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Towards the contribution of the p38MAPK pathway to the dual role of TGFβ in cancer: A boolean model approach

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ABSTRACT

The transforming growth factor-beta (TGF-β) pathway is involved in the regulation of cell growth and differentiation. In normal cells or in the early stages of cancer, this pathway can control proliferation stimuli by inducing cell cycle arrest or apoptosis (through the MAP-kinase protein p38MAPK), while in late stages it seems to act as a tumor promoter. This feature is known as the TGF-β dual role in cancer and it is not completely explained. This seems to arise through the accumulation of mutations in cancer development that affect the normal function of these pathways. In this work we propose a Boolean model of the crosstalk between the TGF-β, p38 MAPK and cell cycle checkpoint pathways which qualitatively describes this dual behavior. The model shows that for the wild type case, TGF-β acts as tumor supressor by inducing cell cycle arrest or apoptosis, as expected. However, the loss of function (LoF) of its two signaling proteins: SMAD2 and SMAD3 has immortalization effects due to the activation of the PI3K/AKT pathway that contributes to inhibit apoptosis. *In silico* mutations of the model elements were compared with cell phenotypes in experiments presenting agreement. In addition, we performed a series of double gene perturbations (that simulate random deleterious mutations) to determine the main regulators of the network. The results suggest that SMAD2/3 and p38MAPK are key players in processing the network input. In addition, when the LoF of SMAD2/3 is combined with the LoF of p38MAPK and p53, cell cycle arrest is completely abrogated. In conclusion, the model allows to visualize, through *in silico* mutations, the dual role of TGF-β: for the wild-type case TGF-β is able to block proliferation, however deleterious mutations can impair cell cycle arrest promoting cellular proliferation.

1. Introduction

The receptors of TGFβ (transforming growth factor-beta) and its signaling pathway perform many functions in the intracellular medium: they are present in activation and inhibition processes of a wide range of proteins, are widely expressed in tissues and play an important role in human diseases [1].

The canonical signaling pathway of TGFβ is initiated by both SMAD2 and SMAD3 (SMAD family members 2 and 3), which are activated through phosphorylation by the type I receptor of TGFβ (Tβ-RI). They form heterotrimeric complexes with SMAD4 (SMAD family member 4) that are translocated to the nucleus [2,3] acting as transcriptional activators. In the non-canonical pathway, where the TGFβ signaling occurs independently of the SMADs proteins, the activated receptor complexes transmit the signal through other factors, e.g. TNF (tumor necrosis factor), p38MAPK (mitogen-activated protein kinase), PI3K (phosphatidylinositol 3′-kinase), AKT (protein kinase B), and others [4–6]. In the case of p38MAPK activation [7,8] by TGFβ, this happens through GADD45β activation [9].

TGFβ signaling can act in a dual way in cells, as a tumor suppressor in normal cells in early phases of carcinogenesis and as a tumor promoter in advanced tumor stage [10,11]. This dual behavior is known as the TGFβ paradox [12], which is not fully understood yet [13,14]. As far as we know, no theoretical model was proposed to explain this paradox. As a tumor suppressor, TGFβ acts by inhibiting the expression of CDKs (cyclin-dependent kinases that regulate cell cycle) through CDK inhibitors such as p15INK4B (cyclin dependent kinase inhibitor 2B), p21 (cyclin dependent kinase inhibitor 1A) and p16INK4a (cyclin dependent kinase inhibitor 2A) [15,16]. However, in tumor cells the TGFβ pathway loses its anti-proliferative response and becomes an oncogenic factor for not performing its function [13]. One of the TGFβ tumor-suppressor mechanisms involves the induction of apoptosis by p38MAPK observed in several murine cells [17,18]. Consequently, if cells are treated with a p38MAPK inhibitor, apoptosis induction by

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Table 1
Short description of the molecular components of the model.

