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A Boolean network model of the FA/BRCA pathway

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ABSTRACT

Motivation: Fanconi anemia (FA) is a chromosomal instability syndrome originated by inherited mutations that impair the Fanconi Anemia/Breast Cancer (FA/BRCA) pathway, which is committed to the repair of DNA interstrand cross-links (ICLs). The disease displays increased spontaneous chromosomal aberrations and hypersensitivity to agents that create DNA interstrand cross-links. In spite of DNA damage, FA/BRCA-deficient cells are able to progress throughout the cell cycle, probably due to the activity of alternative DNA repair pathways, or due to defects in the checkpoints that monitor DNA integrity.

Results: We propose a Boolean network model of the FA/BRCA pathway, Checkpoint proteins and some alternative DNA repair pathways. To our knowledge, this is the largest network model incorporating a DNA repair pathway. Our model is able to simulate the ICL repair process mediated by the FA/BRCA pathway, the activation of Checkpoint proteins observed by recurrent DNA damage, as well as the repair of DNA double-strand breaks and DNA adducts. We generated a series of simulations for mutants, some of which have never been reported and thus constitute predictions about the function of the FA/BRCA pathway. Finally, our model suggests alternative DNA repair pathways that become active whenever the FA/BRCA pathway is defective.

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1 INTRODUCTION

Fanconi anemia (FA) is a chromosomal instability syndrome characterized by bone marrow failure, congenital abnormalities and an increased risk to develop cancer (D'Andrea, 2010; de Winter and Joenje, 2009). Cells of patients with FA display increased chromosomal aberrations, and are hypersensitive to agents that create DNA interstrand cross-links (ICLs) (Auerbach, 2009). The reported prevalence of FA ranges from 1 to 5 cases per one million persons, while the heterozygous carrier frequency is about one case per 300 persons (although the true frequency is probably higher), with a median age at diagnosis of 6.5–8 years (D'Andrea, 2010). FA represents a suitable model to study human DNA repair mechanisms and it is gaining relevance because heterozygous carriers have a high risk of developing malignancies such as breast cancer (Neveling *et al.*, 2009).

FA is a genetically heterogeneous disease, caused by mutations in at least one of the 15 distinct genes, namely: FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCJ/BRIP1/BACH1, FANCL, FANCM, FANCN/PALB2, FANCO/RAD51C and FANCP/SLX4. These genes are involved in the so-called Fanconi Anemia/Breast Cancer (FA/BRCA) pathway, dedicated to the repair of DNA ICLs (de Winter and Joenje, 2009; Kim *et al.*, 2011; Vaz, 2010). Currently, there is no consensus regarding the precise nature of the FA/BRCA pathway, or the network of interactions established with other genes/proteins involved in FA. We hereby propose the regulatory network established among 20 protein complexes that include the aforementioned proteins of the FA/BRCA pathway. Moreover, this network includes nodes to describe the three types of DNA damage commonly observed in FA. We thereafter implement the FA/BRCA network as a discrete dynamical system and study its behavior.

The modeling of regulatory networks of biological interest as discrete dynamical systems is a well-established methodology. Boolean networks, the simplest form of discrete dynamical systems, have been used to model a wide variety of processes such as the genetic control of floral morphogenesis in *Arabidopsis thaliana* (Mendoza *et al.*, 1999), the differentiation process of T-helper lymphocytes (Mendoza, 2006), the control of the mammalian cell cycle (Fauré *et al.*, 2006) and the surviving process of malignant cells in large granular lymphocyte leukemia (Zhang *et al.*, 2009a), to name a few examples. This type of approach is widely used to study the dynamical behaviour of regulatory networks because of its straightforwardness, robustness and compatibility with published qualitative data (Chaves *et al.*, 2005; Christensen *et al.*, 2007; Davidich and Bornholdt, 2008; Mendoza and Pardo, 2010; Wu *et al.*, 2009).

There are some mathematical models that describe DNA repair, see, for example, Crooke and Parl (2010). However, to our knowledge, the model presented here is the first dynamical system proposed specifically for the FA/BRCA DNA repair pathway. This model is able to reproduce the ICLs repair process mediated by the FA/BRCA pathway, and the repair of double-strand breaks (DSBs) and DNA adducts (ADDs). In order to obtain a dynamical behavior that reproduces the experimental data, we propose the

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existence of some interactions not reported in the experimental literature, and therefore they constitute predictions of our model. Specifically, we include the generation of DSBs during ICLs repair by alternative DNA endonucleases, even in FA/BRCA mutants. Second, we propose a rule that enables MRN to act in the absence of FA/BRCA and NHEJ pathways during an ICL-generated DSB repair process. Third, we propose that USP1 is regulated by the activity of FANCM. And fourth, we propose that the Checkpoint Recovery process (CHKREC) negatively regulates 17 nodes of the FA/BRCA pathway.

