Analysis of ATM signaling pathway as an activator of p53 and NF-κB regulatory modules and the role of PPM1D

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Abstract. In this work we presented a mathematical model of the ATM signaling pathway which plays a role of the double-strand breaks (DSB) detector. The proposed model is based on the literature knowledge and our own wet-lab experimental results. Stochastic simulations of the constructed model were performed. The obtained results show that ATM signaling pathway response after the DSB occurrence strongly depends on the PPM1D (Wip1) that can be considered as a gatekeeper in the ATM-p53 regulatory loop.

Keywords: ATM, p53, Wip1(PPM1D), apoptosis, DNA damage detection

1 Introduction

Eukaryotic cells are exposed continuously to the genotoxic stress that may result in the formation of different types of DNA damages, such as double-strand breaks (DSBs). DNA DSBs may be caused by external factors, like ionizing radiation (IR) and some clastogenic drugs [1], or formed endogenously, e.g. during DNA replication [2]. In order to maintain genomic integrity, the DNA damage response (DDR) is activated.

The key components involved in the DDR pathway activated by DSBs induction are p53 [3] and NF-κB [4], [5] transcription factors that regulate hundreds of genes encoding proteins responsible for cell cycle blockade, DNA repair, apoptosis, immune responses or inflammation. Levels of the active forms of these transcription factors are regulated by other cellular components that build the positive and negative feedback loops, e.g. Mdm2 negative loop for p53 activation. DDR activation requires prior DSB detection which is performed by ATM-dependent DSBs detector system [3], [4]. ATM kinase sends the signal about the damage to various mediators, like checkpoint kinases, and regulators, such as p53 and NF-κB.

Incorrect mechanisms of the ATM-dependent p53-NF-κB pathway may lead to pathological changes transmitted to daughter cells, uncontrolled proliferation

and tumor growth. The proper functioning of this pathway is essential to enhance the cellular survival. Therefore, it is important to study interactions between its different components and to model the cellular response to genotoxic stress.

The problem of the theoretical prediction of the cellular behavior based on the ATM-dependent DDR pathway has already been studied [6], [7], [8], [9], [10]. However, to our knowledge, the existing works involving the effect of the DNA damage on the p53 core describe the simplified pathway without considering various compartments or macromolecules directly involved in the activation of ATM, p53 or NF-κB modules, which we found to be essential during cellular damage response and for simulation analysis of this pathway.

In recent works [7], [8], the authors use ordinary differential equations (ODE) in order to describe the dynamics occurring in the single cell after the treatment with IR. Both of the models use molar concentrations equations. The model described in [8] focuses mainly on G2 phase of cell cycle instead of trying to simulate the behavior of the cell in every phase. None of the described mathematical models involve NF-κB pathway and PTEN component of p53 positive feedback loop. Moreover, the original model based on ATM [10] treats this kinase as a direct measure of DNA damage, without considering the oscillations of this module.

In our opinion, intermediate molecules omitted in existing models provide important delays to the system dynamics and stochastic effects are crucial for cell fate decision after the DSB occurs. Therefore, in order to study these interactions and effects we developed a stochastic model of the ATM signaling pathway. Furthermore, we combined presented model with our previously constructed p53-NF-κB model [11]. Because of the space limits in this paper, we describe only the new ATM part. The detail description of the p53-NF-κB signaling pathway model and its biological background can be found in [11], [12] and in the references within.

In short, the ATM system response to the DSB occurrence could be described as follow: The ATM-dependent DDR pathway is activated upon DNA DSBs lesions that are recognized by multi-protein MRN complex (Mre11-Rad51-Nbs1) [14]. The DSBs can be also recognized directly by ATM that exists in cell as an inactive homodimer. After the DSBs occurrence, the elements of this homodimer autophosphorylate each other in Ser-1981, what leads to the homodimer decay, activation of ATM monomers and signal amplification [14].

