A novel ataxia-telangiectasia mutated autoregulatory feedback mechanism in murine embryonic stem cells

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Ataxia-telangiectasia mutated (ATM) is known to play a central role in effecting the DNA damage response that protects somatic cells from potentially harmful mutations, and in this role it is a key anti-cancer agent. However, it also promotes repair of therapeutic damage (e.g. radiotherapy) and so frustrates the efficacy of some treatments. A better understanding of the mechanisms of ATM regulation is therefore important both in prevention and treatment of disease. While progress has been made in elucidating the key signal transduction pathways that mediate damage response in somatic cells, relatively little is known about whether these function similarly in pluripotent embryonic stem (ES) cells where ATM is also implicated in our understanding of adult stem cell ageing and in improvements in regenerative medicine. There is some evidence that different mechanisms may operate in ES cells and that our understanding of the mechanisms of ATM regulation is therefore incomplete. We investigated the behaviour of the damage response signalling pathway in mouse ES cells. We subjected the cells to the DNA-damaging agent doxorubicin, a drug that induces double-strand breaks, and measured ATM expression levels. We found that basal ATM gene expression was unaffected by doxorubicin treatment. However, following ATM kinase inhibition using a specific ATM inhibitor, we observed a significant increase in ATM and ataxia-telangiectasia and Rad3 related transcription. We demonstrate the use of a dynamical modelling approach to show that these results cannot be explained in terms of known mechanisms. Furthermore, we show that the modelling approach can be used to identify a novel feedback process that may underlie the anomalies in the data. The predictions of the model are consistent both with our *in vitro* experiments and with *in vivo* studies of ATM expression in somatic cells in mice, and we hypothesize that this feedback operates in both somatic and ES cells *in vivo*. The results point to a possible new target for ATM inhibition that overcomes the restorative potential of the proposed feedback.

Keywords: model; mathematics; DNA damage; cancer; drug discovery;
ataxia-telangiectasia mutated inhibition

1. INTRODUCTION

The p53 pathway is central to the cellular response to environmental stresses including viral infection and

DNA damage (Lavin & Kozlov 2007). The pathway is negatively controlled by the protein degradation system that includes the Mdm2 E3 ligase and is positively activated by the ataxia-telangiectasia mutated (ATM) protein kinase. The balance between these inhibitory and activating branches regulates the

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specific activity of p53. The integrated behaviour of the negative feedback loop between Mdm2 and p53 has been extensively studied and this has provided important physiological insight into p53 control (Momand *et al.* 2000; Moll & Petrenko 2003). The ATM pathway on the other hand has not been studied at the same level of detail, and so the positive activation of p53 and the processes leading up to it are less well understood.

ATM plays a central role in the coordinated activation of DNA damage response (DDR) signalling pathways that protect somatic cells from the deleterious effects of fixed mutations, forming a potent anti-cancer mechanism. DDR pathways are initiated by phosphoinositide 3-kinase-like kinase family (PIKK) members, including ATM and ataxia-telangiectasia and Rad3 related (ATR), which recognize distinct types of DNA damage. ATM is activated by double-stranded DNA breaks (DSBs) induced by exogenous genotoxins, including chemotherapeutic drugs and ionizing radiation, and endogenous sources such as increased oxidative stress during ageing. ATM is recruited to the sites of DSBs and is activated by Ser-1981 autophosphorylation and binding to the heterotrimeric Mre11/Rad 50/Nbs1 (MRN) adaptor complex, resulting in phosphorylation of downstream targets, including Chk2 and p53, and activation of cell cycle checkpoints (Lavin & Kozlov 2007). ATR is activated by agents that inhibit DNA replication, including ultraviolet radiation and hydroxyurea. ATR is activated following binding to ssDNA-protein complexes, such as form at stalled replication forks, and the main ATR target is the Chk1 checkpoint kinase (Paulsen & Cimprich 2007). Damage-dependent activation of ATM and ATR initiates signal transduction pathways that activate cellular tumour suppressor, checkpoint and damage repair mechanisms. Initially, the ATM and ATR signalling pathways were thought to be parallel, but there is increasing evidence of cross-talk at the signalling level (Hurley & Bunz 2007).

Asymmetric division of stem cell populations maintain tissue homeostasis, and efficient DDR mechanisms within stem cell compartments influence cell number and mutation state to maintain optimum tissue function. Pluripotent embryonic stem (ES) cells provide a good system to investigate DDR mechanisms within stem cells. Compared to somatic cells, ES cells have a reduced mutation rate that protects the organism from deleterious germline fixed mutations and developmental disease (Hong *et al.* 2007), indicating that a functional change in DDR signalling networks occurs within this specialized cell type. Elucidating specific ES cell DDR mechanisms has relevance to adult stem cell ageing, as well as regenerative medicine, although specific modifications to established DDR mechanisms may form part of the protection mechanism in ES cells. A few studies have investigated DDR signalling in ES cells, and reports indicate that a lack of p53-dependent p21 transcription is associated with a loss of the G1 checkpoint. Furthermore, Chk2 mislocalization to centromeres contributes to a lack of G2/M checkpoint. These checkpoint defects are thought to explain the predominant apoptotic response to cellular damage in ES cells

responsible for the low mutation rate and maintenance of genomic integrity.

