I used a software for parameter estimation to reproduce experimental data. With a working model in my hands, I tested its predictive nature by formulating new experiments and reproducing experimental results not used for model definition.

Based on the model's results, I propose ways to avoid adaptation. Some of these have been already tested experimentally and results support model simulations and *vice versa*. Hence, my results may be relevant to optimize the effect of anti-cancer drugs. As such, the research carried out has an obvious potential social impact for the treatment of cancer patients.

Introduction

The complex nature of biological systems demands a systematic approach to understand and predict their behavior. Mathematical models play a pivotal role in the field of computational biology, offering powerful means of analyzing the complex processes governing living organisms.

Computational biology seeks to uncover the underlying principles that drive biological phenomena. Mathematical models allow to simulate, analyze and manipulate biological processes *in silico*, providing valuable insights that might be challenging or impossible to obtain through experimentation alone.

Models serve a dual purpose in computational biology, as they not only enhance our comprehension of intricate biological systems but also provide a valuable tool for predicting outcomes by simulating biological processes. Hence, *in silico* simulations offer rapid means of designing experiments, predicting the system's behaviour and identifying new conditions to explore in experiments.

In my work, I proposed to understand how cells manage to escape the mitotic arrest, by building a stochastic mathematical model. I tested the hypothesis that mitotic checkpoint activity can be described with a positive feedback loop and adaptation is a noise-induced phenomenon in mitotic arrested cells. The model is fitted and tested on experimental data.

This topic is relevant in finding new ways to avoid the emergence of drug-resistant cancer cells. The mathematical model can help in understanding and predicting the behaviour of the real biological system, and identifying a weakness of drugs targeting microtubules, which is a precondition for improving the efficacy of such drugs commonly used for treating cancer patients.

Literature review

2.1 Biological background

2.1.1 Chromosome segregation

The cell cycle is a strictly regulated process that controls the growth and division of cells. It consists of several phases [1]. The proper progression through these phases is essential for maintaining the integrity of DNA and ensuring that cells divide accurately. Disruption at any point in the cell cycle can lead to various issues, including DNA damage, aneuploidy (abnormal chromosome number) and uncontrolled cell growth [2].

The main function of mitosis is to segregate the two sets of chromosomes that are present in the cell after S-phase [1]. Chromosome segregation is carried out by a complex machine called the mitotic spindle, which pulls apart the sister chromatids, which are held together by cohesin complexes [1]. During anaphase, cohesin needs to be removed from chromosomes. This process is executed by a specific protease called separase, which allows chromosome separation when its inhibitor, securin is degraded [3].

Securin degradation is driven by an E3 ubiquitin ligase, called the ‘Anaphase Promoting Complex’ (APC). This is a multiprotein complex, which initiates anaphase and mitotic exit. When bound to its co-factor Cdc20, APC targets securin [1]. The APC/C^{Cdc20} complex also targets Cyclin B for degradation, thus leading to CDK1 inactivation and exit from mitosis (Figure 2.1). Both securin and Cyclin B degradation occur when cells enter anaphase. Cells that do not express Cdc20 or carry APC mutations cannot enter anaphase [4][5].

After securin is degraded chromosomes can progress to segregation, which requires microtubules and kinetochores. Microtubules are molecular machines that build the mitotic spindle and are responsible for dragging chromosomes to the daughter cells [1]. Kinetochores instead are macromolecular structures that create an interface for the key interaction between chromosomes and microtubules of the mitotic spindle [1] [6]. Chromosome movements are mainly dependent on kinetochores [7].

When chromosomes are not attached properly to microtubules, chromosomes can be segre-

gated to the same daughter cell, generating aneuploid progenies. This condition is potentially dangerous and typical of cancer cells [2].

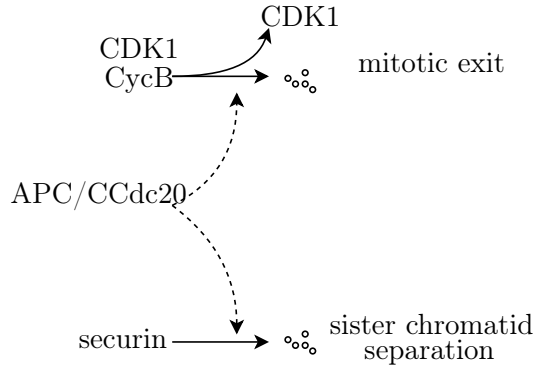


Figure 2.1: Anaphase promoting complex together with its co-factor Cdc20 drives degradation of securin and Cyclin B, leading to CDK1 inactivation. Chromosomes are separated when securin is degraded. Cyclin B degradation initiates mitotic exit.

2.1.2 Mitotic checkpoint

To avoid chromosome missegregation, the process of metaphase-anaphase transition is closely watched by the mitotic checkpoint [1] [8], also called the spindle assembly checkpoint (SAC), which is an important evolutionary conserved surveillance mechanism [1], and has been investigated for decades [8][9].

The main role of the checkpoint is to monitor the attachment between kinetochores and spindle microtubules [7]. Indeed, the checkpoint is activated by the signal sent by unattached kinetochores, when chromosomes are not properly attached to microtubules [1]. In this condition, the checkpoint arrests cells in mitosis, giving cells time to find the correct attachment. Ideally the SAC remains active until the last chromosome has aligned. After this moment, Cyclin B and securin start to be degraded by APC/C^{Cdc20} . The timing of their degradation then is dictated by the dynamics of kinetochore-microtubule attachment [10].

Genetic screening showed that there are several genes in *Saccharomyces cerevisiae* (budding yeast) which arrest cells in mitosis, when microtubule dynamics is impaired: MAD (mitotic-arrest deficient) genes *MAD1*, *MAD2* and *MAD3* and BUB (budding uninhibited by benzimidazole) genes *BUB1* and *BUB3*. These genes are the main components of the spindle assembly checkpoint and are conserved in all eukaryotes [11][8][9][12].

To prevent mitotic exit and chromosome segregation, the mitotic checkpoint proteins inhibit the anaphase promoting complex/ cyclosome and its coactivator Cdc20. In particular, checkpoint activation leads to a form of APC/C^{Cdc20} in which APC/C is inactive [5]. In this form, Cdc20 is first bound to *MAD2* and *MAD3* to form the so-called mitotic checkpoint complex or MCC.

The latter can bind APC/C^{Cdc20} to form the inhibited APC^{MCC} . Importantly, MCC formation is energetically favoured but slow, the rate limiting step being the binding of Cdc20 to Mad2. MCC formation is catalyzed by the MAD1:MAD2 complex which sits at kinetochores when microtubules are not attached [13][14][15][16].

As mentioned, the anaphase promoting complex when bound to MCC is unable to ubiquitinate two key substrates Cyclin B and securin, thereby the progression into anaphase is prevented. The inhibited APC:MCC complex contains two Cdc20 molecules [17][18][19][20]. Importantly, the arrest is not static, since APC:MCC is continuously disassembled by the degradation of Cdc20. The latter separates APC from Cdc20 and Mad2 [21]. These components then bind again to produce APC:MCC, giving rise to a futile cycle.

2.1.3 Adaptation to the mitotic checkpoint

Cells can maintain an arrest for several hours, but this condition does not last indefinitely. Eventually cells adapt or slip out from the arrest, despite conditions that activate the SAC. This phenomenon is referred to as ‘adaptation’ to the mitotic checkpoint or ‘slippage’ [10].

Brito and Rieder [22] studied mitotic checkpoint slippage in mammalian single cells with GFP-tagged Cyclin B. They observed that escape from mitosis occurs when the checkpoint is still signalling and Cyclin B is degraded. In particular, they show that in cells Cyclin B is slowly and continuously degraded independently of SAC activity, eventually driving cells out of mitosis.

Bonaiuti et al. [23] investigated if yeast cells adapt to the mitotic checkpoint with an active SAC by observing Cyclin B (Clb2) and Mad2. They used nocodazole to arrest cells in mitosis and tagged Mad2 with GFP and Clb2 with mCherry to monitor mitotic checkpoint signaling and anaphase transition under a microscope. Clb2 degradation indicated APC/C^{Cdc20} activation and anaphase onset. Cells were tracked for several hours to determine if Mad2 was localized when Clb2 degraded and the duration of mitotic arrest. They found that Cyclin B degradation often occurred with localized Mad2, indicating that cells enter anaphase with an active checkpoint (adaptation). The data showed an exponential distribution of adaptation times, suggesting a consistent probability of adaptation across cells. The study also suggested that low levels of proteins and complexes like MCC and APC:MCC might contribute to adaptation due to fluctuations.

In summary, the transition to anaphase requires the degradation of key proteins, Cyclin B and securin, by $APC/CCdc20$. Once securin is degraded, chromosomes can segregate. Chromosome segregation is dependent on the proper attachment of chromosomes to microtubules via kinetochores. Failure in this attachment can lead to aneuploidy. To avoid it, the spindle

assembly checkpoint stops APC activity, when unattached kinetochores generate a signal that prevents anaphase. In this case, Mad1:Mad2 complexes bind to kinetochores, accelerating the formation of MCC and APC:MCC, which further inhibits APC/C^{Cdc20} activity and cells are arrested in mitosis. Eventually cells manage to adapt to the checkpoint, leaving metaphase with an active checkpoint, possibly due to fluctuations in the levels of checkpoint-related proteins and complexes like MCC and APC:MCC.

2.2 Models of the mitotic checkpoint

Computational modeling has a great significance in understanding how complex biological systems, like the spindle assembly checkpoint work. Several models of the mitotic checkpoint have already been proposed.

The Novak model [24] explores how the metaphase-anaphase switch is regulated, suggesting that system-level feedback mechanisms ensure this transition is irreversible. They propose that feedback loops create a one-way system, making the anaphase transition a bistable switch. The model posits that CDK^{CycB} and APC/C^{Cdc20} form a positive feedback loop through mutual inhibition. Chromosome tension is a key parameter: it indicates correct chromatid orientation, while lack of tension activates the mitotic checkpoint. While the authors propose a bistable switch based on the mutual antagonism between CDK^{CycB} and APC/C^{Cdc20} , their model is not built on a realistic network and does not account for adaptation. The positive feedback loop is not experimentally validated, and the model uses dimensionless concentrations, ignoring realistic values. The model's behavior is unaffected by parameter scaling, which is not biologically plausible.

The Bonaiuti model [23] offers detailed molecular accuracy by introducing spindle assembly checkpoint components, APC, and Cdc20 interactions. It posits that cells can proceed to anaphase through APC/C^{Cdc20} activity even when the mitotic checkpoint is active. This is modeled as a stochastic event where APC/C^{Cdc20} levels fluctuate around a steady-state value below a critical anaphase threshold due to SAC. Random fluctuations can push levels past this threshold, triggering anaphase. The model uses mostly realistic parameters, either measured directly or sourced from existing data. Figure 2.2 from Bonaiuti et al. [23] illustrates how adaptation was determined by an arbitrary APC/C^{Cdc20} threshold. Although the model is realistic, the anaphase threshold is an artificial, ad-hoc component that greatly limits the model.

