BMI1 REDUCES ATM AND ATR ACTIVATION

BMI1 REDUCES ATM AND ATR ACTIVATION DURING DNA DAMAGE RESPONSE THROUGH BINDING TO NBS1 AND TOPBP1

By

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ABSTRACT

DNA damage response (DDR) maintains genome integrity through checkpoint activation and lesion repair. While ATM and ATR are essential in DDR, mechanisms regulating their activation remain unclear. BMI1 is a component of the polycomb repressive complex 1 (PRC1), and contributes to PRC1's E3 ubiquitin (E3-Ub) ligase activity though binding the catalytic subunit RING2. BMI1 binds RING2 through its ring finger (RF) domain. The E3-Ub ligase activity contributes to BMI1-deirved facilitation of the homologous recombination-based repair of DNA double-stranded breaks (DSBs).

My research demonstrates that BMI1 reduces ATM and ATR activation during DDR. DSBs and single-strand DNA (ssDNA) lesions respectively activate ATM and ATR. ATM subsequently phosphorylates CHK2 at threonine 68 (CHK2pT68) and induces G2/M arrest. ATR produces CHK1pS345 and S-phase arrest. Both kinases phosphorylate histone H2AX at serine 139 (γH2AX) to prepare for lesion repair. Hydroxyurea initiates DDR via producing ssDNA lesions, and increases ATR activation (phosphorylation of T1989/ATR pT1989), CHK1pS345, γH2AX, and S-phase arrest. These events were significantly reduced and enhanced following the respective BMI1 overexpression and BMI1 knockdown in MCF7 and DU145 cells. BMI1 also displays similar effects towards ATM during DDR induced by etoposide-caused DSBs.

Activation of ATM and ATR requires the formation of the ATM-NBS1 and ATR-TOPBP1 complexes. We observed that BMI1 interacted with NBS1 or TOPBP1. Deletion of the RF domain from BMI1 did not affect the associations and also had no effects on BMI1's activity in reducing ATM activation and ATR-mediated CHK1 pS345.

Collectively, our research suggests that BMI1 attenuates ATM and ATR signaling independently of the E3-Ub ligase activity.

Genotoxic treatments elicit DDR in cells that are directly exposed and also in cells that are not exposed, a phenomenon known as bystander effect (BE). However, it remains unclear what mediates the BE. Microvesicles are small membrane-enclosed sacks that are shed from donor cells and communicate specific messages to recipient cells. We demonstrated that microvesicles isolated from cells treated with etoposide and ultraviolet induced BE in recipient cells. Neutralization of microvesicles through annexin V reduced the microvesicles-associated BE.

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PREFACE

This thesis is prepared in a "sandwich" thesis format as outlined in the "Guide for the preparation of Master's and Doctoral Theses" available through the School of Graduate Studies at McMaster University. Chapter 1 provides a general introduction and overall objectives of this thesis. Chapter 2 to 4 each consists of an independent study organized into a complete manuscript, preceding with a description of the study, author's contributions and relation to this thesis. While Chapter 2 and Chapter 4 have been published, Chapter 3 has been submitted as a revised manuscript at the time of this thesis submission. The Ph.D. candidate is the first/co-first author of all submitted publications and manuscripts contained in this thesis. References cited within all the manuscripts are formatted consistently with the rest of this thesis. Lastly, Chapter 5 discusses the overall implications of the three studies, future directions and the clinical relevance. Permissions to reprint manuscripts of published work are included in the final Appendix section.

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AAD	ATR activation domain
AKT/PKB	Protein kinase B
ATP	Adenosine triphosphate
ADP-ribose	Adenosine diphosphate ribose
ATLD	Ataxia telangiectasia-like disorder
ATM	Ataxia telangiectasia mutated
ATM pS1981	Autophosphorylation of ATM at serine 1981
ATR pT1989	Autophosphorylation of ATR at threonine 1989
ATRIP	ATR-interacting protein
AP site	apurinic/apyrimidinic site**,** also known as abasic site
XLF	XRCC4-like factor
BE	Bystander effect
BER	Base excision repair
BRCA1	Breast cancer 1, early onset
BRCT	Breast cancer carboxyl terminus
B-NHEJ	Backup/alternative NHEJ
cDNA	Complementary deoxyribonucleic acid
CDK	Cyclin dependent kinase
CHK1 pS345	Phosphorylation of CHK1 at serine 345
CHK2 pT68	Phosphorylation of CHK2 at threonine 68
CLM	calicheamicin
DAPI	4',6-diamidino-2-phenylindole
DDR	DNA damage response
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
D-NHEJ	DNA-PKcs dependent NHEJ
dsDNA	Double-stranded DNA
EBRT	External beam radiation therapy
ECM	Extracellular matrix
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ERCC1	Excision repair cross-complementing protein 1
EGFR	Epidermal growth factor receptor

EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
EM	Electron microscopy
EtOH	Ethanol
F-12	Ham's F12 nutrient medium
FAT	FRAP-ATM-TRRAP motif
FATC	FAT carboxy-terminal domain
GBM	Glioblastoma multiforme cells
HEK 293T	Human embryonic kidney 293T
HEAT	Huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast kinase TOR1.
HR	Homologous recombination
HRP	Horseradish peroxidase
hTERT	Telomerase reverse transcriptase
IL-6	Interleukin 6
IL-8	Interleukin 8
IgG	Immunoglobulin G
IP	Immunoprecipitation
IR	Ionizing radiation
IRIF	Ionizing radiation-induced foci
LIG4	DNA ligase IV
LIG3	DNA ligase III
MAPK	Mitogen-activated protein kinase
MEFs	Mouse embryonic fibroblasts
MDM2	Mouse double minute 2 homolog
MDC1	Mediator of DNA damage checkpoint protein 1
M phase	Mitotic phase
mRNA	Messenger ribonucleic acid
MRN	Mre11/Rad50/Nbs1
MVs	microvesicles
NaCl	Sodium chloride
NCS	neocarcinostain
NER	Nucleotide excision repair
NHEJ	Non-homologous end-joining
NLS	Nuclear localization signal
P53 pS15	Phosphorylation of p53 at serine 15
PAGE	Polyacrylamide gel electrophoresis

PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCNA	Proliferating cell nuclear antigen
PcG	Polycomb group
PEST	a peptide sequence that is rich in proline (P), glutamic acid (E), serine (S), and threonine (T)
PI	Phosphatidylinositol
PI3K	Phosphoinositide-3-kinase
PI3P	Phosphatidylinositol-3-phosphate
PIKKs	PI3K related kinases
PIN	Prostatic intraepithelial neoplasia
PIP_2	Phosphotidylinositol-4, 5-bisphosphate
PIP_3	Phosphotidylinositol-3, 4, 5-triphosphate
PMSF	Phenylmethanesulfonyl fluoride
PRD	PIK regulatory domain
PRC	Polycomb repressive complex
PREs	Polycomb response elements
PTEN	Phosphatase and tensin homolog deleted on chromosome ten
qRT-PCR	Real-time polymerase chain reaction analysis
Rb	Retinoblastoma protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPA	Replication protein A
SDS	Sodium dodecyl sulfate
SE	Standard error
ssDNA	Single-stranded DNA
SSBs	Single-stranded DNA breaks
shRNA	Short hairpin ribonucleic acid
TGF-β	Transforming growth factor-β
TNFα	Tumor necrosis factor α
TFIIH	Transcription factor II H
TOPBP1	Topoisomerase-binding protein
tTG	transgulutaminase
UV	Ultraviolet

VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WHO	World Health Organization
XRCC4	X-ray cross complementing 4
γH2AX	H2A histone family, member X, phosphorylated at S139
53BP1	P-53 binding protein
9-1-1	Rad9-Rad1-Hus1

CHAPTER ONE

INTRODUCTION AND OVERALL OBJECTIVES

1.0 INTRODUCTION

1.1 DNA damage response (DDR)

1.1.1 Introduction of DNA damage response (DDR)

Maintaining of genome stability is a central issue of cell biology, owing to the need to reserve the genetic information and faithfully pass the DNA sequences to the daughter cells. This task is important as DNA is continually exposed to a series of insults including those come from environment and generated from inside cells. Exogenous genotoxins include the ultraviolet (UV) component of sunlight, cigarette smoke and ionizing radiation (IR). Endogenous sources of DNA damage involve reactive oxygen species (ROS) produced during metabolism and errors occurred during DNA replication. Intriguingly, cells can be experienced DNA damage without being directly exposed to genotoxins, a phenomenon known as bystander effect (BE). Furthermore, DNA damage is also a physiological and development process, such as the V(D)J recombination. Mirroring to the existence of complex genotoxins, a variety of types of DNA lesions are produced, including base mismatches, base alkylation, single-stranded breaks (SSBs) and double-stranded breaks (DSBs) (Lord and Ashworth 2012). Erroneous rejoining of DSBs can lead to chromosomal aberrations and gene mutations, making DSBs the most toxic form of DNA lesions and thus the most potent threat to genome integrity (Vamvakas, Vock et al. 1997, Khanna and Jackson 2001). Regardless how DNA lesions are induced, all lesions need to be repaired.

Given the potentially devastating effects of genomic instability, mammalian cells have evolved a complex process, DNA damage response (DDR), to repair DNA lesions in a coordinated manner in order to maintain genome integrity. Owing to the extensive research effort performed in the past decades, our understanding on the pathways regulating DDR has been substantially advanced. Mechanisms underlying DDR regulation are highly conserved. Different types of DNA lesions are initially detected by a set of unique sensors, which subsequently relay specific signals to downstream targets to activate specific checkpoints to hold cell cycle progression and to repair DNA lesions. During this process, cells will also make a decision to undergo apoptosis if the damage is irreparable (Harper and Elledge 2007). Among a variety of sensors are the poly (ADP-ribose) polymerase (PARP) family and three PI3 kinase related kinases (PIKKs), ATM (ataxia telangiectasia mutated) kinase, ATR (ATM- and RAD3-related) kinase, and DNA-PKcs (the catalytic subunit of DNA-dependent protein kinase). Activation of these kinases results in phosphorylation of their specific targets, which act as mediators/transducers to assemble lesion repair complexes by recruiting additional proteins and also to initiate checkpoint activation. Figure 1.1 illustrates the function of ATM in the process of checkpoint activation and repair of DSBs in response to DSBs caused by genotoxic materials. In this regard, different primary types of DNA lesions will initiate different types of DDR. For example, single-strand DNA lesions primarily activate ATR (Cimprich and Cortez 2008) (Fig 1.3). In the rest of this chapter, I will briefly discuss DNA lesion repair, ATM and ATR activation, checkpoint activation, and BMI1-involved regulation of ATM and ATR activation.

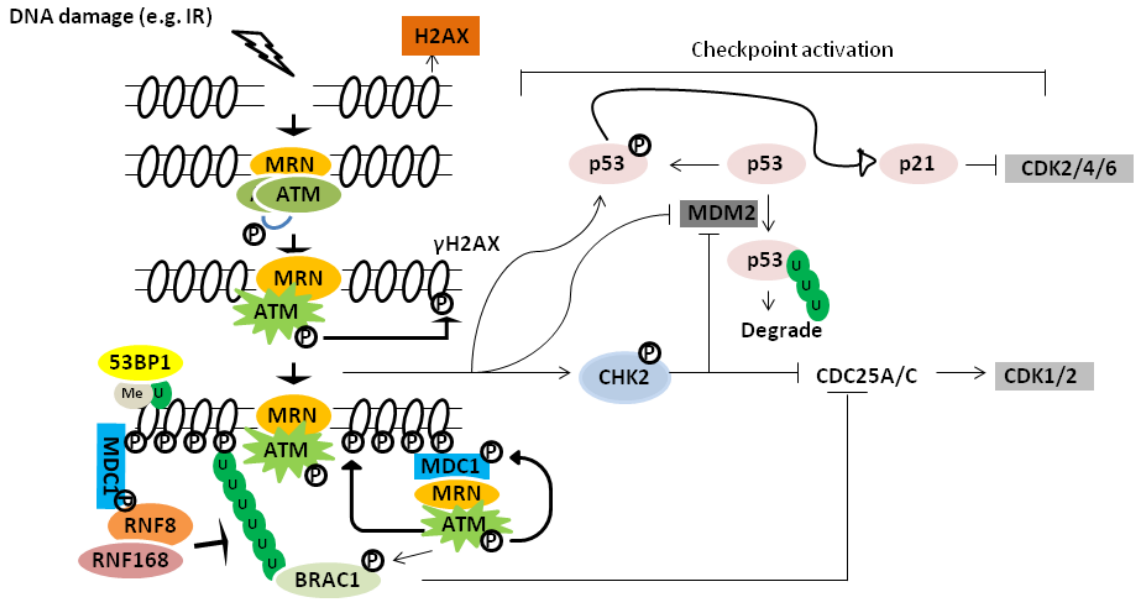

Figure 1.1 Schematic illustration of double-stranded DNA breaks (DSBs)-induced ATM signaling. IR induces a double strand breaks (DSBs), to which the MRN complex together with ATM is recruited, followed by ATM autophosphorylation at S1981 (ATM pS1981) and activation. ATM then phosphorylates S139 of H2AX (γH2AX), T68 of CHK2 (CHK2 pT68) and S15 of P53 (P53 pS15). The actions of CHK2 and P53 subsequently inhibit CDKs' activation via the indicated factors. γH2AX recruits MDC1; the latter binds the MRN complex through NBS1; through MRN, ATM is recruited; and ATM then produces additional γH2AX. In this manner, γH2AX spreads across a long stretch region surrounding a DSB. The association of the MRN-ATM complex with MDC1 leads to ATM-mediated phosphorylation of MDC1 and the subsequent recruitment of RNF8 and RNF168. RNF8 and RNF168 coordinately ubiquitinate histone H2A/H2AX, paving the way for BRCA1 recruitment. The ubiquitination of H2A at K15 together with H4 K20 methylation contribute to 53BP1 recruitment. The recruitment of 53BP1 and BRCA1 (note: both may not be recruited at the same DSBs) sets a stage for DSBs repair.

1.1.2 DNA lesion repair

The choice of repair mechanism is largely defined by the type of lesion and the stage of the cell cycle (Lord and Ashworth 2012). Most of the subtle changes to the DNA that do not strongly disturb the DNA double-helix structure, such as alkylation products and

SSBs, are substrates for Base Excision Repair (BER) (Giglia-Mari, Zotter et al. 2011). In BER, damaged bases are removed from the sugar-phosphate backbone by lesion-specific DNA glycosylases and the resulting abasic (AP) site is incised by AP-endonucleases, producing SSBs (Giglia-Mari, Zotter et al. 2011). These SSBs and other SSBs that are directly caused by ROS (reactive oxygen species) are sensed by poly-ADP-Ribose-Polymerase (PARP), which recruits repair factors to fill the single nucleotide gap with newly synthesized DNA (Caldecott 2007). Whereas bases with small chemical alterations are repaired by BER, bulky DNA lesions that distort the DNA helical structure, such as those caused by UV-irradiation, are removed by Nucleotide Excision Repair (NER) (Lord and Ashworth 2012). There are two different modes of NER: transcription-coupled NER, where damage is detected by elongating RNA polymerase, and global genome NER, in which the lesion is detected not as part of a blocked transcription but anywhere in the genome (Giglia-Mari, Zotter et al. 2011). Although these two processes sense lesions using different mechanisms, the subsequent steps converge into a common mechanism: the damaged DNA segment is opened by the NER/basal transcription factor TFIIH and incised by excision repair cross-complementing protein 1 (ERCC1); the resulting single strand gap is filled in by normal DNA replication proteins (Giglia-Mari, Zotter et al. 2011). Base mismatches are also recognized as DNA lesions because they can form 'insertion and deletion' loops that distort the helical structure of DNA, and they are repaired by mismatch repair proteins encoded by *MSH2 and MLH1* (Lord and Ashworth 2012).

The most toxic form of DNA lesions is double-strand breaks. Under the physiological processes of V(D)J recombination and recombination in meiosis, DSBs are produced. Genotoxic stimuli IR and ROS induce DSBs. Additionally, DSBs can also be produced when DNA replication forks encounter DNA single-stranded breaks (Khanna and Jackson 2001). There are two distinct mechanisms for DSBs repair - homologous recombination (HR) and non-homologous end-joining (NHEJ). The NHEJ can be subdivided in DNA-PK dependent NHEJ (D-NHEJ) and alternative/backup NHEJ (B-NHEJ) (Mladenov, Magin et al. 2013). In HR, the DNA ends first bind Mre11/Rad50/Nbs1 (MRN) complex, which hold the broken pieces together and catalyzes 5' to 3' end resection with exonuclease I on the broken DNA ends (Giglia-Mari, Zotter et al. 2011). The resulting 3' single-strand DNA (ssDNA) tails initially bind replication protein A (RPA) and subsequently replace it with Rad51 recombinase, forming a Rad51 nucleo-protein filament (Mehta and Haber 2014). This Rad51-filament is crucial for 3' ssDNA to invade the homologous sister chromatid and use the undamaged partner as a template for the synthesis of new DNA for DSBs repair (Mehta and Haber 2014). Additionally, BRCA1 and BRCA2 are also important in HR, providing mediator function: through physical interaction with RPA coated ssDNA and Rad51, BRCA2 enables Rad51 to gain access to ssDNA (Holloman 2011). Collectively, HR requires alignment of the broken DNA fragment with a homologous region on the sister chromatid, and is an accurate mechanism to restore DNA sequence. HR predominantly occurs in the S/G2 phase of the cell cycle (Symington and Gautier 2011). NHEJ repairs DSBs by directly ligating broken ends together without using a homologous DNA

template to guide the repair. Within D-NHEJ, DNA breaks are recognized by Ku70/80 heterodimer that subsequent recruits and activates the catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs), forming the active DNA-PK holoenzyme (Mladenov and Iliakis 2011). Formation of this complex leads to autophosphorylation of DNA-PKcs, which results in a conformation change in the holoenzyme. This allows DNA ends to be processed by DNA polymerase and endonuclease Artemis (to make ends compatible); the ends will then be joined by DNA ligase IV (LIG4), the cofactor X-ray cross complementing 4 (XRCC4), and the auxiliary factor XLF (Mladenov and Iliakis 2011, Mladenov, Magin et al. 2013). The repairing process is potentially associated with additions and deletions of nucleotides, making D-NHEJ an error-prone process. Collectively, D-NHEJ restores DNA integrity efficiently with compromising repair fidelity. Although it is not restricted to specific phases of cell cycle, DSBs in G1 are repaired by D-NHEJ (Rothkamm, Kruger et al. 2003), considering that HR does not occur in G1 in part attributable to cyclin-dependent kinase 1 (CDK1) being inactive. CDK1 activity is required for 5' to 3' DNA ends resection and for the recruitment of RPA and Rad51 in HR (Ira, Pellicioli et al. 2004). Additionally, DNA-PK blocks HR in G1 phase (Cui, Yu et al. 2005).

During the past decades, a second pathway which operates NHEJ in a slower kinetics and mainly act as a backup for D-NHEJ (B-NHEJ) was discovered (Mladenov, Magin et al. 2013). B-NHEJ involves PARP-1 for DNA end-binding, MRN complex for processing the DNA ends, and DNA ligase III and its cofactor XRCC1 for joining the ends (Mansour, Rhein et al. 2010, Della-Maria, Zhou et al. 2011). Although it is not

required, microhomology was reported to facilitate B-NHEJ, which may explain why its activity is increased during S and G2 phase (Mansour, Rhein et al. 2010, Decottignies 2013). More importantly, B-NHEJ is more error-prone with increase in translocations and other genomic rearrangement compared to D-NHEJ (Mladenov and Iliakis 2011). B-NHEJ involves single–strand annealing, which is associated with intrachromosomal deletions; B-NHEJ is executed via the removal of Ku proteins, which increases the probability of joining wrong DNA ends (Mladenov and Iliakis 2011).

The use of two different systems, HR and NHEJ, in mammalian cells to repair DSBs meets the need to repair DSBs efficiently and serves specific purposes (Shrivastav, De Haro et al. 2008). For example, while HR is the dominant mechanism of DSB repair in yeast, NHEJ is the major pathway for DSB repair in higher eukaryotes. For V(D)J recombination, DNA-PK-mediated NHEJ is essential. There is a complex relationship between the two pathways. On the one hand, evidence suggests competitions between both, as the efficiency of HR increases in the absence of Ku (required for D-NHEJ) (Clerici, Mantiero et al. 2008). On the other hand, loss of HR proteins does not increase D-NHEJ efficiency, suggesting that D-NHEJ acts first in DSBs repair (Clerici, Mantiero et al. 2008, Symington and Gautier 2011). In consistent with this model, abundant Ku rapidly binds to all DSBs, suggesting D-NHEJ being the primary choice for cells to repair DSBs (Shibata, Conrad et al. 2011). Both pathways are required for DSB repair as the individual pathway functions in different phases of cell cycle. HR works efficiently in the late of the G2 phase when DNA replication is completed and the sister chromatid is available as a repair template. Consistently, the end resection is promoted by CDK1

activity in S phase through the phosphorylation of multiple resection proteins (Chiruvella, Liang et al. 2013). As CDK1 is inactive in the G1 phase, D-NHEJ is thus more prevalent in the G1 phase. With D-NHEJ being more efficient than HR, most DSBs are repaired by D-NHEJ (Beucher, Birraux et al. 2009). However, complex DSBs delays D-NHEJ and subsequently are taken over by HR (Shibata, Conrad et al. 2011). Recent studies also show crosstalk between DNA-PK and ATM regarding the regulation of DSBs repair pathway choice. Another level of regulation of HR/D-NHEJ pathway choice involves the balance between p53 binding protein (53BP1) and BRCA1. 53BP1 blocks DNA resection and favors the repair by NHEJ (Bunting, Callen et al. 2010), while BRCA1 ensures HR via promoting 53BP1 dephosphorylation (Isono, Niimi et al. 2017). Finally, recent advances have shown a competitive relationship between B-NHEJ and HR in human cells. Polθ, a polymerase that functions in B-NHEJ, binds and inhibits RAD51(Ceccaldi, Liu et al. 2015). B-NHEJ may also exploit failures in HR, as the MRN complex facilitates both processes which appear to share a common initial resection mechanism, and end resection prevents D-NHEJ repair (Chiruvella, Liang et al. 2013). Therefore, B-NHEJ promotes cancer development through enhancing mutations when D-NHEJ or HR is compromised (Mladenov, Magin et al. 2013).

1.1.3 ATR, ATM and DNA-PKcs activation in DNA damage response

As discussed above, cell cycle distribution affects how DSBs are repaired. Regulation in cell cycle progression thus constitutes a core component of DDR. To

coordinately regulate both processes, mammalian cells use three apical PIKKs, ATM, ATR and DNA-PKcs (Zhou and Elledge 2000). While both ATM and DNA-PKcs are primarily activated by DSBs, ATR is activated primarily by single strand DNA (ssDNA) lesion (Lin, Ojo et al. 2015). Consistent with being members of the PIKKs family, these three kinases share structural and functional similarities.

ATM and ATR, DNA-PKcs transduce signals to downstream targets through protein phosphorylation. While each kinase has a unique set of target proteins, these proteins all contain a conserved phosphorylation Ser/Thr-Glu (S/T-Q) motif; the S and T residues in this sequence context are phosphorylated by ATM, ATR and DNA-PKcs. Both ATM and ATR phosphorylate hundreds of target proteins, while DNA-PKcs regulates a smaller number of targets and is critical in the repair of DSBs by non-homologous end joining (NHEJ) (Dobbs, Tainer et al. 2010, Marechal and Zou 2013). The large number of target proteins for both ATM and ATR reflects their contributions to checkpoint activation during DDR.

ATM, ATR and DNA-PKcs are large serine/threonine kinases of the PIKKs family, and share the typical structure features of PIKKs, including a kinase domain that is flanked by a C-terminal FATC and N-terminal FRAP-ATM-TRRAP (FAT) motifs, the latter of which is part of a long poorly conserved N-terminal region that folds into an alpha-helical HEAT repeat structure (Cimprich and Cortez 2008, Blackford and Jackson 2017) (Fig 1.2). This structural similarity supports a general pattern governing their activation, i.e., binding to specific regulatory partners in the presence of unique DNA lesions (Burrows and Elledge 2008). As a result of their large sizes, limited high-

resolution structures of these three kinases are available, with the crystal structure of DNA-PKcs being the highest resolution for them (Sibanda, Chirgadze et al. 2010, Sibanda, Chirgadze et al. 2017). DNA-PKcs adopts a central cradle structure formed by alpha-helical HEAT repeats and sitting atop these repeats is kinase domain flanked by FAT and FATC domains. Electron microscopy (EM) studies of ATM and ATR also revealed an arm region formed by the HEAT repeats, suggesting structural resemblance to DNA-PKcs (Llorca, Rivera-Calzada et al. 2003, Sawicka, Wanrooij et al. 2016). The HEAT repeats are predicted to form superhelical scaffolding matrices and mediate interactions with DNA and their unique regulatory co-factors, such as ATRIP (for ATR) and NBS1 (for ATM) (You, Chahwan et al. 2005, Chen, Zhao et al. 2007). On the other hand FATC domain is highly conserved among PIKKs and is required for the kinase activity probably through stabilization of the kinase domain (Mordes, Glick et al. 2008). In contrast to FATC domain, the PIK regulatory domain (PRD) between the kinase and FATC domains is not required for basal kinase activity but important for further activation (Sun, Xu et al. 2007, Mordes, Glick et al. 2008). For example, mutations in ATR PRD (ATR K2589E) decreased the association with TOPBP1 and failed to support CHK1 activation as well as checkpoint activation during DDR (Mordes, Glick et al. 2008).

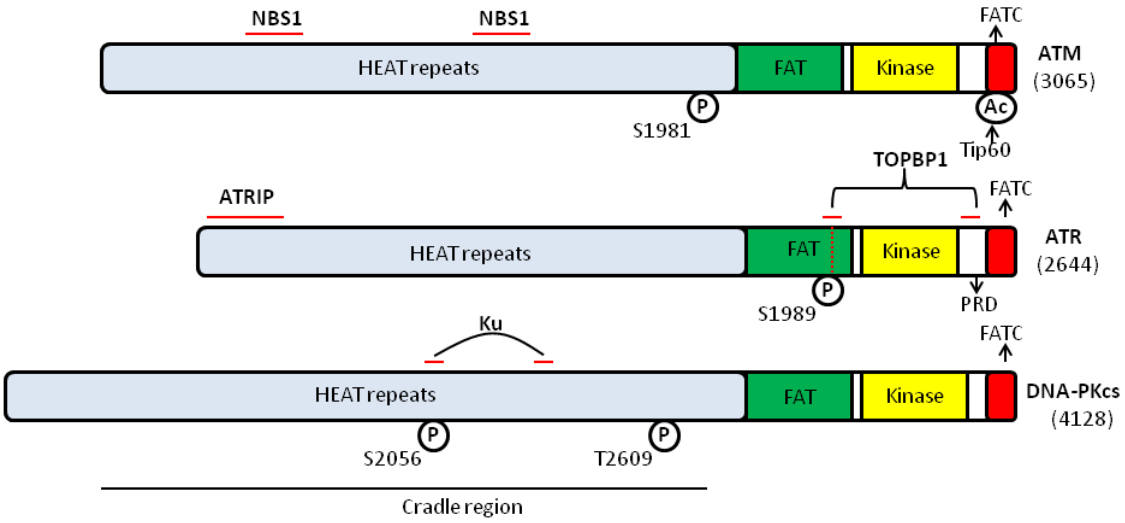

Figure 1.2 Structural features of ATR, ATM and DNA-PKcs. Heat repeats and other domains are indicated. The regions responsible for the binding of NBS1, ATRIP, and Ku subunits to ATM, ATR and DNA-PKcs, respectively, are shown. A PIKK regulatory domain (PRD) between the kinase and FATC domains in ATR is included; the PRD motif regulates ATR activation. Tip60 acetylated region, which contributes to ATM activation, is also indicated.

1.1.3.1 ATM activation and its signaling events

ATM is recruited to DSBs through the interaction with the MER11/RAD50/NBS1 (MRN) complex via directing binding to the C terminus of NBS1 (Lee and Paull 2004, Difilippantonio, Celeste et al. 2005, You, Chahwan et al. 2005, Lin, Ojo et al. 2015), resulting in the conversion of inactive ATM dimers to active monomers via S1981 autophosphorylation (ATM pS1981) (Bakkenist and Kastan 2003) (Fig 1.1). This autophosphorylation stabilizes the association of ATM and DSBs (So, Davis et al. 2009). ATM activation subsequently phosphorylates numerous targets to coordinate checkpoint activation and DNA damage repair, including H2AX at S139 (γH2AX), P53 at S15 and

CHK2 at T68 (Zhou and Elledge 2000). MRN complex is the key regulator of ATM activation and is required for optimal ATM signaling process. MRN directly binds to DSBs and stimulates ATM kinase activity towards p53, CHK2 and H2AX in vitro (Lee and Paull 2005). There is strong genetic evidence supporting the essential roles of the MRN complex in mediating ATM activation. Patients with mutations in *ATM* (ataxia telangiectasia), *MRE11* [(ataxia-telangiectasia like disorder (ATLD)], or *NBS1* (Nijmegen breakage syndrome) are sensitive to ionized radiation and have a similar clinical presentation, including immunodeficiency and cancer predisposition (Shiloh 1997, Stewart, Maser et al. 1999). Additionally, cells defective in MRE11 and in NBS1 significantly reduce ATM pS1981 and phosphorylation of ATM downstream substrates in response to DNA damage induced by neocarcinostain (NCS) (Lavin and Kozlov 2007).

ATM activation initiates chain events in DSB repair and checkpoint activation. Two critical substrates in checkpoint activation are CHK2 and P53 (Fig 1.1). ATM phosphorylates P53 at S15, which likely reduces P53's association with MDM2, and thereby prevents MDM2-mediated ubiquitination of P53 (Shieh, Ikeda et al. 1997). As a result, P53 is stabilized and its activities are enhanced. To ensure P53 stabilization, ATM also phosphorylates MDM2 on multiple sites near its RING domain, and inhibits its E3 ubiquitin ligase activity towards P53 (Cheng and Chen 2010). Additionally, through phosphorylation of MDM2 at Ser395, ATM interferes with MDM2's nucleo-cytoplasmic transportation (Khosravi, Maya et al. 1999, Maya, Balass et al. 2001). Collectively, ATM uses comprehensive actions on the P53/MDM2 complex to ensure P53 stabilization and elevations of P53 signaling. P53 plays multiple roles in checkpoint activation. One of P53

targets is $p21^{CIPI}$, an inhibitor of CDK2, CDK4 and CDK6. These kinases are required for the progression of G1 and S phase (Sherr and Roberts 1999). P53 can also downregulate genes required for transition from G2 to M phase (Stark and Taylor 2006).

A critical role of ATM in checkpoint activation is through CHK2 activation. ATM phosphorylates CHK2 at T68. CHK2 in turn phosphorylates CDC25C at S216, which produces a docking site on CDC25C for the protein 14-3-3; the interaction blocks CDC25C to associate with the CDK1-cyclin B complex (Peng, Graves et al. 1997, Sanchez, Wong et al. 1997). CDC25C is a dual specificity phosphatase that dephosphorylates the phosphotyrosine 15 (pY15) and phosphorthreonine (pT14) of CDK1, an event that is required for CDK1 activation (Peng, Graves et al. 1997, Sanchez, Wong et al. 1997, Matsuoka, Huang et al. 1998). CDK1 activity is essential for G2 and M phase progression. Apart from CDC25C, CHK2 also inactivates CDC25A in a similar manner, leading to activation of G1/S and G2/M checkpoints (Boutros, Dozier et al. 2006). Collectively, by phosphorylation and activation of CHK2, ATM induces G2/M arrest. Moreover, BRAC1, one of ATM's substrate, is also involved in inactivating CDC25C by regulating the level of 14-3-3 (Lavin and Kozlov 2007). Furthermore, CHK2 contributes to checkpoint activation through phosphorylation of P53 at Ser20, which prevents P53 binding MDM2 (Bartek, Falck et al. 2001) and thus facilitates P53-mediated G1/S phase checkpoint activation.

Finally, ATM is instrumental in the formation of γH2AX foci, an event that is essential in DSB repair (Shiloh 2003) (Fig 1.1). ATM triggers a signaling cascade involving phosphorylation, ubiquitylation and other post-translational modifications on

damaged chromatin, the key event of which is the phosphorylation of H2AX at S139 (γH2AX). γH2AX is bound by MDC1 through BRCT (breast cancer carboxyl terminus) domain (Fig 1.1), leading to full-sized γH2AX foci formation (Marechal and Zou 2013). Phosphorylated and stabilized by ATM on chromatin, MDC1 is recognized by the BRCT domain of NBS1, promoting further MRN and ATM recruitment containing γH2AX (Blackford and Jackson 2017). Besides MRN complex, γH2AX foci is essential for recruitment of 53BP1, RAD51 and BRAC1, providing amplification of signals and spreading the assembly along chromatin (Fernandez-Capetillo, Chen et al. 2002, Blackford and Jackson 2017).

1.1.3.2 ATR activation and its signaling events

ATR is primarily activated by replication protein A (RPA)-coated single-stranded DNA (ssDNA), and plays essential roles in the stabilization of replication folks or preventing collapse of replication forks during DNA synthesis (Cimprich and Cortez 2008). ATR is thus required for cell proliferation. ATR is recruited to RPA-ssDNA, which can be generated by DNA end resection and by DNA unwinding ahead of the replication fork when DNA polymerases stall. In addition to RPA-coated ssDNA, ATR activation requires ssDNA/dsDNA junctions and the interaction with ATRIP (ATR-interacting protein) and TOPBP1 (topoisomerase-binding protein 1) (Fig 1.3). ATR exists in complex with ATRIP; the complex is recruited to RPA-ssDNA via the interaction between ATRIP and RPA (Cortez, Guntuku et al. 2001, Ball, Ehrhardt et al. 2007, Burrows and Elledge 2008). ATR signalling also depends on co-localization of the

ATR/ATRIP complex with that of Rad9-Rad1-Hus1 (9-1-1). The 9-1-1 complex is related to the replicative sliding camp PCNA and recognizes a double strand DNA end that is adjacent to a stretch of RPA-ssDNA (Zou, Liu et al. 2003, Cimprich and Cortez 2008). The 9-1-1complex subsequently recruits TOPBP1 to the DNA lesions (Lee, Kumagai et al. 2007) (Fig 1.3), which allows TOPBP1 to bind ATRIP and the ATR PRD domain at the site of DNA lesions and significantly enhances ATR activity (Kumagai, Lee et al. 2006, Mordes, Glick et al. 2008). However, recent studies also show that instead of the 9-1-1 complex, MRN is required for recruitment of TOPBP1 (Duursma, Driscoll et al. 2013). They propose a new model where MRN, 9-1-1 complex and ATR/ATRIP are first loaded to an ssDNA/dsDNA junction and MRN then recruits TOPBP1; TOPBP1 subsequently binds RAD9 within the 9-1-1 complex and results in the exposure of ATR activating domain (AAD) of TOPBP1. It is likely localization of TOPBP1 to DNA lesions is a complex process which requires multiple protein interactions. Nonetheless, TOPBP1 is critical for ATR activation as TOPBP1 loss or inactivating in its AAD is lethal in mammalian cells (Zhou, Liu et al. 2013). Moreover, the independent recruitment of these proteins may be designed to ensure that checkpoint is activated only when it is needed. Unlike ATM or DNA-PKcs, none of the post-translational modifications has been identified as a reliable indicator of ATR activation until 2011 when autophosphorylation of ATR at Thr-1989 (ATRpT1989) was identified as a regulatory event for ATR activation (Liu, Shiotani et al. 2011, Nam, Zhao et al. 2011). Their results suggest that the full activation of ATR following DNA damage is sequentially driven by RPA-ssDNA, ATR autophosphorylation on T1989, and TOPBP1;

TOPBP1 then recognizes ATRpT1989 and subsequently engages ATR-ATRIP and facilitates ATR substrate recognition (Liu, Shiotani et al. 2011). ATR activation coordinates a global cellular response to prevent cell cycle progression into M phase (S arrest or apoptosis) and initiate DNA damage repair by producing γH2AX, P53 pS15 and CHK1 pS345. CHK1 activation performs similar actions as CHK2 activation with respect to inhibition of CDC25 activity (Cimprich and Cortez 2008). In addition to preventing premature mitotic entry, ATR is also critical for regulating replication by inhibiting origin firing and stabilization of replisome (Cortez 2015).

1.1.3.3 Brief discussion of DNA-PKcs activation

Activity of DNA-PKcs is substantially stimulated after being recruited to DSBs via binding to the Ku70/80 heterodimer. DNA-PKcs phosphorylates itself at multiple sites including S/T-Q as well as non-S/T-Q sites, among which phosphorylation of DNA-PKcs at S2056 (in PQR cluster) and T2609 (flanked by ABCDE cluster) are used as surrogate markers of DNA-PKcs activation (Chen, Chan et al. 2005). However, neither of them is required for DNA-PKcs kinase activity, but they are important for DNA repair as they contribute to the dissociation of DNA-PKcs from DSBs to allow subsequent DNA ligation to occur (Jette and Lees-Miller 2015).

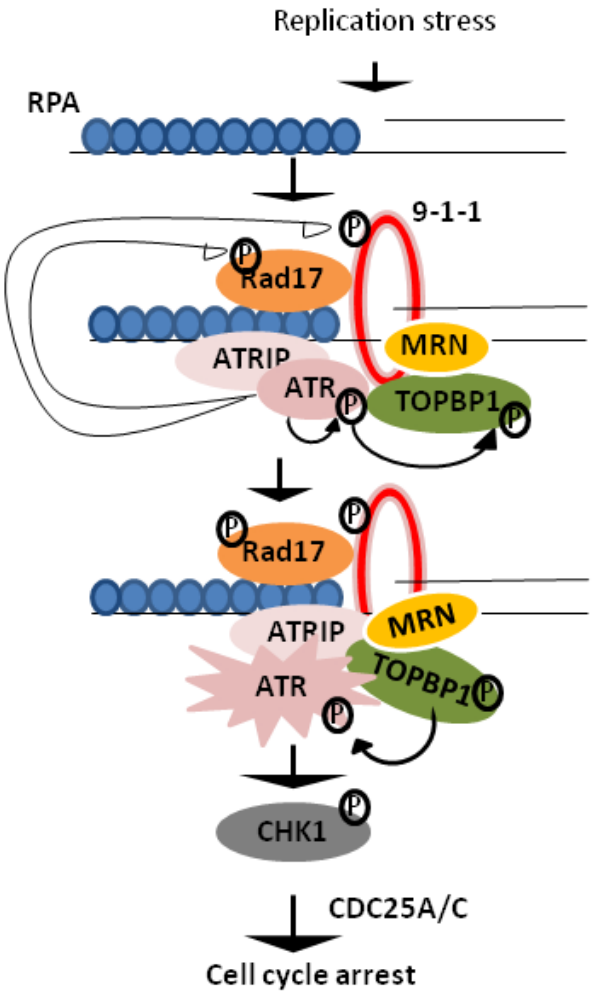

Figure 1.3 Simplified model of ATR activation at stalled replication forks. In response to stalled replication forks, ssDNA is associated with RPA. This structure recruits ATR-ATRIP, RAD17/RFC and the MRN complexes. RAD17 subsequently loads the 9-1-1 clamp complex. The 9-1-1 and possibly MRN complexes mediate TOPBP1 recruitment. The association of TOPBP1 with ATR stimulates ATR kinase activity. ATR phosphorylates 9-1-1, RAD17, TOPBP1, and CHK1. CHK1 in turn inhibits cell cycle progression.

