

**Original Paper**

# Dynamic Coregulatory Complex Containing BRCA1, E2F1 and CtIP Controls ATM Transcription

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**Key Words**

ATM • BRCA1 • Transcription • DNA damage • Prostate cancer

**Abstract**

Chromosomal instability is a key feature in cancer progression. Recently we have reported that BRCA1 regulates the transcription of several genes in prostate cancer, including ATM (ataxia telangiectasia mutated). Although it is well accepted that ATM is a pivotal mediator in genotoxic stress, it is unknown whether ATM transcription is regulated during the molecular response to DNA damage. Here we investigate ATM transcription regulation in human prostate tumor PC3 cell line. We have found that doxorubicin and mitoxantrone repress ATM transcription in PC3 cells but etoposide and methotrexate do not affect ATM expression. We have demonstrated that BRCA1 binds to ATM promoter and after doxorubicin exposure, it is released. BRCA1 overexpression increases ATM transcription and this enhancement is abolished by BRCA1 depletion. Moreover, BRCA1-BRCT domain loss impairs the ability of BRCA1 to regulate ATM promoter activity, strongly suggesting that BRCT domain is essential for ATM regulation by BRCA1. BRCA1-overexpressing PC3 cells exposed to KU55933 ATM kinase inhibitor showed significant decreased ATM promoter activity compared to untreated cells, suggesting that ATM transcriptional regulation by BRCA1 is partially mediated by the ATM kinase activity. In addition, we have demonstrated E2F1 binding to ATM promoter before and after doxorubicin exposure. E2F1 overexpression diminishes ATM transcription after doxorubicin exposure which is impaired by E2F1 dominant negative mutants. Finally, the co-regulator of transcription CtIP increases ATM transcription. Altogether, BRCA1/E2F1/CtIP binding to ATM promoter activates ATM transcription. Doxorubicin exposure releases BRCA1 and CtIP from ATM promoter still keeping E2F1 recruited and, in turn, represses ATM expression.

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## Introduction

Prostate cancer (PCa) is one of the most common cancers and the second leading cause of cancer-related death affecting men in the world [1]. It is well known that both environmental and genetic factors are involved in its etiology [2], making PCa of a heterogeneous nature and extremely difficult to design an appropriate and effective treatment [3]. Survival, proliferation and dissemination are the hallmarks of cancer, features acquired mainly by genomic instability that generates random mutation and chromosomal rearrangements in tumor cells. This condition allows cancerous cells to accelerate the process of mutagenesis making these cells able to be uncontrollably prolific [4].

ATM (ataxia telangiectasia mutated) is a member of the PI-3 kinase family and a component of the DNA damage repair system [5]. In response to DNA double-strand breaks, the inactive ATM dimers autophosphorylate at Ser<sup>1981</sup>, generating active monomers that trigger stabilization of the p53 tumor suppressor protein and up-regulation of p53-dependent genes, finally inducing cell cycle arrest, DNA repair and/or activation of cell death pathways [6]. In PCa, altered p53 expression has been correlated with a higher Gleason score and poorer prognosis [7]. Besides several reports have indicated that loss of ATM function leads to genome instability and to an increased risk of cancer among other diseases [8-10].

There is poor knowledge about ATM transcription regulation in normal or tumor cells. It has been previously reported that ATM is transcriptionally regulated by E2F1 [11] and EGF [12]. However, the factors that govern ATM expression and its response to different genotoxic insults still have to be investigated.

The tumor suppressor gene BRCA1 (Breast susceptibility cancer gene 1) is involved in many important cellular functions. It participates in DNA repair, cell cycle regulation and transcriptional regulation [13]. Along its structure, BRCA1 contains multiple functional domains and interacts with a wide range of proteins, including RNA pol II, the transcription factor E2F1, p53, c-Myc, STAT1, c-jun, estrogen receptors and transcription co-regulators, such as CtIP-CtBP, as well as several members of the acetyl- and deacetyl-histone family, p300 and HDAC 1-2 [14-17]. BRCA1 contains two tandem BRCT (BRCA1 C-terminal) motifs at its carboxi-terminal domain which are critical to its function [18].

We have recently demonstrated that BRCA1 associates to ATM promoter in Cell line is T Jurkat and LNCaP cells [17]. To gain insight into the role of the regulation of ATM transcription in DNA damage response, in this study we have investigated ATM transcription regulation in the prostate tumor cell line, PC3, under different stimuli. We have found that BRCA1, E2F1 and CtIP are critical ATM transcription regulators in response to DNA damage, suggesting a new manner for ATM regulation that may be important for DNA damage control in PCa.

## Materials and Methods

### *Cell culture and reagents*

PC3 (ATCC: CRL-1435<sup>TM</sup>) prostate cancer cells were grown in RPMI 1640 with 10% fetal bovine serum (FBS) in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. PC3 stable transfected cell lines (pcDNA3, pcDNA3 BRCA1, shRNA scramble and shRNA BRCA1) were previously described [19]. Doxorubicin hydrochloride (Rontag S.A.) was prepared in DMSO and used at a final concentration of 1 µM for 24 h. Actinomycin D (Invitrogen) was prepared in DMSO and cells were pre-treated for 1 h (5 µg/ml) before adding doxorubicin. KU 55933 [2-(4-Morpholinyl)-6-(1-thianthrenyl)-4H-pyran-4-one, Trocrist Bioscience] was prepared in DMSO and used at a final concentration of 10 µM for 24 h with 1 h pre-treatment. Mitoxantrone (Micrlev<sup>®</sup>, Ivax Argentina) was used at a final concentration of 1 µM for 24 h. Methotrexate (Ervemi<sup>®</sup>, Ivax Argentina) was used at a final concentration of 200 µM for 24 h. Etoposide (Sigma-Aldrich) was used at a final concentration of 5 µM for 24 h.