The Gross, Bonaiuti model [25] extends the Bonaiuti model by analyzing alternative pathways from Mad2-Cdc20 binding to APC/C inhibition. While the model is molecularly detailed and thoroughly examines the checkpoint signaling network, it does not explain how cells adapt to the mitotic checkpoint, as it lacks stochastic simulations.

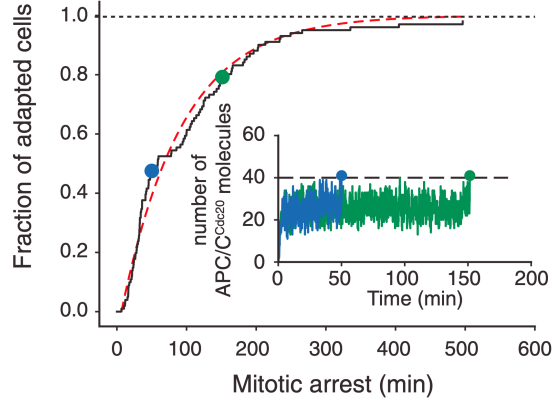


Figure 2.2: APC/C^{Cdc20} fluctuations define transition to anaphase by crossing an arbitrary threshold. (Source: Bonaiuti et al, 2018, Current Biology, Figure 2F [23])

The Henze model [26] focuses on mitotic checkpoint silencing in human cells, presenting a detailed molecular network and spatially stochastic simulations. It uses experimental data from checkpoint component mutants to test switch-like behavior, but only in a simplified version. Parameters and concentrations are arbitrary, and the model does not address adaptation to the checkpoint.

The Vernieri model [27] introduces bistability and addresses the question of understanding how cells adapt in the presence of an active checkpoint. They introduce a simple model for M/A transition in adaptation, however important molecular details, like the mitotic checkpoint components are not introduced.

In conclusion, the two most relevant works are the Bonaiuti [23] and Novak [24] models. The former one lies on detailed and realistic molecular grounds and is the only model so far which dealt with adaptation, but includes a rough approximation. The latter one introduces the idea of positive feedback loops controlling the entry of cells into anaphase, but neither provides experimental evidence for the presence of a positive feedback loop nor describes the system in realistic molecular details.

2.3 Problem statement

None of the models available so far gives an explanation for adaptation or include realistic molecular details and manages to reproduce extensive experimental data with stochastic simulations. Previous models either oversimplify and neglect molecular components, or use and generate artificial unmeasured data. In my work I proposed to model the adaptation to the mitotic checkpoint while satisfying all these requirements.

Results

3.1 Evidence of a positive feedback loop

To improve the Bonaiuti et al. [23] model, we hypothesize that adaptation can be explained as a transition between steady states. This idea is inspired by the Novak [24] model, but I plan to introduce bistability based on tested experimental results. Indeed, experimental data carried out by the host lab ¹ support the presence of a positive feedback loop that reinforces SAC activity.

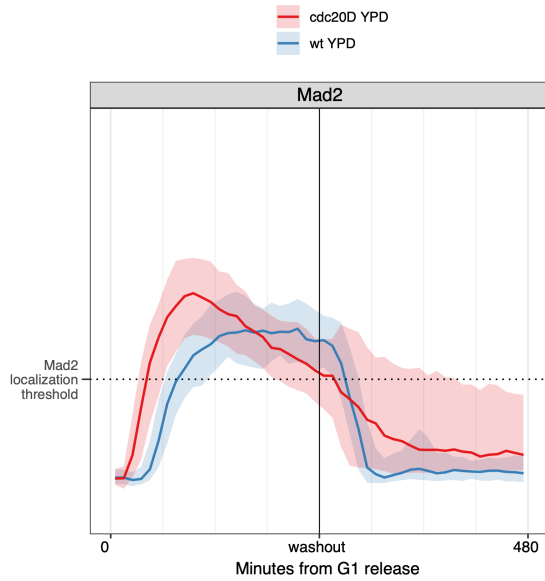


Figure 3.1: Experimental result providing evidence for the presence of a positive feedback loop that supports SAC activity. Mad2 delocalization as a function of time. In red Cdc20D YPD, while in blue wild-type YPD cells are shown. The experiment was carried out by Paolo Bonaiuti.

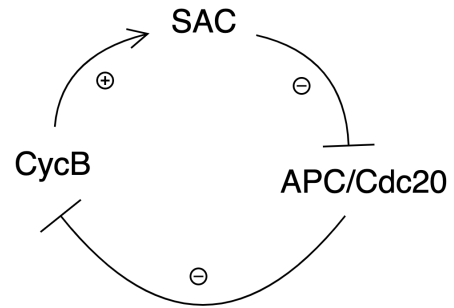


Figure 3.2: Positive feedback loop proposed in the process of checkpoint activation: Cyclin B activates the checkpoint. The SAC inhibits APC/C^{Cdc20} , which inhibits Cyclin B, thus closing the positive feedback loop.

In the experiment, during a nocodazole-induced arrest, the synthesis of Cdc20 was switched off. 240 minutes after G1 release, the drug was removed, and Mad2 delocalization from kinetochores was followed. Results showed that Mad2 delocalization is slower when Cdc20 is not produced (Fig. 3.1) than in wild-type cells, where APC/C^{Cdc20} is active and degrades Cyclin B.

¹Quantitative Biology of Cell Division, Instituto Firc di Oncologia Molecolare, IFOM, Milan, Italy

When Cdc20 is switched off, Cyclin B is stable. Thus one interpretation of the results is that CDK/Cyclin B supports SAC activity, which can be seen from the slow removal of Mad2 from kinetochores. It is worth reminding that kinetochore-bound Mad2 is the active form of this protein. CDK/CycB supporting the SAC would give rise to the positive feedback loop shown in Figure 3.2. A similar loop has been proposed and modeled already by the Novak group [24].

Here, based on this positive feedback loop we propose that mitotic cells can have two states: mitotic checkpoint active (SAC ON) or inactive (SAC OFF). In the first, mitotic cyclin (Cyclin B) is high, and so are the forms of inhibited Cdc20 (MCC, APC:MCC), while APC/C^{Cdc20} is low. In the second, mitotic cyclin and inhibited Cdc20 are low and APC/C^{Cdc20} is high. We propose that adaptation occurs as a random transition from the SAC active to the SAC inactive state. The small number of proteins and complexes would play a critical part in the process.

3.2 Deterministic model

The goal was to build a model describing adaptation to the mitotic checkpoint based on a bistable switch instead of an arbitrary threshold and to introduce more molecular details.

3.2.1 Positive feedback loop in the model

In the developed model the main aspects were: introducing synthesis and degradation of Cyclin B and Cyclin B activating the mitotic checkpoint components (marked with Mad2a and Mad2i - corresponding to active and inactive forms, respectively). Besides Cyclin B, the unattached kinetochores (nUK - number of unattached kinetochores) also catalyze the transition from inactive to active Mad2. We assumed that both are required: neither Cdk1 activity alone, nor nUK alone can sustain SAC activity.

Activated Mad2 binds to Cdc20 forming the mitotic checkpoint complex core (MCC). Cdc20 also binds to the anaphase promoting complex, the binding resulting in activation of APC. The activated APC/C^{Cdc20} degrades Cyclin B and its inhibition is driven by the mitotic checkpoint complex core, resulting in inactive APC:MCC. The latter degrades Cdc20 and breaks into Cdc20, APC/C and inactive Mad2.

The positive feedback loop in the model is similar to what was described in Figure ?? : Cyclin B activates the checkpoint, active Mad2 indirectly inhibits APC/C^{Cdc20} , active APC/C^{Cdc20} degrades Cyclin B.

In Figure 3.3 I show the wiring diagram describing the model.

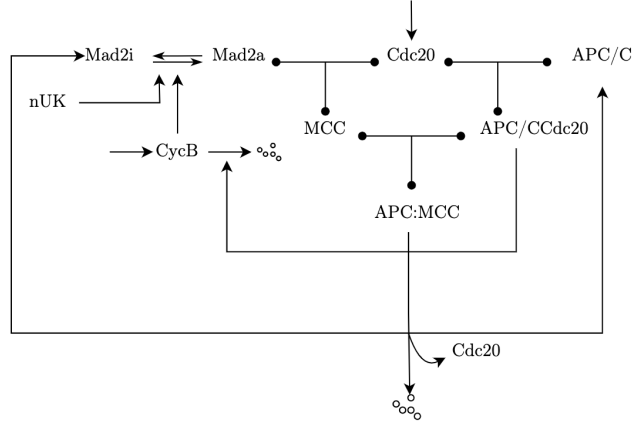


Figure 3.3: Wiring diagram of the new model. APC/C^{Cdc20} degrades Cyclin B, Cyclin B activates the checkpoint. Active Mad2 indirectly inhibits APC/C^{Cdc20} . This last step closes the positive feedback loop.

3.2.2 Mathematical framework for the model

The deterministic version of the model can be translated into the following set of ordinary differential equations, involving 9 variables (A , $Mad2_i$, $Mad2_a$, C , AC , MC , $ACMC$, $CycB$, nUK). The model is defined by 6 differential equations:

1. Cyclin B is synthesized and degraded dependently ($k_{deg_{CycB}}$) and independently ($k_{deg_{BGCycB}}$) from APC/C^{Cdc20} :

$$\frac{dCycB}{dt} = k_{syn_{CycB}} - k_{deg_{CycB}} \cdot CycB \cdot AC - k_{deg_{BGCycB}} \cdot CycB$$

2. The checkpoint component Mad2 is activated via Cyclin B and unattached kinetochores. Aravamudhan et al. [28] showed that as the number of signaling kinetochores in a cell increases, the cumulative number of SAC proteins recruited by these kinetochores reaches saturation. Thus the fraction of active SAC components can be described with the formula $\frac{k' \cdot nUK}{J_n + nUK}$. Hence, similarly to the three-component-model, a Michaelis-Menten constant was introduced to both for Mad2 activation and Mad2 inactivation. Active Mad2 associates with Cdc20 to form the mitotic checkpoint complex (MC), this being a reversible reaction (dissociation of MC leads to active form of M):

$$\begin{aligned} \frac{dMad2_a}{dt} = & k_{act} \cdot CycB \cdot \frac{k' \cdot nUK}{J_n + nUK} \cdot \frac{Mad2_i}{J + Mad2_i} - k_{inact} \cdot \frac{Mad2_a}{J + Mad2_a} \\ & - k_{assMC} \cdot Mad2_a \cdot C + k_{dissMC} \cdot MC \end{aligned}$$

3. APC/C^{Cdc20} (AC in the model) is formed by association of APC/C and its coactivator

Cdc20, which is a reversible reaction (dissociation of AC). AC also takes part in the formation of the inhibited APC complex (ACMC), associating with the mitotic checkpoint complex (MC). APC/C^{Cdc20} is degraded via background degradation. The differential equation is:

$$\frac{dAC}{dt} = k_{assAC} \cdot A \cdot C - k_{dissAC} \cdot AC - k_{assACMC} \cdot AC \cdot MC + k_{dissACMC} \cdot ACMC - k_{degBG} \cdot AC$$

