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Brit1/Mcph1 Mediates The Dna Damage Response By Inducing P53 Stability And Promoting Atr Signaling

Edward Wang

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BRIT1/MCPH1 MEDIATES THE DNA DAMAGE RESPONSE BY INDUCING P53

STABILITY AND PROMOTING ATR SIGNALING

by

Edward Wang, B.S.

APPROVED:

_____
Shiaw-Yih Lin, Ph.D.
Supervisory Professor

_____
Ju-Seog Lee, Ph.D.

_____
Mong-Hong Lee, Ph.D.

_____
Hui-Kuan Lin, Ph.D.

_____
Zahid H. Siddik, Ph.D.

APPROVED:

_____
Dean, The University of Texas
Graduate School of Biomedical Sciences

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A

DISSERTATION

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by

Edward Wang, B.S.

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DEDICATION

I would like to dedicate this dissertation to my parents,

Wen-Chin 'Bill' Wang and Ming-Chiun 'Amy' Wang,

who have always encouraged me to believe in myself,

and to believe in goodness.

To my brother, Frank Wang,

for always reminding me that life should be fun.

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BRIT1/MCPH1 MEDIATES THE DNA DAMAGE RESPONSE BY INDUCING P53 STABILITY AND PROMOTING ATR SIGNALING

Edward Wang, B.S.

Supervisory Professor: Shiaw-Yih Lin, Ph.D.

The BRCT-repeat inhibitor of *hTERT* (BRIT1)/MCPH1 protein promotes the process of homologous recombination (HR) to repair DNA double strand breaks (DSBs). In response to DSBs, BRIT1 foci form at damaged sites, and recruits downstream repair proteins including 53BP1, MDC1, NBS1, and the SWI/SNF complex to the DSB region to promote DNA repair. *BRIT1* copy number deficiency correlates with increased genomic instability in ovarian cancer specimens and breast cancer cell lines. Here, we propose that additional functions of BRIT1 include a direct interaction with the p53 tumor suppressor protein to promote p53 stability, and binding and recruitment of TopBP1 to sites of replication stress to maintain ATR signaling.

The stability of p53 is largely dependent on its negative regulator, the MDM2 ubiquitin E3 ligase. Here, we provide evidence that in addition to its role as an upstream regulator of DNA damage response, BRIT1 is able to directly interact with p53 to promote p53 stability. Our results suggest that BRIT1 protects p53 from degradation, and this occurs even in the absence of DNA damage. The level of BRIT1 expression proportionally reduces MDM2-p53 interaction, increases MDM2 ubiquitylation and degradation, while decrease the ubiquitylation of p53. We show that these findings are clinically relevant, as BRIT1 deficiency increases breast cancer cell proliferation and transformation *in vitro*, while BRIT1 expression reduces breast tumor growth *in vivo*.

The ataxia telangiectasia mutated and Rad3-related (ATR) kinase is a major mediator of DNA damage repair and replication stress response pathways. The key function of ATR is to induce S and G2 phase checkpoints through its interaction and phosphorylation of several

proteins, including Chk1. We demonstrate here that BRIT1 is required to maintain ATR activation for cellular recovery from DNA damage and replication stress. We found that BRIT1 directly interacts with TopBP1. BRIT1 deficiency compromises TopBP1 foci formation, and reduces activation of ATR targets. We also found that the BRIT1-TopBP1 interaction is dependent on ATM/ATR-mediated phosphorylation of BRIT1 at Ser322, suggesting that ATM/ATR instigate a feed-forward mechanism through BRIT1, allowing long-term activation of DNA repair. Together, our work provides insight into novel mechanism for how BRIT1 promotes genome stability.

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Chapter 1

INTRODUCTION AND BACKGROUND

Genomic instability and cancer

In their seminal review published in 2000, Hanahan and Weinberg described six acquired characteristics, or 'hallmarks', that may exist among most or all cancers (Hanahan and Weinberg, 2000). These characteristics include self-sufficiency in growth signals, insensitivity to anti-growth signals, tissue invasion and metastasis, limitless replicative potential, sustained angiogenesis and evading apoptosis. They explain that there must exist a vast number of genetic abnormalities in a cell that allow these characteristics to take root. However, it is unlikely that these abnormalities sequentially occur naturally over the course of a person's lifetime. It is more likely that these abnormalities arise from defects in the cell cycle checkpoint and DNA repair machinery that function together to maintain the genome. These defects lead to a condition where mutations that would normally be repaired efficiently, remain unrepaired and passed on to daughter cells, allowing mutations to accumulate. Since these mutations stem from defects in the cell cycle checkpoint and DNA repair machinery, mutational frequency is high, driving acquisition of the six characteristics of cancer. The term 'genomic instability' describes the characteristic of high genetic abnormalities existing in a cell, likely caused by defects in genome maintenance machinery. Appropriately, genomic instability was termed an 'enabling characteristic', because it provides the means by which the six characteristics of cancer are acquired (Hanahan and Weinberg, 2000). In 2011, an updated review on the topic of cancer 'hallmarks' was published, suggesting further characteristics shared among cancer cells, namely deregulated cellular energetics and avoiding immune destruction. However, it

remains unchanged that genomic instability is an enabling characteristic that drives the acquisition of all other characteristics.

To maintain genomic stability, cells have evolved mechanisms to deal with stress factors, e.g. ionizing radiation, replication stress, oncogene-induced hyperproliferation, etc., which may compromise the genome. Integral mechanisms include the DNA damage response (DDR) and the replication stress response (RSR) (Curtin, 2012). In recent years, the BRIT1 (BRCT repeat inhibitor of *hTERT*) protein has been described to play an important role in the DDR, due to its DNA damage-sensing role in DNA repair. In this work, we show that BRIT1 has an additional role in DDR, which involves maintaining the expression level of the p53 tumor suppressor protein. Furthermore, we show that the role of BRIT1 in DNA repair extends beyond DNA damage-sensing, and also involves cell cycle checkpoint activation, and therefore a role in RSR.

DNA damage response maintains genome stability

DNA damage response consists of cell cycle checkpoint and DNA repair

DNA damage comes from many sources, and these are often categorized as either exogenous to the cell, such as ionizing radiation, or endogenous, such as replication fork collapse and reactive oxygen species arising from metabolic byproducts. The types of damage include chemical modifications of bases or DNA adducts that alter the backbone structure of DNA, and single- and double-strand breaks. Sensor proteins that recognize the damaged DNA induce a checkpoint response that inhibits progression of the cell cycle, which gives the DNA repair system time to repair the DNA. Based on the cell cycle phase in which the DNA repair system is activated, either of two major repair pathways will be activated; homologous recombination (HR) activated from S-phase through G2, and non-homologous end joining (NHEJ) activated throughout the cell cycle (Branzei and Foiani, 2008). When double strand breaks occur in the G1 phase, NHEJ is the main mechanism of repair, however HR is the

favored mechanism when cells have duplicated its genome in the S and G2 phases, because HR is an error-free mechanism. Upstream proteins in HR proteins also function to induce cell cycle checkpoint. Together, cell cycle checkpoint and DNA repair are two major pathways within DNA damage response (Harper and Elledge, 2007).

DNA damage-induced cell cycle checkpoint

Cell cycle checkpoints coordinate DNA repair with cell cycle progression. When DNA is damaged, sensor proteins that recognize the damage and initiate repair are also the proteins that initiate the checkpoint response to halt cell cycle progression until repair is complete. Two major sensors of DNA damage are ATM (*Ataxia telangiectasia mutated*) and ATR (*ATM and Rad3-related*), members of the phosphatidylinositol-3-kinase-like kinase (PIKK) family (Lovejoy and Cortez, 2009). ATM is commonly associated with response to double-strand DNA breaks, while ATR is associated with response to single-strand DNA breaks or stalled replication forks (Bartek and Lukas, 2007; Shiloh, 2003) (Figure 1). ATM and ATR together phosphorylate hundreds of targets, and several of their targets are secondary kinases, e.g. Chk1 and Chk2, which have their own set of targets. Together, the cell cycle checkpoint is a network linked through phosphorylation signaling cascades (Matsuoka *et al.*, 2007). These kinases activate unique as well as overlapping downstream targets to create a cross-talking system that achieves the same purpose of cell cycle checkpoint activation, regardless of cell cycle phase (Shiloh, 2006).

Single strand breaks occur approximately 1000x more frequently than double strand breaks in mammalian cells (Haber, 1999). However, double strand breaks are much more dangerous in terms of their effect on genetic instability if left unrepaired, or if errors occur during repair (Betermier *et al.*, 2014; Ferguson and Alt, 2001). HR, as mentioned, is the preferred method of double strand break repair in S and G2 phases. How does HR lead to checkpoint activation? Double strand break formation is followed first by the MRE11-Rad50-NBS1 (MRN) complex localizing at the break site. This is followed by recruitment of ATM
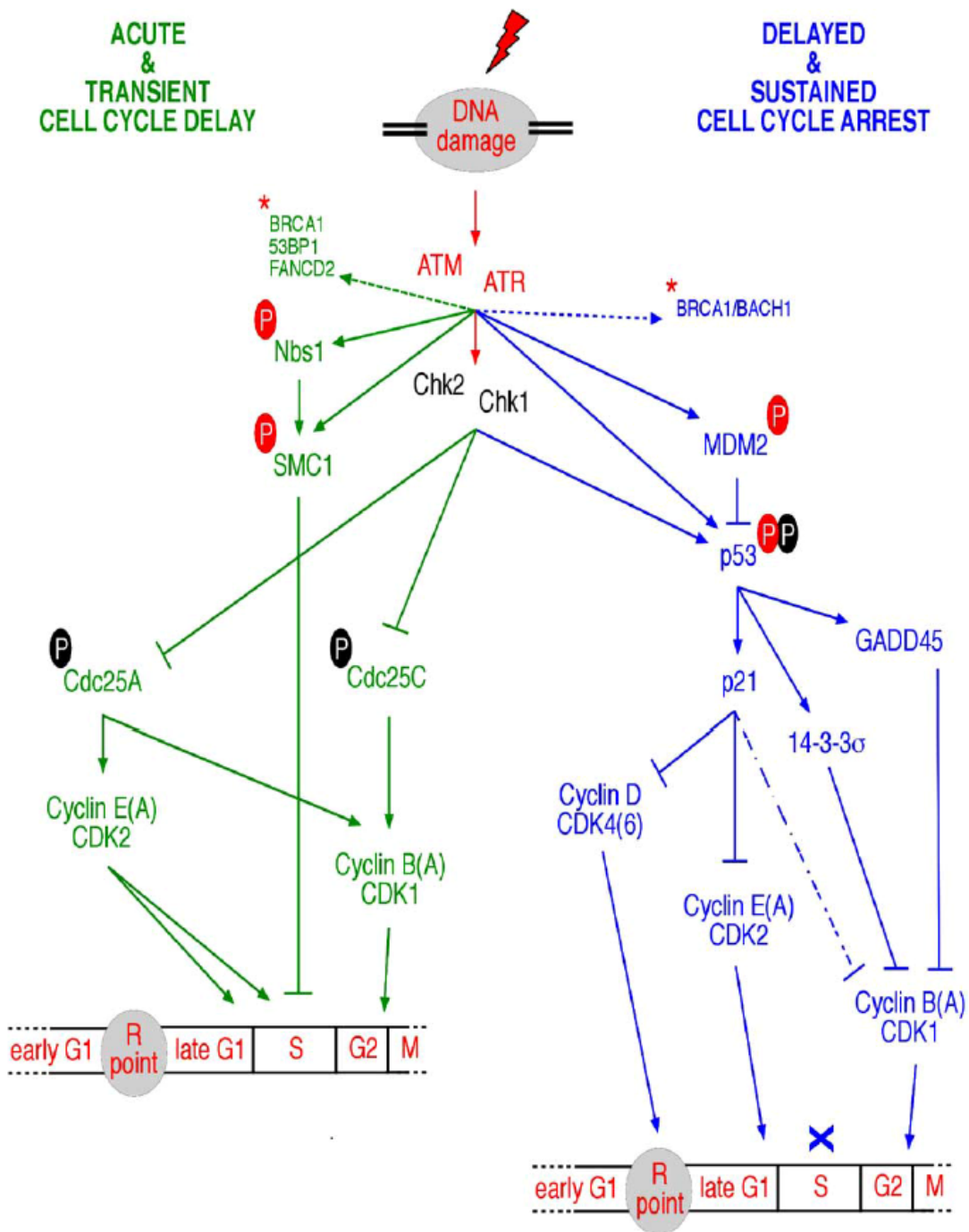
Figure 1. Mammalian cell cycle checkpoints

A schematic representation of molecular pathways involved in transmitting the signal from the sites of DNA damage to delay (green) or arrest (blue) the cell cycle progression at multiple transitions. The apical kinases ATM/ATR (red) and Chk2/Chk1 (black) together with their major substrates (P) are indicated. Note that the 'delayed & sustained' part of the checkpoint response lacks the intra-S-phase component (X). Asterisk indicates that other proteins, all direct targets of ATM and ATR kinases, also participate in the 'acute and transient' cell cycle delay during S phase (BRCA1, 53 BP1, FANCD2) and at the G2/M boundary (BRCA1, 53BP1). *Adapted from Lukas, J., Lukas, C., Bartek, J., DNA Repair **3**, 997-107 (2004) with permission from Elsevier.*

through a direct interaction with Nbs1 C-terminus, which activates ATM (Buscemi *et al.*, 2001; Horejsi *et al.*, 2004). Of the many targets of ATM, CtIP (CtBP-interaction protein) in particular plays an important role in double strand DNA resection to generate single strand DNA (You *et al.*, 2009). Support for this concept comes from studies showing that CtIP depletion abrogates RPA (replication protein A) coating of ssDNA, and thus compromises ATR activation and Chk1 phosphorylation (Huertas and Jackson, 2009; Sartori *et al.*, 2007; Yu and Chen, 2004). Generation of ssDNA is often considered the signal for ATR signaling and subsequent checkpoint activation (Costanzo *et al.*, 2003; Zou and Elledge, 2003). The presence of ssDNA recruits RPA to directly bind and protect ssDNA (Byun *et al.*, 2005). The ATRIP-ATR complex recognizes ssDNA-bound RPA through ATRIP binding to RPA (Ball *et al.*, 2005). At this point, ATR is active, and is able to undergo *trans* autophosphorylation at T1989 of the ATR dimer (Liu *et al.*, 2011). ATR also phosphorylates Rad9 of the Rad9-Hus1-Rad1 (9-1-1) complex (Delacroix *et al.*, 2007). The 9-1-1 complex, or clamp, is loaded onto dsDNA adjacent ssDNA via the Rad17 clamp loader (Lee and Dunphy, 2010). Phosphorylated 9-1-1 creates a binding site for TopBP1 (Topoisomerase (DNA) II binding protein 1). TopBP1 is a protein that contains eight BRCT domains, and through its two N-terminal BRCT domains, binds to phospho-Rad9 (Lee *et al.*, 2007). TopBP1 also binds directly to ATRIP via both N- and C-terminal ends. This interaction then allows TopBP1 to interact with ATR through its ATR activation domain (AAD) located between BRCT6 and 7 (Kumagai *et al.*, 2006). ATR is fully active and able to phosphorylate all its downstream targets only when bound to TopBP1 (Choi *et al.*, 2010). A major downstream target of ATR is Chk1, which directly induces the cell cycle checkpoint through its kinase activity. ATR phosphorylates Chk1 at S317 and S345, leading to Chk1 auto-phosphorylation at S296 (Liu *et al.*, 2000; Zhao and Piwnica-Worms, 2001). ATR phosphorylation of Chk1 allows Chk1 to dissociate from chromatin to target downstream proteins such as Cdc25b. Therefore, ATR phosphorylation of Chk1 may not directly affect Chk1 activity, but rather, displaces Chk1 to act on its targets (Smits *et al.*, 2006). Activated Chk1 maintains Cdc25b at low levels by promoting Cdc25b degradation (Kramer *et al.*, 2004). High

levels of Wee1 kinase through S and G2 phases promote Tyr15 phosphorylation and inhibition of Cdc2 and Cdk2 (Watanabe *et al.*, 1995). If Chk1 keeps Cdc25b at low levels during checkpoint activation, then Cdk/cylin complexes remain at low activity since Cdc25b is not able to dephosphorylate the complexes (Sorensen and Syljuasen, 2012). Collectively, we see how the DNA damage response encompasses the sensing of a single or double strand DNA break, to generating a single stranded DNA that is recognized by ATR, which through several phosphorylation events, leads to checkpoint activation. These events are activated upon ssDNA and dsDNA breaks as well as during replication stress. While the checkpoint system is activated, the DNA repair machinery is working together to repair the DNA.

Double strand DNA break repair by homologous recombination

The DNA damage response, which elicits repair and checkpoint mechanisms, is a complex process that is only activated when DNA is severely damaged, such as when double strand breaks occur (Bekker-Jensen and Mailand, 2010; Wyman and Kanaar, 2006). With respect to the DNA repair process itself, the process can be simplified by categorizing the components of the DNA repair machinery generally into sensor, transducer and effector proteins (Polo and Jackson, 2011). As mentioned, sensor proteins consist of the upstream components that localize immediately to the damage site. The Ku70 and Ku80 heterodimer is the sensor protein for NHEJ, while the MRN complex and ATM can be considered sensors of DNA damage within the HR pathway (Lieber and Wilson, 2010). We describe in this section, the HR pathway since it is in this pathway that the BRIT1 protein is mostly known to function.

As mentioned, MRN complex sits atop the DSB repair chain and constitutes the DNA damage sensor. The MRE11 subunit along with CtIP, are required for DNA resection to form single stranded DNA (Sartori *et al.*, 2007), which sets the stage for ATR recruitment and checkpoint activation as described. Via the NBS1 subunit, ATM is recruited to the damaged site, and this step sets the stage for DNA repair. ATM phosphorylation of histone H2AX at Ser139 to form γH2AX is a well-known event marking the presence of a double strand break

(Yuan *et al.*, 2010). This phosphorylation, however, is not unique to ATM, as DNA-PKcs and ATR also target this site (Bonner *et al.*, 2008). This phosphorylation event occurs within minutes after double strand break induction, e.g. after ionizing radiation, and their localization at damaged sites can be observed as IR-induced foci (IRIF) under immunofluorescence (Bonner *et al.*, 2008). Numerous repair proteins depend on the γH2AX signal for recruitment to the damaged site to form IRIF themselves. One of these is BRIT1, which forms IRIF within a couple of minutes after DNA damage, and promotes recruitment of several downstream proteins, discussed in more detail in a later section (Wood et al., 2007b). Suffice to mention for now that BRIT1 promotes downstream processes that aid in chromatin remodeling to increase accessibility of repair proteins to the site of damage (Peng *et al.*, 2009). Just downstream of γH2AX and BRIT1 is the MDC1 protein, which binds directly to Ser139- γH2AX through its BRCT domains (Stucki *et al.*, 2005). CK2-mediated phosphorylation of MDC1 at SDTDXD/E clusters leads to recruitment of NBS1. NBS1 binds to p-MDC1 through its FHA and tandem BRCT repeats (Wu *et al.*, 2008). Since NBS1 then recruits ATM via direct binding, MDC1 can be regarded as a point of damage signal amplification, since ATM recruitment downstream of MDC1 leads to increased formation of γH2AX adjacent to the damage site. Besides phosphorylation, ubiquitylation is another post-translational modification that plays a significant role in the DNA repair pathway. RNF8 is a RING finger ubiquitin E3 ligase that, like NBS1, also binds via its FHA domain to p-MDC1 (Huen *et al.*, 2007). Huen *et al.* showed that this binding to p-MC1 and subsequent ubiquitylation of H2AX promotes recruitment of RAP80-BRCA1. RNF8 ubiquitylates H2AX, creating a binding site for RAP80, through its ubiquitin interacting motif (UIM). RAP80 then recruits BRCA1 to the damaged DNA site (Huen *et al.*, 2007; Kim *et al.*, 2007). This process is aided by yet another RING protein, RNF168, which localizes to ubiquitylated H2A and H2AX, in an RNF8-dependent manner. Together, RNF8 and RNF168 promotes BRCA1 and 53BP1 recruitment by ubiquitylating H2A and H2AX (Doil *et al.*, 2009).

The downstream DNA damage response proteins that are recruited to the damaged DNA aid the repair process. For example, BRCA2 recruitment is required for Rad51 to perform

the homologous recombination step (Moynahan and Jasin, 2010; Yang *et al.*, 2002). While Mre11 is responsible for initial and limited DNA resection, BLM (Bloom's syndrome protein) helicase and Exo1 (exodeoxyribonuclease I) interaction mediates further resection (Mimitou and Symington, 2008). Rad51 uses the resected DNA for strand invasion in HR (Nimonkar *et al.*, 2008). However, Rad51 activity requires direct interaction with BRCA2. BRCA2 contains eight central BRC (Galkin *et al.*, 2005) repeats that form the basis of interaction with Rad51. Binding between the two proteins Rad51 to bind to ssDNA, and not dsDNA, in the presence of ATP (Carreira *et al.*, 2009). The interaction between BRCA2 and Rad51 is regulated by Cdk-mediated phosphorylation of BRCA2 at S3291. Phosphorylation at this site blocks BRCA2 interaction with Rad51. Under DNA damage conditions in S and G2, Cdk activity is low, and BRCA2 and Rad51 are able to interact to process damaged DNA (Esashi *et al.*, 2005). CDK activity also regulates CtIP activity. Here, Cdk targeting of CtIP at T847 promotes resection in S-phase where resection during DNA replication is frequent. However, after resection and during checkpoint activation, Cdk activity is low until damage is repaired (Huertas and Jackson, 2009). Collectively, DNA repair by HR is regulated by post-translational modifications such as phosphorylation and ubiquitylation of proteins to aid in their interaction, as well as by the cell cycle itself, which allows HR to initiate and come to completion before moving toward mitosis.

Replication stress response is a barrier to genomic instability.

During DNA replication, the region of DNA unwinding and bi-directional replication is called the replication fork (Figure 2). At the fork, many proteins work in concert to maintain continuous replication along the template DNA. When replication encounters challenges that impede normal synthesis, the replication process becomes temporarily stalled, and the condition is referred to as replication fork stalling (Branzei and Foiani, 2010). During fork stalling, the cell undergoes replication stress response (RSR), which activates the replication checkpoint to allow the cell time to recover from the stress that causes the replication fork to
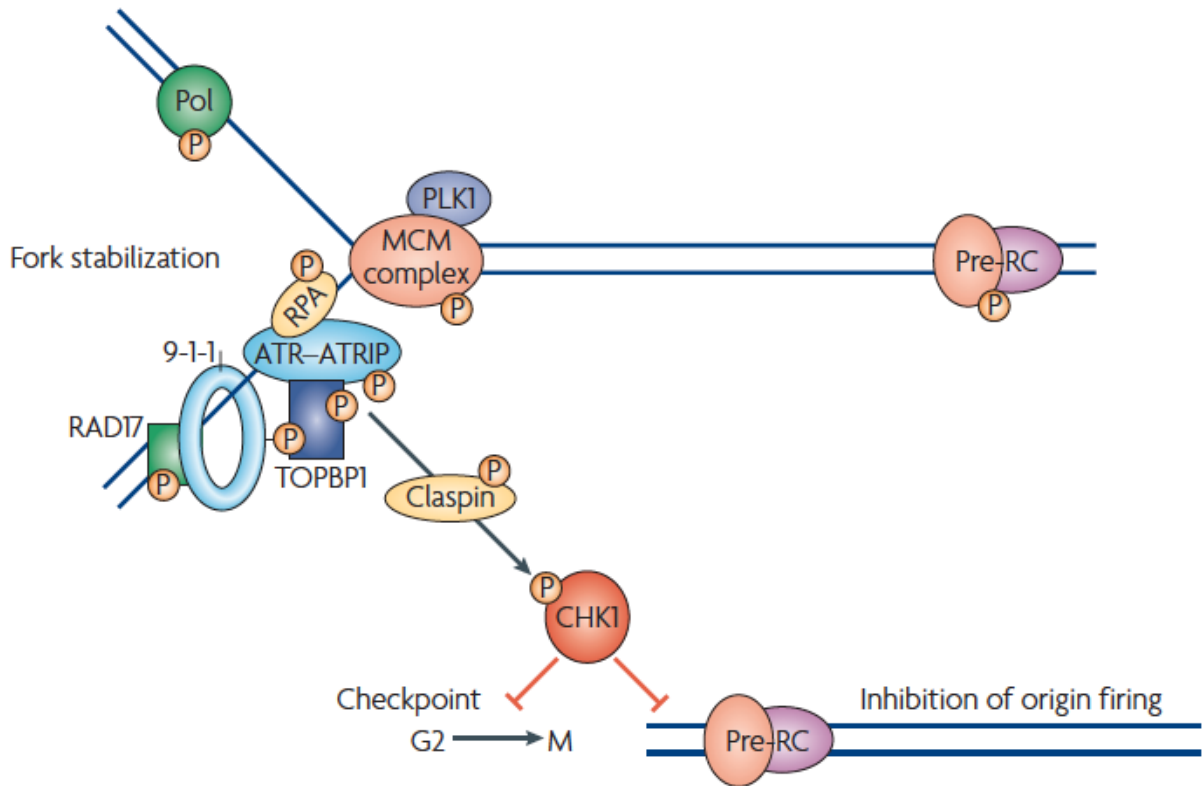
Figure 2. ATR signaling regulates replication and cell-cycle transitions.

ATR phosphorylates numerous substrates to regulate replication and cell-cycle transitions. A major ataxia-telangiectasia mutated and RAD3-related (ATR) kinase substrate is checkpoint kinase-1 (CHK1). Phosphorylation of CHK1 releases it from chromatin and increases its kinase activity. CHK1 has numerous substrates, some of which regulate cell-cycle transitions and replication-origin firing. Many ATR substrates, including minichromosome maintenance protein-2 (MCM2), MCM3, replication protein A (RPA), RAD9 (which is part of the 9□1□1 complex), topoisomerase-binding protein-1 (TOPBP1), RAD17 and CHK1, are at the replication fork. ATRIP, ATR-interacting protein; Pol, polymerase; Pre-RC, pre-replicative complex. *Adapted from Cimprich, K.A., Cortez, D., Nat Rev Mol Cell Biol* **9***, 616-627 (2008) with permission from Nature Publishing Group.*

stall. The high fidelity of DNA replication is attributed in part to this response system. When a cell is unable to recover, the DNA may undergo replication fork collapse, which leads to a double strand break. The RSR functions to prevent genomic instability that could result from significant damages to the DNA (Branzei and Foiani, 2010).

DNA replication or synthesis initiates from one of many origins of replication across a single chromosome. The double-stranded DNA is unwound bi-directionally away from the origin, as the two replication forks pull away from each other to elongate the nascent strand of DNA along its template. Major players in DNA synthesis reside at the replication fork and together form the replisome machinery. The replisome contains all the proteins and complexes required for DNA synthesis. These include: topoisomerase which breaks the phosphodiester bond to unwind double strand DNA (Pommier, 2013), the MCM (minichromosome maintenance) helicase enzyme complex which separates the annealed double strand DNA (Tanaka and Araki, 2013), the leading and lagging-strand DNA polymerases (Heitzer and Tomlinson, 2014), the RPA (replication protein A) trimer that binds and protects single-strand DNA from digestion (Prakash and Borgstahl, 2012), and the PCNA (proliferating cell nuclear antigen) clamp protein which is loaded onto double strand DNA via RFC (replication factor C) and aids in the processivity of DNA polymerases (Mailand *et al.*, 2013), as well as others. The high processivity of DNA polymerases and the linked helicase enzyme allows efficient DNA replication. However, impediments to replication occur frequently, and they must be removed in order to return to a state of high processivity.

There are many causes of replication fork stalling. MCM helicase and DNA polymerases are tethered in such a way that as single strand DNA emerges from MCM-mediated double strand DNA separation, DNA polymerase follows immediate to extend the nascent DNA chain (Aparicio *et al.*, 1997; Zhu *et al.*, 2007). When MCM and DNA polymerase uncouple, MCM may continue to separate double strand DNA even as DNA polymerase lags behind. Uncoupling causes a stretch of ssDNA to become exposed, signaling RPA binding to protect ssDNA. This constitutes the initial step of replication stress response, leading to the

intra-S phase checkpoint to stall the replication fork and inhibit further synthesis until the replisome is repaired (Byun *et al.*, 2005). Uncoupling of leading and lagging strand DNA polymerases also leads to ssDNA exposure, triggering fork stalling (Sogo *et al.*, 2002). Replication progress may also be blocked by bulky lesions intercalated within the DNA, by aberrant DNA structures such thymine dimers, by protein-DNA structures such as tRNA-protein structures present during transcription occurring adjacent to replicating DNA, as well as by dNTP depletion such as when cells are treated with hydroxyurea (Matsuoka *et al.*, 2007). Checkpoints that are activated by stalled forks are often removed once the impediment is resolved, such as by the convergence of adjacent origins of replication that complete replication (McGlynn and Lloyd, 2002a, b; Torres *et al.*, 2004). However, when such adjacent origins are unavailable, and lesions are not resolved, then the replication fork may collapse, leading to a double strand break, which would then activate recombination repair (Branzei and Foiani, 2005).

While replication forks are stalled, the replication stress response checkpoint stabilizes the fork until repair is complete. This replication checkpoint is essentially the same checkpoint that is activated by double strand breaks induced, for instance, by ionizing radiation. This makes sense when considering that DNA resection after double strand breaks exposes ssDNA in the same manner as helicase-polymerase uncoupling during replication fork stalling. In both instances, ssDNA is bound by RPA complexes, which stabilize the ssDNA, followed by recruitment of ATR. Activated ATR phosphorylates several downstream targets such as the MCM helicase, RPA, PCNA, claspin, Chk1, and others, to induce the replication checkpoint (Cimprich and Cortez, 2008).

The role of p53 in maintaining genome stability

A hallmark of cancer is the ability to evade apoptosis, the process of programmed cell death (Hanahan and Weinberg, 2000). The p53 tumor suppressor protein responds to a range

of stress signals, which include DNA damage, oncogene-induced hyperproliferation, replication stress and hypoxia (Gudkov and Komarova, 2003; Meek, 2004; Michael and Oren, 2003; Vousden and Lu, 2002). The well-studied response to these stressors makes p53 an established regulator of cell cycle checkpoint and apoptosis. Apoptotic signals are detected by either of two categories of sensor proteins, either membrane-bound or intracellular. Membrane-bound sensors or receptors signal the extrinsic apoptotic pathway while intracellular sensors such as p53 signal the intrinsic apoptotic pathway. The p53 pathway can be divided into the various sequences of events starting from, 1) negative p53 regulation under normal conditions to prevent cell cycle arrest and apoptosis to 2), stress-induced stabilization of p53 and finally 3) p53-mediated activation of cell cycle checkpoint and apoptosis. In this section, we provide an overview of this sequence of events to summarize the tumor suppressor roles of p53 in mediating genome stability after cellular stress.