Important new insight into the negative regulation of p53 by Mdm2 has been achieved by using mathematical models to accommodate the complexity arising from the number of associated components and the nonlinear nature of their interaction (Lev Bar-Or *et al.* 2000; Lahav *et al.* 2004; Ciliberto *et al.* 2005; Geva-Zatorsky *et al.* 2006; Ramalingam *et al.* 2007). By comparison, analysis of the dynamical behaviour of ATM regulation and the associated mathematical synthesis has not reached the same level and, where work has been done, it has tended to be in relation to the links between ATM and the p53–Mdm2 loop (Ma *et al.* 2005; Wagner *et al.* 2005). Here, we aim to address this imbalance in part by building on our understanding of ATM in somatic cells through complementary studies in ES cells. Also, while previous studies have shown that a loss of the ATM gene is associated with altered gene expression profiles in response to different damaging agents (Heinloth *et al.* 2003), ATM-mediated repression of ATM and ATR transcription has not previously been identified. The adopted approach uses measurements of the dynamical response of ATM gene expression in ES cells subject to perturbations induced by addition of a DNA-damaging agent, doxorubicin, and an inhibitor of ATM, KU-55933. We demonstrate the potential of mathematical models of the ATM–ATR complex for improving understanding by providing quantitative tests of existing hypotheses and for generating new testable hypotheses about the regulation of ATM in ES cells. Finally, we discuss the generalization of the results to somatic cells and the potential impact of our findings for interventions to regulate the levels of ATM in cells.

2. EXPERIMENTAL METHODOLOGY

2.1. Reagents and antibodies

Doxorubicin hydrochloride and wortmannin were obtained from Sigma-Aldrich. ATM inhibitor, KU-55933, and DNA-PK inhibitor, NU7441 (Leahy *et al.* 2004), were obtained from KuDOS Pharmaceuticals, Cambridge, UK.

2.2. Cell culture

E14 murine ES cells were cultured in antibiotic-free Glasgow minimal essential media (GMEM) supplemented with 2-mercaptoethanol, non-essential amino acids, sodium bicarbonate, 10 per cent foetal calf serum and 100 units ml⁻¹ recombinant leukaemia inhibitory factor, on gelatinized tissue culture flasks, and incubated at 37°C and 5 per cent CO₂. ES cells were treated with genotoxic agents at a confluency of 60–70%. Cells were treated with kinase inhibitors at 10 µM KU-55933, 1 µM NU-7441 or 10 µM wortmannin, either alone or for 1 hour prior to 0.5 µM doxorubicin treatment.

2.3. Immunoblotting

Cell pellets were lysed in two to three volumes urea lysis buffer (containing 7 M urea, 0.1 M DTT, 0.05% triton

X-100, 25 mM NaCl and 20 mM Hepes, pH 7.6), incubated on ice for 30 min, then clarified at 13 000*g* for 10 min. Protein concentration was determined by the Bradford method, samples were separated using 10 per cent polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane. Proteins were visualized using ECL (Amersham) and autoradiography.

2.4. Microarray

RNA was isolated from treated cell pellets with a Qiagen RNeasy kit according to the manufacturer's instructions. Low density gene expression analysis was done using the focused oligo GEArray Q series Mouse p53 Signalling Pathway Gene Array (Superarray MM-027) according to the manufacturer's instructions, and data were analysed using Superarray GEArray Expression Analysis Suite Software.

2.5. Real-time RT-PCR

Total RNA was isolated using a Qiagen RNeasy mini kit. Real-time PCR primers were: mATM forward 5'-ggttctttgtacgatccactctt-3'; mATM reverse 5'-tcagctactttgttgaaactctgg-3'; mATR forward 5'-ggg atcagagagaacctttaatga-3'; mATR reverse 5'-gatcaca ccttgtagtcgtgttc-3'; mGAPDH forward 5'-actatgtcg tggactctatgg-3'; mGAPDH reverse 5'-tgagttgtca tatttctgtgggt-3'. RT-PCR was done using the QuantiTect SYBR Green RT-PCR kit (Qiagen), and the reaction mixtures contained 0.5 μ M primers and 40 ng of total mRNA. RT-PCR conditions were: 50°C for 30 min; 95°C for 15 min; 45 cycles of 95°C for 15 s; 55°C for 30 s; and 72°C for 30 s.