Node	Description
TGFbeta	Transforming growth factor-beta
ATM_ATR	Ataxia telangiectasia mutated protein and Ataxia telangiectasia and Rad3 related protein
CHEK1_CHEK2	Checkpoint kinase 1 protein and Checkpoint kinase 2 protein
SMAD2/3	SMAD family member 2 and 3
GADD45beta	Growth arrest and DNA damage inducible beta
PTEN	Tumor supressor phosphatase and tensin homolog
p15INK4b	Cyclin dependent kinase inhibitor 2B (from CDKN2B locus)
p38MAPK	Mitogen-activated protein kinase 14
P13K_AKT	Phosphoinositide 3-kinase and Serine/threonine kinase (also known as protein kinase B or PKB)
p16INK4a	Cyclin depedent kinase inhibitor 2A (from CDKN2A locus)
p14ARF	Alternate reading frame (ARF) protein (from CDKN2A locus)
Mdm2	Murine double minute 2 also known as E3 ubiquitin-protein ligase Mdm2
p53	Tumor protein p53
CdkCyclin	Proteins complexes that promote cell cycle
Cdc25ABC	Cell division Cycle 25 protein Family
p21	Cyclin-dependent kinase inhibitor 1A protein
BCL2	B-cell lymphoma 2, apoptosis regulator
pRB	Retinoblastoma 1 protein
E2F	E2F transcription factor family of proteins (E2F1, E2F2,E2F3)

TGFβ will be blocked. A list of molecules involved in the process is listed in Table 1.

The PI3K/AKT pathway (see Table 1), also regulated by TGFβ, seems to induce tumor suppression in non-malignant cells. In malignant cells this pathway acts as a tumor promoter by inhibiting apoptosis [19]. Studies demonstrate that the TGFβ tumor-suppression function is halted by the accumulation of oncogenic mutations affecting the normal function of the pathway. Among such mutations, there are those that include the activation of the PI3K/AKT signaling, antagonizing the anti-proliferative effects of TGFβ [19,20].

The aim of the present study is to propose a Boolean model of the crosstalk between the TGFβ signaling and the regulation of the cellular checkpoint by DNA damage, taking into account the effect of mutations that lead TGFβ to present its dual behavior. We resort to the boolean approach due to the lack of detailed quantitative information regarding the biochemical reactions that describe the process.

2. Methods

A Boolean model of a regulatory network (G, K) is defined by a set of discrete regulatory components $G = (g_1, g_2, ...g_n)$, where each g_i can take values 0 or 1. The components may represent concentrations of molecules or biological states, biological processes or phenotypes. The input components are not regulated and represent external conditions in the environment.

A vector $g = [g_1, g_2, ...g_n]$ represents a state of the model in the state space S. A logical function K_i defines the values of each g_i in terms of activating or inhibiting interactions that connect the g_i, characterizing a directed graph. The transition function K is defined as $K(g) = (K_1(g), ...K_n(g))$.

The most common updating schemes of Boolean models are the completely deterministic synchronous and the potentially non-deterministic asynchronous method. As molecular networks have stochastic features we use the asynchronous scheme.

The approach allows the simulation of perturbations, which are experimentally known as loss-of-function (LoF) or gain-of-function (GoF). Those perturbations consist on fixing a variable on its lower or higher levels, respectively. More details about logical modeling methods such as Boolean and non-Boolean can be found in Ref. [21].

A variety of modeling frameworks were designed for logical modeling. In this work we use the GINsim 2.9.5 (Gene Interaction Network Simulation) tool. The model used in this work is available in the Supplementary materials as S1 file. GINsim was developed in Java and consists of a simulator of qualitative models of genetic regulatory networks based on the logical (discrete) formalism [21–23]. Among many functionalities, GINsim allows to determine the stable attractors for all possible initial conditions. It can be downloaded from: compbio.igc.gulbenkian.pt/nmd/node/82.

The interaction network in Fig. 1 considers TGFβ and the DNA damage signaling (which can be a consequence of different factors, such as: ionizing radiation or oxidative stress). Cell fate decisions happen at the cellular checkpoint and its activation in the model can lead to different cellular phenotypes: proliferation, cell cycle arrest or apoptosis. The network contemplates most of the interactions reported in the literature among the proteins comprising the pathways.

3. Results and discussion

3.1. Molecular mechanisms

The molecular mechanisms shown in Fig. 1 can be summarized as follows: after DNA damage, Ataxia-telangiectasia mutated (ATM) and Ataxia-telangiectasia Rad3-related (ATR) are activated by phosphorylation. CHEK1 (checkpoint kinase 1 protein) and CHEK2 (checkpoint kinase 2 protein) inhibit Cdc25ABC (cell division cycle protein family) initiating the cycle arrest, since they are required to induce the cyclin-dependent kinases (CdkCyclin) complexes that promote cell cycle. The latter, when deactivated lead to the cycle arrest.