Regulation of p53 expression and activity

Due to negative regulation by MDM2 (Hdm2 in human), p53 protein expression is normally kept at low levels (Figure 3). However, elevated Mdm2 expression due to gene amplification or hyperactivation of Mdm2 due to loss of an Mdm2-negative regulator, e.g. ARF, leads to p53 inactivation. This abrogates the checkpoint- and apoptosis-inducing capabilities of p53 (Tovar *et al.*, 2006). Mdm2 overexpression has been observed in over 40 types of malignancies, correlates with a worse prognosis, and so is a clinically relevant target in cancer (Rayburn *et al.*, 2005). Besides p53, the Mdm2 oncogene has also been shown to target other tumor suppressors such as Rb and ARF (Meek and Knippschild, 2003). Mdm2 is a ubiquitin E3 ligase that binds to the N-terminal transactivation domain of p53 and promotes ubiquitylation at the C-terminal region of p53, leading to proteasomal degradation (Michael and Oren, 2003). This ubiquitin ligase activity depends on the RING and zinc finger domains of Mdm2 (Lindstrom *et al.*, 2007). This activity requires direct binding between Mdm2 and p300, a co-activator of p53 transcriptional activity
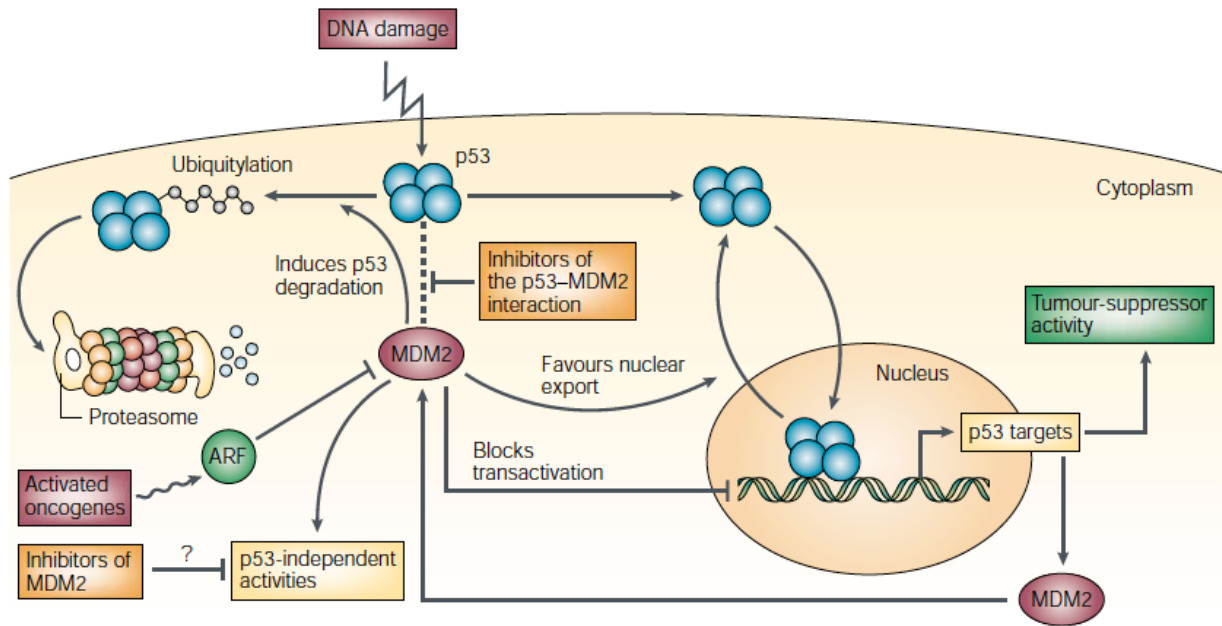
Figure 3. Regulation of p53 by MDM2

p53 and MDM2 form an auto-regulatory feedback loop. p53 stimulates the expression of MDM2; MDM2 inhibits p53 activity because it blocks its transcriptional activity, favours its nuclear export and stimulates its degradation. Different cellular signals, such as DNA-damage or oncogene activation, induce p53 activation. DNA damage favors p53 phosphorylation, preventing its association with MDM2. Activated oncogenes activate the ARF protein, which prevents the MDM2-mediated degradation of p53. Similarly, inhibitors of the p53–MDM2 interaction should activate p53 tumour-suppressor activity in tumour cells that express wild-type p53. These compounds, because they bind to MDM2, could also affect the p53- independent activities of MDM2. *Adapted from Chène P., Nat Rev Cancer 3, 102-109 (2003) with permission from Nature Publishing Group.*

(Zhu *et al.*, 2001). MDM2 mediates multiple monoubiquitin attachment to p53 while p300 mediates subsequent polyubiquitylation (Lai *et al.*, 2001). Additionally, Mdm2 binds to the N-terminal transactivation domain of p53, which inhibits p53 binding to DNA (Momand *et al.*, 1992). This interaction also promotes p53 nuclear export (Roth *et al.*, 1998), and promotes interaction with histone deacetylase 1 (HDAC1), which deacetylates p53 at its C-terminus, making it more prone to ubiquitylation and degradation (Ito *et al.*, 2002; Meek, 2004). In the same family as Mdm2, MdmX (Mdm4) is a cytoplasmic protein that is bound by Mdm2. Mdm2 binding to Mdm4 translocates the complex into the nucleus and importantly, stabilizes Mdm2 to allow negative regulation of p53 (Tovar *et al.*, 2006). Finally, casein kinase I (CK1) also directly interacts with and phosphorylates Mdm2 to promote p53 regulation by Mdm2. CkI inhibition leads to the same phenotype of increased apoptosis as treating cells with Nutlin-3, an Mdm2 inhibitor (Huart *et al.*, 2009).

DNA damage induces p53 stabilization and activity

Under a variety of stimuli including DNA damage, ionizing radiation, genome instability, transforming growth factors, and overexpressed oncogenes, the negative regulation of Mdm2 on p53 is relieved in order to stabilize p53 (Lavin and Gueven, 2006). Proteins that promote p53 stability include ARF, ATM/Chk2 and ATR/Chk1 tumor suppressors (Banin *et al.*, 1998; Chehab *et al.*, 2000; Orlando *et al.*, 2014; Shieh *et al.*, 2000; Tibbetts *et al.*, 1999). p53 contains three major domains: an N-terminal transactivation domain, a central DNA binding domain, and a C-terminal tetramerization domain. Under cellular stress, in order to stabilize p53, several kinases within the DNA damage pathway phosphorylate the N-terminal p53 domain (ATM/ATR - Ser15, Chk1/Chk2/ - Ser20) (Shiloh, 2003). These phosphorylation events alleviate MDM2 binding to p53, promotes p53 stability, and allows p53 to respond to cellular stress. The response can lead to inhibiting cell proliferation temporarily to allow repair, or arrest them permanently (senescence), or activate the apoptotic pathway. While ATM phosphorylates p53 at Ser15, it also phosphorylates MDM2 at Ser395, which blocks Mdm2-mediated exported

of p53, and is an important part in p53 induction. C-Abl phosphorylates adjacent to this site at Ser394, inhibiting Mdm2's ability to mediate degrade p53 (Meek, 2004). In addition to phosphorylation, dephosphorylation of p53 also occurs in an ATM-dependent manner. Dephosphorylation of p53 at S376 leads to increased interaction between p53 and 14-3-3σ, which promotes p53 binding to DNA (Waterman *et al.*, 1998). When p53 is stabilized through these various mechanisms, it forms a tetramer and is able to promote transcriptional upregulation of its target genes. Interestingly, Mdm2 is a target gene for p53, which creates a feedback loop where Mdm2 negatively regulates p53 at the transcriptional level, while p53 upregulates Mdm2 at the transcriptional level (Wu *et al.*, 1993).

Role of p53 in cell cycle checkpoint

When p53 is activated by DNA damage, p53 transcriptional activity targets genes that can be categorized into cell cycle checkpoint or apoptosis-related genes (Benchimol, 2001). Cell cycle checkpoint genes activated by p53 inhibit entry into S-phase and mitosis, to prevent synthesis of damaged DNA and proliferation of cell with damaged DNA, respectively. In apoptosis, p53 is mainly involved in the mitochondrial-mediated or 'intrinsic' pathway, but also plays a role in the death receptor-mediated 'extrinsic' pathway.

p21

A few of the major p53 targets that regulate cell cycle checkpoint include p21 (WAF1/CIP-1), 14-3-3σ and GADD45 (Harper *et al.*, 1993; Hermeking *et al.*, 1997; Papathanasiou *et al.*, 1991) (Figure 3). p21 is a cyclin-dependent kinase inhibitor (CKI) that binds to Cdk proteins throughout the cell cycle. The various Cdk proteins (Cdk4, Cdk2 and Cdc2) bind to their respective cyclin proteins (Cyclin D, E, A and B) to promote transition through the various cell cycle phases. Therefore, when sensors of cellular stress signal for checkpoint activation, inhibition of Cdk-cyclin complexes are the key events that prevent cells from progressing past the checkpoint. The particular Cdk-cyclin complex that needs to be inhibited is cell cycle phase-specific. For the G1-S phase transition, Cdk4-Cyclin D and Cdk2-

Cyclin E are inhibited, in the S-phase, Cdk2-Cyclin A is inhibited, and for the G2-M transition, Cdc2-Cyclin A and Cdc2-Cyclin B are inhibited (He *et al.*, 2005)**.** Binding of p21 to the various Cdk proteins inhibits the Cdk-cyclin kinase activity from functioning in their respective cell cycle transitions in the presence of DNA damage or cellular stress. Which checkpoint becomes activated depends not simply on the phase at which damage occurs, but also depends on the type of damage. In the case of DNA damage, different checkpoints or Cdk-Cyclins are inhibited depending on the specific DNA lesion (Attardi *et al.*, 2004; Buscemi *et al.*, 2004). For example, cells treated with cisplatin, which causes DNA adducts to form DNA intrastrand crosslink adducts leading to DNA strand breaks, causes a G2 arrest, while it's analog diaminocyclohexane (trans-diacetato)(dichloro) platinum (IV) (DAP) causes a G1 arrest (He *et al.*, 2005; Siddik, 2003). Both p53 and p21 are important for arrest in G1 and G2 following DNA damage (Bunz *et al.*, 1998; Li *et al.*, 1994). Cells treated with ionizing radiation typically arrest in G1 and G2. In the absence of either p53 or p21, cells treated with ionizing radiation failed to remain arrested in G2, and progressed into mitosis (Bunz *et al.*, 1998). The G1 arrest, such as that elicited by DAP, is regulated by p53 through its transcriptional activation of p21, which blocks both Cdk4-CyclinD and Cdk2-Cyclin E kinase activity (Deng *et al.*, 1995; He *et al.*, 2005; Waldman *et al.*, 1995). Therefore, the conclusion here is that p53 is required for p21-mediated inhibition of both Cdk4 and Cdk2 activity to induce G1 arrest. Since checkpoint activation is an important process to regulate to maintain genome stability, there are alternate mechanisms to inhibit Cdks. In addition to p21 inhibition of Cdk2, 14-3-3 σ, another p53 transcriptional targ*et al*so maintain Cdk2 inhibition (Chan *et al.*, 1999; Mailand *et al.*, 2000).

14-3-3σ and Cdc25

Cdk activity is regulated through both activating and inhibitory phosphorylation at different sites of the kinase. The wee1 and Myt1 kinases add inhibitory phosphates on Thr14 and Thr15 on Cdc2 (Booher *et al.*, 1997; Parker *et al.*, 1991). 14-3-3σ is one of seven isoforms of the 14-3-3 family of proteins. 14-3-3σ is a cell cycle regulator and transcriptional target of p53. Under checkpoint conditions, 14-3-3σ inhibits the G2-M transition by binding to and

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maintaining Cdc2-CyclinB in the cytoplasm. However, cells that were treated with ionizing radiation or Adriamycin, to induce DNA damage, arrest temporarily in G2, but continue into mitosis shortly after arrest and the cells displayed mitotic catastrophe. 14-3-3σ WT cells however, remained in G2 arrest, as the Cdc2-CyclinB complex remains in the cytoplasm throughout interphase, until it enters the nucleus upon initiation of mitosis (Chan *et al.*, 1999).

Another mechanism whereby the 14-3-3 family of proteins maintains Cdk inhibition is through binding to Cdc25C. The Cdc25 family of phosphatases, which includes Cdc25A/B/C, removes both of the inhibitory Thr14 and Thr15 phosphates to promote cell cycle progression. Therefore, another mechanism to inhibit Cdk-Cyclin complexes upon cell cycle activation is to inhibit this family of Cdc25 phosphatases from removing the inhibitory phosphate from Cdks. When a checkpoint is turned on, inhibiting Cdc25 involves initial phosphorylation by Chk1 or Chk2. The phosphorylated Cdc25 is recognized by a 14-3-3 protein, which binds to p-Cdc25, and sequesters Cdc25 in the cytoplasm, which doesn't necessarily change the activity of Cdc25, but does effectively abolish activity against Cdks (Meek, 2004). As long as 14-3-3 maintains cytoplasmic sequestration of Cdc25, then this maintains Cdk inhibition and prevents premature entry into mitosis (Benchimol, 2001).

GADD45

Like 14-3-3σ, GADD45 is another p53 transcriptional target that prevents premature entry into mitosis (Benchimol, 2001). Growth arrest DNA damage 45 (GADD45) includes GADD45α (GADD45), GADD45β (MyD118) and GADD45γ (CR6), with differential expression in various organs in humans (Zhang *et al.*, 1999). All three GADD45 isoforms are elevated after DNA damage, where they promote cell cycle arrest, apoptosis and DNA repair (Tamura *et al.*, 2012). GADD45 is a DNA damage-inducible gene whose expression is elevated by p53 and BRCA1 (Fornace *et al.*, 1988; Jin *et al.*, 2000; Kastan *et al.*, 1992). p53 binds directly to the third intron of GADD45 (Hollander *et al.*, 1993). The GADD45 interacting proteins include PCNA, p21, Cdc2-CyclinB, and p38 kinase, amongst others (Tamura *et al.*, 2012). In cell cycle regulation, GADD45 interacts directly with Cdc2 to inhibit the kinase activity of the Cdc2-

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CyclinB complex and inhibit the G2-M transition (Zhan *et al.*, 1999). GADD45 also inhibits the G1-S transition, however the mechanism depends on nuclear co-localization with CRIF1 (CR6-interacting factor 1) and inhibits the kinase activity of Cdk2-CyclinE (Chung *et al.*, 2003). GADD45 expression is elevated during G1, but drops precipitously in S-phae (Tamura *et al.*, 2012). In terms of DNA repair, GADD45α and GADD45β participate in DNA repair through their interaction with PCNA to promote nucleotide excision repair and by promoting recruitment of repair proteins to the damaged sites (Smith *et al.*, 1994; Tamura *et al.*, 2012).

Role of p53 in apoptosis

When the intrinsic apoptosis pathway is activated, p53 increases the transcription of the BH3-only proteins, Puma and Noxa, which in turn inhibit the anti-apoptotic proteins Bcl-2, Bcl-XL and Mcl-1 (Benchimol, 2001). This allows Bax and Bak to permeabilize the outer mitochondrial membrane, and induce efflux of cytochrome C (Figure 4) (Zhang *et al.*, 2013). Cytosolic cytochrome C is a signaling molecule that binds to APAF-1. APAF-1 contains three domains: a CARD domain, a NOD domain and a cytochrome C-binding WD40 repeat domain (Riedl and Salvesen, 2007). Cytochrome C binding to Apaf-1 triggers the formation of the apoptosome, which is a wheel-shaped complex of seven cytochrome C-bound Apaf-1 subunits joined in the center by oligomerization of the CARD domains from the seven Apaf-1 proteins (Riedl and Salvesen, 2007). Formation of the apoptosome begins the recruitment and activation of caspases. Caspases are cysteine-dependent aspartate-specific proteases. Procaspase-9, also known as the initiator caspase, is recruited to and binds the active apoptosome through its own CARD domain in a homotypic interaction (Bratton and Salvesen, 2010). Procaspase-9 then undergoes conformational changes to become active caspase-9. Caspase-9 then cleaves downstream procaspase-3 into active caspase-3. Caspase-3 is the terminal caspase that initiates cellular degradation (LeBlanc and Ashkenazi, 2003; Riedl and Salvesen, 2007).
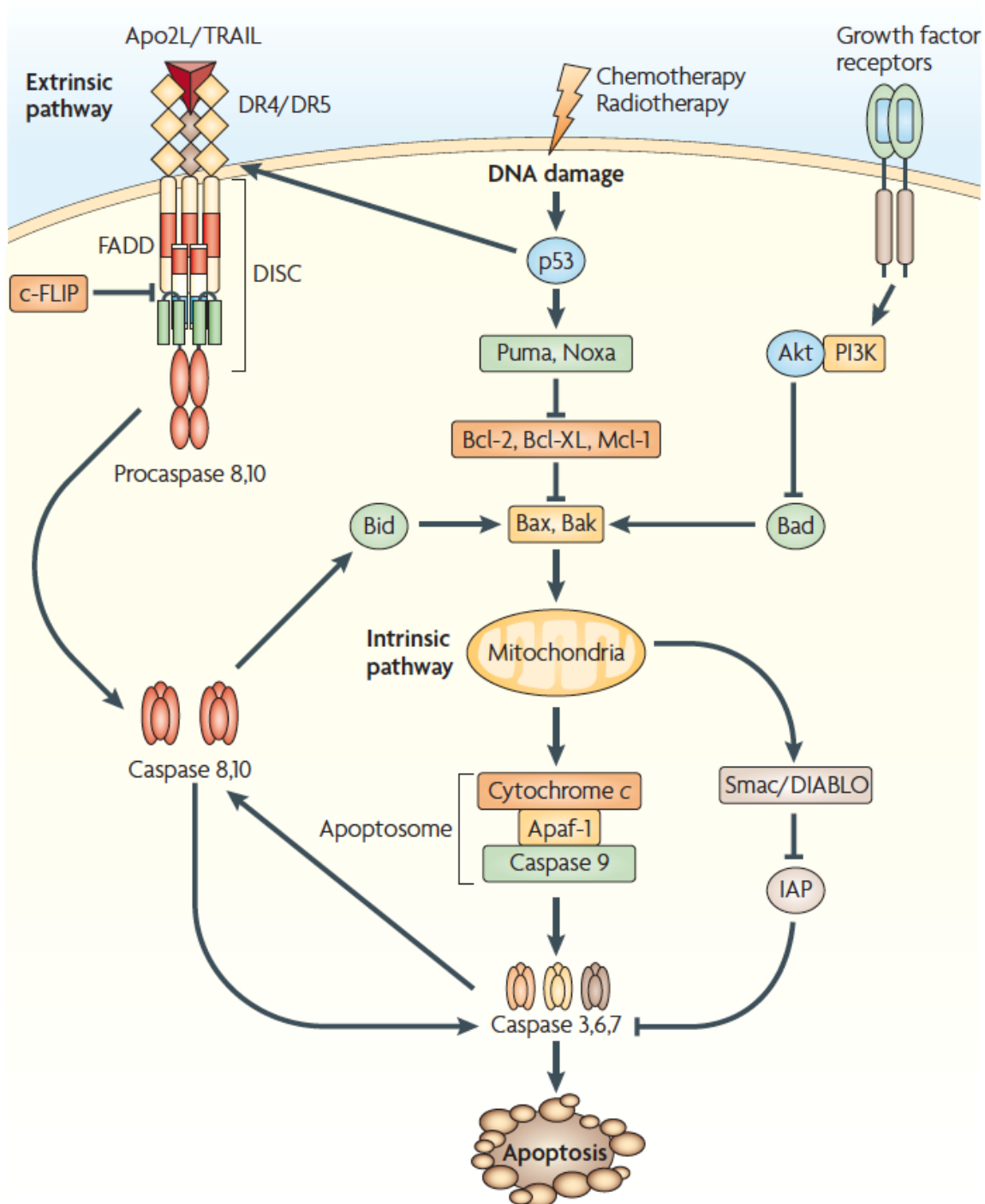
Figure 4. Cellular stress activates the p53 tumor-suppressor protein

p53 initiates the intrinsic pathway by upregulating Puma and Noxa, which in turn activate Bax and Bak. Bax and Bak permeabilize the outer mitochondrial membrane, resulting in efflux of cytochrome c, which binds to the adaptor Apaf 1 to recruit the initiator procaspase 9 into a signalling complex termed the apoptosome. Activated caspase 9 then cleaves and activates the effector caspases 3, 6 and 7 to trigger apoptosis. Cross-talk between the pathways: although the extrinsic and intrinsic pathways can function separately, they often interact. p53 mainly stimulates the intrinsic pathway, but it also upregulates some of the pro-apoptotic receptors such as DR5 and augments extrinsic signaling. *Adapted from Ashkenazi, A., Nat Rev Drug Discov **7**, 1001-1012 (2008) with permission from Nature Publishing Group.*

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In the extrinsic pathway, the mitochondria and cytochrome C signaling is not required. Instead of p53, the apoptotic sensors for the extrinsic pathway reside at the cellular membrane and includes TNF-R1 binding to its ligand TNF-alpha, TRAF-receptor binding TRAF, five receptors (TRAIL-R1-4 and osteoprotegerin) binding TRAIL and FasR binding FasL (Hanahan and Weinberg, 2000; LeBlanc and Ashkenazi, 2003). Transmembrane receptors in the extrinsic pathway activate caspase-8/10. These caspases are able to bypass the mitochondria and directly cleave procaspase-3 to activate caspase-3 and the initiation of cellular degradation through proteolysis (LeBlanc and Ashkenazi, 2003). Even though the extrinsic pathway doesn't require the p53-mediated mitochondrial pathway, p53 however has been shown to upregulate transcription of death receptors such as DR5 (TRAIL-RII) and promote extrinsic signaling (Ashkenazi, 2008). The physiological outcome of apoptosis includes nuclear and DNA fragmentation, chromatin condensation (pyknosis) and cellular blebbing leading to cell shrinkage and apoptotic bodies pinching off from the cell and becoming phagocytosed (Kihlmark *et al.*, 2001; Nagata, 2000; Susin *et al.*, 2000). Within 30-120 min of apoptotic induction, the physiological effects take place, and within 24 hr, the cellular remnants disappear (Wyllie *et al.*, 1980).

Functional roles of BRIT1 in microcephaly and tumor suppression

BRIT1 is a disease gene responsible for microcephaly

The *MCPH1* or *microcephalin* gene, whose transcriptional product is known as MCPH1 or microcephalin, is associated with the neurological disease, microcephaly. Affected individuals have reduced brain and head sizes and are characterized by a head circumference that is >3 standard deviations below the mean of those with the same age and gender (Barkovich *et al.*, 1998; Woods, 2004). Microcephaly is divided into primary and secondary microcephaly. Primary or true microcephaly refers to a statically reduced brain size present in early fetal life and noticeable at birth, where the only neurological disorder is severe mental

deficiency. Secondary microcephaly is characterized by deceleration or degeneration of brain growth caused by neurological disorders occurring in late fetal, perinatal or postnatal stages (Woods, 2004). *MCPH1* is located on chromosome 8p23.1, is organized into 14 exons and codes for an 835 amino acid protein containing three BRCA1 C-terminal (BRCT) domains (Jackson et al., 2002b). To date, *MCPH1* is one of ten genes found to associate with microcephaly (Alsiary *et al.*, 2014).

BRIT1 suppresses telomerase expression

Clues for cellular functions of MCPH1 were initially obtained in a genetic screen in search of genes that repress the expression of human telomerase reverse transcriptase (hTERT), the catalytic subunit of the telomerase enzyme responsible for telomere maintenance (Morin, 1989). Amongst the candidate genes, Lin and Elledge identified a gene that they called *BRIT1* (BRCT repeat inhibitor of *hTERT*) because the sequence contained BRCT domains and inhibited *hTERT* expression (Lin and Elledge, 2003). The *BRIT1* gene was later matched to *MCPH1*, and their names are interchangeable. BRIT1 repression of *hTERT* was later supported by evidence that BRIT1 binds directly to the proximal region of the *hTERT* promoter, with BRIT1 overexpression reducing hTERT promoter activity by nearly 50% compared to empty vector (Shi *et al.*, 2012). This provided the first evidence supporting a tumor suppressor function for BRIT1.

BRIT1 is a DNA damage response protein involved in both cell cycle regulation and DNA repair pathways

BRCT domains are commonly found within DNA damage response proteins, where this domain interacts with phosphorylated sequences to promote protein-protein interactions (Yu *et al.*, 2003). A BRCT domain within BRCA1 interacts with p-BACH1 (Yu *et al.*, 2003), MDC1 with γ-H2AX (Stucki *et al.*, 2005), NBS1 with p-MDC1 (Chapman and Jackson, 2008; Wu *et al.*, 2008) and TopBP1 with p-Rad9 (Lee *et al.*, 2007), to name a few. Within BRIT1, a single N-

terminal BRCT domain spans amino acids 7-83, and two C-terminal domains span amino acids 642-720 and 753-823 (Jackson et al., 2002b). The presence of these BRCT domains suggested potential roles in DNA damage response. Since cell cycle checkpoint and DNA repair are two major pathways activated as part of the DNA damage response, BRIT1 was investigated for roles in both of these pathways.

In response to double strand breaks, the G2/M checkpoint arrests cells in G2 to allow time for repair before progression into mitosis (Lukas *et al.*, 2004). However, when BRIT1 is knocked down, this checkpoint is poorly activated in response to IR, resulting in increased G1 cell population compared to siRNA control-treated cells, as analyzed by flow cytometry. In addition, there was an increase in the apoptotic (sub-G1) cell population, suggesting increased sensitivity to IR after BRIT1 knockdown, which was supported by a decreased colony forming capability after IR (Lin *et al.*, 2005)**.** These results suggest that BRIT1 is important for G2 arrest in response to DNA damage. In search of mechanisms to explain this novel function, it was found that BRCA1 and Chk1 protein expression was reduced in BRIT1 knockdown cells. BRCA1 expression and interaction with BACH1 is required for G2 checkpoint activation, while Chk1 expression and phosphorylation by ATR is also key (Liu *et al.*, 2000; Yu *et al.*, 2003). Reduced BRCA1 and Chk1 expression after BRIT1 knockdown occurred independent of cell cycle changes, since BRIT1 knockdown alone had no significant impact on cell cycle. Additionally, siRNA-resistant BRIT1 was able to rescue BRCA1 and Chk1 protein expression. These data, and data from others, provide evidence that BRIT1 regulation of cell cycle checkpoint in response to DNA damage occurs, in part, through promoting BRCA1 and Chk1 expression (Lin *et al.*, 2005; Xu *et al.*, 2004).

BRIT1 promotes localization of DDR proteins

BRCT-containing proteins in DDR are often phosphorylated by ATM or ATR, and form nuclear foci at DNA damage sites. Since BRIT1 contains BRCT domains, Lin *et al.* tested whether BRIT1 also forms foci. Indeed, BRIT1 co-localized with γ-H2AX at double strand

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breaks and formed foci, which was later shown to occur within just a few minutes post-IR (Lin et al., 2005; Rai et al., 2006; Wood et al., 2007b). Rai *et al.* went on to show that siRNA-mediated BRIT1 knockdown compromised foci formation of early DDR proteins including 53BP1, MDC1 and p-ATM. These observations suggested that BRIT1 is a proximal factor in the DDR pathway, and regulates the recruitment of downstream repair factors.

The mechanism whereby BRIT1 promotes downstream recruitment of repair proteins was shown to involve the chromatin remodeling complex SWI/SNF (SWItch/Sucrose NonFermentable) (Neigeborn and Carlson, 1984; Peng *et al.*, 2009; Stern *et al.*, 1984). SWI/SNF is a complex of *SWI* and *SNF* gene products that together, disrupt histone-DNA interaction through an ATP-dependent mechanism, leading to chromatin relaxation and increased accessibility to DNA (Pazin and Kadonaga, 1997). Peng *et al.* found that a direct interaction between BRIT1 and subunits BAF170 and BAF155 of SWI/SNF mediates recruitment of this complex to chromatin. The BRIT1-BAF170 occurs through ATM-mediated phosphorylation of BAF170 at Ser969. When chromatin relaxation is impaired after BRIT1 knockdown, wild-type BRIT1, but not a SWI/SNF-binding defective mutant of BRIT1, is able to rescue this defect (Peng *et al.*, 2009). The currently accepted model of early DNA damage repair includes BRIT1-mediated recruitment of SWI/SNF to relax the chromatin surrounding DNA damage, to promote efficient localization of repair factors (Lukas *et al.*, 2011) (Figure 5).

BRIT functions explain its roles in microcephaly and maintaining genome stability

BRIT1 regulation of DNA damage-induced cell cycle checkpoint, especially through Chk1 expression, may help explain why BRIT1 is a disease gene for microcephaly. Throughout interphase, Chk1 localizes in the cytoplasm, and can be found at the centrosome, where Chk1 targets Cdc25b phosphatase for proteasomal degradation (Kramer *et al.*, 2004). Low Cdc25b activity maintains the inhibitory phosphate on Cdc2 at Tyr15 to keep the Cdc2/CyclinB complex in an inactive state, unable to serve the function of promoting cellular entry into mitosis (Kramer *et al.*, 2004). Interestingly, not only does BRIT1 regulate Chk1 protein expression levels, but

BRIT1 has also been shown to localize at the centrosome which depends on its N-terminal domain (Rai *et al.*, 2008; Zhong *et al.*, 2006), interact with pericentrin, and recruit Chk1 to the centrosome (Tibelius *et al.*, 2009). N-terminal deletion of BRIT1 abrogates centrosomal localization and directly causes premature chromosome condensation (PCC), a condition where chromosomes condense in interphase, resulting in premature mitotic entry (Trimborn *et al.*, 2004; Wood *et al.*, 2008). When BRIT1 is depleted, or mutant, inhibitory Cdc2 phosphorylation at Tyr15 is low since Chk1 is not recruited to the centrosome. This allows Cdc2/CyclinB to be active in G2, allowing premature entry into mitosis, and premature chromosome condensation (Alderton *et al.*, 2006). Therefore, BRIT1 localization to centrosomes and regulation of Chk1 expression and recruitment to the centrosomes, ultimately aid in preventing PCC, a condition observed in microcephaly.

Additionally, work with BRIT1-/- mice shows that compared to control mice, BRIT1 knockout mice exhibited reduced brain size and less neuroprogenitor cell proliferation (Gruber *et al.*, 2011). Neurospheres from the BRIT1-/- cells exhibited premature mitotic entry, which uncouples mitosis and the centrosome cycle that is important for symmetrical division of daughter nuclei. BRIT1-/- neurospheres were further characterized to determine their response to DNA damage. Zhou *et al.* showed that BRIT1 knockout compromised homologous repair in the neuroprogenitor cells, which increases genomic instability, and increases apoptosis as supported by Gruber *et al.* (Gruber *et al.*, 2011; Zhou *et al.*, 2013). Therefore, increased apoptosis, along with asymmetrical mitotic division both reduce neuroprogenitor cell development and lead to microcephaly.

Several, if not all, BRIT1 functions arguably play a role in tumor suppression. The finding that BRIT1 is a repressor of hTERT expression is one such example (Lin and Elledge, 2003). In normal somatic cells, telomerase activity is suppressed through inhibiting the expression of its catalytic subunit, hTERT. After a certain number of rounds through the cell cycle, differentiated cells reach their Hayflick limit and undergo replicative senescence (Hayflick and Moorhead, 1961). However, in nearly all cancers, hTERT expression and therefore

telomerase activity remain at high levels after differentiation, leading to cellular immortalization and tumorigenesis (Kim *et al.*, 1994). Due to the importance of inhibiting telomerase activity in somatic cells, it's not surprising that several proteins inhibit hTERT expression, including menin (Hashimoto *et al.*, 2008) mad (Oh *et al.*, 2000), and as mentioned, BRIT1, through direct inhibition of the promoter (Lin and Elledge, 2003; Shi *et al.*, 2012). In addition to directly inhibiting the *hTERT* promoter, BRIT1 may also regulate hTERT expression indirectly through its regulation of BRCA1 expression. BRCA1 binds to c-myc at the region of myc-max interaction, therefore inhibiting c-myc transcriptional activity (Wang *et al.*, 1998). One of the transcriptional targets of c-myc is *hTERT* (Wu *et al.*, 1999). Corroborating these observations, BRCA1 binding to c-myc was shown to inhibit hTERT expression (Xiong *et al.*, 2003; Zhou and Liu, 2003). Therefore, the finding that BRIT1 promotes BRCA1 expression not only has implications in cell cycle regulation, but also in hTERT repression. These direct and indirect mechanisms of hTERT repression help BRIT1 maintain genome stability and suppress tumorigenesis.

BRIT1 regulation of the DNA damage response is also a means of maintaining genome stability and promoting tumor suppression. When BRIT1 was found to promote localization of repair proteins to sites of DNA damage, this suggested that BRIT1 deficiency would increase genetic abnormalities. Indeed, BRIT1 knockdown followed by metaphase arrest using colcemid led to increased chromosome breaks and dicentric chromosomes in HMEC, HeLa and other cell types. Chromosome abnormalities were observed in 25% of cells after BRIT1 knockdown, and this number was significantly elevated after treatment with IR (Rai *et al.*, 2006). These results suggest that the BRIT1's function in response to IR-induced DNA damage is crucial to maintaining genome stability. In the following section, we discuss the work of several groups showing that BRIT1 deficiency correlates with tumor progression in various types of cancers.