3. RESULTS

3.1. The ATM pathway is active in DNA-damaged ES cells

In order to determine whether the ATM DDR pathway operates in ES cells, we first set up controls to determine whether ATM could be induced by doxorubicin. This drug is an anthracycline which indirectly causes DSBs and activates ATM-dependent signalling (Kurz *et al.* 2004). We first showed that doxorubicin induced p53 serine-18 phosphorylation at the ATM site in murine ES cells (figure 1*a*). Interestingly, the lower doxorubicin concentration (0.5 μ M) induced a more robust and prolonged phosphorylation than the higher concentration (5 μ M), which may reflect increased cell death at the higher dose. Using a dose titration, we showed that 0.25–0.5 μ M doxorubicin optimally induced both p53 protein and phosphorylation of two major damage response sites, serine-18 and serine-389 (figure 1*b*, lanes 4 and 5 versus lane 1). These data are consistent with the possibility that ATM is indeed active in doxorubicin-damaged cells, although other related kinases are known to target this site on p53. The p53 serine-18 site (human serine-15 equivalent) is phosphorylated *in vitro* by several PIKKs including ATM (Banin *et al.* 1998; Canman *et al.* 1998), DNA-PK (Lees-Miller *et al.* 1992), ATR (Tibbetts *et al.* 1999)

Figure 1. (a) Time course of p53 serine-18 induction following either 0.5 μ M or 5 μ M doxorubicin treatment. (b) Effect of a doxorubicin dose titration (0–2.0 μ M) on p53 protein levels (CM5) and site-specific phosphorylation at serine-18 and serine-389 at a fixed 2 hours time point. (c) E14 murine ES cells were treated with 0.5 μ M doxorubicin for 2 or 4 hours in the absence of inhibitor (lanes 5 and 6, respectively) or in the presence of 10 μ M KU-55933 (ATM inhibitor, lane 7 and 10), 1 μ M NU7441 (DNA-PK inhibitor, lanes 8 and 11) or 10 μ M wortmannin (PIKK inhibitor, lanes 9 and 12). Cells were harvested at the indicated times of 2 or 4 hours post-damage, and lysates were blotted for phosphoserine-18 p53, phosphoserine-389 p53, total p53 protein (CM5) and Mdm2 (2A10 and 4B2).

and SMG-1 (Yamashita *et al.* 2001). We used specific ATM and DNA-PK inhibitors and the broad-spectrum PIKK inhibitor (wortmannin) to determine whether ATM was the PIKK mediating the doxorubicin-induced p53 phosphorylation in ES cells. Previous studies have shown that 10 μ M wortmannin will inhibit both ATM and DNA-PK, but not ATR, in intact cells (Sarkaria *et al.* 1998). We found that doxorubicin-induced p53 serine-18 was completely blocked by KU-55933, a specific ATM kinase inhibitor (figure 1*c*, lane 7 versus lane 5), and wortmannin (figure 1*c*, lane 9 versus lane 5), but not by DNA-PK inhibition (figure 1*c*, lane 8 versus lane 5) at 2 hours post-treatment. The damage-induced serine-389 phosphorylation was attenuated by ATM kinase inhibition at this time point (figure 1*c*, lane 9 versus lane 5 and lane 6), as this phosphorylation site is controlled by the

Table 1. p53 pathway-related genes identified to exhibit more than 2-fold (upregulated) or less than 0.5-fold (downregulated) expression changes in response to doxorubicin or ATM inhibition by KU55933 treatment, as determined by superarray screening 2 hours after damage.

upregulated by doxorubicin		downregulated by doxorubicin		upregulated by ATM inhibition (relative to Dox alone)		downregulated by ATM inhibition (relative to Dox alone)	
PUMA	25.13	CSNK1A1	0.48	p21	496.00	cyclin H	0.00
Gadd45	22.88	ATM	0.42	ACTA1	433.00		
B99	16.99	TNF α	0.40	ATR	75.6		
DAXX	15.79	c-myc	0.23	ATM	47.00		
TNFAIP	12.81	p63	0.17	CDKN2A	6.98		
Mdm2	12.67	Hoxa5	0.17	APR-3	6.76		
cyclin G1	11.53	ATR	0.17	BAP1	5.97		
TRAF1	11.02	Faf1	0.15	Caspase 9	3.68		
cathepsin D	6.57			MDR1	2.42		
Bax	5.48			Apaf-1	2.10		
MDR1	4.80						
p73	4.79						
APR3	3.90						

upstream master regulator ATM. We have therefore established that ATM mediates doxorubicin-induced DDR signalling in ES cells.