#### Plasmids and RNAi

BRCA1 expression vectors (pcDNA3 BRCA1 and pcDNA3 BRCA1  $\Delta$ BRCT) and shRNA (shRNA scramble control and shRNA BRCA1) have been previously described [17]. Plasmids expressing E2F1 *wild type*, E2F1 (E132) and E2F-1  $\Delta$  (1–363) have been previously described [17]. AR expression vector has been kindly provided by Dr. Guido Jenster (Department of Urology, Josephine Nefkens Institute, Erasmus, The Netherlands). p300 expression vector has been previously described [20]. CtBP1 plasmid expression vector has been generously provided by Dr. Richard H. Goodman (Vollum Institute, Oregon Health & Sciences University Portland). CtIP *wild type*, CtIP DC and CtIP EK plasmids have been generously provided by Dr. Ju-Ming Wang (Institute of Bioinformatics and Biosignal transduction NCKU, Taiwan). Rb expression vector has been kindly provided by Dr. Martin Monte (Department of Biological Chemistry, University of Buenos Aires, Argentina). ATM promoter luciferase (ATM-luc) reporter plasmid has been provided by Switch gear (USA).

#### Reporters

PC3 cells were transiently transfected using lipofectamine 2000 (Invitrogen) with 1  $\mu$ g of ATM-luc plasmid and co-transfected with 1  $\mu$ g of pcDNA3 empty vector or the above mentioned expressing vectors. After 24 h, cells were exposed to genotoxic agents as indicated, harvested and lysed with 40  $\mu$ l of Steady Glomax Luciferase System (Promega). Luciferase activity was measured in Luminometer (Glomax Multi Detection System, Promega). Data were normalized to total protein.

#### RNA isolation, cDNA synthesis and qPCR

PC3 cells were exposed to different treatments and total RNA was isolated using TriReagent (Genbiotech). cDNA was retrotranscribed from 2  $\mu$ g of RNA using RevertAid First Strand (Fermentas). Real time PCR (qPCR) was performed as previously described [17] using Taq Polymerase (Fermentas) in a DNA Engine Opticon (MJ Research, BioRad). Figures show media and standard deviations from 3 biological independent experiments. Data were normalized to Actin B (ACTB) expression and vehicle treated control. Primer sequences were: ATM: 5'- CCGCGTTGATACTACTTTGACC-3' and 5'- GCAGCA GGGTGACAATAACAAGTAA -3'; ACTB: 5'-AAGATCATTGCTCCTCCTGAGC-3' and 5'-CATACTCCTGCTTGCTGATCCA-3'.

#### Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation (ChIP) was performed from PC3 cells exposed to doxorubicin (1  $\mu$ M, 24 h) or vehicle (0.1% DMSO in complete medium) as previously described [17] using anti-CtIP (Santa Cruz Biotechnologies), anti-E2F1 (Santa Cruz Biotechnologies) or anti-BRCA1 [17] antibodies. Anti-Gal4 antibody from Santa Cruz Biotechnologies was used as nonspecific control. ChIP-DNA was amplified by qPCR using primers mapping at 3.5 Kb, 1.5 Kb, 1 Kb and 0.5 Kb upstream or 1 Kb downstream from ATM transcription start site (TSS) respectively. Primer sequences were: ATM -3.5 Kb: 5'- CCTTCTGTGCTCTCTACTG-3' and 5'- AATATGGCTGCTTCTCCTG-3'; ATM -1.5 Kb: 5'-AAGCAGGAAGTCAGCAGAGTAG-3' and 5'-AGAAAGCCTAAGAAAGCAGTATC-3'; ATM -1 Kb: 5'- TGACCCACAAACAATCCCTCCTC-3' and 5'-TTCTCCATCCTCCACGCAATACG-3'; ATM -0.5 Kb: 5'- AGGAACCACAATAAGGAACAAG -3' and 5'- AAATTGCCGCGAGTTCAG -3'; ATM +1 Kb: 5'- GTGGATGATAATGTATGTGGTGATAGG-3' and 5'- CCAAGGTAACACTG CGAGGTC-3'. Fold enrichment was calculated normalizing data to input as previously described [21].

#### Immunoblot analysis

PC3 cells were exposed to different treatments, lysed and immunoblotted as previously described [19] using antibodies against ATM (5C2) and Actin B (I19) proteins from Santa Cruz Biotechnology or phosphorylated histone  $\gamma$ H2AX (Millipore). Reactions were detected by horseradish peroxidase conjugated secondary antibodies and enhanced chemiluminescence (Pierce, Rockford, IL) following manufacturer's directions. Protein quantitation was determined using Image J 1.41 software.

#### Statistical analysis

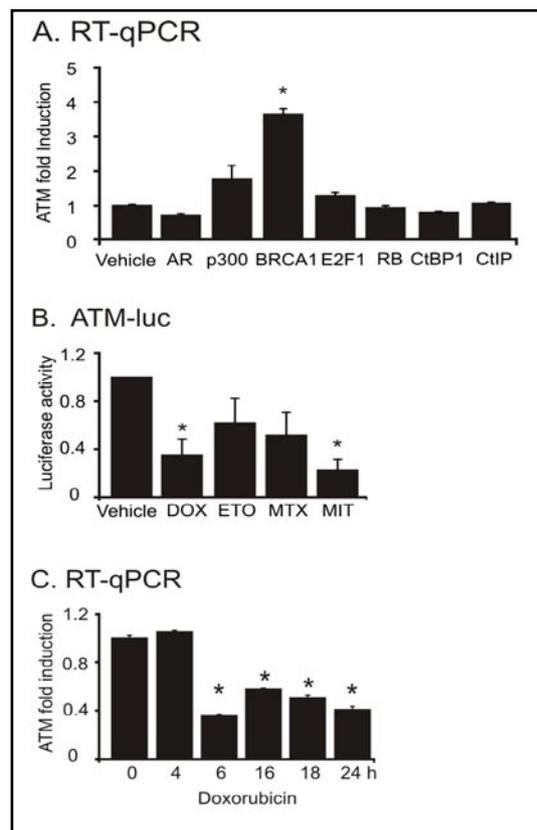
All results are given as mean  $\pm$  standard deviation of 3 separate independent experiments. Students' t tests were used to ascertain statistical significance with a threshold of  $P < 0.05$ .