1.1.3.4 Connections among the activation of ATM, ATR, and DNA-PKcs

Although ATR is also activated in response to ionizing radiation (IR)-induced DSBs, the activation differs from that of ATM; ATM is rapidly activated in response to

IR irrespective of the cell cycle distribution, while ATR is activated more slowly compared to ATM and predominantly in S and G2 phase (Jazayeri, Falck et al. 2006). Although there is an overlap in substrate specificity between ATM and ATR such as P53, BRCA1 and NBS1 (Tibbetts, Cortez et al. 2000, Stiff, Reis et al. 2005), CHK1 and CHK2 may be the substrates exclusively of ATR and ATM respectively. The crosstalk between ATR-CHK1 and ATM-CHK2 pathway may be largely due to the conversation between ssDNA and DSBs during the repairing process. While end resection of DSB produces a single strand region that activates ATR, nucleases can cleave ssDNA to yield DSBs that initiate ATM activation (Cimprich and Cortez 2008). This explains well that ATM controls most of G1/S checkpoint and both ATM and ATR contribute to intra-S and G2/M checkpoint activation in response to DSBs (Jazayeri, Falck et al. 2006).

Crosstalk between ATM and DNA-PK may be related to the choice between HR and NHEJ. First, DNA-PK phosphorylates partially overlapping substrates with ATM, such as γH2AX and 53BP1. Second, ATM is required for DNA end processing in NHEJ, whereas DNA-PK inhibits HR. ATM phosphorylates DNA-PKcs at T2609 which is essential for the full DNA-PK activation and the subsequent DSBs repair, suggesting a role of ATM in NHEJ (Chen, Uematsu et al. 2007). Additionally, phosphorylation of DNA-PKcs by ATM is required for DNA-PK to regulate end-processing by promoting the recruitment of the Artemis endonuclease (Jiang, Crowe et al. 2015). Recent studies indicate that DNA-PK negatively regulates ATM activity through phosphorylation and these modifications repress ATM signaling upon DNA damage (Zhou, Lee et al. 2017). Consistent with this observation, ATM is hyperactive when DNA-PK activity is inhibited

and pre-incubation of ATM with active DNA-PK impairs ATM activity (Finzel, Grybowski et al. 2016, Zhou, Lee et al. 2017). However, it should be emphasized that the critical role of DNA-PKcs in NHEJ is well established, whereas its physiological contributions to HR need further investigations.

ATR also displays connection with DNA-PK. ATR can phosphorylate DNA-PKcs at T2609 but not S2056 upon UV irradiation which is different from the response to IR induction (ATM-mediated response) (Yajima, Lee et al. 2006), suggesting T2609 being phosphorylated by ATM or ATR for a cellular response besides NHEJ, in which DNA-PKcs is likely being involved. In fact, while phosphorylation of DNA-PKcs at S2056 is indicated to block DNA end processing, phosphorylation at T2609 is predicted to promote HR (Cui, Yu et al. 2005). These two reciprocal actions provide a mechanism to prevent excessive end modification, and more importantly, a mechanism to regulate the choice of DSBs repair pathways.

The major conserved features with respect to activation of the three PIKKs include their similar domain structure, substrate specificity, and the essential elements required for their activation, i.e. specific DNA lesions and protein association. For instance, binding of NBS1, TOPBP1, and the Ku subunits is required for the respective activation of ATM, ATR, and DNA-PKcs. Nonetheless, besides this understanding, our knowledge regarding mechanisms and factors regulating their activation remains limited. Considering the essential roles of these kinases in maintaining genome stability and genome instability being the driving force of tumorigenesis, investigation of these unknown mechanisms and factors will significantly advance our understanding not only

on DDR regulation but also on the etiology of tumorigenesis. In this regard, it is interesting to see that recent developments revealed a role of BMI1 in DDR regulation (Ismail, Andrin et al. 2010, Ginjala, Nacerddine et al. 2011, Lin, Ojo et al. 2015). BMI1 is a well-regarded oncogenic protein (Valk-Lingbeek, Bruggeman et al. 2004, Gieni, Ismail et al. 2011).

1.2 BMI1

1.2.1 BMI1 mediates tumorigenesis and stem cell self-renewal

BMI1 is a member of the Polycomb group (PcG) of transcription repressors that are involved in the maintenance of embryonic and adult stem cells and also in promoting cancer development (Valk-Lingbeek, Bruggeman et al. 2004). The PcG *Bmi1*gene was identified as an oncogene that induced B or T cell leukemia in collaboration with *c-Myc* (Haupt, Alexander et al. 1991, van Lohuizen, Verbeek et al. 1991); BMI1 inhibits Myc-induced apoptosis in part through suppressing the expression of the *Ink4a/Arf* locus (Jacobs, Scheijen et al. 1999). This locus encodes two tumor suppressors, $p16^{Ink4a}$ and $p19^{Arf}$ in mice or $p14^{Arf}$ in human, by differential splicing and the use of alternative reading frames (Sharpless and DePinho 1999). $P16^{Ink4a}$ inhibits CDK4/6 activation by preventing cyclin D from binding to CDK4 and CDK6. Inhibition of CDK4 and CDK6 activation allows pRb to repress E2F1-mediated transcription activity, and thereby results in G1 arrest (Sharpless and DePinho 1999). While $P19/14^{Arf}$ does not directly inhibit

CDKs, it binds MDM2, and prevents MDM2 from inducing P53 degradation (Quelle, Zindy et al. 1995, Sharpless and DePinho 1999). P53 subsequently inhibits cell cycle progression by induction of $p21^{CIPI}$ expression, and promotes apoptosis through a variety of mechanisms including induction of Bax expression (Hudson, Morris et al. 2016). In line with its role in suppression of the *Ink4a/Arf* locus, BMI1 is required for the maintenance of self-renewal of hematopoietic and neural stem cells (Molofsky, Pardal et al. 2003, Park, Qian et al. 2003). *Bmi1*$^{-/-}$ hematopoietic progenitors expressed increased levels of INK4A and ARF, and the senescence marker SA-β-gal (Chagraoui, Niessen et al. 2006). Mice deficient in BMI1 display postnatal growth retardation, neurological defects (van der Lugt, Domen et al. 1994) and severe proliferative defects in lymphoid tissue, along with upregulation of $p16^{Ink4a}$ and $p19/14^{Arf}$ and defects in self-renewal of neural and hematopoietic cells. (Alkema, Jacobs et al. 1997, Lessard and Sauvageau 2003). These defects are partially alleviated in mice with loss of *Ink4a/Arf* locus, demonstrating that upregulation of $p16^{Ink4a}$ and $p19/14^{Arf}$ is in part attributable to BMI1-mediated self-renewal of neural and hematopoietic stem cells (Jacobs, Kieboom et al. 1999, Molofsky, Pardal et al. 2003, Bruggeman, Valk-Lingbeek et al. 2005, Molofsky, He et al. 2005). However, mice deficient in both *Bmi1* and *Ink4a/Arf* are smaller than wild type mice and their overall survival is not significantly improved from *Bmi1*$^{-/-}$ mice (Molofsky, He et al. 2005), suggesting that BMI1 also regulates other pathways. Treatment with an antioxidant or interruption of DDR by *Chk2* deletion improves survival in *Bmi1*$^{-/-}$ mice, indicating a role of BMI1 in regulating ROS homeostasis and DDR (Liu, Cao et al. 2009). Indeed, BMI1 was found to enhance antioxidant response,

contributing to the resistance to docetaxel-based chemotherapy in prostate cancer (Crea, Duhagon Serrat et al. 2011) and to cisplatin-based chemotherapy in ovarian cancer (Wang, Bhattacharyya et al. 2011).

Consistent with its role in repressing the *Ink4a/Arf* locus, accumulating evidence also demonstrates that BMI1 promotes tumorigenesis. Overexpression of BMI1 immortalized fibroblasts (Jacobs, Kieboom et al. 1999, Itahana, Zou et al. 2003), and enhanced the expression of human telomerase reverse transcriptase (*hTERT)* in mammary epithelial cells (MECs) and thus extends the replicative life span of MECs (Dimri, Martinez et al. 2002). Overexpression of BMI1 can cause neoplastic transformation and induce lymphomagenesis (Haupt, Bath et al. 1993, Alkema, Jacobs et al. 1997). Elevation of BMI1 occurs in all primary myeloid leukemia (Lessard and Sauvageau 2003) and mantle cell lymphomas (Bea, Tort et al. 2001). Additionally, recent developments demonstrated the upregulation of BMI1 in human non-small cell lung cancer (Vonlanthen, Heighway et al. 2001, Koren, Rijavec et al. 2017), breast cancer (Kim, Yoon et al. 2004), colon cancer (Kim, Yoon et al. 2004), human medulloblastomas (Leung, Lingbeek et al. 2004), and prostate cancer (Berezovska, Glinskii et al. 2006, van Leenders, Dukers et al. 2007, Fan, He et al. 2008). Prostate cancer patients with a stem cell-like expression profile of the 11-gene signature, which contains BMI1, are more likely to have a short interval to disease recurrence, distant metastasis and death after therapy (Glinsky, Berezovska et al. 2005). EZH2, another PcG protein involved in progression of prostate cancer (Varambally, Dhanasekaran et al. 2002), and BMI1 double positive prostate carcinomas are associated with metastasis with an increasing likelihood

of therapy failure and disease relapse (Berezovska, Glinskii et al. 2006). In accordance with these observations, our lab has previously reported that the BMI1 protein was expressed at high levels in PINs (precancerous lesion of prostate cancer) and prostate carcinomas together with reductions of $p16^{Ink4a}$ and $p19/14^{Arf}$ (Fan, He et al. 2008). Moreover, BMI1's oncogenic activity displays a relationship with PTEN, a well-established tumor suppressor. It was reported that the nuclear PTEN inhibits BMI1's ability to suppress $p16^{Ink4a}$ and $p19/14^{Arf}$, and to upregulate hTERT in DU145 prostate cancer cells (Fan, He et al. 2009).

1.2.2 BMI1 contributes to tumorigenesis in part through epigenetically regulating gene expression

The PcG family consists of two complexes, polycomb repressive complex 1 (PRC1) and PRC2, and plays a critical epigenetic role through regulating chromatin structure (Lund and van Lohuizen 2004). PRC2 is recruited to the polycomb response elements (PREs), and methylates histone H3 at lysine residues 9 and 27 (Zhang, Cao et al. 2004). The modifications facilitate the binding of PRC1 to chromatin (Zhang, Cao et al. 2004). H3 lysine 27 trimethylation (H3K27me3) is recognized by PRC1, which creates a silenced chromatin structure via blocking chromatin remodelling mediated by the SWI/SNF complex, thereby preventing the subsequent recruitment of transcription factors and thus inhibiting gene transcription (Levine, Weiss et al. 2002, Levine, King et al. 2004).

BMI1 is a subunit of PRC1. The complex possesses an intrinsic E3 ubiquitin ligase activity and ubiquitinates histone H2AX at lysine 119 (H2AK119ub) (Wang, Wang et al. 2004). Consistent with the ring finger domain (RF) being a signature motif of E3 ubiquitin ligase, the mammalian PRC1 has three RF-containing proteins RING1/RING1A, RING2/RING1B and BMI1 (Cao, Tsukada et al. 2005). Both RING1/RING1A and RING2/RING1B play a role in development by maintaining ubiquitylation of histone H2A (de Napoles, Mermoud et al. 2004). These observations shed light on how PRC1 participates in silencing gene expression. Although BMI1 does not possess ubiquitin E3 ligase activity, its association with RING1B through its RF domain greatly stimulates RING1B-derived E3 ligase activity (Li, Cao et al. 2006), and thus promotes H2A ubiquitylation (Cao, Tsukada et al. 2005). The BMI1-enhanced E3 ubiquitin ligase activity has important implications in understanding the molecular mechanisms by which BMI1 functions in stem cell self-renewal and tumorigenesis.

In addition to its role in gene repression, H2A ubiquitination is implicated as one important post-translational modification in DDR regulation. H2A-K119 ubiquitination by PRC1 is induced at sites of DNA damage and plays an essential role in the recruitment of repair factors during DDR, possibly through chromatin remodeling to establish a barrier of silenced transcription at the break site (Bergink, Salomons et al. 2006, Uckelmann and Sixma 2017). This suggests an involvement of BMI1 in DDR. This possibility is supported by nuclear PTEN-mediated inhibition of BMI1 function (Fan, He et al. 2009). PTEN-mediated tumor suppression is largely attributable to its PIP3 (phosphatidylinositol (3,4,5)–triphosphate) phosphatase activity in which cytosolic PTEN

is recruited to the plasma membrane to dephosphorylate PIP3 to PIP2 (phosphatidylinositol (4,5)), resulting in inhibition of PI3K-mediated tumorigenic activities (Cantley and Neel 1999). On the other hand, nuclear PTEN suppresses tumorigenesis independent of its PIP3 phosphatase activity through maintaining chromosome stability (Shen, Balajee et al. 2007, Jacob, Romigh et al. 2009). The genetic observation that concurrent *Chk2* knockout improves the survival of *Bmi1*$^{-/-}$ mice also indicates a role of BMI1 in DDR regulation.

1.2.3. BMI1 enhances DSB repair by promoting histone H2A and γH2AX ubiquitination

Note: this section is directly derived from our recent publication (Lin, Ojo et al. 2015).

Author's contribution
The section 1.2.3 is a proportion of our review paper published in *Biomolecules* 2015, 5:3396-3415 by Xiaozeng Lin, Diane Ojo, Fengxiang Wei, Nicholas Wong, Yan Gu, and Damu Tang. All authors have reviewed the literature. X. Lin, D. Ojo, and Dr. D. Tang organized and prepared the manuscript. X. Lin, N. Wong, and Y. Gu edited the manuscript. All authors proofread the manuscript.

Accumulative evidence clearly demonstrates the critical functions of histone ubiquitination in DSBs repair through HR and NHEJ. In agreement with this concept, BMI1 has been recently reported to play a role in histone H2A and H2AX ubiquitination through the BMI1/RIN1b E3 ubiquitin ligase, and thus contributes to DSB repair.

PRC1 affects chromatin structure through ubiquitination of histone H2A at K119 using the BMI1/RING1B E3 ubiquitin ligase activity (de Napoles, Mermoud et al. 2004, Wang, Wang et al. 2004, Cao, Tsukada et al. 2005, Li, Cao et al. 2006). This is a well

demonstrated mechanism in suppressing gene transcription; additionally, recent developments add a new role for this modification in facilitating DSB repair. BMI1 was detected being rapidly recruited to DNA lesions caused by local micro-irradiation using UV laser beam, ionizing irradiation (IR), and hydroxyurea (HU) in a set of cell types, including U2OS, mouse embryonic fibroblasts (MEFs), Hela, and CD133+ glioblastoma multiforme (GBM) cells (Facchino, Abdouh et al. 2010, Ismail, Andrin et al. 2010, Chagraoui, Hebert et al. 2011, Ginjala, Nacerddine et al. 2011, Pan, Peng et al. 2011). The recruitment of the BMI1/RING1B E3 ubiquitin ligase is required for the mono-ubiquitination of γH2AX and H2A likely at K119 (H2AK119ub) in the DSB regions in U2OS cells and MEFs, as downregulation of BMI1 abolished the modification (Ismail, Andrin et al. 2010, Ginjala, Nacerddine et al. 2011). In agreement with histone ubiquitination being critical in DSB repair, the presence of BMI1 in IRIF (ionizing radiation-induced foci) contributes to DSB repair. For instance, BMI1 deficient MEFs display a two-fold reduction in repair of DSBs induced by calicheamicin (CLM) at 5 hour post treatment and the defects are rescued upon re-expression of BMI1 (Ismail, Andrin et al. 2010). Furthermore, BMI1 downregulation compromises the survival of U2OS, Hela, and GBM cells with respect to IR treatment (Facchino, Abdouh et al. 2010, Ismail, Andrin et al. 2010, Ginjala, Nacerddine et al. 2011, Pan, Peng et al. 2011), likely results from a reduction in DSB repair due to BMI1 downregulation, thereby indirectly supporting the notion that BMI1 facilitates DSB repair.

BMI1 enhances DSB repair at least in part through HR. Using an I-Scel-based in vivo HR assay in 293T cells, knockdown of BMI1 reduced HR-mediated DSB repair

(Chagraoui, Hebert et al. 2011), an observation that is in accordance with BMI1-facilitated BRCA1 recruitment (Ismail, Andrin et al. 2010). BRCA1 is essential for the commitment of cells to repair DSB using HR (Chapman, Taylor et al. 2012, Lin, Yan et al. 2013). Nonetheless, evidence also supports a role of BMI1 in promoting non-homologous end joint (NHEJ)-mediated DSB repair. For example, NHEJ requires the recruitment of 53BP1 to DSBs (Chapman, Taylor et al. 2012, Lin, Yan et al. 2013). BMI1 enhances this recruitment and may physically interact with 53BP1 (Facchino, Abdouh et al. 2010, Ismail, Andrin et al. 2010). Direct evidence supporting a major role of BMI1/RING1B in promoting NHEJ was obtained under the situation of dysfunctional telomere-initiated NHEJ. Knockdown of either RING1B or BMI1 significantly reduced NHEJ-mediated telomere fusion (Bartocci, Diedrich et al. 2014). However, RING1B deficiency in MEFs did not affect the repair of DSBs caused by gamma irradiation and only transiently decreased NHEJ-derived DSB repair at heterochromatin loci (Bartocci, Diedrich et al. 2014). In aggregate, evidence supports the contributions of BMI1-assocaited E3 ubiquitin ligase to DSB repair (Gieni, Ismail et al. 2011).

Additionally, the involvement of the ligase activity in DSB repair is supported by the requirement of RF domain for BMI1 recruitment to DSBs (Ismail, Andrin et al. 2010, Ginjala, Nacerddine et al. 2011). RF mediates BMI1 association with the catalytic subunit RING2/RING1B, and is thus essential for BMI1-associated E3 ubiquitin ligase activity (Hemenway, Halligan et al. 1998, Li, Cao et al. 2006). With this knowledge in mind, it might be worth to determine whether re-expression of the RF-deleted BMI1 mutant is able to rescue the defects in repairing CLM-caused DSB in $BMI1^{-/-}$ MEFs; this

will provide an additional support that the observed rescue using wild type BMI1 being attributable to its-associated E3 ubiquitin ligase activity (Ismail, Andrin et al. 2010).

Structural analysis also supports the concept that the BMI1-associated ligase activity is important to its activity in facilitating DSB repair (Mattiroli, Vissers et al. 2012). The RNF168 and BMI1/RING1B, but not RNF8, E3 ubiquitin ligases are able to conjugate ubiquitin to the nucleosomal contents of H2A/H2AX, an activity that is attributable to the positively charged residues R57 in RNF168 and K93 in RING1B; whereas the conserved residue for RNF8 is a negatively charged residue D443 (Mattiroli, Vissers et al. 2012). Substitution of either R57 of RNF168 or K93 of RING1B to a negatively charged D residue abolishes their ability to ubiquitinate nucleosomal H2A/H2AX at K13/15 for RNF168 and K118/K119 for BMI1/RING1B, respectively (Mattiroli, Vissers et al. 2012). On the substrate side, H2A/H2AX contains a nucleosome acidic patch (E61, D90, and E92) that is required for both E3 ubiquitin ligases to ubiquitinate the respective lysine residues (Chen, Alpert et al. 2013, Leung, Agarwal et al. 2014). In support of these observations, a recent crystallized structure of BMI1/RING1B has revealed that the structural interface of RING1B for the H2A nucleosome acidic patch included R98, K93, and K97 with R98 being most critical (McGinty, Henrici et al. 2014). R98 inserted into the acidic pocket formed by E61, D90, and D92 of H2A, and made contacts to each of the side chains (McGinty, Henrici et al. 2014). Consistent with these structural roles, substitution of R98 with alanine led to a 50-fold decrease in nucleosomal ubiquitination and affinity to bind nucleosome (McGinty, Henrici et al. 2014). In comparison to the dominant role of RING1B in PRC1's

association with nucleosome, BMI1 does not make a significant contribution to the nucleosome binding of PRC1 (McGinty, Henrici et al. 2014). While it is likely that these structural details are involved in BMI1/RING1B-derived ubiquitination of H2A/H2AX at K118/K119 under DDR, this has yet to be demonstrated.

It also remains unclear what proportion of DSB repair is contributed by BMI1-associated E3 ubiquitin ligase activity. Nonetheless, it seems that a significant proportion occurs without a major contribution from BMI1. Although $BMI1^{-/-}$ MEFs contain 2-fold more DSBs during a course of 5-hour repair of CLM-induced DSBs in comparison to control cells, approximately 60% of DSBs in $BMI1^{-/-}$ MEFs are repaired (Ismail, Andrin et al. 2010). Will more DSBs be repaired in $BMI1^{-/-}$ MEFs if sufficient time is given? This seems likely, as knockdown of BMI1 in U2OS and Hela cells only modestly reduced cell survival in response to IR exposure (Ismail, Andrin et al. 2010, Ginjala, Nacerddine et al. 2011). In support of this possibility, DSBs repair in $BMI1^{-/-}$ MEFs was only delayed compared to wild type MEFs (Bartocci, Diedrich et al. 2014) and the same was also reported in U2OS cells (Kakarougkas, Ismail et al. 2014). Alternatively, there might be factors waiting to be discovered in regulating BMI1-facilitated DSB repair. AKT has been shown to phosphorylate BMI1, which contributes to BMI1's ability to cause the accumulation of mono-ubiquitinated H2A in IRIF in MEFs treated with a UV laser scissors (Nacerddine, Beaudry et al. 2012). Inhibition of AKT activation was without effects on either BMI1 recruitment to DSBs or the recruitment of 53BP1, indicating that NHEJ-mediated DSB repair may not be dramatically affected (Nacerddine, Beaudry et al. 2012).

1.3 Microvesicles contribute to the bystander effect of DDR

1.3.1 Bystander effect

It was generally accepted that direct exposure to genotoxic reagents cause DDR, which contributes to carcinogenesis. The World Health Organization has thoroughly documented that radiation induces cancer (El Ghissassi, Baan et al. 2009). A key consequence of radiation is that direct damage occurs to DNA within the nucleus of the cell (Morgan 2003, Morgan 2012); this induces DDR in the cell evident by cell cycle arrest and or apoptosis as well as DNA lesion repair (Zhou and Elledge 2000, Jackson 2001). However, DDR was also reported in neighboring cells that have not been directly exposed, a phenomenon that is known as bystander effect (Mothersill and Seymour 2003, Mothersill and Seymour 2004, Mothersill and Seymour 2004, Mothersill, Moriarty et al. 2004).

Radiation-induced bystander effect has major implications in cancer therapy. While radiotherapy, a major therapeutic approach for cancer patients, can be very effective in treating the primary tumors, it also induces a wide variety of chronic complications and secondary malignancies which has been clearly observed in patients treated with external beam radiation therapy (EBRT) for cervical cancer and breast cancer (Choy, Barr et al. 1993, Wijnmaalen, van Ooijen et al. 1993, Kleinerman, Boice et al. 1995, Senkus, Konefka et al. 2000). In a review of the data from a 7-year follow up of 269,069 prostate

cancer patients with radiotherapy, 9.9% were diagnosed with a heterochronous secondary cancer (Moon, Stukenborg et al. 2006), and men who received ERRT significantly increase their chances of developing secondary cancers in the upper body such as brain and stomach compared to those who didn't receive radiation therapy (Moon, Stukenborg et al. 2006, Sountoulides, Koletsas et al. 2010).

Accumulating evidence reveals that bystander factors released from irradiated cells play critical roles in radiotherapy-associated secondary carcinogenesis (Mothersill and Seymour 2004, Prise and O'Sullivan 2009). It has been reported that clastogenic factors can be detected not only in the plasma of people from Chernobyl nuclear reactor accident (Emerit, Levy et al. 1994) but also of the radiotherapy patients (Parsons, Watkins et al. 1954, Goh and Sumner 1968, Hollowell and Littlefield 1968). These factors can cause genome destabilization and ultimately carcinogenesis. More importantly, conditioned medium harvested from irradiated cells can increase neoplastic transformation, reduce cloning efficiency, and induce genome instability in recipient cells (Mothersill and Seymour 1997, Seymour and Mothersill 1997, Lorimore, Kadhim et al. 1998, Zhou, Randers-Pehrson et al. 2000, Nagasawa and Little 2002), indicating that the clastogenic factors detected in the blood of radiotherapy-treated patients were released from irradiated cells. In supporting this concept, it was reported that radiation exposure to one-half of the body caused DNA damage in other side of lead-shielded body (Koturbash, Rugo et al. 2006) and local cranial irradiation induced distance bystander effect DNA damage in mice in the lead-shield spleen and testes (Koturbash, Loree et al. 2008, Tamminga, Koturbash et al. 2008). Furthermore, irradiation of the lower body induced

medulloblastoma in the shielded brain of *Patched 1* heterozygous mice, accompanied by DSBs and apoptotic cell death (Mancuso, Pasquali et al. 2008).

Therefore, it is becoming clear that factors released from irradiated cells (bystander factors) have crucial roles in causing DDR. In addition, bystander DDR is not limited to radiation but also be detected in chemotherapy induced DNA damage (Chinnadurai, Chidambaram et al. 2011). The mechanisms may involve cell-cell signaling through gap junction and release of factors into the medium (Mancuso, Pasquali et al. 2008, Prise and O'Sullivan 2009). However, the identities of bystander factors remain largely unknown, although reactive oxygen species (ROS), reactive nitrogen species, TNFα, TGFβ1, Il-6, and IL8 have been implicated to facilitate bystander response (Prise and O'Sullivan 2009).

1.3.2 Microvesicles (MVs)

Microvesicles (MVs) are small membrane-enclosed sacs generated by the outward budding and fission of membrane vesicles from the cell surface, a shedding process that happens in a variety of cells and more frequently in tumor cells (Muralidharan-Chari, Clancy et al. 2010) or cells under stress. The function of MVs seems to be dependent on the unique set of cargo they carry, such as RNAs, microRNAs, proteins and bioactive lipids (Cocucci, Racchetti et al. 2009, Ratajczak and Ratajczak 2016). MVs transfer the specific messages to the acceptor or non-stress cells in a horizontal manner (Camussi, Deregibus et al. 2010, Muralidharan-Chari, Clancy et al. 2010, Rak 2010). For example, MVs secreted by normal endothelial cells have been implicated in angiogenesis (Morel,

Toti et al. 2004), whereas skeletal cells-derived MVs can play a role in bone mineralization (Anderson, Garimella et al. 2005). Except for its role in several physiological processes, MVs has been also reported to play vital roles in cancer progression including invasion and metastases (D'Souza-Schorey and Clancy 2012), inflammation (Cocucci, Racchetti et al. 2009), escaping from immune surveillance (Valenti, Huber et al. 2007), angiogenesis (Al-Nedawi, Meehan et al. 2009), and coagulation (Kawamoto, Ohga et al. 2012).

Several reports indicate that MVs of cancer cells can contribute to the acquisition of aggressive cancerous phenotypes. For example, MVs derived from cancer cells (MDAMB231 breast carcinoma cells and U87 glioma cells) can afford normal fibroblasts and epithelial cells the transformed characteristics of cancer cells by transferring tissue transgulutaminase (tTG) and fibronectin (Antonyak, Li et al. 2011). On the other hand, the oncogenic form of epidermal growth factor receptor EGFRvIII was found to transferred from a subset of aggressive glioma cells to a population of non-aggressive tumor cells through MVs, leading to the activation of two signaling pathways (MAPK and Akt), morphological transformation and an increase in anchorage-independent growth (Al-Nedawi, Meehan et al. 2008). Furthermore, MVs-delivered EGFRvIII is able to induce VEGF expression and VEGFR activation in endothelial cells (Al-Nedawi, Meehan et al. 2009). In addition, MVs shed from tumor cells have also been reported to facilitate ECM (extracellular matrix) invasion and metastases (Dolo, Ginestra et al. 1999) and evasion of immune response (Valenti, Huber et al. 2007). Taken together, MVs play a vital role in cell-to-cell communication and thus are ideal vesicles to carry the bystander

DNA damage messages to recipient cells. Indeed, the involvement of MVs in DDR-induced BE has been recently implicated (Carroll, Pulkoski-Gross et al. 2016).

1.4 Overall Objectives

DDR is required to maintain genome integrity through checkpoint activation and DNA lesion repair, and its disruption is one of the most pervasive characteristics of cancer. While ATM and ATR are essential in DDR, mechanisms regulating their activation remain unclear. BMI1 is a component of PRC1 and contributes to PRC1's E3 ubiquitin ligase activity through binding to the catalytic subunit RING2. This E3 ubiquitin ligase activity contributes to BMI1-derived suppression of the *Ink4a/Arf* locus. Nonetheless, genetic evidence suggest that repression of the *Ink4a/Arf* locus is unlikely to be BMI1's only or even major mechanism in human tumorigenesis. In human cancers, this locus is one of the most frequently mutated loci (Lowe and Sherr 2003) and BMI1 is commonly upregulated (Proctor, Waghray et al. 2013). In supporting this possibility, BMI1 was recently demonstrated to function in DSB repair, a process that requires BMI1-associated E3 ubiquitin ligase activity. However, whether BMI1 contributes to other aspect of DDR regulation remains unclear.

The main objectives of my research were to determine the role of BMI1 in regulating ATM and ATR activation during DSB and ssDNA lesion-induced DDR, respectively. This was achieved through overexpression or knockdown of BMI1 in MCF7 breast cancer and DU145 prostate cancer cell lines. These lines were then treated with etoposide (ETOP) or hydroxyurea (HU) to respectively produce DSBs and ssDNA

lesions in the cells, leading to specific DDR processes. The impacts of BMI1 modulations on ATM and ATR activation as well as the subsequent checkpoint activation were determined. A set of BMI1 mutants with deletion of the RF and other domains were constructed; their effects on the ATM and ATR-mediated signaling events have also been studied.

An intriguing aspect of DDR is the bystander effect (BE), i.e. cells display DDR without being directly exposed to genotoxic stresses. This area has not been thoroughly studied. Microvesicles (MVs) are small membrane-enclosed sacks that are shed from donor cells to carry specific messages to recipient cells. We proposed that MVs contribute to BE. One of goals in this research is to examine the contributions of MVs to BE DDR and whether ATM and/or ATR play a role in BE.

The thesis is organized into 5 chapters. Chapter 2 focuses on the investigation of the contributions of BMI1 in ETOP-induced ATM signaling and the underlying mechanisms by which BMI1 reduces ATM activation. Chapter 3 demonstrates that BMI1 attenuates HU-induced ATR activation and illustrates the mechanisms responsible for this attenuation. Chapter 4 identifies MVs as bystander factors. MVs isolated from cells stimulated with ETOP or UV induced BE in recipient cells. Finally, Chapter 5 will discuss the overall significance of this research and propose some future research.

CHAPTER TWO

BMI1 attenuates etoposide-induced G2/M checkpoints via reducing ATM activation

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Preamble

This manuscript examines the roles of BMI1 in ATM activation. ETOP induces DSBs, resulting in ATM activation and ATM-mediated activation of the G2/M checkpoint. Here, we demonstrated that BMI1 reduced ETOP-induced G2/M checkpoints via reducing ATM activation. Ectopic expression of BM1I in MCF7 and DU145 cell lines significantly reduced ETOP-induced G2/M arrest, while knockdown of BMI1 enhanced this arrest. Additionally, overexpression and knockdown of BM1I respectively reduced and enhanced ETOP-induced ATM signaling, evident by ATM autophosphorylation on S1981 (ATM pS1981) and the phosphorylation of ATM targets, including γH2AX, P53 pS15, CHK2 pT68. Activation of ATM requires ATM to associate with NBS1. We were able to show that BM1I binds NBS1 and this interaction was not affected by deletion of the RF domain. Furthermore, deletion of the RF domain did not affect BMI1's ability to reduce ATM activation in response to ETOP treatment. Collectively, evidence supports that BMI1 reduces ATM activation induced by DSBs independent of BMI1-associated E3 ubiquitin ligase activity.

Author's contribution

Dr. D. Tang, F. Wei, D. Ojo and X. Lin designed the experiment. X. Lin constructed all BMI1 mutants, established the MCF7 cell lines stably expressing the individual mutants, treated these lines with ETOP and examined the effects of these mutants on ETOP-induced activation of the ATM signaling (ATM pS1981, P53 pS15, CHK2 pT68 and γH2AX), and determined the association of the individual mutants with NBS1. X. Lin

also reintroduced mouse BMI1 into MCF7 shBMI1 cells and studied ETOP-induced activation of the ATM signaling in the lines. Furthermore, X. Lin overexpressed BMI1 in MCF10A cells and investigated ATM signaling in these lines following ETOP treatment. X. Lin helped to examine ETOP-induced G2/M arrest. Specifically, X. Lin's contributions resulted in Fig 5, Fig 6c, Fig 6d, and Figs 6-9. F. Wei established BMI1 and shBMI1 stable lines, examined the role of BMI1 in reducing ETOP-induced ATM signaling and determined the role of BMI1 in G2/M arrest in response to ETOP. F. Wei and D. Ojo investigated the interaction of NBS1 with BMI1. D. Ojo determined the association between ATM and NBS1 in empty vector (EV) and BMI1 overexpression MCF7 cells with and without ETOP treatment. D. Ojo also contributed to subcloning of BMI1 mutants. L. He contributed to the co-immunoprecipitation between NBS1 and BMI1. N. Wong, J. Yan, S. Xu, P. Major contributed to data acquisition. Dr. D. Tang, X. Lin, F. Wei and D. Ojo analyzed the data, and prepared the manuscript.

Relationship to the theme of my Ph.D. research

ATM plays a pivotal role in DDR regulation. DDR is essential in maintaining genome stability. Reductions in ATM function lead to genome instability and are a major oncogenic factor. BM1I is a well-established oncogenic protein, and facilitates HR-based repair of DSBs. However, it remains unclear whether there is a relationship between BMI1 and ATM. This research demonstrated for the first time an inhibitory activity of BMI1 towards ATM activation in DSB-induced DDR. This work is in line with our current understanding with respect to 1) downregulation of ATM signaling contributes to genome instability, and as a result tumorigenesis; 2) BMI1 is a well-established oncogenic protein. Nonetheless, this research provides a novel and innovative connection between ATM and BMI1. The major theme of my research includes investigation of BMI1's role in regulating ATM and ATR activation. The current research thus directly contributes to the research theme.

BMI1 attenuates etoposide-induced G2/M checkpoints via reducing ATM activation

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Running Title: BMI1 reduces etoposide-induced ATM activation

Key words: BMI1, DNA damage response, ATM, G2/M checkpoint activation

Abstract

The BMI1 protein contributes to stem cell pluripotency and oncogenesis via multiple functions, including its newly identified role in DNA damage response (DDR). While evidence clearly demonstrates that BMI1 facilitates the repair of double stranded breaks (DSBs) via homologous recombination (HR), it remains unclear how BMI1 regulates checkpoint activation during DDR. We report here that BMI1 plays a role in G2/M checkpoint activation in response to etoposide (ETOP) treatment. Ectopic expression of BMI1 in MCF7 breast cancer and DU145 prostate cancer cells significantly reduced ETOP-induced G2/M arrest. Conversely, knockdown of BMI1 in both lines enhanced the arrest. Consistent with ETOP induced activation of the G2/M checkpoints via the ATM pathway, overexpression and knockdown of BMI1 respectively reduced and enhanced ETOP-induced phosphorylation of ATM at serine 1981 (ATM pS1981). Furthermore, phosphorylation of ATM targets, including γH2AX, threonine 68 (T68) on CHK2 (CHK2 pT68), and serine 15 (S15) on p53 were decreased in overexpression and increased in knockdown BMI1 cells in response to ETOP. In line with the requirement of NBS1 in ATM activation, we were able to show that BMI1 associates with NBS1 and that this interaction altered the binding of NBS1 with ATM. BMI1 consists of a ring finger (RF), helix-turn-helix-turn-helix-turn (HT), proline/serine (PS) domain and two nuclear localization signals (NLS). While deletion of either RF or HT did not affect the association of BMI1 with NBS1, the individual deletions of PS and one NLS (KRMK) robustly reduced the interaction. Stable expression of these BMI1 mutants decreased ETOP-induced ATM pS1981 and CHK2 pT68, but not ETOP-elicited γH2AX in MCF7

cells. Furthermore, ectopic expression of BMI1 in non-transformed breast epithelial MCF10A cells also compromised ETOP-initiated ATM pS1981 and γH2AX. Taken together, we provide compelling evidence that BMI1 decreases ETOP-induced G2/M checkpoint activation via reducing NBS1-mediated ATM activation.

Introduction

The polycomb group (PcG) protein BMI1 plays an essential role in maintaining the self-renewal potential of hematopoietic and neural stem cells (Molofsky, Pardal et al. 2003, Park, Qian et al. 2003). This is at least in part attributable to its role in suppressing the expression of cell proliferation inhibitors, including $p16^{INK4A}$, $p19^{ARF}$/$p14^{ARF}$, and E4F1 (Bruggeman, Valk-Lingbeek et al. 2005, Molofsky, He et al. 2005, Chagraoui, Niessen et al. 2006, Akala, Park et al. 2008). Consistent with INK4A and ARF being critical tumor suppressors (Quelle, Zindy et al. 1995, Sherr 1998), the *BMI1* gene was initially isolated as an oncogene based on its collaboration with c-Myc in leukemogenesis (Haupt, Alexander et al. 1991, van Lohuizen, Verbeek et al. 1991). Overexpression of *BMI1* transformed lymphocytes (Haupt, Bath et al. 1993, Alkema, Jacobs et al. 1997); elevation of BMI1 was detected in lymphomas (Bea, Tort et al. 2001), and is associated with unfavorable prognosis in patients with lymphomas (Mihara, Chowdhury et al. 2006, van Galen, Muris et al. 2007). Additionally, BMI1 also promotes tumorigenesis in numerous malignancies, including non-small cell lung cancer (NSCLC) (Vonlanthen, Heighway et al. 2001), colon cancer (Kim, Yoon et al. 2004), breast cancer (Kim, Yoon et al. 2004), and nasopharyngeal carcinoma (Song, Zeng et al. 2006).

While the detailed mechanisms responsible for BMI1's maintenance of stemness and promotion of tumorigenesis remain incompletely understood, the impact of BMI1 on epigenetically-mediated gene expression is widely regarded to contribute to these processes. BMI1 belongs to the polycomb repressive complex 1 (PRC1) (Levine, King et al. 2004), which contains an E3 ubiquitin ligase that mediates ubiquitination of histone

H2A at lysine 119, thereby resulting in PRC1-medaited gene silencing. The E3 ligase is formed when BMI1 binds to the catalytic subunit RING2 (de Napoles, Mermoud et al. 2004, Levine, King et al. 2004, Cao, Tsukada et al. 2005, Li, Cao et al. 2006). This E3 ubiquitin ligase activity contributes to BMI1's function in stem cell biology and tumorigenesis.

Adding to its role in stem cells and tumorigenesis was the recent demonstration that BMI1 functions in DNA damage response (DDR). This response plays an important role in maintaining genome integrity and ensuring accurate transmission of genetic materials to daughter cells (Zhou and Elledge 2000). Attenuation of DDR promotes tumorigenesis (Lengauer, Kinzler et al. 1998, Zhou and Elledge 2000, Hoeijmakers 2001, Rouse and Jackson 2002). DDR is coordinated by activation of the apical kinases ATM, ATR (ATM- and Rad3-related), and DNAPK, leading to the activation of DNA damage checkpoints to arrest cell cycle progression as well as to repair DNA lesions before renewing cell proliferation (Lengauer, Kinzler et al. 1998, Zhou and Elledge 2000, Hoeijmakers 2001, Rouse and Jackson 2002, Shiloh 2003). The most lethal form of DNA lesions are double stranded DNA breaks (DSBs), which are mainly repaired by homologous recombination (HR) and non-homologous end joining (NHEJ). In response to ionizing radiation (IR), BMI1 is rapidly recruited to DSBs, and contributes to the ubiquitination of γH2AX (Ismail, Andrin et al. 2010, Chagraoui, Hebert et al. 2011, Ginjala, Nacerddine et al. 2011). Recruitment of γH2AX to DSBs (γH2AX foci) facilitates the assembly of repair factors to facilitate DSB repair (Paull, Rogakou et al. 2000, Celeste, Petersen et al. 2002). Therefore, by facilitating HR-mediated repair of

DSBs, BMI1 contributes to maintaining genome integrity and resistance to genotoxic therapeutic reagents (Facchino, Abdouh et al. 2010, Chagraoui, Hebert et al. 2011, Crea, Duhagon Serrat et al. 2011, Gieni, Ismail et al. 2011, Liu, Liu et al. 2012).