3.2. ATM promoter activity increases after ATM inhibitor treatment of ES cells

ES cells are almost unique in the sense that it has been reported that the downstream targets of p53 are inactive (Aladjem *et al.* 1998). This is despite the fact that ATM is active and p53 can be induced by ATM after DNA damage (figure 1). However, we found that expression of the p53 transcriptional target Mdm2 was induced 4 hours after damage (figure 1c, lane 6 versus lane 1), suggesting that the p53 pathway could be active. Thus, to understand the role of ATM–p53 signalling in mediating ES cell DDR, we next identified damage-responsive gene expression using low-density microarray focused on p53 pathway components (table 1). Most genes in this pathway were upregulated 2 hours after damage, although mRNA expression of these p53 targets was largely unaffected by ATM inhibition, thereby showing that p53 serine-18 phosphorylation and p53-dependent transcriptional activity are uncoupled in ES cells (data not shown). These data suggest that, as reported (Aladjem *et al.* 1998), the p53 pathway is attenuated in ES cells. For example, Mdm2 protein induction by doxorubicin is delayed in the presence of the inhibitor (figure 1c, lane 6 and 7 versus lane 10). In addition to our analysis of p53-responsive genes, we found that several p53 pathway genes were downregulated in response to DNA damage including the PIKKs, ATM and ATR (2.4-fold and 5.8-fold, respectively). However, in the presence of both the ATM inhibitor, KU-55933, and doxorubicin, both ATM and ATR gene expression were strikingly upregulated (figure 2a). A time course demonstrates that this induction occurs within 30 min but cycles over the time course used (figure 2b). The data suggest that these cells have evolved a mechanism to sense ATM inhibition that results in rapid induction of the ATM to

Figure 2. (a) ATM and ATR mRNA expression levels were measured in ES cells treated with 10 μ M KU-55933 or DMSO vehicle control, or after treatment with 0.25 μ M doxorubicin following 1 hour pre-treatment with 10 μ M KU-55933. Data are presented normalized to GAPDH mRNA expression levels. Bars indicate expression relative to control treatment (blue bars, ATM; violet bars, ATR). (b) Expression dynamics of ATM was studied over time (0–4 hours) following treatment with 10 μ M KU-55933. Points represent measured expression levels relative to the control treatment, and the line is a smooth polynomial fit to the points.

compensate for this loss of ATM function. The duration of our inhibitor time-course experiments is limited by the half-life of the drug and the survival time of ES cells in tissue culture conditions. As such, we do not know whether chronic ATM inhibition would lead to constitutively higher ATM promoter activity to

Figure 3. Network diagram for the mathematical model. ATM-i and ATM-a denote inactive and active forms of ATM, respectively, and ATR-i and ATR-a are the corresponding forms of ATR. We denote the activated form of the hypothesized repressor as REP-a, and REP-i is the inactivated form. Deactivation of the repressor is assumed to be actioned by a phosphatase, denoted UP1. (Black stars, degradation; dashed lines with arrows, positive regulation; dashed lines with bars, inhibition.)

compensate. However, there was previously identified a striking upregulation of the ATM reporter in ATM-knockout mice (Gueven *et al.* 2005), which suggests in fact that cells can respond over a long time frame to ATM inhibition by stimulating the ATM promoter. Since our data are consistent with the biological data of Gueven *et al.* (2005), we set up a modelling framework to form a foundation for further study on ATM promoter control.

3.3. Summary of conclusions from the experimental work

The results from the experiments show a range of ATM and ATR promoter activity when the ATM protein is inhibited. The key experimental results from the point of view of a dynamical analysis of the system are the following.

- (i) Both ATM and ATR promoter activities are significantly increased at the 2 hours time point when the ATM protein is inhibited.
- (ii) Doxorubicin alone has no effect on ATM or ATR promoter activity.
- (iii) The levels of ATM promoter activity oscillate when observed over a time course when the ATM protein is inhibited.

4. INTERPRETATION OF THE RESULTS USING MATHEMATICAL MODELLING

4.1. Model formulation

The experimental results suggest that there is some mechanism whereby ATM can control its own expression level. There is also evidence of interaction between ATM and ATR since ATR expression

increases when ATM is inhibited. These phenomena could be explained by the ATM protein blocking access to promoters, deactivating transcription factors or by activating a repressor. In the model presented here, the last has been chosen as the most likely for two reasons. First, ATM is unlikely to block its own promoter since there is no known mechanism for this, and there are no physical interactions currently known between ATM and its possible transcription factor, p63. Second, oscillations are evident in the data (figure 2b) and this kind of behaviour is consistent with some form of negative feedback, e.g. an additional mechanism, possibly a repressor. The alternative explanations for the observed behaviour remain possible, however.

To model the system, an interaction network was first defined which included all of the protein species being investigated as well as the external agents being applied, namely the ATM specific inhibitor, KU55933 (KU), and the DNA-damaging agent, doxorubicin (DOX). Also included is a representative form of the repressor mechanism referred to above. The simplest network, consistent with the data, was chosen and this is shown in figure 3.

Each molecular species is described by a differential equation that characterizes its behaviour as a function of the concentrations of the species it is linked to. For proteins, equations describe how concentrations of each species change with time as a function of the concentrations of RNAs and of the other proteins that it is linked to. For the RNAs, equations describe transcription rates as a sigmoidal function of the concentration of proteins acting as repressors.