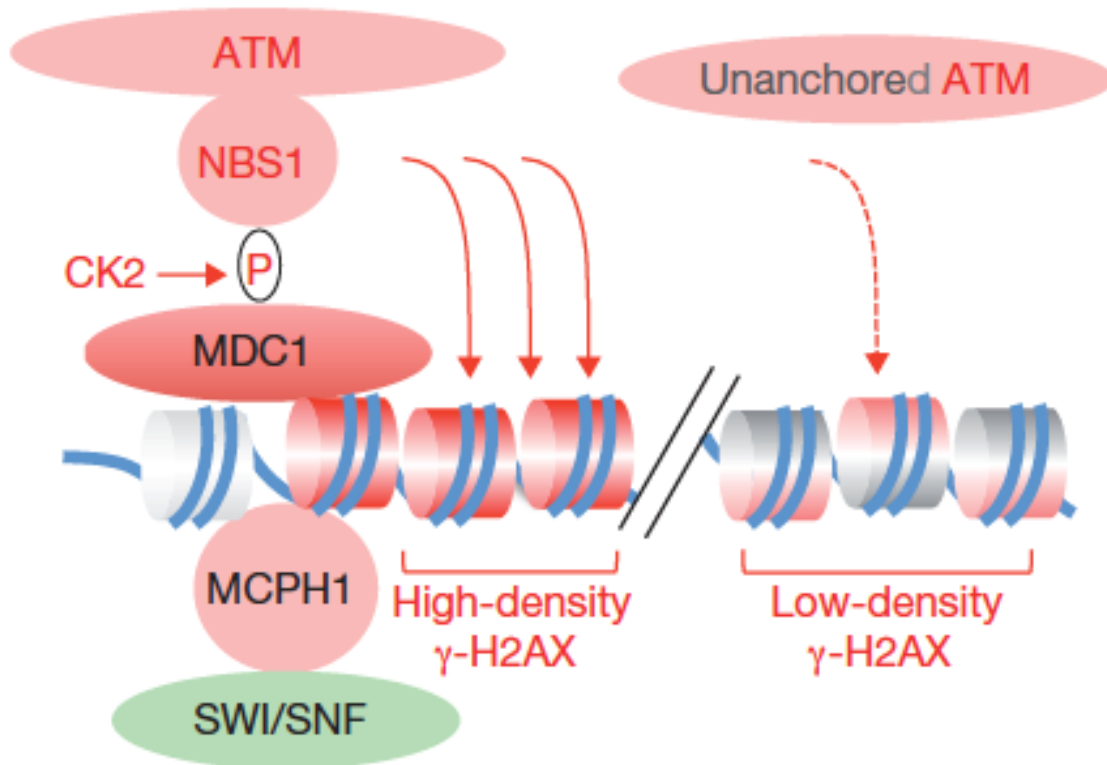**Figure 5. BRIT1/MCPH1 promotes DNA damage response**

BRIT1/MCPH1 interacts directly with subunits of the SWI/SNF chromatin remodeling complex. Recruitment of SWI/SNF relaxes the chromatin around damaged DNA and promotes recruitment of the repair factors shown. *Adapted from Lukas, J., Lukas, C., Bartek, J., Nat Cell Biol* **13**, *1161-1169 (2011) with permission from Nature Publishing Group.*

BRIT1 expression correlates with tumor progression in several cancer types.

Following the observation that BRIT1 deficiency increases genetic abnormalities (Rai *et al.*, 2006), Rai *et al.* sought a correlation between these genetic abnormalities and cancer progression associated with BRIT1 deficiency. They found that 72% of the 54 breast cancer cell lines evaluated had reduced BRIT1 DNA copy number (Rai *et al.*, 2006). And in comparing several cancer cell lines and non-transformed breast cell lines, the cancer cells had significantly lower BRIT1 mRNA and protein expression. The group went on to show that BRIT1 RNA transcript levels correlated with time-to-metastasis in breast cancer. That is, lower BRIT1 levels associated with a shorter time to metastasis. This early link found between BRIT1 expression and breast cancer progression prompted other groups to investigate this relationship.

In a large-scale analysis of the relationship between BRIT1 and breast cancer, Richardson *et al.* performed tissue microarray on 319 breast tumor specimens (Richardson *et al.*, 2011). They found low BRIT1 expression in 29% of tumors, where 35% or less cells staining positive for BRIT1 in a sample indicates reduced expression, and greatest difference in survival. Lower BRIT1 expression correlated with higher tumor grade, where BRIT1 expression in Grade 2 tumors was 61%, compared to 50% in Grade 3 tumors ($P < 0.003$). The study goes on to associate low BRIT1 expression with ER or PR negative and triple negative tumors. This expectedly leads the authors to demonstrate that low BRIT1 expression correlates with decreased survival of ductal breast cancer patients, where mean survival for low BRIT1 expression was 128 months, compared to 146 months ($P < 0.05$) for high BRIT1 expression. These findings suggest that BRIT1 is a predictor of breast cancer survival.

Another group also studied BRIT1 expression in breast cancer specimens, and found a mean 49-fold reduction in BRIT1 RNA expression in these samples compared to normal breast cell lines, explained at least in part by the observed 41% frequency of deletion within the BRIT1 loci of the breast cancer specimens (Bhattacharya *et al.*, 2013). And in a study of 82 breast cancer samples, 43 (52%) of the samples showed low BRIT1 nuclear staining. And from these

samples, high cytoplasmic staining, but not low nuclear staining, associated with high tumor grade (Jo *et al.*, 2013).

In addition to breast cancer, other cancer cell types were analyzed to investigate BRIT1 expression and its association with cancer progression. A recent study in cervical carcinomas shows reduced BRIT1 mRNA (61.3% of cases) and protein (69.8% of cases) expression compared to non-tumorigenic cervical cells (Mai *et al.*, 2014). And amongst the tumor cells, BRIT1 expression inversely correlated with tumor grade (1 vs. 3 $P = 0.013$; 2 vs. 3 $P = 0.047$). Even when BRIT1 is expressed in the tumor cells, immunostaining shows reduced nuclear BRIT1, a phenomenon observed in other tumor types as well (Richardson *et al.*, 2011). The data prompted the group to study the effect of BRIT1 on cervical cell proliferation. They showed that stable overexpression of BRIT1 reduced the proliferation rate in three cervical cancer cell lines (HeLa, SiHa, CaSki), while at least one of these showed reduced capacity for invasion and migration (Mai *et al.*, 2014).

In a study of aberrant BRIT1 expression in human cancer, ovarian cancer specimens were found to have significantly reduced BRIT1 mRNA expression compared to benign ovarian samples (Rai *et al.*, 2006). This study also performed comparative genomic hybridization (CGH) testing of advanced ovarian cancers, and found that 35 of 87 (40%) samples have reduced BRIT1 DNA copy number, which correlated with increased genomic instability. A large-scale study of 294 epithelial ovarian cancer tissue samples support of these findings (Alsiary *et al.*, 2014). Of the 294 samples evaluated in this study, 89 (30%) have low BRIT1 protein expression, and more importantly, 48% of these 89 samples were categorized as Grade 3 tumors. The correlation between low BRIT1 copy number or expression and ovarian cancer found in these studies suggest that BRIT1 may prove to be a useful biomarker for ovarian cancer.

BRIT1 expression was also reduced in prostate specimens. Immuno-histochemical analysis showed progressively lower BRIT1 staining in cancerous prostate tissue compared to normal prostate and benign prostate hypertrophy (Rai *et al.*, 2006).

These studies consistently demonstrate that reduced BRIT1 expression correlates with tumor progression. Based on the well studied role of BRIT1 in maintaining genome stability through promoting DNA repair, it makes sense that BRIT1 deficiency would lead to a compromised repair system, allowing defective DNA to persist. Because reduced BRIT1 expression occurs in several cancer types, and is a cause of defective DNA repair, an intriguing study would be to test whether BRIT1 deficiency sensitizes cancer cells to DNA damaging therapeutics. The rationale being that although genomic instability is a characteristic and promoter of cancer, accumulation of significant DNA damage caused by a defective repair system would trigger the cell death pathway, assuming the pathway is intact. In essence, BRIT1 deficiency may be exploited to increase the DNA damaging potential of chemotherapeutics.

Chapter 2

MATERIALS AND METHODS

Cell culture

Osteosarcoma (U2OS) cells and breast cancer cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The U2OS cells were maintained in McCoy's 5A medium (Cellgro, Manassas, VA, USA) supplemented with 10% fetal bovine serum (FBS). MCF10A cells were maintained in mammary epithelial cell growth medium (Clonetics) containing insulin, hydrocortisone, epidermal growth factor and bovine pituitary extract (Lonza, Basel, Switzerland), supplemented with 5% horse serum. MCF7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Cellgro) supplemented with 15% FBS. Lymphoblastoid control cell line and 2 microcephaly cell lines [MCPH#1 (C74G); MCPH#2 (G321C) (Peng *et al.*, 2009) were grown as a suspension culture in RPMI 1640 medium supplemented with 20% fetal bovine serum. Cells were incubated at 37°C in a humidified incubator with 5% CO_2.

Plasmids and cell culture transfection

The p3xFLAG-CMV vector encoding full-length BRIT1 was previously generated in our lab (Rai et al., 2006). The deletions of BRIT1 were generated from FLAG-BRIT1 plasmids via polymerase chain reaction using primers with restriction sites and subcloned into the N-terminal p3xFLAG-CMV plasmids in frame. MDM2 wild-type and deletion plasmids Δ9 (Iwatsuki et al., 1995), Δ58-89 (Chen et al., 1993), Δ212-296 and Δ295-417 (Lukashchuk and Vousden, 2007) were kindly provided by Dr. Karen Vousden (The Beatson Institute for Cancer Research). The identity of the plasmids was confirmed by sequencing at The University of Texas MD Anderson Cancer Center DNA Core Sequencing Facility. TopBP1 wild-type and deletion plasmids Δ1-Δ8 and ΔAD (Wang et al., 2011) were kindly provided by Dr. Junjie Chen (The University of Texas

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MD Anderson Cancer Center). FLAG-tagged ATM, ATR, ATM-KD (catalytic dead) and ATR-KD plasmids were generously provided by Dr. M. Kastan (St. Jude Children's Research Hospital), Dr. K. Cimprich (Stanford University) and Dr. L. Zou (Harvard University) (Kim et al., 1999). GST-p53 (P3113, #10852) and GST-MDM2 (pGEX-4T, #16237) were purchased from Addgene. Cell culture transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), FuGENE 6 (Roche), and Oligofectamine (Invitrogen) following the manufacturers' protocols.

RNA interference

BRIT1 small interfering RNA (siRNA) sequence, control siRNA and the procedures for BRIT1 transient knockdown were all previously described (Rai *et al.*, 2006). TopBP1 siRNA#1 and #2, and control siRNA were described previously (Kim *et al.*, 2005; Lin *et al.*, 2005). On-target smart pool siRNA against p53 and non-target control siRNA were purchased from Dharmacon Research (Thermo Fisher Scientific, Lafayette, CO, USA). BRIT1 stable knockdown was achieved by RNA interference using a lentiviral vector-based MISSION small hairpin RNA (shRNA) (Sigma-Aldrich, St Louis, MO, USA). Lentiviral particles corresponding to the MISSION shRNA BRIT1-target set were used as well as the MISSION nontarget shRNA control. Specificity and efficacy of the shRNA BRIT1 procedure were controlled by Western blotting after transduction and puromycin selection in MCF10A cells. BRIT1 siRNAs,

Antibodies and Reagents

Rabbit anti-BRIT1 antibody was generated as previously described (Rai *et al.*, 2006). Anti-FLAG M2 affinity gel, anti-FLAG M2 and anti-β-actin were purchased from Sigma-Aldrich. Anti-p53 (DO–1), anti-MDM2 (SMP14) and HRP-conjugated anti-p53 (FL–393) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-TopBP1 and anti-phospho-RPA32 were purchased from Bethyl Laboratories. Anti-H2AX was purchased from Calbiochem. Anti-phospho-H2AX was purchased from Millipore. Anti-RPA (Ab-1) was purchased from

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NeoMarkers. Anti-ubiquitin, anti-phospho-p53 (Ser15), anti-phospho-CHK1 (ser345), anti-phospho-(Ser/Thr) ATM/ATR substrate, anti-ATM, anti-ATR, anti-phospho-histone-H3 (ser10) and anti-GST were purchased from Cell Signaling. Anti-phospho-MCM (BM28) was purchased from BD Transduction Laboratories. Geneticin (G418) was purchased from Roche Applied Science. Puromycin was purchased from Sigma-Aldrich. Cycloheximide (CHX) was obtained from Sigma-Aldrich and used at a concentration of 10 µg/ml. MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucine) was obtained from EMD Biosciences (Billerica, MA, USA) and used at a concentration of 10 µM. Hydroxyurea (HU) was obtained from Sigma-Aldrich and used at a concentration of 1-2 mM. Nocodazole was obtained from Sigma-Aldrich and used at a concentration of 1 µg/mL.

Immunoblotting and immunoprecipitation

Cells were washed in phosphate buffered saline (PBS), and whole cellular extracts were prepared with urea buffer (8 M urea, 50 mM Tris-HCl pH7.4, and 150 mM 2-Mercaptoethanol) or modified radio-immunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 1% NP40, 150 mM NaCl, 1 mM EDTA, 0.25% sodium deoxycholate, freshly added with 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF, 1 µg/mL aprotinin, 1 µg/mL leupeptin, and 1 µg/mL pepstatin) for 30 minutes on ice. Lysates were cleared by centrifugation and proteins were separated by gel electrophoresis. Membranes were blocked in Tris-buffered saline-0.1% Tween-20 (TBST) with 5% (w/v) nonfat, dry milk for one hour at room temperature. Membranes were then incubated with primary antibodies diluted in PBS 5% bovine serum albumin (BSA) for two hours at room temperature. Subsequently, membranes were washed with TBST and incubated with horseradish peroxidase secondary antibody (1:5000) (Sigma-Aldrich) diluted in TBST with 5% nonfat, dry milk. Membranes were washed in TBST and bound antibody was detected by enhanced chemiluminescence (GE Healthcare, Pittsburgh, PA, USA).

For co-immunoprecipitation, U2OS cells were transiently co-transfected with FLAG-BRIT1 wild-type plasmids and MDM2 wild-type/deletions plasmids or with MDM2 wild-type plasmids

and FLAG-BRIT1 wild-type/deletions plasmids. Or, U2OS cells were transiently co-transfected with HA-BRIT1 wild-type plasmids and FLAG-TopBP1 wild-type/deletion plasmids or with TopBP1 wild-type plasmids and FLAG-BRIT1 wild-type/deletion plasmids. Cellular proteins were extracted in RIPA buffer and immunoprecipitated with anti-FLAG M2 affinity gel (Sigma-Aldrich) overnight. Bead-bound immunocomplexes were eluted with 3×FLAG peptide (Sigma-Aldrich) and subjected to SDS-PAGE. For reciprocal immunoprecipitation, the whole cellular extracts were prepared in RIPA buffer and were subjected to incubation with antibody for two hours followed by incubation with Protein A/G PLUS Agarose beads (Santa Cruz Biotechnology) overnight at 4°C. The precipitates were washed three times with RIPA buffer, eluted in 3xloading buffer by boiling at 95°C for five minutes and resolved by SDS-PAGE followed by immunoblotting. For DNA digestion prior to immunoprecipitation, samples were treated with 10U/ml DNase I in 1X reaction buffer (Promega) overnight at 4°C, followed by clarification using centrifugation to remove debris.

Chromatin fractionation

The procedure for chromatin fractionation was performed as previously described (2005 Lin). In short, cells were lysed in 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.34 M sucrose, 10% glycerol, 1 mM dithiothreitol, and 0.1% Triton X-100, and nuclear extracts were lysed in 3 mM EDTA, 0.2 mM EGTA and 1 mM dithiothreitol. Samples were clarified and pellets were resuspended in SDS sample buffer.

In vitro proliferation assay, colony-forming assay and soft agar assay

Standard 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT)-based cell proliferation assay was used to measure cell proliferation. In short, 500 to 1000 cells were plated in 96-well plates and MTT substrate (2 mg/ml) (Sigma-Aldrich) was added to the culture medium. Four hours later, the optical density was measured spectrophotometrically at 570 nm. For the colony-forming assay, 250 to 500 cells were seeded in six-well plates and 0.001%

crystal violet was used to stain the colonies 10 to 14 days later. The forming colonies were counted and analysed. For the soft agar assay, cells were suspended in corresponding medium containing 0.5% low-melting agarose (GenePure LE, ISC BioExpress, Pittsburgh, PA, USA) and 10% FBS. Then cells were seeded onto a coating of 1% low-melting agarose in medium containing 10% FBS. Colonies were scored three to four weeks after preparation. Colonies larger than 0.1 mm in diameter were scored as positive.

Reverse transcriptase-polymerase chain reaction

cDNA was transcribed using SuperScript III RT (Invitrogen) following the manufacturer's instructions. p53 was amplified by polymerase chain reaction using the primers 5'-GCGCACAGAGGAAGAGAATC-3' (Forward) and, 5'-CCTCATTCAGCTCTCGGAAC-3' (Reverse).

Purification of GST-fusion proteins and GST pulldown

BL21 bacteria containing indicated plasmids were allowed to grow for 6 hours after addition of IPTG. Cell pellets were resuspended in lysis buffer and sonicated. The supernatant was incubated with Glutathione Sepharose 4B beads (GE Healthcare) at 4°C overnight. After washing, GST fusion proteins were eluted with glutathione. For pulldown, GST and GST-BRCT5 expressed in BL21 were purified using Glutathione Sepharose 4B beads, and incubated with 293T lysate containing FLAG-BRIT1. The mixture was incubated in 4°C with gentle rotation for 1 hr and the beads were washed 3X with 1X PBS before addition of SDS sample buffer.

In vitro ATM and ATR kinase assay

293T cells were transfected with 8 µg of Flag-tagged ATM, ATR, ATM-KD or ATR-KD plasmids. Cell extracts were prepared in standard lysis buffer for immunoprecipitation (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Tween 20, 0.3% Nonidet P-40, 1 mM sodium fluoride, 1 mM

Na$_3$VO$_4$, 1 mM phenylmethylsulfonyl fluoride, 50 mM glycerophosphate, 1 mM DTT, 1 mM EGTA, 10% glycerol and protease inhibitor mixture from Roche Molecular Biochemicals). Cleared supernatants were immunoprecipitated with anti-Flag M2 antibody (Sigma). After washing with lysis buffer and kinase buffer (20 mM HEPES, pH 7.5, 50 mM NaCl, 10 mM MgCl$_2$, 1 mM dithiothreitol, and 10 mM MnCl$_2$) (Kim et al., 1999) 5 times, the immunoprecipitates was resuspended in 50 μL of kinase buffer containing 10 μCi of [-^{32}P] ATP, 10 μM ATP, 1 mM sodium fluoride, 1 mM Na$_3$VO$_4$, 20 mM glycerophosphate and 1 μg of GST fusion substrate. The kinase reaction was performed at 30℃ for 20 minutes and stopped by the addition of SDS sample buffer, as previously described (Kim et al., 1999). Proteins were separated on SDS PAGE gel and transferred to PVDF membrane. Radiolabeled proteins were visualized by autoradiography.

HU recovery and checkpoint assay

For HU followed by nocodazole, two days after siRNA transfection and rescue transfection, cells were incubated in medium with or without HU (2 mM) for 24 hours. Nocodazole was added to the medium when the HU was removed, and cells were harvested at 0, 8, and 16 hours after release. Harvested cells were fixed in ethanol, stained with propidium iodide, and analyzed by FACS. For HU followed by Taxol, cells were untreated or treated with HU (2 mM) for 24 hours. Cells were then fixed in ethanol and stained using phospho-histone H3 (Ser10)-specific antibody (p-H3) and propidium iodide (PI).

Immunofluorescent staining for foci formation

Cells cultured on coverslips were washed twice in PBS, incubated in cytoskeleton buffer (piperazine-N,N0-bis[2-ethanesulfonic acid], pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1 mM ethylene glycol bis-2-aminoethyl ether-N, N', N'', n'-tetraacetic acid [EGTA], and 0.5% Triton X-100) for 3 minutes on ice. The cells were then washed with ice-cold PBS 3 times and incubated in stripping buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl$_2$, 1%

Tween 20, and 0.25% sodiumdeoxycholate) for 3 minutes on ice. After another 3 washes with ice-cold PBS, cells were fixed with 4% paraformaldehyde at 4°C for 30 minutes, permeabilized in 1% Triton X-100 and 0.5% NP-40 for another 30 minutes, blocked with 1% bovine serum albumin, and incubated with primary antibody for 2 hours and secondary antibody (fluorescein isothiocyanate or rhodamine) for 1 hour. Cells were then stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize nuclear DNA. The coverslips were mounted on glass slides with anti-fade solution and visualized using a fluorescence microscope.

Microscopy

Immunofluorescent images were captured using a microscope (Nikon, Eclipse E800), equipped with a 60X oil objective lens (Plan Fluor, NA 1.3), and a cooled CCD camera (QImaging, Qiclick F-M-12). Images were acquired using NIS-Elements (Nikon). Fluorochrome used include Alexa Fluor 488 and 594 antibody conjugates (Life Technologies) and DAPI.

Tumor growth in nude mice

All animal studies were conducted in compliance with animal protocols approved by the MD Anderson Institutional Animal Care and Use Committee. Before injecting the mice with MCF7 cells, we subcutaneously implanted 6-week-old female nude mice with 0.36 mg of 17β-estradiol 60-day release pellets (SE121; Innovative Research of America, Sarasota, FL, USA). Then mice were injected in the mammary glands with 2×10^6 cells from various cell lines in 100 μl of culture medium. Each cell line was tested in ten mammary glands of five different animals. After two to three weeks, tumors were measured every three to five days. Volume was calculated as $W^2 \times L \times 0.52$.

BrdU incorporation assay

BrdU cell proliferation assay kit was used to detect proliferative activity of cells based on the commercial protocol from Calbiochem. Briefly, cells were transfected with indicated plasmids.

The next day cells were seeded in 96-well plate at 10^4 to 10^6 cells / well. 48 hrs later, BrdU labelling reagent was added and incubated for 2 or 24 hrs. After fixation and denaturation, cells were washed and incubated with anti-BrdU antibody for 1 hour and peroxidase goat anti-mouse IgG horseradish peroxidase conjugate for 30 minutes. Then cells were incubated with the fluorogenic substrate working solution. The horseradish peroxidase catalyzes the conversion of the fluorogenic substrate to a blue fluorescent product. The blue fluorescent product was quantified using a fluorometer.

Apoptosis assay

Apoptotic cells were detected by FITC Annexin V Apoptosis Detection Kit (BD Pharmingen) according to manufacturer's instruction. Briefly, cells were transfected with indicated plasmids. The next day cells were exposed to UV (50 J/m^2). Cells were harvested for apoptosis analysis 72 hrs after UV treatment. Cells in late apoptosis were analyzed, which were both FITC Annexin V and PI positive. Cells were washed with cold PBS and resuspend in 1x binding buffer at a concentration of 10^6 cells. 100 ul of cell suspension was transferred to a 5 ml culture tube and added 5 ul FITC Annexin V and 5 ul PI. Cells were incubated 15 min before flow cytometry analysis.

Ubiquitylation assay

Endogenous p53 or MDM2 are immunoprecipitated with anti-p53 (DO-1) or anti-MDM2 (SMP-14), respectively. General IP protocol was followed to purify p53- and MDM2-antibody conjugates. Samples were run on SDS-PAGE and ubiquitylation was detected using an anti-ubiquitin antibody. For input protein detection, an anti-MDM2 antibody was used, or an HRP-conjugated anti-p53 antibody was used to avoid non-specific detection of antibody heavy-chain.

Data analysis

All data were shown as mean ± standard deviation. Differences among groups was analysed by Student-Newman-Keuls (SNK)-*q* test using SPSS 12.0 for Windows software. Statistical significant was defined as * $P \le 0.05$ and ** $P \le 0.01$. Quantification of protein levels was done using NIH Image J software.

Chapter 3

BRIT1 REGULATES P53 STABILITY AND FUNCTIONS AS A TUMOR SUPPRESSOR IN BREAST CANCER

RESULTS

BRIT1 Regulation of p53 stability is independent of DNA damage

In response to DNA damage, BRIT1 has been shown to play a role in recruiting chromatin-remodeling complexes to relax chromatin-bound DNA and allow localization of downstream repair proteins to sites of damaged DNA. Although these upstream functions of BRIT1 have been documented, it is currently unclear whether BRIT1 directly promotes downstream processes that follow DNA damage recognition. Cell cycle checkpoint is one such downstream process, which halts cell cycle progression to allow DNA repair to take place. The p53 tumor suppressor protein plays a crucial role in mediating the cell cycle checkpoint through its transcriptional activity. Although deubiquitylating enzymes such as USP7 (HAUSP) and USP42 have been implicated in promoting p53 stability after DNA damage (Hock *et al.*, 2011; Sheng *et al.*, 2006), there is limited knowledge regarding whether DNA damage response proteins directly interact with and stabilize p53. To this end, we analyzed p53 stability with respect to BRIT1 expression to see if there is a direct relationship between these two DNA damage response proteins. We used BRIT1 siRNA to knock down BRIT1 in non-transformed breast epithelial MCF10A cells. After knockdown, cells were treated with UV radiation to induce p53 stabilization and activation. Cells were collected at 0, 3 and 6 hours to determine p53 expression post-UV radiation. Western blot shows elevated p53 expression after UV as expected, but lower p53 expression under BRIT1 knockdown conditions for all time points relative to mock and control (non-targeting siRNA) cells (Figure 6A). In addition to UV treatment, we also treated MCF10A cells with IR after BRIT1 knockdown since both UV and IR lead to p53 stabilization (Siliciano *et al.*, 1997). Similar to the results of UV treatment, IR-

induced p53 expression is reduced under BRIT1 knockdown conditions at all time points relative to mock and control cells, suggesting that BRIT1 expression promotes p53 stability (Figure 6B). Since p53 expression is reduced after BRIT1 knockdown even at T=0 after UV and IR treatment, this suggests that the effect of BRIT1 on p53 stability is independent of DNA damage. In addition to transient BRIT1 knockdown, we generated three stable BRIT1 knockdown MCF10A cell lines using BRIT1 shRNAs and lentiviral particles for infection. Each of these three cell lines were treated with UV or IR and collected at 0, 3 and 6 hours post-treatment. As in the transient BRIT1 knockdown cells, p53 expression was decreased for all time points compared to mock and control (non-targeting shRNA) cells (Figure 7A, B). The observation that BRIT1 regulates basal level p53 expression suggests a tumor suppressor function for BRIT1 that is independent of DNA damage signaling.

To provide further evidence that BRIT1 regulates p53 basal expression, we show that MCF10A cells treated with BRIT1 siRNA exhibit reduced p53 expression in the absence of DNA damage induction (Figure 8A), and this phenomena is also observed in MCF10A cells with stable BRIT1 knockdown (Figure 8B). The decrease in p53 expression could be due to decreased protein stability or reduced mRNA expression. To determine whether BRIT1 affects p53 mRNA expression, we performed reverse transcription-polymerase chain reaction (RT-PCR) in the three MCF10A cell lines with stable BRIT1 knockdown using a p53 primer set. Results show that p53 mRNA levels remain stable in all three stable knockdown cell lines (Figure 8C). In addition, we transiently knock down BRIT1 in U2OS, MCF10A and MCF7 cells and performed quantitative PCR (qPCR) to see whether BRIT1 knockdown affects p53 mRNA expression in any of the cell lines used in this study. We found that BRIT1 knockdown does not significantly alter p53 mRNA expression in any of the cell lines (Figure 8D).

Since transient and stable BRIT1 knockdown decreases p53 expression as we have shown, we suspected that BRIT1 overexpression would promote p53 stability. To investigate this, we generated a U2OS cell line that stably expresses FLAG-BRIT1 under G418 selection.

As expected, Western blot shows that p53 protein expression is higher in the FLAG-BRIT1 overexpression cells, compared to U2OS cells with 3xFLAG overexpression (Figure 9).

We have ruled out the possibility that BRIT1 affects p53 at the mRNA expression level (Figure 8C, D), and next determined whether BRIT1 affected the protein turnover rate of p53. We compared p53 turnover rate between three MCF10A cell lines with stable BRIT1 knockdown versus control MCF10A cells expressing non-targeting shRNA. Cells were treated with cycloheximide (CHX) over 120 minutes to inhibit *de novo* protein synthesis. Western blots show that BRIT1 deficiency lowered p53 half-life from 105 to <35 min (Figure 10A). Corollary to this, U2OS cells with stable BRIT1 overexpression exhibited an increase in p53 half-life from 30 to 75 minutes, compared to control (Figure 10B). These results suggest that BRIT1 regulates p53 by stabilizing p53 protein expression.

A.
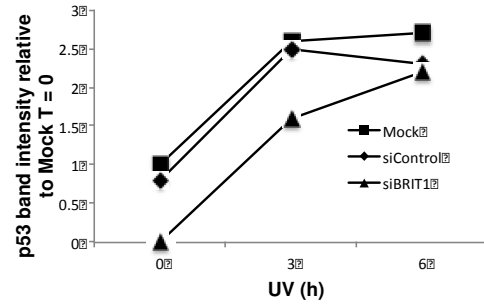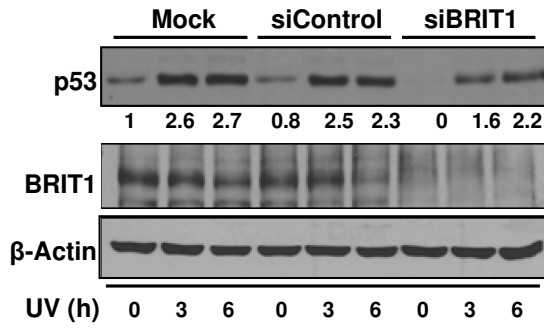
B.
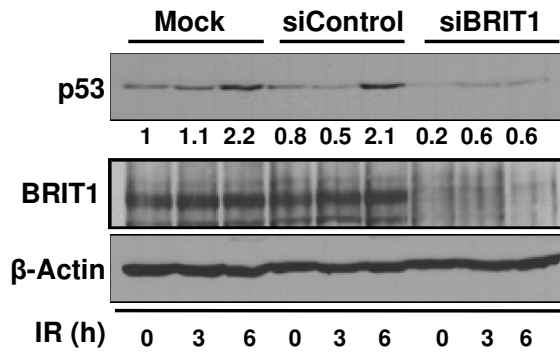
Figure 6. Transient BRIT1 knockdown decreases p53 expression after UV and IR.

(A) MCF10A cells were transiently transfected with mock, control or BRIT1 siRNA and treated with ultraviolet radiation (UV; 50 J/m^2). (B) MCF10A cells transfected as in (A), but treated with γ radiation (IR; 10 Gy). Cell lysates were collected at times 0, 3 and 6 hours after treatment and analyzed by immunoblotting. Band intensity was quantified using Image-J software and graphed. Note that BRIT1 deficiency reduces p53 expression at the basal level (T = 0 hr), as well as after DNA damage.

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A.
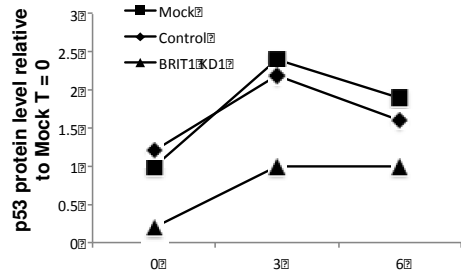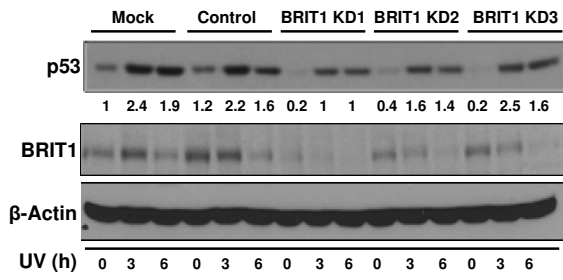
B.