In addition to DSB repair, checkpoint activation is also an essential aspect of DDR (Zhou and Elledge 2000, Shiloh 2003). DSBs activate ATM, which then leads to phosphorylation and activation of downstream targets including H2AX, CHK2, and p53. Activation of CHK2 and p53 results in the arrest of cells in G2/M phase (Zhou and Elledge 2000, Shiloh 2003). Attenuation of checkpoint activation therefore allows cancer cells to proliferate in the presence of DNA lesions which further contribute to genome instability, a hallmark of cancer (Sieber, Heinimann et al. 2003, Kops, Weaver et al. 2005). Consistent with this concept, cancers commonly inactivate or attenuate checkpoint proteins, including ATM, CHK1, NBS1, and p53 (O'Driscoll and Jeggo 2006). Although BMI1 plays a role in HR repair of DSBs, its impact on checkpoint activation remains largely unknown.

We report here that BMI1 reduces ETOP-induced activation of G2/M checkpoints. ETOP causes DSBs via inhibition of topoisomerase II, thereby activating G2/M checkpoints (Wei, Xie et al. 2010). We demonstrate that overexpression of BMI1 reduces, and knockdown of BMI1 enhances ETOP-induced ATM activation as well as phosphorylation of ATM targets including H2AX, CHK2, and p53, in MCF7 and DU145 cells. This accordingly leads to inhibition and sensitization of ETOP-induced G2/M arrest. Furthermore, BMI1 was co-precipitated with NBS1 in MCF7 cells. This association occurs independently of BMI1's ring finger, a motif that is essential to

facilitate HR (Ismail, Andrin et al. 2010, Chagraoui, Hebert et al. 2011, Ginjala, Nacerddine et al. 2011), and alters the interaction between NBS1 and ATM. Taken together, we demonstrate that BMI1 reduces ETOP-induced G2/M arrest via potentially affecting NBS1-mediated ATM activation.

Materials and Methods

Materials, cell lines, plasmids, and cell cycle determination

Propidium iodide (PI), etoposide, and neocarcinostain (NCS) were purchased from Sigma. Etoposide was prepared in DMSO. Cell cycle distribution was determined by individualizing cells using PBS containing 0.02% EDTA, followed by examination of cell cycle profile according to our published procedure (Tang, Wu et al. 2002).

Western blot and immunoprecipitation (IP)

Cells were lysed in a buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 μg /ml leupeptin and 10μg /ml aprotinin. 50 μg of total cell lysates was separated on SDS-PAGE gel and transferred onto Hybond ECL nitrocellulose membranes (Amersham). Membranes were blocked with 5% skim milk, followed by incubation with the indicated antibodies at room temperature for 1 hour. Signals were detected using an ECL Western Blotting Kit (Amersham). Primary antibodies and concentrations used were: polyclonal anti-BMI1 (1:200, Santa Cruz Biotechnology), monoclonal anti-FLAG (M2, 1:500, Sigma), anti-p53 (FL-353 at 1 μg /ml, Santa Cruz), anti-phospho-p53(S15) (1:1000, Cell Signaling), anti-H2AX (1:1000, Santa Cruz), anti-H2AX (1:1000, Upstate), anti-phospho-ATM (S1981) (1:500, Rockland), anti-ATM (1:500, Rockland), anti-phosph-CHK2 (T68) (1:500, Cell

Signaling), anti-CHK2 (1:1000, Cell Signaling), anti-NBS1 (1:1000, Cell Signaling), and anti-actin (1:1000, Santa Cruz).

Immunoprecipitation was performed by incubating cell lysates containing 1mg of protein with specific antibodies plus Protein G agarose beads (Invitrogen) at 4^{o}C overnight, followed by 8 washes with a buffer containing 50 mM Tris (PH 7.5), 100 mM NaCl, 7.5 mM EGTA, and 0.1% Triton X-100. Antibodies used for IP were monoclonal anti-FLAG (M2, Sigma, 1 μg) and polyclonal anti-BMI1 (Santa Cruz, 1 μg). The IP was analyzed by western blot using anti-BMI1 (1:1000, Invitrogen), anti-NBS1 (1:1000, Genetex), and polyclonal anti-FLAG (1:500, Sigma).

Immunofluorescence

Immunofluorescence staining was carried out by fixing cells with prechilled (-20^{o}C) acetone-methanol for 15 minutes. The primary antibodies, anti-H2AX (Upstate, 0.5 μg/ml), anti-phospho-ATM (S1981) (Cell Signaling, 1:100), and anti-histone H3 S10 phosphorylation (Upstate, 1:250) were then added to the slides and incubated at 4ºC overnight. After washing, secondary antibodies FITC-Donkey anti-mouse IgG (1:200, Jackson Immuno Research Lab) and Rhodamine-Donkey anti-rabbit IgG (1:200, Jackson Immuno Research Lab) were then applied for 1 hour at room temperature. The slide was then covered with VECTASHIELD mounting medium with DAPI (VECTOR Lab Inc.). Images were taken with a fluorescent microscope (Carl Zeiss, Axiovert 200).

Quantification of γH2AX positive nuclei

More than 200 nuclei in several randomly selected areas were imaged using a fluorescent microscope (Carl Zeiss, Axiovert 200) at 100x magnification. Nuclei with ≥ 10 γH2AX foci and those with < 10 γH2AX foci were defined as positive and negative for γH2AX, respectively.

Generation of BMI1 mutants

BMI1 ΔRF, BMI1 ΔHT, BMI1 ΔNLS and BMI1 ΔPS mutants were produced by PCR amplification and subsequent ligation into pCDNA3 C-FLAG vector to produce C-FLAG tagged BMI1 mutants. The linker and primers used to generate these mutants are included in Supplementary Table 1.

Cell survival assay

MCF7 EV, BMI1, Ctrl shRNA, and BMI1 shRNA cells were seeded at $2x10^4$/well in 6-well plates for 24 hours. Cells were then treated with DMSO (mock treatment) or ETOP at the designated doses for 8 hours, followed by removal of ETOP. Cells were maintained for two weeks before the mock-treated cells became confluent. Surviving cells were stained with crystal violet (0.5%); the staining was subsequently dissolved in 33% acetic acid and absorbance was read by a spectrophotometer at 550 nm.

Retroviral Infection

Retroviral infection was performed following our previously published procedure (Tang, Okada et al. 2001). Briefly, a gag-pol expressing vector and an envelope-expressing

vector (VSV-G) (Stratagene) were transiently co-transfected with a designed retroviral plasmid into 293T cells. After 48 hours, the virus-containing medium was harvested, filtered through a 0.45 μM filter, and centrifuged at 50,000g for 90 minutes to concentrate the retrovirus. Following the addition of 10 μg/ml of polybrene (Sigma), the medium was used to infect cells. Infection for pBabe-based constructs was selected using puromycin, while infection for pLHCX-based constructs was selected using hygromycin.

Real-time PCR analysis

Total RNA was isolated using TRIZOL (Life Technologies, Burlington, ON). Reverse transcription was carried out using superscript III (Life Technologies, Burlington, ON) according to the manufacturer's instruction. Real time PCR primers used BMI1 and actin are listed in Supplementary Table 2.1. Real-time PCR was performed using the ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Burlington, ON) in the presence of SYBR-green according to the manufacturer's instructions (Applied Biosystems, Burlington, ON). Briefly, each reaction consisted of 1μL cDNA, 0.25 μL forward primer (10 μM), 0.25 μL reverse primer (10 μM), 4.75 μL H_2O and 6.25 μL of SYBR green master mix. The PCR reaction was carried out in a 96 well plate at 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. All samples were run in triplicate.

Knockdown of BMI1 in MCF7 and DU145 cells

Short hairpin-based control and BMI1 shRNA plasmids were purchased from Santa Cruz. Murine BMI1 was obtained from MGC (cat. #MMM1013-202859863), subcloned into pLNCX retroviral vector, and used to reintroduce BMI1 into MCF7 BMI1 shRNA cells.

Statistical analysis

Analysis was performed using student t-test (2-tails). A p-value < 0.05 was considered statistically significant.

Results

BMI1 reduces ETOP-induced production of γH2AX

BMI1 has been reported to enhance HR-mediated repair of DSBs (Ismail, Andrin et al. 2010, Chagraoui, Hebert et al. 2011, Ginjala, Nacerddine et al. 2011). Etoposide (ETOP) induces G2/M checkpoints (Wei, Xie et al. 2010). To investigate other aspects of BMI1 in DDR, we determined the role of BMI1 in ETOP-induced activation of G2/M checkpoints. As production of γH2AX and formation of H2AX nuclear foci play an important role in the activation of DNA damage-induced checkpoints, we first determined the impact of BMI1 in these events. BMI1 was ectopically expressed in MCF7 and DU145 cells (Fig 2.1a, Supplementary Fig 2.1a). Ectopic BMI1 significantly reduced ETOP-induced production of γH2AX (Fig 2.1a, c) as well as the formation of γH2AX nuclear foci in MCF7 cells (Fig 2.1b, d). Conversely, knockdown of BMI1 in MCF7 cells dramatically enhanced γH2AX and its nuclear foci in response to ETOP (Fig 2.2). These reactions are not unique to MCF7 cells, as overexpression and knockdown of BMI1 in DU145 cells also reduced and enhanced ETOP-induced γH2AX nuclear foci, respectively (Supplementary Figs 2.1, 2.2).

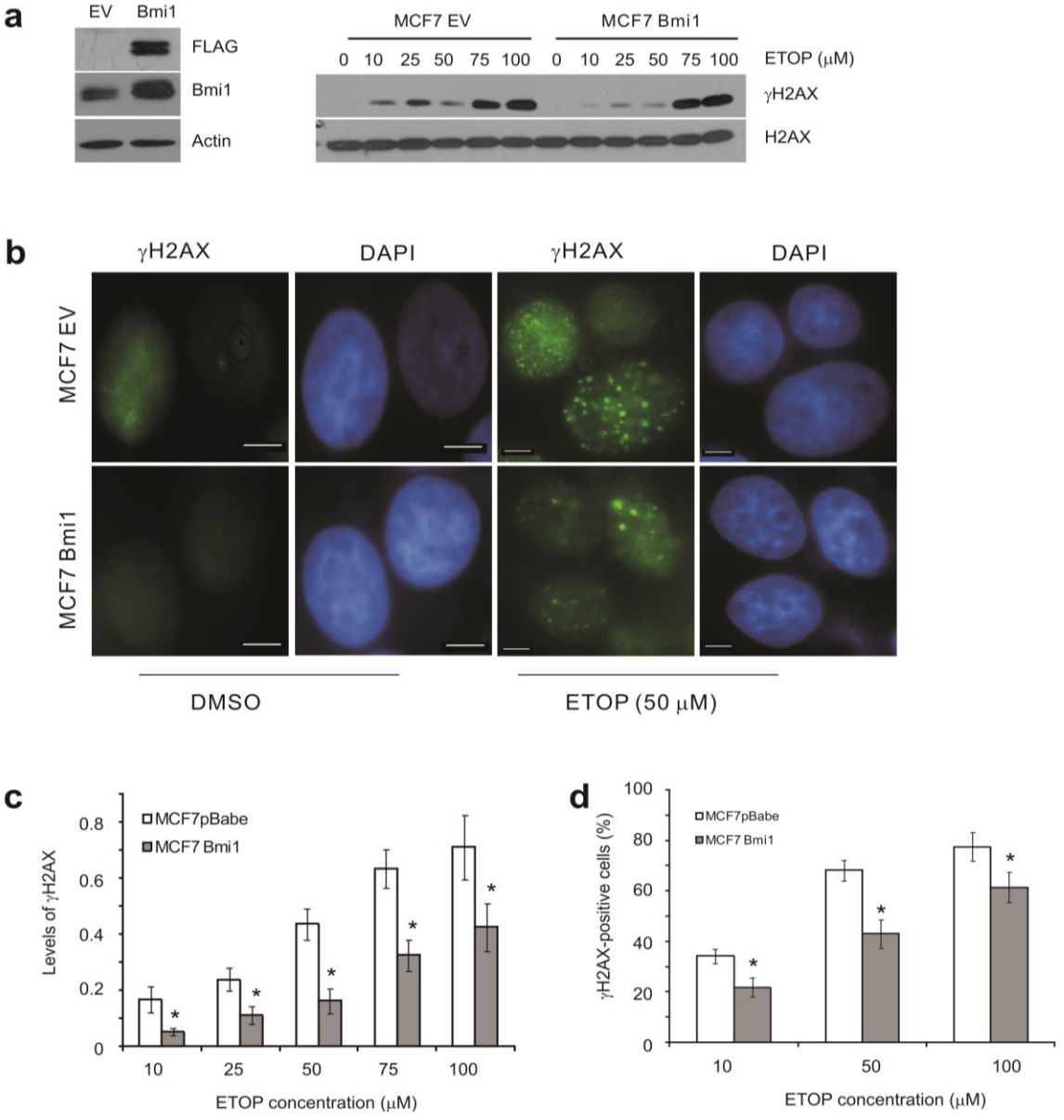

Figure 2.1. Elevation of BMI1 inhibits ETOP-induced production of γH2AX. (**a**) Establishing empty vector (EV) and BM1 stable lines using EV and FLAG-tagged BMI1 retrovirus (left panels). MCF7 EV and BMI1 cells were treated with the indicated doses of ETOP for 2 hours, followed by the examination of γH2AX or H2AX by western blot

using specific antibodies. (**b**) The indicated cell lines were either mock-treated (DMSO) or treated with ETOP for 2 hours, followed by immunofluorescence (IF) staining for γH2AX. Nuclei were counterstained with DAPI (blue). Scale bars represent 5 μm. (**c**) Three independent repeats as shown in (**a)** were quantified for γH2AX [means ± SE (standard errors)]. The levels of γH2AX were normalized to the respective H2AX. * $p<0.05$ (2 tailed Student *t* test) in comparison to the respective EV (pBabe) cells. (**d**) MCF7 EV and BMI1 cells were treated with the indicated doses of ETOP for 2 hours, followed by IF for γH2AX. More than 200 nuclei in four randomly selected fields were counted and the % of γH2AX-postitive nuclei was determined. Experiments were repeated three times. Means ± SE were graphed. * $p<0.05$ (2 tailed Student *t* test) in comparison to the respective EV (pBabe) cells.

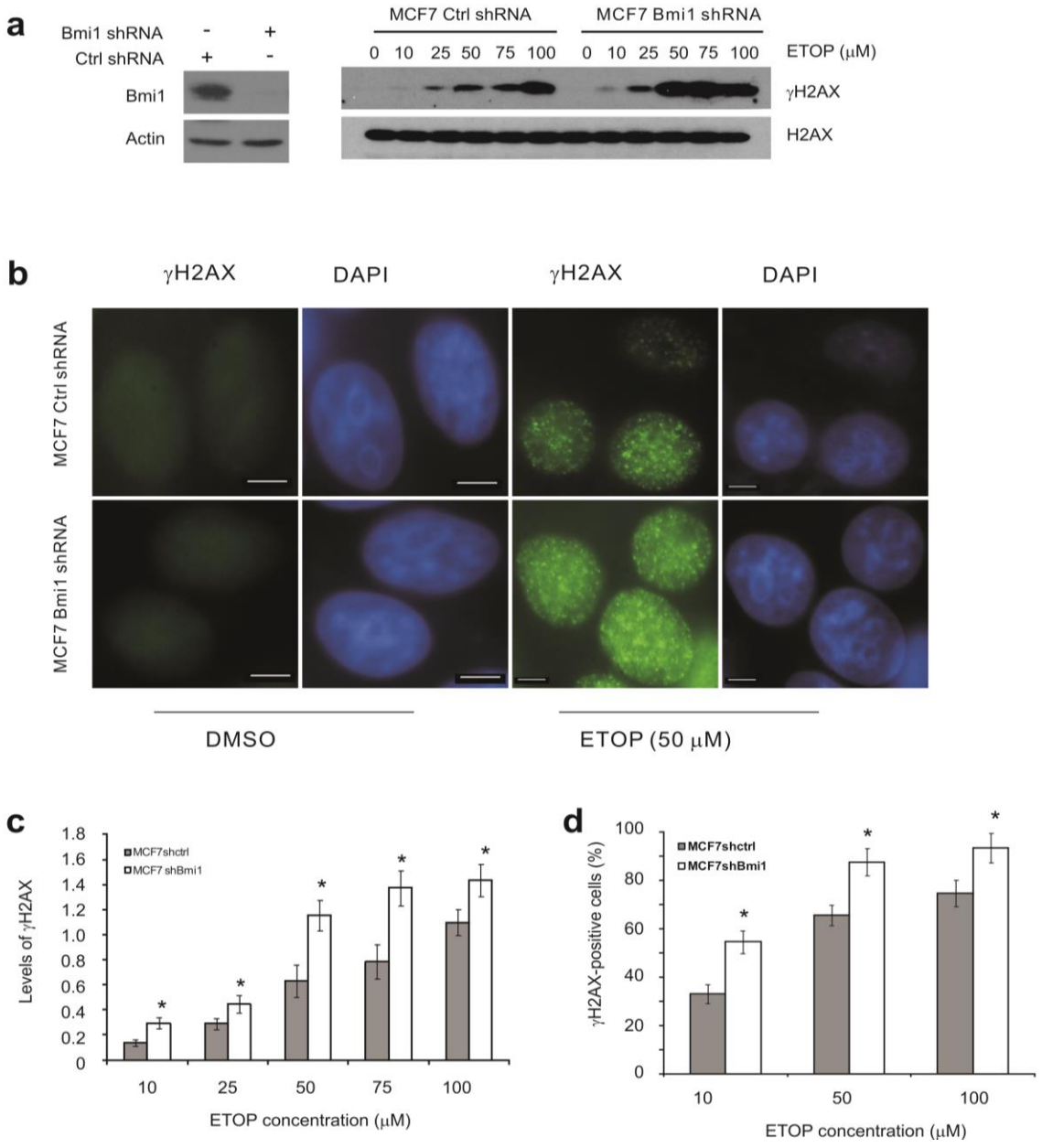

Figure 2.2. Knockdown of BMI1 sensitizes ETOP-induced production of γH2AX. (**a**, **c**) Establishment of MCF7 Ctrl (control) and BMI1 shRNA cell lines (**a,** left panel). MCF7 Ctrl shRNA and MCF7 BMI1 shRNA cells were treated with ETOP for 2 hours and examined for γH2AX by western blot. γH2AX was normalized to the respective H2AX. Experiments were repeated three times. Typical results from a single experiment are

shown (**a**). Means ± SE were graphed (**c**). * $p<0.05$ (2 tailed Student *t* test) in comparison to the respective Ctrl shRNA cells. (**b**, **d**) MCF7 Ctrl shRNA and MCF7 BMI1 shRNA cells were treated with DMSO or ETOP for 2 hours, followed by IF staining for γH2AX. Experiments were repeated three times. Typical images from a single experiment are shown. At least 200 nuclei in four randomly selected fields were counted and the % of γH2AX-postitive nuclei was determined. Means ± SE were graphed. * $p<0.05$ (2 tailed Student *t* test) in comparison to the respective Ctrl shRNA cells.

BMI1 reduces ETOP-induced ATM activation

As γH2AX is a target of ATM, and because ETOP is known to activate the ATM pathway (Zhou and Elledge 2000, Wei, Xie et al. 2010), the above observations indicate that BMI1 may reduce ETOP-initiated ATM activation. To address this possibility, we were able to show that ETOP dose-dependently induced the autophosphorylation of ATM at serine 1981 (S1981), the commonly used surrogate marker of ATM activation (Bakkenist and Kastan 2003), in MCF7 EV cells. This observation was substantially reduced in MCF7 BMI1 cells (Fig 2.3a, c). Conversely, knockdown of BMI1 in MCF7 cells significantly enhanced the kinetics of ETOP-induced ATM activation (Fig 2.3b, d).

In line with BMI1 reducing ETOP-induced ATM activation, BMI1 also attenuated the recruitment of active ATM to DSBs. The number of ATM S1981 phosphorylation (ATM pS1981) foci was significantly reduced in BMI1 overexpression cells compared to EV cells upon ETOP treatment (Supplementary Fig 2.3a). On the other hand, knockdown of BMI1 increased ETOP-induced ATM pS1981 foci in comparison to Ctrl shRNA cells (Supplementary Fig 2.3b). The number of nuclei (cells) with readily detected ATM pS1981 foci in response to ETOP treatment was also reduced and increased in BMI1 overexpression and knockdown cells, respectively (Fig 2.3e, f). Similar results were also

observed in DU145 cells in which BMI1 was either ectopically expressed or knocked down (Supplementary Figs 2.1, 2.2). The apparent co-localization between the foci of γH2AX and those of ATM pS1981 in DU145 (Supplementary Figs 2.1b, 2.2b) and MCF7 cells (data not shown) further supports that BMI1 compromises the recruitment of active ATM to ETOP-induced DSBs. Taken together, the above observations reveal that BMI1 attenuates ETOP-initiated ATM activation.

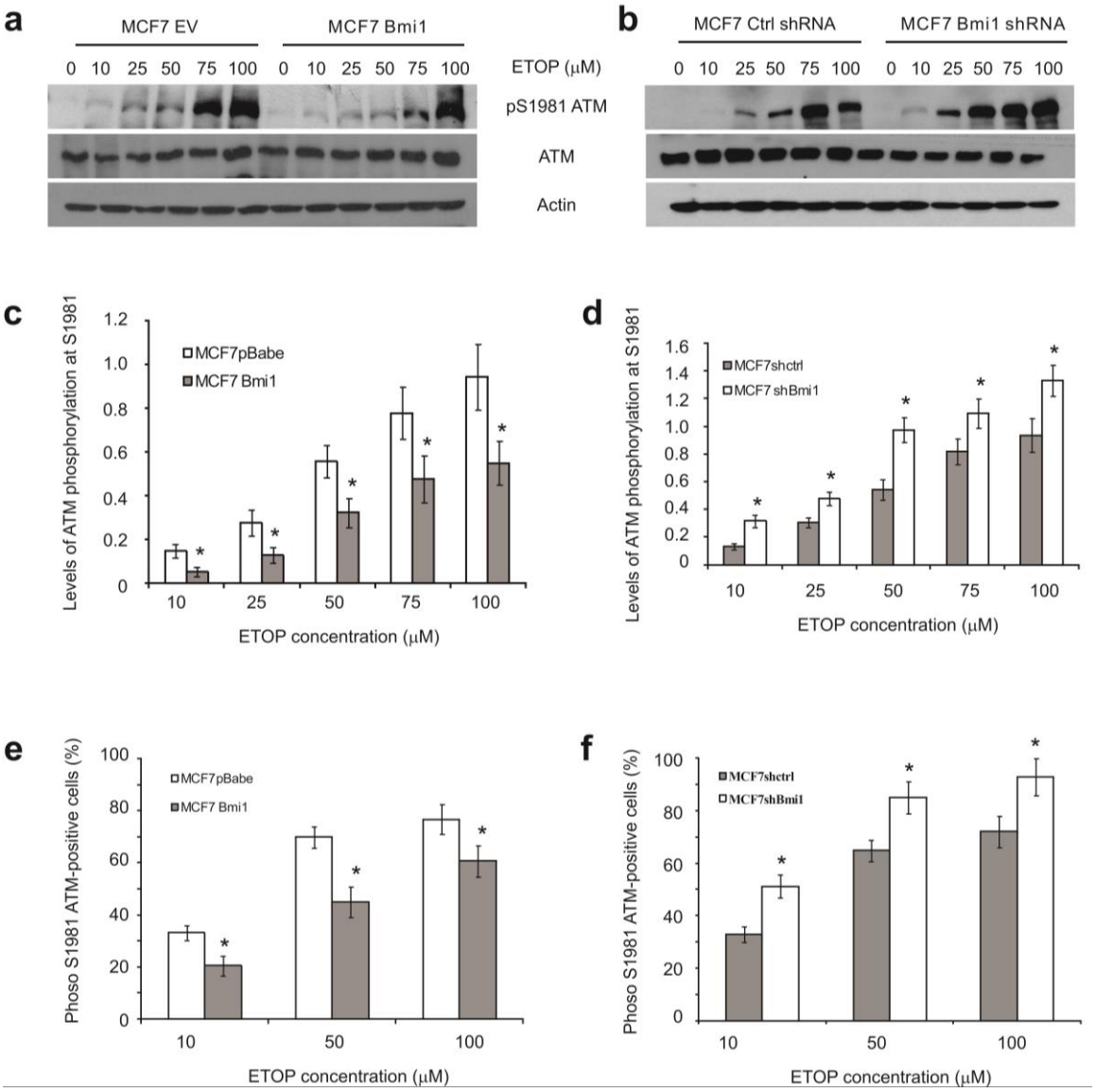

Figure 2.3. BMI1 inhibits ETOP-induced ATM activation. MCF7 EV and BMI1 cells (**a**) as well as MCF7 Ctrl shRNA and BMI1 shRNA cells (**b**) were treated with the indicated doses of ETOP for 2 hours, followed by western blot examination of S1981 phosphorylated ATM (phos-S1981 ATM), ATM, and actin using specific antibodies. (**c**, **d**) Experiments in panels (**a**) and (**b**) were repeated three times. The levels of S1981-phosphorylated ATM were normalized to the respective ATM. Means ± SE were graphed. * $p<0.05$ (2 tailed Student *t* test) in comparison to the respective EV (**a**) or Ctrl

shRNA (**b**) cells. (**e**, **f**) The indicated MCF7 cell lines were treated with ETOP as indicated. IF for S1981-phosphorylated ATM was then performed. Nuclei were counter stained with DAPI. Experiments were repeated three time (see Supplementary Fig 3). At least 200 nuclei in four randomly selected fields were counted and the % of nuclei positive for S1981-phosphorylated ATM was determined. Means ± SE were graphed. * $p<0.05$ (2 tailed Student *t* test) in comparison to the respective EV (**e**) or Ctrl shRNA (**f**) nuclei.

BMI1 reduces the phosphorylation of ATM targets

ATM activation is required to activate DSB-induced G2/M checkpoints (Dasika, Lin et al. 1999, Kurz and Lees-Miller 2004). Our observations that BMI1 attenuates ETOP-induced ATM activation suggest that BMI1 also reduces the phosphorylation of critical downstream targets of ATM, which play a role in activation of G2/M checkpoints. Among these targets are CHK2 and p53. As expected, ETOP dose-dependently induced CHK2 T68 and p53 S15 phosphorylation, two well established ATM targets (Kurz and Lees-Miller 2004), in MCF7 EV cells. The kinetics of these two events were significantly reduced in ETOP-treated MCF7 BMI1 cells (Fig 2.4a, c, d). Conversely, in comparison to Ctrl shRNA cells, knockdown of BMI1 increased CHK2 T68 and p53 S15 phosphorylation in response to ETOP exposure (Fig 2.4b, e, f). However, modulation of BMI1 apparently does not affect ETOP-induced p53 stabilization (Fig 2.4a), an observation that is in accordance with p53 stabilization being regulated by multiple post-translational modifications (Lavin and Gueven 2006). These observations thus suggest that S15 phosphorylation does not play a major role in p53 stabilization in response to ETOP-induced DNA damage in MCF7 cells. In supporting this possibility, the expression of $p21^{CIP1}$, a well regarded p53 target (Zhou and Elledge 2000), was not altered in BMI1-

modulated and ETOP-treated cells (Fig 2.4a). Nonetheless, the observed effects of BMI1 modulation on ETOP-induced p53 S15 phosphorylation support a role of BMI1 in reducing activation of the ATM pathway in response to etoposide treatment.

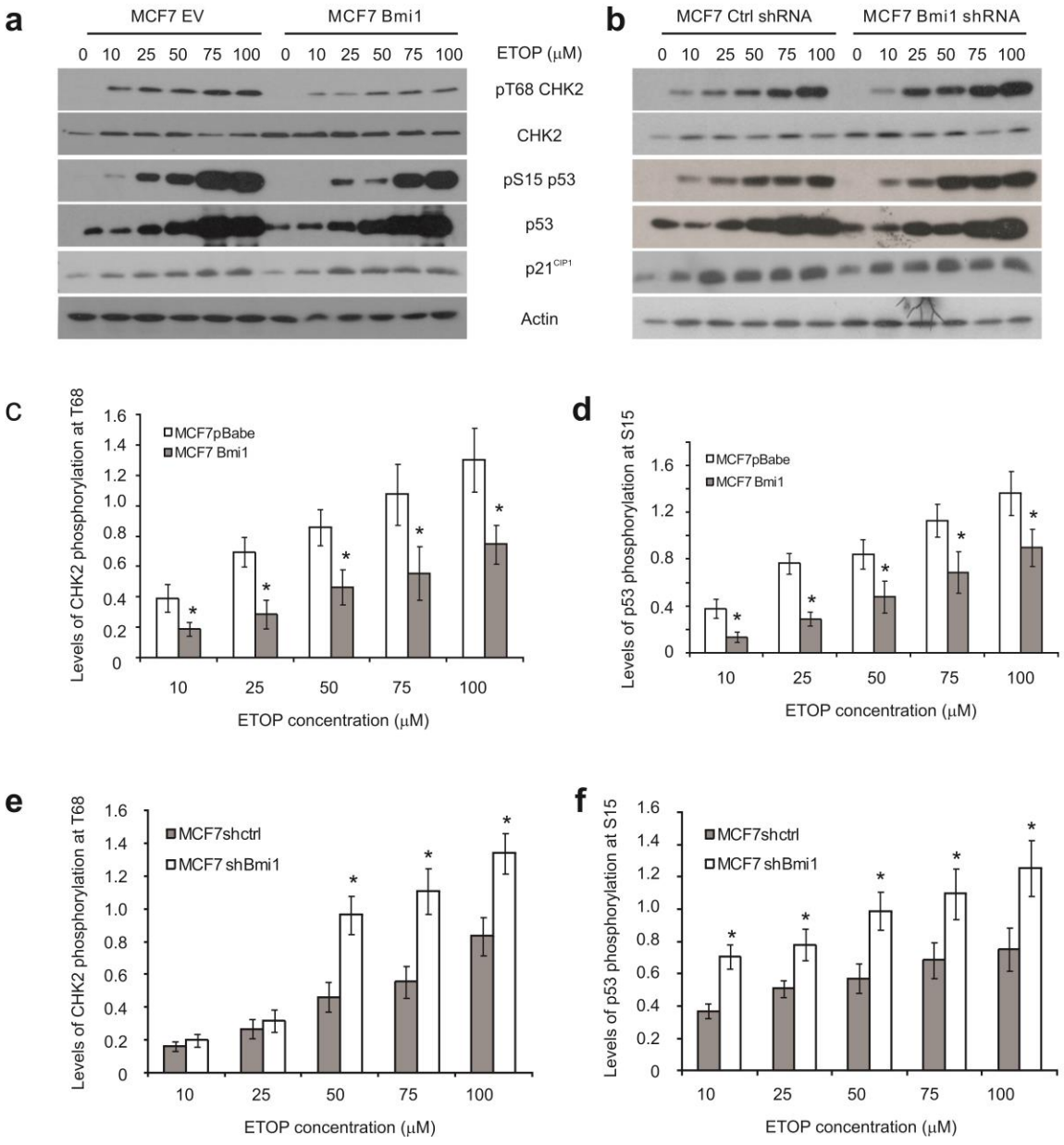

Figure 2.4. BMI1 reduces phosphorylation of CHK2 T68 and p53 S15. MCF7 EV and BMI1 cells (**a**) as well as MCF7 Ctrl shRNA and BMI1 shRNA cells (**b**) were treated with either DMSO (0) or the indicated doses of ETOP for 2 hours, followed by western blot analysis of T68 phosphorylated CHK2 (Phos-T68 CHK2), Chk2, S15 phosphorylated p53 (phos-S15 p53), p53, $p21^{CIP1}$, and actin. (**c**, **d**) Experiments in panel (**a**) were repeated three times. CHK2 T68 phosphorylation (**c**) and p53 S15

phosphorylation (**d**) were normalized to the respective CHK2 and p53, respectively. Means ± SE were graphed. * $p<0.05$ (2 tailed Student *t* test) in comparison to the respective EV (pBabe) cells. (**e**, **f**) Experiments in panel (**b**) were repeated three times. CHK2 T68 phosphorylation (**e**) and p53 S15 phosphorylation (**f**) were normalized to the respective CHK2 and p53, respectively. Means ± SE were graphed. * $p<0.05$ (2 tailed Student *t* test) in comparison to the respective Ctrl shRNA cells.

To exclude potential off-target effects of BMI1 shRNA, murine BMI1 was reintroduced into MCF7 BMI1 shRNA cells (Fig 2.5a). The ectopic murine BMI1 was confirmed using an anti-FLAG antibody (data not shown). In comparison to MCF7 Ctrl shRNA cells, MCF7 BMI1 shRNA+EV cells remained sensitized to ETOP-induced activation of the ATM pathway (data not shown). However, re-expression of murine BMI1 (MCF7 BMI1 shRNA+BMI1 cells) reduced ETOP-induced phosphorylation of ATM, CHK2, p53, and H2AX (γH2AX) (Fig 2.5b) in comparison to MCF7 BMI1 shRNA+EV cells. Collectively, these results reaffirmed the role of BMI1 in reducing ETOP-initiated activation of the ATM pathway.

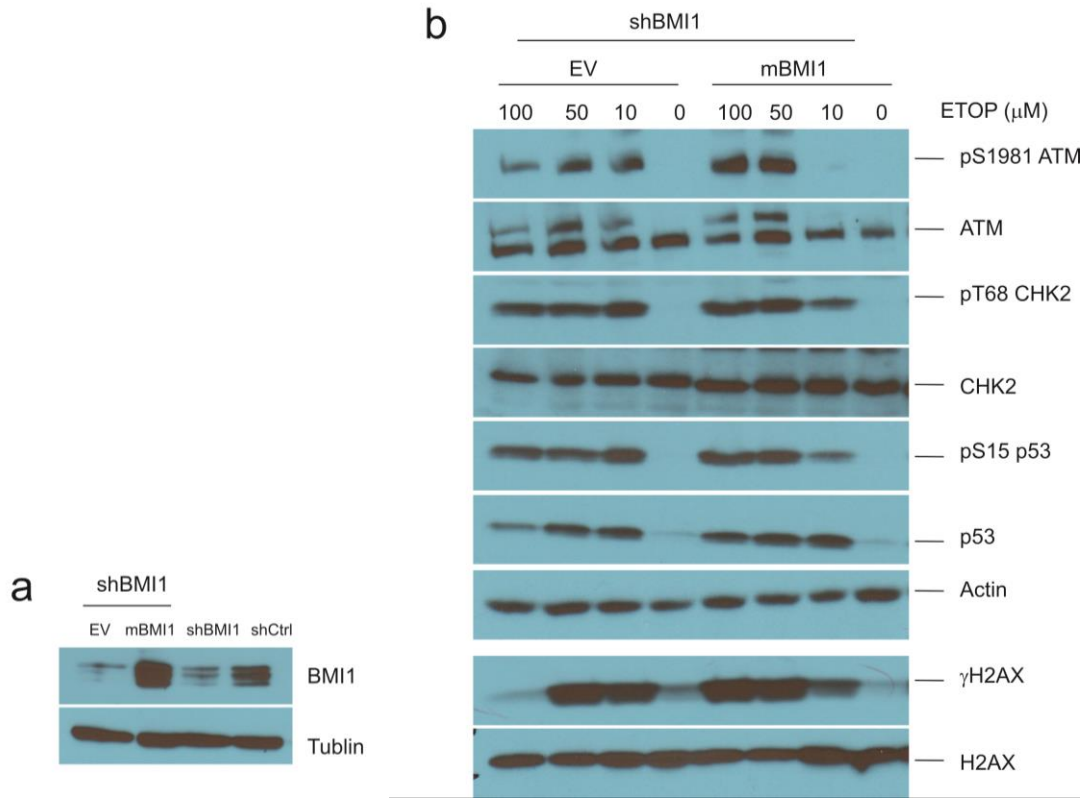

Figure 2.5. Re-expression of BMI1 in MCF7 BMI1 shRNA cells decreases ETOP-triggered ATM activation. (**a**) MCF7 BMI1 shRNA cells were infected by either an EV or murine BMI1 (mBMI1) retrovirus. The indicated cell lines were examined for BMI1 expression by western blot using an anti-BMI1 antibody. (**b**) MCF7 BMI1 shRNA expressing EV or mBMI1 were treated with either DMSO or ETOP for the indicated doses for 2 hours, followed by western blot examination for ATM S1981 phosphorylation, CHK2 T68 phosphorylation, p53 S15 phosphorylation, and γH2AX. Experiments were repeated three times; typical images from a single repeat are shown.

BMI1 attenuates ETOP-induced G2/M arrest

Activation of the ATM pathway in response to DSBs plays a critical role in G2/M arrest. To examine whether modulation of BMI1 affects ETOP-induced G2/M arrest, we have treated MCF7 EV and BMI1 overexpression cells with different doses of ETOP. In

comparison to DMSO (mock treatment), ETOP clearly induced typical G2/M arrest in EV cells (Fig 2.6a; Table 2.1) and ETOP-induced G2/M arrest was significantly reduced in MCF7 BMI1 cells (Fig 2.6a; Table 2.1). As expected, knockdown of BMI1 sensitized the arrest in MCF7 cells (Fig 2.6b; Table 2.2). Similar observations were also obtained in DU145 cells in which BMI1 expression was modulated (Supplementary Fig 2.4).

Taken together, the above observations reveal that BMI1 attenuates ETOP-initiated G2/M arrest. This action would thus be expected to facilitate entry into mitosis in the presence of ETOP. To examine this scenario, cells were halted during mitosis by nocodazole and mitotic cells were examined for the presence of histone H3 serine 10 (S10) phosphorylation, a well regarded mitotic marker (Goto, Tomono et al. 1999, Prigent and Dimitrov 2003). An 8 hour nocodazole treatment revealed approximately 20-25% of cells positive for histone H3 S10 phosphorylation in MCF7 EV, BMI1, Ctrl shRNA and BMI1 shRNA cells (Fig 2.6c, Supplementary Fig 2.5) and the addition of 6 μM ETOP significantly reduced mitotic entry in all cells (Fig 6c, Supplementary Fig 2.5). However, in comparison to EV cells, ectopic BMI1 significantly attenuated the prevention of mitotic entry (Fig 2.6c, left panel; Supplementary Fig 2.5, top two panels). Knockdown of BMI1, on the other hand, sensitized ETOP-induced blockage of mitotic entry (Fig 2.6c, right panel; Supplementary Fig 2.5, bottom two panels). These observations provide additional support that BMI1 reduces G2/M checkpoint activation in MCF7 cells.

Attenuation of checkpoint activation in response to DNA damage may impact a cell's ability to survive DNA lesions. To investigate this possibility, we have

demonstrated that MCF7 BMI1 cells survived better than MCF7 EV cells when treated with 10 μM ETOP for 8 hours (Fig 2.6d, left panel; Supplementary Fig 2.6, top two panels) and that knockdown of BMI1 sensitized cells to this cytotoxicity (Fig 2.6d, right panel; Supplementary Fig 2.6, bottom two panels). Taken together, the above results demonstrate a role of BMI1 in the attenuation of ETOP-induced G2/M checkpoint activation. This activity is in line with BMI1's role in promoting tumorigenesis (see Discussion for details).