With reference to figure 3, the system is assumed to be in a steady state until such time as KU and/or doxorubicin are introduced. In figure 3, ATM-i refers to the state of the ATM protein prior to autophosphorylation

and ATM-a refers to the state after. ATR-i and ATR-a similarly refer to the states of ATR protein. It is proposed that ATM has a, as yet unknown, target, here referred to as REP-i that may be a transcription factor acting as a repressor for both ATM and ATR transcription. REP is presumed to exist in two states, first in an inactivated unphosphorylated state, referred to here as REP-i, and second in a phosphorylated active state, here referred to as REP-a. In this active state, it is assumed to be capable of restricting the transcription of both ATM and ATR. The equilibrium status of REP-i/REP-a (appendix A) is therefore controlled by the levels of ATM protein in both its states. This is assumed to be the case since, during the experiments, doxorubicin alone did not affect mRNA expression and it is, therefore, concluded that the autophosphorylation of ATM does not affect the equilibrium status of the repressor. However, the equilibrium is disturbed when ATM protein activity is restricted by irreversible binding to the ATM-specific inhibitor KU55933. It is assumed that binding occurs in both ATM protein states and this in turn results in a reduction in the ability of the ATM protein to maintain REP in a substantially activated state, a process that is counteracted by an as yet undesignated phosphatase, here referred to as UP1. The deactivation of REP results in a restriction on the ability of REP to oppose transcription of both ATM and ATR, resulting in the increased promoter activity observed in the experiments. It is not known at this stage whether ATR transcription is controlled by the same mechanism as ATM, as assumed here. Also, it is not known whether inhibition of ATR protein would result in similar effects to that modelled here for ATM. Both of these possibilities would have to be tested by further experiment.

4.2. Describing and solving the equations

The mathematical model is detailed in appendix A and comprises 11 coupled nonlinear ordinary differential equations (ODEs) based on the interaction network shown in figure 3. The abbreviations used in the equations are defined in table 2. The initial steady-state condition is defined and represents the reference state for qualitative changes in the system. The system of equations, $dx/dt=f(x, c)$, where c is the parameter set and $x(t)$ is the set of concentrations of the different species at a given time t , was solved for c by fitting the values of $x(t)$ to the values observed in the experiments. Solutions for c were found using an adaptation to the MATLAB ode15s routine combined with a genetic algorithm search process that minimized the sum of the squares of the errors between the model predictions and the experimental dataset. Table 3 gives the values for the parameter set used in the simulations shown in figure 4.

5. RESULTS

The agreement between the model and experimental results displayed in figure 4 shows that the proposed repressor mechanism detailed in figure 3 is consistent with the behaviour of the system observed in the experiments. More significantly, however, the model

can be used to generate a number of testable hypotheses to further probe the proposed feedback. For example, the increased promoter activity in relation to ATM and ATR shown in the experiments and modelled here (figures 2a and 4a) is predicted to be associated with increased protein production in both cases (figure 4b). While the current experiments on which the model is based cannot confirm whether or not protein levels do increase, in a recent paper (Gueven *et al.* 2005), a number of experiments were described where it was possible to identify ATM promoter activity *in vivo* and so it is possible to test this hypothesis. First, using a cloning strategy, Gueven *et al.* (2005) developed a transgenic mouse that carried the transgene expressing the ATM promoter luciferase. This enabled ATM promoter activity to be identified using biophotonics. They also developed an ATM double mutant that carried the same transgene. Using these animals, they firstly compared ATM promoter basal activity in whole animals and then in separate tissues. They found very significant increases in the ATM protein levels ranging from twofold in the case of heart tissue to 17.5-fold in the case of the thymus, thus demonstrating that ATM is not only regulated at the protein level by autophosphorylation but also at the promoter level. This accords to some extent with the model that has been developed here which shows a two- to threefold increase in ATM promoter activity in an ATM double mutant (figure 4c).

Inhibition of ATM is predicted to result in reduced levels of free ATM as might be expected (figure 4d). However, this only applies in the early stages and over time the levels of ATM recover. This would indicate that the introduction of an ATM-specific bound inhibitor is not likely to be an effective way of inhibiting ATM protein activity over a sustained period. The reduction in free ATM, which arises when the ATM inhibitor KU55933 is introduced, is accompanied by a related increase in the level of ATR. If in fact ATM and ATR are linked (Hurley & Bunz 2007), the protein level as well as the transcriptional level would increase ATR which could result in further compensation, for example through Chk1 phosphorylation, for the effect of any ATM-specific inhibition. This possibility is supported by the fact that both ATM and ATR phosphorylate p53 at serine 15 and 37 and, according to the model at least, inhibition of ATM alone will not prevent activation of the DNA correction pathway if ATR is activated in some way. The model, however, does not indicate how this might occur.

According to the model, successful inhibition of ATM can only be carried out in one way, i.e. by inhibiting the undesignated phosphatase, referred to as UP1 in the model (figure 4e). There is a possibility that ATM could be inhibited by blocking ATM transcription. However, according to the model, this would appear to result in increases in ATR which could compensate for ATM losses in some ways depending on the type of DNA damage to which the cell is subjected. Additionally, inhibition of ATR will result in increased ATM/ATR expression. This could be checked in future in an experiment by restricting the ATR protein in some way.