2 METHODS

2.1 Reconstruction of FA/BRCA network

The experimental literature on FA was thoroughly analyzed, so as to reconstruct the connectivity among key components of the FA/BRCA pathway. Briefly, a DNA ICL is able to arrest the DNA synthesis process; the FANCM/FAAP24 protein complex (FANCM) recognizes the damage and recruits the FA core (FAcore) complex (Gari *et al.*, 2008). Then, the FAcore complex monoubiquitinates the FANCD2I complex (Kim *et al.*, 2011), which in turn recruits endonucleases such as FAN1 (MacKay *et al.*, 2010; Smogorzewska *et al.*, 2010), XPF/ERCC1 (Bhagwat *et al.*, 2009) and MUS81/EME1 (MUS81) (Hanada *et al.*, 2006) to generate a DSB and a DNA adduct. The FA/BRCA proteins funnel the DSB to be repaired by the homologous recombination repair (HRR) pathway mediated by Breast Cancer 1 (BRCA1) (Bekker-Jensen *et al.*, 2006), FANCN (Xia *et al.*, 2007), FANCD1 (San Filippo *et al.*, 2006), FANCJ (Gong *et al.*, 2010) and the RAD51 complex (San Filippo *et al.*, 2006). The blockage of the DNA replication by the presence of an ADD can be bypassed with the help of the proliferation cell nuclear antigen (PCNA) and translesion synthesis (TLS) polymerases and then repaired by the nucleotide excision repair (NER) proteins (Chang and Cimprich, 2009; Garg *et al.*, 2005). As a final step, the ubiquitin-specific protease 1 (USP1) protein inactivates the FAcore complex and PCNA proteins by deubiquitination, turning the signaling process off (Huang and D'Andrea, 2006; Niimi *et al.*, 2008). Finally, it is important to note that the FA/BRCA pathway establishes a cross talk with the ataxia telangiectasia and Rad3-related (ATR) Checkpoint protein and its effector kinase, Checkpoint kinase 1 (CHK1), which are required for the activation of the FA/BRCA pathway and cell cycle arrest. The reader may find a more detailed explanation of the whole process in Supplementary Material.

Our proposed network for the FA/BRCA pathway, shown in Figure 1, Supplementary Figure S1 and Supplementary Material (FA_BRCA_network.pdf), incorporates DNA damage recognition proteins, DNA damage Checkpoint proteins and DNA repair effector proteins. At least 51, proteins were found to be relevant for the reconstruction of the network; however, some of these proteins act as functional complexes, and therefore they were collapsed into single functional nodes to facilitate the dynamical analysis. Furthermore, three nodes representing different types of DNA damage were included into the model, namely ICL, DSB and ADD. As a result, the network is made up of 28 nodes and 122 regulatory interactions, 80 of them positive and 42 negative (Fig. 1). The regulatory rules of this network is presented in Table 1. A detailed description of the information used to reconstruct the connectivity and the logical rules of such network can be found as Supplementary Material.

Not all interactions of the FA/BRCA network, as shown in Figure 1, have been reported in the experimental literature or appear in specialized databases. Indeed, while our regulatory network includes some of the elements of the FA/BRCA pathway as included in the KEGG database, our network incorporates more nodes, specifically ATM, CHK1 CHK2 and some pathways like the non-homologous end joining. Because of this characteristic, our network is unique in incorporating a cross talk with alternative DNA repair pathways.

Of the 122 regulatory interactions present in our model, 16 were included so as to obtain a dynamical behavior that reproduce the experimental data. These proposed regulatory interactions are divided into four groups. First, we included a positive interaction of XPF over DSB to ensure the generation of DSBs during ICL repair by the activity of alternative DNA endonucleases even in FA/BRCA-deficient cells. Second, we incorporated negative regulations of MRN from FANCD2I, KU, RAD51 and CHKREC, so that during the ICL-generated DSB repair process the MRN exonuclease acts only in the absence of FA/BRCA and NHEJ pathways. Third, we propose that USP1 is under positive control from FANCD1N, and under negative control from FANCM, to indicate that the USP1 deubiquitinating enzyme is directly or indirectly regulated by the presence of FANCD1/BRCA2, FANCN and FANCM. And fourth, we propose that CHKREC negatively regulates 11 nodes (see Fig. 1, Table 1 and Supplementary Fig. S2). The selection of these 16 unreported interactions was based exclusively in the recovery of the known attractors for the wild type and the mutants. This was the minimal set of new interactions that we found consistent with the experimental data.

2.2 The FA/BRCA network as a discrete dynamical system

We modeled the reconstructed FA/BRCA pathway as a Boolean network, where each node is described by a variable x that takes the value of 0 or 1 to represent its inactive or active state of activation, respectively. The activation state of the whole network is then represented by a vector with the set of Boolean variables $x_1, x_2, ..., x_n$, where x_i is the state of activation of the i-th node. The state of activation of each node changes in discrete time steps according to $x_i(t+1) = F_i(x_1(t), x_2(t), ..., x_n(t))$, where $x_1(t), x_2(t), ..., x_n(t)$ is the state of the regulators of node x_i at time t, and F_i is a Boolean function, also called a logical rule, determining the state of node x_i at the next time step. The specific Boolean functions for each node are shown in Table 1. These equations describe a deterministic dynamical system, with a total of $2^{28} = 268435456$ possible activation states. We exhaustively tested the dynamical behavior of the FA/BRCA network by starting the simulations from all possible initial conditions with BoolNet (Müssel *et al.*, 2010) under synchronous updating, until the system reached a stationary pattern of activation, also referred to as an attractor. Finally, to simulate loss- and gain-of-function mutants, the corresponding variable was fixed to 0 or 1, respectively. The file containing the FA/BRCA network in the BoolNet format is available as the Supplementary Material (FA_for_BoolNet.txt).

Given the large number of possible activation states of the FA/BRCA network, it is computationally unfeasible to fully analyze its dynamical behavior using an asynchronous updating. However, it is instructive to compare certain key results under both synchronous and asynchronous updating. Therefore, the attractors of the wild type, FAcore null mutant, and FANCD1N null mutant models, were obtained by using BoolNet with asynchronous updating starting from 10 000 random initial states.