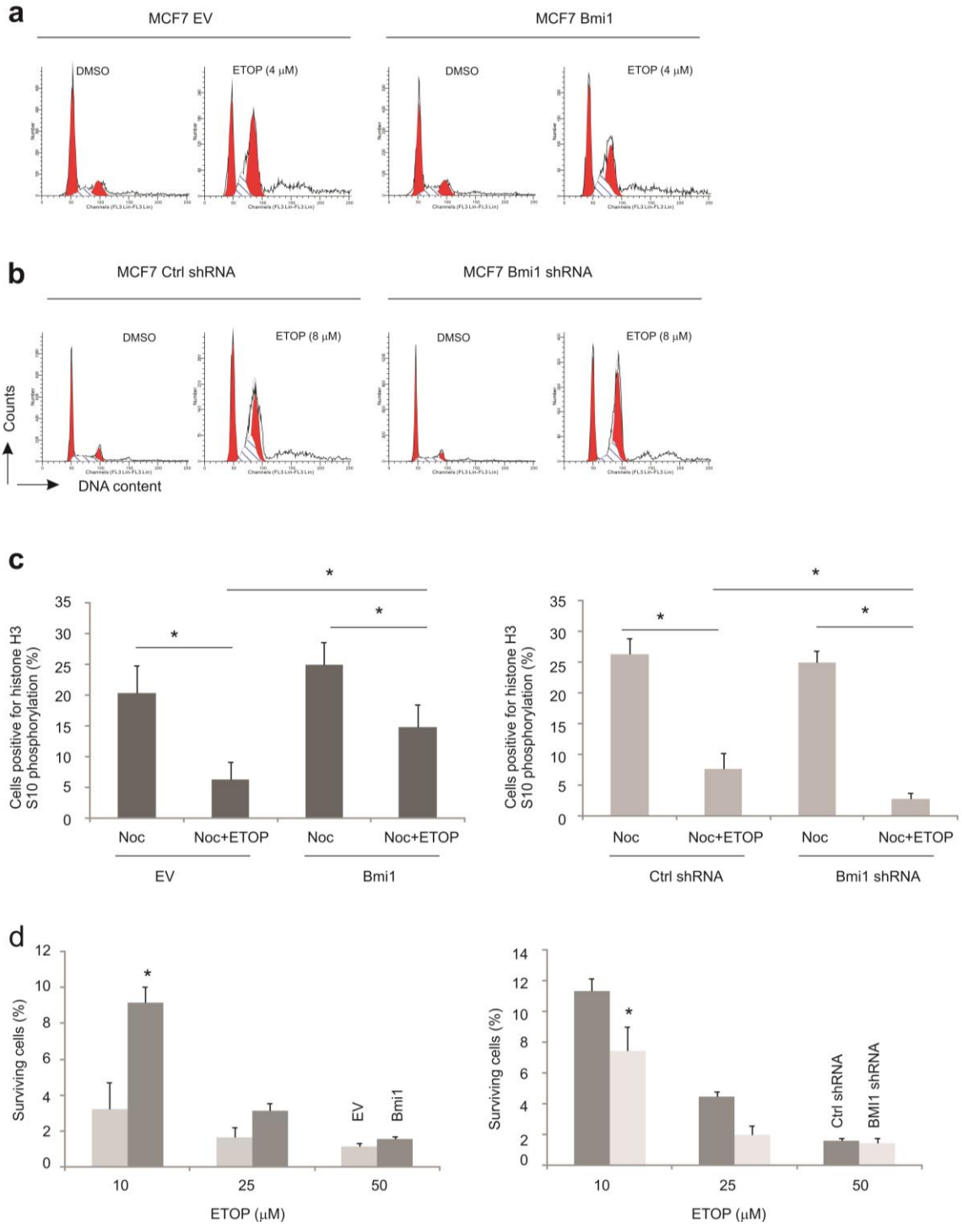
a
MCF7 EV
MCF7 Bmi1
DMSO
ETOP (4 μM)
DMSO
ETOP (4 μM)
b
MCF7 Ctrl shRNA
MCF7 Bmi1 shRNA
DMSO
ETOP (8 μM)
DMSO
ETOP (8 μM)
Counts
DNA content
c
Cells positive for histone H3 S10 phosphorylation (%)
Noc
Noc+ETOP
Noc
Noc+ETOP
EV
Bmi1
Cells positive for histone H3 S10 phosphorylation (%)
Noc
Noc+ETOP
Noc
Noc+ETOP
Ctrl shRNA
Bmi1 shRNA
d
Surviving cells (%)
EV
Bmi1
10
25
50
ETOP (μM)
Surviving cells (%)
Ctrl shRNA
BMI1 shRNA
10
25
50
ETOP (μM)

Figure 2.6. Modulation of BMI1 expression in MCF7 cells accordingly affects ETOP-induced G2/M arrest. MCF7 EV and BMI1 cells (**a**) as well as MCF7 Ctrl shRNA and BMI1 shRNA cells (**b**) were treated with either DMSO or ETOP for 24 hours, followed by the analysis of cell cycle distribution by a flow cytometer. (**c**) MCF7 EV, BMI1, Ctrl shRNA and BMI1 shRNA cells were treated with nocodazole (Noc) (0.1 μg/ml) for 8 hours with or without addition of etoposide (ETOP, 6 μM) for the last 4 hours, followed by immunofluorescence staining for histone H3 S10 phosphorylation. More than 2,000 nuclei from 10 randomly selected areas per slide were counted to determine the percentage of nuclei positive for histone H3 S10 phosphorylation. Experiments were repeated at least 3 times (see Supplementary Fig 5 for typical images). Means ± SD were graphed; * $p < 0.05$ (2 tailed Student *t* test) in the indicated comparisons. (**d**) MCF7 EV, BMI1, Ctrl shRNA and BMI1 shRNA cells were seeded in 6-well plates at 2 x 10^4/well for one day, followed by the indicated treatment for 8 hours. Media were then changed and cells were cultured for approximately two weeks before staining with crystal violet (see Supplementary Figure 6 for typical images). The staining was dissolved in 33% acetic acid and absorbance was read by a spectrophotometer at 550nm. The amount of staining was expressed as a percentage of the staining obtained in the respective mock-treated cells. Experiments were repeated three times. Means ± SD were graphed; * $p < 0.05$ (2 tailed Student *t* test) in the indicated comparison to EV or Ctrl shRNA cells.

Association of BMI1 with NBS1

Activation of ATM requires NBS1 (Difilippantonio, Celeste et al. 2005, Falck, Coates et al. 2005). To address the potential mechanisms responsible for BMI1-derived reduction of ATM activation, we were able to show that NBS1 can be co-immunoprecipitated with BMI1 not only in MCF7 cells expressing ectopic NBS1 but also in MCF7 EV and MCF7 parental cells (Fig 2.7a, b). This demonstrated an association between endogenous BMI1 and endogenous NBS1. However, ectopic expression of BMI1 did not increase its association with NBS1, and DNA damage does not seem to affect this interaction (Fig 2.7a) (see Discussion for details). The association of BMI1 and NBS1 does not appear to be mediated by their association with DNA, as the presence of 50 μg of ethidium

bromide in cell lysates prepared from MCF7 cell with and without ETOP treatment had no effect on this interaction (Fig 2.7b).

We subsequently determined whether binding to BMI1 affects NBS1's ability to associate with ATM. While it is well established that interaction with NBS1 is critical for DSB-induced ATM activation, demonstration of this association via immunoprecipitation (IP) of the endogenous ATM-NBS1 complex is a challenging task. We also had a difficult time trying to IP this complex in MCF7 EV cells with or without ETOP treatment (Fig 2.7c). Intriguingly, this complex was readily precipitated in ETOP-treated MCF7 BMI1 cells (Fig 2.7c). Although the reasons as to why ectopic BMI1 enhanced the ETOP-induced association of ATM and NBS1 are unknown, it is clear that BMI1 overexpression affected their interaction. Collectively these observations support the possibility that by associating with NBS1, BMI1 interferes with NBS1-mediated ATM activation.

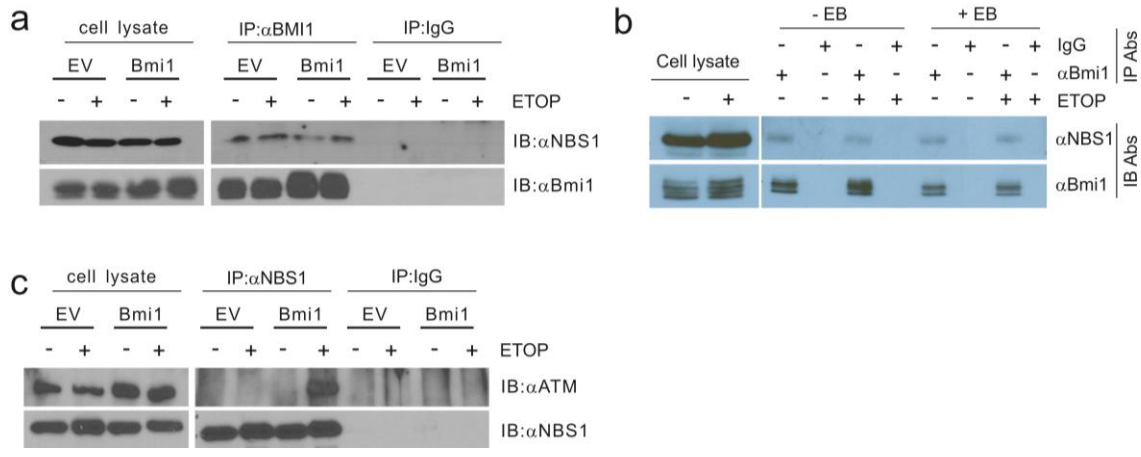

Figure 2.7. Association of BMI1 with NBS1 affects the interaction between NBS1 and ATM. (**a**) MCF7 EV and BMI1 cells were treated with ETOP (50 μm) for 2 hours, followed by immunoprecipitation of the BMI1-NBS1 complex. (**b**) MCF7 cells were treated with DMSO (-) or ETOP at 50 μm for 2 hours, followed by immunoprecipitation of the BMI1-NBS1 complex in the presence or absence of 50 μg/ml ethidium bromide (EB) (note: cell lysates were pre-incubated with EB for 10 minutes on ice prior to IP). (**c**) MCF7 EV and BMI1 cells were treated with ETOP (50 μm) for 2 hours, followed by IP of the ATM-NBS1 complex.

Characterization of BMI1-mediated attenuation of ATM activation

BMI1 contains several motifs, including a ring finger (RF), helix-turn-helix-turn-helix-turn (HT), nuclear localization signals (NLS), and a proline/serine (PS) rich region/PEST domain (Haupt, Alexander et al. 1991, van Lohuizen, Verbeek et al. 1991, Cohen, Hanna et al. 1996). To further characterize the interaction of BMI1 and NBS1, a set of C-terminal FLAG tagged BMI1 mutants with deletion of the RF, HT, PS domain, or one of two NLS sites (232-KRMK-235) were generated. Upon co-expression with NBS1 in 293T cells, NBS1 was co-immunoprecipitated with BMI1, BMI1 ΔRF, and BMI1 ΔHT at a comparable efficiency (Fig 8a), indicating that neither the RF nor the HT domain play major roles in mediating the binding of BMI1 and NBS1. In contrast, removal of the PS domain or the NLS residues (232-KRMK-235) substantially reduced the association (Fig 8a), suggesting a major role of either motif in mediating BMI1's association with NBS1.

To determine the contributions of the above structural elements to BMI1-mediated attenuation of ATM activation in response to ETOP treatment, we stably expressed BMI1 ΔHT, BMI1 ΔNLS (232-KRMK-235) (ΔNLS), BMI1 ΔPS, and BMI1 ΔRF individually in MCF7 cells (Fig 2.8b, Supplementary Fig 2.7). Although real-time PCR analysis indicated a high level of BMI1 ΔRF in MCF7 ΔRF cells (Fig 2.8b), we were unable to

detect the mutant protein in cell lysates via western blot (Supplementary Fig 2.7, the middle panel). However, the mutant protein was successfully observed following an IP-western blot procedure (Supplementary Fig 2.7, the right panel). It is thus likely that deletion of the RF domain caused the mutant to become unstable in MCF7 cells.

With the availability of these stable lines, we have examined the impact of the respective BMI1 mutants on ETOP-induced ATM activation. Consistent with the observed inhibition of ATM activation in ETOP-treated cells by BMI1, ectopic expression of all mutants significantly decreased ETOP-initiated ATM pS1981, indicative of ATM activation (Fig 2.8c). Phosphorylation of CHK2 at T68, but not p53 at S15 (data not shown), was also reduced in MCF7 cells expressing individual BMI1 mutants upon ETOP treatment (Fig 2.8c). Unlike MCF7 cells ectopically expressing BMI1 (Fig 2.1), overexpression of the individual mutants either had no effect (ΔNLS) on or increased ETOP-induced γH2AX expression (Fig 2.8c). As γH2AX is a surrogate marker of DSBs, the above results strongly suggest that BMI1 is able to reduce ETOP-induced ATM activation independently of its role in facilitating HR-mediated DSB repair (see Discussion for details).

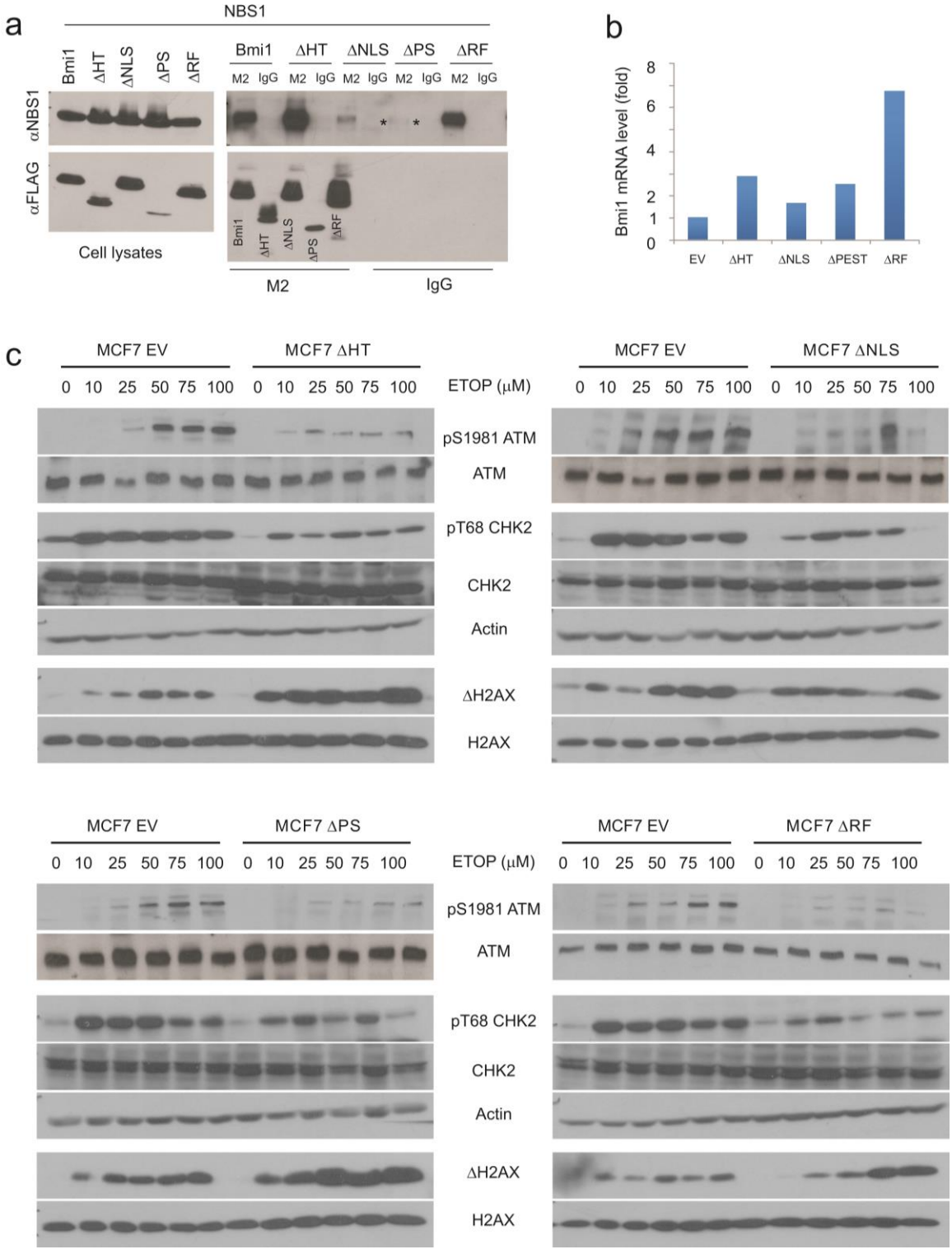
a
NBS1
Bmi1
ΔHT
ΔNLS
ΔPS
ΔRF
M2
IgG
αNBS1
αFLAG
Cell lysates
b
Bmi1 mRNA level (fold)
EV
ΔHT
ΔNLS
ΔPEST
ΔRF
c
MCF7 EV
MCF7 ΔHT
MCF7 ΔNLS
MCF7 ΔPS
MCF7 ΔRF
ETOP (μM)
0 10 25 50 75 100
pS1981 ATM
ATM
pT68 CHK2
CHK2
Actin
ΔH2AX
H2AX

Figure 2.8. Characterization of BMI1-derived inhibition of ATM activation in ETOP-treated MCF7 cells. (**a**) 293T cells were transiently transfected with NBS1 and FLAG-tagged BMI1 or the indicated BMI1 mutants. Cell lysates were prepared and used for the immunoprecipitation (IP) as indicated. The precipitates and cell lysates were analyzed by immunoblotting with the indicated antibodies. M2: monoclonal anti-FLAG antibody (Sigma); * indicates a low level of NBS1 being IP with ΔPS. Note: in comparison to 293T cells overexpression of NBS1 in cells transfected with NBS1 was confirmed (data not shown). The IP results presented in the top and bottom panels were from a single experiment but were analyzed in a different manner. (**b**) Real-PCR (in triplicate) analysis of BMI1 and the respective BMI1 mutants in MCF7 cells stably expressing EV or the indicated BMI1 mutants. The reaction also amplified endogenous BMI1. Results were normalized to actin, and expressed as fold change in reference to BMI1 in EV cells. (**c**) The indicated lines were treated as indicated for 2 hours, followed by western blot analysis for the indicated proteins. Experiments were repeated multiple times by two individuals. Typical results from a single experiment are shown.

BMI1 attenuates ETOP-induced ATM activation in MCF10A cells

To examine whether BMI1-reduced ATM activation in response to ETOP treatment is associated with transformation status, BMI1 was ectopically expressed in MCF10A cells (Fig 2.9a). While ETOP robustly induced ATM activation in MCF10A EV cells, evidenced by the rapid kinetics of increasing ATM pS1981, this ATM activation was clearly reduced in MCF10A BMI1 cells (Fig 2.9b). In comparison to MCF10A EV cells, ETOP-induced γH2AX was also decreased in MCF10A BMI1 cells (Fig 2.9c). However, phosphorylation of two ATM targets, CHK2 T68 and p53 S15, was not affected (Fig 2.9c) (see Discussion for detail interpretations). Collectively, these observations demonstrate that BMI1 decreases ETOP-induced ATM activation in both cancerous and non-cancerous cells.

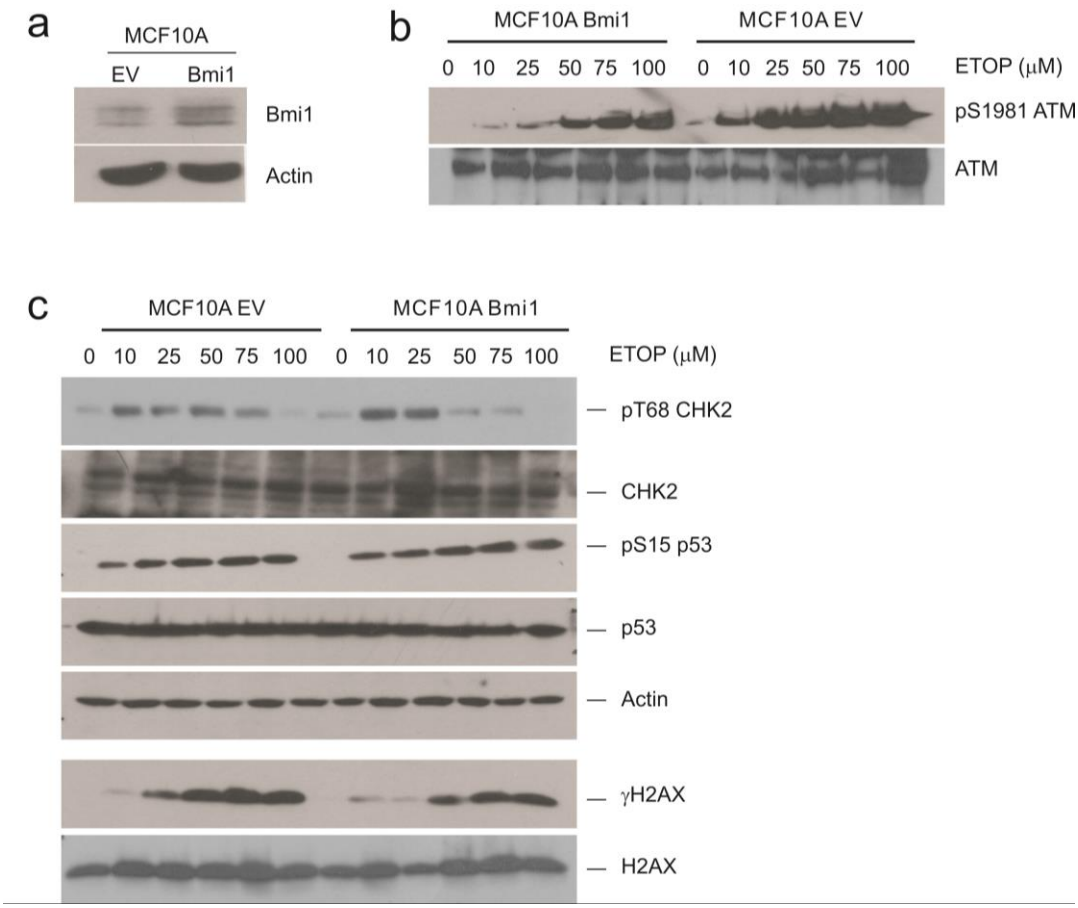

Figure 2.9. BMI1 reduces ETOP-initiated ATM activation in MCF10A cells. (**a**) MCF10A cells were stably transfected with pLNCX (EV) and pLNCX-BMI1 (BMIL1) retrovirus, followed by western blot analysis using anti-BMI1 and anti-Actin antibodies. (**b, c**) The indicated lines were treated for 2 hours with ETOP at the indicated doses, and analyzed for the indicated events by western blot.

Discussion

While accumulating evidence reveals a critical role of BMI1 in maintaining stemness and promoting tumorigenesis in a variety of tumors, largely by modulating gene expression via epigenetic mechanisms, recent developments have revealed a role of BMI1 in DDR. By enhancing a cell's ability to repair DSBs via HR, BMI1 may contribute to a cancer cell's resistance to DNA damage inducing chemotherapeutic reagents (Facchino, Abdouh et al. 2010, Chagraoui, Hebert et al. 2011, Crea, Duhagon Serrat et al. 2011, Gieni, Ismail et al. 2011, Liu, Liu et al. 2012). In addition to facilitating HR-mediated repair of DSBs, we provide evidence that in response to ETOP, BMI1 reduces activation of the G2/M checkpoints in both MCF7 and DU145 cells. BMI1-derived attenuation of ATM activation is not limited to ETOP. In comparison to EV cells, neocarcinostain (NCS) induced less ATM activation in MCF7 BMI1 cells (Supplementary Fig 2.8).

Our research may reveal an additional mechanism by which BMI1 promotes tumorigenesis, via compromising DSB-induced G2/M arrest. BMI1 plays a role in facilitating cancer cell proliferation in the presence of DNA damage. This may enhance genome instability, and prime cancer cells for mutations which subsequently promote cancer progression. This possibility agrees well with our current knowledge that cancer cells are commonly associated with genome instability (Sieber, Heinimann et al. 2003, Kops, Weaver et al. 2005), and that cancer cells frequently display elevated DNA damage (Bartkova, Rezaei et al. 2006, Halazonetis, Gorgoulis et al. 2008). Furthermore, BMI1 was reported to associate with cancer progression; the 11 gene signature that predicts poor prognosis in prostate cancer patients is associated with BMI1-positive prostate

carcinoma (Glinsky, Berezovska et al. 2005). BMI1 is linked to more advanced prostate cancers in both humans and mice (Nacerddine, Beaudry et al. 2012) and high levels of BMI1 predict poor prognosis for numerous cancers including neuroblastomas, prostate, breast, lung, ovarian, and many others (Siddique and Saleem 2012). While it is certain that BMI1 promotes the progression of different types of cancers via different mechanisms, our research suggests that one of these is to attenuate DSB-induced checkpoint activation.

BMI1 has been shown to enhance HR-mediated repair of DSBs in mouse embryonic fibroblast (MEFs), HCT116, and U2OS cells in response to IR (Ismail, Andrin et al. 2010, Chagraoui, Hebert et al. 2011). As HR is an error-free repair process for DSBs, BMI1 therefore functions in maintaining genome integrity. Our research on the other hand suggests that BMI1 enhances genome instability as a result of BMI1-mediated attenuation of G2/M arrest in ETOP-treated cells. The inconsistencies between this and published research may be due to different types of cells being studied, different DNA damage stimuli used (IR versus ETOP), or separate aspects of DDR being investigated. However, we would like to stress that our observations are not mutually exclusive from published research. While cancer cells are commonly associated with genome instability, certain levels of genome integrity need to be maintained. BMI1, therefore, may play a role in both processes. Intriguingly, this possibility is supported by our observations that while BMI1 attenuates ETOP-induced G2/M arrest, it also modestly enhances MCF7 cell's survival of ETOP-derived cytotoxicity. The fact that BMI1-mediated reduction of G2/M checkpoint activation in ETOP-treated MCF7 cells is not associated with an

enhancement of cytotoxicity supports a role of BMI1 in facilitating the repair of DNA lesions.

BMI1 has been reported to enhance IR-induced activation of ATM and its downstream targets CHK2 and H2AX (van Lohuizen, Verbeek et al. 1991, Quelle, Zindy et al. 1995), observations that are consistent with activation of ATM contributing to HR-mediated repair of DSBs (Chagraoui, Hebert et al. 2011). However, the relationship between BMI1 and DNA damage-induced ATM activation is complex. While IR causes BMI1 to be recruited onto DSBs, BMI1 does not apparently affect activation of the ATM pathway in MEFs (Ismail, Andrin et al. 2010). In normal human keratinocytes, BMI1-enhanced DSB repair was associated with reduced accumulation of γH2AX nuclear foci (Dong, Oh et al. 2011). Furthermore, knockdown of BMI1 in ovarian cancer cells enhanced cisplatin-induced CHK2 activation and γH2AX (Wang, Bhattacharyya et al. 2011). Our results demonstrate that in MCF7 breast cancer and DU145 prostate cancer cells. BMI1 reduces ETOP-initiated activation of the ATM pathway. It is therefore possible that the impact of BMI1 on DSB-induced G2/M checkpoint activation depends on cell contents. This concept is supported by our observations that while BMI1 attenuated ETOP-induced ATM pS1981, CHK2 pT68, p53 pS15, and γH2AX in MCF7 cells (Figs 1-4), it reduced ETOP-initiated ATM pS1981 and γH2AX but not CHK2 pT68 and p53 pS15 in MCF10A cells (Fig 2.9). However, we cannot exclude the possibility that the differences observed in MCF7 and MCF10A cells were caused by the different levels of ectopic BMI1 in the two lines.

The mechanism by which BMI1 reduces ETOP-induced ATM activation needs further investigation. DSBs activate ATM, a process that requires ATM to bind NBS1 (Difilippantonio, Celeste et al. 2005, Falck, Coates et al. 2005). However, the association of ATM with NBS1 does not require the presence of DSBs (Gatei, Young et al. 2000). While we have co-precipitated NBS1 through BMI1, indicative of a high affinity association between the two proteins either directly or indirectly (Fig 2.7a), overexpression of BMI1 did not enhance their co-precipitation (Fig 2.7a). This suggests that mechanisms in addition to the high affinity association exist which may play a role in BMI1-mediated attenuation of ATM activation. Although high levels of BMI1 did not lead to increases in its high affinity interaction with NBS1, this does not exclude the possibility that low affinity-mediated dynamic association between the two proteins is affected, which may reduce NBS1-initiated ATM activation.

While our observations that BMI1 reduces DSB-induced activation of the ATM pathway can potentially result from BMI1-mediated robust repair of DSB via homologous recombination, we prefer a possibility that BMI1 affects HR and G2/M checkpoint activation via separate mechanisms. BMI1 is a component of the polycomb repressive complex 1 (PRC1), a complex with E3 ubiquitin ligase activity (Li, Cao et al. 2006). While BMI1 alone does not possess this enzymatic activity, its association with the E3 ligase RING2/RING1B significantly enhances the E3 ligase activity (Li, Cao et al. 2006). This association is mediated by the ring finger (RF) domain of both BMI1 and RING1B (Hemenway, Halligan et al. 1998). BMI1 enhances HR via ubiquitination of γH2AX and H2AX (Ismail, Andrin et al. 2010, Ginjala, Nacerddine et al. 2011), a

process likely dependent on the RF domain of BMI1, as the recruitment of RING2/RING1B to DNA lesions is mediated by BMI1 and deletion of RF abolishes BMI1 recruitment (Ismail, Andrin et al. 2010, Ginjala, Nacerddine et al. 2011). Intriguingly, deletion of RF has no effect on BMI1's association with NBS1, a protein that is required for ATM activation, and BMI1 ΔRF potently reduced ETOP-induced ATM activation (Fig 2.8c). While BMI1 ΔHT, BMI1 ΔRF, BMI1 ΔPS, and BMI1 ΔNLS all inhibit ATM activation in cells treated with ETOP, observations that are in line with the effect of BMI1 on ETOP-induced ATM activation, these mutants unlike BMI1 elevate γH2AX in ETOP-treated MCF7 cells (Fig 2.8c). These results can be interpreted as a potential interference of these mutants on BMI1's ability to facilitate HR-derived DSB repair. Therefore, there is the likelihood that BMI1 affects DDR at multiple levels.

Conflict of interest

The authors declare no conflict of interest

Acknowledgments

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Table 2.1. Bmi1 reduces ETOP-induced G2/M arrest in MCF7 cells

	DMSO	2 μM	4 μM		6 μM		8 μM		10 μM
	EV	EV	EV		EV		EV		EV
	Bmi1	Bmi1	Bmi1		Bmi1		Bmi1		Bmi1
G1	65.9±1.7	67.5±2.3	47.9±2.5	63.8±1.5	46.3±0.3	63.2±2.1	42.9±3.4	57.7±2.3*	46.1±0.4	57.4±1.8*	55.6±0.6*	55.8±0.5*
S	15.2±1.3	6.9±0.6	14.4±2.1	17.9±2.1	13.4±1.9	12.1±2.2	13.9±3.9	14.5±3.5	9.9±0.6	12.5±2.8	12.9±0.6	12.1±0.3*
G2/M	18.9±0.5	25.6±1.8	37.7±2.4	18.2±0.8	40.3±1.7	24.7±2.3	43.2±1.3	27.8±1.8*	44.0±1.0	30.1±1.0*	31.5±0.7*	32.1±0.2*

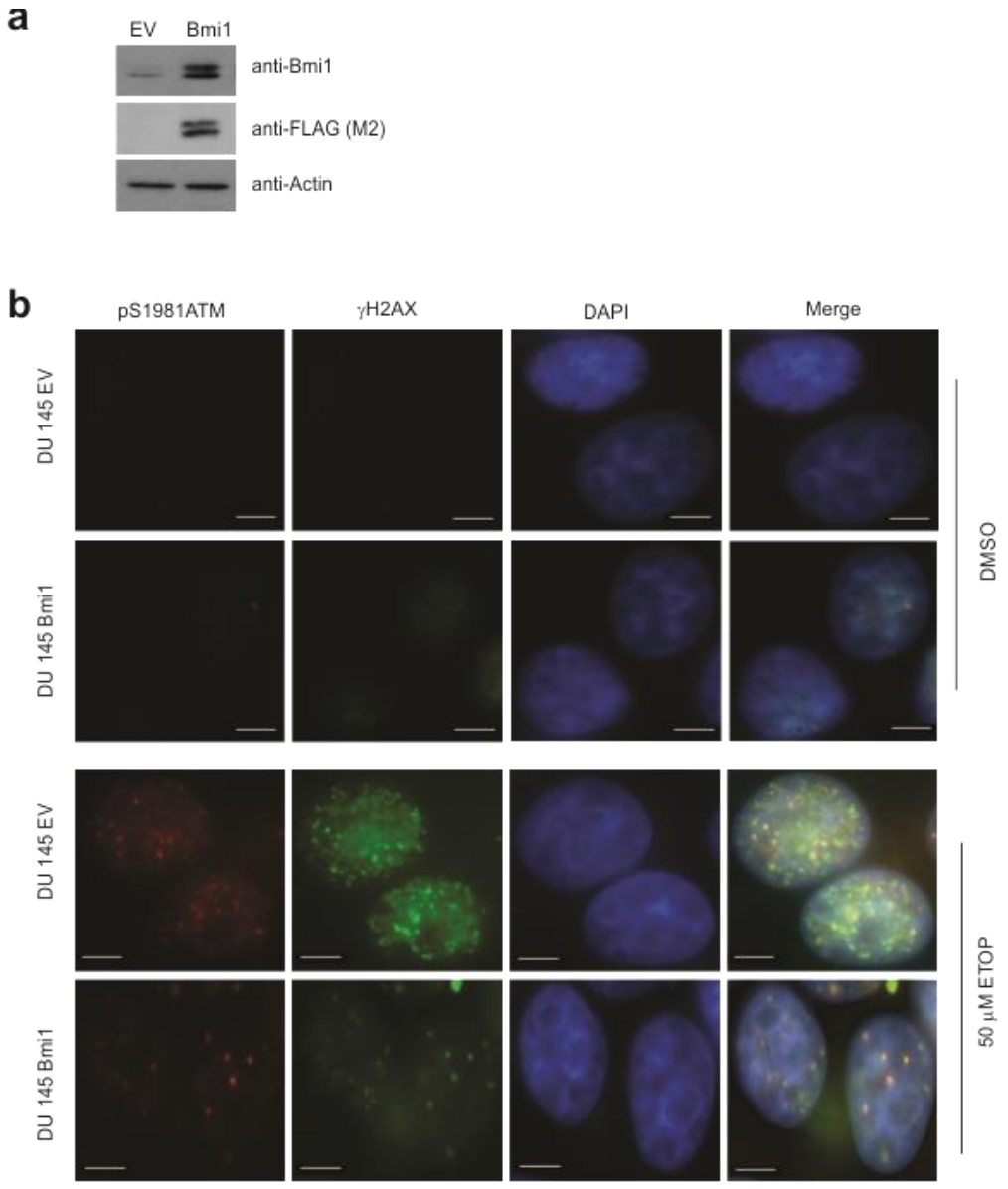
Note: Cell cycle distributions were derived from three independent experiments and were presented as means±SE; Ctrl: control; * $p < 0.05$ (in comparison to the respective cell cycle distributions of EV cells)

Table 2.2. Knockdown of Bmi1 enhances ETOP-induced G2/M arrest in MCF7 cells

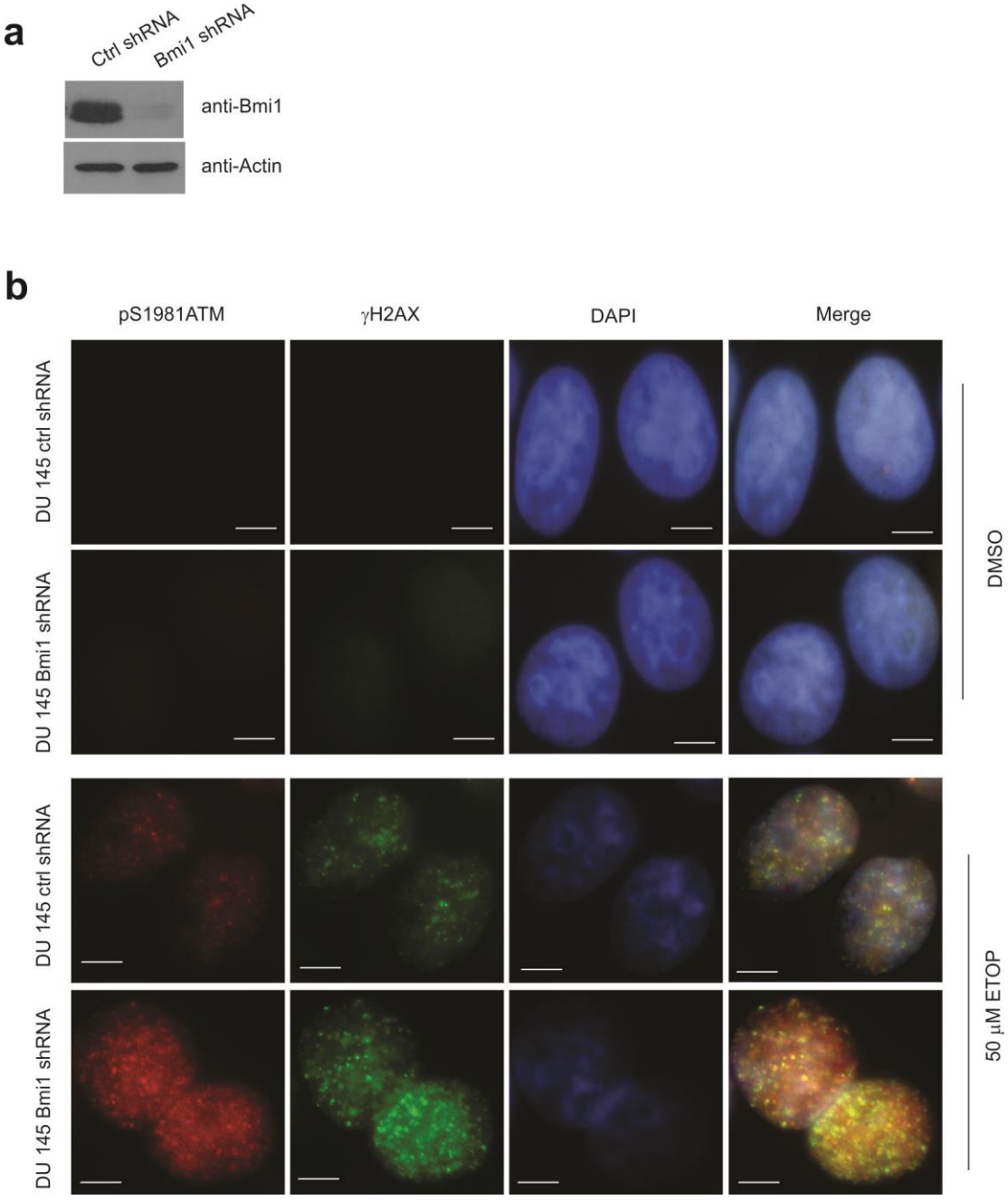
	DMSO	2 μM	4 μM	6 μM	8 μM	10 μM
	Ctrl shRNA	Ctrl shRNA	Ctrl shRNA	Ctrl shRNA	Ctrl shRNA	Ctrl shRNA
	Bmi1 shRNA	Bmi1 shRNA	Bmi1 shRNA	Bmi1 shRNA	Bmi1 shRNA	Bmi1 shRNA
G1	67.4±2.5	69.1±1.5	54.0±2.7	46.3±0.3	44.2±3.5	42.9±3.5
	67.2±2.5	59.2±1.2*	38.6±3.5*	35.5±0.9*	35.6±0.2	35.3±0.4
S	16.7±1.3	10.5±4.6	10.3±1.7	13.4±1.9	12.6±3.3	13.5±3.1
	16.5±2.0	14.7±3.5	13.4±2.6	13.7±0.6	11.8±0.5	10.3±0.2
G2/M	16.9±1.5	20.4±3.1	35.7±1.1	40.3±1.7	43.2±0.3	43.6±0.6
	16.2±0.7	25.9±2.3	47.9±1.7*	50.7±0.4*	2.6±0.5*	54.5±0.4*