Figure 4. Simulations generated by the model. (a) ATM and ATR mRNA expression levels over time when the ATM inhibitor KU55933 is introduced. Points represent the data for ATM and ATR expression levels that were used to establish the model parameters. (b) Predicted long-term protein behaviour following introduction of the ATM inhibitor KU55933. As ATM falls there is a corresponding rapid rise in ATR, although over time the situation stabilizes with both ATM and ATR returning to their equilibrium position. (c) Predicted ATM promoter activity in an ATM ($-/-$) mutant, showing behaviour similar to that observed *in vitro* (see text). (d) Predicted protein levels following introduction of KU55933, showing the overall increase in the level of ATM to compensate for the introduction of the inhibitor. The level of ATM-i recovers to its pre-inhibition status. (e) Predicted elimination of ATM by inhibition of the undesignated phosphatase UP1. The level of REP-a maximizes hence preventing ATM mRNA expression with the result that ATM protein decays over time.

6. CONCLUSIONS

The successful inhibition of ATM could be a useful tool in cancer therapy, particularly in the case of radiotherapy and certain types of chemotherapy

where prevention of DNA synthesis and DNA damage are involved. However, the development of effective inhibitors must eventually depend on a greater understanding of how ATM and ATR are regulated

at both the transcriptional and protein levels. To this end, we believe that modelling approaches such as the one adopted here will be of assistance. Using the model, we have shown that inhibition of ATM alone does not reduce protein activity, and that the novel feedback in the regulatory network described in figure 3 can explain the observed behaviour. This represents a new testable hypothesis and offers a starting point for future experimentation and model refinement until the representation is sufficiently robust to predict the required intervention.

The p53 pathway responds quantitatively to damage and provides a physiologically relevant model to develop a system's approach to understanding tumour suppression. In our initial analysis of the ATM pathway in ES cells, we have confirmed that ATM is active in damaged ES cells and that this cell can provide a good model to develop quantitative studies on signalling. We have unexpectedly identified a feedback loop that allows ATM to control its own promoter expression.

Mathematical analysis of signal transduction opens the door to developing novel insights into how cells have evolved regulatory networks that interact. This is

affirmed in this study, where we show that the ATM pathway has evolved a sensing system that responds to ATM inhibition by stimulating ATM promoter activity. These data are supported by independent *in vivo* studies, where ATM-inactive mice with an integrated ATM-promoter reporter have enhanced reporter activity in many tissues. The proposed repressor mechanism of ATM control shows how a relatively small number of protein species can interact to create an extremely robust system. This may be typical of how many of the cell's regulatory processes have evolved in ways that result in molecular mechanisms that return the cell to homeostasis when challenged with intervention procedures. If this is in fact the case, then it may be one of the reasons why so many targeted drug regimes find such strong resistance. In the current situation regarding the DDR pathway, the effect of the introduction of a small molecule inhibitor was not anticipated and further mathematical analysis invoked a novel target. It may be that the type of mathematical modelling demonstrated here should be a necessary precursor to identifying potential drug targets.

APPENDIX A. MATHEMATICAL MODEL OF THE ATM/ATR REPRESSOR MECHANISM

The mathematical model uses a system of nonlinear differential equations to describe the concentration changes over time of the molecules and complexes included in the network shown in figure 3.

The abbreviations used in the model are detailed in table 2.

Table 2. Abbreviations.

abbreviation	description	unit
mRNA : ATM	messenger RNA for ATM	mol. cell ⁻¹
ATM-i	dimeric inactive form of ATM	complex cell ⁻¹
ATM-a	monomeric active form of ATM	complex cell ⁻¹
REP-a	active form of ATM transcription repressor	mol. cell ⁻¹
mRNA : ATR	messenger RNA for ATR	mol. cell ⁻¹
ATR-i	inactive form of ATR	mol. cell ⁻¹
ATR-a	active form of ATR	mol. cell ⁻¹
total_ATM	total amount of ATM complex bound and unbound	complex cell ⁻¹
ATM-a : KU	dimer formed by ATM-a and KU55933	complex cell ⁻¹
ATM-i : KU	heterodimer formed by ATM-i and KU55933	complex cell ⁻¹
Dox	doxorubicin	mol. cell ⁻¹
KU	ATM-specific inhibitor KU55933	complex cell ⁻¹

A.1. Differential equations

Transcription of ATM and ATR is described by equations (A 1) and (A 2), where $G1$ is a sigmoidal function that determines the ability of REP-a to restrict the synthesis of mRNA:ATM and mRNA:ATR. $G1$ exists in the open interval $]0, 1[$ and is here defined for $0 \leq \text{REP-a} \leq 4800$. For $\text{REP-a} > 4800$, $G1$ is valued as 0, i.e. no transcription occurs. mRNA degradation is described by a negative exponential. Thus,

$$d/dt[\text{mRNA : ATM}] = G1 \cdot k_{S1} - k_{D1} \cdot [\text{mRNA : ATM}], \quad (\text{A } 1)$$

$$d/dt[\text{mRNA : ATR}] = G1 \cdot k_{S2} - k_{D2} \cdot [\text{mRNA : ATR}], \quad (\text{A } 2)$$

where $G1 = 0.5 \cdot ((-m/((m^2) + 1))^{0.5} + 1)$ where $m = ([\text{REP-a}] - k_{T2})/k_{T1}$.