In addition to its role in the activation of SMADs proteins, TGFβ participates in the activation of GADD45β (growth arrest and DNA damage inducible beta) [18] that activates p38MAPK, which can also be activated indirectly by ATM and ATR [24,25]. Amongst several functions performed by p38MAPK, an important one is the ability to induce the apoptotic state in cells through tumor suppressors such as p53 and p14ARF. In this model activation of apoptosis is a consequence of p53 activation which is experimentally observed [26].

The network contemplates both the canonical and non-canonical pathways of TGFβ signaling [3,5]. These pathways can activate cell cycle inhibitors evincing its antiproliferative function [2]. In the opposite way, they can activate PTEN (phosphatase and tensin homolog deleted on chromosome 10), which makes a negative control of the PI3/AKT pathway that stimulates cellular growth and proliferation by inhibiting apoptosis in response to extracellular signals [27]. In the network, this function is represented by the apoptosis inhibitor BCL2 (B-cell lymphoma 2) [28,29].

3.2. The wild-type case

The model results for the wild-type case can be seen in Fig. 2. Table 2 presents the logical rules controlling the interactions among network elements. There are 4 different input combinations (2 for Damage × 2 for TGFbeta), consequently we should expect 4 different dynamical states.

The model predicts 3 stable states and one cyclic attractor (see below). As expected, there is no checkpoint activation in the absence of DNA damage [30], which means that the checkpoint arrest was not activated and the cell cycle regulatory proteins are induced: CdkCyclin, Cdc25ABC and E2F. In the presence of DNA damage and/or expression of TGFβ, the checkpoints and p53 are activated, the latter induces apoptosis in the model. Cycle arrest is activated by the CDK inhibitors: p21 and p15INK4b (see Table 1). p21 is activated by ATM_ATR [24] due to DNA damage or by SMAD2/3 [25] through TGFβ. P15INK4b is induced by p38MAPK and TGFβ, respectively. However, when damage is present in the absence of TGFβ, we find a cyclic attractor induced by the negative circuit p53-Mdm2, which can also be associated to a transient cycle arrest as shown in experiments [31].