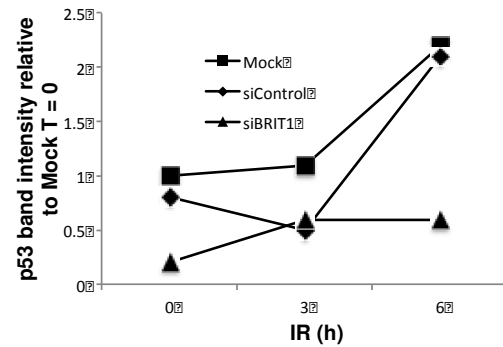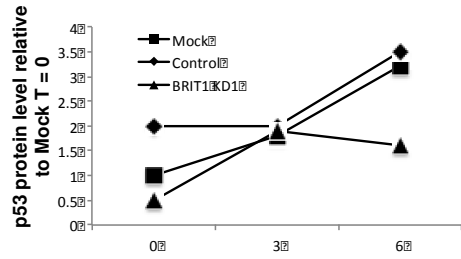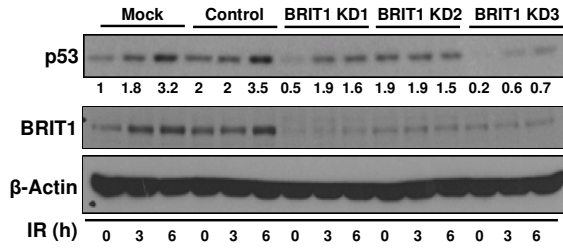**Figure 7. Stable BRIT1 knockdown decreases p53 expression after UV and IR.**

(A) Mock, control and BRIT1 stable knockdown MCF10A cells (BRIT1 KD1, BRIT1 KD2, and BRIT1 KD3) were treated with ultraviolet (UV; 50 J/m^2). (B) The same cells used in (A) were treated with γ radiation (IR; 10Gy). Cell lysates were collected at times 0, 3 and 6 hours after treatment and analyzed by immunoblotting. Band intensity on the Western blot was quantified using Image-J software. A representative knockdown (BRIT1 KD1) was plotted against mock and control conditions. As in the transient BRIT1 knockdown experiment (Figure 1), BRIT1 deficiency after stable knockdown reduced p53 expression at both the basal level and after DNA damage.
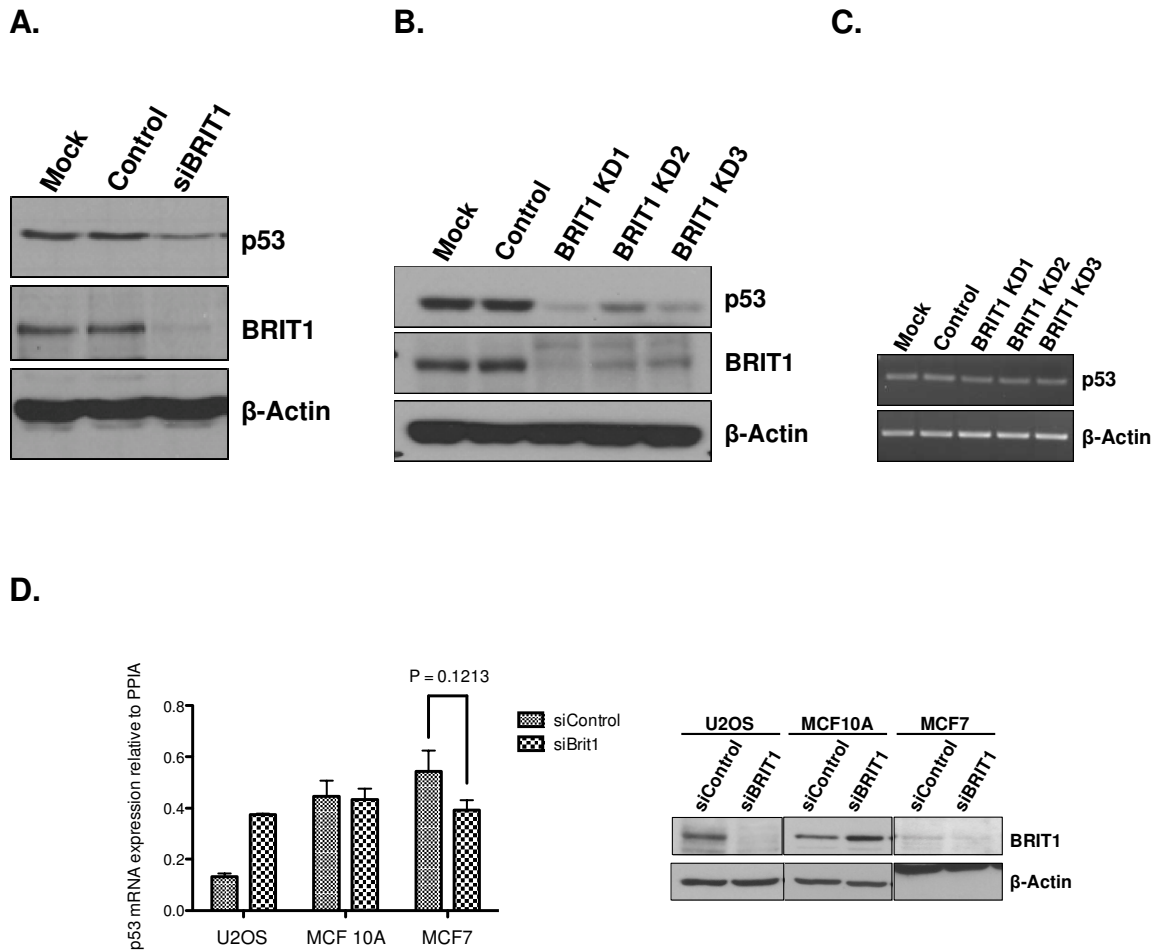
Figure 8. BRIT1 deficiency reduces basal p53 protein expression.

(A) MCF10A cells were transiently transfected with mock, control or BRIT1 siRNA. 48 hours post-transfection, cell lysates were collected for immunoblotting. (B) Mock, control and BRIT1 stable knockdown (BRIT1 KD1-3) MCF10A cell lysates were harvested for immunoblotting. Note that BRIT1 deficiency in both (A) and (B) reduced basal p53 protein expression. (C) Mock, control and BRIT1 KD1-3 MCF10A cells were collected for semi-quantitative PCR to compare p53 mRNA expression. Note that p53 mRNA expression levels are similar between all samples. (D) U2OS, MCF10A and MCF7 cells were transfected with control or BRIT1 siRNA. 48 hours post-transfection, cells were collected for Western blotting and quantitative-PCR (q-PCR). Western blotting confirms BRIT1 knockdown, while qPCR result shows that BRIT1 knockdown does not reduce p53 mRNA expression in any of the cell lines.
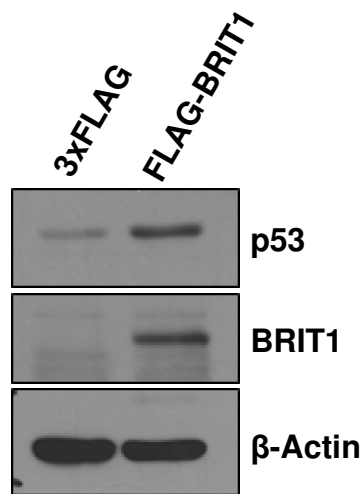
Figure 9. BRIT1 overexpression promotes p53 protein expression.

Lysate from U2OS control and cells with stable BRIT1 overexpression were collected for Western blotting. Note that BRIT1 expression correlates with increased p53 expression.

A.
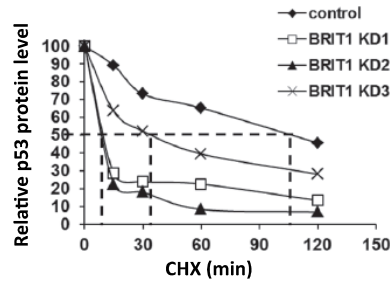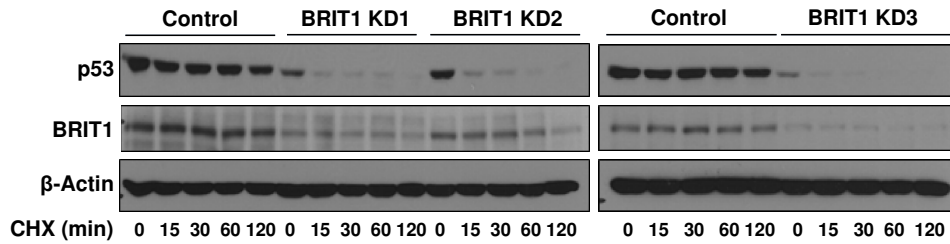
B.
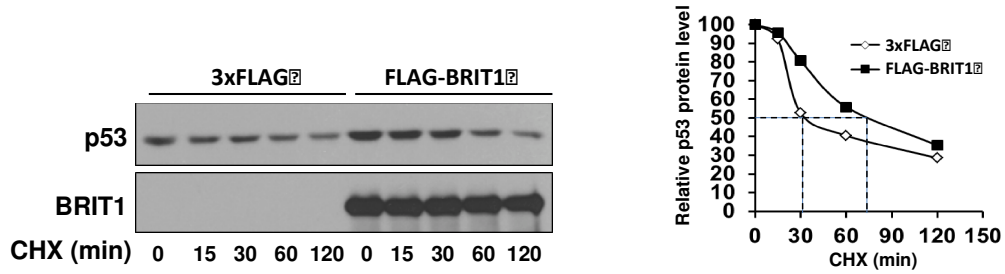
Figure 10. BRIT1 stabilizes p53 protein expression.

(A) Three MCF10A cell lines with stable BRIT1 knockdown or control cells expressing non-targeting shRNA. 48 hours post-transfection, cells were treated with cycloheximide (CHX) and the lysate were collected over the indicated time points. Samples were run on Western blot with the bands quantified using Image-J and plotted on a graph as shown. Note the reduced p53 half-life in cells deficient in BRIT1. (B) Control and stable BRIT1 overexpression U2OS cells treated with CHX and processed as in (A). Note that BRIT1 expression increased the relative half-life of p53.

BRIT1 binds to p53 and MDM2

To understand the mechanism mediating BRIT1 regulation of p53 expression, we conducted cellular binding experiments to test for an interaction between these two proteins. Western blot results show that p53 was co-immunoprecipitated with FLAG-BRIT1 from U2OS cells (Figure 11A). Interestingly, we found that MDM2, a binding partner and regulator of p53 stability, also co-immunoprecipitated with FLAG-BRIT1 (Figure 11B). To show that these interactions occur endogenously, we immunoprecipitated endogenous p53 and MDM2 from MCF10A cells, and found that BRIT1 was pulled down in complex with both p53 (Figure 11C) and MDM2 (Figure 11D). Since p53 and MDM2 are known to have a direct interaction, we needed to determine whether BRIT1 interaction with either of the two proteins depends on both of the proteins, or whether BRIT1 interacts with both proteins independently of each other. *In vitro* binding shows that BRIT1 indeed interacts with both p53 and MDM2 independently of each other (Figure 12).
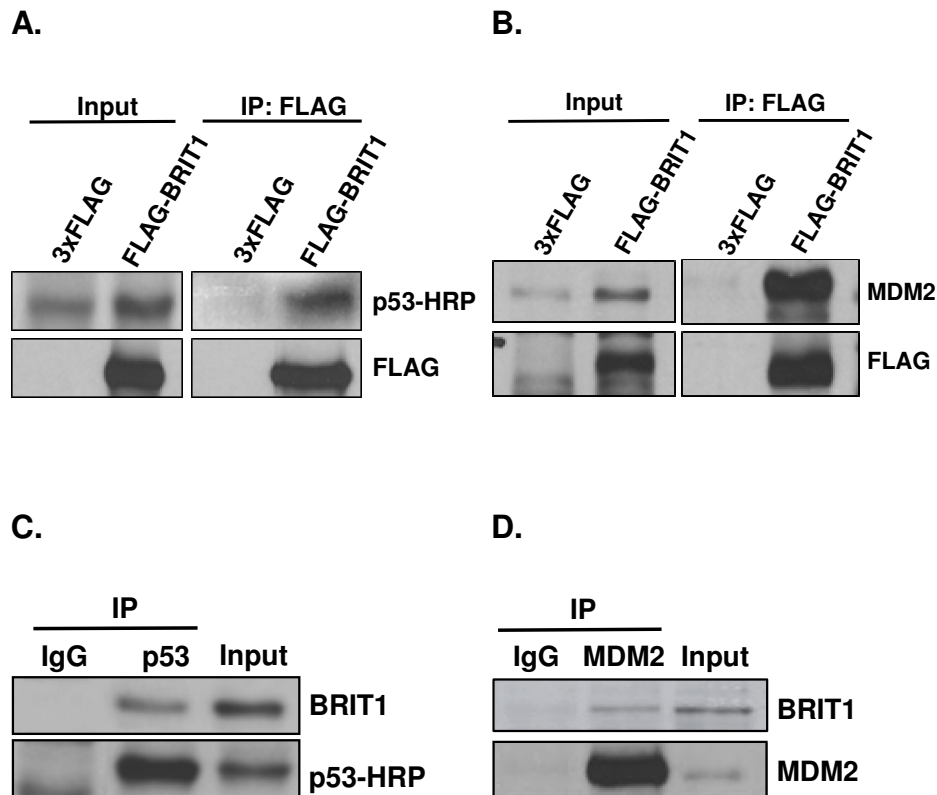
Figure 11. BRIT1 interacts with p53 and MDM2.

(A) and (B) U2OS cells were transfected with 3xFLAG or FLAG-BRIT1 expression vectors. 48 hours post-transfection, cell lysates were collected for immunoprecipitation (IP). 3 mg lysate was incubated with anti-FLAG (M2) affinity gel and analyzed by Western blot using the indicated antibodies. (C) and (D) MCF10A cell lysate was collected for IP and incubated with either anti-p53 (C) or anti-MDM2 (D) along with their respective control IgG antibodies, and analyzed by Western blot. Input was 3% of the amount used for IP. HRP: horseradish peroxidase.
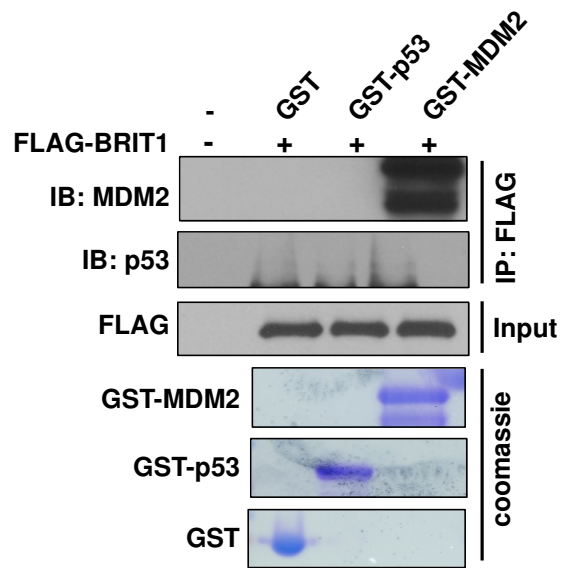
Figure 12. BRIT1 interacts with p53 and MDM2 independently of each other.

FLAG-BRIT1 was purified from 293T cells using anti-FLAG (M2) affinity gel and eluted using 3xFLAG peptide. GST empty vector, GST-p53 and GST-MDM2 were expresssed and purified from BL-21 bacteria cells using glutathione beads and eluted using L-glutathione. FLAG-BRIT1 was incubated with the individual GST-tagged proteins and an anti-FLAG antibody was used for IP, and samples were run on Western blot using the indicated antibodies. Input was run on Western blot (FLAG) or coomassie (GST-tagged proteins). Note that FLAG-BRIT1 binds to both p53 and MDM2, independently of each other.

BRIT1 regulates p53 proteasomal degradation

Since BRIT1 stabilizes p53 expression, we asked whether the direct BRIT1-p53 interaction acts to block MDM2-mediated p53 ubiquitylation and degradation. To answer this question, we first determined whether the decrease in p53 expression upon BRIT1 knockdown, as previously observed, is due to increased p53 proteasomal degradation. When we transiently knocked down BRIT1 in MCF10A cells, we noticed a decrease in p53 expression as expected. However, when these cells were treated with the proteasome inhibitor MG132, we noticed that p53 expression was rescued to levels observed in control cells (Figure 13A). This phenomenon was also observed in MCF10A cells with stable BRIT1 knockdown (Figure 13B). This suggests that BRIT1 deficiency leads to accelerated p53 proteasomal degradation. Since protein ubiquitylation is a prerequisite for proteasomal degradation, we expect that if p53 degradation is dependent on BRIT1, then the ubiquitylation status of p53 is also dependent on BRIT1. Indeed, we found that after MG132 treatment, the level of p53 poly-ubiquitylation was significantly elevated in BRIT1 stable knockdown cells compared to control (Figure 13C). Surprisingly, BRIT1 deficiency not only increased p53 ubiquitylation, but it also decreased MDM2 ubiquitylation (Figure 13D). This suggests that BRIT1 promotes MDM2 ubiquitylation and degradation. Since BRIT1 binds to p53 and MDM2, these results provide a mechanism of p53 stabilization where BRIT1 binding to p53 relieves p53 binding to MDM2, thus preventing MDM2-mediated degradation. Additionally, MDM2 ubiquitylation and degradation also contributes to p53 stabilization. To further show that BRIT1 contributes to MDM2 degradation, we overexpressed BRIT1 to see the effect on MDM2 expression. Expectedly, Western blot shows that BRIT1 expression reduced MDM2 expression, which was rescued by MG132 (Figure 14A). Additionally, we show that BRIT1 deficiency leads to increased pulldown of p53 with MDM2, which further shows that BRIT1 binding to p53 protects p53 from MDM2-mediated degradation (Figure 14B). To better understand the binding dynamics involving BRIT1, p53 and MDM2, we performed an *in vitro* binding experiment to see how the interaction between p53 and MDM2 changes with increasing FLAG-BRIT1 in the reaction. Remarkably, we observed

50

that increasing concentrations of FLAG-BRIT1 decreased MDM2-p53 interaction while increasing FLAG-BRIT1 binding to MDM2 (Figure 14C). These results suggest competitive binding between the three proteins, such that BRIT1 competes with MDM2 for antagonistic effects against p53. Together, these results support a model where BRIT1 binding to p53 and MDM2 causes both reduced MDM2 binding to p53, which leads to reduced p53 ubiquitylation and degradation, as well as increased MDM2 ubiquitylation and degradation (Figure 14D).

A.

B.

C.

D.
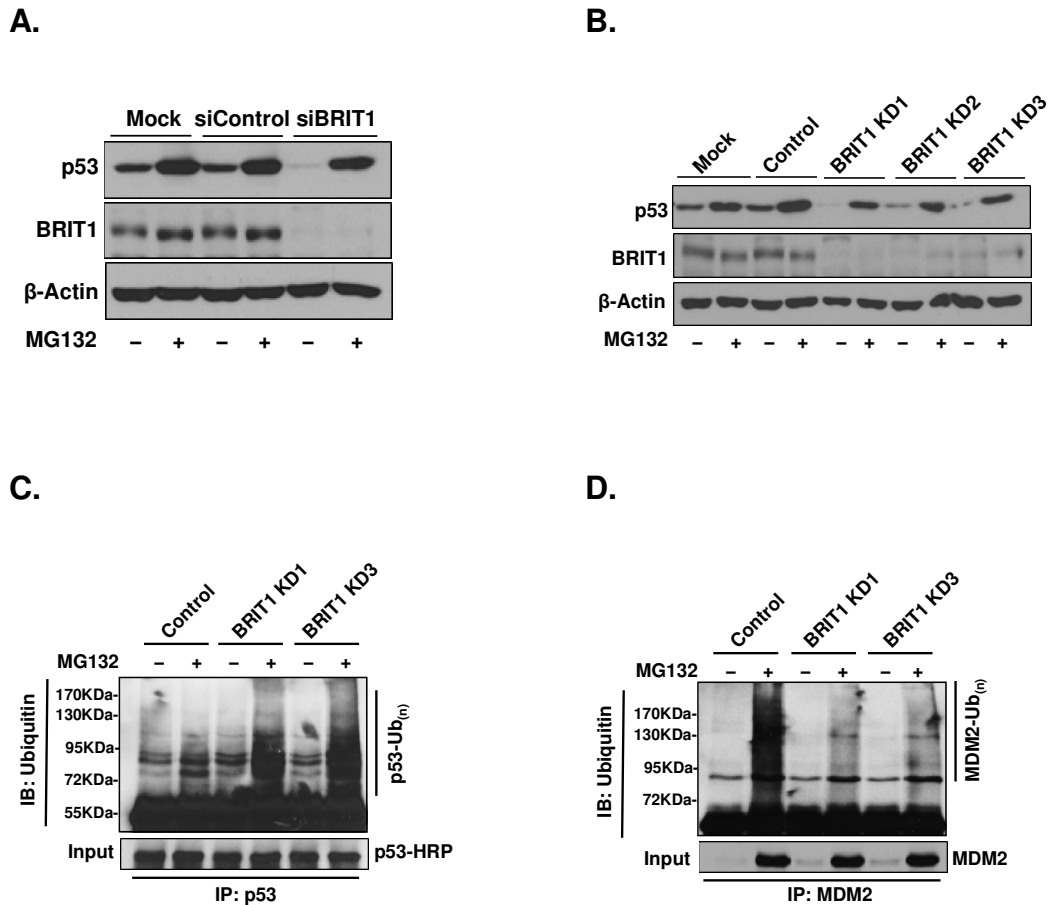
Figure 13. BRIT1 regulates p53 proteasomal degradation.

(A) MCF10A cells were transfected with mock, control or BRIT1 siRNA. 48 hours post-transfection, cells were untreated or treated with 10 µM MG132 for six hours and lysate was collected for immunoblotting. (B) Mock, control and stable BRIT knockdown MCF10A cells were treated as in (A) for immunoblotting. Note that MG132 rescues the reduction of p53 protein expression in samples with BRIT1 deficiency. (C) Control or BRIT1 stable knockdown MCF10A cells (BRIT1 KD1 and BRIT KD3) were untreated or treated with 10 µM MG132 for six hours and then collected for IP using anti-p53 antibody. The IP product was run on Western blot and probed using anti-ubiquitin antibody to detect ubiquitylated p53. p53 in the input was detected using an HRP-conjugated anti-p53 antibody. (D) MCF10A cells treated as in (C), but lysate was immunoprecipitated using anti-Mdm2 antibody and the Western blot was probed with anti-ubiquitin antibody to detect ubiquitylated MDM2. Note that while p53 ubiquitylation is increased in cells with BRIT1 deficiency, MDM2 ubiquitylation is decreased.

A.

MG132	-	-	+
FLAG-BRIT1	-	+	+

MDM2

β-Actin

B.

IP: MDM2

siBRIT1	-	+

p53

BRIT1

MDM2

MDM2   } Input

β-Actin

C.

IP: MDM2

FLAG-BRIT1	-	-	◢		
GST-p53	-	+	+	+	+
GST-MDM2	+	+	+	+	+

IB: BRIT1

IB: p53

IB: MDM2

FLAG-BRIT1]

GST-p53] Input

GST-MDM2]

D.
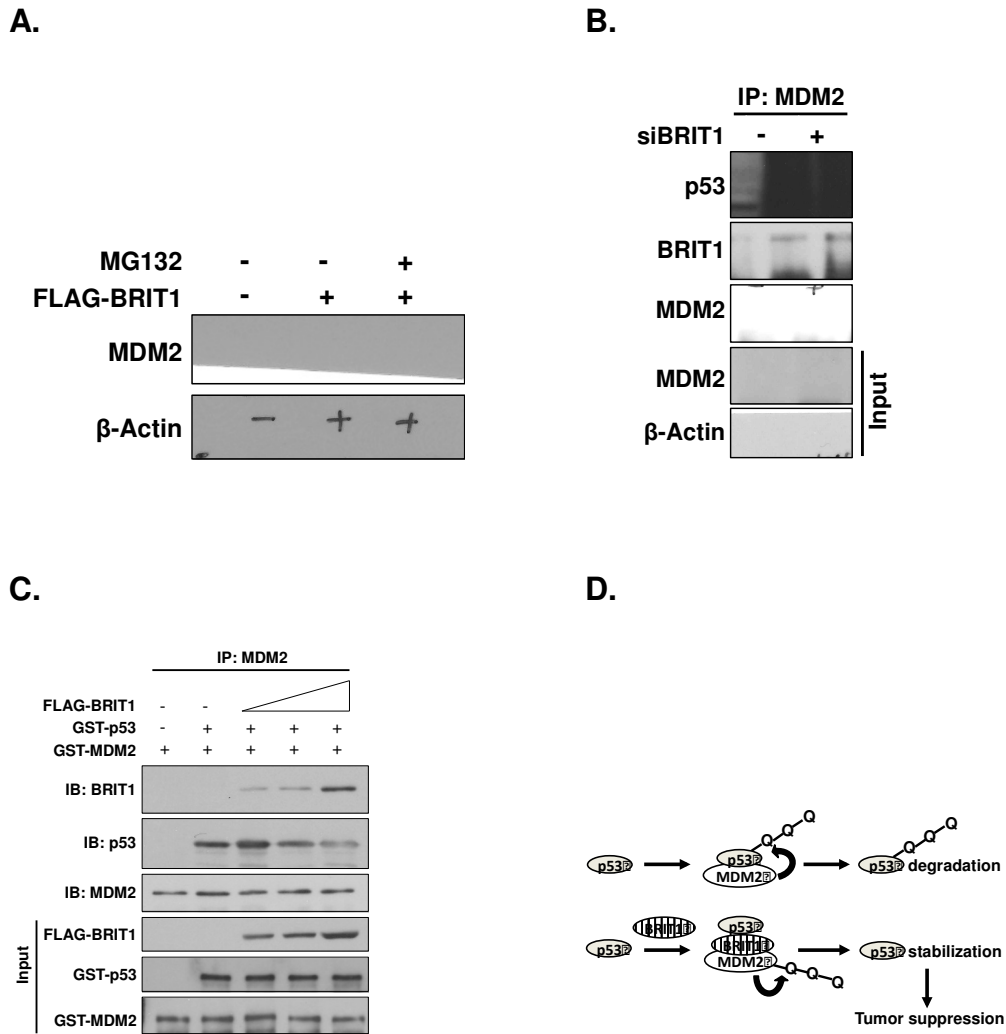
Figure 14. BRIT1 competes with MDM2 to bind and stabilize p53.

(A) U2OS cells were transfected with FLAG vector or FLAG-BRIT1. 48 hours post-transfection, cells were untreated or treated with 10 µM MG132 for six hours, and cell lysate was collected. Immunoblot shows that MDM2 protein expression was reduced in samples with BRIT1 overexpression (lane 2), however this was rescued with MG132 (lane 3). (B) U2OS cells transfected with control or BRIT1 siRNA. 48 hours post-transfection, lysate was collected for IP using anti-MDM2 antibody. Western blot was performed using the antibodies shown. Note that BRIT1 deficiency resulted in increased p53 binding to MDM2. (C) *In vitro* binding experiment. FLAG-BRIT1 was expressed in 293T cells and purified using anti-FLAG (M2) affinity gel, and eluted using 3xFLAG peptide. GST-p53 and GST-MDM2 were expressed in BL21 cells and purified using glutathione beads. Reactions were set up as shown, importantly with increasing FLAG-BRIT1 incubated with GST-p53 and GST-MDM2. Note that the amount of p53 co-immunoprecipitated with MDM2 is inversely related to the amount of BRIT1 binding to MDM2. (D) A proposed model based on data presented so far. p53 is bound to MDM2 and ubiquitylated for proteasomal degradation. BRIT1 serves to protect p53 from degradation, possibly by blocking p53-MDM2 binding, which stabilizes p53 and decrease MDM2 stability.

BRIT1 interaction with MDM2 is required to stabilize p53

p53 stability is largely MDM2-dependent. We have shown that BRIT1 blocks p53-MDM2 interaction through its competitive binding to both p53 and MDM2, however we wanted to clearly show that BRIT1 binding to MDM2 is sufficient for stabilizing p53 expression. To do this, it was necessary to map the binding domain between BRIT1 and MDM2. Our laboratory has previously generated FLAG-BRIT1 constructs consisting of full-length and various deletion constructs, notably missing one or more BRCT domains (Figure 15A). MDM2 constructs were kindly provided by Dr. Karen Vousden (Beatson Institute for Cancer Research) (Figure 15B). To determine the region of BRIT1 required for MDM2-binding, we co-overexpressed full-length MDM2 with wild-type or deletion mutants of FLAG-BRIT1 into U2OS cells. The co-immunoprecipitation result shows that deletion of either the BRCT2 ('BRIT1Δ2') or BRCT3 ('BRIT1Δ3') domain of FLAG-BRIT1 significantly reduces C-terminal deletions of FLAG-BRIT1, while deletion of the entire C-terminus containing BRCT2 and BRCT3 ('BRIT1Δ2,3') results in nearly complete loss of binding to MDM2 (Figure 15C), suggesting that BRIT1 C-terminus is required for binding MDM2. Conversely, to determine the region of MDM2 required to bind BRIT1, we co-expressed full-length BRIT1 with MDM2 wild-type or deletion constructs into U2OS cells. The co-immunoprecipitation result shows that the zinc finger domain of MDM2 significantly reduces the binding between BRIT1 and MDM2, suggesting this domain is critical for interaction (Figure 15D). Given the results of these binding experiments, we set out to determine whether the interaction between BRIT1 and MDM2 is sufficient to stabilize p53. If it is sufficient, we expect that loss of the MDM2-binding domain of BRIT1 would lead to decreased p53 protein expression due to increased p53-MDM2 binding and subsequent p53 degradation. To test this, we expressed wild-type and various C-terminal deletion constructs of FLAG-BRIT1 into U2OS cells. Western blot result shows that BRIT1 (WT) stabilizes p53 compared to control, however expression of BRIT1Δ2,3 doesn't increase p53 stability (Figure 16A). Since BRIT1 binds to both p53 and MDM2, we needed to show that the inability of BRIT1Δ2,3 to stabilize p53 is specific to changes in the BRIT1-MDM2 interaction. To do this,

we expressed either BRIT1 (WT) or BRIT1Δ2,3 in U2OS and treated the cells with UV to induce p53 expression. We found that under BRIT1 (WT) expression, p53 binding to MDM2 is reduced, and p53 expression is stabilized compared to control. However, under BRIT1Δ2,3 expression, p53 expression is not stabilized to control, likely explained by the observation that the p53-MDM2 interaction is not reduced under BRIT1Δ2,3 expression (Figure 16B). Together, these results suggest that the binding between BRIT1 and MDM2 plays a significant role in reducing the interaction between p53 and MDM2, which leads to stable expression of p53.
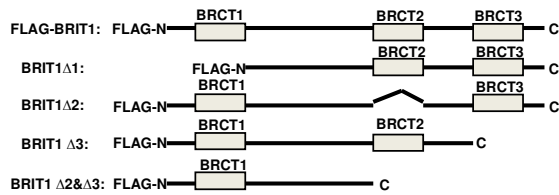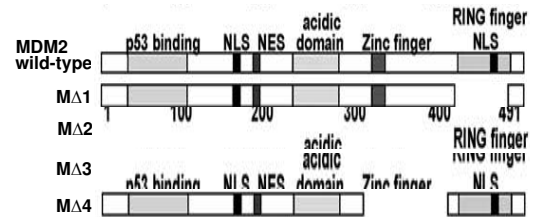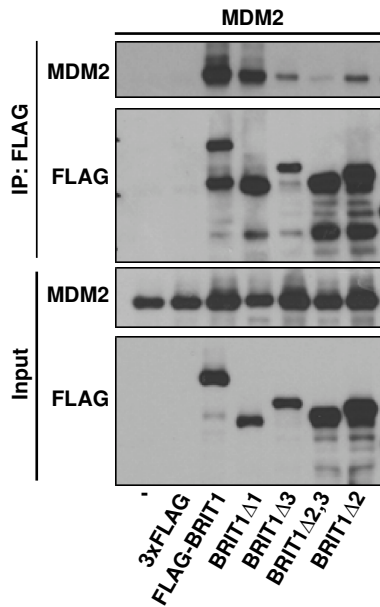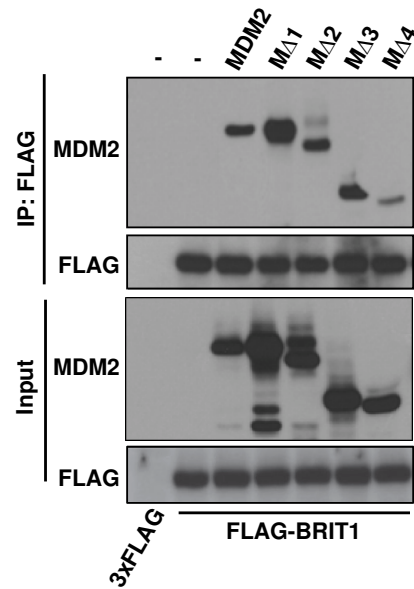
Figure 15. BRIT1 C-terminus and MDM2 zinc finger domains required for BRIT1-MDM2 interaction.