3 RESULTS AND DISCUSSION

A great proportion of FA cells in culture die when exposed to ICL inducing agents, such as mitomycin C (MMC) or diepoxybutane (DEB), due to severe DNA repair deficiencies. However, some cells survive exhibiting unrepaired DSBs and cytogenetic evidence of erroneous DNA repair such as radial exchange figures and a normal rate of sister chromatid exchanges (SCEs) (Latt *et al.*, 1975). Our aim was to understand the mechanism by which FA cells use alternative DNA repair pathways when exposed to ICL-inducing agents. To accomplish this, we developed a Boolean network model of the FA/BRCA pathway, which incorporates the known interactions of FA proteins with other pathways that participate in the preservation of genome stability. The network has three nodes that can be used as input, so as to model different types of DNA damage signals that

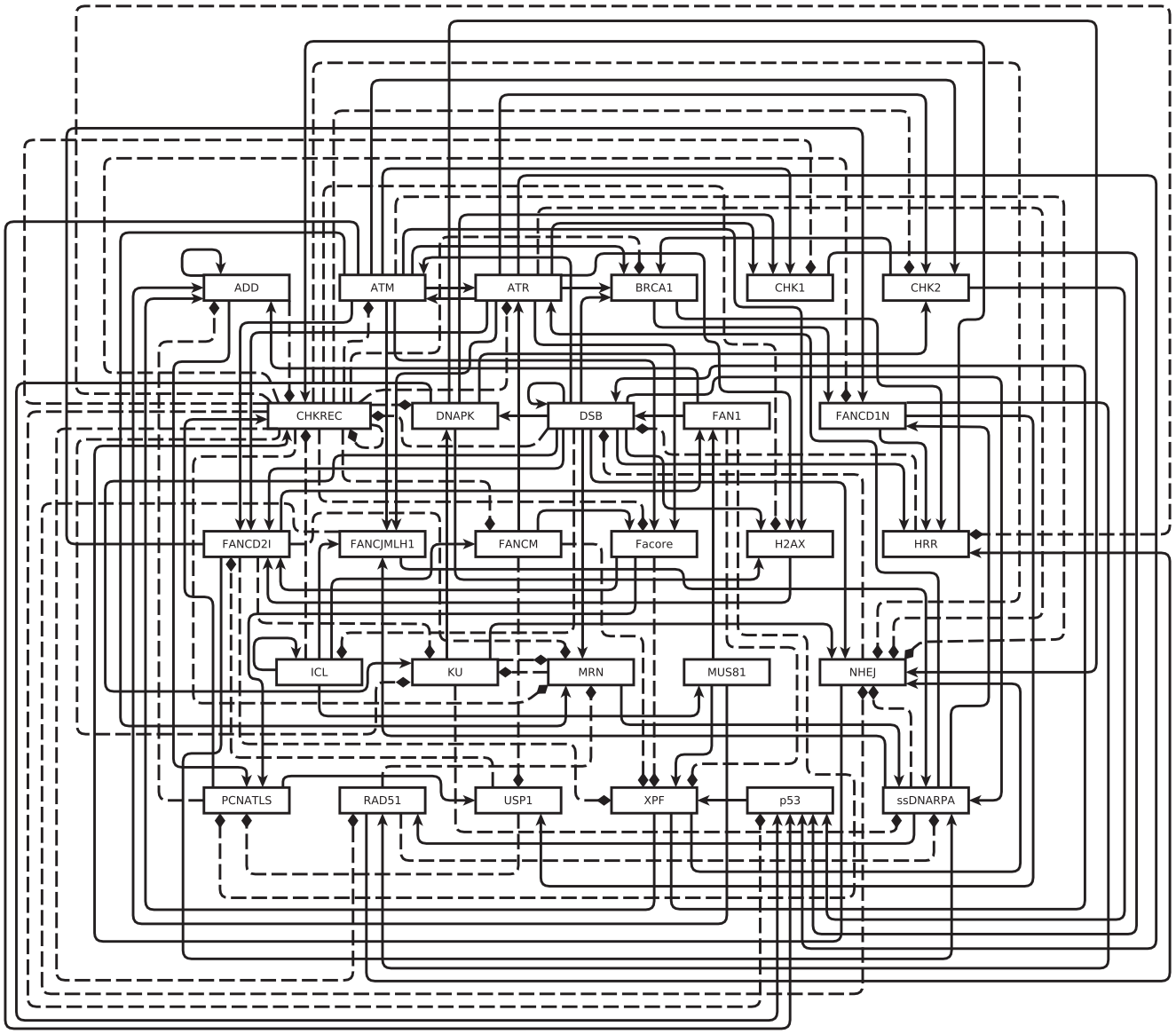

Fig. 1. The FA/BRCA network. Rectangles represent proteins or protein complexes, pointed arrows are positive regulatory interactions, and dashed lines with blunt arrows are negative regulatory interactions.

activate the DNA repair process, namely ICL, DSB and ADD. The reason to include these nodes in the network is that FA cells are highly sensitive to ICLs (D'Andrea, 2010; de Winter and Joenje, 2009), whereas DSBs and ADDs are found during ICLs repair (Kee and D'Andrea, 2010).

3.1 The FA/BRCA network

The reconstructed FA/BRCA regulatory network (Fig. 1) and its associated logical functions (Table 1) integrate the vast majority of experimental knowledge about the FA/BRCA pathway and its interactions with other genes/proteins involved in FA. The resulting discrete dynamical system was exhaustively analyzed so as to obtain the attractors of the system, particularly when starting in the presence of the three types of DNA damage: ICLs, DSBs and ADDs, under wild type and mutant conditions. With the inclusion of some inferred, but not yet reported, interactions the model recovers the main known features of the wild type FA/BRCA pathway. Since, to our knowledge, there are no published reports about these interactions, they constitute veritable predictions of our model that require additional research in order to be validated.

