

The Homologous Recombination Mathematical Model and the Role of miRNAs in ATM/ATR-dependent and BRCA1/BRCA2-dependent DSBs Repair Pathway

Outline of the Project

Katarzyna Jonak, Monika Kurpas and Krzysztof Puszyński
Systems Engineering Group, Institute of Automatic Control, Silesian University of Technology,
ul. Akademicka 16A, 44-100 Gliwice, Poland

1 INTRODUCTION

Eukaryotic cells are exposed continuously to the genotoxic stresses caused by various sources, what may result in formation of DNA double strand breaks (DSBs) or single strand breaks (SSBs). DSBs are known to be one of the most cytotoxic lesions, caused by exposure to ionizing radiation (IR), clastogenic drugs (Lindahl and Barnes, 2012), but also formed endogenously during DNA replication or even as an effect of reactive oxygen species (ROS) (Lopez-Contreras and Fernandez-Capetillo, 2012). In order to maintain genomic integrity, the DNA damage response is activated. This biological signaling pathway is a cascade of the signals from different types of macromolecules: detectors that recognize DSBs, proteins mediating signal transduction, and effectors responsible for activation of damage response.

DSBs are detected indirectly by ataxia telangiectasia mutated (ATM) that stabilizes and activates repair pathways, such as homologous recombination (HR) or non-homologous end joining (NHEJ). The proper functioning of repair pathways is essential to enhance the cellular survival.

For better understanding of the molecular mechanisms of DNA repair pathways, the useful approach is presented by systems biology. It describes complex systems of interactions between macromolecules with loops of positive and negative feedbacks in a form of mathematical models. Such modeling allows to not only understand the complex interactions between various components of the regulatory pathways, but also allows to investigate the impact of DNA-damaging agents on cells that can lead to such diseases as neurological disorders or cancerogenesis. Mathematical models can be used for preliminary analysis of the experimental

hypotheses, as well as for putting the hypotheses on possible treatment at the level of the cellular signaling pathways.

2 STAGE OF THE RESEARCH

2.1 DSBs Detector Module

ATM functions as a DSBs detector that sends the signal about the damage to different mediators and effectors. We have developed a mathematical model, where the consequences of ATM activation on two transcription factors, tumor antigen p53 and NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), are presented. Both nuclear factors control several physiological processes from cell cycle arrest through DNA repair and adaptive immune response to apoptosis. The model is based on our previous model of p53-NF- κ B interaction (Puszynski et al., 2009).

Major DNA damage response regulators play an essential role in ATM-p53-NF- κ B pathway. Mdm2 (E3 ubiquitin-protein ligase) facilitates p53 degradation, checkpoint kinase 2 (Chk2) inhibits p53 ubiquitination and degradation, and cellular transcription factor CREB transcriptionally activates ATM. Moreover, in this model we linked ATM-p53-NF- κ B pathway components with protein phosphatase Wip1 that regulates dephosphorylation events (inactivation of the most of the pathway components).

The mathematical model is presented as a set of stochastic and deterministic equations according to the Haseltine-Rawlins postulate. Stochastic description and following Gillespie direct method based simulation were used for slow reactions, like states of genes change, while deterministic

description based on ordinary differential equations (ODE) and following Runge-Kutta 4th order simulation method were used for description of quick reactions, like activation or degradation of the proteins involved in repair pathway.

The model is activated upon IR induction and TNF α (tumor necrosis factor alpha). The signaling pathway is also stimulated continuously by the small number of damages that occur spontaneously. The components of ATM-p53-NF- κ B model are presented mostly in two main states: active and inactive. Moreover, most of the proteins considered in the model contain their transcriptional forms (mRNA). The model has an assumption that each gene has two copies and among them one can be in an active state, both, or none.

The output of the model is p53 level that determinates cell fate. In this model cell death is recognized as a permanently increase of p53 level for more than 6 hours, then cell is considered as an apoptotic and its elements are degraded.

We simulated the cellular response to the damage combining all of the described elements.

The obtained results shown that ATM pathway is an effective system for DSBs detection with strong amplification signal and quick response. Furthermore, we observed the strong dependence of the cellular response to the DNA damage on Wip1, what leads to the conclusion that it plays a role as a gatekeeper in the ATM-Mdm2-p53 regulatory loops, essential in the process of DNA damage repair.