(A) Schematic diagram of the BRIT1 protein and BRIT1 deletions. (B) Schematic diagram of the MDM2 protein and MDM2 deletions. NLS: nucleolar localization signal. NES: nuclear export signal. (C) Mapping the MDM2-binding domain on BRIT1. U2OS cells were transfected with 3xFLAG or the indicated FLAG-BRIT1 constructs. Lysates were collected and immunoprecipitated using anti-FLAG (M2) affinity gel and analyzed by immunoblotting. Note that the C-terminal deletion construct (BRIT1Δ2,3), which lacks the BRCT2 and BRCT3 domains, has significantly reduced binding with MDM2 compared to full-length BRIT1. (D) Mapping the BRIT1-binding domain on MDM2. FLAG-BRIT1 was co-transfected with the various MDM2 constructs shown. Samples were collected and processed as in (C). Note that the MDM2 construct lacking the Zinc finger domain (MΔ4) has significantly reduced binding with BRIT1 compared to full-length MDM2.

A.
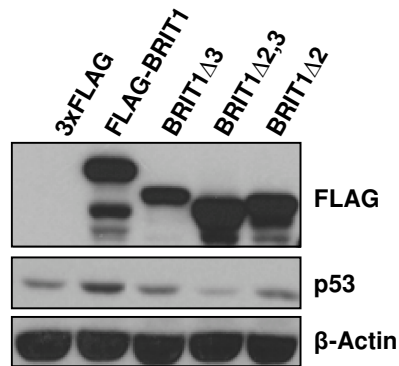
B.
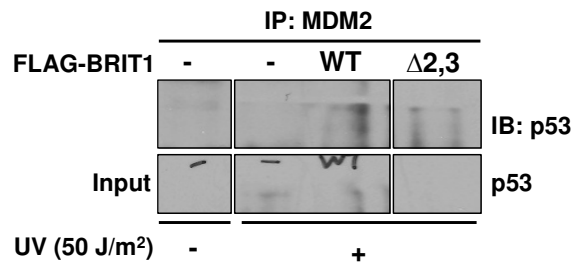
Figure 16. BRIT1 binding to MDM2 directly stabilizes p53.

(A) U2OS cells were transfected with the indicated BRIT1 constructs. 48 hours post-transfection, lysate was collected for immunoblotting. Note that p53 protein expression is lowest in the sample expressing BRIT1Δ2,3, which is the construct with reduced binding to MDM2 (as shown in Figure 10C). (B) U2OS cells were untransfected or transfected with FLAG-BRIT1 (WT or Δ2,3). 48 hours post-transfection, cells were untreated or treated with UV (50 J/m^2). Lysate was collected 5 hours after UV treatment for immunoblotting. Note that FLAG-BRIT1 WT, but not Δ2,3, stabilizes p53 expression compared to control.

BRIT1 functions as a breast tumor suppressor through p53-dependent and independent mechanisms

The role of BRIT1 in promoting DNA damage response categorizes BRIT1 as a tumor suppressor gene. Our study showing that BRIT1 stabilizes p53 provides an alternative mechanism whereby BRIT1 acts as a tumor suppressor protein. Since p53 is a frequently mutated gene in breast cancer, and BRIT1 expression is reduced in a significant number of human breast tumors (Rai *et al.*, 2006), we wanted to test whether BRIT1 has p53-dependent tumor suppressor functions in breast cancer. To establish a system that shows that BRIT1 suppresses breast cancer growth, we used the stable BRIT1-knockdown MCF10A non-transformed breast cancer cell lines used previously in this study. Cell proliferation experiments using MTT show that BRIT1 knockdown leads to higher cell proliferation compared to control MCF10A cells (Figure 17A). Additionally, BRIT1 knockdown cells displayed increased colony formation in soft agar compared to control cells, suggesting that BRIT1 deficiency promotes anchorage-independent growth; a characteristic of cellular transformation (Figure 17B). Consistently, BRIT1 knockdown promoted MCF10A survival as assessed by colony formation assay (Figure 17C). These results suggest that loss of BRIT1 provides a growth advantage to breast cancer cells, supporting our hypothesis that BRIT1 acts as a tumor suppressor in breast cancer.

We next wanted to test whether the BRIT1 tumor suppressor function in breast cancer is dependent on p53 status. We first established a stable overexpression of FLAG-BRIT1 in the transformed MCF7 breast cancer cell line (Figure 18A). Corollary to our observation that BRIT1 deficiency increased MCF10A proliferation (Figure 17A), BRIT1 overexpression in MCF7 cells effectively reduced cell proliferation compared to control (Figure 18B), as well as decreased cell survival (Figure 18C). If BRIT1 reduces cell proliferation through p53 as presumed, then we expect that this reduction is caused by increased cellular apoptosis due to p53 activity. To test this, we compared the level of apoptosis between MCF7 with stable FLAG-BRIT1 compared to

control. As expected, FLAG-BRIT1 expression led to increased levels of apoptosis (Figure 19). To further test for BRIT1's dependency on p53, we next established stable FLAG-BRIT1 overexpression in MCF7 in the presence of stable control of p53 knockdown. FLAG-BRIT1 overexpression in the presence of p53 knockdown in MCF7 was confirmed by Western blotting (Figure 18D). Expression of FLAG-BRIT1 in the presence of p53 knockdown reduced cell proliferation and survival as in MCF7 with normal p53 expression (Figure 18B, C), however the effect was not as significant (Figure 18E, F). These results suggest that BRIT1 suppression of breast cancer cell proliferation and survival is dependent on the expression status of p53.

To provide *in vivo* support of these observations, we first tested for tumor growth abilities of MCF7 control and stable FLAG-BRIT1 overexpression cells in nude mice. As our *in vitro* results would suggest, the tumor volume of FLAG-BRIT1 overexpression MCF7 cells was significantly lower than control MCF7 cells (Figure 19A). When FLAG-BRIT1 was expressed under the stable p53 knockdown condition, the tumor volume in these mice measured smaller than control cells without FLAG-BRIT1 expression (Figure 19B), however this difference is not as significant as the difference in tumor volume for cells without p53 knockdown (control vs. FLAG-BRIT1 expression) (Figure 19A). These *in vivo* results support our *in vitro* results that together suggest that the tumor suppressor abilities of BRIT1 indeed depends on p53 expression. Mechanistically, this can be explained by the observation that BRIT1 binds to MDM2 and relieves p53 from MDM2-mediated degradation, thus stabilizing p53 to function as a suppressor of cancer cell growth.

A.

B.

C.
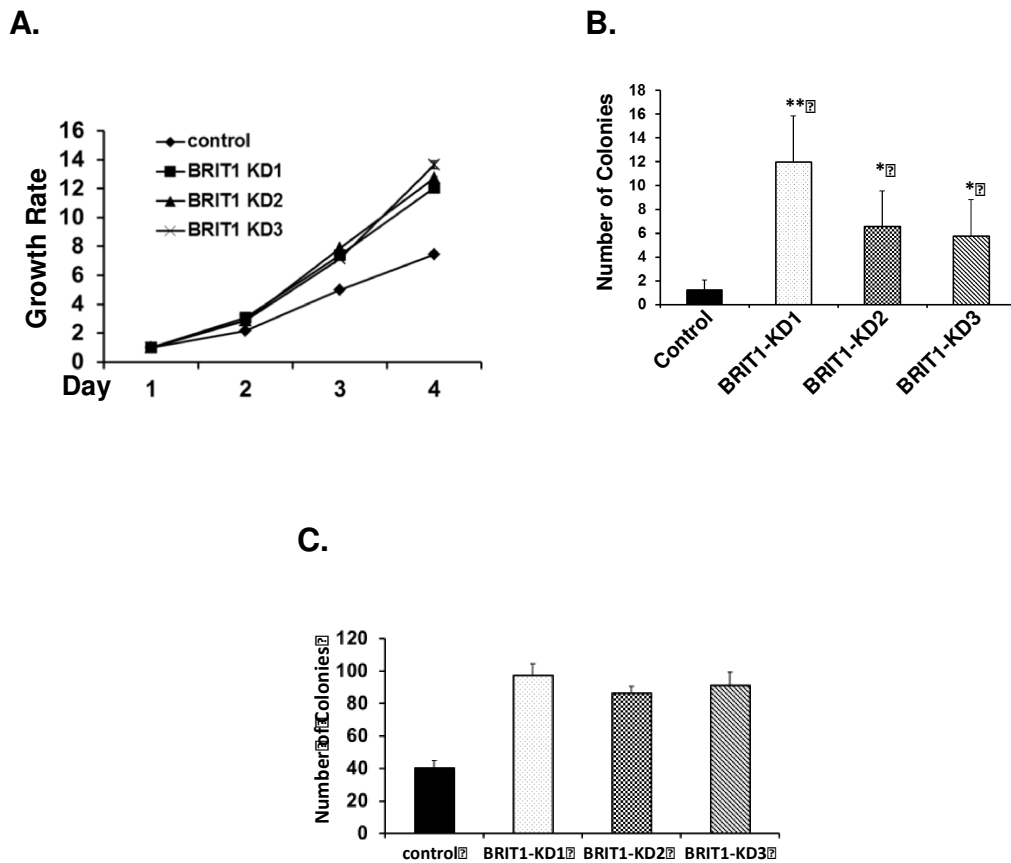
Figure 17. BRIT1 deficiency promotes breast cell proliferation and transformation.

(A) BRIT1 knockdown enhances the proliferation of MCF10A cells. Control or BRIT1 stable knockdown MCF10A cells (BRIT1 KD1, BRIT1 KD2, and BRIT1 KD3) were seeded in a 96-well plate at $1x10^3$ cells/well. Cell proliferation was measured by MTT assay over four days. Note that BRIT1 KD1-3 increased cell proliferation compared to control. (B) BRIT1 knockdown induces anchorage-independent growth of MCF10A cells. Viable colonies were counted 4 days after seeding onto soft agar. (C) BRIT1 knockdown enhances the survival ability of MCF10A cells. Control or BRIT1 stable knockdown MCF10A cells (BRIT1 KD1, BRIT1 KD2, and BRIT1 KD3) were seeded in a six-well plate at 250 cells/well. Viable colonies were stained using crystal violet and counted. *$P \leq 0.05$, **$P \leq 0.01$. Data shown as mean ± SD of three independent experiments.

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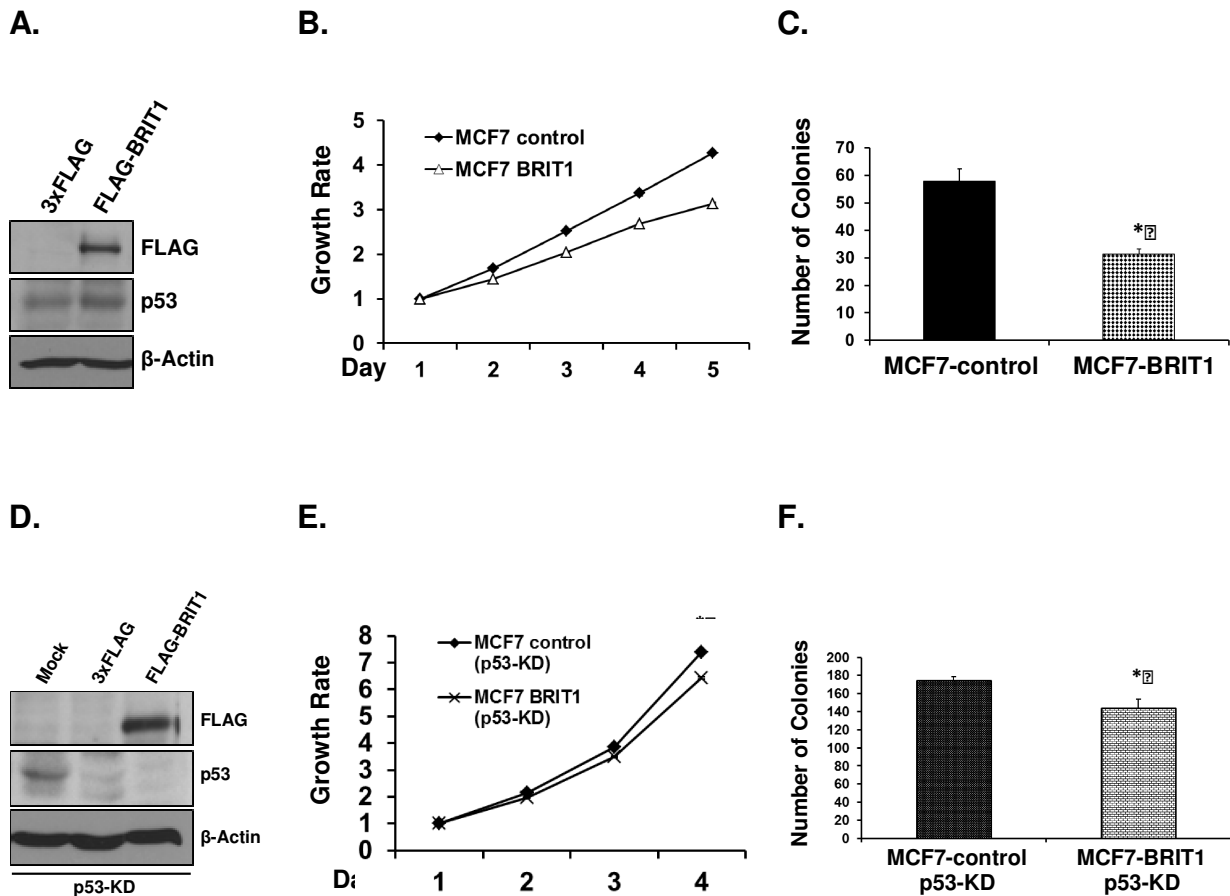
Figure 18. Tumor suppressor function of BRIT1 is dependent on p53.

(A) BRIT1 stable overexpressing MCF7 cells established using FLAG-BRIT1 expression vector and G418 selection. BRIT1 and p53 protein levels were analyzed by Western blotting. (B) BRIT1 overexpression suppresses the proliferation of MCF7 cells. Control or BRIT1 stable-overexpression MCF7 cells were seeded in a 96-well plate at $1x10^3$ cells/well. Cell proliferation was measured by MTT assay for five days. (C) BRIT1 overexpression represses MCF7 cell survival. Control or BRIT1 stable overexpressing MCF7 cells were seeded in a six-well plate at 250 cells/well. Viable colonies were stained by crystal violet and counted. (D) MCF7 p53-knockdown cells were used to establish FLAG-BRIT1 overexpressing cells using G418. Overexpression of BRIT1 was confirmed by Western blotting. (E) MTT assay was performed in control MCF7 p53-knockdown cell and BRIT1 overexpressing MCF7 p53-knockdown cells. Note that although BRIT1 overexpression correlates with reduced proliferation, this was not as significant as BRIT1 overexpression in cells with normal p53 (B). (F) Colony formation assay was performed in control MCF7 p53-knockdown cells and BRIT1 overexpression MCF7 p53-knockdown cells. p53-KD: p53 knockdown. *$P \leq 0.05$. Data shown as mean ± SD of three independent experiments.
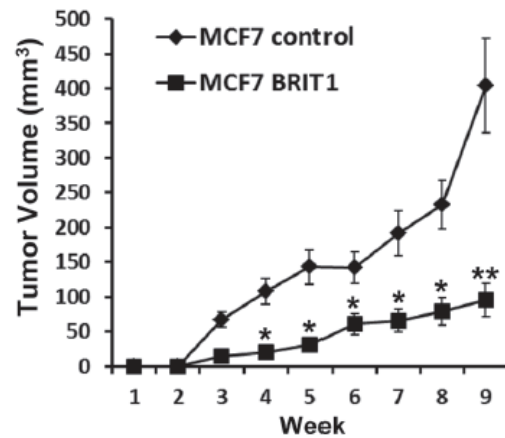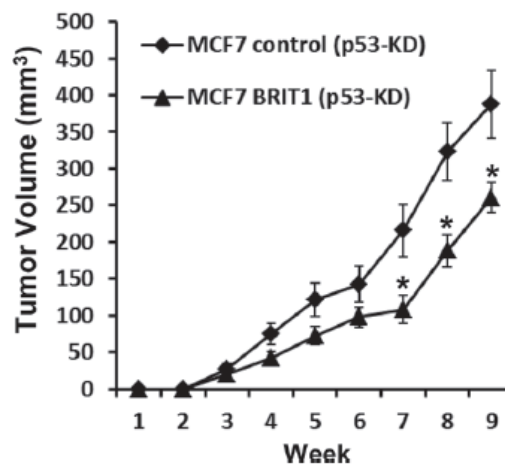
Figure 19. BRIT1 reduction of breast tumor volume is p53-dependent.

(A) BRIT1-overexpressing or vector control MCF7 cells with wild-type p53 were injected into the fat pads of nude mice and tumor volumes were measured over the time-course indicated. (B) BRIT1-overexpressing or vector control MCF7 cells with p53-knockdown were injected into the fat pads of nude mice and tumor volumes were measured over the time-course indicated. p53-KD: p53 knockdown. * $P \leq 0.05$, ** $P \leq 0.01$. Each cell line was tested in 10 mammary glands of $N = 5$ mice.

DISCUSSION

p53 stability is tightly regulated by the MDM2 ubiquitin E3 ligase, and is required for the timely induction of apoptosis, and so is relevant to cell death and survival (Manfredi, 2010). MDM2 knockout mice are embryonic lethal because of deregulated p53-mediated apoptosis (Jones *et al.*, 1995; Montes de Oca Luna *et al.*, 1995), while p53 knockout mice survive, possibility due to p53-independent mechanisms of DNA repair (Frenkel *et al.*, 1999), however these alternative mechanisms are not adequate for normal physiological development, as p53 knockout female mice may develop exencephaly, a condition where neural developmental defects lead to outgrowth of the brain (Armstrong *et al.*, 1995). Also, even though p53 knockout mice may remain viable after birth, they have a high incidence of tumor formation, with p53-/- mice developing tumors earlier than p53-/+ (Donehower, 1996; Donehower and Lozano, 2009; Yamamoto *et al.*, 2000). These findings demonstrate the importance of both p53 regulation by MDM2, as well as understanding the mechanisms that limit MDM2-mediated degradation of p53.

In previous studies from our lab, we have shown that BRIT1 forms foci in response to DNA damage, and that this is intimately connected to other proteins that form IR-induced foci upon DNA damage, as their ability to form foci depends on BRIT1 (Rai *et al.*, 2006). This is one of the tumor suppressor functions of BRIT1, which promotes genome stability by regulating DNA damage detection, cell cycle checkpoint activation and repair. In this current study, we show that BRIT1 regulates the stability of p53. One of the major tumor suppressor functions of p53 is in relaying the apoptotic signal from sensor proteins to effector proteins involved in mitochondria-mediated apoptotic pathway (Erster *et al.*, 2004). Our findings suggest that BRIT1 not only maintains genome stability through its role in DNA damage repair via promoting protein recruitment, but also through its ability to regulate p53 protein expression and thus the induction of apoptosis.

Regulation of p53 and MDM2 stability

Our early experiments showed that the positive regulation of p53 stability provided by BRIT1 occurs at the basal level (Figure 6 and 7). That is, even though we saw reduced p53 expression under UV and IR treatment when BRIT1 is deficient, the reduction was evident even without induction of DNA damage. This suggests that a basal level of p53 may be protected from MDM2 partly through BRIT1. We further show that BRIT1 promotes p53 stability by performing cycloheximide blocking experiments (Figure 10). An alternative method to determine protein half-life is the pulse-chase method. In short, radioactive ^{35}S-labeled methionine or cysteine is supplemented to media during the 'pulse' phase, to allow the protein-of-interest to become radio-labeled. During the wash or 'chase' phase, the radioactive media is replaced with media containing an excess of non-radioactive molecules. Cells are harvested at various time points and the protein-of-interest is immunoprecipitated and subjected to SDS-PAGE. The half-life of the protein corresponds to the time required for the protein concentration to be reduced to half the concentration at the start of the 'chase' phase.

When DNA damage occurs, p53 activation occurs in phases, where early activation promotes expression of cell cycle-related genes such as p21, 14-3-3σ and GADD45, followed by late expression of apoptotic genes such as Noxa, Bax and Puma (Zhang *et al.*, 2011). Since p53 is processed for degradation under normal conditions, it seems likely that there would be some mechanism to protect a basal level of p53 from degradation. This basal level of p53 would provide a target for DNA damage-activated ATM and ATR, leading to phosphorylated and activated p53, and a timely response to DNA damage in the form of cell cycle checkpoint activation. Another factor that stabilizes p53 is p300/CBP. The P300/CBP acetyltransferase complex acetylates p53, and this is indispensable for p53 activation. This acetylation is promoted by p53 phosphorylation at Thr18 and Ser15/20 after DNA damage to induce p53 activation, but under normal conditions, MDM2 P300/CBP-p53 is in a ternary complex, and acetylation is inhibited by MDM2 (Ito *et al.*, 2001; Kobet *et al.*, 2000; Lee *et al.*, 2010). Like the P300/CBP-p53 interaction, our binding data suggests that the interaction between BRIT1 and p53 also occurs under basal conditions, independent of DNA damage (Figure 11). Also, just as

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has been shown that overexpression of p300/CBP stabilizes p53 (Ito *et al.*, 2001), we have also shown that BRIT1 overexpression stabilizes p53 in the absence of DNA damage (Figure 9). In search of a mechanism whereby BRIT1 stabilizes p53, we studied the effect of BRIT1 on p53 and MDM2 ubiquitylation. Like many E3 ligases, MDM2 undergoes autoubiquitylation (Fang *et al.*, 2000). Although it was originally thought that MDM2 autoubiquitylation promotes autodegradation, it has recently been shown that this may not be the case, but rather, autoubiquitylation may actually activate the E3 ligase activity of MDM2, and promote p53 targeting (Itahana *et al.*, 2007; Ranaweera and Yang, 2013). This is proposed to occur through recruitment of E3 conjugating enzyme to the poly-ubiquitin chain on MDM2. Our data shows that BRIT1 deficiency leads to increased p53 polyubiquitylation, suggesting BRIT1 stabilizes p53. Interestingly however, we also observed that BRIT1 deficiency decreased MDM2 polyubiquitylation (Figure 13). Based on what has been learned regarding MDM2 ubiquitylation, it seems that our findings would suggest that BRIT1 promotes ubiquitylation and activation of MDM2 against p53. However, we believe this is not the case. We show that BRIT1 overexpression actually reduces MDM2 protein expression (Figure 14), which is rescued by MG132, which supports our hypothesis that BRIT1 acts to increase MDM2 ubiquitylation for degradation. Even though MDM2 autoubiquitylation may not lead to autodegradation, BRIT1 may act to promote MDM2 ubiquitylation via other proteins and/or at alternate amino acids or linkages that would lead to MDM2 degradation. This is supported by Itahana *et al.*, who have demonstrated that a deactivating mutation in MDM2 did not increase MDM2 protein expression, compared to wild-type MDM2, and that polyubiquitylation of MDM2 occurred in both wild-type and E3 ligase-dead mutants (Itahana *et al.*, 2007). This strongly suggests that there are other modes of MDM2 regulation that may be affected by BRIT1. As mentioned by Itahana *et al.*, the PCAF acetyltransferase mediates MDM2 ubiquitylation and degradation (Linares *et al.*, 2007). Another mode of MDM2 regulation involves β-TRCP, an F-box/WD40 repeat protein involved in cell cycle regulation. Inuzuka et al. showed that β-TRCP mediates ubiquitylation and degradation of MDM2 (Inuzuka et al., 2010). Therefore, BRIT1 may promote MDM2

Regardless of the effect that BRIT1 has on MDM2 stability, our results demonstrate that BRIT1 reduces the interaction between p53 and MDM2 and stabilizes p53 protein expression. Figure 16A shows that loss of the BRCT2,3 domains of BRIT1 leads to decreased p53 protein expression. Since Figure 10C shows that the BRCT2,3 domains are required for BRIT1 to interact with MDM2, the result shown in Figure 16A suggests that the reduced p53 protein expression is due to a reduced interaction between BRIT1 and MDM2, allowing MDM2 to interact with and degrade p53. We demonstrate this further by showing that expression of wild-type BRIT1 reduces p53 binding to MDM2, and therefore leads to increased p53 protein expression, whereas expression of the ΔBRCT2,3 BRIT1 construct does not reduce p53 binding to MDM2, nor does it stabilize p53 protein expression (Figure 16B). This data, together with Figure 16A, strongly suggest that the decreased p53 protein expression observed in lane 4 of Figure 16A is due to an insufficiency of the mutant BRIT1 to protect p53 from MDM2-mediated degradation.

Clinical relevance of BRIT1 regulation of p53

Our research shows that BRIT1 has tumor suppressor functions in breast cancer. Previous work from our lab and others show a correlation between BRIT1 expression and breast cancer progression (Bhattacharya *et al.*, 2013; Rai *et al.*, 2006; Richardson *et al.*, 2011). However here, using MCF10A and MCF7 cells, we show that BRIT1 directly affects cell transformation *in vitro* and tumor formation *in vivo*. Specifically, we show that BRIT1 deficiency increases the transformation of MCF10A cells (Figure 17) leading to more rapid proliferation and anchorage independent growth. We also show that BRIT1 overexpression in MCF7 tumor cells *in vivo* decreased tumor growth, and that the degree of growth inhibition is dependent on p53 expression. That is, BRIT1-mediated tumor growth suppression occurs in both p53-dependent and independent manners, though the effect is stronger in tumors with wild-type p53. It is worth noting that this negative effect on tumor growth reveals a previously unknown

tumor suppressor function of BRIT1. That is, BRIT1 is mostly known for maintaining genome stability through its role in DNA damage response. Only when damage occurs, is BRIT1 recruited to the site of DNA damage for repair. However, we show here that BRIT1 maintains genome stability through p53 regulation at the basal level, maintaining p53 expression in the absence of induced DNA damage, as well as after DNA damage. This demonstrates that BRIT1 operates at multiple layers of genome maintenance. BRIT1 appears to activate p53-mediated checkpoint activation by recruiting upstream proteins, including kinases such as ATM and ATR, which in turn promote p53 stability through phosphorylation. Furthermore, BRIT1 may also directly interact with p53 to alleviate MDM2-mediation degradation. Given our finding that BRIT1 mediates its breast tumor suppressor functions partly through p53 stabilization, it prompted us investigate whether there is a clinical correlation between BRIT1 versus p53 expression in breast patient samples. Since disease-related mutations in BRIT1 are rare (Ghani-Kakhki *et al.*, 2012), we only looked for changes in p53 protein (or mRNA) expression relative to decreases in BRIT1 DNA copy number. We downloaded mRNA (RNASeq) and RPPA level 3 data publicly available from the Cancer Genome Atlas Project (TCGA; http://tcga-data.nci.nih.gov/) from patients with breast invasive carcinoma. Putative copy number data as generated by GISTIC algorithm was obtained from cbio portal (http://www.cbioportal.org/public-portal/). Data were categorized by differences in BRIT1 DNA copy number: homozygous deletion, single-copy deletion, diploid and greater than two copy numbers. Overall changes in BRIT1 appears to affect p53 protein expression ($P < 0.01$), however there doesn't appear to be a visible decrease in p53 protein expression relative to loss of BRIT1 DNA copy number from diploid to homozygous deletion (Figure 20A). When only diploid and >Diploid BRIT1 DNA copy numbers were analyzed, p53 protein expression was not significantly altered ($P = 0.2759$), although in the diploid samples, there appears to be a higher percentage with lower p53 protein expression compared to the >Diploid samples, where only 1 out of 38 samples had considerably lower p53 expression (Figure 20A). This observation is consistent with our hypothesis that BRIT1 expression protects p53 from MDM2-mediated degradation. To be sure

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that this trend is limited to p53 protein expression, BRIT1 DNA copy number was plotted against p53 mRNA. Results show that even in the >Diploid BRIT1 copy number condition, there was a significant percentage of samples with low p53 mRNA expression (Figure 20B). This suggests that protection of p53 expression by increased BRIT1 expression is limited to the protein level.

Our *in vivo* studies (Figure 18) demonstrated that BRIT1 overexpression reduces tumor growth, and we figured that this phenomenon occurred in a p53-dependent pathway, since p53 KO reduced the effects on MCF7 tumor growth inhibition. Therefore, we expected that patient data would show that p53 protein expression would decrease in proportion with BRIT1 DNA copy number (Figure 20A). However, this was not readily observed. Perhaps there are other factors to consider in the relationship between BRIT1 and p53. It has been reported that besides MDM2-mediated inactivation of p53, other mechanisms include viral-induced inactivation and degradation, cytoplasmic sequestration, and importantly, mutational inactivation of p53 frequently observed in many cancer types (Davidoff *et al.*, 1991; Gasco *et al.*, 2002; Moll *et al.*, 1992; Pharoah *et al.*, 1999). In these situations, despite BRIT1-mediated stabilization of p53 protein expression, p53 activity may still be compromised. Additionally, it would be interesting to see whether there are differences in BRIT1 regulation of p53 expression relative to the various subtypes of breast cancer.

A.
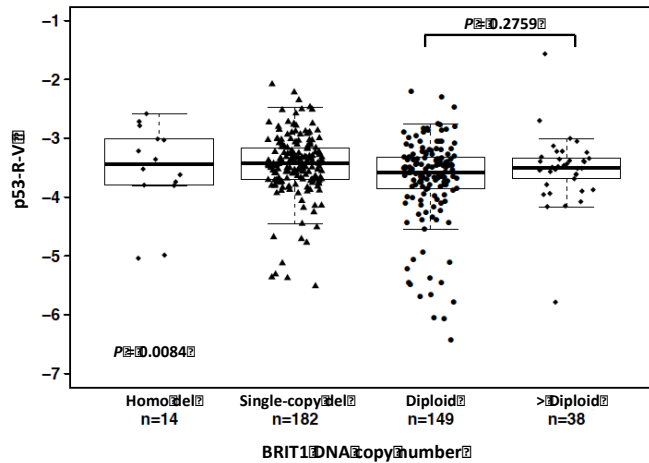
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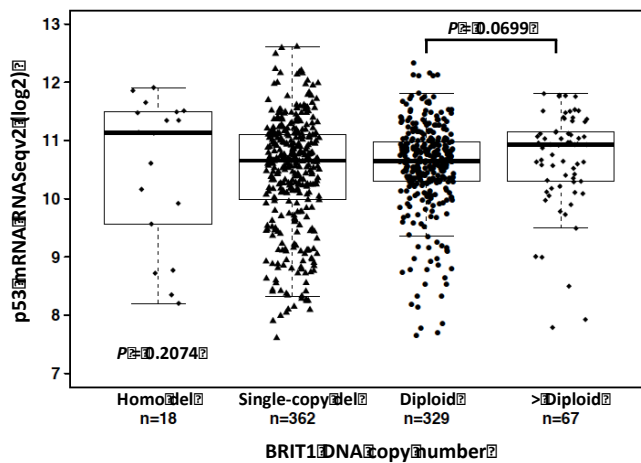
Data analysis and graphs kindly generated by Dr. Cristina Ivan.

Figure 20. BRIT1 DNA copy number compared to p53 protein and mRNA expression in breast cancer specimens.