3.1.1 The generation of DSBs during ICL repair is assured by the activity of FA/BRCA pathway endonucleases It has been reported that primary FA-C cells are not defective in the generation of DSBs during the initial steps of ICL repair (Rothfuss and Grompe, 2004). Since the generation of DSBs during the processing of ICLs is not absolutely abolished in FA, then there must exist alternative endonucleases that become active even when the FA/BRCA pathway

Table 1. Boolean functions for the nodes in the FA/BRCA network

Rules
ICL ← ICL ∧ ¬ DSB
FANCM ← ICL ∧ ¬ CHKREC
FAcore ← FANCM ∧ (ATR ∨ ATM) ∧ ¬ CHKREC
FANCD2I ← FAcore ∧ ((ATM ∨ ATR) ∨ (H2AX ∧ DSB)) ∧ ¬ USP1
MUS81 ← ICL
FANCJBRCA1 ← (ICL ∨ ssDNARPA) ∧ (ATM ∨ ATR)
XPF ← (MUS81 ∧ ¬ FANCM) ∨ (MUS81 ∧ p53 ∧ ¬ (FAcore ∧ FANCD2I ∧ FAN1))
FAN1 ← MUS81 ∧ FANCD2I
ADD ← (ADD ∨ (MUS81 ∧ (FAN1 ∨ XPF))) ∧ ¬ PCNATLS
DSB ← (DSB ∨ FAN1 ∨ XPF) ∧ ¬ (NHEJ ∨ HRR)
PCNATLS ← (ADD ∨ (ADD ∧ FAcore)) ∧ ¬ (USP1 ∨ FAN1)
MRN ← DSB ∧ ATM ∧ ¬ ((KU ∧ FANCD2I) ∨ RAD51 ∨ CHKREC)
BRCA1 ← DSB ∧ (ATM ∨ CHK2 ∨ ATR) ∧ ¬ CHKREC
ssDNARPA ← DSB ∧ ((FANCD2I ∧ FANCJBRCA1) ∨ MRN) ∧ ¬ (RAD51 ∨ KU)
FANCD1N ← (ssDNARPA ∧ BRCA1) ∨ (FANCD2I ∧ ssDNARPA) ∧ ¬ CHKREC
RAD51 ← ssDNARPA ∧ FANCD1N ∧ ¬ CHKREC
HRR ← DSB ∧ RAD51 ∧ FANCD1N ∧ BRCA1 ∧ ¬ CHKREC
USP1 ← ((FANCD1N ∧ FANCD2I) ∨ PCNATLS) ∧ ¬ FANCM
KU ← DSB ∧ ¬ (MRN ∨ FANCD2I ∨ CHKREC)
DNAPK ← (DSB ∧ KU) ∧ ¬ CHKREC
NHEJ ← (DSB ∧ DNAPK ∧ XPF ∧ ¬ ((FANCJBRCA1 ∧ ssDNARPA) ∨ CHKREC)) ∨ ((DSB ∧ DNAPK ∧ KU) ∧ ¬ (ATM ∧ ATR))
ATR ← (ssDNARPA ∨ FANCM ∨ ATM) ∧ ¬ CHKREC
ATM ← (ATR ∨ DSB) ∧ ¬ CHKREC
p53 ← (((ATM ∧ CHK2) ∨ (ATR ∧ CHK1)) ∨ DNAPK) ∧ ¬ CHKREC
CHK1 ← (ATM ∨ ATR ∨ DNAPK) ∧ ¬ CHKREC
CHK2 ← (ATM ∨ ATR ∨ DNAPK) ∧ ¬ CHKREC
H2AX ← DSB ∧ (ATM ∨ ATR ∨ DNAPK) ∧ ¬ CHKREC
CHKREC ← ((PCNATLS ∨ NHEJ ∨ HRR) ∧ ¬ DSB) ∨ ((¬ ADD) ∧ (¬ ICL) ∧ (¬ DSB) ∧ ¬ CHKREC)

References regarding the literature used for the inference of this rules can be found in the Supplementary Material.

is defective. A likely candidate is XPF/ERCC1 with 5′ endonuclease activity (Kuraoka *et al.*,, 2000), which has a similar function to FAN1 (MacKay *et al.*, 2010). We hereby propose that the activity of this alternative endonuclease is promoted by MUS81 proteins, very probably in concert with the newly described SLX4 (FANCP) (Crossan *et al.*, 2011; Kim *et al.*, 2011). Alternatively, XPF/ERCC1 might be activated by a p53-dependent pathway. Under wild-type conditions, the activity of this alternative endonuclease must be inhibited in order to avoid a possible interference with the FA/BRCA pathway. In our model, this inhibition was accomplished by the activation of the FANCM or FANCD2I complexes in association with FAN1 (Supplementary Fig. S3). This is an issue that deserves additional research because it does not exist information about any direct or indirect active inhibition of any endonuclease by the FA/BRCA proteins. Very likely, these alternative endonucleases are present as a secondary pathway whose effect becomes evident whenever the FA/BRCA pathway is defective.