2.2 SSBs Detector Module

Another detector system which was important in our work was ATR module (ataxia telangiectasia and Rad3-related protein) responsible for detection of DNA SSBs caused by, for example, ultraviolet radiation (UV) or replication fork arrest (Lopez-Contreras and Fernandez-Capetillo, 2012). The main subject of the study was to develop a mathematical model for p53 regulatory pathway with ATR as a main detector system, perform simulations and then linked to NF- κ B regulatory module, ATM detection module and deactivation agent Wip1.

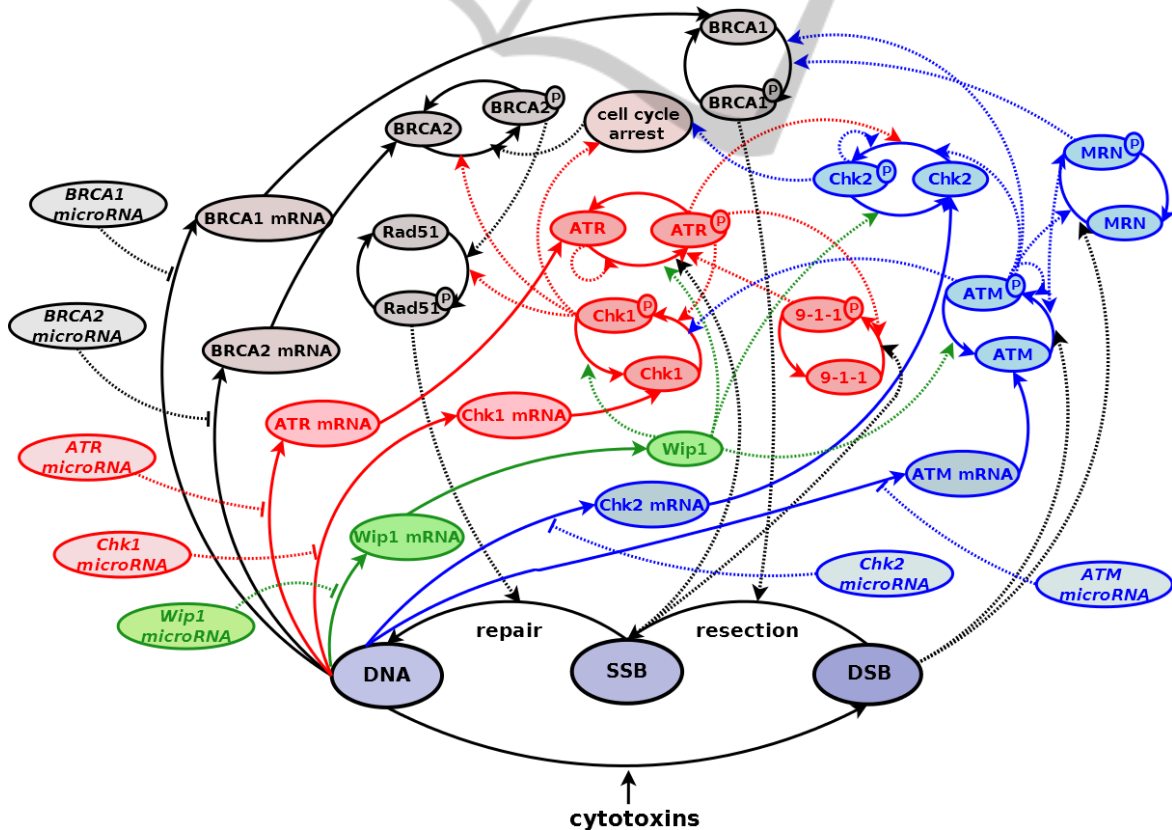


Figure 1: Schematic model of HR repair pathway with ATM and ATR as major detector systems of DSBs and SSBs. The letter "P" next to the protein name indicates phosphorylated form of this protein. Solid lines are transitions between states of the HR components, dotted lines with arrow-heads are positive regulation (acceleration), and dotted lines with hammer-heads are negative regulations (inhibition).

In ATR-p53 model the first stage of SSBs detection is an activation of Rad9-Rad1-Hus1 protein complex (called 9-1-1) and activation of the ATR-ATRIP (ATR interacting protein) complex. Rad9 subunit phosphorylated by protein ATR recruits TopBP1 (topoisomerase II binding protein 1), which presence is necessary for full activation of ATR. Then, ATR activates checkpoint kinase 1 (Chk1) that activates p53 and increase degradation rate of Mdm2.