The rates of formation of the ATM protein dimer and the ATR protein are taken in each case to be proportional to the respective mRNA available and degradation, in each case, is described by a negative exponential. Activation of ATM-i by doxorubicin, and the formation of the ATM-a monomer, is described by Michaelian kinetics and the association of ATM protein with the inhibitor KU is considered to be proportional to the quantities of protein and KU available. UV is included in the model with a view to future experiments involving the activation of ATR-i and is described here by the Michaelian kinetics. Thus,

$$d/dt[\text{ATM-i}] = k_{S3} \cdot [\text{mRNA : ATM}] - k_{D3} \cdot [\text{ATM-i}] - [\text{Dox}] \cdot [\text{ATM-i}] \cdot k_{V5} / (k_{M5} + [\text{ATM-i}]) - [\text{ATM-i}] \cdot [\text{KU}] \cdot k_{A1}, \quad (\text{A } 3)$$

$$d/dt[\text{ATM-a}] = [\text{Dox}] \cdot [\text{ATM-i}] \cdot k_{V5} / (k_{M5} + [\text{ATM-i}]) - ([\text{ATM-a}]^2) \cdot k_{D4} - [\text{KU}] \cdot ([\text{ATM-a}]^2) \cdot k_{A2}, \quad (\text{A } 4)$$

$$d/dt[\text{ATR-i}] = [\text{mRNA : ATR}] \cdot k_{S4} - [\text{ATR-i}] \cdot k_{D7} - k_{C3} \cdot [\text{ATR-i}] \cdot k_{V6} / (k_{M6} + [\text{ATR-i}]), \quad (\text{A } 5)$$

$$d/dt[\text{ATR-a}] = k_{C3} \cdot [\text{ATR-i}] \cdot k_{V6} / (k_{M6} + [\text{ATR-i}]) - k_{D8} \cdot [\text{ATR-a}], \quad (\text{A } 6)$$

$$d/dt[\text{ATM-a : KU}] = [\text{ATM-a}]^2 \cdot [\text{KU}] \cdot k_{A2} - [\text{ATM-a : KU}] \cdot k_{D6}, \quad (\text{A } 7)$$

$$d/dt[\text{ATM-i : KU}] = [\text{ATM-i}] \cdot [\text{KU}] \cdot k_{A1} - [\text{ATM-i : KU}] \cdot k_{D5}, \quad (\text{A } 8)$$

$$d/dt[\text{KU}] = -[\text{ATM-a}]^2 \cdot [\text{KU}] \cdot k_{A2} - [\text{ATM-i}] \cdot [\text{KU}] \cdot k_{A1} - [\text{KU}] \cdot k_{D9}. \quad (\text{A } 9)$$

The inactive form of the repressor, REP-i, is considered to be a substrate of ATM both in its dimeric form, ATM-i, and in its monomeric form, ATM-a. REP-i is also considered to be a substrate of ATR in both its inactive and active forms. The deactivation of REP-a is assumed to be carried out by an, as yet, undesignated phosphatase UP1 defined in equation (A 10) by parameter k_{C2} . Activation and deactivation of REP are described by the Michaelian kinetics. Thus,

$$\begin{aligned} d/dt[\text{REP-a}] = & (k_{C1} - [\text{REP-a}]) \cdot [\text{ATM-i}] \cdot k_{V1} / (k_{M1} + k_{C1} - [\text{REP-a}]) + (k_{C1} - [\text{REP-a}]) \\ & \cdot [\text{ATM-a}] \cdot k_{V2} / (k_{M2} + k_{C1} - [\text{REP-a}]) + (k_{C1} - [\text{REP-a}]) \cdot [\text{ATR-i}] \\ & \cdot k_{V3} / (k_{M3} + k_{C1} - [\text{REP-a}]) + (k_{C1} - [\text{REP-a}]) \cdot [\text{ATR-a}] \cdot k_{V4} / (k_{M4} + k_{C1} - [\text{REP-a}]) \\ & - [\text{REP-a}] \cdot k_{C2} \cdot k_{V7} / (k_{M7} + [\text{REP-a}]). \end{aligned} \quad (\text{A } 10)$$

The drug doxorubicin is assumed to decay in the system according to the equation

$$d/dt[\text{Dox}] = -0.000693147 \cdot [\text{Dox}] \text{ where parameter unit is } \text{min}^{-1}. \quad (\text{A } 11)$$

The total quantity of ATM complex, i.e. in both monomeric and dimeric forms and bound to KU, is given by the equation

$$\text{Total\_ATM} = \text{ATM-i} + \text{ATM-a} + \text{ATM-a : KU} + \text{ATM-i : KU}.$$

A.2. Default parameter set

The parameter values listed in [table 3](#) were determined using a genetic algorithm in combination with an ODE solver for the above set of differential equations, and are to be taken as characterization factors for the model rather than in terms of the literal descriptions of the respective values. The model is qualitative only at this stage and the parameter values arise in part from the initial conditions chosen. The descriptions given indicate how the parameters have been included in the differential equations.