4. The equation for the checkpoint complex (MC) shows the association of active Mad2 and Cdc20 and the reverse reaction, dissociation of MC. MC forms the inhibited APC/C and is degraded similarly to APC/C^{Cdc20} via background degradation:

$$\begin{aligned} \frac{dMC}{dt} = & k_{assMC} \cdot Mad2_i \cdot C - k_{dissMC} \cdot MC - k_{assACMC} \cdot AC \cdot MC \\ & + k_{dissACMC} \cdot ACMC - k_{degBG} \cdot MC \end{aligned}$$

5. The equation for the inhibited form of APC/C^{Cdc20} (ACMC) is the following:

$$\frac{dACMC}{dt} = k_{assACMC} \cdot AC \cdot MC - k_{dissACMC} \cdot ACMC - k_{deg} \cdot ACMC \cdot MC - k_{degBG} \cdot ACMC$$

6. Cdc20 is synthesized, used in formation of MC and AC (reversible reactions), produced by ACMC degradation and degraded via background degradation and via APC:MCC-catalyzed degradation:

$$\begin{aligned} \frac{dC}{dt} = & k_{synC20} - k_{assAC} \cdot A \cdot C + k_{dissAC} \cdot AC - k_{assMC} \cdot Mad2_a \cdot C + k_{dissMC} \cdot MC \\ & + k_{deg} \cdot ACMC - k_{degBG} \cdot C \end{aligned}$$

Since the total amounts of APC/C and checkpoint proteins are assumed to be constant, the APC/C and inactive M levels can be calculated from the total amounts:

$$A = A_{total} - (AC + ACMC)$$

$$Mad2_i = Mad2_{total} - (Mad2_a + MC + ACMC)$$

3.2.3 Introduction of kinetochore attachment dynamics

We used the model to reproduce published experimental data [23] of adaptation under constant checkpoint activation and new data about drug-washout (an experimental condition where kine-

tochores attach asynchronously to microtubulues in consequence of drug removal, discussed later in section 3.6).

For this reason, we introduced a new variable nUK (number of unattached kinetochores) in the model. Number of unattached kinetochores can vary between 0 and 32, because our experimental model system is budding yeast, which has 16 chromosomes and 32 kinetochores. Having 32 value for nUK, means all kinetochores are unattached, this state corresponding to the activated checkpoint state. Having 0 nUK corresponds to the checkpoint being inactive, where all kinetochores are attached and the cells have silenced the checkpoint. Any value above 1 was suggested to be sufficient to activate the checkpoint, albeit with different strength [29].

The attachment process is modeled by the following reaction: $nUK \xrightarrow{k_{nUK_{att}}} \emptyset$, with $\frac{dnUK}{dt} = -k_{nUK_{att}} \cdot nUK$.

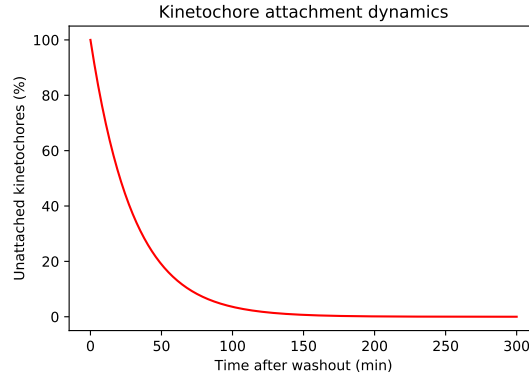


Figure 3.4: Kinetochore attachment dynamics, percentage of unattached kinetochores against time (minutes). Washout started at time = 0.

In Figure 3.4, I simulate the effect of washing out a drug, which induces the attachment of all kinetochores. As a consequence the system goes from 32 to 0 unattached kinetochores. The vertical axes shows the percentage of unattached kinetochores, which changes between 0 and 100% (100% corresponding to all kinetochores being unattached - at 0 timepoint). Time $t = 0$ corresponds to the time of the washout.

3.2.4 Parameters

Parameters chosen for the model are largely based on experimental results. The parameters were fitted in order for the model to reproduce quantitatively experimental data.

Concentrations - total amounts

Bonaiuti et al. [23] carried out two experiments relevant for determining total amount concentrations. Cdc20 and Cdc23 were tagged with the same tag. Cdc23 is a subunit of APC/C, thus

it can be used as a proxy for APC/C levels. Tagging was followed by performing a western blot in the same gel (Fig 2E [23]). This allowed a direct comparison of Cdc23 (APC/C) and Cdc20. The readout shows that their concentration levels are very similar.

Another experiment, where fluorescence correlation spectroscopy (FCS) was used, measured the concentration of Cdc23 (Fig 2D [23]). It was observed that it is ~ 60 nM. Based on these two experiments we assumed that Cdc20 is also ~ 60 nM. In the same paper also Mad2 and Mad3 levels were measured (figure 2D [23]). Mad3 was also found to be limiting to ~ 60 nM.

In agreement with these data, in the model we set as total amounts $APC_{total} = 60$ nM, $Mad_{total} = 70$ nM. In stochastic simulations, concentration values must be converted into molecules per cell. Since all reactions occur in the nucleus, not in the whole cell, we calculated the conversion factor using a haploid yeast cell's mean nuclear radius of $1\text{ }\mu\text{m}$ [30], giving a nuclear volume of $4.2\text{ }\mu\text{m}^3$ (approximating the nucleus as a sphere). Concentration is measured in nanomolar (nM), with 1 nM equal to 1 nanomole per liter. Using Avogadro's number (6.022×10^{23} molecules per mole), we determined the ratio of molecule numbers to molar concentration. The nuclear volume of $4.2\text{ }\mu\text{m}^3$ and the Avogadro number yield a conversion factor of 2.5 molecules/nM. This led to the following values for number of molecules: $Cdc20_{total} = 150$ molecules/cell, $APC/C_{total} = 150$ molecules/cell, $Mad_{total} = 175$ molecules/cell.

Cyclin B was not measured in these experiments. For defining a plausible value for number of Cyclin B molecules, we used protein levels based on mass spectrometry data from Matafora et al. [31]. These experiments were done also on mitotic arrested cells. They showed that Cyclin B concentration levels are around ~ 1200 molecules/cell. In order to be consistent with the previous values, we compared the levels of Mad2 molecules from Matafora et al. [31] to what Bonaiuti et al [23] measured. A three-fold overestimation of Matafora et al. [31] can be assumed. Therefore for Cyclin B we expect 400 molecules/cell.

Concentrations - species

From fluorescence cross-correlation spectroscopy (FCCS) measurements (unpublished) the following values of protein complexes during mitotic arrest are known: mitotic checkpoint complex $MC = 36$ molecules/cell, anaphase promoting complex $A = 60$ molecules/cell, inhibited APC/C^{Cdc20} $ACMC = 30$ molecules/cell. These imply active APC, $AC = 60$ molecules/cell and thus an almost-zero free Cdc20 value during an arrest.

In my model, the use of measured concentration values was an important aspect. In Figure 3.5 the concentrations from the model (i.e., steady states during SAC ON) are shown (green crosses). Blue circles show the above mentioned measured experimental values. Red bars show

the two-fold interval in which the optimization algorithm was accepting values during parameter fitting (see section 4.2). APC total and Mad total were fixed to the values described above.

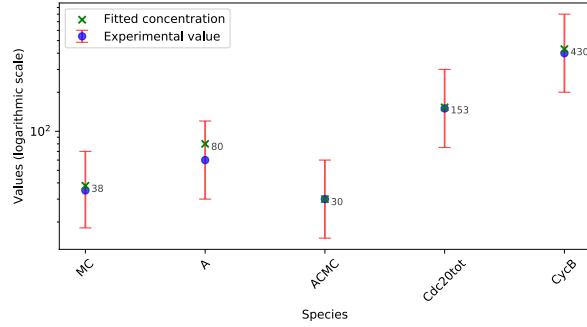


Figure 3.5: Concentration values in the model (green crosses) compared to experimental measured values (blue circles), and the range (red bars) in which concentrations were accepted by the optimization algorithm.

Reaction rates

The rates of synthesis and degradation of Cdc20, and the rates of association and dissociation of AC, MC and APMC were chosen in line with the Bonaiuti model [23] (see Appendix A.1). Activation and inactivation rate of Mads, as also the rates of Cyclin B synthesis and degradation were chosen in order to fit the measured concentration levels. Apart from having biologically plausible concentrations and reaction rates, we expected the model to fit other constraints. In particular, we asked the flux of main degradation of Cdc20 and Cylin B to be significantly higher than the flux of the corresponding background degradation (we expected ~ 10 larger values). All the values for rates and molecule numbers used in the model with experimental references and explanations are uploaded to the github repository linked in Appendix A.2.

Other parameters in the model are k_{nuk} and J_1 which describe the function of signaling kinetochores ($\frac{k' \cdot nUK}{J_1 + nUK}$) in agreement with Aravamudhan et al. [28]. Based on their observations, the Michaelis-Menten constant J_1 , (i.e., the number of unattached kinetochores where checkpoint proteins reach the half of their maximum concentration value (Michaelis, Menten, 1913)), is ~ 3 . The J_1 has a direct effect on the position of the saddle node. For the model we intended to use J_1 value which is in the same order of magnitude which Aravamudhan et al. reported, and allows a saddle node value at $nUK = \sim 1$. This requirement implies that the SAC ON state exists until 1 unattached kinetochore.

Parameter fitting

After introducing the constraints to the model, I executed an optimization algorithm to obtain the parameters which fit the data (see Chapter 4.2). To explore the parameter space, I performed

an analysis which provided a hundred individual parameter sets (a parameter ensemble), all fulfilling the desired characteristics and constraints.

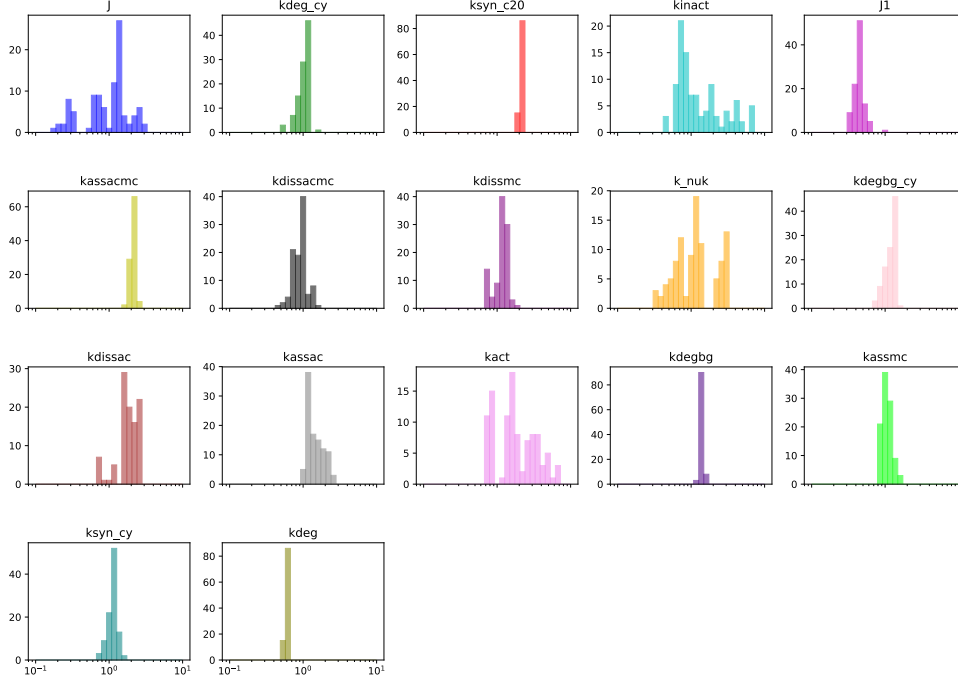


Figure 3.6: Parameter ensemble results: distribution of the parameters of the model which were fitted during optimization algorithm.