## Results

### DNA damage and co-regulator proteins modulate ATM transcription

ATM is a kinase protein that is a pivotal mediator in genotoxic stress, however, it is unknown if the regulation of ATM transcription plays a role in the DNA damage response.

**Fig. 1.** ATM transcription is modulated by DNA damage and co-regulatory proteins. A. PC3 cells were transiently transfected with expression vectors (pcDNA3, AR, p300, BRCA1, E2F1, Rb, CtIP and CtBP) and total RNA was isolated to perform RT-qPCR. ATM mRNA expression levels were determined for each condition. Data were normalized to ACTB and control. B. PC3 cells were transfected with the ATM-luc reporter plasmid and then treated with DNA damage agents for 24 h (DOX = Doxorubicin 1 $\mu$ M, ETO = Etoposide 5 $\mu$ M, MTX = Methotrexate 200 $\mu$ M, MIT = Mitoxantrone 1 $\mu$ M). Cells were harvested and luciferase activity was determined. Data were normalized to total proteins and control. C. ATM RT-qPCR from PC3 cells treated with doxorubicin 1 $\mu$ M for the indicated times (0 to 24 h). Data were normalized to ACTB. Media and standard deviations from three independent experiments are shown. \* =  $p < 0.05$ .



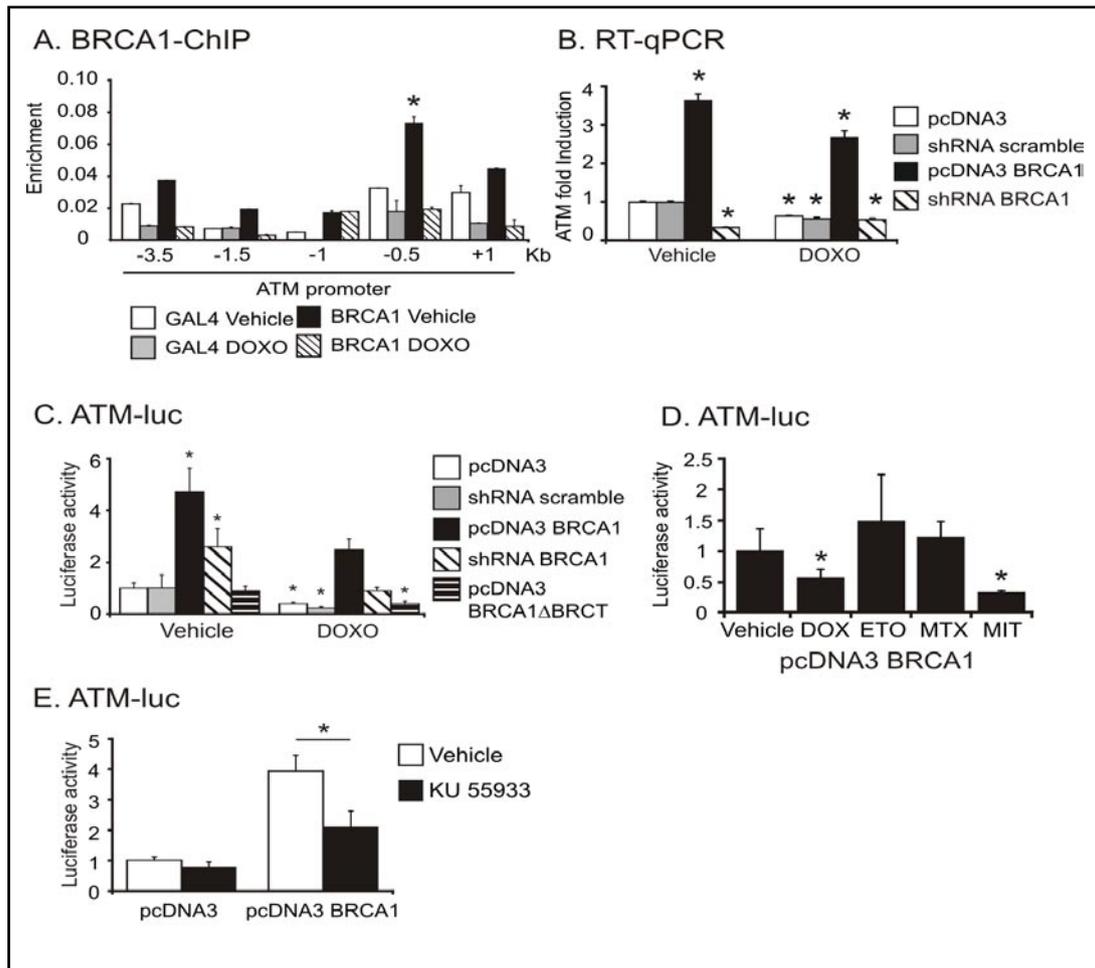
We have investigated the effect of some transcriptional co-regulators over ATM expression: androgen receptor (AR, transcription factor essential for PCa cells proliferation), histone acetyl-transferase p300, BRCA1 (tumor suppressor gene involved in genome stability and DNA damage response), E2F1 (transcription factor with crucial role in cell cycle regulation), Rb (tumor suppressor important in the control of the cell cycle progression), C-terminal interacting protein (CtIP, tumor suppressor involved in DNA damage response and cell cycle regulation) and C-terminal binding protein (CtBP, co-repressor with important roles during development and oncogenesis). We have found that BRCA1 overexpression significantly induced ATM transcription (Fig. 1A) while other factors, AR, p300, E2F1, Rb, CtBP1 and CtIP overexpression has no effect on ATM transcription (Fig. 1A).