The activation of MRN complex leads to the fully activation of phosphorylated ATM monomers, which in turn phosphorylate their substrates: the components of MRN complex (Nbs1 and Mre11) [15], Mdm2 [16], Chk2 [17], CREB [18], Akt [19] and KSRP [20]. ATM mediated phosphorylation of Mdm2 leads to the stabilization of p53-Mdm2 auto-regulatory feedback loop [21], and activated form of CREB positively regulates transcription of ATM [22] and Wip1 [23]. ATM phosphorylates p53 in Ser-15 and Ser-20 directly and indirectly via Chk2, what results in inability to form a p53-Mdm2 complex, and as an effect enable to activate p53 protein [24], [25]. In turn, the expression of Chk2 gene is negatively regulated by active forms of p53 [26].

ATM is also essential for NF-κB activation in response to DSBs and this activity is mediated via the IkB kinase complex [27].

When the process of DNA damage repair is finished, Wip1 dephosphorylates ATM in Ser-1981 (autophosphorylation target). That event results in inactivation of ATM [28]. Wip1, as a main deactivation agent of ATM-dependent p53-NF-κB pathway, dephosphorylates also p53 in Ser-15 [29], and Chk2 in Tyr-68 [30]. For Mdm2 Wip1 acts as an activator from the multiphosphorylated form. Wip1 dephosphorylation of Mdm2 in Ser-395 (ATM phosphorylation target) results in its stabilization [21]. Wip1 is also a phosphatase of Ser-536 of the p65 subunit of NF-κB, what leads to the inability to form an active NF-κB-p300 (transcriptional co-activator) complex [31].

Like Mdm2, Wip1 gene is a transcriptional target of p53, and p53 induction of Wip1 expression may limit the p53-dependent DNA damage response [32].

2 The Model

Scheme of the proposed model is presented in Fig. 1. It includes NF-κB module (pale grey), p53 module (dark grey) and ATM/Wip1 part (green and violet). Numerical implementation of our model is based on Haseltine-Rawlings postulate [44], in which all reactions are divided into slow (gene switching and DSB formation and repair) and fast (remaining reactions). Slow reactions are described by reaction propensities, while fast by ODE.

2.1 System Activation

Courilleau et al. in [45] provide U2-OS cells based experimental data for DSB formation and repair. They noticed that radiation dose of 2 Gy results in $\sim$19 DSBs, while 8 Gy in $\sim$29 DSBs. This combined with our own experimental results indicating $\sim$24 DSB for 4 Gy leads to the conclusion that the DSB formation, which is an effect of treatment with IR, has to be nonlinear function with saturation. Because of the fact that detailed DNA DSB repair description was not subject of our work but it was necessary for cell fate determination, we assumed simple DSB repair mechanism as presented in [12]. As a result probabilities of the DSB formation (r_1) and repair processes (r_2) are equal to:

$$r_1(t) = ma_1 \frac{IR(t)}{IR(t) + mm4} \tag{1}$$

$$r_2(t) = mc_1 \frac{DSB(t)}{DSB(t) + mm_1} P53^2_{pn}(t) \tag{2}$$

2.2 Gene Switching and Transcription

Stochasticity in our model occurs mainly because of the stochastic gene switching. ATM (ra_1), Chk2 (ra_2) and Wip1 (ra_3) genes are activated with the probability proportional to the amount of the proper transcription factors, while deactivation processes are spontaneous.