The resulting parameter space is shown in Figure 3.6. It shows the distribution of all parameters of the model, which were fitted during optimization. This analysis shows to what extent each parameter can be varied without altering the behavior of the model, and thus the robustness of the system. The most sensitive parameters are synthesis and degradation of Cdc20 ($k_{syn_{c20}}$, k_{degBG}).

3.2.5 Bistability underlies SAC silencing in the model

Bistability is a consequence of the positive feedback loop. To illustrate bistability I created a bifurcation diagram (Fig. 3.7). On the diagram APC/C^{Cdc20} (AC) is plotted against the percentage of unattached kinetochores. I remind that APC/C^{Cdc20} is the target of the checkpoint, which inhibits it.

Starting from high values on the horizontal axes, there is a large number of unattached kinetochores, which keeps the checkpoint active, and APC/C^{Cdc20} at the lower steady state. The saddle node is the value of nUK where the SAC ON state is lost.

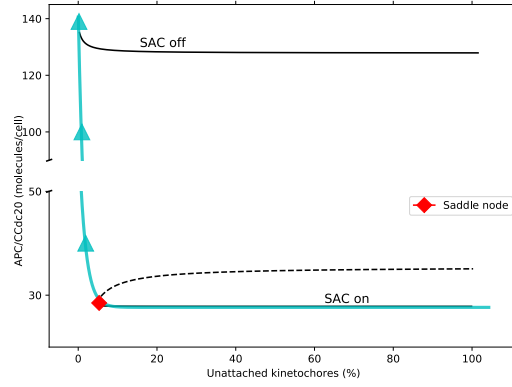


Figure 3.7: Bifurcation diagram: APC/C^{Cdc20} vs. percentage of unattached kinetochores, in cyan: APC/C^{Cdc20} dynamics upon attachment of all chromosomes.

As the nUK value decreases, the saddle node (red marker) is reached and the system jumps to the upper steady state, where the checkpoint is inactivated. In the same figure (Fig. 3.7) APC/C^{Cdc20} dynamics upon attachment of all chromosomes is shown, superimposed to the bifurcation diagram. An APC/C^{Cdc20} trajectory is plotted in cyan blue to illustrate checkpoint silencing (transition from lower steady state to the upper steady state) taking place when all kinetochores attach.

3.2.6 Two-dimensional bifurcation analysis

In order to explore how different parameters modify the model's behavior, I performed two-dimensional bifurcation analysis. Studying the two-dimensional bifurcation diagrams (parameters of the model vs. number of unattached kinetochores) allows to observe how the position of the saddle node (from the starting one-parameter bifurcation diagram) changes. With the help of these diagrams, two classes of parameters can be identified: those which move the lower saddle node to the left (towards smaller number of unattached kinetochores) thus helping the checkpoint ON steady state and those having the opposite effect.

In Figure 3.8 I show an example for both classes. On the upper plot (in red), we followed the rate of APC/C^{Cdc20} formation. As it increases, the saddle node moves to the right so the checkpoint ON state is weakened. In green, on the lower plot, the opposite effect is presented: increasing AC dissociation, the saddle node value decreases, making the checkpoint stronger.

In the table in Fig. 3.9 we present the two classes of parameters, those helping (Pro SAC) or weakening (Anti SAC) the checkpoint.

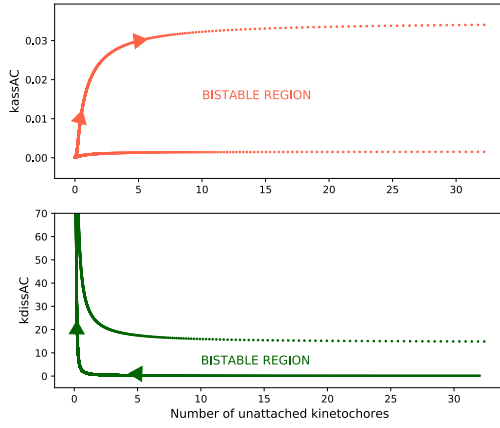


Figure 3.8: Two dimensional bifurcation analysis for APC/C^{Cdc20} association (in red) and dissociation (in green) of APC/C^{Cdc20} versus the number of unattached kinetochores.

Pro SAC	Anti SAC
Mad2 activation rate	Mad2 inactivation rate
Cyclin B synthesis rate	Cyclin B degradation rate
Cdc20 degradation rate	Cdc20 synthesis rate
AC dissociation rate	AC formation rate
MC association rate	MC dissociation rate
ACMC association rate	ACMC dissociation rate

Figure 3.9: Two dimensional bifurcation analysis results - classification of parameters of the model based on whether they help the checkpoint arrested steady state or not.

3.3 Stochasticity in the model

It has been shown that key proteins of the biological system under consideration are present in order of a few dozens, which leaves room for relevant stochastic fluctuations [23]. By introducing stochasticity, the aim was to make the model more realistic, since noise is present in every biological system. We asked whether such fluctuations may drive SAC silencing and spontaneous adaptation to the checkpoint. To test this possibility I performed stochastic simulations.

Here, adaptation connotes the ability of cells to transit to the checkpoint OFF steady state in the presence of unattached kinetochores, while the checkpoint is still active (see Chapter 2.1.3). On the bifurcation diagram adaptation occurs with a switch from the SAC ON to the SAC OFF state with number of unattached kinetochores higher than the saddle node. In Figure 3.10 I superimposed stochastic APC/C^{Cdc20} dynamics to the bifurcation diagram, when kinetochores are being attached (i.e., the trajectory in time moves from right to left). In contrast to the deterministic dynamics shown in Figure 3.7, here it is visible how fluctuations drive the transition to the upper steady state before the saddle node is reached and the SAC ON state is lost.

Stochastic simulations make possible distinguishing between adaptation (slippage) and regular exit from the checkpoint arrest once the checkpoint is silenced. Such regular exit happens when cells transit to the SAC OFF steady state upon attachment of all kinetochores. As we shall see, with decreasing number of unattached kinetochores the possibility of cells to adapt increases, since it becomes easier for them to change steady state driven by noise.

To highlight the difference between the deterministic and stochastic model, in Figure 3.11a I show results from a deterministic simulation. The SAC ON state is simulated until 300 minutes

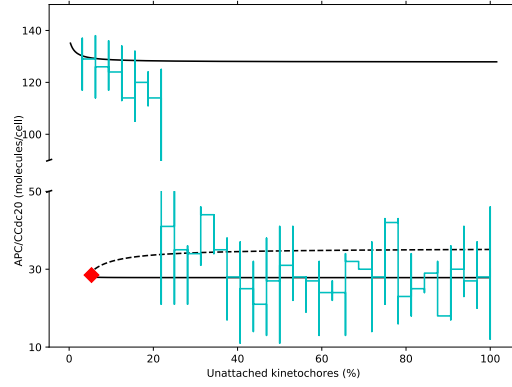


Figure 3.10: Bifurcation diagram: APC/C^{Cdc20} vs. percentage of unattached kinetochores, in cyan: stochastic APC/C^{Cdc20} dynamics. Fluctuations drive the transition to the upper steady state.

(starting from the corresponding initial conditions and setting all kinetochores unattached). At $t = 300$ minutes I turn off the checkpoint by setting the Mad2 active components to 0, thus inducing the SAC OFF steady state. In the SAC OFF state concentration levels change as expected: APC/C^{Cdc20} is active (its value increases) and degrades Cyclin B whose levels drop. Cdc20 is released from the checkpoint core complex so its values rapidly increase.

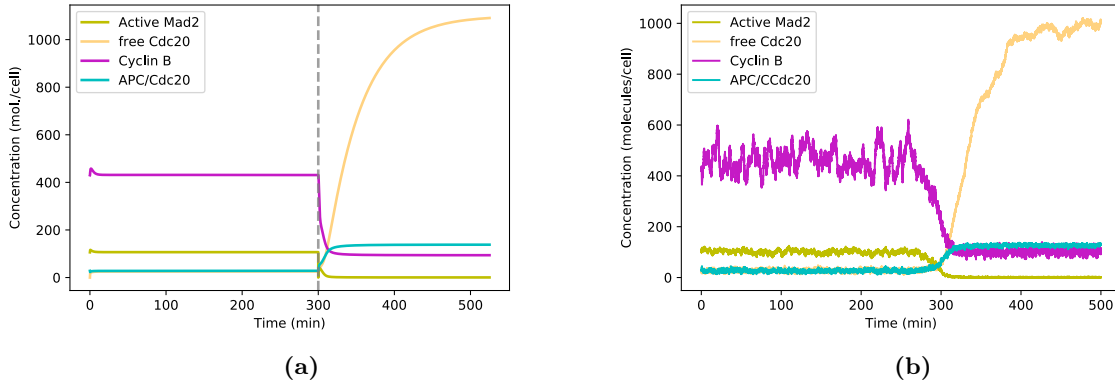


Figure 3.11: Deterministic (a) and stochastic (b) simulations results: concentrations over time for the labeled species. (a) Checkpoint is turned off at $t = 300$ min (by changing initial condition for Mad2). Transition to the SAC OFF steady state is visible for labeled species. The dashed vertical line shows the moment of changing the Mad2 active value. (b) Stochastic adaptation happens at $t \sim 300$ minutes with constant number of unattached kinetochores. Transition to the SAC OFF steady state is visible for all species.

In Figure 3.11b a result of a stochastic simulation is shown. One cell is simulated under constant checkpoint arrest, which eventually shows stochastic adaptation after ~ 300 time units as a consequence of fluctuations in molecule levels. In this case, I did not need to set ad-hoc one species to zero, but the natural variability caused adaptation.

The active checkpoint is simulated in the model by having 100% of unattached kinetochores. As explained, adaptation is identified in the model as transition of each species from the SAC

ON steady state to the SAC OFF steady state when $nUK > 1$. The timepoint of adaptation can be determined by looking at the time where the transition in the concentration level of the model's species takes place and can be calculated from the SAC ON and SAC OFF steady states: $threshold = SAC_{ONSS} + \frac{SAC_{OFFSS} - SAC_{ONSS}}{2}$. The threshold can be defined for any of the species of the model. Any calculated threshold can be used for determining the time of adaptation and number of unattached kinetochores at that timepoint, and it would give the same result (with insignificant variance), due to the fact that all species change their concentration levels (steady states) synchronously.