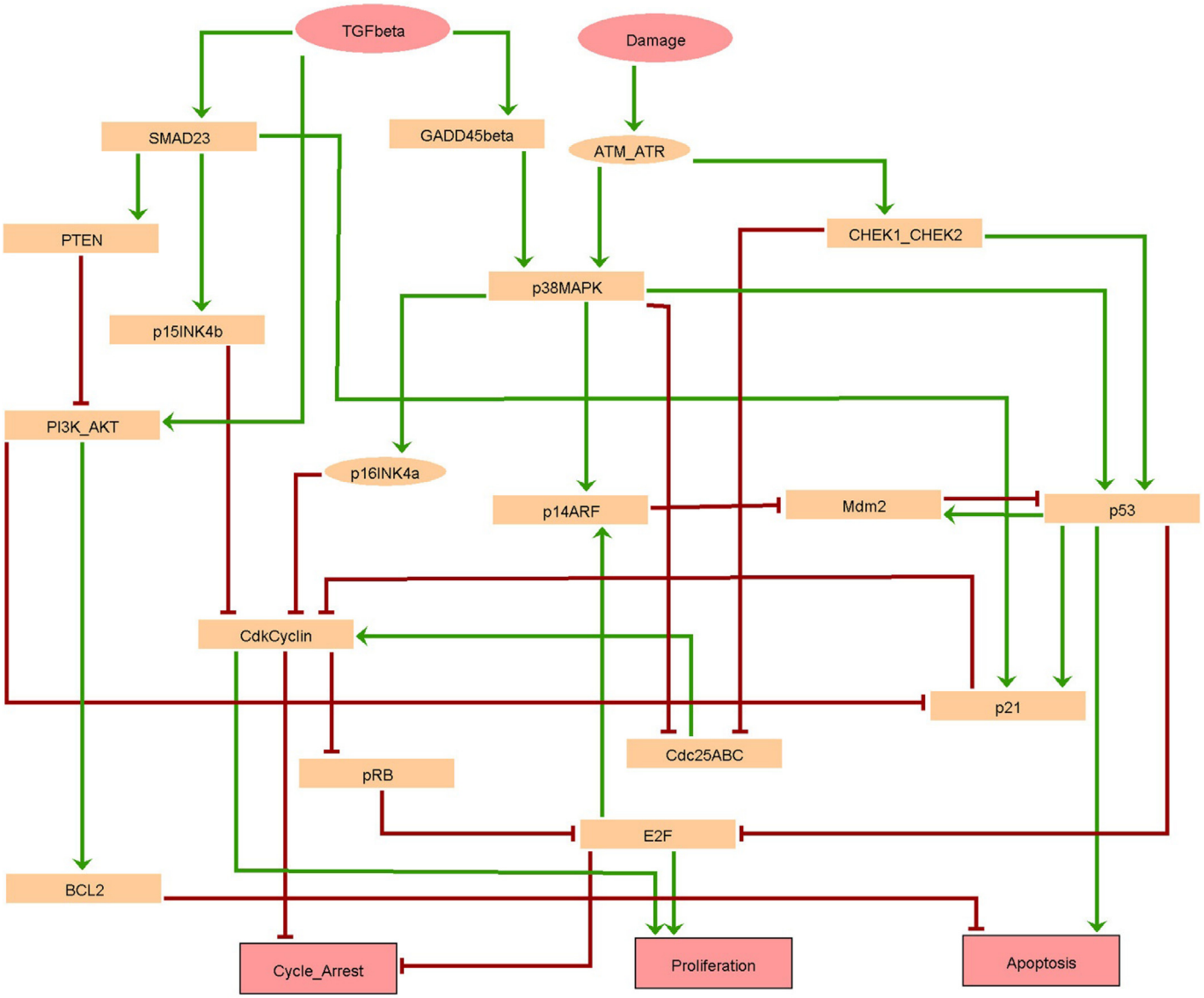

Fig. 1. Interaction network of the model. The model inputs consider damage to the DNA and the TGFβ signaling equal to zero where there is no damage to DNA and/or no TGFβ signaling and equal to one when there is DNA damage and/or TGFβ signaling. The genotoxic stimuli are the inputs in the upper part of the network (nodes represented by pink color and elliptical shape), and these signals are transmitted and processed in the intermediate part of the network (nodes represented by yellow color and rectangular shape). The signals are connected to the output proteins in the lower part of the network. In the model, these signals are represented by the following phenotypes: proliferation, cellular cycle arrest and apoptosis, which are represented by pink color and rectangular shape. Green lines depict activation interactions and the red lines depict inhibitions.

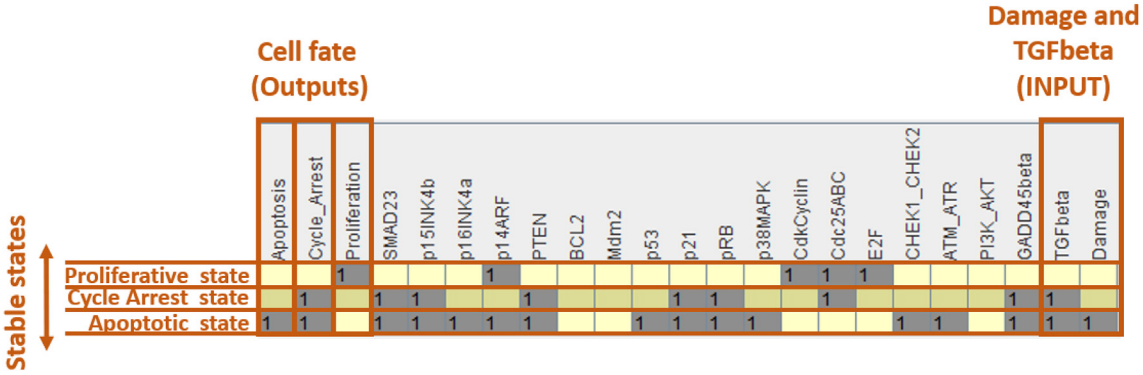

Fig. 2. Network states of the wild-type case results considering 3 possible input combinations of TGFβ and DNA damage, each one leading to one possible cellular fate. Empty boxes correspond to variable state value zero. When damage is present in the absence of TGFβ, we find a complex cyclic attractor induced by the negative circuit p53-Mdm2 (see text).

Table 2
Logical rules used to control the network depicted in Fig. 1. The logical operators AND, OR and NOT are used to determine the rules of each node in the proposed network.