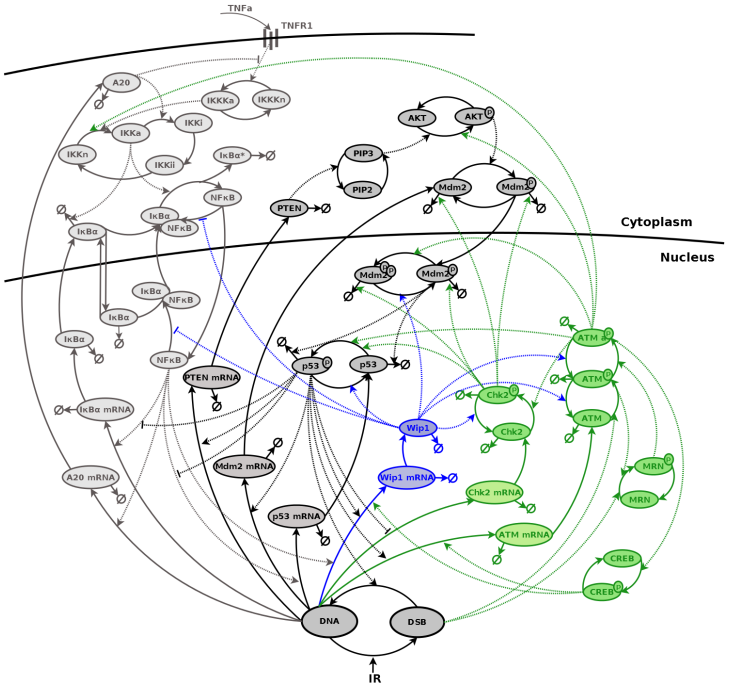

Fig. 1. Diagram of the proposed model. Pale gray part - NFκB regulatory unit. Dark grey - p53 regulatory unit. Green - ATM detection module. Violet - Wip1 (PPM1D) part. Solid lines stand for transition, while dotted ones for influences. Solid arrows mean positive influence, while hammerhead negative ones.

$$ra_1(t) = q^a_{ATM_0} + q^a_{ATM_1} P53^2_{pn}(t) + q^a_{ATM_2} CREB_{pn}(t) \quad (3)$$

$$rd_1(t) = q^d_{ATM} \quad (4)$$

$$ra_2(t) = q^a_{CHK2} \quad (5)$$

$$rd_2(t) = q^d_{CHK2} \quad (6)$$

$$ra_3(t) = q^a_{WIP_0} + q^a_{WIP_1} P53^2_{pn}(t) + q^a_{WIP_2} CREB_{pn}(t) + q^a_{WIP_3} NFKB_n(t) \quad (7)$$

$$rd_3(t) = q^d_{WIP} \quad (8)$$

Transcription rate is a function of the appriopriate gene state (0, 1 or 2). Please notice that following [26] Chk2 transcription rate is negatively regulated by phospho-p53.

$$\frac{d}{dt} ATM_t(t) = ms_1 G_{ATM}(t) - md_1 ATM_t(t) \quad (9)$$

$$\frac{d}{dt} CHK2_t(t) = ms_2 G_{CHK2}(t) \frac{pm_3}{pm_3 + P53_{pn}(t)} - md_3 CHK2_t(t) \quad (10)$$

$$\frac{d}{dt}WIP1_t(t) = ws_1 G_{WIP1}(t) - wd_1 WIP1_t(t) \tag{11}$$

2.3 Signal Transduction

Inactive ATM in the nucleus. Subsequent terms stand for: ATM translation, phospho-ATM dephosphorylation by Wip1, ATM phosphorylation by DSBs and degradation:

$$\begin{aligned}\frac{d}{dt}ATM_n(t) = \quad & mt_1 ATM_t(t) + mc_2 WIP1_n(t) ATM_{pn}(t) \\ & -ma_3 ATM_n(t) \tfrac{DSB(t)}{DSB(t)+mm_2} - md_2 ATM_n(t)\end{aligned} \tag{12}$$

Phospho-ATM in nucleus: phosphorylation upon DSBs induction, deactivation of the fully activated ATM by Wip1, full activation by KMRN (MRN complex), dephosphorylation by WIP1 and degradation:

$$\begin{aligned}\frac{d}{dt}ATM_{pn}(t) = {} & ma_3 ATM_n(t) \tfrac{DSB(t)}{DSB(t)+mm_2} + mc_2 WIP1_n(t) ATM_{an}(t) \\ & -ma_4 ATM_{pn}(t) KMRN_{pn}(t) \\ & -mc_2 * WIP1_n(t) ATM_{pn}(t) - md_2 ATM_{pn}(t)\end{aligned} \tag{13}$$

Fully active ATM: activation by KMRN, inactivation by Wip1 and degradation:

$$\begin{aligned}\frac{d}{dt}ATM_{an}(t) = \quad & ma_4 ATM_{pn}(t) KMRN_{pn}(t) \\ & -mc_2 WIP1_n(t) ATM_{an}(t) - md_2 ATM_{an}(t)\end{aligned} \tag{14}$$