Since ATM is activated mainly in response to DNA double-strand breaks, we have measured ATM transcription after PC3 cells exposure to different DNA damage agents. We have transfected PC3 cells with ATM-luc plasmid and exposed them to doxorubicin (topoisomerase II inhibitor), mitoxantrone (doxorubicin analog and topoisomerase I and II inhibitor), etoposide (DNA interstrand breaks inducer) and methotrexate (DNA synthesis inhibitor). As shown in Figure 1B, mitoxantrone and doxorubicin have significantly diminished ATM transcription. A time course exposure of the cells to doxorubicin has demonstrated that 6 h to 24 h of treatment is effective to repress ATM transcription (Fig. 1C).

#### *BRCA1 binds to ATM proximal promoter activating its transcription*

To assess whether ATM regulation of transcription by BRCA1 is due to BRCA1 recruitment to ATM promoter, we have performed BRCA1-ChIP-qPCR assay using primers spanning every 500 bp along ATM promoter. As shown in Figure 2A, BRCA1 associates to ATM promoter at 0.5 Kb from the TSS. After doxorubicin exposure, BRCA1 protein is released from this region.

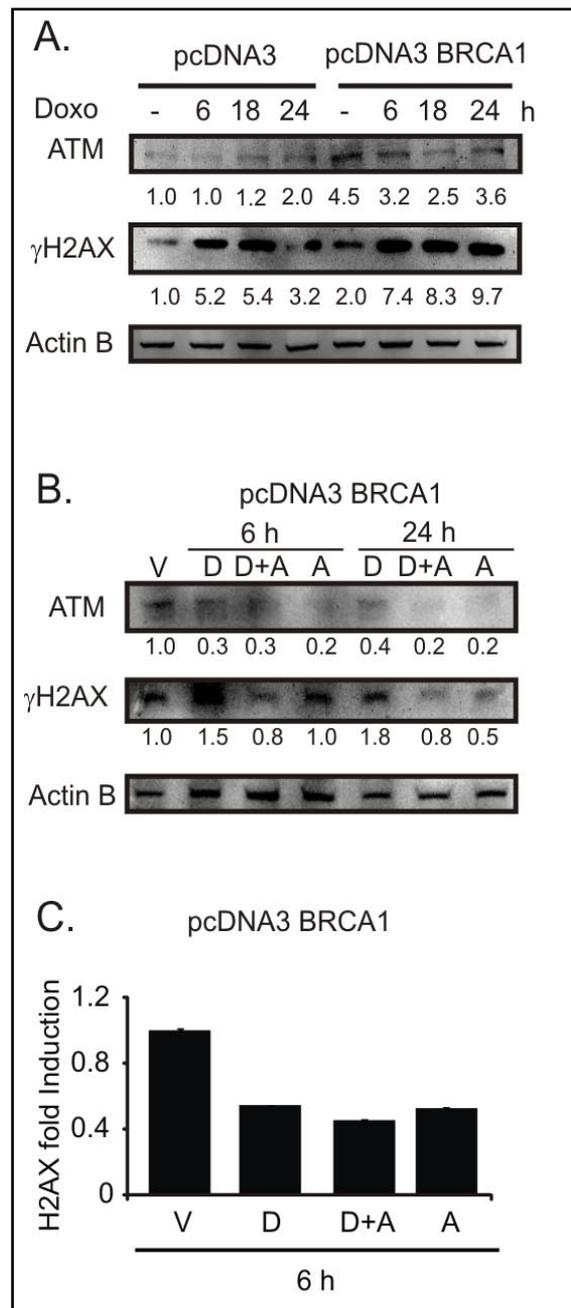
To determine the effect of BRCA1 and doxorubicin on ATM transcription, we have measured ATM mRNA levels after BRCA1 overexpression or depletion. We have found that



**Fig. 2.** BRCA1 binds and regulates ATM promoter in PC3 prostate cancer cell line. A. PC3 cells were exposed to doxorubicin (DOXO 1  $\mu$ M) or vehicle and ChIP analysis using BRCA1-specific or nonspecific (GAL4) antibodies was performed. DNA-ChIP was analyzed by qPCR using primers located at 3.5 Kb, 1.5 Kb, 1 Kb and 0.5 Kb upstream or 1 Kb downstream from ATM TSS. Fold enrichment was calculated normalizing data to input. B. ATM mRNA expression levels were determined by RT-qPCR from BRCA1-overexpressing (pcDNA3 BRCA1), BRCA1-depleted (shRNA BRCA1) or controls (pcDNA3, shRNA scramble) PC3 cells. Cells were exposed to doxorubicin (1 $\mu$ M for 24 h) or vehicle. Data were normalized to ACTB. Mean and standard deviations from 3 biological independent experiments is shown. C. PC3 stable cell lines (pcDNA BRCA1, shRNA BRCA1 and controls) were transfected with ATM-luc plasmid. PC3 cells were transiently co-transfected with pcDNA3  $\Delta$ BRCT and ATM-luc plasmids. After 24 h cells were exposed to doxorubicin for 24 h, harvested, and luciferase activity was measured. Data were normalized to total protein and control. Mean and standard deviations from 5 biological independent experiments is shown. D. PC3 pcDNA3 BRCA1 cell lines were transiently transfected with ATM-luc reporter plasmid and treated for 24 h with different DNA damage agents (as in Fig. 1B). Luciferase activity was measured and data were normalized to total protein and control. E. PC3 stable cell lines were transiently transfected with ATM-luc reporter and exposed to the ATM kinase inhibitor KU55933 for 24 h. Luciferase activity was normalized to total protein. All reporter assays were done in triplicate. Mean and standard deviations of at least 3 independent experiments are shown. \* =  $p < 0.05$ .