3.1.2 During the repair of an ICL-intermediate DSB the MRN exonuclease activity acts in the absence of FA/BRCA and NHEJ pathways When a DSB is generated, proteins of different DNA repair pathways such as HRR and NHEJ surround the region of DNA damage. The co-localization of these proteins is considered as evidence of interaction between both DNA repair pathways (Bekker-Jensen *et al.*, 2006; Kass and Jasin, 2010). Now, the MRN complex, a heterotrimeric complex with exonuclease activity, is able to process DSBs generating DNA overhangs that are funneled to the HRR process. In normal cells, the presence of the MRN complex is considered as essential for FANCD2 stability (Roques *et al.*, 2009); moreover, recent studies have suggested that FANCD2 also possesses exonuclease activity (Pace *et al.*, 2010). Therefore, the exonuclease activity of the MRN complex may not be necessary when the FA/BRCA pathway functions properly. The same group also found that the KU70, a NHEJ protein, corrupts the DNA repair process and changes the DNA repair choice to the error-prone NHEJ pathway in FA cells. They also observed that the FANCA/KU70 double mutant is able to repair the DNA damage by the HRR pathway. Moreover, a series of reports have shown that cells deficient in the FA/BRCA pathway generate sister chromatid exchanges (SCEs) when treated with MMC (Latt *et al.*, 1975; Wilson *et al.*, 2001). Now, as SCEs are considered a result of HRR with crossing-over, this could mean that FA cells are still able to perform HRR by a FA/BRCA-independent pathway. Interestingly, downstream FA/BRCA pathway mutant cells, such as RAD51 and FANCD1/BRCA2, are not able to generate SCEs when exposed to ICL-inducing agents (Sonoda *et al.*, 1999; Takata *et al.*, 2001).

Taking all the previous information into account, we believe that the MRN complex is a likely candidate to direct the HRR process in the absence of FA/BRCA and NHEJ proteins. According to this, the first mechanism to repair an ICL-intermediate DSB would be the HRR mediated by FA/BRCA and then the NHEJ proteins. If both these pathways are defective, the candidate to initiate an alternative

HRR and generate SCE would be the MRN complex. Our model proposes that KU70 in conjunction with the FANCD2I complex act inhibiting MRN (Supplementary Fig. S4). Then, when the inhibitors are absent, the activation of ATM and the presence of a DSB is enough to activate MRN. This might explain why the DNA repair is improved in FANCA/KU70 double mutants (Pace *et al.*, 2010). Alternatively, a point of inhibition over MRN could be the activation of RAD51 and CHKREC, which would turn the activity of the MRN complex off.

The USP1 deubiquitinating enzyme is directly or indirectly regulated by the presence of FANCM To date, it is known that the USP1 deubiquitinating protein is able to inactivate the FANCD2I complex (Cohn *et al.*, 2009; Nijman *et al.*, 2005) and TLS proteins (Huang and D'Andrea, 2006; Niimi *et al.*, 2008), although it is not yet clear which is the mechanism that activates USP1 itself. In our model, the activation of USP1 cannot occur unless both the FANCD2I and FANCD1N complexes or TLS are active (Supplementary Fig. S5). Also, the activation of USP1 is restricted since it does not occur unless FANCM is turned off. This behavior ensures that the activity of the FA/BRCA pathway continues until the ICL has been processed.

3.1.3 The Checkpoint recovery process is critical for the FA/BRCA pathway regulation We found that negative feedback mediated by the CHKREC is indispensable to obtain the experimentally observed behavior of the FA/BRCA network. In our model, we propose that CHKREC is able to inactivate 10 nodes; namely, MRN, BRCA, FANCD1N, RAD51, HRR, DNAPK, NHEJ, ATR, p53 and CHKREC itself. These 10 out of 17 regulatory interactions of CHKREC are not reported in the experimental literature, they are predictions of our model. It is important to mention that the CHKREC process includes several proteins able to inactivate key DNA repair and Checkpoint proteins allowing the progression of the cell cycle (Bartek and Lukas, 2007; Carlessi *et al.*, 2010; Douglas *et al.*, 2010; Fiscella *et al.*, 1997).

3.2 Dynamics of the FA/BRCA boolean model

3.2.1 Dynamics of the model in the absence of damage The FA/BRCA network model has only one attractor, regardless of the initial state of the network, that we refer to as the cell cycle progression (CCP) attractor, characterized by a periodic oscillation between two network states (Fig. 2, specifically the last two time periods in Panels A–C). This attractor is reached in an average of ∼10 time steps from its initial state (see the distribution of values in Supplementary Fig. S6). Once in the CCP attractor all nodes are inactive, except for CHKREC that oscillates between the on and off states. This period-2 attractor can be interpreted biologically as the periodical transition in the cell cycle that starts with the activation of proteins that release the G_2/M Checkpoint, which verifies the DNA integrity and allow the progression of the cell cycle. Once a new cell cycle starts, the CHKREC proteins turn off, only to be active again when the cell reaches the G_2 phase of a new cell cycle. Hence, the proteins involved in this cyclic behavior might include Cyclin-dependent kinases (Cdks), which have an active/inactive transitional behavior controlling the progression through the eukaryotic cell cycle (Bloom and Cross, 2007). Importantly, this period-2 attractor is also obtained if the network is studied with an asynchronous updating.