TCGA data of breast cancer patient specimens were analyzed. The Shapiro-Wilk test was applied and verified that p53 RPPA/mRNA data in different groups according to BRIT1 DNA putative copy number don't follow a normal distribution. Accordingly, Kruskal-Wallis test followed by a Nemenyi post-hoc test, was applied to assess the relationship between p53 RPPA/mRNA and BRIT1 copy number. A box-and-whisker plot (Box plot represents first (lower bound) and third (upper bound) quartiles, whiskers represent 1.5 times the interquartile range) was used to visualize the data (log2). Analyses were carried out in R statistical environment (version 3.0.1) (http:///www.r-project.org/). All tests were two-sided and considered statistical significant at the 0.05 level. (A) p53 RPPA levels in different groups according to BRIT1 DNA putative copy number. *P = 0.0084 is given by the Kruskal-Wallis test and shows that p53 protein expression has different distributions in different groups. However, when the test is applied to the 'Diploid' and '>Diploid' groups, the result is non-significant.* (B) p53 mRNA levels in different groups according to BRIT1 DNA putative copy number. *P = 0.2074 is given by the Kruskal-Wallis test.* R-V, rabbit antibody validated for RPPA.

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Chapter 4

BRIT1 AMPLIFIES ATR SIGNALING BY COORDINATING TOPBP1 RECRUITMENT

RESULTS

BRIT1 associates with TopBP1 in a DNA damage- and stress-dependent manner

Since the identification of BRCT repeats within the BRIT1 sequence, there has been much work describing the role that BRIT1 plays in DNA damage response (Lin and Elledge, 2003; Peng *et al.*, 2009; Rai *et al.*, 2006). The bulk of early evidence supports BRIT1 as an upstream recruiter of DDR proteins to sites of damaged DNA (Rai *et al.*, 2006), however it remains unknown whether BRIT1 plays a role in the downstream processes of DNA damage repair, such as damage signal amplification or activation of transducer proteins involved in repair. To shed light on this matter, a proteomic analysis was performed to identify BRIT1 binding partners (Peng *et al.*, 2009). One of the identified proteins was TopBP1, a known activator of ATR signaling. To confirm this finding, FLAG-BRIT1 was expressed and immunoprecipitated from U2OS cells. Western blot shows that endogenous TopBP1 was pulled down with FLAG-BRIT1 (Figure 21A). Reverse binding was also performed by immunoprecipitating endogenous TopBP1, which pulled down BRIT1. Interesting, more BRIT1 was pulled down when cells are treated with hydroxyurea (HU), suggesting that replication stress promotes the BRIT1 and TopBP1 interaction (Figure 21B). To further confirm replication stress-dependent binding, FLAG-BRIT1 was expressed an immunoprecipitated from U2OS after control or UV treatment. Western blot shows increased TopBP1 pull down in the UV conditions (Figure 21C). To further support BRIT1-TopBP1 interaction, immunofluorescence shows that BRIT1 and TopBP1 co-localize after UV treatment (Figure 21D). Because BRIT1 and TopBP1 are both chromatin interacting proteins, we wanted to test whether the observed interaction was DNA dependent, that is, whether the proteins were co-immunoprecipitated indirectly due to their association with DNA. Immunoprecipitation of TopBP1 was performed

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after cellular lysate was left untreated or treated with DNase I to digest DNA. Results show that similar amounts of BRIT1 were pulled down irrespective of DNase I treatment (Figure 22). These results reveal TopBP1 as a novel binding partner of BRIT1, and that their interaction is replication stress dependent.
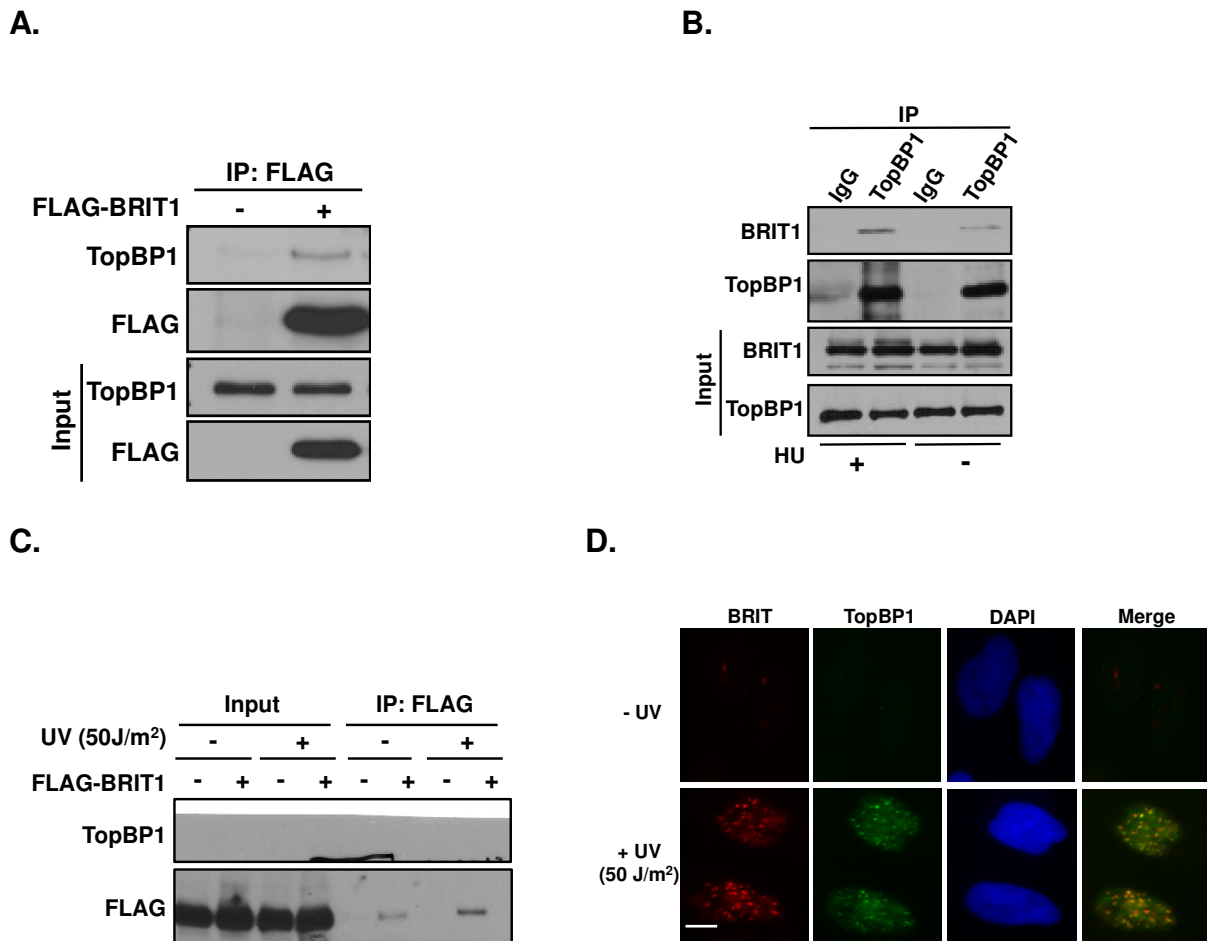
Figure 21. BRIT1 associates with TopBP1 in a Replication stress-dependent manner.

(A) U2OS cells were transfected with 3xFLAG or FLAG-BRIT1. 48 hours post-transfection, cell lysate was collected for IP using anti-FLAG (M2) affinity gel overnight. Samples were washed and analyzed by Western blotting. Input was 3% of the amount used for IP. (B) U2OS cells were untreated or treated with hydroxyurea (HU) (2 mM) for 3 hours, and lysate was collected for immunoprecipitation using IgG or anti-TopBP1 antibody. Samples were analyzed by Western blotting and the indicated proteins were probed. Note that BRIT1 co-immunoprecipitated with TopBP1 endogenously, and this interaction was increased in the presence of HU. (C) U2OS cells were transfected with 3xFLAG or FLAG-BRIT1. 48 hours post-transfection, cells were untreated or treated with UV (50 J/m^2) for 3 hours. Cell lysates (3 mg) were collected for immunoprecipitation using anti-FLAG (M2) affinity gel and immunoblotted with anti-FLAG or anti-TopBP1 antibody. The input was 3% of that used in the immunoprecipitation. Note that TopBP1 co-immunoprecipitation with FLAG-BRIT1 was elevated after UV treatment. (B) and (C) together suggest that replication stress increases BRIT1 binding with TopBP1. (D) U2OS cells were treated with UV (50 J/m^2) for 3 hours. cells were then fixed and immune-stained with anti-BRIT1 and anti-TopBP1 antibodies. Scale bar, 10 μm.
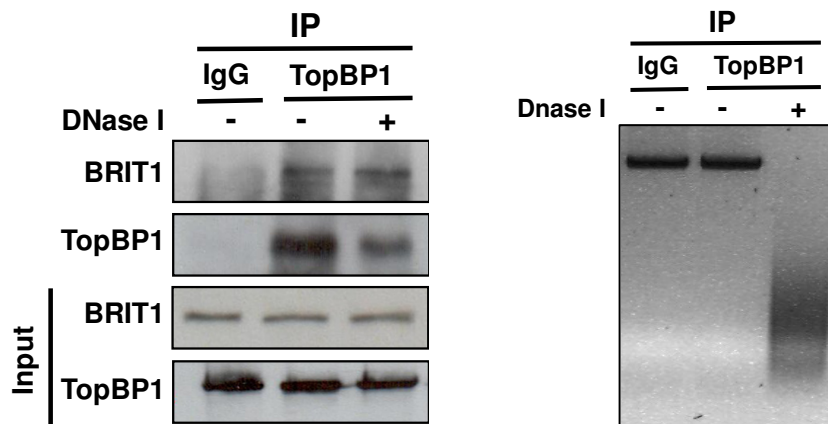
Figure 22. BRIT1 associates with TopBP1 independent of DNA.

U2OS cells were collected and lysed using a modified RIPA buffer with gentle rotation at 4°C for 1 hour. Samples were either untreated or treated with DNase I for an additional hour at 4°C with gentle rotation. Samples were then separated for DNA extraction or for immunoprecipitation using normal IgG or anti-TopBP1 antibodies. Immunoblotting was performed to detect the indicated proteins (left panel). DNA extraction was performed using Trizol reagent to confirm digestion of DNA (bottom panel). This result shows that BRIT1 interacts with TopBP1, and this interaction occurs independent of crosslinking with DNA.

BRIT1 promotes TopBP1 recruitment to damaged DNA

Since we have shown that BRIT1 binds and co-localizes with TopBP1 at sites of DNA damage, we asked whether BRIT1 is required for TopBP1 recruitment to damaged DNA. First, since BRIT1 is known to localize at double-strand DNA break, we tested whether BRIT1 recruitment to IR-induced DNA damage sites is TopBP1-dependent. Immunofluorescence shows that TopBP1 knockdown doesn't affect BRIT1 IRIF formation (Figure 23). Next, to show whether TopBP1 recruitment to replication stress sites is BRIT1-dependent, we used patient-derived cells containing homozygous loss-of-function truncation mutations to BRIT1 (a gift from Dr. Andrew P. Jackson) (Jackson et al., 2002a). These mutants contain a C74->G nucleotide mutation resulting in a premature stop codon. We refer to two of these mutant cells as 'MCPH1#1' and 'MCPH1#2' (from the alternative name of BRIT1, microcephalin), with 'control' cells derived from unaffected members of the same family. After two hours of HU treatment, control cells form TopBP1 foci at presumed sites of replication stress. However in the BRIT1 mutant cells, TopBP1 foci formation was defective, with the same phenomenon observed after UV treatment (Figure 24A, B). Defective recruitment of TopBP1 is expected to correlate with impaired ATR signaling since TopBP1 play an important role in ATR activation. Indeed, we show that BRIT1 knockdown compromised p-MCM2 levels after UV treatment (Figure 24C). Together, these results suggest that the interaction between BRIT1 and TopBP1 allows BRIT1 to promote TopBP1 recruitment to sites of replication stress or DNA damage.
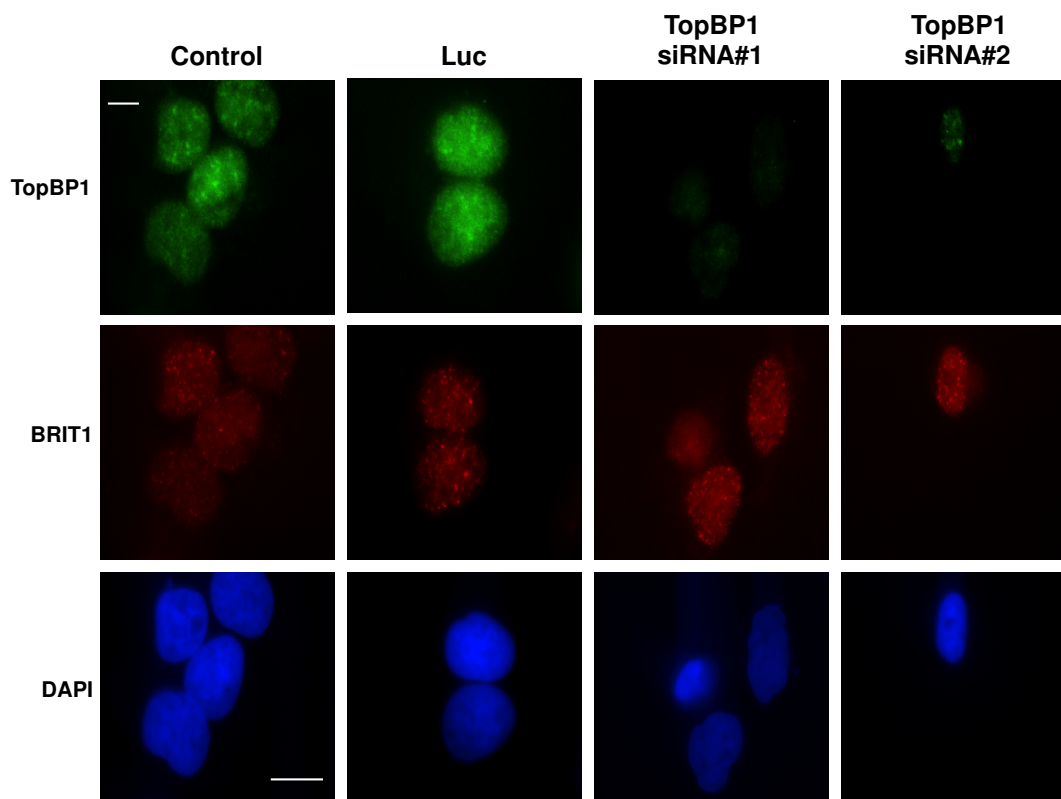
Figure 23. TopBP1 is not required for BRIT1 IRIF formation.

U2OS cells were transfected with control, luciferase-specific (Luc) or TopBP1-specific siRNA (#1 and #2). Cells were then treated with IR (10 Gy). 1 hour later, cells were fixed and immunostained with anti-TopBP1 and anti-BRIT1 antibodies. Scale bar, 10 μm. Note that there isn't a noticeable change in BRIT1 foci forming ability when TopBP1 is deficient.

A.
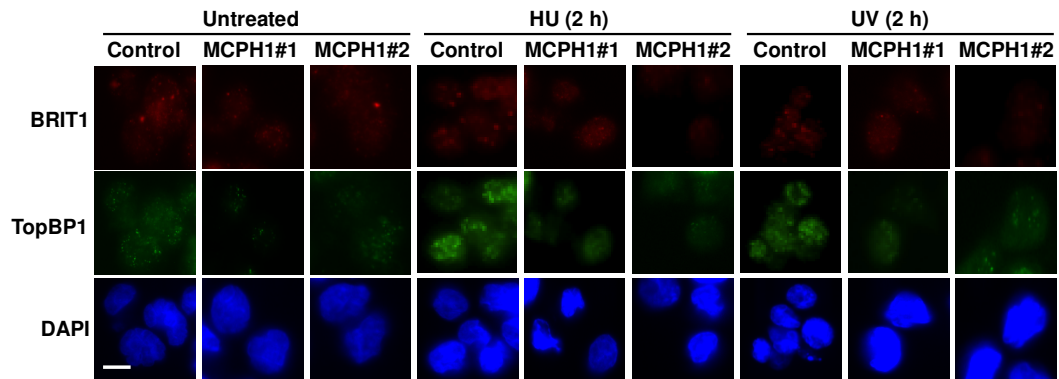
B.
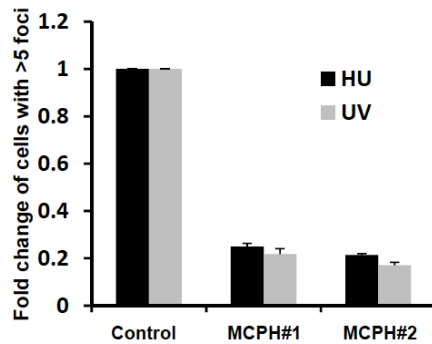
C.
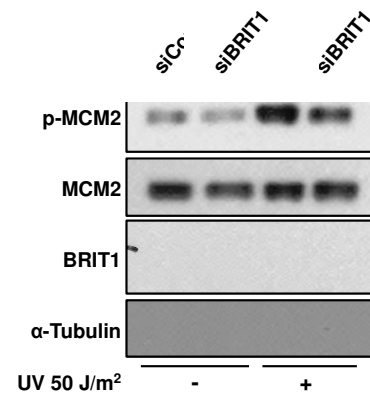
Figure 24. BRIT1 promotes TopBP1 recruitment to sites of DNA replication stress.

(A) BRIT1-proficient human cells (Control) and BRIT1-deficient cells from patients with microcephaly (MCPH1#1 and MCPH1#2) were treated with HU (2 mM) or UV (50 J/m^2) for 2 hours. Cells were fixed and immunostained with anti-BRIT1 and anti-TopBP1 antibodies. Scale bar, 10 μm. (B) Quantitative results from (A), plotting the fold-change of the number of cells with more than 5 foci. Control group was set as 1. Data are presented as mean ± standard deviation of 3 independent experiments. At least 50 cells were scored in each sample. Note that TopBP1 foci was significantly reduced in cells deficient for BRIT1. (C) U2OS cells were transfected with control or BRIT1-specific siRNA. 48 hours post-transfection, cells were untreated or treated with UV (50J/m^2) for 3 hours. Western blotting of the cellular lysate was performed to observe the expression of the indicated proteins. Note that BRIT1 deficiency reduced the expression of an ATR phosphorylation target, MCM (minichromosome maintenance), after UV treatment.

BRIT1 amplifies ATR signaling

The data so far point to BRIT1 as a mediator of ATR signaling. We looked further into the effect that BRIT1 has on ATR target activation, to better understand the extent that BRIT1 affects ATR signaling through TopBP1 recruitment. Under replication stress, ATR phosphorylates the RPA32 subunit of RPA at S33. We look at S33-RPA32 phosphorylation status after TopBP1 knockdown, which we expect to be reduced. As expected, U2OS cells with TopBP1 deficiency resulted in dramatically decreased S33-RPA32 phosphorylation after UV treatment (Figure 25A). However, BRIT1 knockdown only led a slight decrease in pS33-RPA32 after UV or HU treatment (Figure 25B). We had expected that if BRIT1 affects TopBP1 recruitment, and thus ATR activation, then BRIT1 knockdown would compromise ATR signaling. We decided to test the kinetics of ATR signaling by harvesting cells at various time points of UV and HU exposure under BRIT1 knockdown conditions. Western blot shows that as in Figure 25B, BRIT1 knockdown did not have a significant effect on S33-RPA32 phosphorylation after one hour exposure to HU. However, over three hours, the BRIT1 knockdown cells clearly displayed lower levels of pS33-RPA32 (Figure 25C). We tested for this phenomenon in BRIT1 mutant cells, where control or MCPH1#1 cells were untreated or treated with HU for up to six hours. Similar to BRIT1 knockdown cells in U2OS, MCPH1#1 cells displayed a more noticeable reduction in pS33-RPA32 signal over time (Figure 25D). When the HU-treated cells were stained with anti-pS33-RPA32 for immunofluorescence, results show that BRIT1 knockdown cells displayed fewer numbers of foci after the one hour time point compared to control cells (Figure 25E). Since the amount of RPA protein bound to DNA depends on the availability of ssDNA, we tested whether the reduction in pS33-RPA32 after BRIT1 knockdown or in MCPH1#1 cells is actually due to a decrease in S33-RPA32 phosphorylation, or a decrease in the amount of chromatin-bound RPA. We performed chromatin fraction of cells expressing BRIT1 siRNA, and the Western blot for RPA shows that BRIT1 deficiency does not affect the level of chromatin-bound RPA after UV or HU treatment

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(Figure 25F). Together, these results demonstrate that BRIT1 may be dispensable for activation of ATR signaling, but plays a role in amplifying or sustaining ATR signaling.

A.

siControl | Luc | siTopBP1 #1 | siTopBP1 #2 | siControl | Luc | siTopBP1 #1 | siTopBP1 #2

TopBP1

pS33-RPA32

RPA32

β-Actin

− UV   + UV

B.

siControl | siBRIT1 | siControl | siBRIT1 | siControl | siBRIT1

BRIT1

pS33-RPA32

RPA32

β-Actin

Untreated   UV   HU

C.

siControl   siBRIT1

HU (h)   0   0.5   1   2   3   0   0.5   1   2   3

pS33-RPA32

RPA32

BRIT1

β-Actin

D.

Control   MCPH1#1

HU (h)   0   1   3   6   0   1   3   6

pS33-RPA32

RPA32

β-Actin

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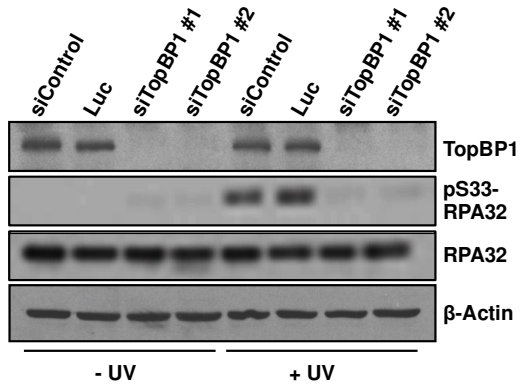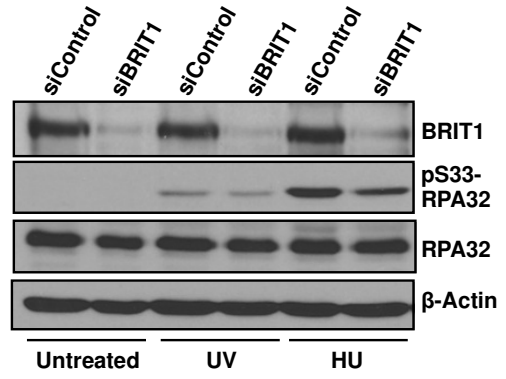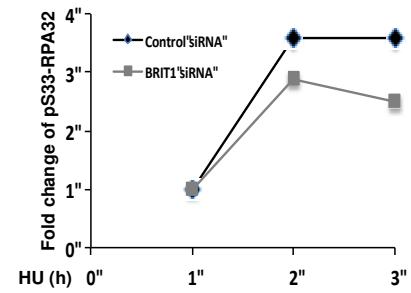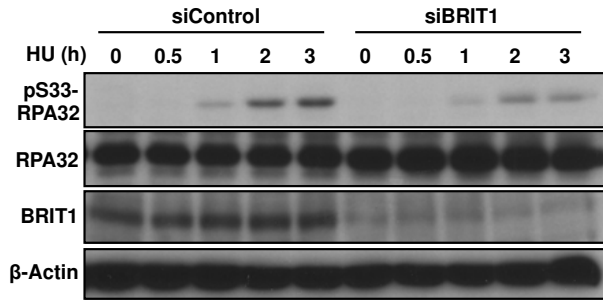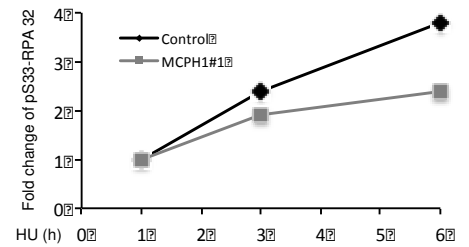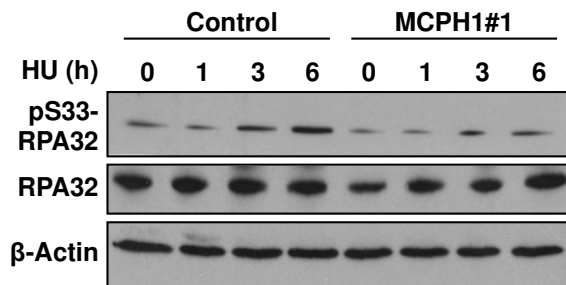
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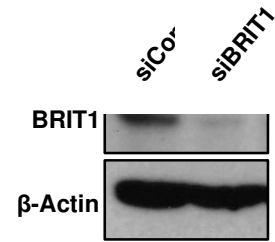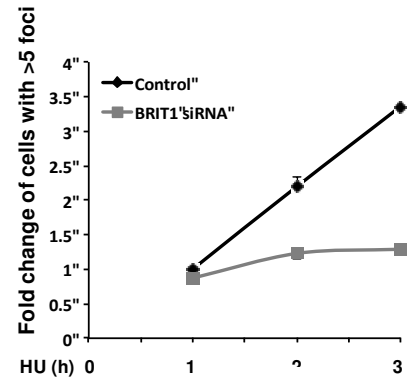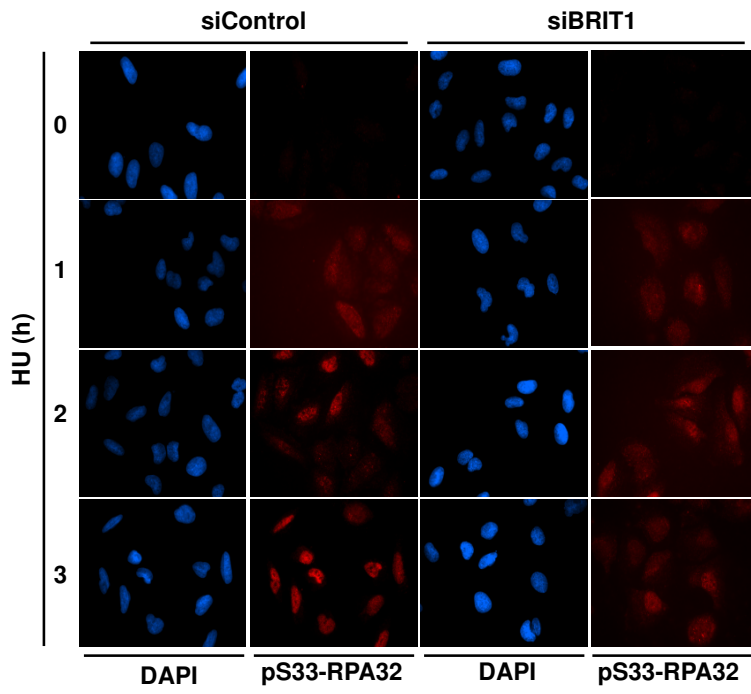
F.
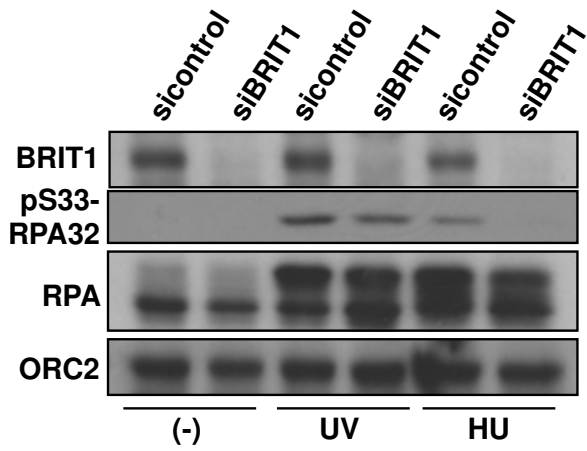
Figure 25. BRIT1 amplifies ATR signaling.

(A) U2OS cells were transfected with control, luciferase-specific (Luc) or TopBP1-specific siRNA (#1 and #2). 48-72 hours post-transfection, cells were untreated or treated with UV (50 J/m^2). One hour post-radiation, cell lysate was collected and the expression of TopBP1, pS33-RPA, and total RPA was detected by Western blotting. TopBP1 deficiency reduced pS33-RPA32 signal as expected, since TopBP1 is important for ATR-mediated phosphorylation. (B) MCF10A cells were transfected with control siRNA or BRIT1-specific siRNA. 48-72 hours later, cells were treated with UV (50 J/m^2) or HU (2 mM). One hour post-treatment, cell lysate was collected and expression of TopBP1, pS33-RPA, and total RPA was detected by Western blotting. Note that BRIT1 deficiency caused no noticeable change in pS33-RPA32 signal after UV treatment, and only a slight reduction after HU treatment. (C) U2OS cells were transfected with control siRNA or BRIT1-specific siRNA. 48-72 hours post-transfection, cells were treated with HU (2 mM) and harvested at the indicated time points. Expression of TopBP1, pS33-RPA32, and total RPA was detected by Western blotting. Image J software was used to quantify the pS33-RPA32 bands to plot a graph using Excel software. Note that a trend in reduced pS33-RPA32 signal over the three-hour time course is observed after HU treatment. (D) BRIT1-deficient lymphoblastoid cells from patients with microcephaly (MCPH1#1) and BRIT1-proficient cells (Control) were treated with HU (2 mM) and harvested at the indicated time points. Cellular lysate was collected for immunoblotting to compare levels of pS33-RPA32 (left panel), and the bands were quantified using Image J software and plotted on a graph using Excel software (bottom panel). As in the BRIT1 knockdown cells (C), these BRIT1-deficient cells also displayed a trend in reduced pS33-RPA32 over the time-course after HU treatment. (E) U2OS cells were transfected with control or BRIT1-specific siRNA. 48 hours post-transfection, cells were exposed to HU (2 mM) for the indicated times. pS33-RPA32 was detected using immunofluorescent staining. Image J software was used to quantify the pS33-RPA32 bands to plot a graph using Excel software. Lysate was run on Western blot and immunoblotted with anti-BRIT1 antibody. Correlating with decreased pS33-RPA32 band observed after BRIT1 knockdown (C and D), there is a decrease in pS33-RPA32 foci after HU treatment. (F) U2OS cells were transfected with control or BRIT1-specific siRNA. 48 hours post-transfection, cells were either untreated, or treated with HU (2 mM) or UV (50 J/m^2) for 6 hours. Cells were then harvested for chromatin fractionation and immunoblotted to detect the indicated proteins. Note that there is no noticeable difference in the level of chromatin-bound RPA after BRIT1 knockdown, however there is a reduction in pS33-RPA32.

BRIT1 bp250-500 and BRCT5-TopBP1 are required for BRIT1-TopBP1 interaction.

As we have already shown that BRIT1 is a binding partner of TopBP1, we used wild-type and deletion constructs of FLAG-BRIT1 to map the region required for TopBP1 binding (Figure 26A). Myc-TopBP1 was co-expressed with FLAG-BRIT1 constructs in U2OS cells. Western blot shows that a region between bp250-500 is required for interaction with TopBP1 (Figure 26B). To confirm that this region is important for binding, we generated a new FLAG-BRIT1 construct lacking bp250-50 (BΔ7) (Figure 26C). When FLAG-BRIT1 (WT) or BΔ7 were expressed in U2OS cells, we found that BΔ7 binding with TopBP1 was significantly reduced compared to FLAG-BRIT1 (WT) binding to TopBP1 (Figure 26D). This confirms that the BRIT1 region between bp250-500 is required for TopBP1 binding (Figure 26D).
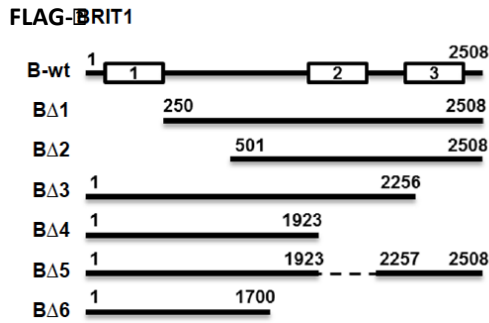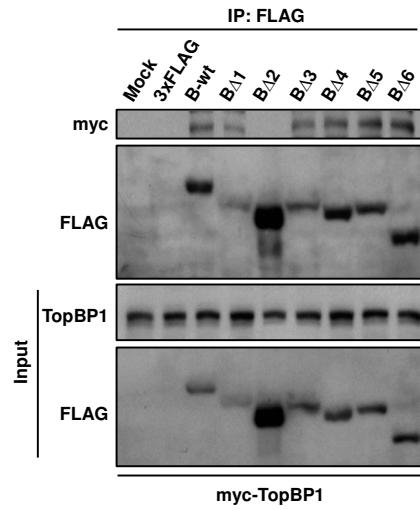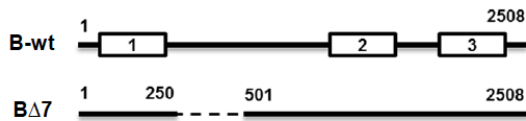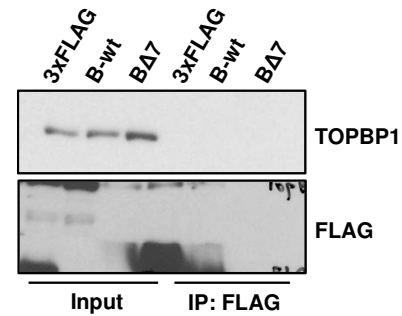
Figure 26. BRIT1 bp250-500 is required for BRIT1-TopBP1 interaction.