BRCA1 overexpression increased both ATM mRNA levels (Fig. 2B) and also promoter activity (Fig. 2C). Accordingly, BRCA1 depletion abolished ATM transcription induction (Fig. 2B-C). Furthermore, BRCA1-BRCT domain loss (BRCA1 $\Delta$ BRCT) have impaired the ability of BRCA1 to regulate ATM promoter activity (Fig. 2C). These results strongly suggest that BRCT domain

**Fig. 3.** ATM transcription induction by BRCA1 correlates with an increase in ATM activity. A. ATM and  $\gamma$ H2AX expression was determined by Western Blot from PC3 stable cell lines exposed to doxorubicin at the indicated times (0 to 24 h) or vehicle. B. PC3 pcDNA3 BRCA1 cell line were pretreated for 1 h with Actinomycin D (5 $\mu$ g/ml), and then exposed for 6 or 24 h to doxorubicin (1 $\mu$ M) as indicated. Total proteins were isolated and ATM and  $\gamma$ H2AX protein levels were determined by Western Blot. V = vehicle; A = Actinomycin D; D = Doxorubicin; A+D = Actinomycin D and doxorubicin. Quantification and normalization to actin B protein levels and control is shown under each band. One representative experiment from three biological replicates is shown. C. PC3 pcDNA3 BRCA1 cells were exposed as in B. Total RNA isolation and RT-qPCR was performed using H2AX and Actin B specific primers. Data were normalized to ACTB and control.



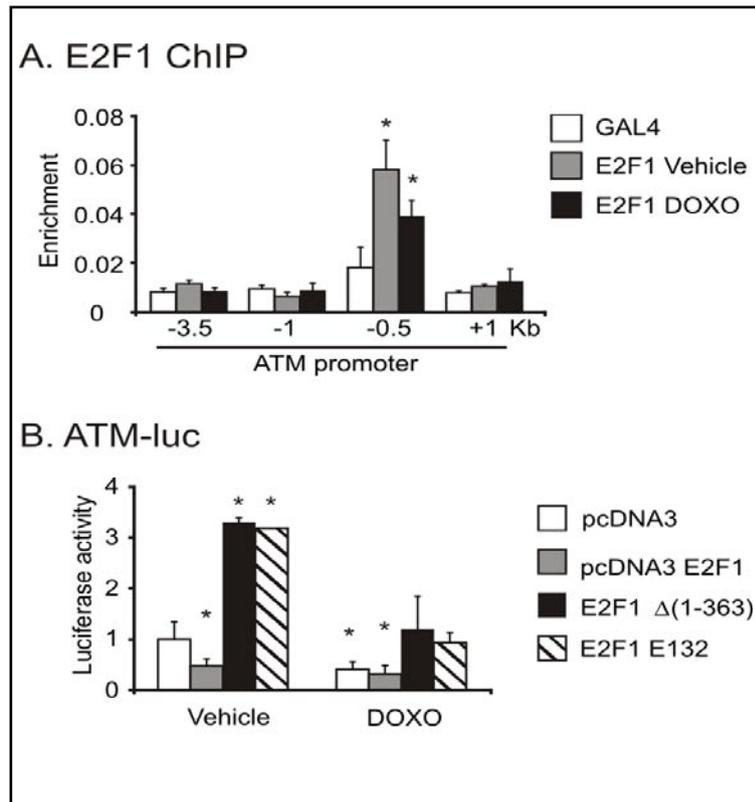
is essential for the ATM transcriptional regulation. In addition, doxorubicin has significantly reduced BRCA1 induction of ATM transcription and promoter activity (Fig. 2B-C). We further analyzed other DNA damaging agents, such as etoposide, methotrexate and the doxorubicin analog, mitoxantrone. As shown in Figure 2D, doxorubicin and mitoxantrone decreased ATM promoter activity.

These data show that BRCA1 binds to ATM promoter activating its transcription and this activation depends on its BRCT domain. However, doxorubicin exposure releases BRCA1 from ATM promoter which in turn diminishes ATM transcription.

*ATM regulates its own expression by a positive feedback loop mediated by BRCA1*

It is well accepted that BRCA1 is phosphorylated by ATM at serine residues (S<sup>1387</sup>-S<sup>1423</sup>-S<sup>1524</sup>) [22]. Considering that BRCA1 regulates ATM transcription, we have later investigated

**Fig. 4.** E2F1 protein associates to ATM proximal promoter region and regulates its transcription. A. PC3 cells were exposed to doxorubicin (DOXO 1  $\mu$ M) or vehicle and ChIP analysis using E2F1-specific or nonspecific (GAL4) antibodies was performed. DNA-ChIP was analyzed by qPCR using primers located at 3.5 Kb, 1 Kb and 0.5 Kb upstream or 1 Kb downstream from ATM TSS. Fold enrichment was calculated normalizing data to input. B. PC3 cell line was co-transfected with ATM-luc plasmid and E2F1 *wild type* (pcDNA3 E2F1) or dominant negative (E2F1 E132 and E2F1  $\Delta$ (1-363)) plasmids. Cells were exposed to doxorubicin for 24 h and then luciferase activity was measured. Data were normalized to total protein and control. \* =  $p < 0.05$ .



BRCA1's ability to activate ATM promoter when ATM kinase activity is abolished. We have exposed BRCA1 overexpressing PC3 cells and controls to ATM kinase inhibitor KU55933 (10  $\mu$ M) [23]. As shown in Figure 2E, KU55933 treatment has partially diminished ATM transcriptional activation by BRCA1. However, ATM promoter still maintains certain activity in spite of being treated with the ATM kinase inhibitor, which suggests that ATM transcriptional regulation by BRCA1 is, at least in part, mediated by ATM kinase activity. However, other factors might be implicated in this regulation.