The model was built using the same assumptions as ATM model, with the usage of stochastic and deterministic approaches. The activation of the ATR-p53 model was performed upon UV radiation at 24 hours after start of the simulations. The effect of different doses of UVC was studied and the apoptotic death threshold was chosen. It was observed *in situ* that with dose of 18 J/m^2 more than half of the cells become apoptotic.

The simulations shown that ATR module acts as an efficient system to detect even a single DNA damage. The SSBs detection module is very fast - the breakage is detected within few seconds after the occurrence of DNA damage. Moreover, ATR and ATM mathematical models explain that the base production and activation level of the p53 protein and its signaling pathway proteins may be caused by persistent cellular stress.

2.3 HR as a Repair Pathway

The interesting subject in the area of modeling DSBs and SSBs detection modules is the role of the different proteins responsible for specific repair pathways. Therefore, now we combine the two detector modules, ATM and ATR, in order to perform further extensions to homologous recombination repair pathway.

We have already investigated the main modules responsible for HR. The main interactions between them are presented in Figure 1. The detector system of DSBs is ATM, which together with MRN complex activates BRCA1 (breast cancer type 1 susceptibility protein) responsible for DNA repair. This activation leads to resection of DSBs and activation of ATR module that detects SSBs. Then, the process of repairing the single strand damages starts and goes through BRCA2 (breast cancer type 2 susceptibility protein) and Rad51. The model includes the effects of the damages on checkpoints proteins, as well as the effect of known specific microRNAs (miRNAs) responsible for regulation of transcription of the proteins involved in HR pathway.

3 OUTLINE OF OBJECTIVES

For a better understanding of the HR pathway and identification of the abnormalities that may occur in this process, it is useful to build a mathematical model describing the dynamics of this module. The model should be based on experimental data in order to correspond to reality, hence the biological model will be built that will allow experimental verification of the HR mathematical model.

The main goal of the project is to examine the impact of different agents that cause DSBs on HR in a single cell as one of the most important repair pathways in eukaryotic organisms. For this purpose the models of ATM and ATR pathways will be combined as detectors active in S/G2 phase of cell cycle without taking into account interactions with p53 and NF- κ B. The whole HR pathway will be described, from detector module to ligation of the DNA strands. Another objective is to identify substances which can cause DSBs without causing SSBs. The impact of the DNA damages on miRNAs will be also investigated with the negative interaction of these macromolecules on other components of HR pathway.

Within the project we anticipated three tasks:

- Construction of the deterministic and stochastic mathematical model of HR repair pathway based on the information from existing models described in the literature, as well as ATM and ATR pathways models developed by the authors.
- Collection of the data from experiments and literature on the parameters of the model, such as activation and inactivation rates, degradation rates, transcription rates, etc.
- Construction and experimental verification of the model.

4 RESEARCH PROBLEM

The main research problem focuses on the interactions between different components of HR pathway during repair process of DNA double strand breaks. Because of the fact that HR occurs only in S/G2 phase, some of the proteins, such as p53, are not active, what should be taken into account during the model development. It is necessary to note that different concentrations of specific detection components, such as MRN, may lead to various repair pathways, for example, NHEJ. This

phenomena should be also considered in the theoretical model.

The impact of miRNAs on HR components is essential during repair. Overexpression of some of the miRNAs may cause cell cycle arrest and may forward the cell into apoptosis pathway through p53 protein in G1 phase. The same result may be obtained by downregulation of BRCA1 or BRCA2 genes and low activation level of these proteins. Therefore, the exact impact of these components should be examined, as well as the impact of Wip1 – the major deactivation agent.

We found necessary to examine which DNA-damaging factors may lead to DSBs and to activation of HR pathway, as well as what dose of these factors may lead to cell cycle arrest and apoptosis.

The main purpose of the existence of the HR model with the DNA damages detector modules is to illustrate the processes occurring during DNA repair. The project will allow to understand the interactions between molecules, probably investigate new components of the HR network, and contribute to putting the hypotheses on possible treatment of different diseases at the level of the cellular pathways.