Note: Cell cycle distributions were derived from three independent experiments and were presented as means±SE; Ctrl: control; * $p < 0.05$ (in comparison to the respective cell cycle distributions of Ctrl shRNA cells)

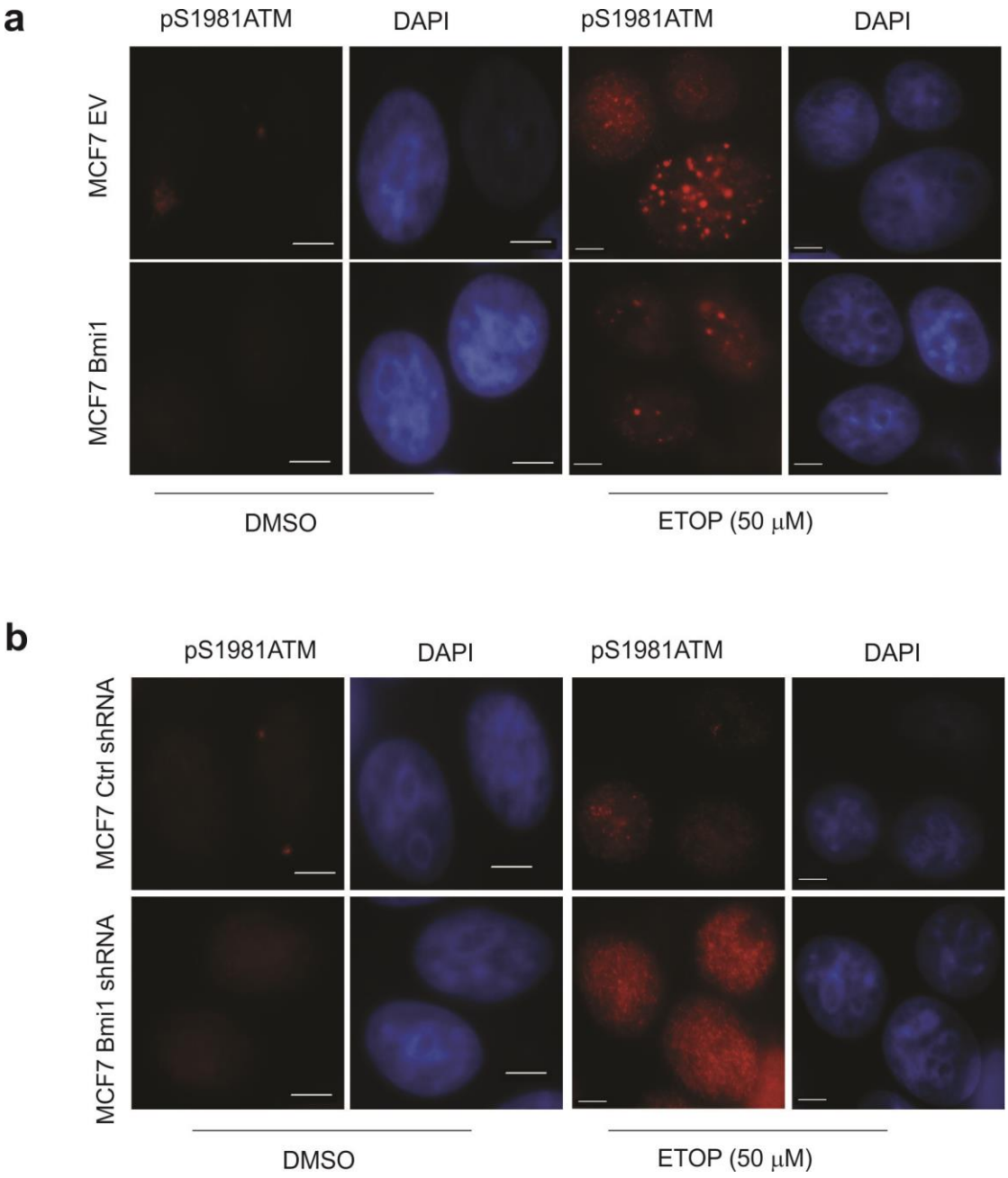
Supplementary figures



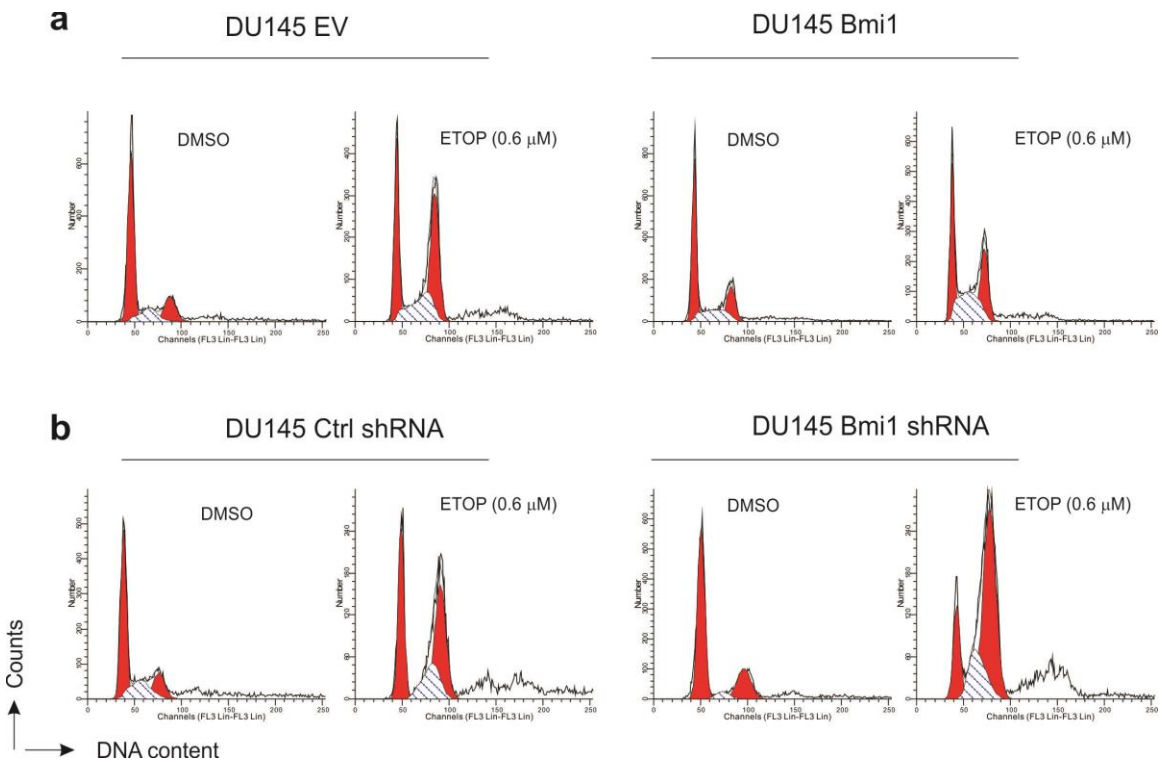
Supplementary Figure 2.1. Increase in BMI1 inhibits ETOP-induced activation of ATM and the production of γH2AX. (a) DU145 cells were stably infected with an empty vector (EV) retrovirus (pBabe) or FLAG-tagged BMI1 retrovirus. The expression of ectopic BMI1 was demonstrated by western blot using anti-BMI1 and anti-FLAG (M2) antibodies. (b) DU145 EV and BMI1 cells were treated with DMSO and ETOP for 2 hours, followed by IF staining for ATM S1981 phosphorylation (red) or for γH2AX (green). Nuclei were counter-stained with DAPI (blue). The merged images are also shown.



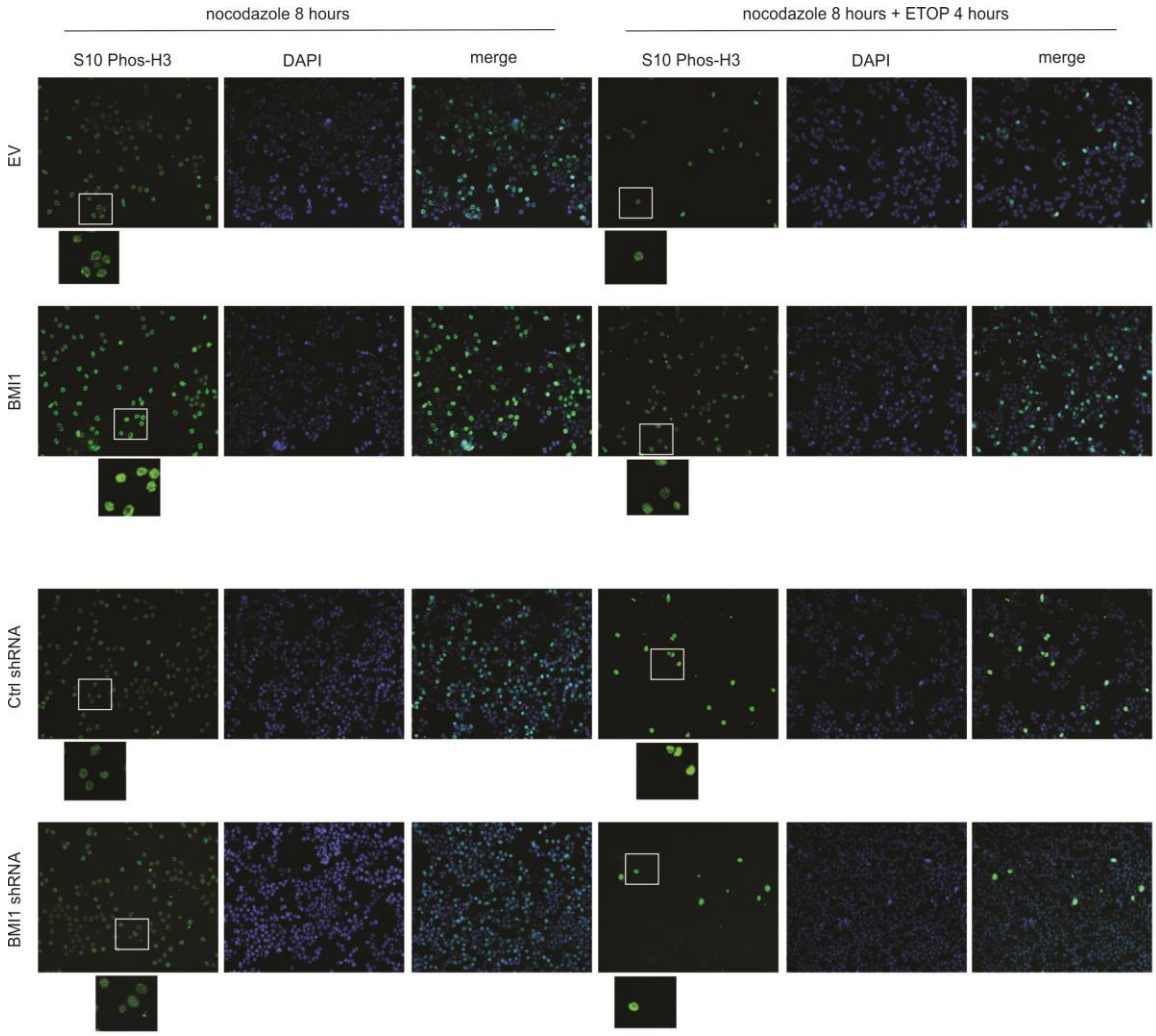
Supplementary Figure 2.2. Knockdown of BMI1 enhances ETOP-induced the activation of ATM and the production of γH2AX. (a) Stably knockdown of BMI1 in DU145 cells using control (Ctrl) or BMI1 shRNA retrovirus. (b) DU145 Ctrl shRNA and BMI1 shRNA cells were treated with DMSO and ETOP for 2 hours, followed by IF staining for ATM S1981 phosphorylation (red) or for γH2AX (green). Nuclei were counter-stained with DAPI (blue). The merged images are also shown.



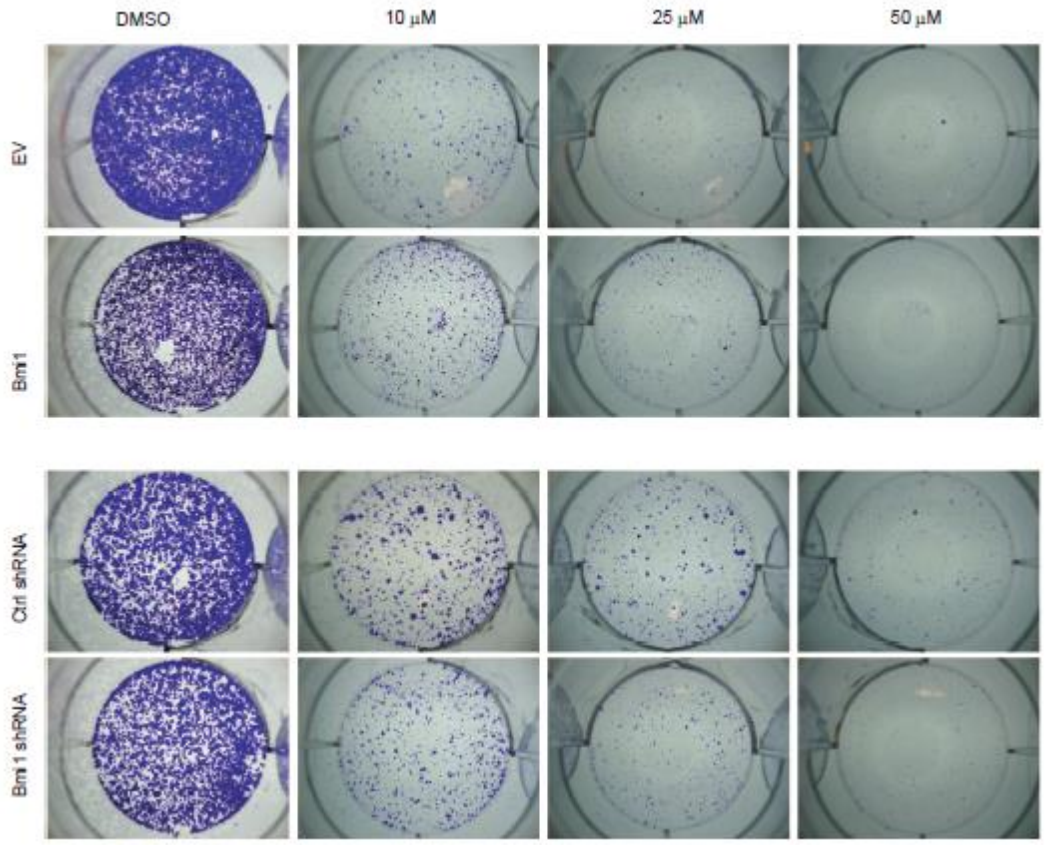
Supplementary Figure 2.3. Modulation of BMI1 expression in MCF7 cells affects the recruitment of active ATM to ETOP-induced DNA lesions. MCF7 cells. MCF7 EV and BMI1 cells (**a**) as well as MCF7 Ctrl shRNA and BMI1 shRNA cells (**b**) were treated with either DMSO or ETOP (50 μM) for 2 hours, followed by IF staining for active ATM (S1981 phosphorylated ATM/phos-S1981 ATM). Nuclei were counter stained with DAPI.



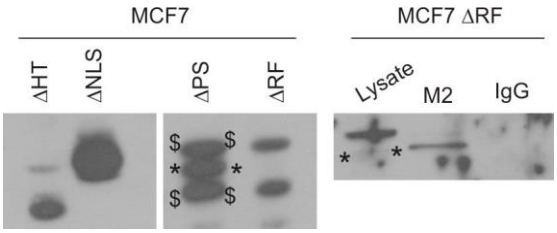
Supplementary Figure 2.4. Modulation of BMI1 expression in DU145 cells accordingly affects ETOP-induced G2/M arrest. DU145 EV and BMI1 cells (**a**) as well as DU145 Ctrl shRNA and BMI1 shRNA cells (**b**) were treated with either DMSO or ETOP for 24 hours, followed by analysis of cell cycle distribution by a flow cytometer.



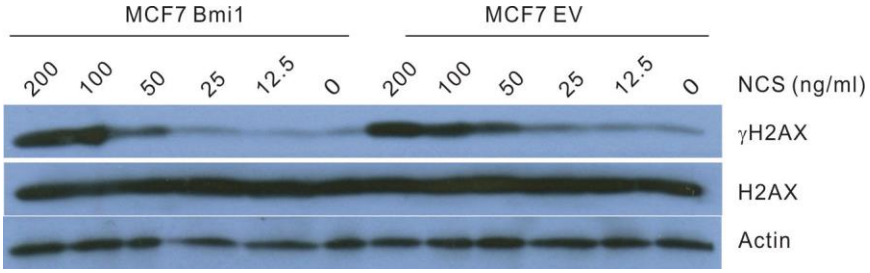
Supplementary Figure 2.5. BMI1 facilitates cell cycle progression into mitosis in the presence of etoposide. MCF7 EV, BMI1, Ctrl shRNA and BMI1 shRNA cells were treated with nocodazole (0.1 μg/ml) for 8 hours without or with addition of etoposide (ETOP, 6 μM) for the last 4 hours, followed by immunefluorescence staining for histone H3 S10 phosphorylation (green) (S10 phos-H3). Nuclei were counter stained with DAPI (blue). Experiments were repeated more than 3 times; typical images from a single repeat are included. The marked areas were enlarged 1.6 fold and placed underneath of the individual panels to reveal the mitotic feature of nuclei positive for histone H3 S10 phosphorylation.



Supplementary Figure 2.6. BMI1 enhances MCF7 cell survival of etoposide-induced cytotoxicity. MCF7 EV, BMI1, Ctrl shRNA and BMI1 shRNA cells were seeded in 6-well plates at 2 x 10^4/well. Cells were treated with DMSO or ETOP at the indicated doses for 8 hours, followed by a medium change. Cells were cultured for approximately two weeks till the mock-treated (DMSO) cells reach to approximately 90% confluency before staining with crystal violet. Experiments were repeated three time; typical images from a single repeat are shown



Supplementary Figure 2.7. Expression of BMI1 mutants in MCF7 cells. Cell lysates from MCF7 cells stably expressing ΔHT, ΔNLS, ΔPS, and ΔRF (the left and middle panels) were analyzed by western blot using anti-FLAG antibody. Note: the BMI1 ΔRF mutant protein was not detected in cell lysate in several trials by two individuals. $: background bands; *: the BMI1 ΔPS protein. MCF7 BMI1 ΔRF cell lysates were IP with anti-FLAG (M2) or control IgG (IgG) (the right panel), followed by western blot examination with a polyclonal anti-FLAG antibody (Sigma); 1/10 of the amount of cell lysate used for IP was also included. *: indicates the potential BMI1 ΔRF protein.



Supplementary Figure 2.8. BMI1 attenuates NCS-induced γH2AX. The indicated cells were treated with NCS at the indicated doses for 2 hours, followed by the examination of γH2AX, H2AX, and actin.

Supplementary Table 2.1. Linkers and primers used to construct the BMI1 deletion mutants, and real time PCR primers

ΔRF - Linker and primers	linker top strand - gatccgccaccatgcatcgaacaacgagaatcaagatcactgagctaaatccccacctgatgc linker bottom strand - tcgagcatcaggtggggatttagctcagtgatcttgattctcgttgttcgatgcatggtggcc primer 1: 5'-ccgctggaggtccaagttcacaagaccagacc-3' primer 2: 5'-gctctagaaccagaagaagttgctgatgacc-3'
ΔHT primers	primer 1: 5'-cgggatccgccaccatgcatcgaacaacgagaatc-3' primer 2: 5'-ccgctggaggtatcttttatcattcacctcctcctta-3' primer 3: 5'-ccgctcgagcctacttgtaaaagaatgaagatcagtc-3' primer 4: 5'-gctctagaaccagaagaagttgctgatgacc-3'
ΔPS primers	primer 1: 5'-cgggatccgccaccatgcatcgaacaacgagaatc-3' primer 2: 5'-gctctagattccagttctccagcatttgtc-3'
ΔNLS primers	primer 1: 5'-cgggatccgccaccatgcatcgaacaacgagaatc-3' primer 2: 5'-ccgctcgagacaagtaggtcgaactctgtatttcaat-3' primer 3: 5'-ccgctcgagatcagtcaccagagagatggactg-3' primer 4: 5'-gctctagaaccagaagaagttgctgatgacc-3'
BMI1 (qPCR)	primer 1: 5'-gttcacaagaccagaccactac-3' primer 2: 5'-gcagaaggatgagctgcatta-3'
Actin (qPCR)	primer 1: 5'-accgagcgcggctacag-3' primer 2: 5'-cttaatgtcacgcacgatttcc-3'

CHAPTER THREE

BMI1 reduces ATR activation and signalling caused by hydroxyurea

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Preamble

While BMI1 facilitates HR-based DSB repair, it remains unknown whether BMI1 functions in DDR induced by ssDNA. In this manuscript we show that BMI1 reduces ssDNA-initiated checkpoint activation through a reduction of ATR activation. ATR subsequently produces γH2AX and CHK1 pS345, leading to S-phase arrest. Overexpression and knockdown of BMI1 respectively reduced and enhanced these events. Mechanistically, ATR activation requires association with TOPBP1. Of note, BMI1 binds TOPBP1 and possibly ATR, suggesting a mechanism by which BMI1 attenuates HU-induced ATR activation. The ring finger (RF) domain is essential for BMI1 to stimulate PRC1-derived E3 ubiquitin ligase activity. Deletion of this domain was without effects on BMI1's association with TOPBP1 and ATR-mediated CHK1 phosphorylation, suggesting that BMI1 attenuates ATR activation independent of the E3 ubiquitin ligase activity.

Author's Contribution

Dr. D. Tang and X. Lin designed the experiment. X. Lin examined 1) the impacts of BMI1 and its mutants on HU-induced ATR activation, 2) the association of BMI1 and TOPBP1, 3) the formation of BMI1/ATR/TOBP1 complex, and 4) S-phase arrest. F. Wei established MCF7 BMI1 overexpression stable line, investigated the contribution of BMI1 to S phase arrest, and determined BMI1-derived reduction in ATR activation. Dr. P. Whyte and Dr. D. Tang supervised this experiment. X. Lin, F. Wei, Dr. P. Whyte, and Dr. D. Tang prepared the manuscript.

Relationship to the theme of my Ph.D. research

ATR is essential for the stabilization of replication forks, which is critical for maintaining genome integrity. This work provides the first demonstration that BMI1 reduces ATR activation induced by ssDNA. The theme of my research is to examine the effects of BMI1 on the activation of ATM and ATR during DDR. The current research thus directly contributes to the research theme.

BMI1 reduces ATR activation and signalling caused by hydroxyurea

Xiaozeng Lin,[1,2,3] Fengxiang Wei,[1,2,3,4] Peter Whyte,[5] and Damu Tang [1,2,3 #]

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Running Title: BMI1 reduces hydroxyurea-induced ATR activation

Key words: BMI1, DNA damage response, ATR, CHK1, S checkpoint activation

Abstract

BMI1 facilitates DNA damage response (DDR) induced by double strand DNA breaks; however, it remains unknown whether BMI1 functions in single strand DNA (ssDNA) lesions-initiated DDR. We report here that BMI1 reduces hydroxyurea-elicited ATR activation, thereby reducing the S-phase checkpoints. Hydroxyurea induces ssDNA lesions, which activate ATR through binding TOPBP1 as evidenced by phosphorylation of ATR at threonine 1989 (ATRpT1989). ATR subsequently phosphorylates H2AX at serine 139 (γH2AX) and CHK1 at serine 345 (CHK1pS345), leading to phosphorylation of CDK1 at tyrosine 15 (CDK1pY15) and S-phase arrest. BMI1 overexpression reduced γH2AX, CHK1pS345, CDK1pY15, S-phase arrest, and ATR activation in HU-treated MCF7 and DU145 cells, whereas BMI1 knockdown enhanced these events. BMI1 contains a ring finger, helix-turn, proline/serine domain and two nuclear localization signals (NLS). Individual deletion of these domains abolished BMI1-derived reduction of ATRpT1989 and γH2AX but not CHK1pS345 in MCF7 cells following HU exposure, suggesting a novel mechanism whereby BMI1 reduces ATR-mediated phosphorylation of CHK1 and inhibits ATR activation. BMI1 interacts with both TOPBP1 and ATR. Furthermore, all BMI1 mutants associate with endogenous TOPBP1, while BMI1(ΔNLS) also binds endogenous ATR. Association of TOPBP1 and ATR is required for ATR activation. In this regard, the observed interactions of BMI1 and its mutants with TOPBP1 and ATR suggest an impact of BMI association with TOPBP1 and ATR on the TOPBP1/ATR complex-mediated ATR activation in response to ssDNA lesions.

Collectively, we provide the first demonstration that BMI1 reduces ATR activation via binding to TOPBP1 and ATR.

Introduction

BMI1 is a polycomb group (PcG) protein of the polycomb repressive complex 1 (PRC1) (Levine, King et al. 2004), and is required for formation of E3 ubiquitin ligase activity of PRC1 via binding to the catalytic subunit RING2 (de Napoles, Mermoud et al. 2004, Wang, Wang et al. 2004, Cao, Tsukada et al. 2005, Li, Cao et al. 2006). The E3 ubiquitin ligase activity underlies PRC1-mediated suppression of gene expression. BMI1 represses the *INK4A/ARF* and *E4F1* loci (Bruggeman, Valk-Lingbeek et al. 2005, Molofsky, He et al. 2005, Chagraoui, Niessen et al. 2006, Akala, Park et al. 2008). The *INK4A/ARF* locus encodes two tumor suppressors, $p16^{INK4A}$ and $p19^{ARF}$/$p14^{ARF}$, via alternative splicing and using differential promoters (Quelle, Zindy et al. 1995, Sherr 1998). E4F1 inhibits cell proliferation, in part, through promoting p53 and CHK1 functions (Le Cam, Linares et al. 2006, Grote, Moison et al. 2015, Rodier, Kirsh et al. 2015). Suppression of these loci contributes to BMI1-derived maintenance of the self-renewal of hematopoietic and neural stem cells (Molofsky, Pardal et al. 2003, Park, Qian et al. 2003, Chagraoui, Niessen et al. 2006). Inhibition of INK4A and ARF-mediated tumor suppression is critical for tumorigenesis (Quelle, Zindy et al. 1995, Sherr 1998). Upregulation of BMI1 occurs in numerous cancer types including non-small cell lung cancer (Vonlanthen, Heighway et al. 2001), colon cancer (Kim, Yoon et al. 2004), breast cancer (Kim, Yoon et al. 2004), and nasopharyngeal carcinoma (Song, Zeng et al. 2006). BMI1 overexpression is able to transform lymphocytes (Haupt, Bath et al. 1993, Alkema, Jacobs et al. 1997) and its upregulation in lymphomas associates with poor prognosis (Bea, Tort et al. 2001, Mihara, Chowdhury et al. 2006, van Galen, Muris et al. 2007).

BMI1 synergizes with c-Myc in transgenic mouse models for leukemogenesis (Haupt, Alexander et al. 1991, van Lohuizen, Verbeek et al. 1991).

In addition to inhibition of the pRB and p53 tumor suppressors through repression of the *INK4A/ARF* locus (Quelle, Zindy et al. 1995, Sherr 1998), BMI1 is involved in DNA damage response (DDR) (Ismail, Andrin et al. 2010, Chagraoui, Hebert et al. 2011, Ginjala, Nacerddine et al. 2011, Wei, Ojo et al. 2015). DDR is essential in maintenance of genomic integrity and accurate passage of genetic materials to the daughter cells (Zhou and Elledge 2000). Compromising DDR leads to genomic instability, a hallmark of cancer (Sieber, Heinimann et al. 2003, Kops, Weaver et al. 2005) and a major cause of tumorigenesis (Lengauer, Kinzler et al. 1998, Hoeijmakers 2001, Rouse and Jackson 2002). Enhancing repair of DDR lesions contributes to therapy resistance in cancer (Burrows, Williams et al. 2016, Gaponova, Deneka et al. 2016). DDR is initiated by a variety of DNA lesions (Mordasini, Ueda et al. 2017) through activation of three apical PI3 kinase-related kinases (PIKKs) ATM, ATR, and DNA-PK (Wei, Yan et al. 2011, Lin, Yan et al. 2013). PIKKs coordinate DDR via checkpoint activation to prevent cell cycle progression and preparation for DNA lesion repair (Zhou and Elledge 2000, Shiloh 2003). Double strand DNA breaks (DSBs) activate ATM, leading to phosphorylation of downstream targets, including CHK2 and γH2AX (Wei, Yan et al. 2011, Lin, Yan et al. 2013). CHK2 activation subsequently results in G2/M arrest (Zhou and Elledge 2000, Shiloh 2003) and the formation of γH2AX nuclear foci around DSBs initiates DSB repair (Paull, Rogakou et al. 2000, Celeste, Petersen et al. 2002). As part of the repair process, BMI1 rapidly associates with DSBs, ubiquitinates γH2AX, and contributes to

homologous recombination (HR)-facilitated DSB repair (Ismail, Andrin et al. 2010, Chagraoui, Hebert et al. 2011, Ginjala, Nacerddine et al. 2011). Additionally, BMI1 also compromises DSB-induced checkpoint activation by reducing ATM activation (Wei, Ojo et al. 2015).

Another major arm of DDR is initiated by single-strand DNA (ssDNA) lesions, which are typically produced by stalled replication forks. These lesions are first coated with replication protein A (RPA). RPA-ssDNA independently recruits the ATR-ATRIP complex and TOPBP1, where TOPBP1 activates ATR through a physical association. ATR subsequently phosphorylates and activates CHK1, leading to S-phase arrest (Cimprich and Cortez 2008, Awasthi, Foiani et al. 2015). In view of these similarities between ATM and ATR activation, we have examined whether BMI1 also decreases ssDNA-initiated ATR activation.

Hydroxyurea (HU) is a potent DNA synthesis inhibitor (Timson 1975), and causes stalled replication forks through depletion of the dNTP pool, leading to accumulation of ssDNA and activation of the ATR-dependent S-phase checkpoints (Ivessa, Lenzmeier et al. 2003). We report here that BMI1 delays S-phase checkpoint activation induced by HU. In MCF7 cells treated with HU, BMI1 overexpression reduced ATR activation, phosphorylation of CHK1, and S-phase arrest, whilst BMI1 knockdown had the opposite effect. BMI1 interacted with TOPBP1 and ATR in co-immunoprecipitation experiments suggesting a possible mechanism.

Materials and Methods

Materials, cell lines, and cell cycle determination

HU and propidium iodide (PI) were purchased from Sigma. MCF7 breast cancer and DU145 prostate cancer cell lines were obtained from ATCC, and cultured in DMEM (MCF7) and MEM (DU145) supplemented with 10% FBS (Sigma Aldrich, Oakville, ON) and 1% Penicillin-Streptomycin (Life Technologies, Carlsbad, CA). Cell cycle distribution was examined according to our published procedure (Tang, Wu et al. 2002).

Immunofluorescence staining

Immunofluorescence staining was performed by fixing cells with prechilled (-20^{o}C) acetone-methanol for 15 minutes, followed by addition of primary antibodies to anti-γH2AX (Cell Signaling, 1:100) or anti-histone H3 S10 phosphorylation (Upstate, 1:250) at 4^{o}C overnight. After rinsing, FITC-Donkey anti-rabbit IgG (1:200, Jackson Immuno Research Lab) was applied for 1 hour at room temperature. Slides were subsequently covered with the DAPI mounting medium (VECTOR Lab Inc.). Images were then acquired with a fluorescent microscope (Carl Zeiss, Axiovert 200).

Quantification of γH2AX-positive nuclei

More than 200 nuclei from several randomly selected fields of focus were analyzed. Nuclei with ≥ 10 γH2AX foci and those with < 10 γH2AX foci were respectively defined as positive and negative.

Western blot

Cell lysates were prepared in a buffer consisting of 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 25 mM sodium pyrophosphate, 1 mM NaF, 1 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM PMSF, 2 μg/ml leupeptin and 10 μg/ml aprotinin. Cell lysates containing 50 μg protein were separated on SDS-PAGE gel, and transferred onto Hybond ECL nitrocellulose membranes (Amersham), followed by treatment with 5% skim milk at room temperature for one hour as well as incubation with individual primary and secondary antibodies. Signals were then developed (ECL Western Blotting Kit, Amersham). Primary antibodies used were: monoclonal anti-BMI1 (1:1000, Invitrogen), anti-H2AX (1:1000, Millpore), anti-γH2AX (1:1000，Cell Signaling), anti-T1989 phosphorylated ATR (1:1000, Abcam), anti-ATR (1:500, Santa Cruz), anti- phospho-CHK1 (S345) (1:500, Cell Signaling), anti-CHK1 (1:1000, Cell signaling), anti-TOPBP1 (1:500, Bethyl), anti-phospho-CDK1 (Y15) (1:1000, Cell Signaling), anti-CDK1 (1:1000, Santa Cruz), anti-tubulin (1:1000, Santa Cruz), and anti-actin (1:1000, Santa Cruz).

Immunoprecipitation (IP)

IP were carried out by incubating 1mg cell lysate proteins with individual antibodies in the presence of Protein G agarose (Invitrogen) or Dynabeads (Invitrogen) overnight at 4^{o}C, and washing 8 times with a buffer [50 mM Tris (PH 7.5), 100 mM NaCl, 7.5 mM EGTA, and 0.1% Triton X-100]. Lysates were treated with Benzonase (Sigma, 1 U/μl)

for one hour on ice. Antibodies used for IP were polyclonal anti-BMI1 (Santa Cruz, 1 μg), anti-M2 (Sigma, 1 μg), and anti-ATR (Santa Cruz, 1 μg). The immunoprecipitations were analyzed by western blot using anti-BMI1 (1:1000, Invitrogen), anti-TOPBP1 (1:500, Bethyl), polyclonal anti-FLAG (Sigma, 1:500) and anti-ATR (1:500, Santa Cruz).

Retroviral Infection

Retroviral infection was performed according to our published procedure (Tang, Okada et al. 2001). In brief, a gag-pol expressing vector and an envelope-expressing plasmid (VSV-G) (Stratagene) were co-transfected with a specific retroviral construct into 293T cells for 48 hours. The virus-containing medium was then filtered (0.45 μM filter) and centrifuged (50,000g for 90 minutes). The viral pellet was resuspended in a specific medium containing 10 μg/ml of polybrene (Sigma) prior to infection of cells. Infection was selected using specific antibiotics.

BMI1 overexpression and knockdown in MCF7 and DU145 cells

MCF7 and DU145 cells were transfected with pBabe and pBabe-based BMI1 retrovirus to establish the respective EV and BMI1 stable cell lines. Control and BMI1 retroviral plasmids expressing short hairpin-based RNAs (shRNA) were purchased from Santa Cruz. Retrovirus was packed and used to generate the respective shCtrl and shBMI1 lines.

Statistical analysis

Student's t-test (2-tails) was used. A p-value < 0.05 was regarded as statistically significant.

Results

BMI1 delays HU-induced activation of the S-phase checkpoints

BMI1 has been reported to enhance HR-mediated DSB repair (Ismail, Andrin et al. 2010, Chagraoui, Hebert et al. 2011, Ginjala, Nacerddine et al. 2011), and reduce DSB-initiated G2/M checkpoints caused by etoposide (Wei, Ojo et al. 2015). To investigate whether BMI1 is involved in ssDNA-stimulated DDR, we have constructed MCF7 breast cancer and DU145 prostate cancer cell lines in which BMI1 was either stably overexpressed or knocked-down (Supplementary Figure 3.1). Using these lines, we examined the impact of BMI1 expression levels on HU-induced S-phase checkpoints. HU causes stalled replication forks as a result of depletion of cellular dNTP pools, leading to activation of the S-phase checkpoints in MCF7 and DU145 cells (Wu, Chen et al. 2006, Wei, Xie et al. 2011) through CHK1 activation. CHK1 contributes to CDK1 inactivation via sustaining CDK1 phosphorylation at tyrosine 15 (Y15) (O'Connell, Raleigh et al. 1997), an event that prevents mitotic entry (Chen and Sanchez 2004). In accordance with this knowledge, HU dose-dependently stimulated CHK1 phosphorylation at serine 345 (CHK1pS345), indicative of CHK1 activation, and CDK1pY15 in both MCF7 (Figure 3.1A, C, and E) and DU145 cells (Supplementary Figure 3.2). Both events were substantially reduced in MCF7 BMI1 (Figure 3.1A, C, and E). Although there was an unexpected high level of CDK1pY15 in DU145 BMI1 cells treated with 0.5mM HU, it is apparent that DU145 BMI1 cells displayed a low kinetics of CDK1pY15 in response to HU exposure compared to DU145 EV cells (Supplementary Figure 3.2). Collectively,

evidence supports that enforced BMI1 expression in both MCF7 and DU145 cells results in reductions in CHK1 activation and CDK1 inactivation following HU treatment. Conversely, knockdown of BMI1 elevated HU-induced CHK1pS345 and CDK1pY15 in MCF7 cells (Figure 3.1B, D, and F). Furthermore, we have stably re-expressed murine BMI1 into MCF7 shBMI1 cells (Wei, Ojo et al. 2015) and confirmed its expression (Fig 3.1G); re-expression of murine BMI1 reversed the elevation of CHK1pS345 in MCF7 shBMI1 cells treated with HU (compare the CHK1pS345 profile in Figure 1B to that in Figure 3.1G), indicating that the increase in CHK1pS345 following BMI1 knockdown in MCF7 cells (Figure 3.1B) was not caused by potential off-target effects. Examination of HU-induced checkpoint activation has been commonly performed by treating cells for 24 hours at concentrations of 1mM or less (Wu, Chen et al. 2006, Wei, Xie et al. 2011). This condition does not result in substantial collapse of the DNA replication forks, as cells renewed proliferation upon releasing from 1mM HU-24 hour treatment with minimal adverse effects in comparison to mock-treated cells (Supplementary Figure 3.3).

To further justify the treatment conditions involving 1mM HU or less for 24 hours, we determined the kinetics of CHK1 activation in HU-treated MCF7 cells. HU elicited early (1-8h) and late phases (14-24h) of CHK1 activation (Supplementary Figure 4). As cell cycle arrest, a major event of checkpoint activation, is commonly examined at 24 hour following treatment (Wu, Chen et al. 2006, Wei, Xie et al. 2011), we thus focused this study on the late phase of CHK1 activation and DDR induced by HU at doses $\leq$ 1mM. Nevertheless, because HU also induces an early onset of CHK1pS345 (Supplementary Figure 3.4), we have determined whether BMI1 affected CHK1pS345 in

this early phase. It was clear that overexpression of BMI1 in both MCF7 and DU145 cells robustly reduced the dose-dependent kinetics of CHK1pS345 during a 2-hour HU treatment (Figure 3.2A, B, left panels), whereas knockdown of BMI1 in both lines elevated this event (Figure 3.2A, B, right panels). Furthermore, knockdown of BMI1 in DU145 cells also reduced HU-induced late-phase CHK1pS345 in compared to DU145 shCtrl cells (Fig 3.2B, bottom right panel). Similar results were also obtained in MCF7 shBMI1 cells (Fig 3.1B, D). BMI1 overexpression in MCF7 and DU145 cells reduced HU-induced CHK1pS345 at 24-hour HU treatment (Fig 3.1A, C; Supplementary Fig 3.2). Nonetheless, the effects of modulation of BMI1 on CHK1pS345 in cells treated with high doses of HU, particularly 1mM HU appeared less robust compared to cells treated with low doses of HU in response to either late (24h) or early phase (2h) treatment (Figs 3.1 and 3.2). These observations indicate that BMI1 attenuates CHK1 activation. Collectively, the above experiments provide a comprehensive set of data demonstrating a role of BMI1 in reducing CHK1 activation following HU treatment.