	Node	Rule	Interpretation
Inputs	Damage	0	No damage
		1	DNA damage
	TGFbeta	0	No signaling
		1	TGFβ signaling
	GADD45beta	1: TGFbeta = 1	
	ATM_ATR	1: Damage = 1	
	CHEK1_CHEK2	1: ATM_ATR = 1	p53 accmulation leading to apoptosis
	p53	1: NOT (Mdm2 = 1) AND (p38MAPK = 1 OR CHEK1_CHEK2 = 1)	
	SMAD2/3	1: TGFbeta = 1	
	PTEN	1: SMAD2/3 = 1	
	p15INK4b	1: SMAD2/3 = 1	p15INK4b accumulation leading to cell cycle arrest
	p14ARF	1: E2F = 1 OR p38MAPK = 1	
	PI3K_AKT	1: TGFbeta = 1 AND NOT PTEN = 1	
	BCL2	1: PI3K_AKT = 1	
	Mdm2	1: p53 = 1 AND NOT p14ARF = 1	
	p38MAPK	1:GADD45beta = 1 AND ATM_ATR = 1	p38MAPK activation (leading to cycle arrest or apoptosis)
	p16INK4a	1: p38MAPK = 1	
	p21	1: (p53 = 1 OR SMAD2/3 = 1) AND NOT PI3K_AKT = 1	
	pRB	1: NOT CdkCyclin = 1	Dephosphorylated pRB bound to E2F
	E2F	1: NOT (pRB = 1) AND NOT (p53 = 1)	
	CdkCyclin	1: (Cdc25ABC = 1) AND NOT (p21 = 1) AND NOT (p16INK4a = 1) AND NOT (p15INK4b = 1)	Cell cycle progression
	Cdc25ABC	1:(CHEK1_CHEK2 = 1) AND NOT (p38MAPK = 1)	
Outputs	Cell Cycle Arrest	1: NOT (CdkCyclin = 1) OR NOT (E2F1 = 1)	
	Proliferation	1: (CdkCyclin = 1) AND E2F1 = 1	
	Apoptosis	1: p53 = 1 AND NOT BCL2 = 1	

3.3. Model validation

In this section we show that our model is consistent with several experimental works related to our study as presented in Table 3. We explain briefly these studies in what follows. Observe that when the values of the inputs are constrained, the number of stable states decrease, accordingly.

Our model agrees with studies [32,33] showing that, in the absence of DNA damage, TGFβ induces growth arrest in melanoma and skin cells in a p53-independent way. To test that we fixed TGFbeta at its highest level in the model. The resulting stable states are shown in supplementary material as S2 file, where we can see that TGFβ can induce cycle arrest without activation of p53.

The role of TGFβ signaling was investigated in combination with the chemotherapeutic drug doxorubicin that produces DNA damage in osteosarcoma and kidney cells [34]. Doxorubicin induces TGFβ and p53 producing an apoptotic phenotype. We tested this behavior in our model using the combined perturbation: TGFbeta E1 + Damage E1, the model result in this case is a single apoptotic stable state.

In human kidney and murine skin cells [35,36] the induction of SMAD2 and SMAD3 favors cycle arrest, while their inhibition favors proliferation consistent with our model results using the perturbation SMAD23 E1. In the same experiment the authors observed that when the induction of p53 was accompanied by the induction of SMAD23, they yielded an apoptotic phenotype [37] also found in the model under the perturbation p53 E1 + SMAD2/3 E1.

In liver cells SMAD2 and SMAD3 inhibition combined with PI3k/AKT induction [19] favors proliferation, also observed in our model with the perturbation SMAD23 knockout (KO) + PI3K/AKT E1.

In several human and murine cell types [17,37–39] the induction of p38MAPK generates an apoptotic phenotype, while its inhibition favors cycle arrest in the presence of TGFβ, as observed in the model under the perturbation p38MAPK E1.

Finally, when TGFβ is induced in p53 deficient melanoma and lung cells [40] they arrest the cell cycle, as predicted by the model with the perturbation TGFbeta E1 + p53 KO.

3.4. TGFβ dual behavior

In the following discussion we analyze the effect of a few combined protein mutations, in order to check their effect on the generated phenotypes. We want to establish how a small number of mutations in these pathways can modify the TGFβ role in the regulation of cell cycle to help elucidate its dual behavior.

According to a study proposed in 2017 [20], the tumor-suppression effect of TGFβ is signaled by SMAD3. In this study the authors observed

Table 3
The model agrees with experimental results. E1 represents GoF and KO represents LoF of the corresponding gene.