5 STATE OF THE ART

5.1 DSBs Detection

Activation of a specific mechanism of DNA repair depends mainly on cell cycle. Thus, initiation of NHEJ is possible during the whole cycle, however usually appears in G1/G0 phases, where HR is limited to late S phase and G2 phase (Langerak and Russell, 2011). DSBs are detected by ATM and multiprotein complex MRN. In G1 phase, the activation of these proteins results in Chk2-mediated p53-dependent cell cycle arrest. DNA damages undergo only minor nucleolytic processing being repaired very fast by NHEJ (Jazayeri et al., 2006). In case of S/G2 phases and repair by HR, the cell cycle arrest is p53-independent and the process of repair is slower than NHEJ (Jazayeri et al., 2006). The choice between HR and NHEJ repair pathways is partially determinate by MRN complex activity: in HR when DSBs require resection the activity of the complex is much higher (Chowdhury et al., 2013). BRCA1 also promotes resection and excludes 53BP1 protein, which is involved in NHEJ process (Chowdhury et al., 2013).

ATM is activated by DSBs directly and

indirectly by MRN complex. ATM is also autophosphorylated by the formation of defects in the chromatin structure (Bakkenist and Kastan, 2003). At the same time exonuclease MRN is activated directly by DSBs and by phosphorylation of one of its component, Nbs1, by ATM (Bakkenist and Kastan, 2003). ATM is involved in cell cycle arrest by phosphorylation of Chk1 and mostly by phosphorylation of Chk2. The signal of DSBs is amplified by autophosphorylation of Chk2 induced by ATM (Ahn et al., 2004).

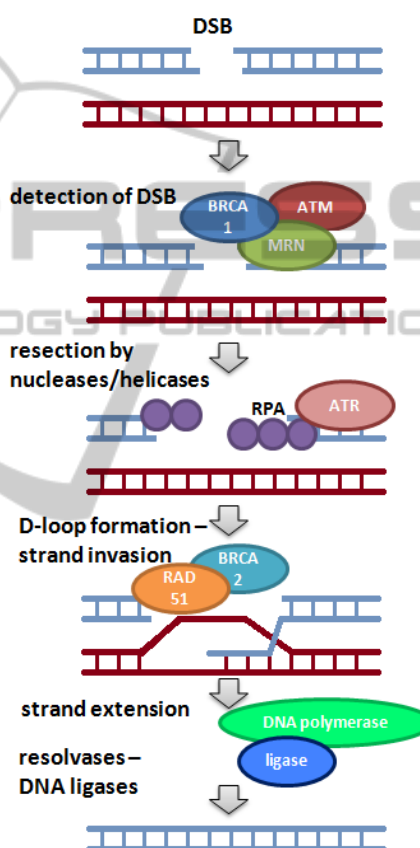


Figure 2: Scheme of HR repair pathway. DNA helix after DSBs induction is presented as a light blue ladder, DNA from sister chromatid is a purple ladder.

5.2 DNA Resection and Repair

MRN together with CtIP protein binds to free ends of DNA molecule and in presence of BRCA1 degrades one of the two helices from 5' end of DNA strand. This process results is resection of DSBs. The resulting free 3' ends are detected and protected by replication protein A complex (RPA) (Filippo et al., 2008). These damages of single stranded DNA (ssDNA) recruits proteins involved in detection of

SSBs, such as ATR. ATRIP is capable of attaching themselves to the RPA-ssDNA, which induces autophosphorylation of ATR (Nam et al., 2011). In addition, ssDNA fragment binds a complex of Rad17-RFC2-5, which allows the attachment of the 9-1-1 complex to the damaged site (Filippo et al., 2008). ATR recruits TopBP1 and then activates several proteins involved in the repair pathway, such as Chk1 and Chk2. Signal strength of the checkpoint cascade is dependent on the length of RPA-ssDNA and the possibility of autophosphorylation of ATR molecules (Filippo et al., 2008).

Activated Chk1 and Chk2 results in cell cycle arrest by inactivation of cyclin-dependent kinases. The reduction of activity of these kinases leads to activation of BRCA2, which recruits Rad51 and interact with RPA-ssDNA to initiate HR process (Filippo et al., 2008). Moreover, activated Chk1 phosphorylates Rad51, what leads the cell to the HR. BRCA2 and RAD51 together with the accompanying proteins catalyze the invasion of the free 3' end to the homologous sequence of sister chromatid. As an effect the D-loop structure is formed, what allows for the rebuilding of a missing DNA fragment by polymerase η (Filippo et al., 2008). The DNA ligase I join the new fragments. Synthesis is finished when two Holliday's structures are formed (Filippo et al., 2008). These structures are then removed by helicases, like BLM, topoisomerases III α or endonucleases, such as Mus81/Eme1 (Filippo et al., 2008). The process of DNA repair by HR is presented in simplified way in **Figure 2**.