In Figure 3.11b, different trajectories represent different molecule concentration levels over time, as labeled. The anticipated switching behavior can be seen for all labeled species. APC/C is activated (switch up), degrades Cyclin B (switch down), Mad2 levels decrease, Cdc20 is not bound anymore so its concentration level highly increases.

To conclude, simulations show that adaptation can be driven by noise in a realistic checkpoint model.

3.4 Reproducing experiments

In my work the main goal was to reproduce qualitatively and as much as possible quantitatively experimental data showing cells overcoming an active mitotic checkpoint.

The experimental data (published and not) are mainly produced by Paolo Bonaiuti².

3.4.1 Evidence of a positive feedback loop - simulation

The experiment which served as an evidence for the presence of a positive feedback loop (described in section 3.1) can be simulated with the model. After some time spent under drug arrest (with high nUK value) I set the number of unattached kinetochores to 0 (simulating the removal of the drug) and observed two different conditions: the wild-type case, where the Cdc20 synthesis is at its normal value versus the mutant when the Cdc20 synthesis is reduced to almost zero. In the simulations I follow the dynamics of active Mad2. The simulation output is in agreement with the experimental results. While in the wild-type case after washout active Mad2 values decrease immediately, when Cdc20 is switched off the Mad2 dynamics is much slower and less synchronous. Figure 3.12 shows the simulation results, for wild-type (blue) and Cdc20 inhibition (red) cells. (Experimental results are shown in Figure 3.1).

²Quantitative Biology of Cell Division, Istituto Firc di Oncologia Molecolare, IFOM, Milan, Italy

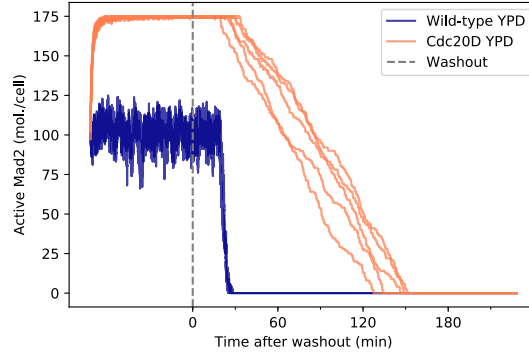


Figure 3.12: Simulation - evidence of a positive feedback loop. Wild-type cell trajectories (in blue) and cell trajectories where Cdc20 synthesis was switched off (in red). Active Mad2 levels are followed in time, during arrest and after washout ($t=0$ - time of the washout). Cdc20 inhibition cells show a slower and less synchronous Mad2 decrease.

3.5 Constant drug arrest experiments

In this section I reproduce two experiments from Bonaiuti et al. [23]. The main difference between my model and the model proposed in [23] is that I reproduce the experimental data by explaining the transition to anaphase with a positive feedback loop rather than using an arbitrary threshold.

3.5.1 Cells adapt when treated with SAC targeting drug

Published experimental data [23] show that cells adapt to the operational mitotic checkpoint in the presence of drugs which activate the SAC by altering microtubule dynamics (e.g. nocodazole). In constant presence of the drug cells can hold an arrest for several hours, however after 600 minutes all cells manage to escape from the arrest. These cells can continue dividing and may become resistant to the drug, which makes them more dangerous (see chapter 2.1.3).

As an experimental readout, the fraction of cells that escaped an arrest are plotted as a function of time spent in mitosis. To reproduce these data, I simulated a hundred individual cell trajectories and plotted the cumulative function of adaptation times (i.e., switch to the SAC OFF steady state). In Figure 3.13a I compare the experimental cumulative function of adaptation times (inset, from [23]) with the cumulative distribution from stochastic simulations (main figure). Simulations produced an exponential distribution similar to experimental data, with quantitatively similar time scale.

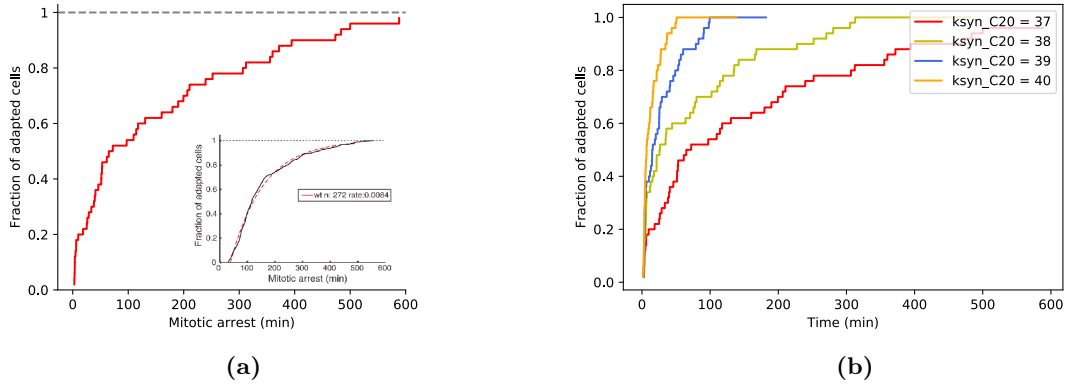


Figure 3.13: (a) Cumulative distribution function of adaptation times under constant nocodazole arrest. Simulation results (main figure) compared with experimental cumulative distribution (inset, source: Bonaiuti et al, 2018, Current Biology, Figure 2A [23]). (b) Cdc20 overexpression simulation results: cumulative distribution functions of adaptation times. Synthesis of Cdc20 was increased as labeled. The red curve shows the wild-type.

3.5.2 Cdc20 overexpression

In the same paper, it has been shown that increasing Cdc20 copy numbers increases the chance of cells to adapt [23]. This is because higher levels of Cdc20 activate the anaphase promoting complex and drive cells to anaphase. To test whether our model is able to reproduce the experiment I simulated cell trajectories for different values of Cdc20 synthesis rate.

The simulation results are in qualitative agreement with experiments. Experimental data show that the effect of Cdc20 overexpression decreases as the overexpression doubles. In the simulations, we see a similar trend but with much smaller increase of Cdc20. This is shown in Figure 3.13b, where I plot the cumulative distribution of adaptation times from different stochastic simulations - varying the Cdc20 synthesis rate as labeled.

3.6 Washout experiments

Since after a few hours, regardless of the constant presence of the drug, all cells adapt to the mitotic checkpoint, one may formulate the question whether it is possible to avoid adaptation by removing the drug before cells manage to escape. To test this idea, the host lab has performed further experiments studying cells in condition of drug-washout. Such data are not yet published, but are used in my work to test the model's predictions.

In particular, new experiments addressed the effect of washing out a microtubule - depolymerizing drug, based on those previously described (section 3.5), where cells were held under constant arrest via nocodazole. These new experiments showed the paradoxical result, that cells undergo adaptation also when the drug is washed out. We called this phenomenon washout induced

adaptation. To keep track of adaptation, in these experiments as well, Mad2 and Clb2 levels were followed, observing Mad2 localization index at the time of Clb2 degradation.

In the washout experiments cells were treated with nocodazole for 180 minutes then the drug was washed out. I simulated these experiments by introducing kinetochore attachment dynamics as mentioned in section 3.2.3. The nUK signal was held constant for 180 time units at 100%, then the washout was simulated by gradually decreasing to 0% unattached kinetochores, meaning that all of the kinetochores become attached, so the checkpoint turns off.

Experimental results show that by washing out the drug in 60.8% of cells Mad2 is still localized at Clb2 degradation and only 39.2% of cells exit regularly, when the checkpoint is not signaling. The simulations were executed on a hundred different trajectories simulating individual cells. The results are similar to experimental statistics: from the simulation 68.75% of the cells undergo slippage and 31.25% of the cells regular mitotic exit. In Figure 3.14a I compare the results of experiments and simulations, while Figure 3.14b shows simulated APC/C^{Cdc20} trajectories in drug washout conditions.

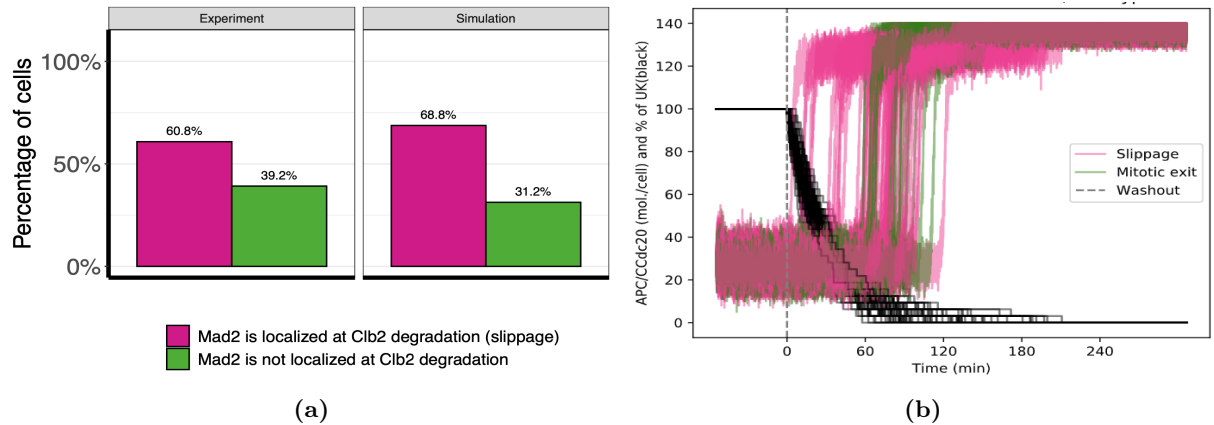


Figure 3.14: (a) Comparison of experimental and simulation results, percentage of wild-type cells which undergo slippage (pink) or mitotic exit (green). (b) Washout simulation in wild-type cells. APC/C^{Cdc20} trajectories vs. time (min). Time 0 represents the time of the washout. Pink trajectories are the cells that escape from the arrest. Green trajectories are the ones which exit mitosis after checkpoint silencing. Black trajectories show stochastic kinetochore attachment dynamics.

As mentioned, experimental results show the paradoxical result that washing out the drug before cells start to adapt does not prevent adaptation, but to the contrary it favors it. Since simulations done with the model reproduce this behavior, we can use the model to explain the results. For that, I compare the value of nUK at adaptation for the washout experiment and when the value of nUK is constant. In Figure 3.15 I show the distribution of number of unattached kinetochores at the time of adaptation for all simulated cells which undergo slippage during the washout (in pink). The gray histogram shows that under arrest, when nUK is constant and all kinetochores are unattached, all cells manage to adapt. In the first case (washout), we can

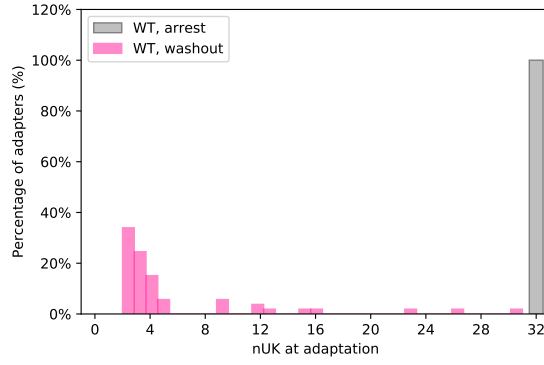


Figure 3.15: Distribution of number of unattached kinetochores at the time of adaptation in wild-type cells, drug-washout (pink) and constant drug arrest (gray) conditions compared.

see that while some cells adapt with higher number of unattached kinetochores, the majority of them adapt with a smaller number of nuk, when adaptation is easier. Hence, the model suggests that when the drug is washed-out, cells go through phases with lower number of unattached kinetochores which makes easier for them to transit to the other steady state.