#### *ATM transcription induction by BRCA1 enhances ATM activity*

In order to determine whether BRCA1 ultimately enhances ATM synthesis and activity, we have analyzed ATM protein levels and H2AX phosphorylation ( $\gamma$ H2AX), a specific ATM phosphorylated target in response to DNA damage. As expected, doxorubicin has increased 5 fold H2AX phosphorylation in PC3 control cells (Fig. 3A). In addition, BRCA1 overexpression provokes an increase in ATM (4.5 fold) and  $\gamma$ H2AX (2 fold) protein levels (Fig. 3A), reinforcing our findings of an enhancement in ATM mRNA and promoter activity by BRCA1. Moreover, we have observed that doxorubicin exposure causes a decrease of ATM protein levels in cells overexpressing BRCA1 (Fig. 3A, lanes 5 to 8), consistent with the data showed in Figure 2B and C. Most notably, doxorubicin exposure has increased  $\gamma$ H2AX; nevertheless, this effect has been significantly higher in BRCA1 overexpressing cells (Fig. 3A).

To further investigate whether ATM modulation and H2AX phosphorylation by BRCA1 after genotoxic stress stimulation depends on ATM synthesis, we have exposed the PC3 overexpressing cells to the transcription inhibitor Actinomycin D. As shown in Figure 3B, we have found that transcription inhibition has significantly decreased doxorubicin-induced  $\gamma$ H2AX. Furthermore, we observed that, at short times (6 h), Actinomycin D exposure has no effect over H2AX phosphorylation (Fig. 3B, lanes 1 versus 4). Nevertheless administration of doxorubicin plus Actinomycin D markedly modified phosphorylation of H2AX (Fig. 3B, lanes

**Table 1.** Occurrence of E2F family TFBSs within -4000 to +400 of the TSS of ATM gene.

Matrix Family	Matrix	Sequence	Position from TSS		
			Start position	End position	Anchor position
V\$E2FF	V\$E2F.03	tagggGCGCggaggcaa	357	373	365
V\$E2FF	V\$E2F.02	acactgccCAAACat	90	106	98
V\$E2FF	V\$E2F3.01	gaaggGCGAgcgcaaa	15	31	23
<b>V\$E2FF</b>	<b>V\$E2F1_DP2.01</b>	<b>ggagGCGGggaggacg</b>	<b>-44</b>	<b>-28</b>	<b>-36</b>
<b>V\$E2FF</b>	<b>V\$E2F1_DP1.01</b>	<b>gaggGCGGgggatgagg</b>	<b>-59</b>	<b>-43</b>	<b>-51</b>
V\$E2FF	V\$E2F3.02	gaaagGCGCgaaatga	-128	-112	-120
V\$E2FF	V\$E2F3.02	tctcgGCGCcttcttt	-131	-115	-123
<b>V\$E2FF</b>	<b>V\$E2F1_DP1.01</b>	<b>aagaGCGGggacaatt</b>	<b>-181</b>	<b>-165</b>	<b>-173</b>
V\$E2FF	V\$E2F4_DP2.01	aactcGCGGcaattgt	-191	-175	-183
V\$E2FF	V\$RB_E2F1_DP1.01	aattgCCGCgagttcag	-194	-178	-186
V\$E2FF	V\$E2F4_DP1.01	aaattgCGCCcaagggt	-692	-676	-684
V\$E2FF	V\$E2F4_DP1.01	accttgGCGCcaatttc	-693	-677	-685
V\$E2FF	V\$E2F3.02	gaaggGCGCcgaggagg	-779	-763	-771
V\$E2FF	V\$E2F3.02	tcttgGCGCcttctaa	-782	-766	-774
<b>V\$E2FF</b>	<b>V\$E2F1_DP2.01</b>	<b>taagGCGGggttattag</b>	<b>-795</b>	<b>-779</b>	<b>-787</b>
V\$E2FF	V\$E2F.01	ttgatcagcGAAAaatt	-929	-913	-921
V\$E2FF	V\$E2F.02	gatcagcgaAAAAtttc	-931	-915	-923
V\$E2FF	V\$E2F.01	ctagggctaGAAAacgt	-976	-960	-968
V\$E2FF	V\$E2F.01	ggtggcagtGAAAatgg	-1905	-1889	-1897
V\$E2FF	V\$E2F.01	gatttgctaGAAAaaaa	-2293	-2277	-2285
V\$E2FF	V\$E2F.01	ctaaggggaGAAAactt	-2365	-2349	-2357
V\$E2FF	V\$E2F.01	ggaatggggGAAAaata	-3620	-3604	-3612

E2F binding site sequences found in ATM gene. Upstream gene regulatory sequences from -4000 to +400 bp relative to the TSS of each gene were extracted with the Gene2promoter module of the GenomatixSuite 3.4.1. (Munich). Matches for transcription factor binding site matrix families were searched using optimized thresholds for each matrix family.

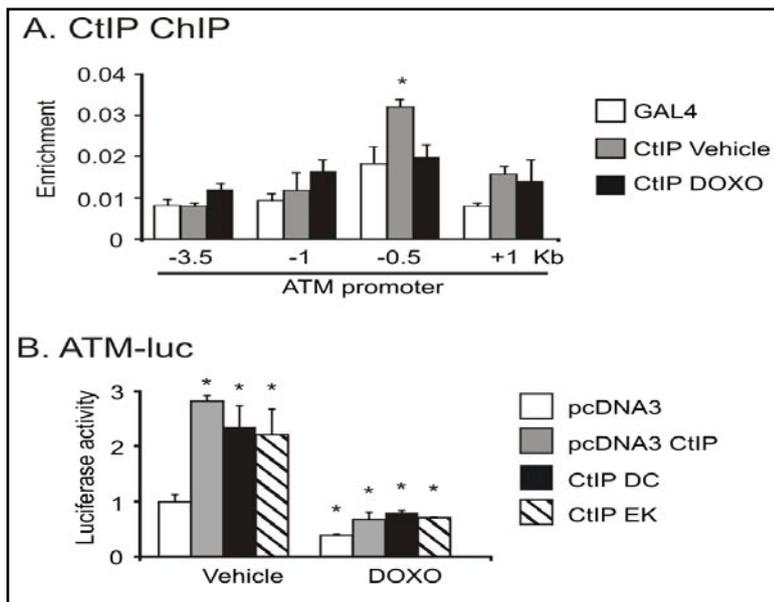
2 versus 3). As shown in Figure 3C, H2AX expression is not significantly affected by Actinomycin D or Doxorubicin treatments.