Inactive Chk2 protein: translation, inactivation by Wip1, activation by ATM and degradation:

$$\begin{aligned}\frac{d}{dt}CHK2_n(t) = \; & mt_2 CHK2_t(t) + mc_3 WIP1_n(t) CHK2_{pn}(t) \\ & -ma_5 ATM_{an}(t) CHK2_n(t) - md_4 CHK2_n(t)\end{aligned} \tag{15}$$

Active Chk2: activation by ATM, inctivation by Wip1 and degradation:

$$\begin{aligned}\frac{d}{dt}CHK2_{pn}(t) = \quad & ma_5 ATM_{an}(t) CHK2_n(t) \\ & -mc_3 WIP1_n(t) CHK2_{pn}(t) - md_4 CHK2_{pn}(t)\end{aligned} \tag{16}$$

Active KMRN (MRN complex): activation by ATM, activation by DSBs and spontaneous deactivation:

$$\begin{aligned}\frac{d}{dt}KMRN_{pn}(t) = \quad & ma_6 ATM_{pn}(t)(KMRN_{tot} - KMRN_{pn}(t)) \\ & +(ma_7 \tfrac{DSB(t)}{DSB(t)+mm_3})(KMRN_{tot} - KMRN_{pn}(t)) \\ & -mc_4 KMRN_{pn}(t) \end{aligned} \tag{17}$$

Active CREB: activation by ATM and spontaneous deactivation:

$$\frac{d}{dt}CREB_{pn}(t) = ma_5 ATM_{an}(t)(CREB_{tot} - CREB_{pn}(t)) - mc_5 CREB_{pn}(t) \tag{18}$$

Wip1 protein: translation and degradation:

$$\frac{d}{dt}WIP1_n(t) = wt_1 WIP1_t(t) - wd_4 WIP1_n(t) \tag{19}$$

2.4 The Parameters of the Model

Our mathematical model of ATM-p53-NF-κB regulatory pathway includes various kinetics parameters obtained from our experiments performed in The Institute of Oncology in Gliwice. The rest of the parameters are taken from the literature or were chosen by us to receive the proper model behavior.

Determination of the amount of the DNA DSBs was based on the analysis of the γ-H2AX foci counting assay. This type of assay has been used in several studies on DNA DSBs, as described in [34], [35]. The U2-OS cancer cells (osteosarcoma) were irradiated with 4 Gy of IR. The average number of foci per cell from about 100 cells were counted under fluorescence microscopy. One foci was determined as a one DNA damage (DSB). The controls were untreated cells. The number of DSBs after induction with IR was a relative percentage of damages to the control cells.

DNA repair rate and apoptosis rate was tested using clonogenic cell survival assay [36], [37]. It allows to determinate how many cells are not able to proliferate, what is equivalent to the exceeding the first threshold described in [13]. In this assay, the apoptotic cells and cells with blocked cell cycle are not distinguishable. U2-OS cells were treated with different doses of IR (2, 4, 6 and 8 Gy). The abortive colonies with less than 50 cells were counted from each plate. The non-proliferated fractions of the treated cells, which we used in the model as apoptotic fractions, were referred to the control of untreated cells.

Degradation rate of the transcripts was determined using quantitative real-time polymerase chain reaction (qRT-PCR), in similar way as described in [38]. U2-OS cells were treated with actinomycin D in order to inhibit the process of transcription. There were several time points where the quantification of the material was performed after adding the inhibitor. The threshold cycle value (Ct) was measured and the delta Ct method was used in order to quantify the level of gene expression, with GAPDH as a reference gene which level we found constant

in the studied cells. The regression analysis with logarithmic approximation was performed with the start point of the highest expression value. The half-life time was quantified as a time when the expression level is reduced to half. Then, the degradation rate was quantified as a quotient of the natural logarithm of two and the half-life time in seconds.