3.7 Avoiding adaptation - mutant experiments

Washout experiments have shown that despite being released from nocodazole, many cells degrade Cyclin B while Mad2 is localized (ie., they adapt). Thus further experiments were carried out by the host lab, in order to test if it is possible to avoid adaptation. Mutations of crucial proteins are expected to influence the qualitative behavior of the spindle assembly checkpoint making adaptation less likely. In particular, two conditions were tested: first an APC mutant, a so-called APC-A mutant, second a strain where Mad2 is overexpressed, referred to as GAL-Mad2 mutant.

3.7.1 APC-A mutant prevents adaptation in constant nocodazole

This experiment aimed to make adaptation less likely by using a mutant that binds less efficiently Cdc20 (APC-A). Using FCCS, it was observed that APC-A produces as much APC/MCC as the wild type, but more MCC. The hypothesis is that it also will make less APC/C^{Cdc20} , thus helping the mitotic checkpoint.

In the model, I obtained the APC-A mutant by making the APC/C^{Cdc20} formation less likely via decreasing the association rate of AC. First, I performed steady state simulations to decrease APC/C-Cdc20 binding to values similar to those measured. I observed that after decreasing by 40% the association rate, the steady state in the SAC ON state was quantitatively close to the data.

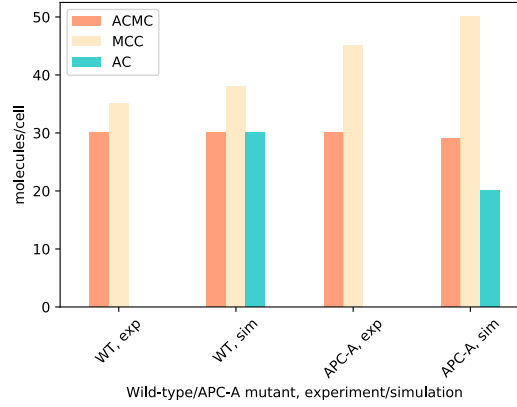


Figure 3.16: Bar plot comparing results from experiment (performed by Paolo Bonaiuti) and simulation, wild-type and APC-A mutant cells. Different bars show measured and simulated concentration levels under different conditions.

In Figure 3.16, I compared MCC and APMC in experimental data to simulation results both in wild-type and in the APC-A mutant cells. In the simulations I also calculated the APC/C^{Cdc20} steady states and, as expected, it was lower in the APC-A mutant case.

Further experiments were done to check whether these APC-A mutant cells could adapt in constant nocodazole and it was observed that they do not. I tested this result on the model by setting the maximum number of unattached kinetochores and decreased the APC/C-Cdc20 affinity. The simulations reproduce the results from experiments: by decreasing the APC/C - Cdc20 association rate no transitions occur with high and constant number of unattached kinetochores. Both the experiment and simulation show that APC-A mutant cells significantly reduce slippage, almost all cells undergoing regular exit from mitosis.

3.7.2 Washout experiment with the APC-A mutant

Finally, I simulated the washout experiment with the APC-A mutant. Experimentally, cells were treated in nocodazole for 180 minutes then the drug was washed out. APC-A mutant reduced slippage, since in 85.9% of cells Mad2 was not localized at the time of Cyclin B degradation and only 14.1% of the cells underwent slippage. I simulated the washout experiment on the modified model with reduced APC/C Cdc20 affinity. In agreement with the experimental data, the simulations showed less slippage (22%) with 78% of the cells exiting regularly when the checkpoint is silenced (Figure 3.17).

In Figure 3.18a I show the empirical cumulative density functions from experimental results, comparing wild-type and APC-A mutant cells during washout experiments. Solid lines show the wild-type behavior while dashed lines represent the cumulative distribution for the APC-A mutant. The colors differentiate between slippage and regular exit. In Figure 3.18b the same

statistics is reproduced for simulation results.

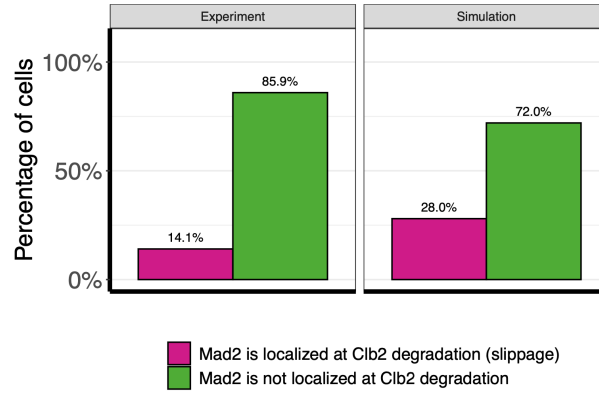


Figure 3.17: Comparison of experimental and simulation results, percentage of APC-A mutant cells which undergo slippage (pink) or mitotic exit (green).

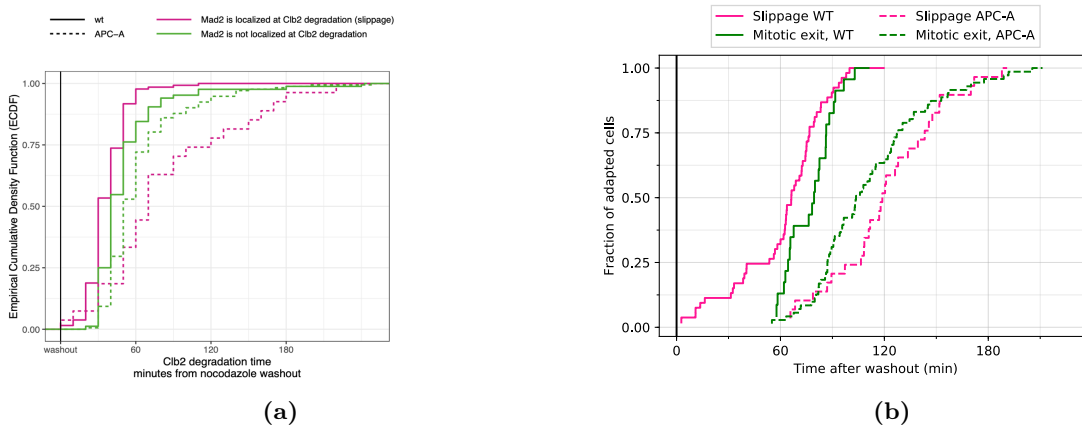


Figure 3.18: Washout simulation in wild-type and APC-A mutant cells. Pink trajectories are the cells that escape from the arrest. Green trajectories are the ones which exit mitosis after checkpoint silencing. (a) - experimental results (Produced by Paolo Bonaiuti), (b) - simulation results.

Both in the experimental and simulation results, it can be observed that the cumulative distribution functions for wild-type and APC-A show different behaviors. The expectation was that the mutant would be slower at adaptation, which indeed is supported by the experiments and simulations as well. However, results show other tendencies specific of the APC-A mutant: the cumulative distributions of adapting cells and of those exiting regularly partially overlap, and slippage can even anticipate regular exit. This behavior is not well understood. Since the model showed similar behaviors, we asked whether it could help to provide an understanding of these phenotypes.

To this aim, I studied the distribution of unattached kinetochores at the time of adaptation for wild-type and APC-A mutant cells in condition of washing out the drug. In Figure 3.19 I distinguish, for both the wild-type and APC-A cells, the early and late adapters, based on the timepoint where the cumulative distribution function of each reaches 50%. Notice, that these

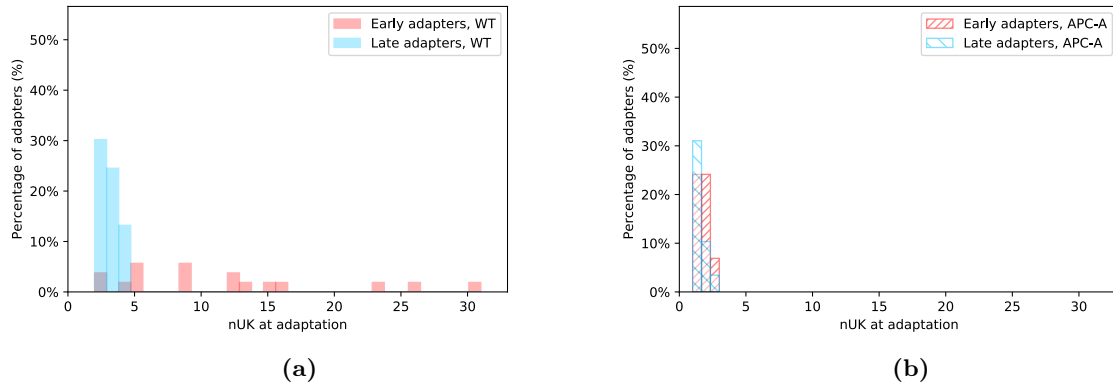


Figure 3.19: Distribution of nUK at adaptation: early adapters (red histograms), late adapters (blue histograms). (a) Wild-type. (b) APC-A mutant.

distributions only represent cells which undergo slippage (100% means all cells that adapted in the simulation, we refer to them as adapters).

From the simulated cumulative distributions (Fig. 3.18), we saw that in wild-type the first cells which leave mitosis always undergo slippage, since the first transitions in the beginning can only happen with a high number unattached kinetochores. This is supported by the nUK distribution of early adapters in the wild-type (red histogram in Figure 3.19a).

Compared to the distribution in wild-type cells, in APC-A cells the distribution of nUK at adaptation (Figure 3.19b) is much more concentrated around small nUK values. Hence, in APC-A early and late adapters cannot be separated based on the value of nUK at adaptation (they only adapt with 1,2 or 3 unattached kinetochores).

Since noise is present in the dynamics of kinetochore attachment, $nUK=0$ is reached at different time for different cells. In this context, variability in attachment plays a key role. It may happen that at a timepoint where one cell exits regularly, another cell can still have a higher number of unattached kinetochores and undergo slippage later. That is why in APC-A the two cumulative distributions tend to overlap. Also in reality some cells could hold on to the arrest with one or two signalling kinetochores for a long time before escaping, by the time other cells have already exited regularly. Our hypothesis is that if the experiment were repeated with larger number of cells, the APC-A mutant curves would tend to overlap more extensively.

Hence, we can also hypothesize that the APC-A mutant only acts on ‘early adapters’, preventing cells with high number of unattached kinetochores from adapting. The model then predicts that the wild-type has cells with many aneuploid chromosomes unlike APC-A.