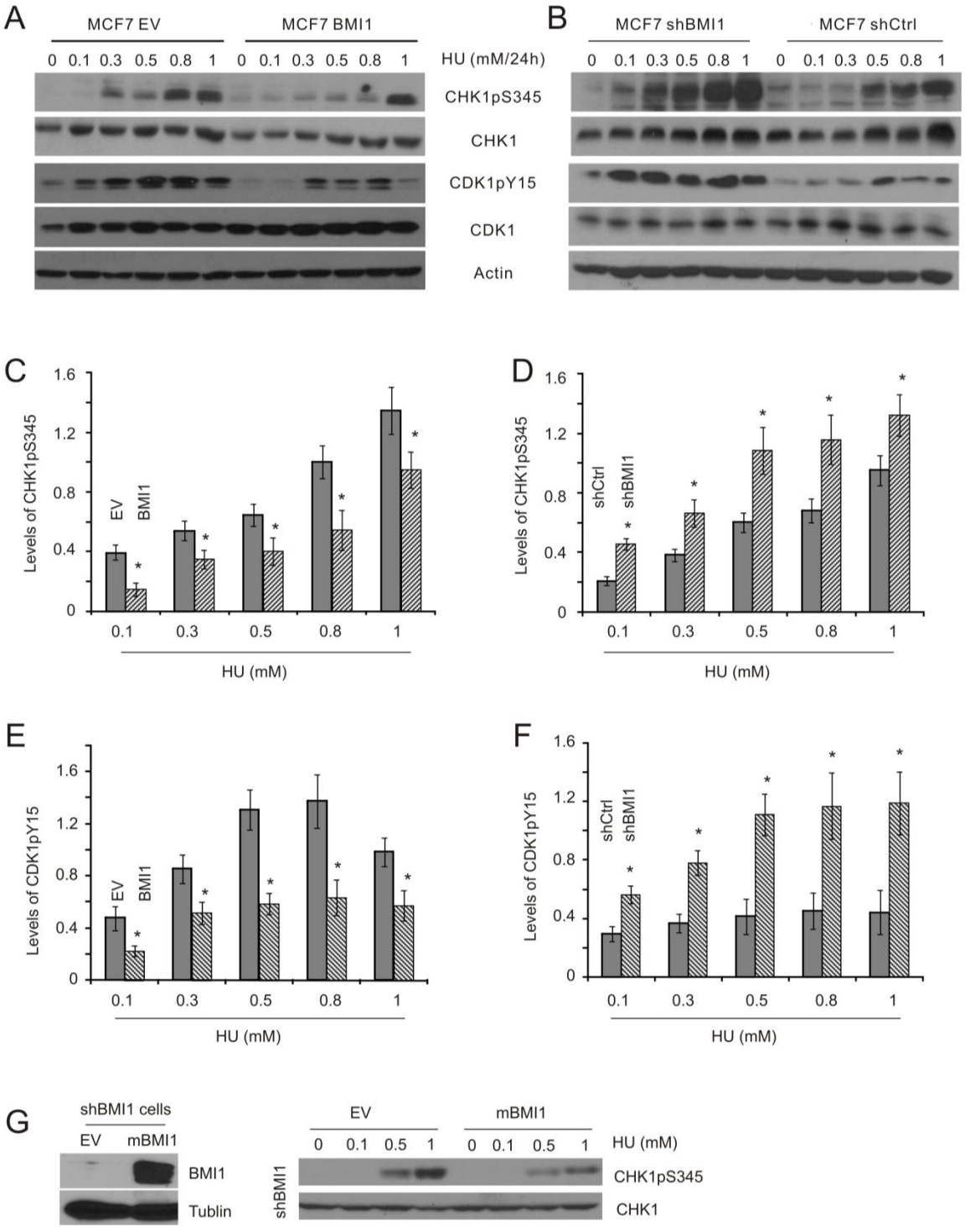
A
MCF7 EV
MCF7 BMI1
0 0.1 0.3 0.5 0.8 1 0 0.1 0.3 0.5 0.8 1
HU (mM/24h)
CHK1pS345
CHK1
CDK1pY15
CDK1
Actin
B
MCF7 shBMI1
MCF7 shCtrl
0 0.1 0.3 0.5 0.8 1 0 0.1 0.3 0.5 0.8 1
C
Levels of CHK1pS345
EV
BMI1
0.1 0.3 0.5 0.8 1
HU (mM)
D
Levels of CHK1pS345
shCtrl
shBMI1
0.1 0.3 0.5 0.8 1
HU (mM)
E
Levels of CDK1pY15
EV
BMI1
0.1 0.3 0.5 0.8 1
HU (mM)
F
Levels of CDK1pY15
shCtrl
shBMI1
0.1 0.3 0.5 0.8 1
HU (mM)
G
shBMI1 cells
EV mBMI1
BMI1
Tublin
EV
mBMI1
0 0.1 0.5 1 0 0.1 0.5 1
HU (mM)
shBMI1
CHK1pS345
CHK1

Figure 3.1. BMI1 decreases HU-induced CHK1 activation in MCF7 cells. MCF7 EV (empty vector), BMI1, shCtrl (Ctrl: control), and shBMI1 stable cell lines were established (see Supplementary Figure 1). These cell lines were treated with HU at the indicated doses for 24 hours, followed by Western blot examination for CHK1 phosphorylation at S345 (CHK1pS345), CHK1, CDK1 phosphorylation at Y15 (CDK1pY15), CDK1, and actin. Experiments were carried out three times; typical results from a single repeat are shown (**A, B**); means ± S.E (standard error) were graphed (**C-F**). *$p < 0.05$ in comparison to the respective control (EV and shCtrl) cells (two-tailed Student's *t*-test). (**G**) MCF7 shBMI1 cells were stably transfected with EV or mouse BMI1 (mBMI1) (left panel), followed by the examination of HU-induced CHK1pS345. The experiments were repeated once; typical images from a single repeat are included.

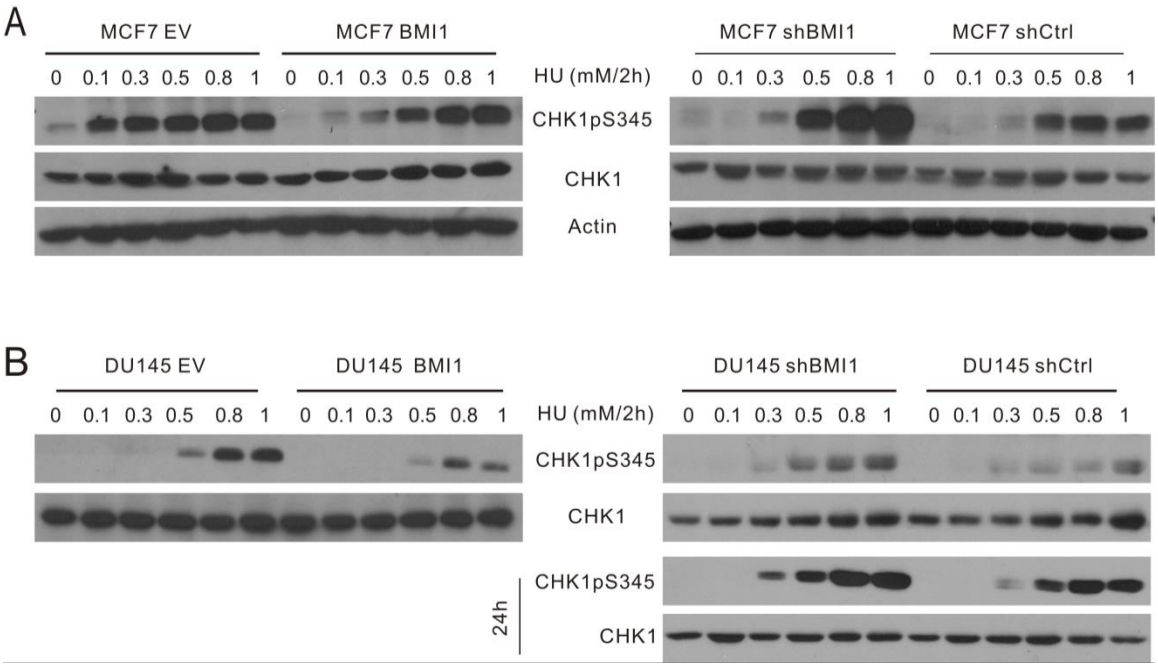

Figure 3.2. BMI1 reduces the early onset of CHK1pS345 caused by HU. The MCF7 and DU145-based EV, BMI1, shCtrl, and shBMI1 cells were treated with the indicated doses of HU for 2 hours, followed by determination of CHK1pS345, CHK1, and actin by Western blot. For DU145 shCtrl and shBMI1 cells, the indicated treatments were also continued for 24 hours (bottom right panel). Experiments were repeated once; typical images from a single repeat are shown.

BMI1 reduces ATR activation caused by HU

The above observations suggest that BMI1 reduces ATR activation caused by HU. ATR activation depends on autophosphorylation at threonine 1989 (ATRpT1989) (Liu, Shiotani et al. 2011). In this regard, BMI1 overexpression reduced ATRpT1989 in HU-treated MCF7 cells (Figure 3A, left panel; Fig 3B), whereas BMI1 knockdown enhanced the event (Figure 3.3A, right panel; Fig 3.3C). These observations support the concept that BMI1 attenuates HU-induced ATR activation.

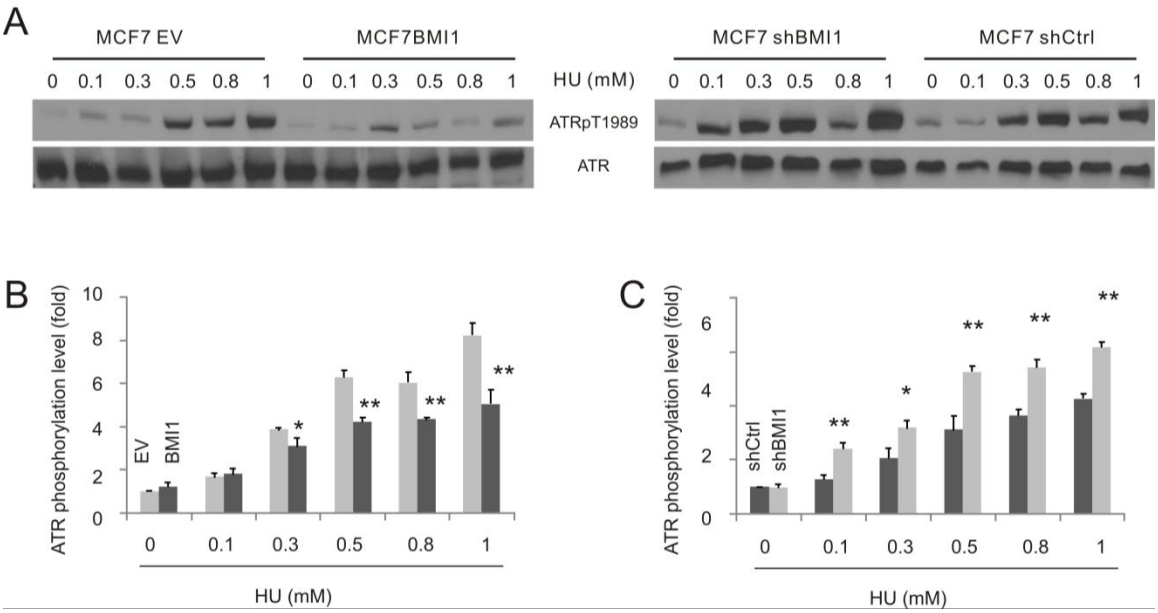

Figure 3.3. BMI1 reduces ATR activation. Examination of phosphorylation of ATR at T1989 (ATRpT1989) in the indicated MCF7 cell lines**.** Experiments were repeated three times; typical images from a single repeat are shown (A). (B, C) ATR phosphorylation was normalized to the respective ATR and expressed as fold changes to the normalized ATR phosphorylation levels in the respective (EV and shCtrl) control (untreated) cells. Means ± SD (standard derivation) were graphed; *p<0.05 and **p<0.01 by 2-tailed Student's t-test in comparison to the respective controls.

This notion is further supported by the effects of BMI1 on γH2AX, a target of ATR, in MCF7 cells (Cimprich and Cortez 2008). Western blot analysis revealed a reduction of γH2AX in MCF7 BMI1 cells in comparison to MCF7 EV cells in response to HU treatment (Figure 3.4A, C). Similar results were also obtained in DU145 BMI1 cells (Figure 3.4B). Conversely, knockdown of BMI1 in both MCF7 and DU145 cells elevated HU-induced γH2AX levels (Figure 3.4A, B, D). Additionally, formation of γH2AX nuclear foci, an apical and essential event for DSB repair (van Attikum and Gasser 2009, Lin, Ojo et al. 2015), was respectively reduced and enhanced in MCF7 BMI1 and shBMI1 cells in comparison to the respective control cells following HU exposure (Figure 3.4E, F; Supplementary Figure 5).

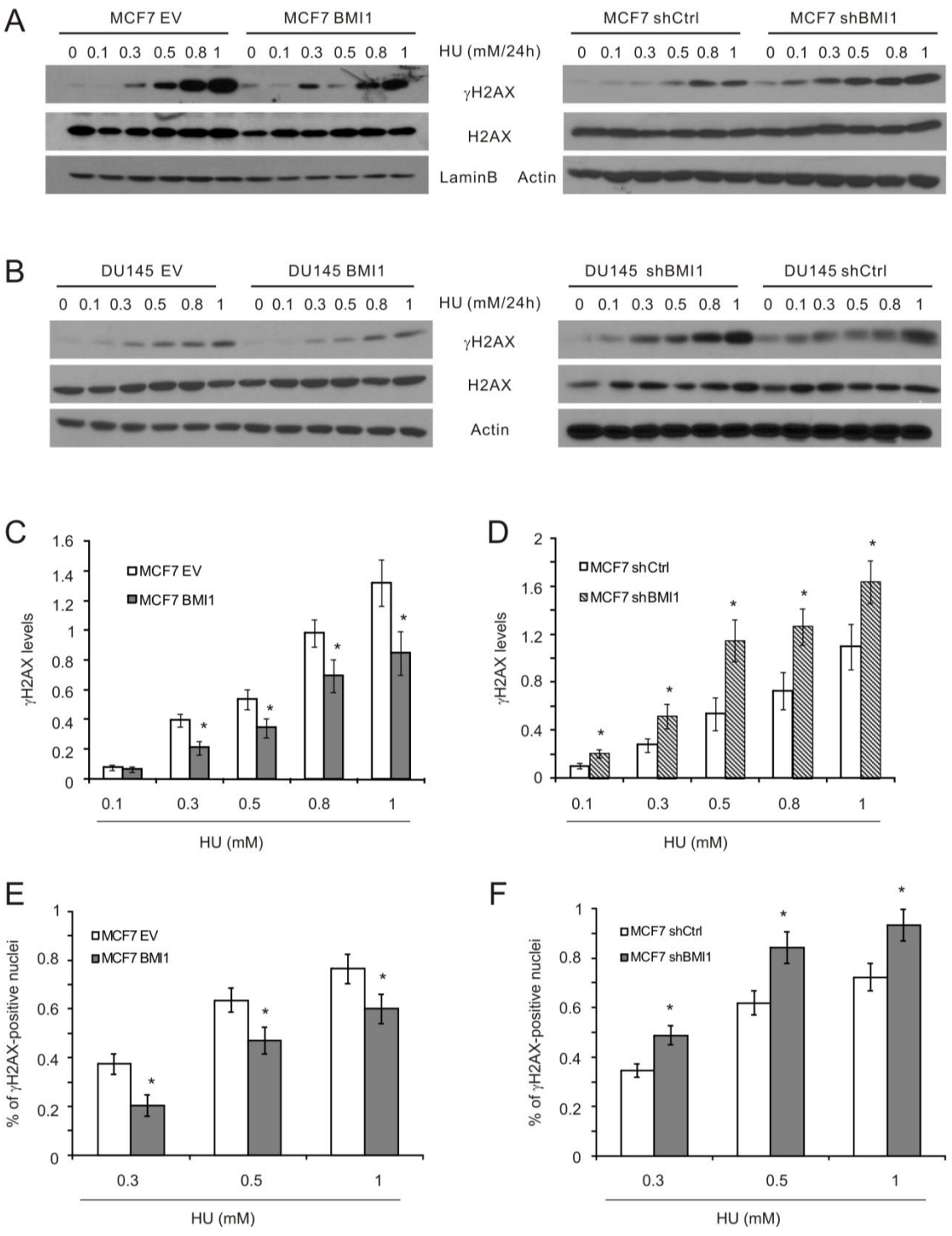
A
MCF7 EV
MCF7 BMI1
MCF7 shCtrl
MCF7 shBMI1
0 0.1 0.3 0.5 0.8 1
HU (mM/24h)
γH2AX
H2AX
LaminB
Actin
B
DU145 EV
DU145 BMI1
DU145 shBMI1
DU145 shCtrl
Actin
C
MCF7 EV
MCF7 BMI1
γH2AX levels
HU (mM)
D
MCF7 shCtrl
MCF7 shBMI1
E
% of γH2AX-positive nuclei
F

Figure 3.4. BMI1 attenuates γH2AX in cells treated with HU. (**A, B**) The indicated MCF7 and DU145 cell lines were exposed to HU at the indicated doses for 24 hours. The indicated events were examined by Western blot. Experiments were repeated three times. Typical results from a single repeat were included (**A, B**). γH2AX results for MCF7 EV, BMI1 (**C**), shCtrl, and shBMI1 (**D**) cells were quantified. Means ± S.E were graphed. *$p < 0.05$ in comparison to the respective control cells (two-tailed Student's *t*-test). (**E, F**) MCF7 EV, BMI1 (**E**), shCtrl, and shBMI1 (**F**) cells were treated with PBS or HU for 24 hours. IF staining for γH2AX was performed. Typical images are presented in Supplementary Figure 5. Experiments were repeated three times; γH2AX-positive nuclei were quantified; mean percentages ± S.E are graphed. *$p < 0.05$ in comparison to the respective control cells (two-tailed Student's *t*-test).

BMI1 attenuates HU-induced S phase arrest

ATR is required for S-phase arrest in response to HU-induced DNA damage (Wu, Chen et al. 2006, Wei, Xie et al. 2011). To determine whether BMI1 affected this process, HU treated cells were examined for S phase cell cycle arrest. In EV cells, HU treatment resulted in the expected dose-dependent accumulation in S phase that is indicative of S phase arrest (Table 3.1; Supplementary Figure 3.6). In the MCF7 cells, the accumulation of S phase cells was significantly reduced (Table 3.1; Supplementary Figure 3.6); whereas, BMI1 knockdown increased the proportion of S phase cells (Table 3.2; Supplementary Figure 3.6) suggesting that BMI1 reduces HU-induced S phase arrest.

To further examine BMI1's influence on S-phase arrest in HU-treated cells, MCF7 cells were blocked in mitosis by nocodazole treatment in combination with HU exposure. In these experiments, BMI1-derived reduction of S phase arrest should allow cell cycle progression to mitosis, despite the presence of HU, resulting in mitotic arrest due to the effects of nocodazole. As expected, nocodazole treatment resulted in the accumulation of mitotic cells with condensed chromosomes detected by phosphorylation of histone H3 at

S10 (Figure 3.5A). HU treatment reduced the number of mitotic nuclei in MCF7 EV cells and this reduction was compromised in MCF7 BMI1 cells, i.e. more mitotic nuclei were observed in HU-treated MCF7 BMI1 cells compared to MCF7 EV cells (Figure 3.5A, B). In contrast, BMI1 knockdown reduced the number of mitotic nuclei in response to HU (Figure 3.5A, B). Collectively, the above observations support the notion that BMI1 reduces S-phase arrest in HU-treated MCF7 cells.

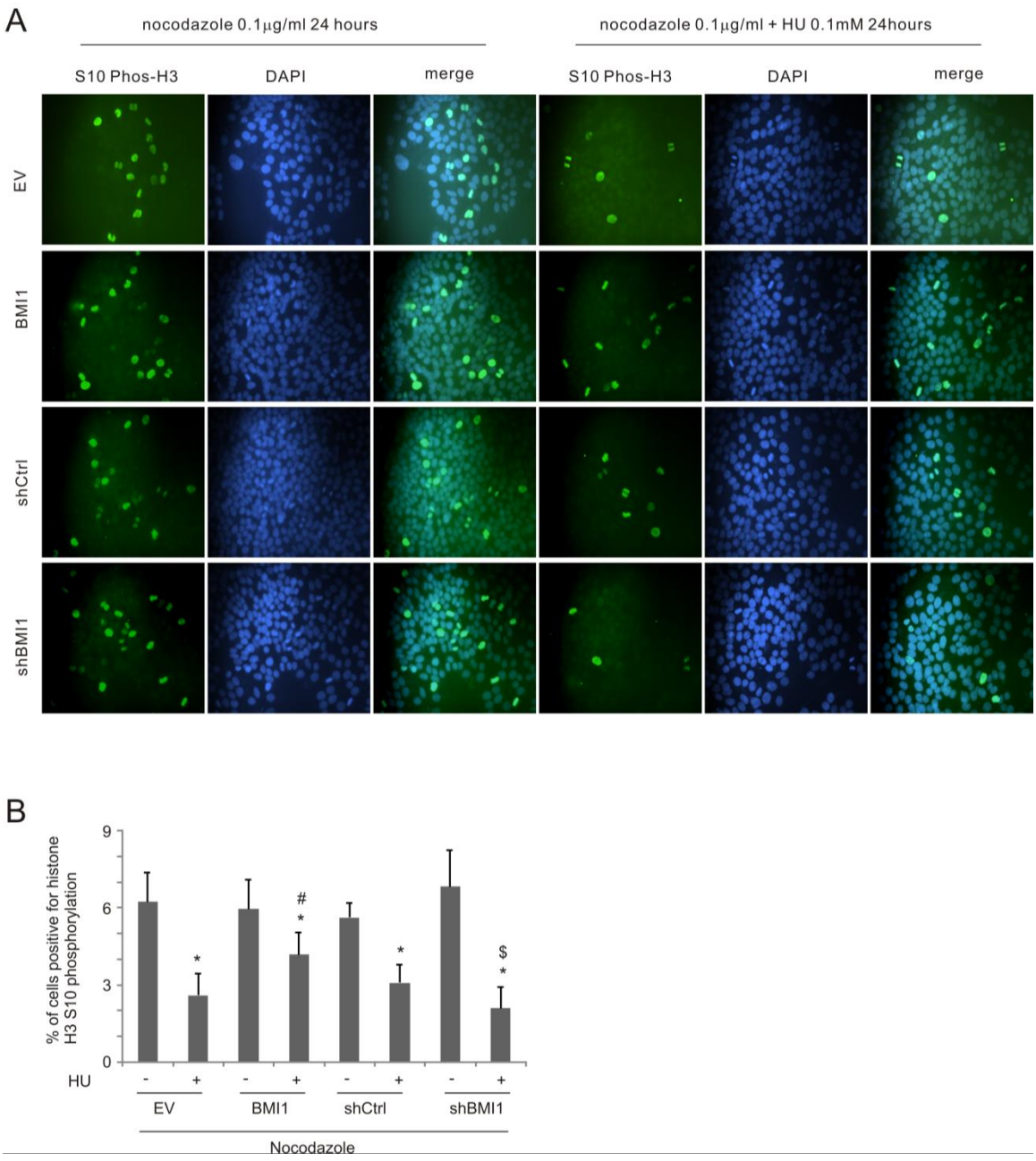

Figure 3.5. BMI1 decreases HU-elicited S-phase arrest. (**A**) MCF7 EV, BMI1, shCtrl, and shBMI1 cells were treated with nocodazole (0.1 μg/ml) without and with HU (0.1 mM) for 24 hours. Histone H3 S10 phosphorylation was determined using IF staining; nuclei were counter-stained with DAPI. Experiments were repeated 3 times; typical images from a single repeat are included (**B**). More than 8,000 nuclei from 12 randomly

selected areas per slide were counted to determine the percentage of nuclei positive for histone H3 S10 phosphorylation. Means ± SD (standard deviation) were graphed; *p < 0.05 (two-tailed Student's *t*-test) in comparison to the individual mock treatments, #p < 0.05 in comparison to HU-treated EV cells; and \$$p$ < 0.05 in comparison to HU-treated shCtrl cells.

The involvement of BMI1's structural elements in HU-elicited ATR activation and ATR signalling

Previously, we have described a set of MCF7 cell lines that stably express individual BMI1 mutants with deletions of the RF, PS, HT, or one of NLS sites (Wei, Ojo et al. 2015). Using these lines, we investigated the generation of ATRpT1989 and two ATR targets (CHK1pS345 and γH2AX) upon HU treatment. While some variation occurs in ATRpT1989 in HU treated MCF7 EV cells, the level of phosphorylation plateaued at 0.5mM HU and remained high at 0.8 and 1mM HU (Figure 3.6). Within this dose range, all mutants had either a minimal or modest effect on HU-induced ATRpT1989 in comparison to MCF7 EV cells (Figure 3.6), suggesting that these domains contribute to BMI1-derived inhibition of ATRpT1989. This notion is supported further by the minimal impact of these mutants on HU-induced γH2AX (Figure 3.6). Nonetheless, all mutants clearly reduced CHK1pS345 in cells treated with HU (Figure 3.6). As CHK1pS345 is the best characterized target of ATR (Zhao and Piwnica-Worms 2001), these domains may not be essential for BMI1 to reduce ATR signalling (see Discussion for details).

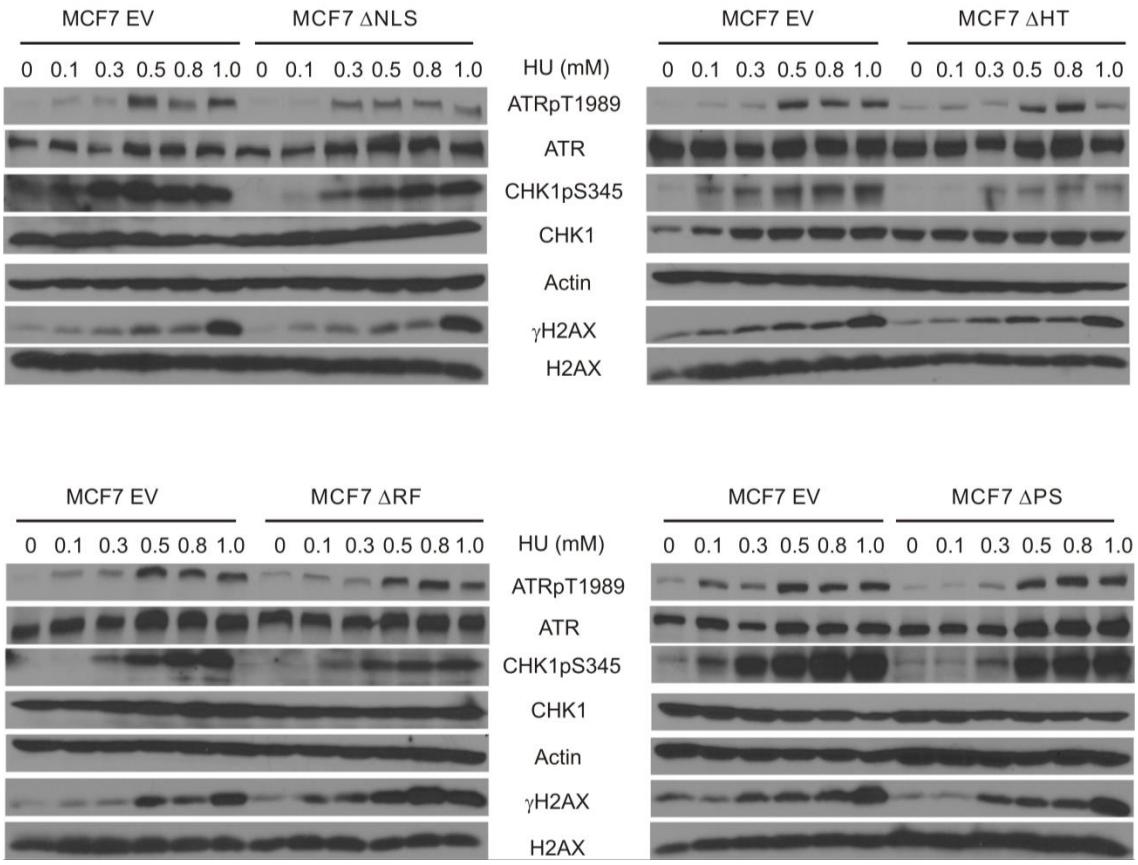

Figure 3.6. Characterization of BMI1-derived inhibition of ATR activation and ATR signalling. MCF7-based EV, BMI1ΔNLS, BMI1ΔHT, BMI1ΔRF, and BMI1ΔPS stable lines have been previously established (Wei, Ojo et al. 2015). These lines were treated as indicated and examined for ATRpT1989, ATR, CHK1pS345, CHK1, actin, γH2AX, and H2AX. Experiments were performed twice. Typical images from a single repeat are shown.

BMI1 binds TOPBP1

ATR activation involves binding to ssDNA and TOPBP1 (Kumagai, Lee et al. 2006, Mordes, Glick et al. 2008, Wei, Yan et al. 2011). The above observations thus suggest that BMI1 may reduce ATR activation via binding to TOPBP1. In 293T cells co-

transfected with BMI1 and TOPBP1, immunoprecipitation (IP) of BMI1 led to co-precipitation of TOPBP1 with and without HU treatment, while HU treatment appeared to reduce the association (Figure 3.7A, see the ethidium bromide/EB minus lanes). The presence of EB (50 µg/ml) (Figure 3.7A), a condition that is commonly used to release DNA-associated proteins from DNA (Wei, Ojo et al. 2015), did not reduce the efficiency of the co-IP (Figure 3.7A, compare the respective EB- and EB+ lanes), making it unlikely that the presence of DNA was the cause for the interaction. In fact, the presence of EB apparently enhanced the association, suggesting that interaction between BMI1 and TOPBP1 is possibly mediated by a hydrophobic force, which is enhanced by the presence of positively charged EB.

We further demonstrated the binding of BMI1 and TOPBP1 using the endogenous proteins. In MCF7 cells, immunoprecipitation of BMI1 resulted in co-precipitation of TOPBP1 in the presence of DNase (benzonase endonuclease), further demonstrating that the association was unlikely to be attributable to the presence of DNA (Figure 3.7B). Similarly, the association between BMI1 and TOPBP1 could be demonstrated with and without HU exposure (Figure 3.7B). The observed decreases in co-immunoprecipitation of TOPBP1 with BMI1 in HU-treated cells compared to non-treated cells (Figure 3.7A, B) indicate that reductions in this interaction free TOPBP1 for ATR activation. Collectively, the above results suggest that BMI1 exists in a protein complex that includes TOPBP1.

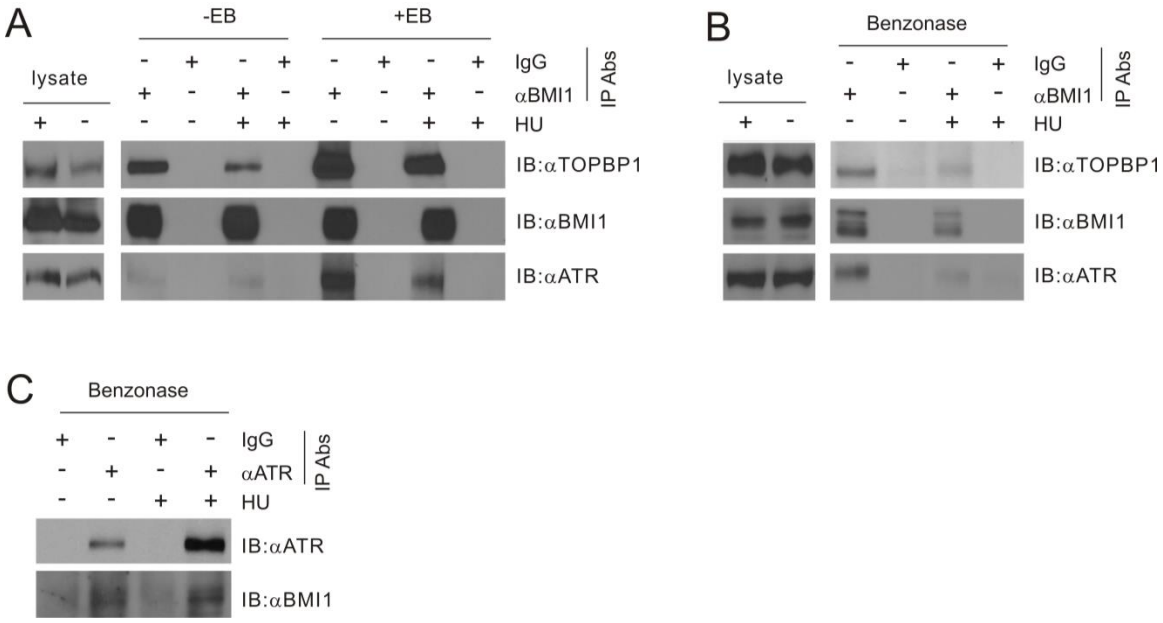

Figure 3.7. BMI1 associates with TOPBP1. (**A**) 293T cells were transiently co-transfected with BMI1 and TOPBP1 for 36 hours, and treated with HU (1 mM for 24 hours). Cell lysates were then immunoprecipitated (IP) with anti-BMI1 (αBMI1) or control IgG (IgG) in the presence or absence of ethidium bromide (EB, 50 μg/ml; note: cell lysates were pre-incubated with EB for 10 minutes on ice prior to IP) and analyzed by Western blot for TOPBP1, BMI1, and ATR. 1/14 of cell lysates used for IP were also analyzed. (**B, C**) MCF7 cells were treated without or with HU (1 mM for 24 hours). Cell lysates were prepared and treated with benzonase at 1 U/μl on ice for 1 hour, followed by IP for BMI1 (**B**) or ATR (**C**), and Western blot examination as indicated. All experiments have been repeated once. Typical images from a single repeat are shown.

Formation of a potential BMI1/TOPBP1/ATR complex

The binding of BMI1 and TOPBP1 raises the possibility of a BMI1 presence in the TOPBP1/ATR complex. This possibility is supported by the co-IP of endogenous BMI1 through IP of endogenous ATR in the presence of benzonase in MCF7 cells (Figure 3.7C). The association was observed with and without HU treatment (Figure 3.7C). Furthermore, the endogenous ATR was co-immunoprecipitated together with the ectopic complex of BM1I-TOPBP1 in 293T cells (Figure 3.7A) and the endogenous BMI1-

TOPBP1 complex in MCF7 cells (Figure 3.7B). Collectively, evidence supports the formation of an endogenous BMI1/TOPBP1/ATR complex.

Characterization of the association of BMI1 and TOPBP1

To further study the interaction between BMI1 and TOPBP1, we took advantage of our established MCF7 cell lines stably expressing BMI1ΔRF, BMI1ΔNLS, BMI1ΔHT, or BMI1ΔPS. The expression of the individual BMI1 mutants has been shown in our previous report (Wei, Ojo et al. 2015) and has been re-demonstrated here (Figure 3.8A-D). As we have reported previously (Wei, Ojo et al. 2015), BMI1ΔRF was expressed at a low level in MCF7 BMI1ΔRF cells, which is likely attributable to the mutant protein being unstable. This made detection of the BMI1ΔRF mutant protein difficult in cell lysates (Wei, Ojo et al. 2015). Consistent with our previous report, we observed the mutant in MCF7 BMI1ΔRF cells by real time PCR (data not shown) (Wei, Ojo et al. 2015) and IP (Figure 3.8A) (Wei, Ojo et al. 2015). Importantly, immunoprecipitation of the individual BMI1 mutants through their FLAG tags (using the M2 antibody) resulted in detection of the individual mutant proteins, as expected, along with endogenous TOPBP1 (Figure 3.8A-D). With the exception of BMI1ΔRF (Figure 3.8A), HU treatment either did not affect the association of BMI1ΔHT and TOPBP1 (Figure 3.8C) or reduced the interaction of TOPBP1 with BMI1ΔNLS (Figure 3.8B) or BMI1ΔPS (Figure 3.8D). Collectively, we demonstrated that all BMI1 mutants are capable of interaction with endogenous TOPBP1.

Although we were unable to demonstrate an interaction between ATR and BMI1ΔHT or BMI1ΔPS, we observed co-IP of ATR through BMI1ΔRF or BMI1ΔNLS in cells without exposure to HU (Figure 3.8A, B). Consistent with HU treatment enhancing BMI1ΔRF binding to TOPBP1 (Figure 3.8A) or reducing TOPBP1 to interact with BMI1ΔNLS (Fig 3.8B), HU exposure respectively elevated the co-IP of ATR via BMI1ΔRF (Figure 3.8A) and reduced the co-IP of ATR through BMI1ΔNLS (Figure 3.8B). Due to a low level of the BMI1ΔRF protein in MCF7 BMI1ΔRF cells (see discussion above for details), the system does not support a reverse co-IP, e.g. co-IP of BMI1ΔRF through ATR. Nonetheless, we have demonstrated co-IP of BMI1ΔNLS through endogenous ATR in MCF7 BMI1ΔNLS cells (Figure 3.8E). Collectively, these observations further support that BMI1 is able to associate with the TOPBP1/ATR complex.

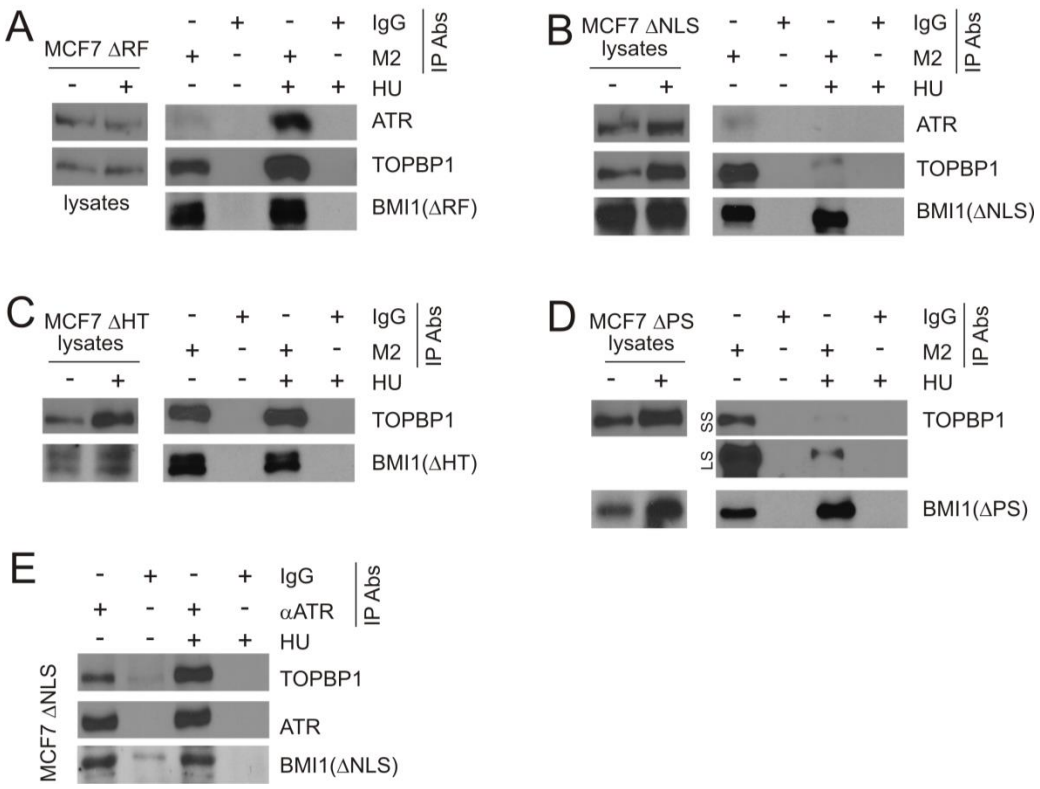

Figure 3.8. Characterization of BMI1 association with TOPBP1 and ATR. Cell lysate from the indicated MCF7 BMI1ΔRF, BMI1ΔNLS, BMI1ΔHT, or BMI1ΔPS cells (**A-E**) were pre-treated with benzonase at 1U/μl on ice for 1 hour, followed by IP with a monoclonal anti-FLAG (M2) (Ndiaye, Ka et al.) or anti-ATR antibodies (**E**). Western blot analyses were performed for ATR, TOPBP1, and the indicated BMI1 mutants (using a polyclonal anti-FLAG antibody). 1/10 of cell lysates used for IP were also analyzed. Experiments were repeated at least three times; typical results from a single repeat are shown. (**D**) LS: long exposure; SS: short exposure.