Altogether, these results suggest that the effect of doxorubicin over  $\gamma$ H2AX is caused by BRCA1 overexpression and mediated by ATM synthesis under DNA damage insults.

#### *E2F1 binds to ATM proximal promoter*

Recently, we have reported that BRCA1 represses its own transcription through E2F1/Rb protein interaction [17]. Furthermore, under genotoxic stress, BRCA1 and E2F1 are released from BRCA1 promoter and it associates to other targets during DNA damage response [17, 19]. Based on these evidences, we have speculated that ATM regulation by BRCA1 might be mediated by E2F1. We have first localized several E2F putative binding sites within ATM promoter using MatInspector (Genomatix) (Table 1). Interestingly, this examination has revealed four E2F1 binding sites at 36, 51, 173 and 787 bp upstream of the ATM TSS. Based on this, we have performed E2F1-ChIP-qPCR assay using primers spanning every 500 bp along ATM promoter. As shown in Figure 4A, E2F1 binds to ATM promoter at 0.5 Kb from the TSS, before and after doxorubicin exposure. In addition, the ChIP assay resolution is

**Fig. 5.** CtIP association with ATM proximal promoter region induces ATM transcription and this regulation is abolished by genotoxic stress. A. PC3 cells were exposed to doxorubicin (DOXO 1  $\mu$ M) or vehicle and ChIP analysis using CtIP-specific or nonspecific (GAL4) antibodies was performed. DNA-ChIP was analyzed by qPCR using primers located at 3.5 Kb, 1 Kb and 0.5 Kb upstream or 1 Kb downstream from ATM TSS. Fold enrichment was calculated normalizing data to input. B. PC3 cell line was co-transfected with ATM-luc plasmid and CtIP *wild type* (pcDNA3 CtIP) or mutated (CtIP DC and CtIP EK) expression vectors. Cells were exposed to doxorubicin (1  $\mu$ M, 24h). Luciferase activity was measured. Data were normalized to total protein and control. \* =  $p < 0.05$ .



considered around 1,000 bp from primer location. This is due to the chromatin shearing by sonication, which was performed to produce fragments between 100 bp and 500 bp long. The putative E2F1 binding sites listed on the Table 1 are located at 36, 51, 173 and 787 bp upstream from the TSS, thus using primers at 500 bp upstream from TSS should detect the E2F1 binding to any of those sites. However, considering that at 1,000 bp upstream from TSS there is no enrichment, it is probably that E2F1 is binding to all the sites except -787.

#### *E2F1 represses ATM transcription during DNA damage response*

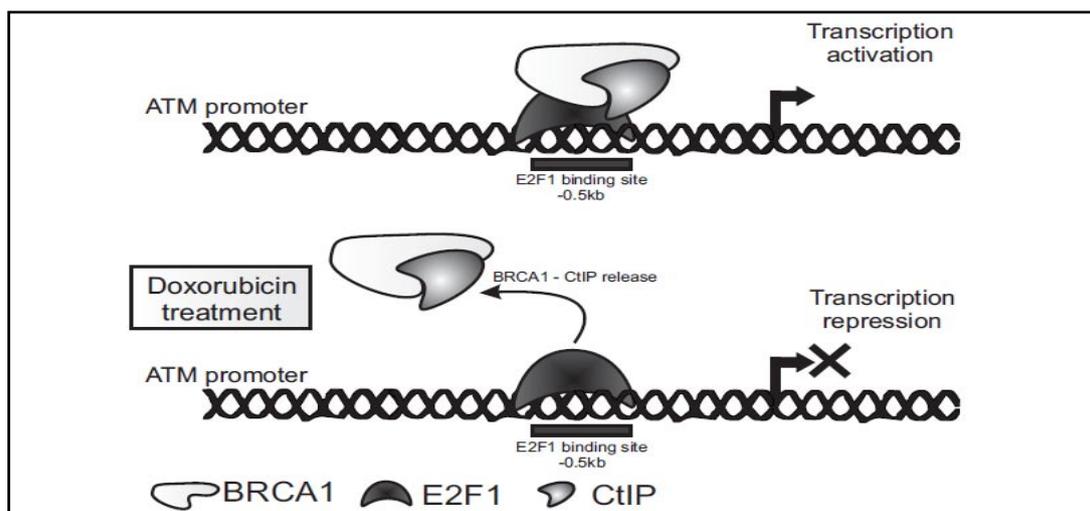
To determine whether E2F1 regulates ATM transcription in PC3 cells we have co-transfected PC3 cells with ATM reporter plasmid and E2F1 *wild type* expression plasmid, E2F1 dominant negative mutant plasmids or control plasmid. As shown in Figure 4B, E2F1 *wild type* represses ATM transcription in both vehicle and doxorubicin exposure. As shown above, we found that E2F1 has no effect over ATM transcription by RT-qPCR in control cells (Fig. 1A). Due to ATM luciferase construct contains only a portion of ATM promoter, we speculate that probably other factors or conformational configurations along ATM promoter might be involved in this regulation.

Co-transfecting ATM reporter plasmid with E2F1 dominant negative vectors, such as E2F1 E132 (E2F1 with a double point mutation in the DNA binding domain) or E2F1  $\Delta$ (1-363) (E2F1 mutant lacking the trans-activation domain), we have found that ATM promoter activity has been highly induced in the absence of doxorubicin (Fig. 4B).

Altogether, these results suggest that BRCA1 and E2F1 bind ATM promoter. However, after doxorubicin treatment BRCA1 is released allowing E2F1 to markedly repress ATM transcription.