3.7.3 Mad2 overexpression by GAL-MAD2 reduces adaptation in constant nocodazole

Overexpression of the crucial checkpoint core component Mad2 is also expected to strengthen the mitotic checkpoint and help avoiding slippage. In molecular terms, Mad2 overexpression induces MCC production thus resulting in less APC/C^{Cdc20} . Experiments were done with strains carrying one copy of GAL-MAD2, an exogenous promoter that increases Mad2 levels.

Since Mad2 is overexpressed, tagging the endogenous is not a useful strategy: it would be diluted among lots of untagged Mad2. Since Mad2 localization could not be followed, slippage and regular exit cannot be distinguished. Hence, here I could only reproduce the fraction of cells degrading Clb2, putting together adaptation and exit.

Cumulative distributions from experimental results comparing Clb2 degradation times in wild-type and GAL-MAD2 mutants are shown in Figure 3.20a.

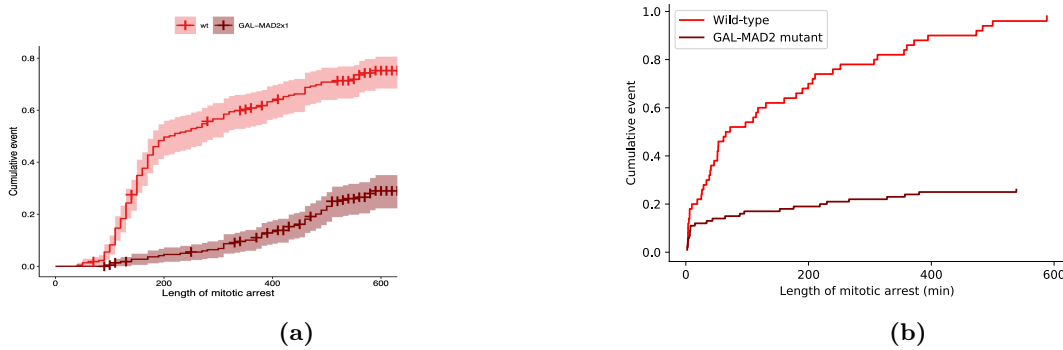


Figure 3.20: Empirical cumulative distribution functions Clb2 degradation times comparing wild-type (in red) and GAL-Mad2 mutant cells (in brown) under constant nocodazole arrest: (a) - experimental result (produced by Paolo Bonaiuti), (b) - simulation result.

For simulating Mad2 overexpression with the model, I increased the total amount of Mad2. I integrated the ODEs of the model to find the steady states. Starting from the modified steady states I simulated cell trajectories under high and constant number of unattached kinetochores. Compared to wild-type, the number of cells exiting the arrest is much lower (20% of all simulated cells). This is in agreement with the experimental data. In Figure 3.20b I plotted the cumulative distribution functions of cyclin degradation times, comparing the wild-type and mutant cells.

3.7.4 Washout experiment with GAL-MAD2 mutant

Adaptation during washout was also tested experimentally in cells where Mad2 is overexpressed. It was expected that cells with a strengthened SAC should adapt less than the wild-type. In the experiment, cells were treated with nocodazole for 240 minutes after G1 release. With extra Mad2, cells do not degrade cyclin B before the drug washout, but after the washout cells

degrade cyclin in a similar fashion to wild-type cells, with a delay (Figure 3.21a). Since Mad2 is not tagged, from these experiments we cannot conclude that cells adapt less. However, other data (not shown) reveal that GAL-Mad2 cells missegregate less chromosomes during a washout. This result implies that they also reduce adaptation.

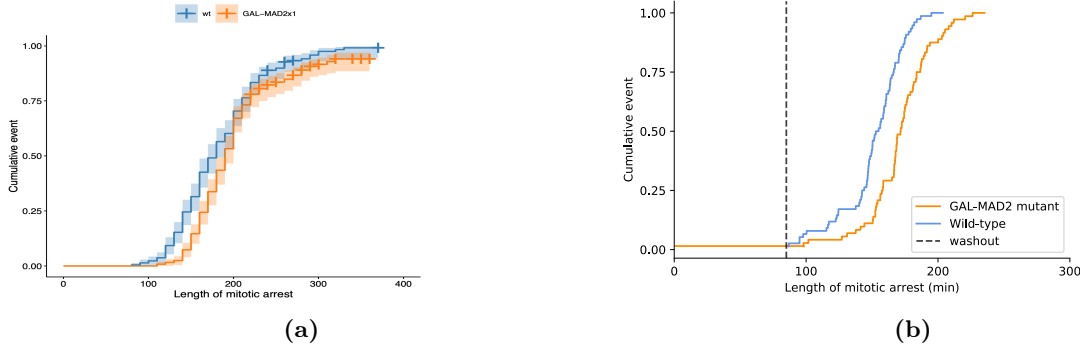


Figure 3.21: Empirical cumulative distribution functions of Clb2 degradation times comparing wild-type (in blue) and GAL-Mad2 mutant cells (in orange) in condition of drug washout: (a) - experimental result (produced by Paolo Bonaiuti), (b) - simulation result.

I simulated the washout experiments by decreasing the number of unattached kinetochores. Simulations are in agreement with experimental data: all cells manage to escape from the checkpoint, in a similar behavior but delayed compared to wild-type cells after washout (Figure 3.21b). Also, the simulated GAL-Mad2 avoids the early adapters, although less than APC-A mutant (see Figure 3.22a: nUK distribution at adaptation for simulated GAL-Mad2 mutant). The fact that GAL-Mad2 cells adapt less, is in agreement with the observation mentioned above that chromosome missegregation is reduced in these cells.

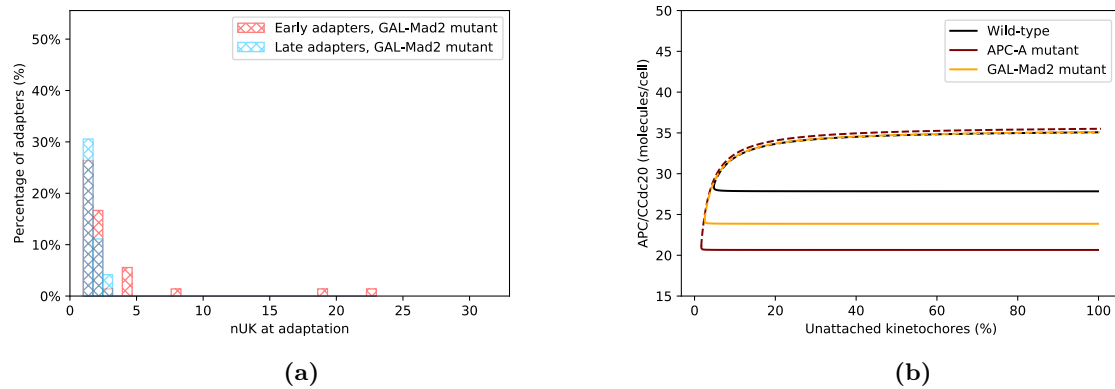


Figure 3.22: (a) Distribution of nUK at adaptation for simulated GAL-Mad2 mutant: early adapters (red histogram), late adapters (blue histogram). (b) Bifurcation diagram (lower steady state and unstable points) for APC-A mutant cells (in brown) and GAL-Mad2 mutant cells (in orange). For comparison wild-type bifurcation diagram is shown (in black).

Notice, that I only simulate cells from a mitotic arrest. In reality, mitotic entry is a stochastic

event and the difference between the time of washout and mitotic entry differs for each cell. In the simulations I assumed that all cells enter mitosis simultaneously, thus the time of washout is the same for every simulated cell, which is only a first approximation: I defined it based on the average difference between washout (same for every cell) and mitotic entry (different for every cell, average number provided by experimental data provided by the host lab).

To understand how the APC-A and GAL-Mad2 mutants work to avoid adaptations in Figure 3.22b, I show the bifurcation diagram (lower steady state and unstable points) for APC/C^{Cdc20} : in the APC-A mutant (in brown), in the GAL-Mad2 mutant (in orange) as well as in the wild-type (in black). One can observe that in the mutants the steady states are lower, meaning that there is less APC/C^{Cdc20} which could drive cells to anaphase. Additionally the lower saddle node is dragged to the left, making transitions more difficult and only possible with lower strength from unattached kinetochores.

In conclusion, washout experiments carried out with the two mutants, show that indeed adaptation is diminished in both conditions. These results suggest that it is possible to treat cells with microtubule-targeting drugs while preventing adaptation.

3.8 Robustness of the model

The model is able to reproduce experimental data and to provide explanations for questions encountered during evaluation of these data. Finally, I tested how robust this system is.

In section 3.2.4, I showed that the parameter set used for simulations was chosen from a parameter ensemble, consisting of a hundred different parameter sets, which was built to fit the constraints. Here, in order to explore the robustness of the model, I tested the parameter sensitivity of the model. I disturbed the parameter values of the final parameter set, one-by-one in a ten-fold logarithmic range and observed how the behavior of the model changes. For each parameter, for each value, I performed a stochastic simulation for 600 minutes and 50 trajectories and kept track of the following: (i) whether the system kept bistability, (ii) if so, how many of the cells adapt to the checkpoint under constant drug arrest, (iii) how the steady state levels (species concentrations) change, (iv) whether the fluxes of background degradations was smaller than the fluxes via the main degradations.

In Figure 3.23 I show the result of the robustness analysis. Each subplot shows how the change of a single parameter on a 10-fold logarithmic scale changes the behavior of the system by showing the percentage of cells which adapt. In the starting parameter set all cells adapt by 600 minutes. On the dashed gray line on each plot, this starting value is marked with a cross, where 100% of cells adapt. The lack of a point means the loss of bistability in the system.

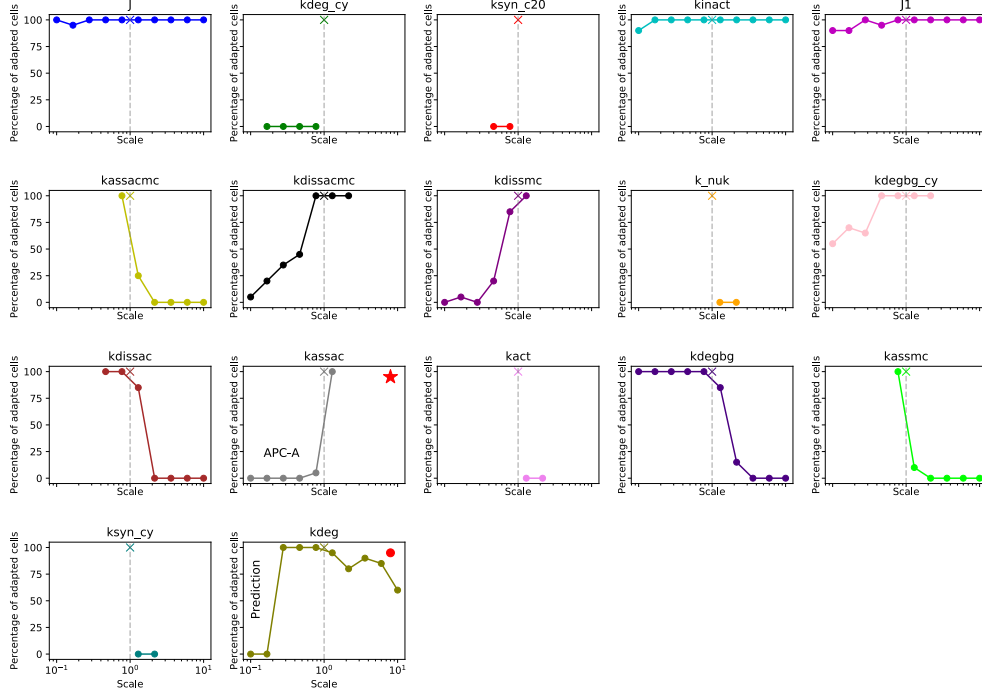


Figure 3.23: Result of parameter robustness test. Subplots show how varying each parameter (as labeled) changes the behavior of the system. For each tested parameter value the percentage of adapted cells is plotted. A lack of a point means the loss of bistability.