Discussion

BMI1 is thought to play an important role in maintaining self-renewal of stem cell populations and it may function in tumorigenesis by repressing tumor suppressor genes through its associated ubiquitin E3 ligase activity. More recent studies have provided evidence of a role for BMI1 in DDR regulation by enhancing HR-mediated DSB repair in mouse embryonic fibroblasts (MEFs), HCT116, and U2OS cells (Ismail, Andrin et al. 2010, Chagraoui, Hebert et al. 2011). This function may contribute to resistance towards genotoxic drug-based cancer therapies (Facchino, Abdouh et al. 2010, Chagraoui, Hebert et al. 2011, Crea, Duhagon Serrat et al. 2011, Gieni, Ismail et al. 2011, Liu, Liu et al. 2012). Additionally, BMI1 may attenuate ATM activation following etoposide-caused DSBs, thereby reducing the duration of the etoposide-initiated G2/M checkpoint (Wei, Ojo et al. 2015). By attenuating DDR signalling, normal levels of BMI1 may favour a return to the cell cycle following DNA lesion repair. In cancer cells with BMI1 upregulation, the elevated levels of BMI1 potentially may compromise DDR, thereby contributing to genome instability, a hallmark of tumorigenesis (Sieber, Heinimann et al. 2003, Kops, Weaver et al. 2005). Our present results extend these observations by demonstrating that BMI1 can downregulate S-phase checkpoints initiated by ssDNA damage induced by HU in MCF7 and DU145 cells. These observations support a broader role for BMI1 in attenuating DNA damage-induced checkpoint activation.

ATM and ATR are related kinases that share a number of structural features such as the FRAP-ATM-TRRAP (FAT) motif, a kinase domain, and a C-terminal FAT (FATC) domain and presumably have similar mechanisms of activation (Cimprich and Cortez

2008, Lempiainen and Halazonetis 2009). While ATM activation requires the presence of DSBs and binding to NBS1, ATR is activated through association with TOPBP1 on RPA-coated ssDNA (Kumagai, Lee et al. 2006, Mordes, Glick et al. 2008, Wei, Yan et al. 2011). BMI1 attenuates ATM activation through binding to NBS1 (Wei, Ojo et al. 2015) and ATR activation through its interaction with TOPBP1 (this study) suggesting that it affects the two kinases through similar mechanisms. In addition, several studies support a role for NBS1 in promoting ATR activation (Manthey, Opiyo et al. 2007, Kobayashi, Hayashi et al. 2013, Shiotani, Nguyen et al. 2013), raising the possibility that BMI1 can influence both ATM and ATR activation through its interaction with NBS1. However, mutational analysis of BMI1 indicates that BMI1 reduces HU-elicited ATR activation in a different manner from its action in inhibiting etopside-induced ATM activation (Wei, Ojo et al. 2015). Individual deletion of RF, HT, PS or NLS does not compromise BMI1's ability to regulate ATM activation nor the subsequent CHK2 phosphorylation under etoposide-initiated DDR (Wei, Ojo et al. 2015), whereas each of the mutants were largely incapable of inhibiting HU-elicited ATRpT1989. Surprisingly, the mutants are competent in inhibiting ATR-mediated phosphorylation of CHK1 at S345, demonstrating specificity of these domains in reducing ATR activation. These results highlight novel properties of BMI1 in inhibiting ATR signalling. It is possible that BMI1 adopts two different structures or uses different structural elements in inhibiting ATR activation and ATR's ability to phosphorylate CHK1. This possibility is not in contrast to the observations that these mutants are incapable of reducing γH2AX in HU-treated MCF7 cells. A possible explanation is that ATM and DNA-PK also produce

γH2AX (Mukherjee, Kessinger et al. 2006, Awasthi, Foiani et al. 2015) and DNA-PK can be activated by HU-induced replication stress (Wang, Huang et al. 2015). Additionally, ATR may phosphorylate S139 of H2AX (γH2AX) in a different manner from phosphorylation of CHK1 at S345. Besides DNA-PK, HU can also activate ATM (Stiff, Walker et al. 2006). ATM and DNA-PK may contribute to CHK1 activation in response to HU treatment; this possibility is in line with the structural similarities among the three PIKKs: ATM, ATR, and DNA-PK (Wei, Yan et al. 2011, Lin, Yan et al. 2013). It is possible that full length and mutant BMI1 proteins reduce HU-induced CHK1 phosphorylation through attenuation of ATM and/or DNA-PK. Evidence supporting this possibility is our demonstrated inhibitory activities of BMI1 towards DSB-induced ATM activation (Wei, Ojo et al. 2015). Nonetheless, despite the above possibilities, ATR is the dominant upstream kinase phosphorylating CHK1 in response to stalled replication forks. In this regard, it is tempting to speculate that BMI1 possesses two properties in reducing ATR activation and decreasing ATR's ability to phosphorylate and activate CHK1.

BMI1 is well studied for its associated E3 ubiquitin ligase activity, a property that requires BMI1's RF domain (de Napoles, Mermoud et al. 2004, Wang, Wang et al. 2004, Cao, Tsukada et al. 2005, Li, Cao et al. 2006). In the DDR process, BMI1 facilitates DSB repair through the E3 ligase activity (Ismail, Andrin et al. 2010, Chagraoui, Hebert et al. 2011, Ginjala, Nacerddine et al. 2011), and reduces ATM activation independently of the enzyme activity (Wei, Ojo et al. 2015). For inhibiting HU-induced ATR activation, the RF motif is required. However, for reducing ATR-mediated CHK1pS345, the RF domain is not essential, which is in accordance with the RF domain being dispensable for BMI1

to associate with either TOPBP1 or ATR. These observations share similarity with RF being not essential in reducing ATM signaling (Wei, Ojo et al. 2015). It is thus possible that BMI1 reduces ATR activation and signalling in E3 ligase-dependent and -independent processes, respectively. Further research will be required to investigate this issue and the structural elements involved in inhibiting ATR activation and ATR signalling.

BMI1 binds TOPBP1 and ATR. While we cannot distinguish whether BMI1 primarily associates with TOPBP1 or ATR, we favour a model that involves the former. This is because the association of BMI1 with TOPBP1 was consistently detected in comparison to its interaction with ATR. However, this issue should be further investigated. Furthermore, this research is essentially based on enforced expression and knockdown of BMI1. Future research should investigate the impact of endogenous BMI1 on ATR activation and functions.

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Conflict of interest

The authors declare no conflict of interest

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Table 3.1. BMI1 reduces HU-induced S arrest in MCF7 cells

	CTRL	0.1mM	0.3mM	0.5mM	0.8mM	1.0mM
	EV BMI1	EV BMI1	EV BMI1	EV BMI1	EV BMI1	EV BMI1
G1	64.1±1.8 65.3±2.5	59.7±0.1 58.7±1.5	57.4±4.2 58.9±3.1	40.0±2.2 52.9±1.5*	33.6±2.9 51.4±1.3*	23.8±1.1 45.1±0.6*
S	15.8±0.5 14.2±1.3	24.5±0.8 18.6±0.4*	31.0±1.8 19.3±0.9*	51.6±2.2 28.7±0.6*	59.7±3.9 33.7±2.2*	68.8±3.1 36.9±0.1*
G2/M	20.1±2.0 20.5±1.9	15.8±0.8 22.7±1.2*	11.6±3.5 21.7±2.2	11.4±1.5 18.4±1.1*	6.7±1.4 14.9±0.9*	7.4±2.1 18.0±0.5*

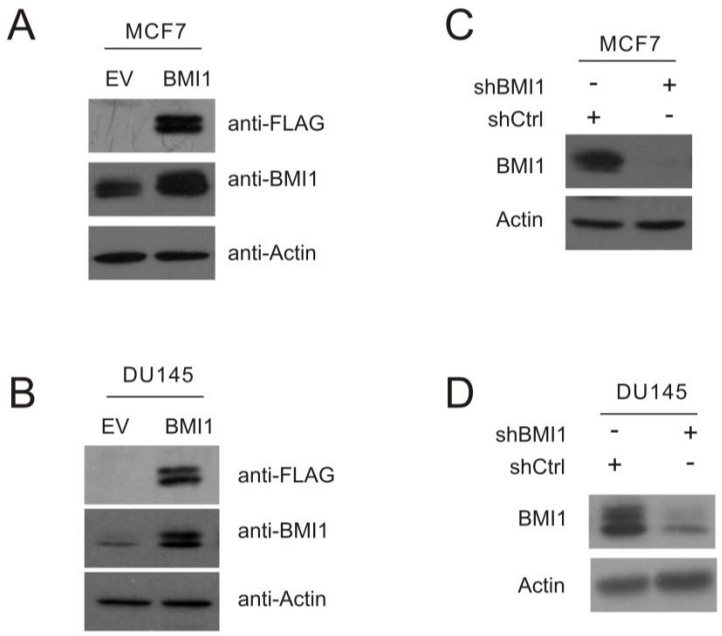
Note: Cell cycle distributions were derived from three independent experiments and were presented as means±S.E.; Ctrl: control; * $p < 0.05$ (in comparison to the respective cell cycle distributions of EV cells)

Table 3.2. Knockdown of BMI1 enhances HU-induced S arrest in MCF7 cells

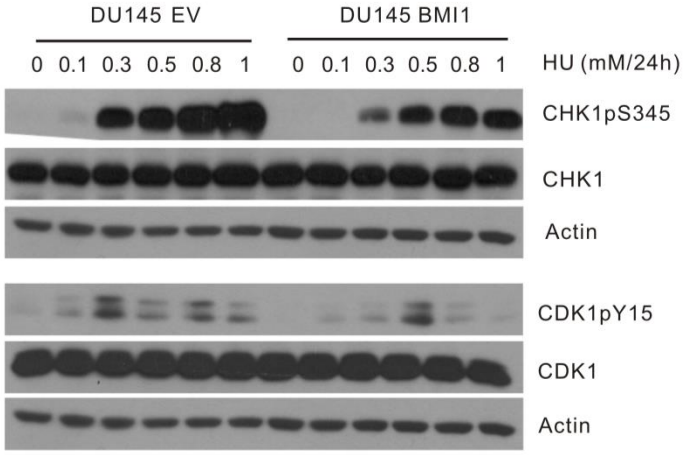
	CTRL	0.1mM	0.3mM	0.5mM	0.8mM	1.0mM
	shCtrl shBMI1	shCtrl shBMI1	shCtrl shBMI1	shCtrl shBMI1	shCtrl shBMI1	shCtrl shBMI1
G1	64.6±2.3 67.8±0.7	59.0±0.8 56.1±3.0	62.3±1.6 26.3±3.7*	44.9±4.6 20.1±2.5*	29.5±4.4 10.0±0.2*	23.3±0.6 9.8±1.9*
S	19.5±3.4 13.5±0.8	24.4±0.8 26.6±1.4	26.6±0.5 48.2±3.1*	43.0±5.6 66.7±1.2*	64.2±4.2 78.1±1.0*	71.2±1.2 80.9±1.4*
G2/M	17.0±1.1 18.7±1.5	16.6±0.8 17.2±2.4*	11.1±1.8 25.5±0.7	12.1±2.0 13.3±2.1*	6.3±1.0 12.0±1.2*	5.5±0.9 9.3±0.5*

Note: Cell cycle distributions were derived from three independent experiments and were presented as means±S.E.; Ctrl: control; * $p < 0.05$ (in comparison to the respective cell cycle distributions of shCtrl cells)

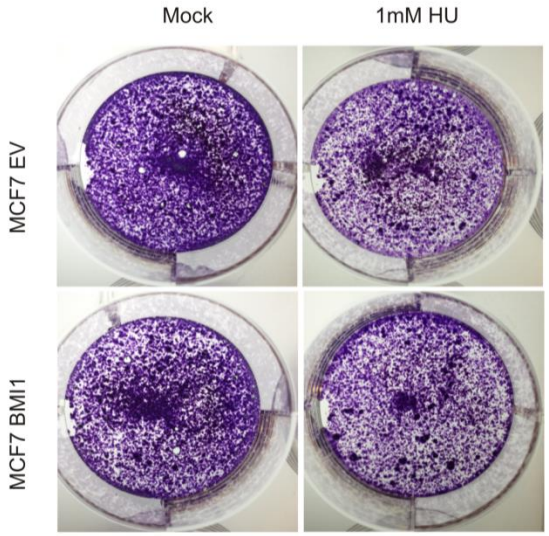
Supplementary Figures



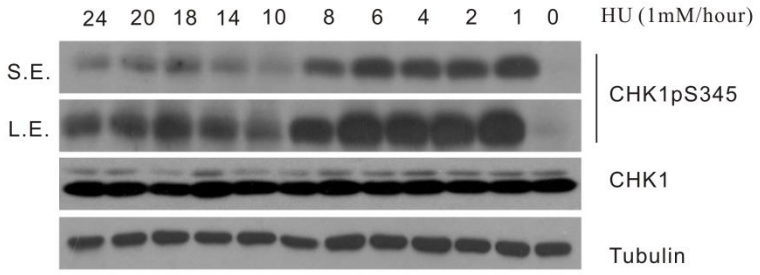
Supplementary Figure 3.1. Generation of MCF7 and DU145 stable cell lines. (**a, b**) Stable expression of FLAG tagged BMI1 in MCF7 and DU145 cell using an empty vector (EV) and BMI1 retrovirus. (**c, d**) Stably knockdown of BMI1 using control shRNA (shCtrl) and shBMI1 retrovirus.



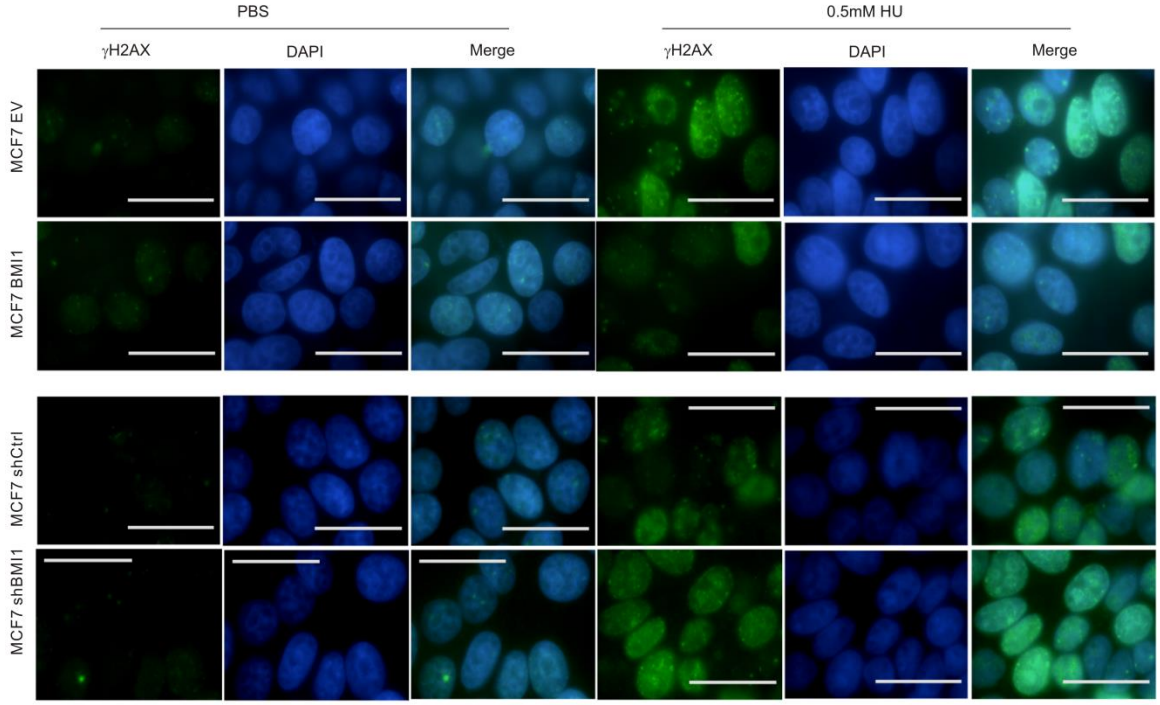
Supplementary Figure 3.2. Increase in BMI1 reduces CHK1 activation induced by HU. DU145 EV and BMI1 cells were treated with HU as indicated. Western blot analysis was performed to examine phosphorylation of CHK1 at S345 (CHK1pS345), CHK1, phosphorylation of CDC2 at Y15 (CDK2pY15), CDK2, and actin. Experiments were performed twice; typical results from a single repeat are included.



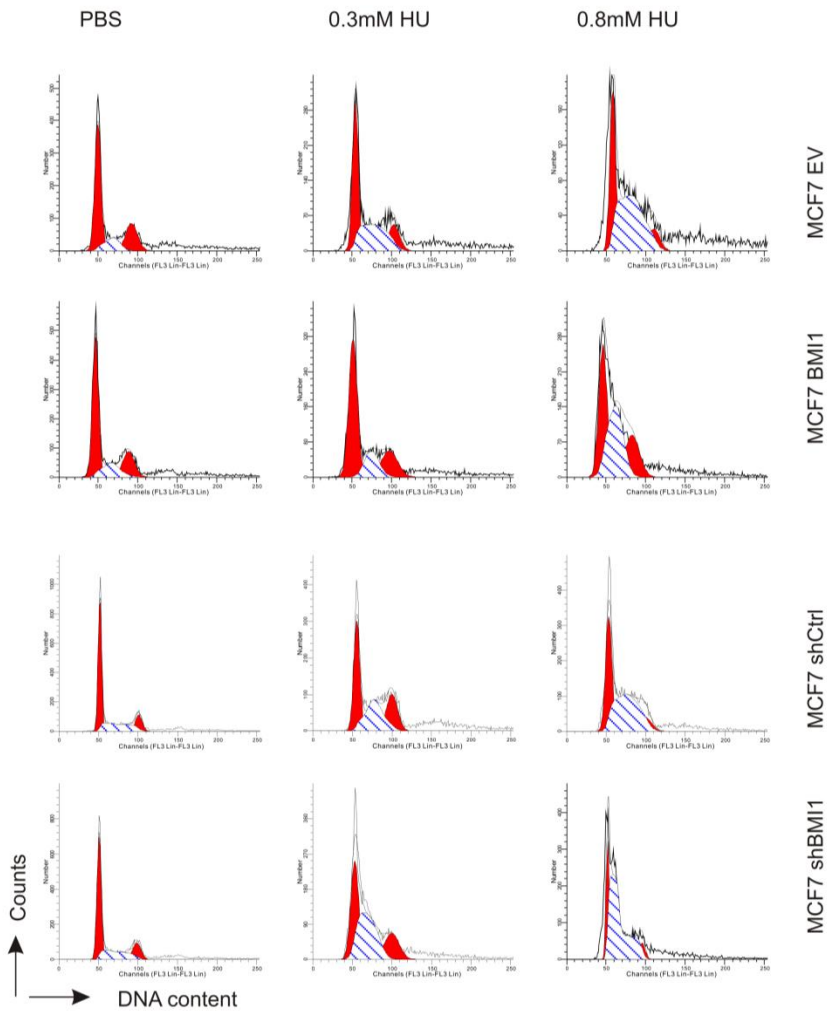
Supplementary Figure 3.3. MCF7 EV or BMI1 cells renew proliferation after HU treatment. The indicated MCF7 cells (104) were mock-treated (PBS) or treated with 1 mM HU for 24 hours. HU was then removed; cells were rinsed and cultured in normal medium for 9 days. Surviving cells were stained with 0.5% crystal violet. Experiments were repeated once. Typical images from a single repeat are shown.



Supplementary Figure 3.4. Kinetics of HU-induced CHK1pS345. MCF7 cells were treated as indicated. Western blot analysis was then performed to examine CHK1pS345, CHK1, and tubulin. The CHK1pS345 signals were obtained by a short exposure (S.E.) and long exposure (L.E.).



Supplementary Figure 3.5. BMI1 attenuates γH2AX in cells treated with HU. MCF7 EV, BMI1, shCtrl, and shBMI1 cells were treated with PBS (control) or HU for 24 hours. IF staining for γH2AX was performed; nuclei were counter stained with DAPI (blue). Scale bars are for 20 μm. Experiments were repeated three times; typical images from a single repeat are shown.



Supplementary Figure 3.6. BMI1 decreases HU-elicited S-phase arrest. MCF7 EV, BMI1, shCtrl, and shBMI1 cells were treated with PBS, 0.3 mM HU, or 0.8 mM HU for 24 hours. Cell cycle distribution was determined using a flow cytometer.

CHAPTER FOUR

Microvesicles Contribute to the Bystander Effect of DNA Damage

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Preamble

DDR was reported in cells adjacent to cells undergoing DDR, a phenomenon known as bystander effect. However, factors responsible for this bystander effect are not entirely clear. Here, we propose that microvesicles (MVs) contribute to the induction of bystander DDR. MVs play a vital role in cell-to-cell communication. We demonstrated that ETOP and UV stimulate MVs production and MVs isolated from these treated cells can induce DDR in recipient cells. Moreover, neutralization of MVs derived from UV-treated cells with annexin V blocked the MVs' activity in BE induction.

Author's Contribution

Xiaozeng Lin, Fengxiang Wei, and Damu Tang designed the study. Xiaozeng Lin, Fengxiang Wei, and Hassan A. Al Saleh performed experiments and obtained data. Damu Tang and Khalid Al-Nedawi supervised the project. Xiaozeng Lin, Fengxiang Wei, Pierre Major, Khalid Al-Nedawi, Hassan A. Al Saleh and Damu Tang carried out data analysis and interpretation, and concluded the results. Xiaozeng Lin, Pierre Major, Khalid Al-Nedawi and Damu Tang prepared the manuscript.

Relationship to the theme of my Ph.D. research

Radiation-induced bystander effect has major implications for cancer therapy. Accumulating evidence suggests bystander factors released from irradiated cells play critical roles in causing DDR, which can lead to radiotherapy-associated secondary carcinogenesis. Our research shows that MVs are a source of bystander factors and that MVs induces BE likely through a pathway upstream of ATM. This research extends my research theme in the examination of DDR.

Microvesicles Contribute to the Bystander Effect of DNA Damage

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Keywords: DNA damage response; microvesicles; γH2AX; ATM; ATR

Abstract

Genotoxic treatments elicit DNA damage response (DDR) not only in cells that are directly exposed but also in cells that are not in the field of treatment (bystander cells), a phenomenon that is commonly referred to as the bystander effect (BE). However, mechanisms underlying the BE remain elusive. We report here that etoposide and ultraviolet (UV) exposure stimulate the production of microvesicles (MVs) in DU145 prostate cancer cells. MVs isolated from UV-treated DU145 and A431 epidermoid carcinoma cells as well as etoposide-treated DU145 cells induced phosphorylation of ataxia-telangiectasia mutated (ATM) at serine 1981 (indicative of ATM activation) and phosphorylation of histone H2AX at serine 139 (γH2AX) in naïve DU145 cells. Importantly, neutralization of MVs derived from UV-treated cells with annexin V significantly reduced the MV-associated BE activities. Etoposide and UV are known to induce DDR primarily through the ATM and ATM- and Rad3-related (ATR) pathways, respectively. In this regard, MV is likely a common source for the DNA damage-induced bystander effect. However, pre-treatment of DU145 naïve cells with an ATM (KU55933) inhibitor does not affect the BE elicited by MVs isolated from etoposide-treated cells, indicating that the BE is induced upstream of ATM actions. Taken together, we provide evidence supporting that MVs are a source of the DNA damage-induced bystander effect.

Introduction

The World Health Organization has thoroughly documented that radiation induces cancer (El Ghissassi, Baan et al. 2009). Major epidemiological studies on A-bomb survivors clearly linked radiation exposure to carcinogenesis for human cancers (Pawel, Preston et al. 2008). Mechanistically, radiation causes DNA damage when traveling through the nucleus of the cell, thereby inducing DNA damage response (DDR) by activating three apical PI3 kinase related kinases (PIKKs): ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-related), and DNA-dependent protein kinase (DNAPK) (Zhou and Elledge 2000, Wei, Yan et al. 2011, Lin, Yan et al. 2013). ATM and ATR subsequently phosphorylate their downstream targets, including check kinase 1 (CHK1) S345 (for ATR), CHK2 T68 (for ATM), and H2AX S139 (γH2AX) (Zhou and Elledge 2000, Jiang, Wang et al. 2016). The actions of ATM, ATR and DNAPK orchestrate check point activation and DNA lesion repair (Zhou and Elledge 2000, Wei, Yan et al. 2011, Lin, Yan et al. 2013).

As a result of being exposed to genotoxic treatment, cells not only elicit DDR but also produce factors that induce DDR in cells that have not been exposed to genotoxic reagents, a process that is commonly known as the bystander effect (BE) of DNA damage (Mothersill and Seymour 2004, Martin, Redon et al. 2011). Bystander factors released from irradiated cells play critical roles in radiotherapy-associated secondary carcinogenesis (Prise and O'Sullivan 2009). These secondary cancers were observed in patients with cervical and breast cancer (Choy, Barr et al. 1993, Wijnmaalen, van Ooijen et al. 1993, Kleinerman, Boice et al. 1995, Ohno, Sakurai et al. 1997, Senkus, Konefka et

al. 2000, Mills, Vinnicombe et al. 2002); the reported latency is 5–15 years for cervical cancer patients receiving external bean radiation (Sountoulides, Koletsas et al. 2010). In a 7-year follow-up of 269,069 prostate cancer patients treated with radiotherapy, approximately 10% experienced secondary cancers (Moon, Stukenborg et al. 2006, Sountoulides, Koletsas et al. 2010). It has been shown that the blood of patients receiving radiotherapy contained activities or clastogenic factors that cause chromosome damage (Parsons, Watkins et al. 1954, Goh and Sumner 1968, Hollowell and Littlefield 1968). Clastogenic factors were also detected in the plasma of individuals exposed to radiation from the Chernobyl nuclear reactor accident (Emerit, Levy et al. 1994). Additionally, conditioned medium harvested from irradiated cells induced cell death, mutation, and genome instability in naive cells (Nagasawa and Little 1992, Deshpande, Goodwin et al. 1996, Mothersill and Seymour 1997, Lorimore, Kadhim et al. 1998, Zhou, Randers-Pehrson et al. 2000, Sawant, Randers-Pehrson et al. 2001, Nagasawa and Little 2002), supporting the concept that the clastogenic factors detected in the plasma of radiotherapy-treated patients were directly released from irradiated cells. Furthermore, local cranial irradiation induced distance BE DNA damage in mice in the lead-shield spleen and testes (Koturbash, Loree et al. 2008, Tamminga, Koturbash et al. 2008) and irradiation of one side of the mouse body caused DNA damage and epigenetic changes in the other side of the body that was shielded by lead (Koturbash, Rugo et al. 2006). More importantly, irradiation of the lower body induced medulloblastoma in the brain of Patched 1 heterozygous mice (Mancuso, Pasquali et al. 2008).

While accumulating evidence demonstrates that bystander DNA damage contributes to radiotherapy-induced secondary cancers, the bystander factors remain unclear. Although a variety of molecules have been implicated in mediating the BE, including reactive oxygen species (ROS), reactive nitrogen species, TNFα, TGFβ1, IL-6, and IL-8 (Prise and O'Sullivan 2009), evidence suggests the existence of additional bystander factors (Mothersill and Seymour 2004).

Microvesicles (MVs) are small membrane-enclosed sacks that are shed from donor cells. MVs carry a unique set of cargo, and communicate the specific messages from donor cells to the acceptor cells (Muralidharan-Chari, Clancy et al. 2010, Rak 2010, Ratajczak and Ratajczak 2016). As bystander factors communicate the stress signals originated from cells undergoing DNA damage response to naïve cells, MVs are ideal vesicles to carry these messages to bystander cells. We thus report here that MV is a source of bystander DNA damage signals.

Materials and Methods

Chemicals, Cell Lines, and Plasmids

Hydroxyurea (HU) and etoposide (ETOP) were purchased from Sigma (Oakville, ON, Canada). ETOP was dissolved in dimethylsulfoxide (DMSO). Annexin V was purchased from BD Biosciences (Mississauga, ON, Canada). DU145 and A431 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in Mininum Essential Medium Eagle (MEM) (DU145) and Dulbecco's Modified Eagle Medium (DMEM) (A431) media supplemented with 10% foetal bovine serum (FBS; Sigma Aldrich, Oakville, ON, Canada) and 1% penicillin–streptomycin (Life Technologies; Burlington, ON, Canada). Membrane-associated GFP (green fluorescent protein) (mGFP) was provided by Addgene (Cambridge, MA, USA) and subcloned into PLPCX retroviral vector. DU145 cells stably expressing mGFP were subsequently constructed using mGFP retrovirus.

Retroviral Infection

Packing retrovirus was performed according to our published procedures (He, Ingram et al. 2010, He, Fan et al. 2011, Yan, Ojo et al. 2016). Briefly, the mGFP retroviral vector, the gag-pol (GP) and an envelope expressing vector (VSV-G) (Stratagene, Mississauga, ON, Canada) vectors were transiently co-transfected into 293T cells using a calcium-phosphate transfection. The virus-containing medium was harvested 2 days later, filtered using a 0.45 μM filter, and centrifuged at 50,000× g for 90 min. The viral pellet was

resuspended in the MEM medium containing 10 μg/mL of polybrene (Sigma) prior to infecting cells. Infection was selected using specific antibiotics.

Immunofluorescence Staining

Immunofluorescence (IF) staining was performed following our published conditions (He, Ingram et al. 2010, He, Fan et al. 2011, Yan, Ojo et al. 2016). Briefly, cells were fixed with prechilled (−20 °C) acetone–methanol for 15 min prior to the addition of primary antibodies anti-γH2AX (1:100, Cell Signaling, Danvers, MA, USA) and anti-phospho-ATM (S1981) (1:100, Cell Signaling) at 4 °C overnight. After rinsing, FITC-Donkey anti-mouse IgG (1:200, Jackson Immuno Research Lab, West Grove, PA, USA) and Rhodamine-Donkey anti-rabbit IgG (1:200, Jackson Immuno Research Lab) were added for 1 h at room temperature. Slides were mounted using the VECTASHIELD mounting medium containing DAPI (VECTOR Lab Inc., Burlington, ON, Canada). VE-281 (Selleckchem, Burlington, ON, Canada) or KU-55933 (Selleckchem) was added to cells 8 h prior to treatment. Images were then acquired with a fluorescent microscope (Axiovert 200, Carl Zeiss,North York, ON, Canada).

Western Blot Analysis

Cells were lysed in a buffer containing Tris (20 mM, pH 7.4), NaCl (150 mM), EDTA (1 mM), EGTA (1 mM), Triton X-100 (1%), sodium pyrophosphate (25 mM), NaF (1 mM), β-glycerophosphate (1 mM), sodium orthovanadate (0.1 mM), PMSF (1 mM), leupeptin (2 μg/mL) and aprotinin (10 μg/mL). An amount of 50 μg of total cell lysate protein was

separated on SDS-PAGE gel, and transferred onto Hybond ECL nitrocellulose membranes (Amersham, UK), which were blocked with 5% skim milk at room temperature (RT) for 1 h, and incubated with individual primary (overnight at 4 °C) and secondary antibodies for 1 h at RT. Signals were subsequently developed using an ECL kit (Amersham, UK). Primary antibodies used were anti-γH2AX (1:100, Cell Signaling); anti-H2AX (1:1000, Millpore, Billerica, MA, USA); anti-phosphorylated DNAPK (1:1000, Abcam, Toronto, ON, Canada); anti-DNAPK (1:1000, Abcam); anti-phosph-CHK1 (S345) (1:500, Cell Signaling); anti-CHK1 (1:1000, Cell Signaling); anti-phospho-CHK2 (T68) (1:1000, Cell Signaling); anti-CHK2 (1:1000, Cell Signaling); anti-Flotillin-1 (1:1000, Cell Signaling); and anti-actin (1:1000, Santa Cruz, Dallas, TX, USA).

Microvesicle Isolation and Treatment of Cells with MVs

Isolation of microvesicles was carried out based on our published procedures (Al-Nedawi, Meehan et al. 2008, Al-Nedawi, Meehan et al. 2009, Gabriel, Ingram et al. 2013). Briefly, conditioned medium was harvested from cells following specific treatments, followed by centrifugations for 5 min at 300× ***g***, 20 min at 12,000× ***g***, and 120 min at 100,000× ***g*.** Supernatant of the first two centrifugations and the pellet of the last centrifugation were collected. The 100,000× *g* centrifugation pellet was washed twice with phosphate buffered saline (PBS), and resuspended in PBS. The exosome proteins were determined using the Bradford assay (Bio-Rad, Mississauga, ON, Canada). Cells

were treated with MVs at 100 μg/μL and MVs that were pre-incubated with Annexin V (BD Pharmingen, Mississauga, ON, Canada) at 2 μg/mL for 1 h.

Statistical Analysis

Student's *t*-test (2-tails) was used for statistical analyses. The significant level was defined as a p-value < 0.05.

Results

DNA Damage Enhances Microvesicles (MV) Formation

ATM and ATR play pivotal roles in the initiation of DDR under different settings of DNA damage. While etoposide (ETOP) primarily activates ATM by the induction of double strand DNA breaks (DSBs) (Tang, Wu et al. 2002, Wei, Xie et al. 2010, Wei, Yan et al. 2011), ultraviolet (UV)-initiated DDR is mediated by ATR (Lagerwerf, Vrouwe et al. 2011, Park and Kang 2016). We thus set up our investigations into the involvement of microvesicles (MVs) in ETOP and UV-induced BE. For this purpose, we first determined the kinetics of UV and etoposide-elicited DDR in DU145 cells. In response to UV at the energy levels ranging from 10 to 50 mJ/cm^2, CHK1 phosphorylation at S345 (p-CHK1) was clearly induced in a dose-dependent manner starting at 10 mJ/cm^2 in DU145 cells, while other typical DDR events, including CHK2 phosphorylation at T68 (p-CHK2), phosphorylation of DNA-PKcs (catalytic subunit) at S2056 (p-DNAPK), and γH2AX production, occurred in response to treatment with higher doses (Figure 4.1A). ATR plays a major role in the initiation of DDR caused by UV exposure (Batista, Kaina et al. 2009); CHK1 is a specific ATR target (Liu, Guntuku et al. 2000, Zhao and Piwnica-Worms 2001, Cimprich and Cortez 2008, Awasthi, Foiani et al. 2015). The kinetics of UV-induced DDR events in DU145 cells are thus in line with this knowledge. On the other hand, etoposide results in DSBs, which primarily induce ATM and DNAPK activation (Mahaney, Meek et al. 2009, Awasthi, Foiani et al. 2015, Paull 2015). In this

regard, ETOP induces DNAPK activation, evidenced by phosphorylation of DNA-PK at S2056, in a dose-dependent manner in DU145 cells (Figure 4.1B).

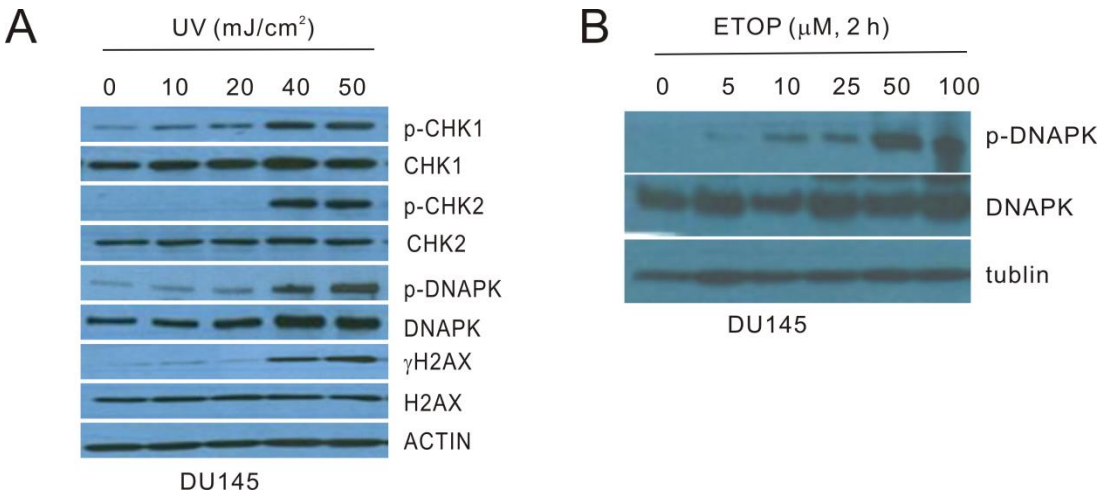

Figure 4.1. Characterization of ultraviolet (UV) and etoposide (ETOP) induced DNA damage response (DDR). (A) DU145 cells were treated with UV at the indicated energy levels and cultured for 6 h. Western blot was performed for the indicated proteins. p-DNAPK: phosphorylation of DNAPK at serine 2056 (S2056); p-CHK1: phosphorylation of CHK1 at S345; p-CHK2: phosphorylation of CHK2 at threonine 68 (T68); (B) DU145 cells were treated with ETOP for 2 h at the indicated doses, followed by Western blot examination for the indicated proteins. All experiments were repeated once; typical images from a single repeat are shown.

We subsequently analyzed the effects of UV and ETOP treatment on MV production. It has been reported previously that expression of a membrane-bound green fluorescent protein (GFP) simplified the process of detecting MVs (Antonyak, Li et al. 2011). Following this system, we have stably expressed membrane-bound GFP in DU145 cells. In comparison to mock-treated cells, UV at 20 mJ/cm^2 and ETOP at concentrations ranging from 5 μM to 25 μM increased the number of MVs per cell (Figure 4.2). Our results are consistent with a report showing an elevation of MV production in MCF7 cells

undergoing doxorubicin-induced DDR (Carroll, Pulkoski-Gross et al. 2016). Etoposide and doxorubicin are well-established DNA topoisomerase II inhibitors (Pommier, Leo et al. 2010).