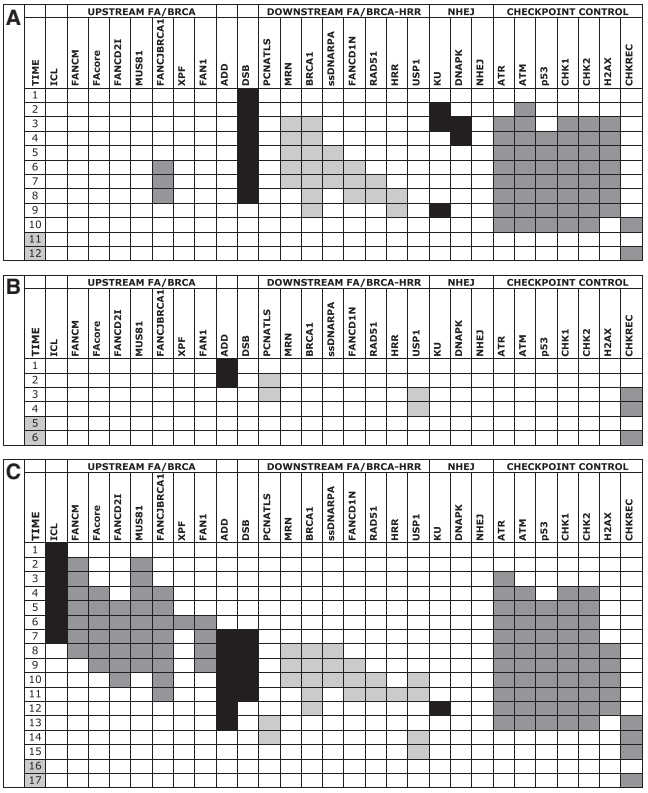

Fig. 2. Dynamical behavior of the FA/BRCA network, simulating the response to three different types of damage. (**A**) In a DSB damage, DNA is repaired by MRN-mediated HRR. (**B**) An ADD is repaired by PCNATLS. (**C**) An ICL is repaired by FA/BRCA pathway via the creation of intermediary DSB and ADD. Time steps in arbitrary units are indicated at the leftmost column. The period-2 attractor of the network is indicated in gray in the time column. Nodes are grouped into functional categories by shades of gray, and also by name at the topmost column.

3.2.2 Dynamics of the model during DSB, ADD and ICL repair The FA/BRCA network recovers the known order of activations leading to the repair of DSBs, ADDs and ICLs. Specifically, when a DSB is introduced in the model (Fig. 2A), the network is able to repair it via the MRN-mediated HRR pathway (Kass and Jasin, 2010). Also, the simulation of an ADD (Fig. 2B) causes the activation of the PCNATLS pathway (Chang and Cimprich, 2009), which repairs the ADD by the mediation of NER. Finally, the FA/BRCA network is able to describe the elimination of an ICL (Fig. 2C) by way of the generation of DSB and ADD intermediates (de Winter and Joenje, 2009). Moreover, the dynamics shown in Figure 2C also includes the recently described inhibition of FA proteins over NHEJ proteins (Bunting *et al.*, 2010; Pace *et al.*, 2010), as well as the activation of the Checkpoint proteins (Bartek and Lukas, 2007).

The cross talk among pathways generate negative feedback loops that create an homeostatic process in the recovering of the system after the DNA damage. In our model, the negative feedback mediated by CHKREC involves NHEJ, MRN, BRCA1, FANCD1N, RAD51, HRR, DNAPK, ATR, p53 and CHKREC itself. Once DNA damages have been eliminated, the network returns to its normal behavior, as represented by the CCP attractor. However, if by any reason the damage is constantly generated, for example by the persistent

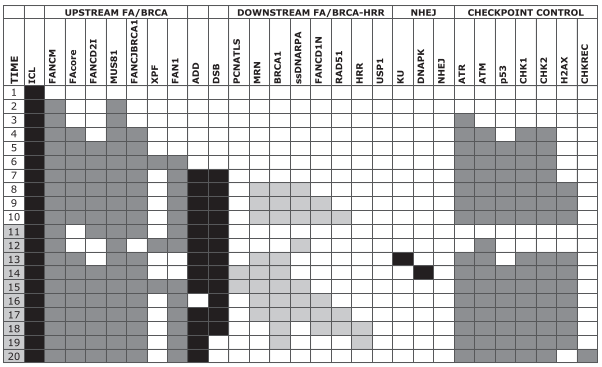

Fig. 3. Dynamical behavior of the FA/BRCA network simulating a persistent ICL damage. The period-10 attractor of the network is indicated in gray in the time column.

presence of MMC or DEB, the network reaches attractors different from the CCP (see further).

3.2.3 Dynamics of mutants of the FA/BRCA network We used the FA/BRCA to study the effects of turning some nodes on or off permanently, thus simulating diverse physiological and mutant conditions. First, we simulated the permanent presence of DNA damage by leaving the ICL node always on. Such a constitutive activation pretends to represent the effect observed when there is a persistent presence of chemicals such as MMC or DEB. Figure 3 (time steps 11–20) shows that under such circumstances the network reaches a period-10 attractor characterized by the persistent activation of Checkpoint, FA/BRCA and HRR pathways. Biologically, this pattern might represent that the cell is performing a DNA repair process without progressing on the cell cycle. Because of this characteristic, we refer to this attractor as the cell cycle arrest (CCA) attractor. If the source of DNA damage is eliminated, i.e. the ICL node is permitted to become inactive, the cell cycle progresses as evidenced by the network reaching the CCP attractor (data not shown).

Following the simulation of the repair of ICLs, DSBs and ADDs, we tested all possible single gain- and loss-of-function mutants and their dynamical behavior (Supplementary Material). Regarding null mutants on the FA/BRCA pathway, our model adequately describes the reported cellular phenotypes including the persistence of unrepaired DSBs, the activation of the NHEJ pathway, the activation of RAD51 in upstream mutants, the inability to activate RAD51 in downstream mutants and the correct activation of Checkpoint proteins that leads to an arrest in CCP. This last point is of relevance, since it argues in favor of a well-conserved G_2/M Checkpoint function of FA/BRCA pathway in mutant cells (Neveling *et al.*, 2009). Interestingly, with the exception of the CHKREC null mutant, every null mutant recovers the CCP attractor, although some mutants also have additional attractors with larger basins (Supplementary Material).