Stimulus/Perturbations	Response/Phenotype	Cancer type	References
TGFβ E1	Induce Arrest at G1/S	human melanoma; human Skin	[32,33]
TGFβ E1 + Damage E1	Induced Apoptosis	human kidney and breast	[34]
SMAD2/3 E1	Induced Arrest at G1/S	human kidney; mouse skin	[34,35]
SMAD2/3 KO	Induced Proliferation	human brain	[36]
SMAD2/3 KO + P13K_AKT E1	Induced Proliferation	liver	[19]
p38MAPK E1	Induced Apoptosis	mouse mammary gland epithelial cell, rat liver, mouse embryonic fibroblast cells	[17,37,38]
p38MAPK KO	Induced Cycle Arrest	human breast	[39]
TGFβ E1 + p53 KO	Induced Cycle Arrest	Melanoma; lung	[40]
p53 E1 + SMAD2/3 E1	Induced Apoptosis	human kidney and breast	[35]

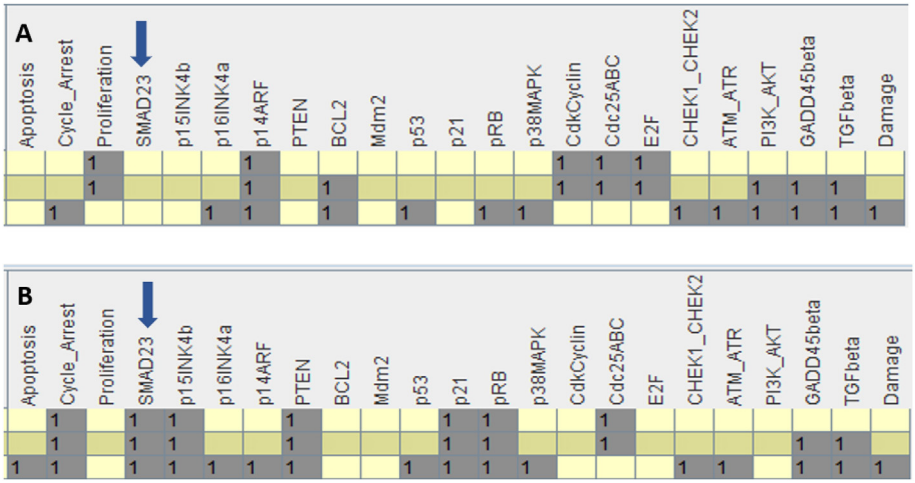

Fig. 3. Stable states of the model for the cases with one mutation only, knockout of SMAD23 or in its active state. **A-** Mutation considering SMAD23 knockout. **B-** Mutation considering SMAD23 overexpressed.

that in SMAD3-deficient cells the PI3k/AKT pathway signaling correlates with an increase in cellular proliferation and apoptosis resistance. In our model we tested this effect and the results are shown in Fig. 3A and B. It is clear that the knockout of SMAD23 leads to the activation of BCL2, since PTEN, the inhibitor of PI3k/AKT, is not induced. In the case of constant activation of SMAD23, we observe an anti-proliferative effect promoted by p15INK4b (Fig. 3B). We also observe that the activation of both p38MAPK and SMAD2/3 are required to induce apoptosis, as reported in several works [9,17,18,28].

In another experimental study [41] the authors observed in tumor cells the dual effect of TGFβ. They describe that a late cancer stage TGFβ signature is characterized by high amounts of this protein and by the reduced signaling of SMAD3, corresponding to the invasive phenotype and increased tumor recurrence. This case corresponds to the second line of Fig. 3A, i.e. SMAD23 KO and TGFbeta E1 whose only result is proliferation in the absence of DNA damage.

The results above stress the important role of SMAD2/3 and TGFβ in cell cycle regulation. However, we still have to investigate the contribution of p38MAPK and p53 to this process. The latter is known to be mutated in 50% of all cancers [26]. We found that by adding only the knockout of p38MAPK to the perturbation SMAD23 KO, is not sufficient to obtain a single proliferative phenotype, as the p53-Mdm2 circuit becomes functional in the presence of DNA damage (Fig. 4A). However, when we add p53 KO knockout to p38MAPK + SMAD23 KO (Fig. 4), the negative circuit p53-Mdm2 becomes not functional and the cyclic attractor disappears, as a result the only stable state is proliferation even in the presence of DNA damage. This shows that the simultaneous disfunction of SMAD2/3, p38MAPK and p53 pathway is sufficient for a proliferative phenotype.