5.3 MiRNAs and Wip1 in HR Pathway

An essential component in regulation of DSBs detection and the process of DNA damages repair is Wip1, which is a deactivation agent for key proteins involved in HR. The main role of this protein is to regulate the level of activated proteins mostly after succeed process of repair, and to unlock the cell cycle. Wip1 transcription is p53-dependend, what makes it connected to DNA damages (Lowe et al., 2012). The most important parts of regulation of the repair pathways by Wip1 is associated with inactivation of DNA damages detector modules: ATM and ATR. Moreover, the cell cycle checkpoints, Chk1 and Chk2, are dephosphorylated by Wip1, as well as p53 and Mdm2 (Lowe et al., 2012).

Small RNAs, called miRNAs, also play important roles in DSBs repair pathway. Both miRNA transcription and maturation processes are

altered in response to damages of DNA strands and repair processes. Biogenesis of these micromolecules is induced in an ATM-dependent manner (Chen X. and Chen T., 2011). Activated ATM phosphorylates KSRP protein (KH-type splicing regulatory protein) what leads to activation of some of the miRNAs (Chen X. and Chen T., 2011). Not all of the miRNAs involved in HR pathway have been discovered yet, however there are some which are known, such as miR-100, miR-101 and miR-421 which suppress ATM, miR-182 which suppress BRCA1 or miR-16 that suppress Wip1 (Chen X. and Chen T., 2011).

5.4 Existing HR Models

The existing models of HR pathway are based mainly on the late phase of the repair process: from RPA coating to the action of DNA ligases.

In the model described in (Taleei et al., 2011) DSBs detection module is treated as a one component: MRN. The authors focus on the effects of resection, without describing the whole DSBs detection with ATM, and without including checkpoint proteins. The most important parts there are MRN, RPA, Rad52 and ligase. Another theoretical model (Rodríguez et al., 2012) is based on Boolean network system for the FA/BRCA pathway involved in HR. The whole model is very expanded and it takes into account also NHEJ repair pathway. However, the model do not contain the effects of miRNAs and is rather focus only on the whole BRCA pathway.

6 METHODOLOGY

The mathematical model of HR repair pathway will be built using set of equations that allow simulating the behavior of one cell treated with different doses of cytotoxins that cause DSBs. For the mathematical model stochastic and deterministic approach will be used as it was performed for the models of ATM and ATR signaling pathways. Moreover, the Michaelis-Menten kinetics and the law of mass action will be used in order to bring the model and its reaction speed to reality.

In order to investigate the effect of other not-yet-known modules on described components of the HR pathway, several biological experiments will be performed. Northern blotting will be used in order to investigate miRNAs that regulate different components of the HR. Western blotting will be used to investigate level of key proteins (total and

phosphorylated forms) involved in the process of DSBs detection and repair, as well as to collect the information concerning the kinetics parameters of the model, such as time of deactivation of the specific protein. Moreover, the analysis of the expression profile of genes involved in HR pathway will be performed with a method of quantitative real-time PCR in stress conditions.

The analysis of the level of double strand breaks of DNA after treatment with specific cytotoxins will be performed with microscope analysis of H2AX foci assay. The amount of foci reflects the amount of DNA breaks. The analysis of the total number of DSBs and SSBs will be performed with comet assay.

The experiments will be performed on mammalian cancer and normal cell lines with active or inactive forms of the proteins involved in HR. The whole methodology is still under development.

7 EXPECTED OUTCOME

The experimental-based mathematical model (stochastic and deterministic) of HR repair pathway will be presented. The HR model, together with ATM and ATR models developed by our group, will create a comprehensive mathematical model describing the dynamics of the interactions occurring in the cell from the inception of DNA damages to making the decisions about cell fate: to direct cell to repair by HR, to arrest cell cycle or direct cell to apoptosis pathway during HR or before the process of repair.

The expected outcome is an identification of at least one new component of the HR pathway which can be even protein or miRNA, and the parameters of the model for most of the pathway components. Moreover, it is expected that the developed model will reflect the experimental data and will be a good tool for simulation of the cell behavior during HR.

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