Most FA patients carry an alteration in the FANCA gene (Auerbach, 2009), hence we hereby show the corresponding simulation, which is the constitutive null mutant of FAcore (Fig. 4). The model of the mutant is able to repair the damage simulated by an ICL pulse, thus reaching the CCP attractor (Fig. 4A, time steps 14–15). Noteworthy, the same attractor is recovered by using an asynchronous updating scheme. In this condition, our model

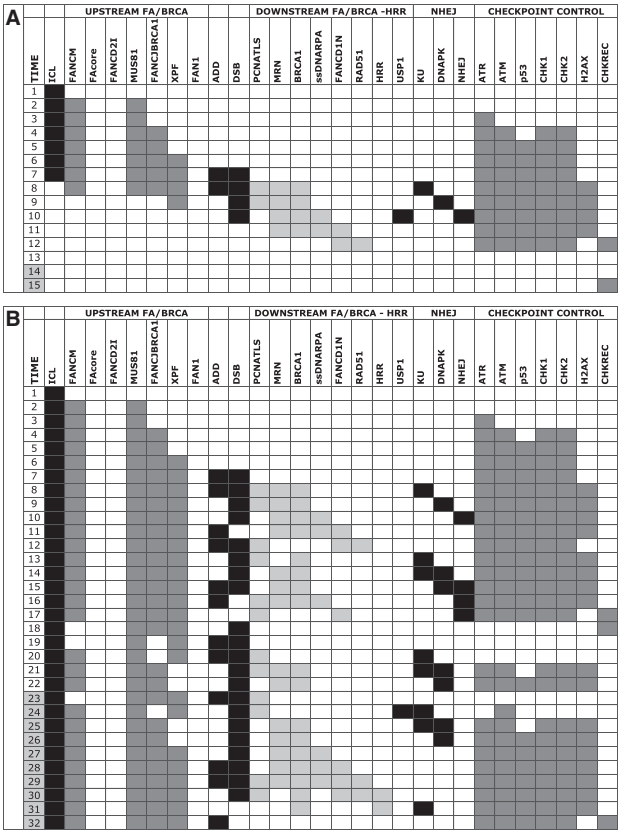

Fig. 4. Simulation of the network with a null mutation in FAcore. (**A**) A single ICL pulse. (**B**) A persistent ICL damage.

shows that the NHEJ pathway repaired the DSB generated as an intermediary. Furthermore, when a constant ICL damage was simulated in the FAcore null mutant, the model reaches a period-10 attractor (Fig. 4B, time steps 23–32) somewhat similar to the CCA attractor (Fig. 3). Interestingly, the model shows a difference in the mechanism of repair, depending on the persistence of the damage. With a single ICL pulse, the NHEJ pathway is the main route to repair the damage, whereas with a constant ICL stimulus there is a combined effort among the NHEJ and HRR paths. This is in marked contrast to the wild-type network, where the HRR—by mediation of the FA/BRCA proteins—is always the first option of repair.

With an intact FA/BRCA pathway, the FAcore collaborates in the activation of the FAN1 endonuclease to generate the necessary DSBs to repair an ICL (MacKay *et al.*, 2010; Smogorzewska *et al.*, 2010) (Fig. 2C). However, in FANCA patients the activity of FAcore does not exist, it is therefore instructive to analyze which DNA endonucleases are responsible for the generation of DSBs in this case. According to our model, in the absence of FAcore, the effect could be achieved by the activation of the FANCM protein and the MUS81 and XPF endonucleases (Fig. 4), with the subsequent activation of FANCJBRCA1 and Checkpoint proteins, such as ATR and ATM kinases. At this point, an ADD and a DSB are generated, a normal TLS/NER process (part of the PCNATLS node) repairs the ADD, and then the NHEJ pathway or the MRN-mediated HRR could repair the DSB. Recent data have shown that a completely functional FA/BRCA pathway will block the NHEJ pathway (Pace

et al., 2010); therefore in FAcore mutants, the KU proteins have the possibility to occupy the DSB and direct the DNA lesion to the NHEJ pathway. Additionally, this mechanism could explain the radial exchange figures seen in FA cell metaphases.

The period-10 attractor observed due to a persistent ICL in FAcore null mutant includes the activation of the HRR pathway, mediated by MRN, BRCA1, ssDNARPA and RAD51 (Fig. 4B). This behaviour could explain the sister chromatid exchanges observed in upstream FA cells, as these SCEs are considered a result of HRR with crossing-over (Hirano *et al.*, 2005). This, in turn, could mean that FA cells are still able to perform HRR by a FA/BRCA-independent pathway. Our model points to MRN as a candidate to direct the HRR process in the absence of some FA proteins.

The simulation in Figure 4B shows that in FAcore null mutant is possible to observe that the activation of the Checkpoint proteins, namely ATR, ATM, CHK1, CHK2 and p53, is not impaired. This implies that the Checkpoint protein activation in the presence of DNA damage should be normal in FA cells, in concordance with experimental data (Heinrich *et al.*, 1998). However, the simulation shows that the Checkpoint module turns off even when the DNA damage nodes are still active, which may explain the presence of unrepaired ICLs, DSBs and ADDs.

We turn now our attention to a different mutant. It has been demonstrated the involvement of FANCD1/BRCA2 and FANCN/PALB2 in the development of breast cancer. These are molecules that act downstream of the FA/BRCA pathway in the DSB repair mediated by HRR (Xia *et al.*, 2007; Zhang *et al.*, 2009b). Patients carrying FA/BRCA downstream null mutations, such as FANCD1/BRCA2 or FANCN/PALB2, are relatively rare and develop malignant solid tumors earlier in life than FA patients carrying FA/BRCA upstream null mutations (Neveling *et al.*, 2009). This characteristic might be due to the inability to use alternative DNA repair pathways; in fact, cells from these patients are unable to perform sister chromatid exchanges (Sonoda *et al.*, 1999; Takata *et al.*, 2001).