The most sensitive parameters are synthesis and degradation of Cyclin B, synthesis of Cdc20, and activation rate of Mad proteins, similarly to the observations made based on the parameter ensemble (Fig. 3.6).

3.9 Predictions for inhibiting adaptation

We asked whether the robustness test can also serve as a method for making predictions with the help of the model, which could be tested experimentally. In Figure 3.23 I marked with a red asterisk the subplot which shows how the number of adapted cells changes when association rate of APC/C^{Cdc20} is varied. By decreasing the APC/C -Cdc20 binding affinity, the number of adapted cells decreases to 0. This is precisely the condition of the APC-A mutant. We then looked for other parameter changes which based on the model's predictions, prevent cells from adapting. One such experiment could be the inhibition of degradation rate (kdeg) which can be achieved experimentally (by inhibiting the proteasome). Based on the model's prediction decreasing it could help to avoid adaptations (the corresponding subplot is marked with a red circle in Figure 3.23). The host lab will test the prediction in the near future.

Methods

4.1 Deterministic model

Deterministic ordinary differential equation model and bifurcation analysis were done using the software XPPAUT [32]. In my simulations I used two numerical integrators: the Runge-Kutta and the CVODE method. Both are methods for solving ordinary differential equations (ODEs). In every simulation I set parameter boundaries to 1000 and integration time to 2000 time units. In case of CVODE method I use the following settings: *relative tolerance* = 0.0001, *absolute tolerance* = 0.0001. Once I found a steady state of the system I used AUTO for bifurcation analysis to track the solution of the system as one parameter is varied. As mentioned in detail in Chapter 3.2, I chose the number of unattached kinetochores as a bifurcation parameter. I used AUTO with the following settings: @ *dsmax*=.5, *dsmin*=.0001, *ds*=-.02, *ntst*=1500, @ *nmax*=2000, *npr*=500, *parmin*=0, *parmax*=32, *autoxmin*=0, *autoxmax*=1, *autoymin*=0, @ *autoymax*=100. The ODE file used in simulations is provided in the github repository A.2.

4.2 Parameter optimization and ensemble analysis

I converted the ODE model into a SBML (Systems Biology Markup Language) [33] model of biochemical reactions that can be used for simulations in Python. Parameter fitting was carried out with the Python package 'SloppyCell' [34]. SloppyCell allows to fit simultaneously multiple experiments and investigate the space of parameters that allows a proper fitting (i.e., tests the robustness of the model). I used SloppyCell to fit the total amount of proteins and the concentrations of the protein complexes to experimental values. SloppyCell allowed me to define plausible intervals for each reaction rate. I also introduced constraints such that the flux of background degradation (see Chapter 3.2.2) of Cdc20 and Cyclin B should be significantly smaller than the flux of the main source of degradation. Once the model was defined and the desired constraints were introduced, SloppyCell was able to optimize the parameters in order to minimize the cost and thus best fit the data (constraints and intervals from experimental values).

In the Optimization module I used the SloppyCell implementation of Levenberg-Marquardt method. It uses analytical derivatives calculated via sensitivity integration [34]. However it does only local optimization, thus in order to further explore the parameter space one can build a parameter ensemble.

Building a parameter ensemble allows to explore the full nonlinear space of parameters that are statistically consistent with the model and data. The built-in algorithm uses an importance-sampled Markov-Chain Monte-Carlo algorithm and creates the ensemble by a random walk through parameter space, converging to the correct probability distribution [34].

With the help of a parameter ensemble I was able to choose a parameter set which fulfilled the introduced constraints and showed the desired behavior of the system (see table containing parameters in Appendix A.2).

4.3 Stochastic simulations

For the stochastic simulations, I used a Stochastic Simulation Module in Python: StochPy package [35]. This package uses a stochastic simulation algorithm called Gillespie (1977). On the set I chose from the built parameter ensemble I ran stochastic simulations in order to simulate the behavior of the biological system. In the simulations we considered 1 simulated time unit as 1/3.33 minute, thus ~ 20 seconds. This factor was chosen based on nocodazole-arrest simulation results fitted to experimental results. All cells manage to adapt after 2000 stochastic time units in simulations and 600 minutes in experiments [23]. Therefore we chose a time conversion factor of 0.3. All the model files and codes I created and used can be accessed via the link provided in Appendix A.2.

4.4 Experimental methods

The host lab conducted single-cell experiments in real-time, arresting cells in G1 with α -factor and synchronously starting the cell cycle upon its removal. Cells, grown in a microfluidic chamber, were imaged using a DeltaVision Elite microscope and analyzed with Phylocell software and custom MATLAB and R scripts. The experiments used cells with various fluorophores and genetic alterations:

Cdc20 $\times$ *N*: Extra copies of the Cdc20 gene to increase protein production.

APC – *A*: Alanine substitutions in Cdc16 and Cdc27 to reduce APC-Cdc20 affinity.

GAL – *Mad2*: Galactose-inducible promoter for extra Mad2 production.

GAL – *Cdc20 cdc20* Δ : Galactose-inducible promoter for Cdc20, with Cdc20 removed.

Summary

5.1 Problem statement

Cells can be arrested during the cell cycle by disrupting essential functions and/or structures. When this happens, cells arrest cell cycle progression to minimize the risks of undergoing faulty divisions. The arrest is driven by specialized pathways known as checkpoints. In particular, in the biological system under consideration, the mitotic checkpoint oversees chromosome segregation arresting cell cycle progression if not all chromosomes are properly attached. However, some cells manage to escape from the mitotic arrest and resume proliferation. In the context of cancer treatments, these are dangerous cells which escape the effect of the drugs and may become resistant to treatment. In this project, we proposed to understand how cells escape the arrest induced by microtubule targeting drugs.

5.2 Results

Biological data proved the presence of a positive feedback loop supporting checkpoint activation. I then hypothesized that adaptation may be explained as the transition between a SAC ON and a SAC OFF state, driven by molecular noise.

I created a model with realistic molecular details. This model showed that adaptation can be described by a transition between two stable steady states, driven by noise, proving that bistability is present. The parameters, reaction rates, concentrations of protein complexes and total protein amounts were based on experimental measurements. A whole parameter ensemble was built, which consists of parameter sets which fulfill the proposed requirements.

The model is able to reproduce experimental data, which was carried out by experimentalists in the hosting lab ¹.

The following experiments were discussed and simulated in a stochastic framework: from Bonaiuti et al. [23] (i) cells treated with SAC targeting drug adapt to the operational mitotic checkpoint and (ii) cells which overexpress Cdc20 adapt with a higher chance. The novelty compared to the

¹Quantitative Biology of Cell Division, Istituto Firc di Oncologia Molecolare, IFOM, Milan, Italy

model proposed in [23], is the introduction of a positive feedback loop controlling the entry to anaphase rather than using an arbitrary threshold.

I also reproduced non-published data produced by Paolo Bonaiuti: (i) Evidence for a positive feedback loop in SAC activation, (ii) SAC-targeting drug-washout favors mitotic slippage, (iii) APC-A mutant prevents cells adapting to the checkpoint, (iv) GAL-Mad2 mutant largely impairs adaptation. Washout experiments were also done on the two mutants.

In conclusion, the model supports the proposed hypothesis, that cells escape the mitotic arrest through a stochastic process. It shows that eventually even in constant arrest induced by microtubule-depolymerising drugs cells are able to escape the arrest, despite an operational checkpoint.

Besides reproducing published and non-published experimental data, the model provides explanation for questions which are difficult to understand based on the measured data, i.e. (i) why washing out the drug favours adaptation, (ii) why do adaptations in APC-A mutant behave differently than in the wild-type, (iii) how do the two proposed mutants influence checkpoint activity. Finally, I tested the model's robustness and used the model to suggest new experiments which could test ways to avoid adaptation of cells.

5.3 Future plans

In my work, I created a working model describing the mitotic checkpoint, which supports qualitatively and to some extent quantitatively experimental data. One possible future approach could be further investigating and improving the model in order to reproduce experimental data more quantitatively.

In the model, I simulate cells in mitosis and I assume that all cells enter mitosis at the same time. One possible improvement could be introducing stochasticity in this context. This could be relevant in the simulation of washout experiments.

Based on the model, I proposed one prediction to be tested experimentally. Further predictions could be made and carried out based on the model.

Finally, in mammalian cells, the same molecules take part in the mitotic checkpoint as described by the model. However the dynamics is slightly different than in yeast. Further research could include understanding how the same network shows different behaviors in mammals and yeast, eventually building a comprehensive model which also describes mammalian cell behaviour.

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Appendix

A.1 Parameters used in the model

In Figure A.1 I compare the reaction rate values from the parameter set used in my model (green cross), to the corresponding values used in Bonaiuti et al. [23] (blue dot). The red bars mark the two-fold interval set for the optimization algorithm.

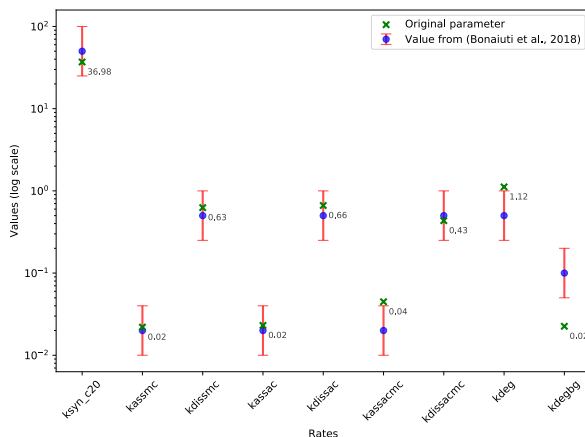


Figure A.1: Reaction rates used in the model compared with values from Bonaiuti et al.[23].

A.2 Parameters, source codes and model files

In the following repository I provide the tables containing parameters of the model with references provided, species concentrations, source codes used to build the parameter ensemble, scripts to perform stochastic simulations and generate the figures I present in this work. It also contains all files required by the scripts and the ODE file used in XPPAUT for deterministic simulations and bifurcation analysis.

The repository can be accessed via: <https://github.com/sbeatrrix/TDK>.