(A) Schematic diagram of wild-type BRIT1 and deletion mutants of BRIT1.
(B) U2OS cells were transfected with myc-TopBP1 and FLAG-BRIT1 (WT) or a one of the indicated FLAG-BRIT1 mutants. Cell lysate was collected and immunoprecipitated using anti-FLAG (M2) affinity gel and analyzed by Western blotting. The input was 3% of that used in the immunoprecipitation. Note that the region of BRIT1 between base pair 250-500 displayed significantly reduced binding to TopBP1. (C) Schematic diagram of wild-type BRIT1 and the BΔ7 deletion mutant of BRIT1. (D) U2OS cells were transfected with 3xFLAG, FLAG-BRIT1 and BΔ7 as indicated. 48 hours later, cells lysate was collected for immunoprecipitation using anti-FLAG (M2) affinity gel and analyzed by Western blotting. The input was 3% of that used in the immunoprecipitation. Note that BΔ7 binding to TopBP1 is reduced compared to FLAG-BRIT1 (WT).

BRIT1 is phosphorylated by ATM and ATR

In response to replication stress, several proteins within the ATR pathway are phosphorylated by ATR, including RPA subunits and Chk1. Since we show here that BRIT1 amplifies ATR signaling through TopBP1, we asked whether BRIT1 is a phosphorylation target of ATR. ATM and ATR substrates are phosphorylated on a S/TQ motif, so we used an antibody targeting this motif to see whether BRIT1 is a potential target. Western blot shows that BRIT1 could be pulled down using this antibody, while phosphatase treatment abolishes the signal, suggesting that BRIT1 is phosphorylated on some S/TQ site. Interestingly, HU treatment resulted in a stronger pulldown signal, suggesting that BRIT1 phosphorylation at S/TQ is induced by replication stress (Figure 27A). As we scanned the BRIT1 sequence for potential phosphorylation sites, we noticed that the sequence downstream of S322 bears homology to the sequence around Ser15 of p53 (Figure 27B). Therefore, to help determine whether S322-BRIT1 is a phosphorylated site, we used an antibody recognizing p-p53 (S15) to test whether this antibody could immunoprecipitate BRIT1. Indeed, Western blot shows that the p-p53 (S15) pulls down BRIT1 even in the absence of HU, suggesting a basal level of BRIT1 phosphorylation, possibly due to endogenous stress or DNA damage. When cells are treated with HU, the phosphorylation signal for BRIT1 is increased, and importantly, an S322A mutation nearly abolishes the BRIT1 signal, which strongly suggests that the S322 site is a target of ATM or ATR (Figure 27C). To further confirm that HU induces phosphorylation at S322-BRIT1, we used the anti-pS/TQ antibody to pulldown BRIT1 wild-type or S322A mutant. Expectedly, only the BRIT1 wild-type was immunoprecipitated after HU treatment (Figure 27D). Together, these results show that S322-BRIT1 is a potential target of ATM or ATR upon replication stress.

To specifically identify ATM and/or ATR as kinases for BRIT1, we performed *in vitro* kinase experiments using a GST-BRIT1 construct comprising of 100 amino acids around S322. We show that wild-type ATM phosphorylates GST-322wt, but not GST-322mut (Figure 28A). Interestingly, we show that wild-type ATR is also able to phosphorylate GST-322wt. However,

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ATR is also able to phosphorylate the GST-322mut construct, suggesting that ATR phosphorylates at BRIT1 sites other than S322 (Figure 28B). This is consistent with the observation that the S322A-BRIT1 mutant doesn't completely abolish phosphorylation after replication stress (Figure 28D). To show that ATR phosphorylates BRIT1 in the cellular setting, we used an ATR inhibitor drug, VE-821, to see the effect on BRIT1 phosphorylation. Western blot shows that ATR inhibition significantly reduces BRIT1 phosphorylation (Figure 28C), suggesting that ATR is a BRIT1 kinase.

A.

B.

BRIT1 322███$ Q E T F█ E

p53   15███$ Q E T█ F S

C.

D.
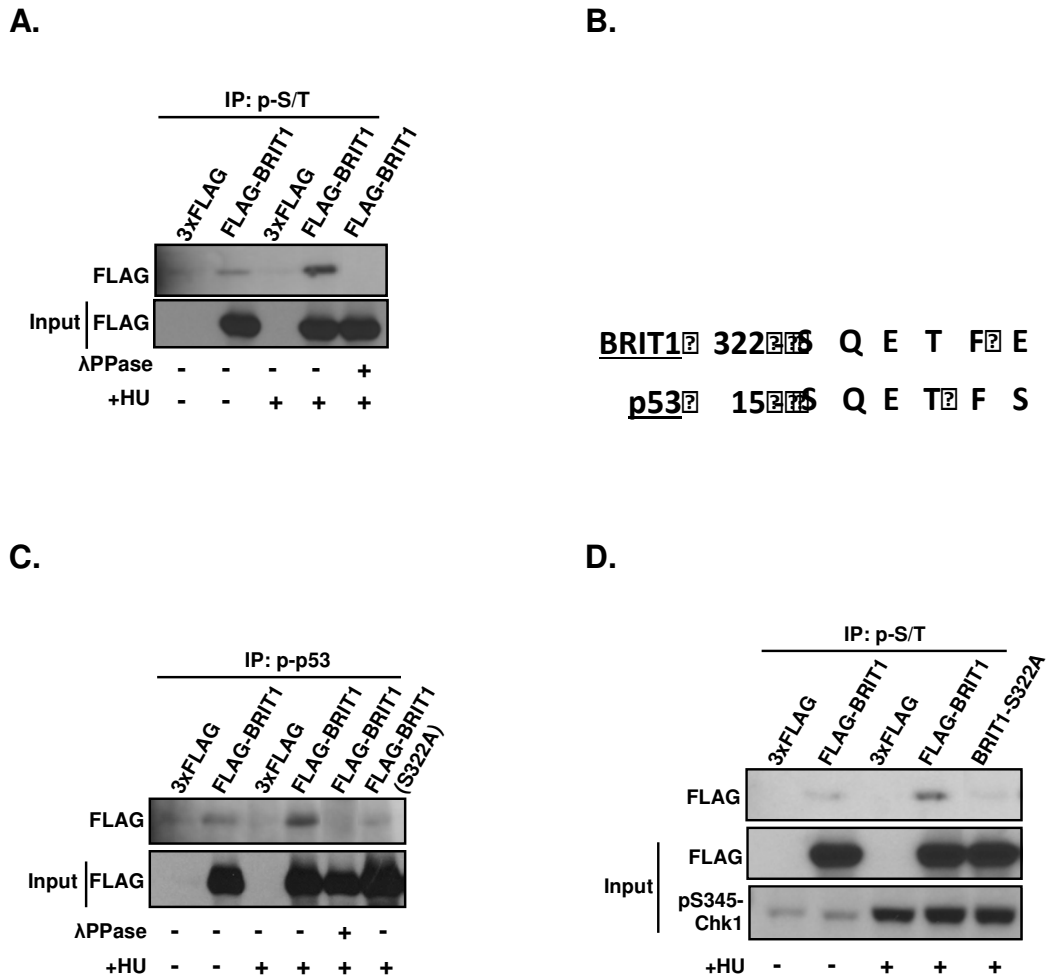
Figure 27. BRIT1 Ser322 is phosphorylated upon replication stress.

(A) U2OS cells were transfected with 3xFLAG or FLAG-BRIT1. 48 hours post-transfection, cells were untreated or treated with HU (2 mM) for 3 hours. Cell lysate was collected for immunoprecipitation using an anti-p-S/T antibody. The precipitates were incubated with or without Lambda PPase for 20 minutes at 30°C, eluted with 3x loading buffer by boiling at 95°C for 5 minutes, and resolved by SDS-PAGE followed by Western blotting. Result suggests that BRIT1 is a phosphorylated protein. (B) Diagram showing the homology between BRIT1 and p53 around S322 and S15, respectively. (C) U2OS cells were transfected with 3xFLAG, FLAG-BRIT1 (WT) or the S322A construct. 48 hours post-transfection, cells were untreated or treated with HU for 3 hours. Immunoprecipitation was conducted using an anti-phospho (p)-p53 antibody. The precipitates were incubated with or without Lambda PPase for 20 minutes at 30°C. (D) U2OS cells were transfected as in (C). 48 hours post-transfection, cells were untreated or treated with HU for 3 hours. Immunoprecipitation was conducted using an anti-p-S/T antibody. pS345-Chk1 was used as a positive control to demonstrate strate activated ATR signaling after HU treatment. Note that the phosphorylation of the BRIT1-S322A mutant is much reduced compared to FLAG-BRIT1 (WT).

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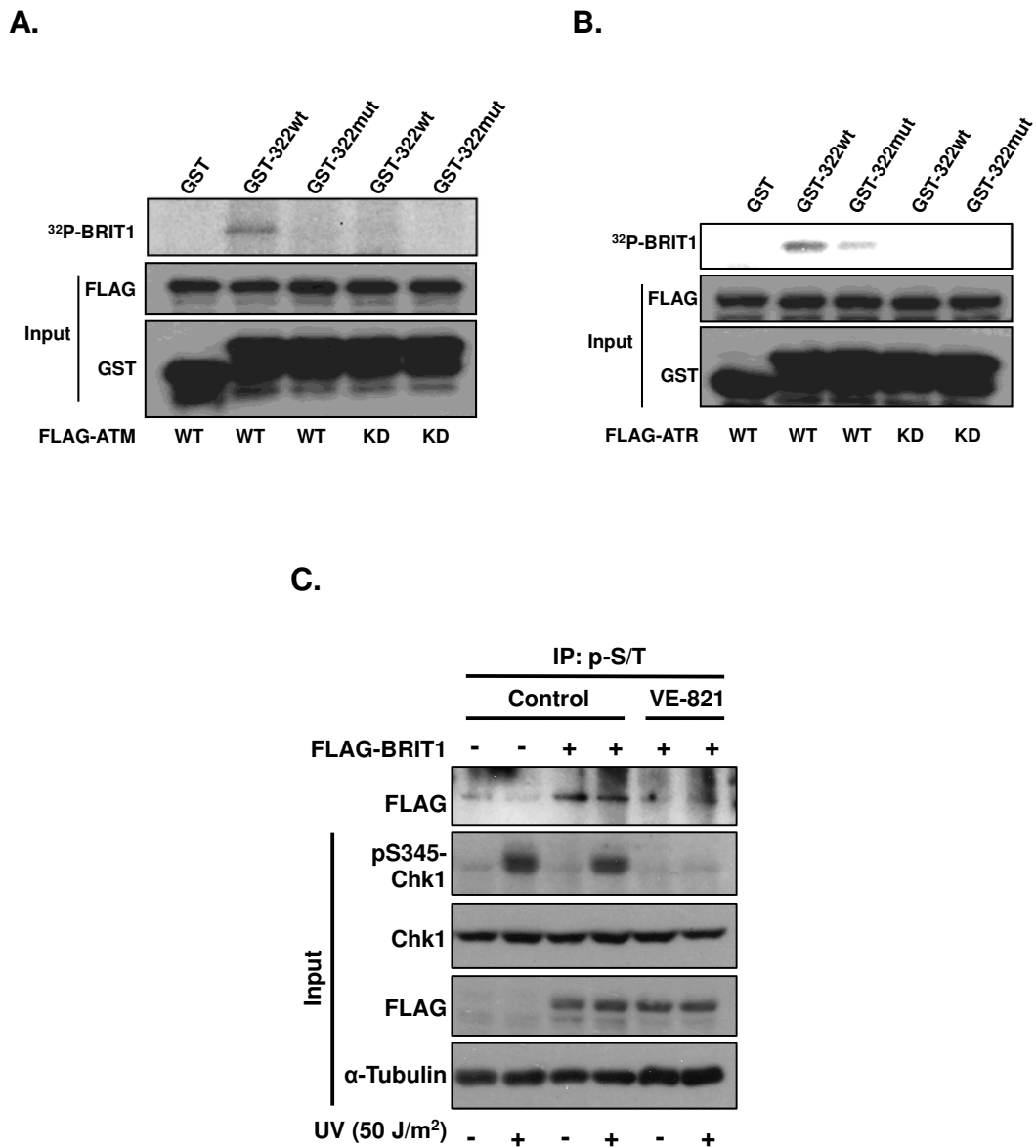
Figure 28. ATM and ATR phosphorylate BRIT1 on S322.

(A) The sequence around BRIT1 S322 was cloned into a GST vector, and the BRIT1 S322A mutant (S322A) was made in this vector. U2OS cells were transfected with wild-type (WT) or kinase-deletion-mutant (KD) FLAG-ATM expression vectors as indicated. Cell lysate was collected for immunoprecipitation using anti-FLAG (M2) affinity gel, incubated overnight, and incubated with 3xFLAG Peptide for 1 hour for elution. The eluted sample was incubated with ATP [γ-^{32}P] and GST or GST-BRIT1 (322wt or 322mut) as indicated for 30 min at 30℃. Samples were run on SDS-PAGE and exposed to x-ray film. Result indicates that ATM phosphorylates 322wt, but not 322mt. (B) U2OS cells were transfected with WT or KD FLAG-ATR expression vectors as purified as in (A). Kinase assay was performed as in (A). (C) U2OS cells were transfected with 3xFLAG or FLAG-BRIT1. 48 post-transfection, cells were either untreated or treated with an ATR inhibitor (VE-821) at 2 µM for 3 hours. Cells were then either untreated or treated with UV (50 J/m^2). Cell lysate was collected and immunoprecipitated with p-S/T-specific antibody, and run on Western blot to detect the indicated proteins.

BRIT1 phosphorylation and interaction with TopBP1 are required for TopBP1 recruitment

We have determined the BRIT1 domain required for binding to TopBP1, and we have found a BRIT1 site of phosphorylation mediated in part by ATR. Using the BΔ7 BRIT1 mutant that either lacks the TopBP1 binding domain, or the S322A phospho-mutant, we sought to determine whether these mutants negatively affect TopBP1 recruitment to sites of replication stress. The overexpression constructs are all siRNA resistant. Under BRIT1 knockdown conditions, TopBP1 accumulation at replication stress sites was rescued by wild-type BRIT1, but not by the BΔ7 or S322A mutants (Figure 29A, B). Expectedly, we observed in these cells that when the levels of ATR signaling is diminished in the BRIT1 knockdown condition – as measured by the level of pS33-RPA32 Western blot signal – only wild-type BRIT1 was able to rescue RPA32 phosphorylation, while the mutants failed to do so (Fig 29C). Similar results supporting the importance of the TopBP1 binding domain and phosphorylation site were observed in MCPH1#1 BRIT1 mutant cells.

A.
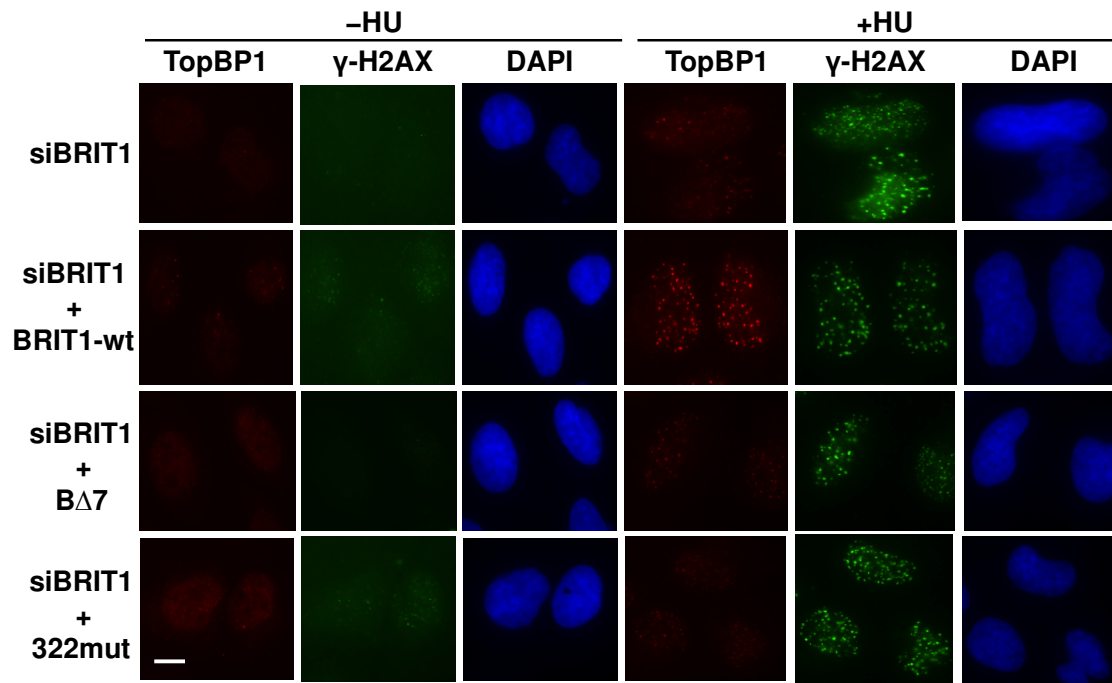
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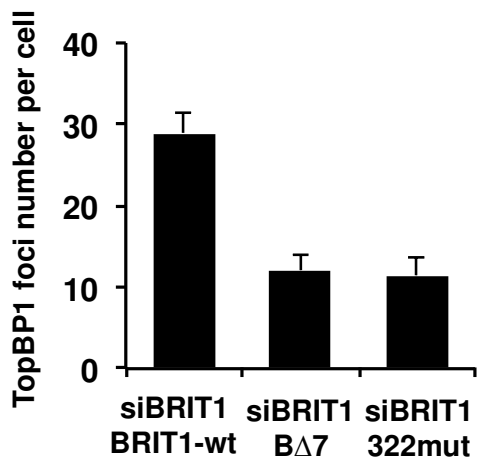
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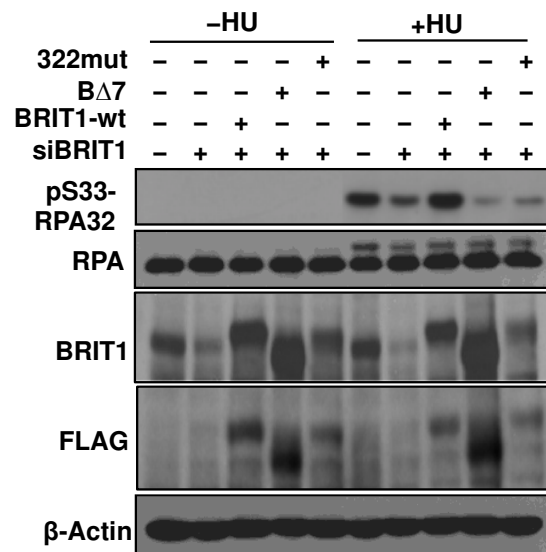
Figure 29. BRIT1 phosphorylation and interaction with TopBP1 are required for TopBP1 accumulation at sites of replication stress.

(A) U2OS cells were transfected with siRNA-resistant BRIT1-wt, BΔ7 or 322mt plasmids as indicated. After 6 hours, cells were transfected with BRIT1-specific siRNA (siBRIT1) as indicated to knock down the expression of BRIT1. 48 hours after siRNA transfection, cells were untreated or treated with HU (2 mM) for 3 hours, fixed and immunostained with anti-TopBP1 and anti-γ-H2AX antibodies. Scale bar, 10 μm. Note that TopBP1 foci formation is rescued after BRIT1-wt, but not BΔ7 or 322mt expression. (B) Quantitative results from (A). The number of TopBP1 foci per cell was quantified. Data are presented as mean ± standard deviation of 3 independent experiments; Student's t-test. At least 50 cells were scored in each sample. (C) U2OS cells were transfected and untreated or treated with HU as in (A). Expression of the indicated proteins was verified by Western blotting. Note that the Western blot results support the immunofluorescence data in (A), since only BRIT1-wt, but not BΔ7 or 322mt rescued the pS33-RPA32 signal.

BRIT1 phosphorylation and interaction with TopBP1 are required for replication stress recovery

We have so far determined that BRIT1 promotes TopBP1 accumulation at sites of replication stress. Since TopBP1 accumulation is a prerequisite for ATR signaling, we next determine whether the interaction between BRIT1 and TopBP1 indeed promotes ATR signaling. As ATR signaling is involved in checkpoint control and cell survival from replication stress, we tested whether BRIT1 knockdown affects cellular sensitivity to HU- and UV-induced stress. Our results show that BRIT1 deficiency leads to reduced cell survival after both HU and UV treatment as measured by colony formation (Figure 30A, B).

In addition to checkpoint activation during S-phase, ATR signaling is essential for recovery from replication stress and complete DNA replication (Mordes *et al.*, 2008). We performed a couple of experiments to determine whether BRIT1, through its interaction with TopBP1, regulates replication stress recovery. First, we measured the cell's ability to recover from replication stress by analyzing their cell cycle profiles after HU treatment. Cells were treated with HU for 24 hours and then released into media containing nocodazole, then collected at various time points. Results show that in BRIT1 knockdown cells, there is a greater accumulation of cells in the G1 phase after HU treatment, suggesting that these cells are not recovering as efficiently from the damage accumulated during replication stress. We show that although wild-type BRIT1 was able to promote recovery out of G1, both the BΔ7 and S3222 mutants of BRIT1 were not able to do so (Figure 30C, D). As an alternative method to test for replication stress recovery, we measured the level of the mitotic marker, phospho-histone H3 (p-H3) Ser10, after cells are treated with HU followed by taxol to allow stressed cells to recover and accumulate in mitosis. We first performed this experiment in BRIT1-mutant MCPH1#1 cells and found that these cells displayed lower levels of p-H3 after HU and taxol treatment, suggesting that BRIT1 deficiency prevents cells from recovering from replication stress and continuing through the cell cycle (Figure 30E). Similar results supporting the role of BRIT1 in replication stress recovery are observed in BRIT1 knockdown cells. Importantly, the level of p-

H3 is rescued in the BRIT1 knockdown cells by expression of wild-type BRIT1, but not the BΔ7 mutant nor the S322A phospho-mutant (Figure 30F). Together, these results suggest that BRIT1 plays an important role in cellular response and recovery from replication stress, and that this function is dependent on the TopBP1 interaction domain of BRIT1 as well as the ATM/ATR-targeted S322 phosphorylation site of BRIT1.

A.

B.

C.

D.

E.

F.

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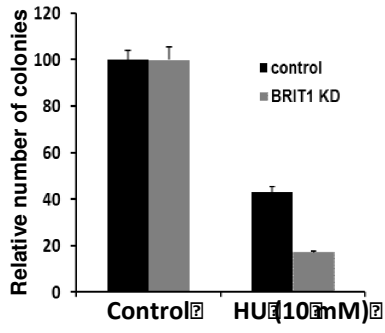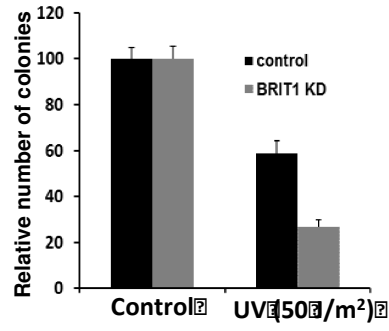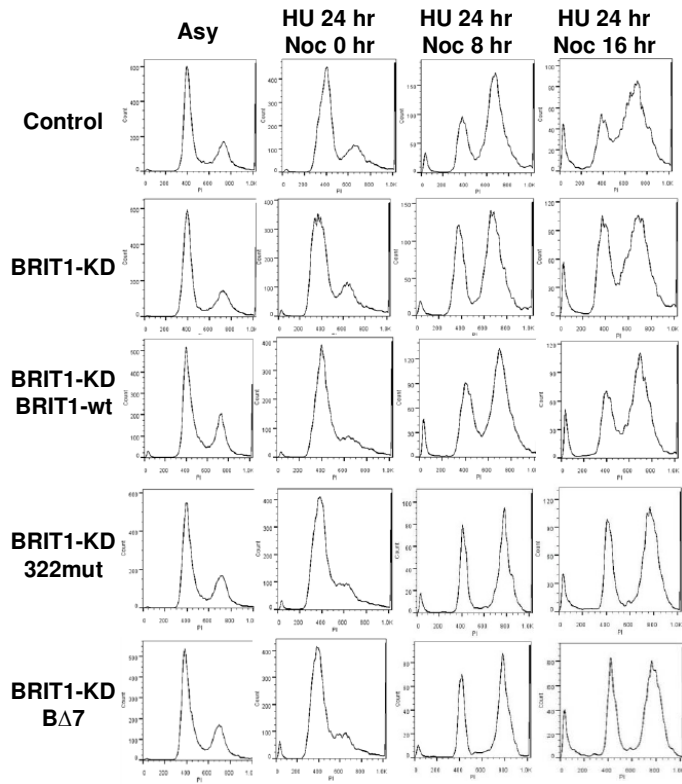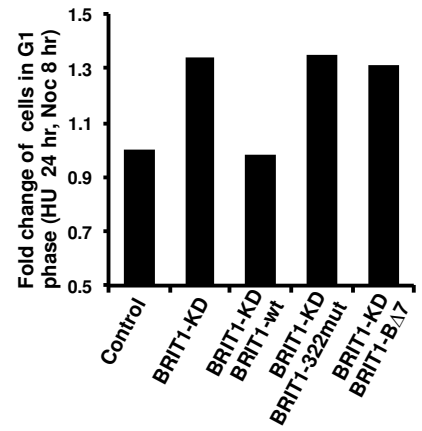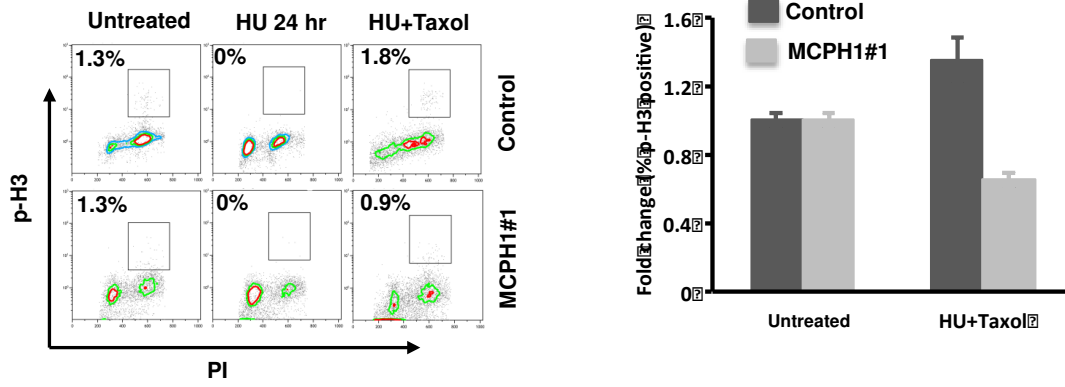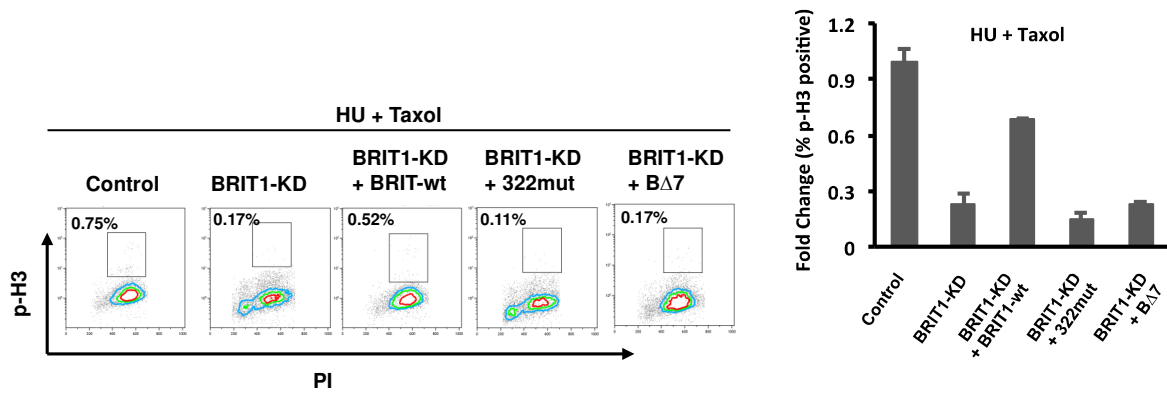
Figure 30. BRIT1 phosphorylation and interaction with TopBP1 are required for replication stress recovery.

(A and B) MCF10A cells were transfected with control or BRIT1-specific siRNA (BRIT1 KD) for 48-72 hours. Cells were then untreated or treated with HU (10 mM) for 24 hours (A) or UV (50 J/m^2) for 3 hours (B). Cells were then seeded in a 6-well plate at 250 cells/well. Viable colonies were stained by crystal violet and counted. Data are presented as mean ± standard deviation (error bars) from 3 independent experiments. (C) U2OS cells were transfected with BRIT1-wt, BΔ7 or 322mt plasmids as indicated. Six hours post-transfection, cells were transfected with BRIT1-specific siRNA (BRIT1-KD) as indicated to knock down the expression of BRIT1. 48 later, cells were incubated in medium with or without HU (2 mM) for 24 hours. Cells were then released into media containing Nocodazole (1 µg/mL). Cells were harvested at 0, 8 and 16 h after release. Harvested cells were fixed in ethanol, stained with propidium iodide, and analyzed by FACS. (D) Quantitative results from (C). The percentage of cells in G1 phase is shown. Data are presented as mean ± standard deviation of 3 independent experiments; Student's *t*-test. (E) BRIT1-proficient cells (Control) cells and BRIT1-deficient cells (MCPH1#1) were untreated or treated with HU (2 mM) for 24 hrs. Cells were then washed to remove HU and released into media with or without Taxol (2 ng/ml) for 10 hours. Cells were then fixed and stained using phospho-Histone H3 (Ser10)-specific antibody (p-H3) and propidium iodide (PI) (left panel). The population of cells that stained positive for p-H3 (Ser10) was plotted on a graph to compare the conditions as shown (right panel). Bar graph represents the average from three independent experiments. (F) U2OS cells were treated with control or BRIT1 siRNA (BRIT1-KD) and reconstituted with the indicated constructs. 48 hours after transfection, cells were treated with HU (2 mM) for 4 hours, and released into taxol for 10 hours. Cells were then fixed and stained using p-H3 antibody and PI (left panel). The population of cells that stained positive for p-H3 was plotted on a graph to compare the conditions as shown (right panel). Bar graph represents the average from three independent experiments.

DISCUSSION

The DNA damage response incorporates DNA repair and cell cycle checkpoint activation (Curtin, 2012). BRIT1 is known to be involved in the DNA repair process through its recruitment of repair factors to the site of DNA damage. In this study however, we provide evidence suggesting that BRIT1 may also play a role in cell cycle checkpoint activation in response to DNA damage. Interestingly, the replication stress response also activates the cell cycle checkpoint, and this functions to promote removal of stress impediment before damage occurs, e.g. in the form of replication fork collapse, leading to double strand DNA breaks. In this sense, the RSR can be viewed as a mechanism of preventative maintenance to safeguard the genome against DNA damage. Since the proteins and signaling events in checkpoint signaling is similar between DNA damage and the RSR, this suggests that BRIT1 may have a role in promoting cell cycle checkpoint signaling after both DNA damage and replication stress.