Table 1. Model parameters. All values, except total protein number, are in $[s^{-1}]$

Name	Value	Name	Value	Name	Value
ma_1	1.1E-1	md_4	3.02E-5	$q^a_{ATM_0}$	3E-3
ma_3	1.5E-3	mm_1	10	$q^a_{ATM_1}$	5.87E-13
ma_4	5E-6	mm_2	1	$q^a_{ATM_2}$	3E-8
ma_5	1E-7	mm_3	1	q^d_{ATM}	3E-3
ma_6	4E-7	mm_4	1.5	q^a_{CHK2}	3E-3
ma_7	5E-5	ms_1	5E-3	q^d_{CHK2}	3E-3
mc_1	2.3E-13	ms_2	1E-2	$q^a_{WIP_0}$	1E-4
mc_2	6E-8	mt_1	5E-3	$q^a_{WIP_1}$	5.87E-13
mc_3	1E-8	mt_2	1E-2	$q^a_{WIP_2}$	3E-8
mc_4	2E-3	pm_3	5.54E+4	$q^a_{WIP_3}$	1.5E-7
mc_5	1E-3	wd_1	6.46E-5	q^d_{WIP}	3E-3
md_1	3.77E-5	wd_4	3.96E-5	$KMRN_{tot}$	1E+4
md_2	4.11E-5	wt_1	3E-2	$CREB_{tot}$	1E+5
md_3	4.18E-5	ws_1	2E-2		

Degradation rate of the proteins was determined using Western blot assay [39], [40]. The proteins obtained from U2-OS cells were treated with cycloheximide in order to inhibit the process of translation. There were several time points where the quantification was performed. The amount of the protein was quantified using the extended version of the application for analysis of the images from Western blot assay [41], developed by the authors. The relative level of the proteins was counted by performing normalization of the material from inhibitor-treated cells to the untreated control in one time, called time zero. The half-life time and degradation rate of the proteins were quantified.

Level change of proteins after treatment with IR was performed using Western blot assay. U2-OS cells were treated with 10 Gy of IR and collected in different time points (2, 8 and 24h) after irradiation. The level of the molecules was quantified using the application developed by the authors [41], and the relative level of the proteins was counted by performing normalization of the proteins from IR-treated cells to untreated controls for each collection time point. The obtained results were used to adjust the other parameters of the mathematical model, such as activation and inactivation rate of the proteins.

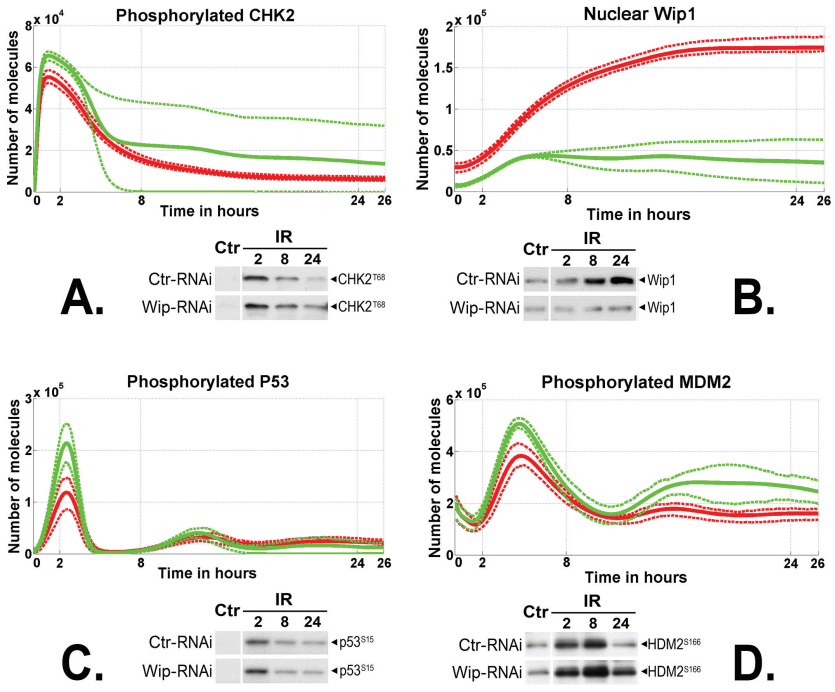

Fig. 2. Cells response to the 10 Gy of IR radiation for two cases: with normal WIP1 level (red) and RNAi caused silencing of WIP1 to $\sim$ 25% (green). The mean, first and third quartile are shown in each plot. Below are the Western blot results for corresponding molecules.