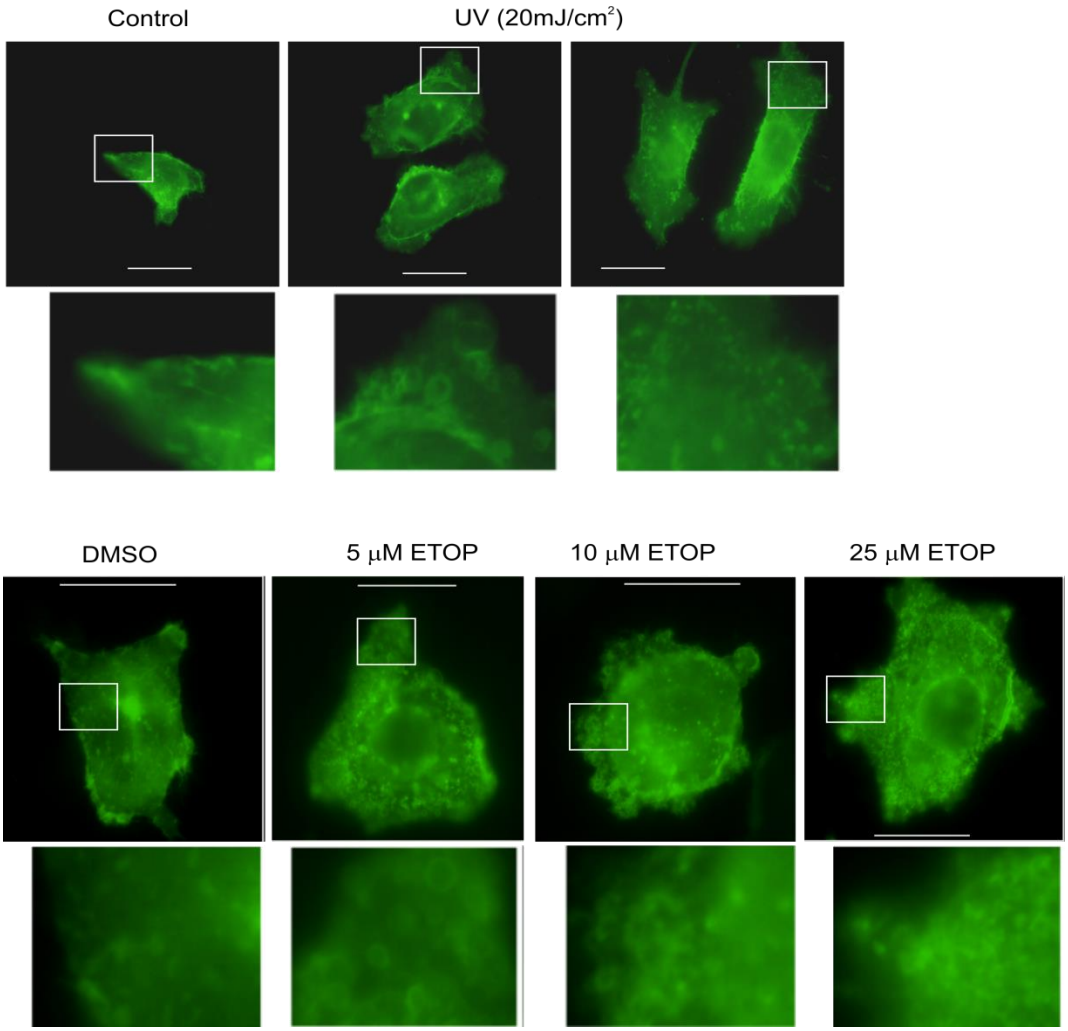

Figure 4.2. UV and ETOP treatments elevate microvesicle production. DU145 cells were stably expressed with a membrane-bound green fluorescent protein (GFP). The cells were treated with UV at 20 mJ/cm2 and cultured for 6 h or with the indicated doses of ETOP for 2 h. Images were taken for control treatment, UV-, DMSO- and ETOP-treated cells. Scale bars indicate 20 μm. The marked regions are enlarged 4-fold and placed underneath the respective panels. Experiments were repeated once; typical images are included.

MVs Contributes to DNA Damage-Induced Bystander Effect (BE)

The observed enhancement of MV production in cells undergoing DDR above strongly suggests that MVs produced by these cells are able to elicit DDR in naïve or bystander cells. To examine this possibility, we isolated MVs from DU145 cells treated with either DMSO or 25 μM ETOP; these isolations were confirmed by the presence of flotillin (Figure 4.3A), a well-established MV protein (Al-Nedawi, Meehan et al. 2008). At 100 μg/mL, MVs derived from ETOP-treated cells (ETOP-MVs) robustly induced γH2AX in naïve DU145 cells in comparison to MVs produced by DMSO-treated cells (control/Ctrl-MVs) (Figure 4.3B). The bystander DDR resulted unlikely from residual ETOP presence in ETOP-MVs, as similar observations were reported when naïve MCF7 cells were incubated with MVs isolated from doxorubicin-treated cells (Carroll, Pulkoski-Gross et al. 2016).

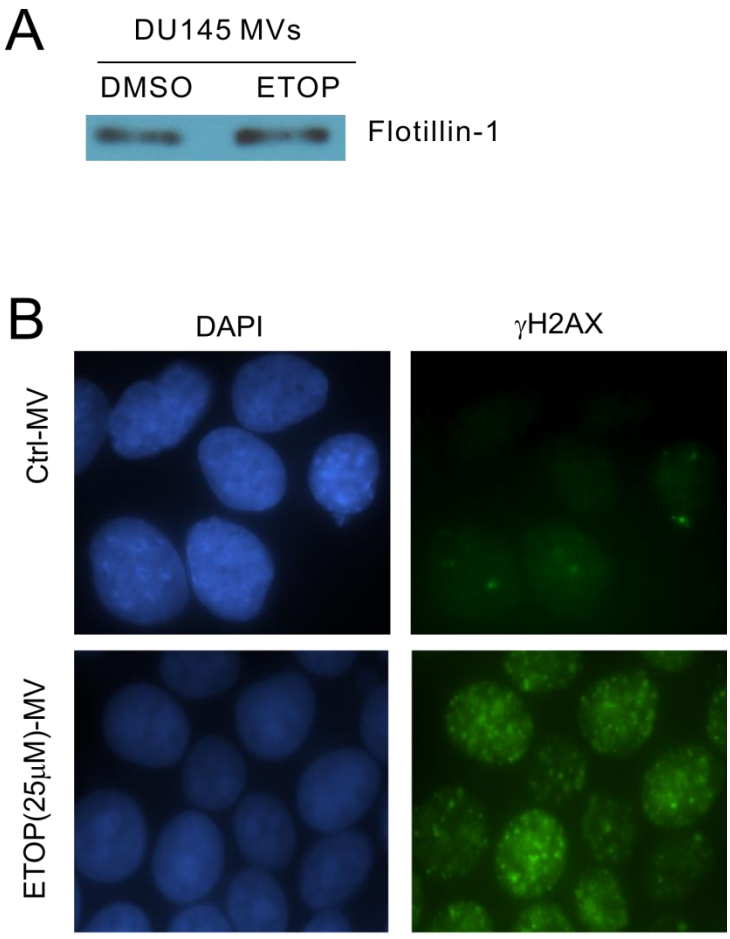

Figure 4.3. Microvesicles (MVs) derived from ETOP-treated DU145 cells induce the bystander effect (BE) in naïve DU145 cells. (A) MVs were isolated from DU145 cells treated with DMSO (control/Ctrl) or 25 μM ETOP for 24 h, followed by Western blot for flotillin-1; (B) DU145 recipient (naïve) cells were incubated with Ctrl-MVs or ETOP-MVs at 100 μg/mL for 24 h, followed by immunofluorescence (IF) staining for γH2AX. Nuclei were counterstained with DAPI. Images were acquired at 63x magnification.

To further study the contributions of MVs to the DNA damage-induced BE in naïve cells, we have treated DU145 cells with UV at either 10 or 20 mJ/cm^2, conditions that resulted in DDR evidenced by elevations of p-CHK1 (Figure 4.1A). In comparison to MVs isolated from control (Ctrl) cells, MVs derived from cells treated with UV at either 10 mJ/cm^2 (UV10) or 20 mJ/cm^2 (UV20) clearly enhanced γH2AX production in DU145

naïve cells (Figure 4.4A). Additionally, MVs derived from UV-treated DU145 cells induced γH2AX nuclear foci in recipient DU145 cells in comparison to Ctrl MVs (Figure 4.4B). These observations further exclude the unlikely possibility that MVs produced from ETOP-treated DU145 initiate the BE through a potential presence of residue ETOP. Furthermore, by taking advantage of the high affinity association between annexin V and MVs (Al-Nedawi, Meehan et al. 2008, Al-Nedawi, Meehan et al. 2009), we were able to demonstrate that incubation of MVs isolated from DU145 cells treated with UV at 40 mJ/cm^2 with annexin V effectively blocked the MV-associated BE activities (Figure 4.4C).

The bystander DDR properties can also be demonstrated in heterogeneous cell types. We first established that UV at 20 mJ/cm^2 induced DDR in A431 cells (Figure 4.5A). Following these conditions, we initiated DDR in A431 cells through UV exposure, and subsequently isolated MVs from Ctrl and UV-treated A431 cells (Figure 4.5B). In comparison to MVs isolated from Ctrl A431 cells, those derived from UV-treated A431 cells enhanced γH2AX production in recipient DU145 cells (Figure 4.5C). Furthermore, MVs produced by UV-treated A431 cells clearly generated γH2AX and p-ATM nuclear foci, indicative of ATM activation, in DU145 naïve cells (Figure 4.5D); co-localization of both types of nuclear foci was also demonstrated (Figure 4.5D). Taken together, we provide evidence supporting that MVs produced from DDR cells possess activities in inducing BE.

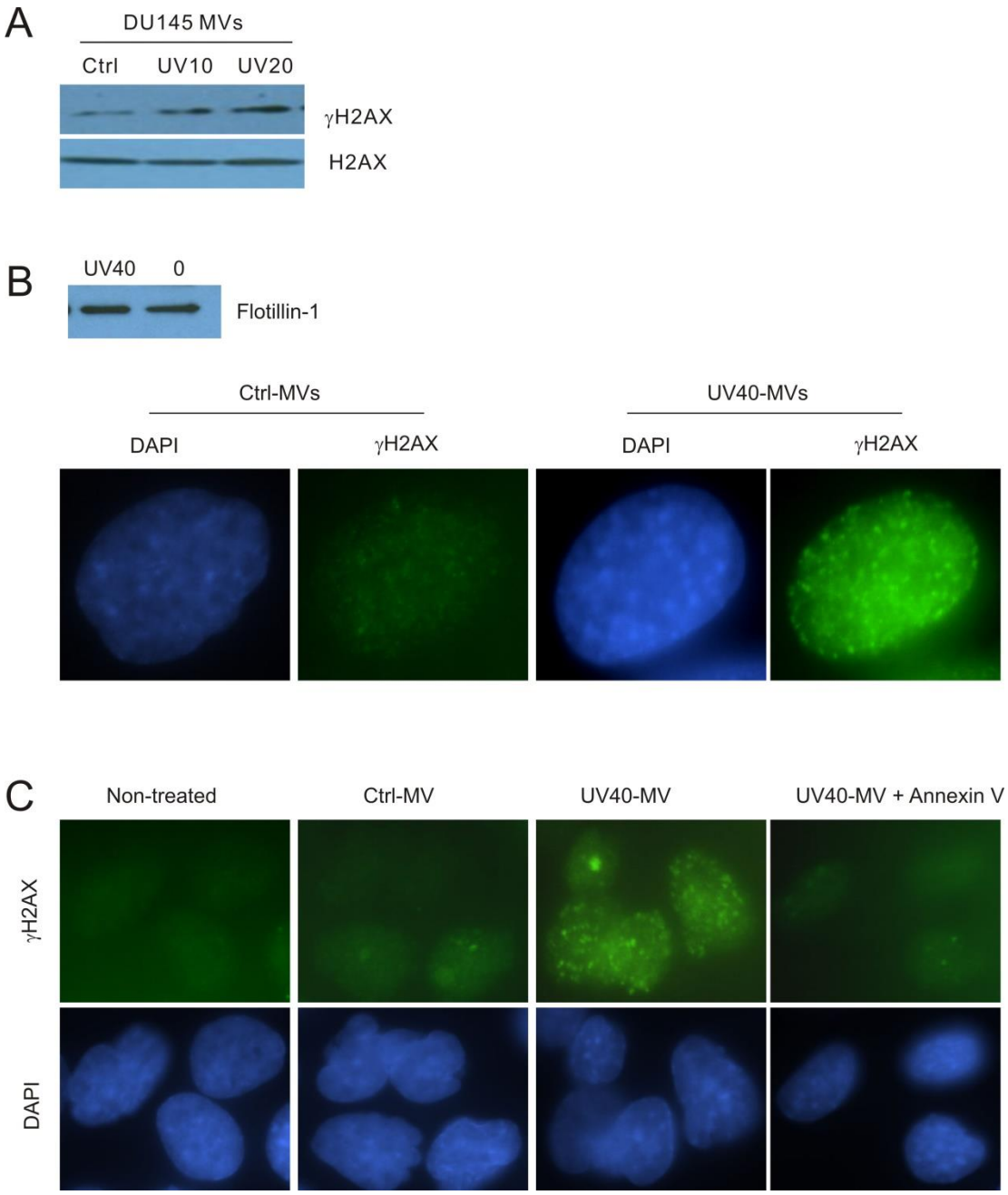

Figure 4.4. MVs produced by UV-treated DU145 cells induce the BE in recipient DU145 cells. (A) MVs were isolated from DU145 cells treated with Ctrl or UV at either 10 mJ/cm2 (UV10) or 20 mJ/cm2 (UV20) and used to incubate with naïve DU145 cells at 100 μg/mL for 24 h, followed by Western blot examination for γH2AX and H2AX. Experiments were repeated once; typical images from a single repeat are included; (B) MVs were isolated from control (0) or UV-treated (40 mJ/cm2; UV40) DU145 cells; MV isolations were confirmed by Western blot for flotillin (up top panel). DU145 naïve cells

were incubated with Ctrl-MVs and UV40-MVs for 24 h, followed by IF examination for γH2AX. Nuclei were counterstained with DAPI. Typical images are shown (bottom panel); Images were taken at the 100x magnification. (C) DU145 naïve cells were either untreated (non-treated) or treated with Ctrl-MVs, UV40-MVs, or UV40-MVs + annexin V. IF was carried out for γH2AX. Nuclei were counterstained with DAPI. Images were acquired at the 63x magnification.

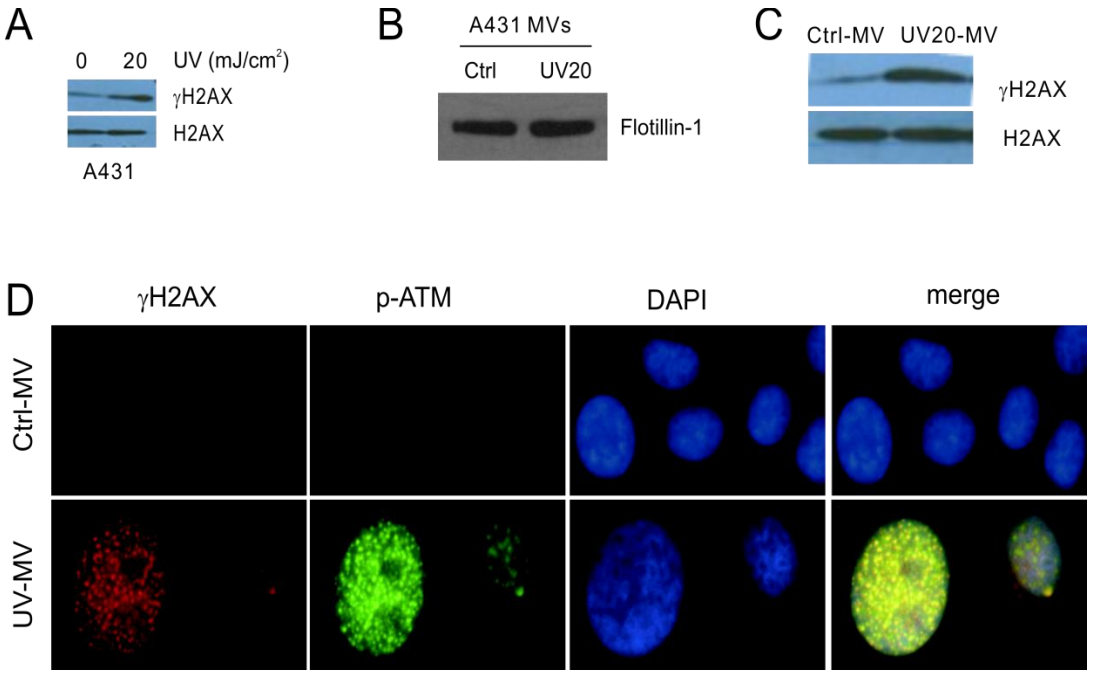

Figure 4.5. MVs produced by UV-treated A431 cells induce the BE in naive DU145 cells; (A) A431 cells were control- or UV-treated as indicated, cultured for 6 h, and examined for γH2AX and H2AX; (B) A431 cells were treated with Ctrl or UV at 20 mJ/cm2 (UV20) and cultured for 6 h. Isolation of MVs was examined for flotillin by Western blot; (C,D) DU145 recipient cells were treated with Ctrl- or UV20-MVs for 24 h at 100 μg/mL, followed by Western blot examination for γH2AX and H2AX (C) and by IF for γH2AX or S1981-phosphorylated ATM (p-ATM). Nuclei were counterstained with DAPI. Images were taken at the 63x magnification (D) .

MV Induces BE in Recipient Cells Upstream of ATM

In view of the respective apical role of ATM and ATR in ETOP and UV-initiated DDR (Tang, Wu et al. 2002, Batista, Kaina et al. 2009, Wei, Xie et al. 2010), we have investigated the respective contributions of ATM and ATR in the ETOP- and UV-MV-induced BE. For this purpose, we first demonstrated that the ATM inhibitor KU55933 at 10 μM substantially reduced ETOP-induced CHK2 phosphorylation, a well-established ATM target (Figure 4.6A). Furthermore, the basal level of CHK2 phosphorylation was also inhibited by KU55933 (Figure 4.6A, comparing p-CHK2 in lane 1 and lane 3). On the other hand, KU55933 does not apparently affect ETOP-induced γH2AX production (Figure 4.6A), consistent with the fact that γH2AX is also produced by ATR and DNAPK (Zhou and Elledge 2000, Jiang, Wang et al. 2016). Collectively, evidence supports that KU55933 at 10 μM inhibits ETOP-initiated ATM activation. Consistent with a major role of ATM activity in DSB repair (Goodarzi and Jeggo 2012, Lin, Yan et al. 2013), the presence of KU55933 sensitized γH2AX formation in DU145 cells treated with DMSO MVs (Figure 4.6B). However, KU55933 did not significantly affect γH2AX nuclear foci in naïve DU145 cells treated with ETOP-MVs (Figure 4.6B). These results do not support a major role of ATM kinase activity in the ETOP MV-induced BE.

Following the same principle, we have examined the involvement of ATR in the UV-MVs-induced BE. By taking advantage of CHK1 being the most specific ATR target in DDR, we were able to show that both the basal and UV-induced p-CHK1 were dramatically reduced by the specific ATR inhibitor VE821 (Figure 4.7A). Surprisingly, VE821 significantly elevated γH2AX production (Figure 4.7A, comparing lane 1 to lane

3) and γH2AX nuclear foci in DU145 recipient cells treated with not only Ctrl-MVs but also UV-MVs (Figure 4.7B). The enhancement was likely attributable to ATR activity being required in preventing the collapse of replication forks during DNA replication; inhibition of ATR activity by VE821 itself will be expected to induce DDR. In this regard, the system was unable to examine the role of ATR in the UV-MV-elicited BE.

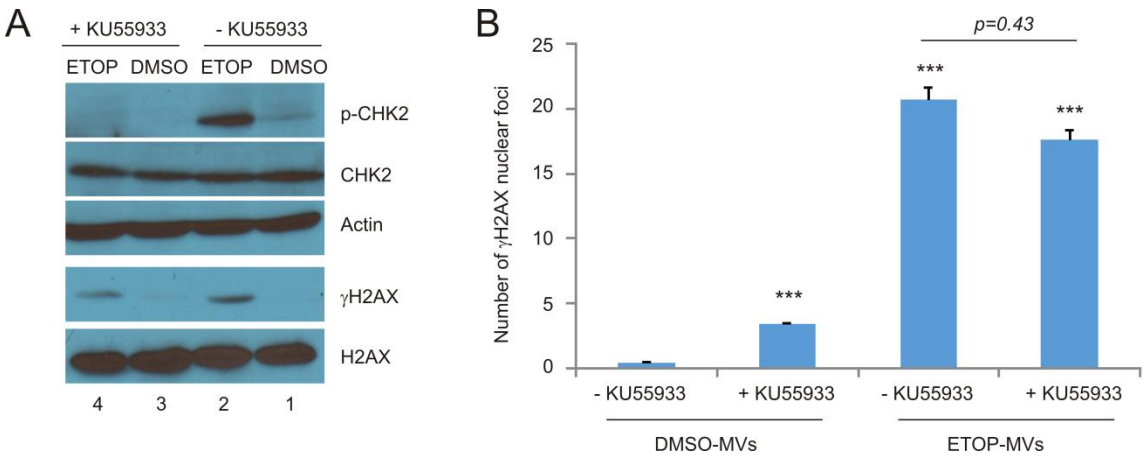

Figure 4.6. Inhibition of ATM activities in recipient DU145 cells does not reduce the BE induced by MVs. (A) DU145 cells were pre-treated with the ATM inhibitor KU55933, followed by mock treatment with DMSO or ETOP at 25 μM for 2 h. Western blot analysis was subsequently performed for the indicated proteins; (B) DU145 cells were pre-incubated with KU55933 for 8 h, followed by treatment with DMSO-MVs or ETOP-MVs (derived from cells treated with 25 μM ETOP for 24 h) for 24 h. IF staining was carried out for γH2AX. Experiments were repeated three times. More than 400 nuclei in several randomly selected fields were counted for γH2AX nuclear foci; means ± SE (standard error of the mean) were graphed. *** $p < 0.0001$ in comparison to naïve DU145 cells treated with DMSO MVs (2-tailed Student t-test).

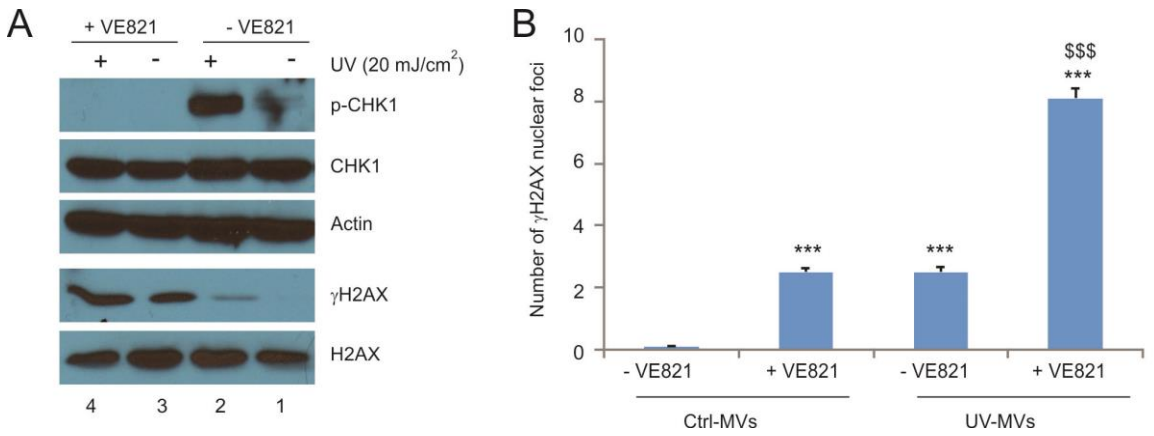

Figure 4.7. Examination of the involvement of ATR activities in naive DU145 cells treated with MVs derived from UV-treated DU145 cells. (A) DU145 cells were pre-treated with the ATR inhibitor VE821 for 8 h, followed by exposure to UV at 20 mJ/cm2 and cultured for 6 h. Western blot was then performed for the indicated events; (B) DU145 naïve cells were pre-incubated with VE821 for 8 h, followed by the treatment of Ctrl-MVs or UV-MVs (derived from DU145 cells treated with UV at 20 mJ/cm2) for 24 h. IF staining was carried out for γH2AX. Experiments were repeated three times. More than 500 nuclei in several randomly selected fields were counted for γH2AX nuclear foci; means ± SE (standard error of the mean) were graphed. *** $p < 0.0001$ in comparison to naïve DU145 cells treated with Ctrl-MVs (2-tailed Student t-test); $$$ $p < 0.0001$ in comparison to naïve DU145 cells treated with UV-MVs (2-tailed Student t-test).

Discussion

A large body of evidence reveals a major impact of the DNA damage-induced BE on human health. Radiotherapy is a major cancer therapy and can effectively control tumors. However, the treatment is associated with a variety of chronic complications, including the well-established cardiovascular diseases (Berkey 2010), and secondary malignancies. These adverse effects occur in both adult and childhood cancer survivors, and are a particular concern for the latter survivors. While technological advances in modern radiotherapy enable 80% of children with cancer to survive for 5 years or longer (Robison, Armstrong et al. 2009, Smith, Seibel et al. 2010), the cumulative incidence of secondary neoplasm was 20.5% among these survivors 30 years later (Friedman, Whitton et al. 2010, Smith, Seibel et al. 2010). Additionally, chronic health conditions affect 73.4% of survivors with 42.4% being life-threatening 30 years after radiotherapy (Oeffinger, Mertens et al. 2006). Despite the BE being a major health issue in cancer patients receiving radiation therapy, the process has not been thoroughly investigated and the underlying mechanisms remain elusive.

We provide clear evidence supporting a role of MVs in inducing the BE. This concept is based on our observations that MVs derived from cells undergoing DDR induce the BE in naïve DU145 cells and that neutralization of these MVs with annexin V significantly reduces the BE activities. The concept of MVs as a DDR messenger is supported by the knowledge that MVs play roles in cell-to-cell communication (Budnik, Ruiz-Canada et al. 2016, Lawson, Vicencio et al. 2016, Ratajczak and Ratajczak 2016). Intriguingly, MVs are a pathological contributor to cardiovascular disease (Lawson,

Vicencio et al. 2016), a well-established adverse effect caused by radiation therapy in cancer patients (Berkey 2010). Furthermore, the involvement of MVs in the DDR-induced BE has also been reported by others very recently (Xu, Wang et al. 2015, Carroll, Pulkoski-Gross et al. 2016).

In line with our observations, Carroll et al. reported an elevation of MV shedding in doxorubicin-treated MCF7 cells (Carroll, Pulkoski-Gross et al. 2016). In addition to etoposide, we also observed an enhancement in MV shedding in DU145 cells treated with UV (Figure 4.2). Based on these observations, it is tempting to propose that upregulation of MV shedding is a general response of cells undergoing DDR and that this upregulation has a functional consequence i.e., the bystander effect.

MVs contain a complex array of materials that are able to mediate cell–cell communications (Ratajczak and Ratajczak 2016). It is thus likely that multiple mechanisms are in play for DDR cell-derived MVs to elicit the BE in naïve cells. ATM, BRCA1, FANCD2, and CHK1 have been indicated to contribute to the BE in naïve cells in response to conditioned medium obtained from cells treated with ionizing radiation (Burdak-Rothkamm, Rothkamm et al. 2015, Ghosh, Ghosh et al. 2015). Transfer of microRNA-21 (miR-21) by MVs was reported to be a cause of ionizing radiation-induced BE (Xu, Wang et al. 2015). In our research, inhibition of ATM activation using KU55933 in recipient cells was without an apparent impact on the formation of γH2AX nuclear foci when treated with MVs produced by etoposide-treated DU145 cells (Figure 4.6B). The nuclear focus of γH2AX is a common surrogate marker of DSBs (Mirzayans, Andrais et al. 2015), suggesting that MVs derived from cells with DNA damage are able to damage

DNA. However, the molecular basis underlying this process remains unknown. Collectively, the exact mechanisms underlying the MV-mediated BE need further investigations.

The BE is observed in recipient DU145 cells treated with MVs isolated from DU145 and A431 cells exposed to either etoposide or UV (this study). MVs also deliver the BE message in MCF7 breast cancer and human lung fibroblast MRC-5 cells in the setting of doxorubicin and ionizing radiation-induced DDR (Xu, Wang et al. 2015, Carroll, Pulkoski-Gross et al. 2016). Taken together, these observations suggest the existence of common BE factors in DDR cell-produced MVs. However, this possibility does not exclude the presence of other BE factors in cells undergoing DDR. Nonetheless, the contributions of MVs to the DDR-induced BE implies an interesting application of annexin V in attenuating the chronic effects of radiation therapy.

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Conflicts of Interest

The authors declare no conflict of interest.

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CHAPTER FIVE

DISSCUSSION AND SIGNIFICANCE

5.0 DISCUSSION

Maintenance of genome stability and integrity is one of the fundamental tasks for cells and organisms. The task is performed by DNA damage response (DDR). Compromising the DDR pathways is a primary cause of cancer, leads to premature aging, and affects the homeostasis of the nervous, reproductive, and immune systems (Ciccia and Elledge 2010). In addition to being a cause of cancer, defects in DDR promotes cancer progression and therapy resistance. On the other hand, DDR is widely explored in cancer therapy and as a result, DDR contributes to the side effects of cancer treatment (Kastan 2008). DDR is a complex process that is coordinated by ATM, ATR, and DNA-PKcs. Despite DDR has being heavily investigated for several decades and significant progress has been made, our knowledge on factors regulating the activation of the above three PIKKs during DDR is incomplete and our understanding on mechanisms responsible to the side-effects of DDR-based radiotherapy and chemotherapy remains limited. This research provides insights on the activation of ATM and ATR as well as factors contribute the DDR therapy-associated adverse effects.

5.1 BMI1 attenuates ATM activation

Our research reveals BMI1 being a novel factor reducing ATM activation during DSB-induced DDR in MCF7 breast cancer and DU145 prostate cancer cells, at least in response to etoposide. Our observations are unlikely cell-type and stimulus-specific. DSBs-initiated DDR is highly conserved, i.e. not being limited to certain cell type and stimulus. Additionally, binding of BMI1 to NBS1 was detected in cells without etoposide

treatment. The formation of NBS1/ATM complex appears to be affected in MCF7 cells ectopically expressing BMI1, suggesting a potential mechanism for BMI1 to reduce ATM activation.

This research sheds light on tumorigenesis affected by both BMI1 and ATM. Activation of ATM occurs in response not only to genotoxins but also to oncogenic activities. Oncogenes commonly activate the ATM-mediated DDR, which suppresses tumorigenesis. In this regard, the ATM-regulated DDR forms a critical anti-tumor barrier which needs to be overcome in order for tumorigenesis to continue (Jia, Gao et al. 2013, Kahn 2014, Krausova and Korinek 2014). Exactly, how this barrier is removed remains unclear. On the other hand, BMI1 is commonly upregulated in a variety of cancers (Bea, Tort et al. 2001, Vonlanthen, Heighway et al. 2001, Lessard and Sauvageau 2003, Kim, Yoon et al. 2004, van Leenders, Dukers et al. 2007, Koren, Rijavec et al. 2017). An intriguing property of BMI1-derived tumorigenesis is its collaboration with a variety of oncogenes. BMI1 was identified as an oncogene based on its collaboration with c-Myc during leukemogenesis (Haupt, Alexander et al. 1991, van Lohuizen, Verbeek et al. 1991). BMI1 is also critical for c-Myc-induced neuroblastoma (Huang, Cheung et al. 2011). Furthermore, c-Myc directly transactivates BMI1 in leukemia, neuroblastoma, and nasopharyngeal carcinoma (Huang, Cheung et al. 2011, Waldron, De Dominici et al. 2012, Wang, Liu et al. 2013). BMI1 also works with other oncogenes, including Ras (Datta, Hoenerhoff et al. 2007, Hoenerhoff, Chu et al. 2009), Abl (Waldron, De Dominici et al. 2012), and hTERT (Tatrai, Szepesi et al. 2012, Miller, Dakic et al. 2013, Qiao, Chen et al. 2013). Our reported relationship between BMI1 and ATM in conjunction with

the collaborative property of BMI1 with other oncogenes implies that BMI1 contributes to tumorigenesis in part through downregulation of the ATM barrier. Although this collaboration is likely in part attributable to BMI1-mediated suppression of the *Ink4a/Arf* locus, clearly this is not the only mechanism. In human cancers, this locus is one of the most frequently mutated loci (Mathieu, Zhang et al. 2011) and BMI1 is commonly upregulated (Wang, Zhu et al. 2014). BMI1 remains oncogenic in cancer cells where the locus is mutated (Wang, Zhu et al. 2014). Furthermore, experimental evidence has shown that BMI1's oncogenic activity can be separated from its transcriptional suppression (Mimeault and Batra 2013). Collectively, it is an appealing possibility that downregulation of ATM function is a part of the collaborative basis on which BMI1 induces tumorigenesis. In this regard, by reduction of ATM function, BMI1 may contribute to genome instability. Since BMI1 apparently does not need the ring finger (RF) domain to attenuate ATM activation, whether the BMI1ΔRF mutant is competent to collaborative with c-Myc and other oncogenes and whether this collaboration is through attenuation of ATM activation can be determined. The above experiments may also shed light on the contributions of repression of the *Ink4a/Arf* locus vs. attenuation of ATM activation to BMI1-mediated tumorigenesis or collaboration. This is because the RF domain is required for BMI1 to stimulate the E3 ubiquitin ligase activity of PRC1 and that the E3 ligase activity is essential for BMI1 to repress the *Ink4a/Arf* locus.

It should be emphasized that BMI1 may also contribute to tumorigenesis and cancer progression via other mechanisms. By enhancing DSB repair, BMI1 can contribute to resistance to radiotherapy and chemotherapy.

5.2. BMI1 reduces ATR function

Our demonstration of BMI1 also attenuating single strand DNA breaks (SSBs)-initiated ATR activation is in accordance with the structural and functional similarities between ATM and ATR. Nonetheless, these similarities should not take away the importance of this research; this is because both kinases initiate different DDR processes and guard genome integrity primarily against DSBs and SSBs, respectively. Rather, these findings solidify BMI's activity in reducing ATM and ATR activation, and thus in contributing to genome instability. Similar to ATM-mediated surveillance of tumorigenesis (the ATM barrier), the ATR pathway is also activated and plays a role in suppression of tumorigenesis. The important physiological cause of ATR activation is the presence of ssDNA or stall of the replication forks during DNA synthesis; ATR is thus essential for DNA synthesis and cell cycle progression (Cimprich and Cortez 2008). In this regard, replication stress (RS) results in ssDNA, thereby activating ATR (Lopez-Contreras and Fernandez-Capetillo 2010). RS can be caused by oncogenes due to the disturbance of cell proliferation, leading to ATR activation. ATR in turn mounts resistance to cancer development. ATR function is thus reduced following tumor progression (Lecona and Fernandez-Capetillo 2014, Awasthi, Foiani et al. 2015). In this regard, whether BMI1 plays a role in reducing the strength of ATR signaling in oncogene-caused RS, thereby facilitating tumorigenesis along with enhancing genome instability, is also an interesting question for future research.

Although BMI1 downregulates ATR activation in a similar manner as it reduces ATM activation, BMI1 likely uses unique mechanisms to reduce ATR function. Similar

to association with NBS1, the RF motif and the other structural features of BMI1 is not essential for association with TOPBP1. However, while BMI1ΔRF retains activity in inhibiting ATM autophosphorylation at S1981, the mutant is incapable of reducing ATR autophosphorylation at T1989. Nonetheless, the BMI1ΔRF mutant similarly reduces ATM-mediated CHK2 phosphorylation and ATR-dependent CHK1 phosphorylation. These observations suggest that BMI1 involves different mechanisms to downregulate ATM and ATR functions. By association with NBS1, BMI1 may directly reduce ATM autophosphorylation. For ATR, BMI1 may interfere with TOPBP1-mediated ATR activation. It was recently reported that autophosphorylation of ATR at T1989 facilitates its association with TOPBP1 and that binding to TOPBP1 further stimulates ATR activation (Liu, Shiotani et al. 2011). It is thus a possibility that BMI1 may reduce TOPBP1 to bind the ATR-ATRIP complex or the subsequent steps by which TOPBP1 induces full strength of ATR activation.

5.3 Future research to examine the mechanisms underlying BMI1-derived attenuation of ATM and ATR activation

It should be emphasized that BMI1 only attenuates or reduces but not inhibits ATM and ATR activation under specific DDR process. While the underlying mechanisms and potential functions for this setting are unknown, it is tempting to propose that attenuation of ATM and ATR activation will give cells, particularly cancer cells, flexibility to modulate the strength of DDR to allow cancer cells to proliferate in the presence of DNA lesions. In this regard, this setting may provide cancer the 'just right' instability and thus

the ability to overcome DDR-associated inhibition of cell proliferation (Burrell, McGranahan et al. 2013).

The research performed on ATM and ATR has several limitations. The work was essentially performed using cell lines. More physiological models will need to be used to examine BMI1's role in reducing ATM and ATR activation. For example, BMI1 or BMI1ΔRF transgenic mice could be generated and examined for DSBs and SSB-initiated ATM and ATR activation, respectively. DSBs and SSBs can be produced using ionizing radiation, etoposide, and HU. With availability of these mice, whether transgenic expression of BMI1ΔRF enhances c-Myc-initiated cancer formation can also be determined.

In our research, we used ATM pS1981 and ATR pT1989 and the phosphorylation of their targets (CHK2 for ATM and CHK1 for ATR) to indicate ATM and ATR activation. The impact of BMI1 on both kinases can be directly studied. ATM and ATR could be immunoprecipitated from MCF7 EV and MCF7 BMI1 cells with and without being exposed with etoposide or HU. ATM and ATR-associated kinase activity could be detected and quantified using an in vitro kinase assay using recombinant GST-P53 (residues 1-40) as a substrate.

Since BMI1 retains its ability to bind either NBS1 or TOPBP1 after individual deletion of the RF, PS, HT, and NLS motifs, it can be explored whether other regions or any combinational removal of these motifs will impair the aforementioned binding. However, it should keep in mind that such deletion approach may affect the overall protein structure and the positive results, i.e. specific deletions abolish the association,

may not reflect the requirement of the deleted regions for BMI1 to bind NBS1 or TOPBP1.

To obtain detailed mechanisms governing BMI1-mediated reductions in ATM and ATR activation, mass spectrometry analysis can be performed on immunoprecipitated BMI1, ATM, and ATR complexes. The analysis may shed light on whether there are specific modifications on the ATM and/or ATR complex and whether there are specific proteins associated with the complexes following modulation of BMI1 expression with and without induction of DNA damage.

5.4 Microvesicles and bystander effects

DDR plays multiple roles in cancer. While its reduction is essential for tumorigenesis and progression, its induction in cancer cells is the major goal in cancer therapy. DDR also contributes to treatment-associated secondary cancer and other side effects (Kastan 2008). Interestingly, as discussed in Chapter 4, these side effects often occur in regions or organs that have not been directly exposed, particularly in the situation of radiotherapy. Mechanisms contributing to these BE-based side effects remain essentially under studied. By demonstration that microvesicles (MVs) contribute to BE, it opens a possible way to reduce BE, and thereby may reduce cancer therapy-associated side effects. It is well established that MVs can be neutralized by annexin V, particularly diannexin (Al-Nedawi, Meehan et al. 2009). Diannexin is a homodimer of annexin V with ability to protect ischemia/reperfusion (I/R) injury (Shen, Ke et al. 2007). Diannexin, also known as Alavita or ASP-8597, has been under clinical trial to prevent

organ rejection in kidney transplantation (clinicaltrials.gov/ct2/show/NCT00615966). It has been reported that irradiation of the lower body induced medulloblastoma in the brain of *Patched 1* heterozygous mice (Mancuso, Pasquali et al. 2008). It is likely that the brain tumors in this animal model is resulted from BE. It will be interesting to examine whether diannexin is able to reduce the process of brain tumor formation using this animal model.

Additionally, MVs-communicated BE DDR should be characterized. For example, will MV derived from DDR cells directly cause DNA breaks or just initiate the DDR signaling events? Based on the appearance of γH2AX nuclear foci, we prefer the scenario of induction of DNA breaks. With this determined, it can be further asked the cause(s) for DNA breaks. The detailed function of ATM and ATR in regulating BE DDR should also be further investigated.

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