ATRIP-ATR recruitment to DNA is followed by ATR *trans* autophosphorylation (Liu *et al.*, 2011). Therefore, initial activation of ATR signaling occurs independent of TopBP1. However, TopBP1 recruitment to the site of ATR localization is a prerequisite for optimal ATR activity. TopBP1 recruitment allows ATR to target several of its downstream targets including claspin, Chk1 and MCM2 (Kumagai *et al.*, 2006; Liu *et al.*, 2006). Furthermore, kinase activity is required to maintain DNA damage signal. For example, in response to DNA damage, ATM makes the initial phosphorylation on Ser15-p53, while ATR phosphorylation at the same site is required to maintain this phosphorylation over several hours (Meek, 2004). Therefore, it is important to study the mechanisms that allow ATR to maintain signaling over a prolonged period during DNA damage or replication stress. The general mechanism of ATR activation and downstream signaling has been well described (Flynn and Zou, 2011), but there remains a gap in understanding how ATR signaling is maintained. Our research aims to help fill this gap by introducing BRIT1 as a regulator of ATR signaling, through recruitment of TopBP1.

Our initial observations that BRIT1 promotes TopBP1 recruitment to sites of replication stress after UV and HU treatment, suggested that BRIT1 might affect ATR signaling through TopBP1 (Figure 24). Indeed, BRIT1 deficiency did have a negative effect on ATR signaling. However this effect was not apparent until two to three hours after UV or HU treatment (Figure 25). We noticed that even though the initial phosphorylation of the ATR target RPA32 wasn't affected, time-course experiments demonstrated that pS33-RPA32 was reduced over several hours in BRIT1 deficient cells. To clearly show that BRIT1 depletion impairs RPA phosphorylation at later time points after initial ATR activation, we first quantified and plotted the pS33-RPA32 bands in Figure 25C to compare ATR activity under siBRIT1 versus siControl conditions. The graph shows that BRIT1 knockdown leads to a shorter activation period compared to control. Second, in Figure 25E, U2OS cells were transfected with siControl or siBRIT1 to compare changes in ATR signaling. 48 hours post-transfection, cells were treated with HU over a period of 3 hours. Cells were then fixed and stained for pS33-RPA32 foci. We show that in cells with BRIT1 knockdown, there are a reduced number of foci as time increased toward 3 hours of HU exposure, compared to cells treated with siControl. Finally, we showed that the relationship between BRIT1 and positive ATR pathway regulation is relevant in BRIT1-deficient patient cells. We used a BRIT1-deficient lymphoblastoid cell line (MCPH1#1) and a BRIT1-proficient 'Control' cell line, treated them with HU and collected samples over a 6-hour time course to quantify and compare pS33-RPA32 levels between the two cell lines. We show that MPCH1#1 cells displayed reduced pS33-RPA32 levels compared to Control cells (Figure 25D). Together, our data support the conclusion that BRIT1 deficiency impairs RPA phosphorylation at later time points after initial ATR activation. These results are consistent with the current knowledge of ATR signaling being a later response to DNA damage, and being maintained over several hours, compared to the initial response by ATM (Appella and Anderson, 2001). Additionally, because we conclude that TopBP1-mediated activation of ATR is compromised by BRIT1 deficiency, we needed to show that this effect was not caused by a decrease in the amount of chromatin-bound RPA after BRIT1 knockdown. To test this

possibility, we conducted a chromatin fractionation experiment. We transfected U2OS cells with control siRNA or BRIT1 siRNA. 48 hours later, cells were either left untreated, or treated with HU (2 mM) or UV (50 J/m^2) for 6 hours. Cells were then harvested for chromatin fractionation to determine whether changes in BRIT1 expression correlates with chromatin-bound RPA. Our results show that the level of chromatin-bound RPA remains unchanged between control and BRIT1 siRNA-treated cells (Figure 25F). This result suggests that the reduced p-RPA in BRIT1-knockdown cells was not due to the reduced amount of RPA binding to chromatin.

For a better understanding of the mechanism of TopBP1 recruitment, we performed binding experiments to see whether TopBP1 recruitment depended on a direct interaction with BRIT1. Our results show that the region from base pair 250-500 of BRIT1 is required for BRIT1 to co-immunoprecipitate with TopBP1. Furthermore, we show that a BRIT1 mutant that lacks this potential binding domain (BΔ7) does not co-IP with TopBP1. Since base pair 250-500 doesn't lie within any of the three BRCT domains of BRIT1, which are known to bind phospho-peptides, it seems unlikely that BRIT1 binds to a phospho-peptide sequence within TopBP1, though this cannot be ruled out until further binding studies are done to determine the region of TopBP1 that binds BRIT1.

Determining whether ATM and/or ATR phosphorylate BRIT1 was important to see if ATR is involved in mediating TopBP1 recruitment through BRIT1. If so, this would suggest another self-propagating mechanism of ATR signaling, where ATR phosphorylates targets that promote its own signaling pathway. This phenomenon has been observed in ATR phosphorylation of RPA and TopBP1 (Liu *et al.*, 2012; Vassin *et al.*, 2009). Through our IP experiments using anti-pS/TQ and anti-pS15-p53 antibodies, we demonstrated that S322-BRIT1 is a candidate phopshorylation site (Figure 27). We then performed *in vitro* kinase experiments to demonstrate that both ATR and ATM are potential kinases for Ser322-BRIT1 (Figure 28). This result is interesting on a couple of levels. First is that the Ser322 of BRIT1 does not lie within the TopBP1-binding domain (bp 250-500) of BRIT1. Finding an ATR phosphorylation site on BRIT1 that lies within this region could have provided a simple

explanation for the BRIT1-TopBP1 interaction. That is, it would suggest that one of the eight BRCT domains within TopBP1 requires Ser322-BRIT1 to be phosphorylated in order for interaction to occur. However, since Ser322 doesn't lie within the TopBP1-binding domain of BRIT1, it remains unclear how phospho-Ser322 aids in BRIT1 interaction with TopBP1, as we go on to show that it certainly does promote this interaction. Second, the kinase experiment results are interesting because we find that both ATM and ATR are both able to phosphorylate BRIT1. That ATM and ATR target the same protein is not a surprise. There have been many documented instances where ATM and ATR share targets, and this crosstalk often functions as backup mechanisms or prerequisites for the two pathways to work properly (Gatei *et al.*, 2001; Stiff *et al.*, 2006; Tibbetts *et al.*, 1999; Ward and Chen, 2001). Thus, ATM phosphorylation of BRIT1 may provide a binding site for an early-phase of recruitment of proteins downstream of BRIT1. And upon DNA resection and ATRIP-ATR recruitment to ssDNA-RPA, ATR mediates a late-phase recruitment of TopBP1 through BRIT1 phosphorylation.

A functional replication stress response allows cells to recover from S-phase arrest and continue into G2 and M-phase. We presumed that reduced ATR signaling due to BRIT1 deficiency would compromise the RSR, and result in slower recovery from S-phase arrest following induction of replication stress. Indeed, our results show that cells with BRIT1 deficiency have a lower percentage of phospho-histone H3 (p-HH3) signaling compared to BRIT-normal cells after release from S-phase arrest (Figure 30E,F). Previous studies from our lab have shown that BRIT1 deficiency does not directly lead to a decrease in S-phase cells (Peng *et al.*, 2014; Peng *et al.*, 2009), and so the reduced p-HH3 staining in BRIT1-deficient cells is not due to a reduction in S-phase cells. Similarly, in our results where BRIT1 deficiency reduced TopBP1 foci (Figure 29A), this is also not due to reduced S-phase cells.

As mentioned in the introduction, BRIT1 is one of ten genes that associate with microcephaly. It's interesting to consider how the current finding that BRIT1 promotes ATR signaling helps to further explain how BRIT1 inhibits microcephaly. Previous findings show that BRIT1 inhibits microcephaly through recruitment of Chk1 to centrosomes, along with promoting

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HR to prevent neuroprogenitor cell apoptosis (Alderton et al., 2006). Along similar lines, ATR signaling is crucial in activating S-phase checkpoint so that stalled replication forks have an opportunity to undergo repair prior to replication restart, and to prevent fork collapse leading to double strand breaks and apoptosis (Flynn and Zou, 2011). The mutual inhibition of apoptosis by ATR and BRIT1 suggests that BRIT1 may inhibit apoptosis, and therefore microcephaly, in part through ATR regulation. This is supported by the fact that hypomorphic mutations in ATR is linked to Seckel syndrome, which shares characteristics with microcephaly (Majewski and Goecke, 1982). Seckel syndrome and microcephaly both display sensitivity to replication stress, and it's been suggested that similarities in the clinical features of these diseases are an effect of deficient ATR activity (Neitzel et al., 2002). Our data suggest that ATR-mediated phosphorylation of BRIT1 may be a mechanism to ensure sustained ATR signaling to stabilize replication forks (Figure 27, 28). Therefore, BRIT1 may contain ATR-independent mechanisms of suppressing microcephaly, e.g. regulation of Chk1 recruitment to centrosomes, while ATR inhibits microcephaly in a BRIT1-dependent mechanism. In addition to its role in replication fork stability, ATR and other Seckel factors participate in centrosome maturation. Others have shown that defective ATR causes supernumerary centrosomes, and impaired cell cycle progression (Alderton et al., 2004). This finding that ATR maintains centrosome structure parallels BRIT1's function of maintaining spindle formation and chromosome alignment during metaphase (Rai et al., 2008). Although our findings suggest that BRIT1 affects ATR signaling at replication stress sites, it's possible that BRIT1 may affect ATR function at centrosomes as well. Defects in centrosome maintenance affect cell proliferation and would therefore have implications in neuroprogenitor cell development (Higginbotham and Gleeson, 2007). Therefore, an interaction between BRIT1 and ATR at centrosomes provides a mechanism of maintaining chromosome stability to inhibit microcephaly.

In summary, our study on BRIT1-mediated TopBP1 recruitment has significance in both DNA damage repair and replication stress response. BRIT1 promotes DNA repair protein recruitment to the sites of DNA damage, and as we present here, also recruits TopBP1 to

promote the long-term activation of ATR signaling. With this new information, we can view BRIT1 as a mediator of early DNA damage response and checkpoint activation, as well as a protein involved in preventing genomic instability by maintaining the replication fork during DNA replication (Figure 31).
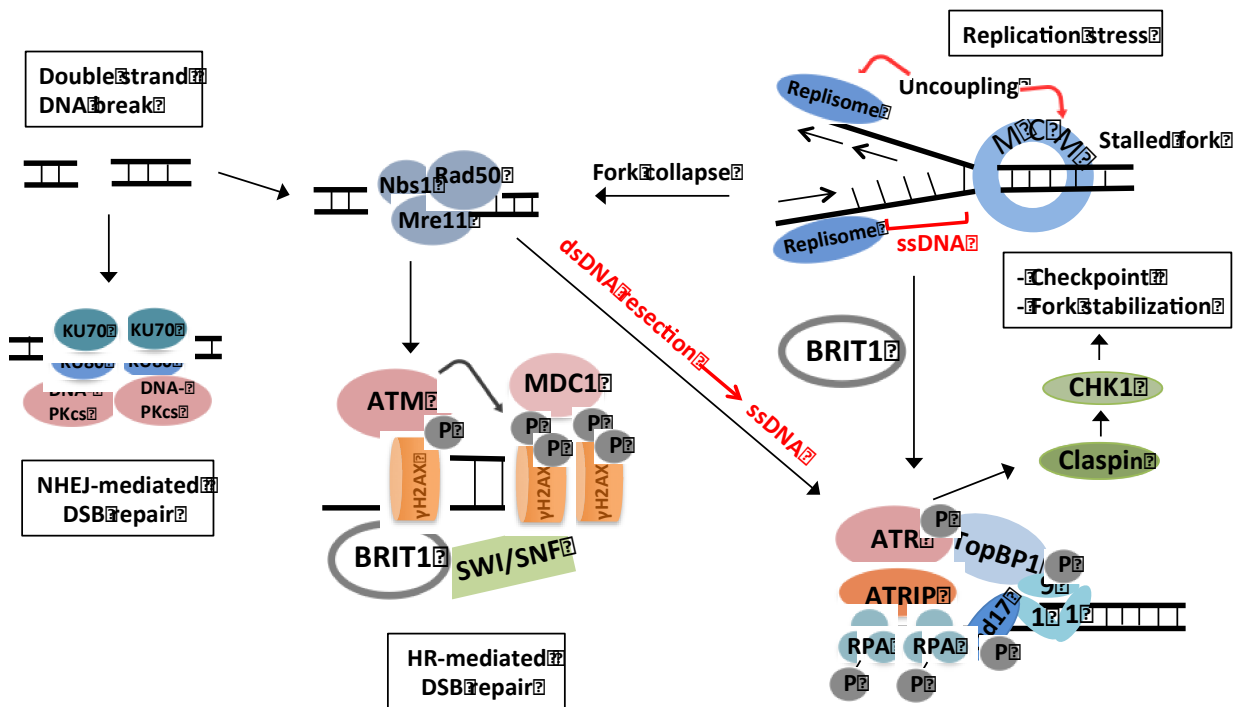
Figure 31. A model of BRIT1 functions in DNA damage repair and replication stress response.

BRIT1 has been shown to play a role in the early process of homologous recombination by recruiting repair proteins to the site of DNA damage. These include the SWI/SNF complex, 53BP1, MDC1 and Nbs1. Here we provide evidence that BRIT1 also is involved in the replication stress response during DNA replication. We propose that BRIT1 recruits TopBP1 to sites of ATR activation, and that this occurs through a direct interaction with TopBP1, and ATM/ATR-dependent phosphorylation of BRIT1 at Ser322. Together, these data demonstrate that BRIT1 promotes checkpoint response, which is required for proper repair of DNA damage and resolution of replication stress.

Chapter 5

CONCLUDING REMARKS

The findings described herein involve two novel functions of BRIT1. First, we have shown that BRIT1 interacts with MDM2 to inhibit MDM2 from targeting p53 for ubiquitylation and degradation. This is the first time that BRIT1 has been shown to play a role in the p53 pathway and to therefore have an effect on cellular apoptosis. Second, we have shown that BRIT1 is involved in the replication stress response through recruitment of TopBP1 to sites of replication stress. This finding provided evidence that BRIT1 not only functions upstream in the DNA damage response, but also functions in the replication stress response, in a manner that promotes the long-term maintenance of ATR signaling. Therefore, both findings are independently significant. However, it is interesting to speculate on the implications that the findings have on each other, as well as on other known functions of BRIT1.

Functional outcomes of replication stress signaling include cell cycle checkpoint activation and induction of apoptosis after a prolonged checkpoint. Since BRIT1 maintains ATR signaling, BRIT1 would therefore be expected to promote p53 transcriptional activity simply due to ATR-mediated phosphorylation and activation of Chk1, which in turn phosphorylates p53 to promote p53 activation. Therefore, our finding that BRIT1 promotes p53 stability through negative regulation of MDM2, adds a layer of complexity to ATR pathway regulation by BRIT1. Our findings suggest that not only does BRIT1 sustain ATR signaling locally at the site of replication stress, but BRIT1 also functions at a distance to relieve p53 from MDM2-mediated ubiquitylation and degradation. The latter may serve to ensure that a pool of p53 molecules are available for ATR or Chk1 targeting, and as suggested earlier, allowing an early p53 response upon replication stress.

We have already discussed the possibility that the ATR-mediated phosphorylation of BRIT1 at Ser322 may aid in BRIT1 binding to TopBP1. This is based on the fact that TopBP1 contains several BRCT domains capable of binding phospho-peptides, and that previous work

has shown that indeed, BRCT5 of TopBP1 binds to MDC1, which is important for TopBP1 recruitment (Wang et al., 2011). As the interaction between BRIT1 and TopBP1 is further studied with respect to BRIT1 phosphorylation, so too should the interaction between BRIT1 and p53 or MDM2 be inspected to determine if BRIT1 phosphorylation aids in p53 stabilization. Could BRIT1 phosphorylation affect its interaction with MDM2? Since BRIT1 is able to interact with MDM2 to stabilize p53 in the absence of DNA damage, we would expect that BRIT1 phosphorylation would not be required to stabilize p53 through the MDM2 inhibition mechanism. Possibly more interesting, is whether BRIT1 phosphorylation by ATR affects p53 transcriptional activity. Since BRIT1 localizes to replication stress sites or DNA damage sites, and we've shown that BRIT1 interacts directly with p53, it would be worth testing the possibility that BRIT1 interacts with and recruits p53 to promote p53 transcriptional activity. Since BRIT1 has previously been shown to interact with the E2F transcription factor to promote E2F target gene transcription (Yang et al., 2008), this sets the precedence for a similar function for BRIT1 and p53.

　　　　BRIT1's has been shown to promote chromatin relaxation through its interaction with the chromatin-remodeling complex, SWI/SNF (Peng et al., 2009). This BRIT1 function is crucial to the recruitment of downstream proteins in response to DNA damage. SWI/SNF has been shown to also promote derepression of DNA response genes upon replication stress (Minard et al., 2011). Therefore, our current findings involving BRIT1-mediated TopBP1 recruitment may also be aided by SWI/SNF recruitment. That is, BRIT1's role in chromatin relaxation may promote multiple mechanisms at the site of DNA damage or stress, including recruitment of double strand break repair proteins (Rai et al., 2006), recruitment of TopBP1 upon replication stress, and recruitment of transcriptional activators including E2F and potentially, p53.

　　　　Our studies have shown that BRIT1 deficiency promotes cell proliferation and transformation in a p53-dependent manner, and that ATR signaling is compromised due to reduced TopBP1 recruitment by BRIT1. We and others have shown that BRIT1 deficiency correlates with breast cancer progression (Rai et al., 2006; Richardson et al., 2011). Previous

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work suggests that BRIT1's role in DNA damage response is significant in such a way that loss of BRIT1 would compromise DNA repair processes, making cells susceptible to genomic instability and cancer. Here, we provide evidence for other tumor suppressor functions of BRIT1, which suggests that BRIT1 deficiency may promote cancer progression in ways besides upstream recruitment of proteins after double strand breaks. We show that BRIT1, by sustaining or promoting ATR signaling, appears to play a preventive maintenance role assuring replication forks are repaired and do not collapse to form DNA double strand breaks. This maintenance role of BRIT1 makes BRIT1 very unique in its ability to prevent DNA damage as well as being able to help repair ensuing DNA damage. The repair process then involves an upstream role in protein recruitment as well as the downstream role of stabilizing p53 by preventing MDM2-mediated degradation. Together, our studies support a multi-faceted approach for BRIT1 in tumor suppression.

Chapter 6

FUTURE WORK

Further explore the interaction between BRIT1 and MDM2/p53.

Our model depicting the interaction between BRIT1 and MDM2/p53 shows a ternary complex formed by these three proteins (Figure 14). We propose this model based on the evidence that BRIT1 binds to both MDM2 and p53 independently of each other (Figure 12) and that increased BRIT1 binding to MDM2 correlates with decreased binding between MDM2 and p53 (Figure 14). To validate this ternary complex, we could perform a double immunoprecipitation. For example, we could overexpress HA-MDM2 in U2OS, and use an anti-HA antibody against cellular lysate. The immunoblot would show bands for MDM2, p53 and BRIT1. However, to show that the three proteins are being pulled down as one complex, and not due to separate interactions between MDM2-p53 and MDM2-BRIT1, we would perform a second immunoprecipitation using an anti-p53 antibody. If the immunoblot shows all three proteins, this would confirm the ternary complex. However, if the immunoblot shows only p53 and MDM2, this means that the three proteins do not exist in a ternary complex. Since we have not performed this double immunoprecipitation experiment, we cannot rule out other potential models of interaction. An alternative model would be one where instead of a ternary complex, BRIT1 binds either MDM2 or p53, separately. In this model, BRIT1 binding to MDM2 effectively prevents MDM2 from binding to and degrading p53. In turn, p53 is stabilized, and the interaction between BRIT1 and p53, as suggested by their co-immunoprecipitation, may serve to further block p53 from binding to MDM2, or may serve purposes beyond protecting p53 from MDM2. A better understanding of the interaction between BRIT1 and p53 may shed light on which of these possibilities is most likely. An initial experiment would be to determine the region of p53 that binds BRIT1. Since MDM2 binds to the N-terminal transactivation domain of p53 (Chen et al., 1993), BRIT1 binding to p53 at the same region would suggest an inhibitory effect

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on MDM2-p53 interaction. Together, these experiments would provide evidence to support our proposed model, or to help generate a revised model for BRIT1-mediated p53 stabilization.

Determine the mechanism by which BRIT1 reduces MDM2 protein expression.

Figures 9-11 together provide evidence that BRIT1 competes with MDM2 to bind and stabilize p53 from degradation, and that BRIT1 overexpression reduces MDM2 expression. However, it is still unclear how BRIT1 reduces MDM2 expression. Our model in Figure 9D shows that, based on our data, BRIT1 promotes MDM2 ubiquitylation, which presumably leads to MDM2 degradation. This would explain the reduced MDM2 expression. However, it is not clear whether MDM2 autoubiquitylation is occurring or whether MDM2 is being targeted for ubqiuitylation. Despite MDM2 being able to undergo autoubiquitylation, this may not be the mechanism for how MDM2 expression is being reduced, considering MDM2 autoubiquitylation doesn't necessarily lead to degradation, as mentioned earlier (Itahana *et al.*, 2007). Therefore, it would be interesting to determine the precise manner in which BRIT1 reduces MDM2 expression. Could BRIT1 promote the interaction between MDM2 and an E3 ligase that regulates its stability, such as the PCAF acetyltransferase or β-TRCP? Experiments can be done to show whether BRIT1 expression correspondingly increases the binding between MDM2 and PCAF or between MDM2 and β-TRCP, and whether this causes changes in MDM2 ubiquitylation pattern. Certainly, it would also be important to determine whether BRIT1 promotes MDM2 activity as might be suggested from the decrease in MDM2 ubiquitylation when BRIT1 is deficient. That is, does BRIT1-mediated MDM2 ubiquitylation promote MDM2 binding with its E2 conjugating enzyme, which would actually increase MDM2 substrate targeting? This possibility is not likely, since BRIT1 reduces MDM2 expression. But based on the recent finding that ubiquitylation affects MDM2 activity, it is worth taking this possibility into consideration. Understanding the mechanism whereby BRIT1 negatively regulates MDM2 is significant because of the effect that this has on p53 stability. For example, when the DNA damage response is activated in cells where BRIT1 is deleted, could there be reduced p53

expression due to loss of BRIT-mediated regulation of MDM2? To test this, BRIT1 deficient

cells, e.g. MCPH1#1 lymphoblastoid cells or cancer cell lines with BRIT1 deficiency, and BRIT1

wild-type cells would be treated with various DNA stress stimuli to induce a DNA damage

response. The cells would then be analyzed for p53 protein expression, expression of p53

transcriptional targets, e.g. p21, expression of MDM2, as well as the cells' ability to induce

apoptosis and cell cycle checkpoint. Although we saw in our studies, BRIT1 regulation of p53

stability even in the absence of stress, it would be important to see how this regulation

translates to conditions of cellular stress.

Further demonstrate that BRIT1 stabilizes p53 expression

The *in vitro* data we have provided demonstrates that BRIT1 deficiency reduces p53

stability in the absence or presence of DNA damage (Figure 6 and 7). *In vivo,* we show that

BRIT1 deficiency promotes normal breast cell transformation, whereas BRIT1 overexpression

suppresses breast cancer growth. To further demonstrate that BRIT1 deficiency reduces p53

stability, we could perform experiments to test for the functional effects of such a reduction. *In*

vitro, we could perform p53 half-life studies after BRIT1 knockdown, and then blot for changes

in not only p53 expression, but also for decreases in expression of p53 target genes such as

p21 and 14-3-3 or cell cycle proteins, and BAX and PUMA for apoptotic proteins. Upon DNA

damage, BRIT1 deficiency is expected to reduce the level of p53-mediated apoptosis. This

reduction can be tested by comparing Annexin V staining between normal and BRIT1-deficient

cels, or by staining for caspase activation. *In vivo,* the tumor specimens from mice can be

sectioned and also stained for proteins downstream of p53 transactivation. The results from

tumor staining would provide evidence that the reduced tumor formation in BRIT1

overexpression cells is due to increased p53-mediated transcriptional activity.

Investigate the clinical applicability for BRIT1 regulation of p53 expression

TCGA data shows no significant correlation between BRIT1 DNA copy number and p53

protein expression in breast cancer (Figure 20). Although we have shown *in vitro* that BRIT1 stabilizes p53 protein expression, and that BRIT1 expression reduces breast tumor size *in vivo* (dependent on p53 status), we have yet to show that the reduction in tumor size is actually caused by more stable p53 expression in the BRIT1 overexpressing tumor cells. To test for this, tumors from both normal BRIT1 and BRIT1 overexpressing conditions would need to be analyzed. Each specimen would be analyzed for p53 protein expression, to check whether BRIT1 overexpression alone had an effect on p53 stability. Also, staining of p53 transcriptional targets would need to be checked to see whether p53 activity is elevated in the BRIT1 overexpressing cells. And in the comparatively smaller tumors where BRIT1 is overexpressing, histological analysis should be checked to determine if there is increased apoptotic cell death.

If after these analyses are performed, and it's observed that BRIT1 expression alone indeed promotes p53 protein expression and activity leading to reduced tumor size, it would then be important to understand why clinical samples do not show a significant correlation between BRIT1 DNA copy number and p53 protein expression. An initial test would be to first test additional cancer cell lines for BRIT1 and p53 correlation *in vitro*, to determine whether the effects we've observed is limited to certain cell lines. In other cell lines, there may be other mechanisms that maintain p53 expression. And even in cell lines where BRIT1 expression is normal, p53 protein expression may be low due to reasons beyond BRIT1 expression. Since p53 is highly mutated in cancer, it wouldn't be surprising that there are other factors that lower p53 expression. For instance, a study of the expression signature of p53 in breast cancer has shown that p53 transcript expression may be reduced in some cancers (Miller *et al.*, 2005). In these instances, even normal BRIT1 copy number may not be able to sufficiently stabilize p53, and there would be no correlation between BRIT1 DNA copy number versus p53 protein expression. However it would be interesting to see whether BRIT1 overexpression would have an effect on p53 protein even in cases where there is no correlation. If BRIT1 overexpression increases p53, this would suggest that BRIT1 is able to stabilize p53, but at diploid levels, the effect may not be significant due to other factors that reduce p53 expression.

It is well known that tumors with wild-type p53 are generally more sensitive to genotoxic agents than tumors with mutant p53 (Brosh and Rotter, 2009; Vogelstein and Kinzler, 2004). Based on our research however, even tumors containing wild-type p53 may be less sensitive to genotoxins if BRIT1 is deficient, as p53 stability and apoptotic activity is compromised. Therefore, it is of interest to conduct further correlation studies between BRIT1 and p53. These studies may aid in effective targeting of BRIT1-deficient tumors.

Further investigate the interaction between BRIT1 and TopBP1

The model for ATR activation through TopBP1 has been well studied (Figure 2). Our research adds to this model a mechanism for maintaining ATR signaling. However there are still details yet to be resolved. We have shown that BRIT1 binds to TopBP1 within the base pair region between 250-500. However we have not yet determined which region of TopBP1 binds to BRIT1. We would need to obtain TopBP1 constructs that lack each of the eight BRCT domains, and test the constructs for binding to BRIT1. It has been shown that the BRCT5 domain of TopBP1 interacts with phosph-MDC1 to promote TopBP1 recruitment (Wang *et al.*, 2011), the N-terminal BRCT domains interact with phospho-Rad9 of the 9-1-1 complex (Delacroix *et al.*, 2007), and several BRCT domains within TopBP1 interact with ATRIP (Kumagai *et al.*, 2006; Xu and Leffak, 2010). Since we found that ATR phosphorylates BRIT1 at Ser322, it seems possible that TopBP1 would bind BRIT1 at this site through one of its BRCT domains.

Even after a better understanding of the interaction between BRIT1 and TopBP1, it is still unclear where the interaction occurs within the cell. Is the complex recruited to the DNA en bloc, or does BRIT1 provide a docking site to recruit TopBP1 to the site replication stress or DNA damage? Figure 16 shows that BRIT1 binds to TopBP1 independent of DNA. This suggests that the proteins may be interacting distant from the site of damage. It would be interesting to see whether separation of the chromatin fraction still allows us to detect binding. Similarly, nuclear fractionation would tell us whether the proteins interact in the cytosol.

Furthermore, how does BRIT1-TopBP1 fit into the current model of ATR signaling? ATR deficiency has been shown to induce γH2AX formation, presumably in response to formation of double strand breaks (Chanoux *et al.*, 2009). Since our work shows that BRIT1 promotes the long-term accumulation of TopBP1, perhaps BRIT1 interaction with TopBP1 does not have an effect on ATR signaling until BRIT1 is able to localize to γH2AX foci. Since unresolved single strand breaks collapse and become double strand breaks, leading to H2AX phosphorylation, it would be interesting to generate a cell line that contains mutant H2AX unable to form γH2AX (Brown *et al.*, 2012), and to see whether BRIT1 is able to promote TopBP1 recruitment. BRIT1 has previously been shown to form foci dependent on γH2AX, so it is possible that TopBP1 recruitment depends on γH2AX formation (Wood et al., 2007a). ATR signaling that is maintained by BRIT1 can be tracked by immunoblotting for ATR target proteins such as p21, Chk1 and MCM.

Test BRIT1-deficient tumors for sensitivity to RSR-inducing therapeutics.

Replication stress inducing therapeutics such as cisplatin and gemcitabine induce DNA damage and genomic instability, leading to apoptosis. However, in cells that are proficient in DNA damage response in repair, the therapeutic effect of the drugs is diminished, as the damage becomes repaired. On the other hand, when either the ATM and/or ATR pathways are defective, drug sensitivity is increased. For example, in cells where ATM is defective (ataxia telangiectasia patient cells), the synergy of cisplatin and an ATR inhibitor is significantly increased (Reaper *et al.*, 2011). This is the case because functional ATM commonly rescues ATR defects, as they share common targets. When ATM is unable to compensate for ATR function, then cells are unable to resolve the damage and cells will more likely undergo apoptosis. In our studies, we showed that BRIT1 promotes the long-term activation of ATR signaling. Looking at this from a therapeutic perspective, BRIT1 deficiency would mimic ATR inactivation. Because of this, it would be interesting to test whether BRIT1 deficiency sensitizes cells to cisplatin or other DNA damaging agents, especially under conditions where the ATM

pathway is compromised. Liang *et al.* have already generated mice models with BRIT1 deficiencies. They have shown that shown that BRIT1 knockout mice were growth-retarded at adulthood, and were more sensitive to ionizing radiation, as all BRIT1-/- mice died within nine days post-IR, while the majority of BRIT1-/+ or +/+ mice survived past 4 weeks (n = 30/genotype) (Liang *et al.*, 2010). Another *in vivo* study demonstrated that BRIT1 deletion caused a defect in HR and increasing genomic instability in mice neuroprogenitor cells (Zhou *et al.*, 2013). Using similar animal models, BRIT1 deficiency can be tested for sensitivity to DNA damaging and stress-inducing therapeutics such as cisplatin and gemcitabine, especially when the ATM pathway is inhibited. If BRIT1 deficient cells do display elevated sensitivity, we could test whether ATR signaling is intact, to check whether drug sensitivity is caused by defects in ATR signaling and checkpoint activation. Together, these studies would help clarify whether BRIT1 promotes the replication stress response through ATR signaling.

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Vita

Edward Wang was born in Taipei, Taiwan on August 6, 1980 the son of Wen-Chin Wang and Ming-Chiun Wang. After graduating from Gunderson high school in San Jose, California in 1999, he attended De Anza Junior College in Cupertino, California. He then finished his undergraduate studies at the University of California at Santa Cruz, California where he obtained a Bachelor of Science degree in Molecular, Cellular and Developmental Biology in 2004. He then worked at Abgenix, Inc. and Amgen, Inc. through 2006 where he worked as a Process Development Associate. He then worked at OncoMed Pharmaceuticals as a Process Development Associate until 2008. In the fall semester of 2008, he entered the Ph.D. graduate program at the University of Texas Health Sciences Center at Houston Graduate School of Biomedical Sciences. In 2011, he joined the lab of Dr. Shiaw-Yih Lin at the University of Texas MD Anderson Cancer Center and studied the mechanisms of DNA damage repair.