#### *CtIP binds and induces ATM promoter*

It has been previously reported that the ZBRK1 promoter contains an authentic E2F-recognition sequence that specifically binds E2F1 together with the chromatin remodeling proteins CtIP and CtBP to form a repression complex that suppresses ZBRK1 transcription [24]. In addition it is known that BRCA1 protein directly interacts with CtIP [25]. To further



**Fig. 6.** ATM transcription regulation proposed model.

investigate CtIP role in ATM regulation by BRCA1/E2F1 proteins, we have first studied CtIP binding to ATM promoter by CtIP-ChIP-qPCR. We have found that CtIP binds to ATM proximal promoter, but after DNA damage CtIP is released (Fig. 5A).

We have co-transfected PC3 cells with ATM reporter plasmid and CtIP *wild type* expression plasmid or control vector and we have found that CtIP significantly induces ATM promoter activity (Fig. 5B). Despite the fact that we found that CtIP has no effect over ATM transcription by RT-qPCR in control cells (Fig. 1A) we believe that these differences might be due to ATM luciferase construct contains only a portion of ATM promoter, suggesting that probably other factors might be involved in this regulation.

To determine if this induction is dependent on Rb or CtBP co-regulators, we have co-transfected PC3 cells with ATM reporter plasmid and CtIP DC (mutant unable to interact with CtBP protein) or CtIP EK (mutant unable to interact with Rb protein) plasmids. As shown in Figure 5B, we have found that CtIP mutants do not change the effect triggered by CtIP *wild type* suggesting that ATM induction by CtIP is independent of the presence of the CtBP and Rb co-regulator proteins.

## Discussion

The cellular response to DNA damage is a complex process that involves a network of interacting signal transduction pathways [26, 27]. It is initiated by several proteins, such as ATM, which detect or sense DNA damage and subsequently transmit a signal by activating a cascade of phosphorylation events. These results in a variety of cellular responses, including activation of cell cycle checkpoints, commencement of transcriptional programs, execution of DNA repair and, in some cases, induction of cell death. Thus, it is tempting to speculate that only post-translational regulation mechanisms of ATM are important for DNA damage response. In the present study we have addressed further questions: is the activation of ATM transcription a relevant process to control DNA damage response? What are the key ATM transcription regulators that lead to aberrant ATM expression in tumor cells? Hence, the main goal of this work has been to identify the factors involved in transcriptional activation of ATM.

Previously, we have identified a novel transcriptional mechanism for BRCA1 auto-regulation of transcription in response to genotoxic insult [17]. We have found that BRCA1 assembles with complexes containing E2F1 and RB to form a repressive multicomponent

transcriptional complex that inhibits BRCA1 transcription. This complex is disrupted by genotoxic stress, resulting in the displacement of BRCA1 protein from the BRCA1 promoter and subsequent upregulation of its transcription [17]. In addition, we have previously reported that BRCA1 plays a central role in the transcriptional response to genotoxic stress in PCa, identifying multiple regulators of genome stability and cell-cycle control as direct transcriptional targets of BRCA1 [19]. In this work, we have defined a potential mechanism for ATM transcriptional regulation in PCa. We have shown that ATM promoter contains an E2F1 binding site that may serve as the platform for the recruitment of the BRCA1/E2F1/CtIP proteins (Table 1, Fig. 2A, 4A and 5A) activating ATM transcription (Fig. 1A, 2B-C). Nevertheless, after doxorubicin exposure, BRCA1 and CtIP are released (Fig. 2A and 5A), keeping E2F1 protein still recruited to repress ATM expression (Fig. 4). A hypothetical model is shown in Figure 6.

The E2F1 transcription factor is a critical downstream target of the tumor suppressor, RB. When activated, E2F1 induces cell proliferation. In addition, deregulation of E2F1 constitutes an oncogenic stress that can induce apoptosis [11]. The protein kinase ATM is a pivotal mediator of the response to another type of stress, genotoxic stress. Previously, Berkovich and Ginsberg [11] reported that E2F1 elevates ATM promoter activity and induces an increase in ATM mRNA and protein levels. This is accompanied by an E2F induced increase in p53 phosphorylation. Our results seem to be opposite; however, in this work we used tumor p53<sup>-/-</sup> cells and the results obtained by Berkovich and Ginsberg [11] were found in a non tumor p53 *wild type* cells.

As we have mentioned, the cellular response to DNA damage is critical to determine whether carcinogenesis, cell death or other deleterious biological effects will ensue [27]. Numerous cellular mechanisms can directly repair damaged DNA and thus reduce potentially harmful effects. DNA damage induces activation of cell cycle checkpoints and, as a consequence, cell cycle is delayed. This allows extra time for the repair before the entry of cells into critical phases of the cell cycle. When damage is excessive, apoptotic cellular suicide mechanisms can be induced. Chemotherapy agents used to treat cancer patients often cause DNA damage. Therefore, alteration of DNA damage response genes and proteins should be considered as an important avenue to improve cancer therapy which has not been fully exploited yet. Numerous clinical trials are progressing in this field. Clearly, the coupling of conventionally established therapies targeting gene expression profiling, especially with respect to damage response elements, might generate extremely valuable tools to treat cancer patients, and perhaps individuals with other diseases as well.

It is important to mention that ATM mutations correlate with high PCa risk [28, 29] and BRCA1 mutations associates with more aggressive PCa phenotype [30, 31]. Based on this and considering the role for BRCA1 and ATM sensing DNA damage response, we believe that mutations on these genes will provide patients a particular chemotherapy response that should be considered to design new therapeutic strategies. Hence, personalized medicine for this type of patients should be considered.

In summary, we demonstrate in this study that DNA damage caused by topoisomerase poisons leads to the repression of ATM transcription mediated by E2F1 binding to the ATM promoter collectively with the BRCA1/CtIP proteins release. Taken together all these data may have implications on the developing of new therapeutic interventions for patients with PCa.

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