3 Results

Stochastic simulations of the 300 cells were performed for two cases: without and with RNAi that results in WIP1 silence to around 25%. The mean, 1st and 3rd quartile were calculated. The simulation results were compared with the experimental results obtained in the wet-lab in The Institute of Oncology in Gliwice (Fig. 2). The simulation results agree with experimental ones. In normal cells after the DSBs induction the levels of ATM and following Chk2 raise quickly (Fig. 2A, red). In turn, Mdm2 is degraded (Fig. 2D, red) and p53 activated (Fig. 2C, red). At this stage, cell cycle is blocked, because of the elevated phospho-p53 and p21 level, and DNA repair occurs. Meanwhile, WIP1 molecules cumulate (Fig. 2B, red). The role of Wip1 is to inactivate proteins involved in the DDR after the successful repair, so the apoptotic signal and the cell cycle blockade signal could be turned off. Silencing of WIP1 with RNAi significantly lowers its level (Fig. 2B, green). As a result, ATM and Chk2 levels are higher and decrease slower (Fig. 2A, green). Also, p53 activation is stronger (Fig. 2C, green) and the levels of p53-dependent proteins, such as Mdm2, are elevated for a long time (Fig. 2D, green). Cells with silenced WIP1 do not turn off the cell cycle blockade

signals as quickly as normal ones, what results in lowered clonogenic activity in the following days (Fig. 3).

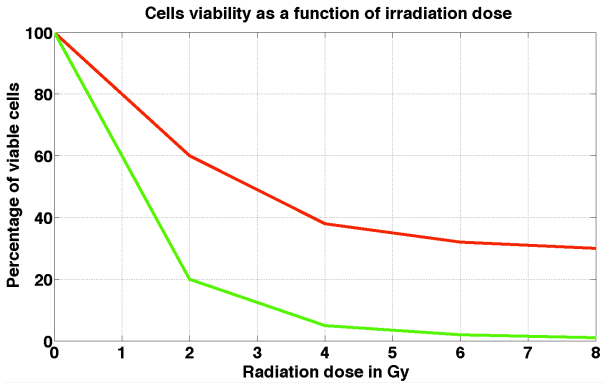

Fig. 3. Cells viability as a function of IR dose. Red - normal cells, green - cells with silenced WIP1. Cells are considered as viable when their p53 and p21 levels do not cross the given threshold.

Although our initial results based on the sub-G1 fraction measurement 24h after the IR treatment suggested that silenced WIP1 lowers the level of apoptotic cells, further results based on the clongenic ability show the opposite. Experimental results (data not shown) suggest that with the increased doses cells have lower ability to form colonies and therefore lower viability. Moreover, silenced WIP1 reduce this ability even more (Fig. 3). Following Kracikova results [13], the cells were considered as viable if their p53 and p21 levels do not cross the given threshold which was set as double of the p53 and p21 levels in untreated cells.

4 Conclusion

Deterministic models, by their definition, are unable to reproduce statistical differences of the model response for different doses or RNAi that results in protein silencing. Therefore, stochastic models, as presented in this paper, have advantage in better description of the complex system dynamics and its output, such as viable/not viable fractions. Proposed model, although generally is in agreement with experimental results, has of course some deficiencies. For example, DSB production and repair are very simplified and could be better described, also cell cycle blockade/apoptotic decision based on Kracikova results [13] could be done with more details. However, the above upgrades were not the subject of our research. They are rather complex and would increase the model complexity what we preferred to avoid. Recent publications suggest also that WIP1

transcript could be under the control of miR-16, which in turn depends on ATM activation [33]. That will be the topic of our future research.

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