A.3. Initial condition

The initial condition vector for the system of 11 differential equations detailed above and used for the simulations shown in [figure 4](#) is

$$[5 \quad 4 \quad 5000 \quad 0 \quad 4000 \quad 0 \quad 0 \quad 0 \quad 5000 \quad 4000 \quad 1000].$$

Table 3. Default parameter set.

par.	description	value	unit
k_{T1}	sharpness of the sigmoidal function	750	mol.
k_{T2}	point of inflection for sigmoidal function	2000	mol.
k_{S1}	rate constant for production of mRNA : ATM	24.5	mol. cell ⁻¹ min ⁻¹
k_{S2}	rate constant for production of mRNA : ATR	3.2	mol. cell ⁻¹ min ⁻¹
k_{S3}	rate constant for production of ATM-i	0.84	complex mol. ⁻¹ min ⁻¹
k_{S4}	rate constant for production of ATR-i	40	min ⁻¹
k_{D1}	rate constant for degradation of mRNA : ATM	0.155993515	min ⁻¹
k_{D2}	rate constant for degradation of mRNA : ATR	0.02546832897	min ⁻¹
k_{D3}	rate constant for degradation of ATM-i	0.00084	min ⁻¹
k_{D4}	rate constant for degradation of ATM-a	0.00007	cell complex ⁻¹ min ⁻¹
k_{D5}	rate constant for degradation of ATM-i : KU	0.00007	min ⁻¹
k_{D6}	rate constant for degradation of ATM-a : KU	0.01	min ⁻¹
k_{D7}	rate constant for degradation of ATR-i	0.04	min ⁻¹
k_{D8}	rate constant for degradation of ATR-a	0.001	min ⁻¹
k_{D9}	rate constant for degradation of KU	0.001	min ⁻¹
k_{C1}	constant quantity of REP available	5000	mol. cell ⁻¹
k_{C2}	constant quantity of undesigned phosphatase UP1 available	5000	mol. cell ⁻¹
k_{C3}	UV applied (equivalent no. of molecules activating ATR-i)	1000	mol. cell ⁻¹
k_{A1}	rate constant for association of KU55933 and ATM-i	0.000009569	cell complex ⁻¹ min ⁻¹
k_{A2}	rate constant for association of KU55933 and ATM-a	0.000009569	cell ² mol. ⁻² min ⁻¹
k_{V1}	Vmax for phosphorylation and activation of REP-i by ATM-i ^a	0.4	mol. complex ⁻¹ min ⁻¹
k_{V2}	Vmax for phosphorylation and activation of REP-i by ATM-a ^a	0.2	mol. complex ⁻¹ min ⁻¹
k_{V3}	Vmax for phosphorylation and activation of REP-i by ATR-i ^a	0.4	mol. complex ⁻¹ min ⁻¹
k_{V4}	Vmax for phosphorylation and activation of REP-i by ATR-a ^a	0.2	mol. complex ⁻¹ min ⁻¹
k_{V5}	Vmax for activation of ATM-i by doxorubicin ^a	0.002	complex mol. ⁻¹ min ⁻¹
k_{V6}	Vmax for activation of ATR-i by UV ^a	0.02	min ⁻¹
k_{V7}	Vmax for dephosphorylation and deactivation of REP-a by UP1 ^a	0.72	min ⁻¹
k_{M1}	Michaelis constant for phosphorylation and activation of REP-i by ATM-i	100	mol. cell ⁻¹
k_{M2}	Michaelis constant for phosphorylation and activation of REP-i by ATM-a	100	mol. cell ⁻¹
k_{M3}	Michaelis constant for phosphorylation and activation of REP-i by ATR-i	100	mol. cell ⁻¹
k_{M4}	Michaelis constant for phosphorylation and activation of REP-i by ATR-a	100	mol. cell ⁻¹
k_{M5}	Michaelis constant for activation of ATM-i by doxorubicin	500	complex cell ⁻¹
k_{M6}	Michaelis constant for activation of ATR-i by UVC	100	mol. cell ⁻¹
k_{M7}	Michaelis constant for dephosphorylation and deactivation of REP-a by UP1	400	mol. cell ⁻¹

^aIt should be understood that the term Vmax is not used here in the conventional way. In the model, the quantities of enzyme are continually subject to change and the Vmax used here, therefore, is a constant that is independent of the enzyme quantity but which, nevertheless, is used to relate the quantity of enzyme and the quantity of substrate in a velocity formula.

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