3.5. Extended Mutation Analysis

In order to get further insight on the role of perturbations on the model dynamics, we performed several double node perturbations. Inputs of the network were not included in the analysis. Here we assume that deleterious mutations are mainly responsible for the transformation of the cell. However, since testing all possible combinations of perturbations is unfeasible, we studied only the subset of 154 double perturbations that abrogate apoptosis, as this is an important feature of cancer. Then, for this set of mutations we determined which of those contribute to or increase proliferation (a set of 14, see supplementary material S3 file). Among this set of 14, we ranked the nodes that appear more frequently inhibited (OFF) to represent the targets of deleterious mutations that are needed for apoptosis abrogation and proliferation increase. This set also includes some trivial mutations, such as SMAD23 KO that produces SMAD23 OFF. The results are presented in Table 4 of the paper. Assuming that the main regulators are the elements upstream in a regulatory pathway, examining the top 10 nodes of the rank we

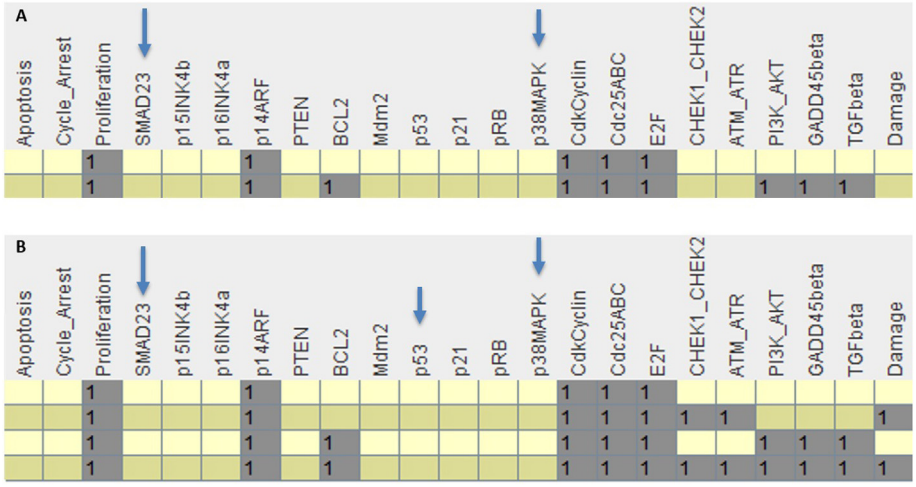

Fig. 4. Stable states of the model with the combined mutation of proteins p53, p38MAPK and SMAD23. **A-** Stable states for the combined mutation of SMAD23 and p38MAPK, both knocked-out. **B-** Stable states generated when taking into account p53 protein knockout together with p38MAPK and SMAD23 knockouts.

Table 4
Rank of most frequently inhibited nodes in mutations that abrogate apoptosis and increase proliferation (see S3 file).

# Inhibitions	Node
41	PTEN, p15INK4b, p21
39	Mdm2, SMAD23
37	p53
36	p16INK4a
35	p38MAPK, Cdc25ABC
34	CHEK1_CHEK2

observe that the most upstream elements are SMAD23, p38MAPK and CHEK1_CHEK2. Although CHEK1_CHEK2 can contribute to the process, its influence is restricted to the DNA damage pathway, while SMAD23 and p38MAPK exert the important crosstalk between both pathways (see Fig. 1). These results are consistent with those in Fig. 3. We think they support our claim that p38MAPK and SMAD2/3 are key elements to explain the TGFbeta paradox.

4. Conclusion

In this work we introduced a Boolean model of the crosstalk between the TGF-β pathway and the cell cycle checkpoints intended to describe the TGF-β dual role in cancer. Our *in silico* results suggest that SMAD2/3 and p38MAPK are the main regulators of the influence of TGF-β on cell fate determination. Based on model perturbations we can summarize the dual behavior of TGF-β: the wild-type SMAD2/3 and p38MAPK pathways regulated by TGF-β can suppress proliferation, however deleterious mutations in these pathways and in the DNA damage response pathway impair TGF-β function promoting cellular proliferation.

Conflicts of interest

There are no conflicts to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.compbiomed.2018.11.025.

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