Figure 5 shows the simulations of the null mutation in FANCD1N. In Figure 5A, it can be observed that the attractor reached is characterized by the activation of the Checkpoint proteins with a concomitant absence of HRR and NHEJ activities. Moreover, such attractor is obtained with either synchronous or asynchronous updating schemes. Now, Figure 5B shows the attractor reached under persistent ICL damage. These activation patterns can be interpreted as resulting from an arrest in the cell cycle due to a lack of repair of the DNA damage. If true, this behavior could partially explain the scarcity of patients with mutations in either FANCD1/BRCA2 or FANCN/PALB2 genes. Clearly, those surviving patients presenting these mutations should present alternative mechanisms of DNA repair allowing them to survive.

The clinical manifestations of FA patients are extremely variable, therefore it has been difficult to establish a clear genotype–phenotype correlation. Nonetheless, there are phenotypic differences when comparing patients with FAcore mutations against those who carry mutations in the downstream genes, as FANCN or FANCD1. Specially the later kind of patients die due to the development of Wilms Tumor or meduloblastome, which typically occur earlier than pancytopenia, and also have more severe congenital malformations. In our model, it is possible to find a clear difference between null mutants in upstream and downstream nodes. The upstream null mutant shows the activation of alternative DNA repair pathways,

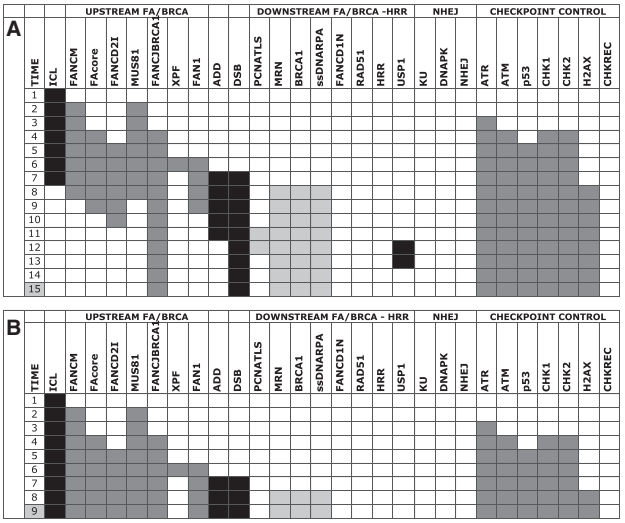

Fig. 5. Simulation of the network with a null mutation in FANCD1N. (**A**) A single ICL pulse. (**B**) A persistent ICL damage.

eventually leading to the system to the CCP attractor (Fig. 4 and Supplementary Material). In contrast, the simulations of downstream null mutant show the limited possibilities of DNA repair, leading the system principally to the CCA attractor (Fig. 5 and Supplementary Material). This implies that cells with downstream null mutations have clearly few possibilities of repair, thus eventually causing cell death. It is important to mention the scarcity of patients carrying mutations somewhere in downstream FA/BRCA, probably due to early cell demise concomitant with an irreversible arrest of CCP, as implied by the CCA attractor.

We described in the previous paragraphs the simulation of some variants of the FA/BRCA network that reflect the mutations of certain type of patients, and the response of their cells to certain type of DNA damage, namely ICLs. The reader may refer to the Supplementary Material for an exhaustive presentation of the simulations for all possible single mutants of the FA/BRCA network.

4 CONCLUSION

We presented in this article the reconstruction of the FA/BRCA network, as well as its implementation as a discrete dynamical system, and its behavior under a number of simulated mutations and DNA damages. To our knowledge, this is the first network model incorporating this DNA repair pathway.

We simulated with the model the dynamical behavior leading to alternative routes of DNA repair that might help explain the phenotype of cells obtained from patients with FA. Our simulations strongly suggest that the use of alternative DNA repair pathways help to safeguard the integrity of the genome, although the choice of pathway does not warrant a proper joining of damaged DNA.

While we did not present a formal robustness analysis of the FA/BRCA network, the results shown here are suggestive of a robust system. The recovery of a small number of attractors, all of them with a clear qualitative biological interpretation, even under the systematic alteration of the network to represent loss- and gain-of-function mutations imply that the network has a strong canalizing dynamical behavior. Moreover, 45 out of the possible 56

mutants conserved the wild-type attractor. Now, most simulations were performed using a synchronous update method, which is unrealistic from the biological point of view. However, we tested a number of key network variants, namely the wild type, the FAcore and the FANCD1N null mutants with both synchronous and asynchronous update schemes, obtaining exactly the same attractors. This invariability favors the interpretation that our results are robust, and are not completely determined by the updating methodology used. We are aware that these results are only suggestive about the robustness of the FA/BRCA network, and therefore the issue has to be further studied properly and systematically.

Our model makes a number of very concrete, testable predictions in the form of trajectories and attractors reached under different circumstances. Testing these predictions in the laboratory can help in the understanding of the mechanisms behind the DNA repair process as mediated by the FA/BRCA pathway. Such knowledge has potential implications in the refinement of FA/BRCA and DNA repair related pathways, with the concomitant improvement in the treatment of patients with FA, with tumors with a FA phenotype, or even with chemo-resistant forms of cancer.

The current model will be improved in the future in several ways. The incorporation of more nodes or interactions may refine the model so that it would explain the cellular behavior of rare complementation groups. Also, the analysis of the network as a stochastic dynamical system could generate information about the probability of cells to make use of alternative DNA repair mechanism, and thus improve the chance to survive to DNA damages. Finally, it is worth exploring the incorporation of interactions with apoptotic pathways, which are highly related to the DNA repair process.

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