

BAP1 loss in mesothelioma induces genome instability through BRCA1- dependent and independent mechanisms

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester

by

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MARCH 2019

DECLARATION

The accompanying thesis submitted for the degree of Doctor of Philosophy, entitled “BAP1 loss in mesothelioma induces genome instability through BRCA1-dependent and independent mechanisms” is based on work conducted by the author in the Department of Molecular and Cell Biology at the University of Leicester mainly during the period between April 2015 and March 2019. All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references. IHC samples were stained by Aarti Gaba at NHS and scored by Dr. Michael Sheaff. None of the work has been submitted for another degree in this or any other University.

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Summary

BRCA1 associated protein 1 (BAP1) is a tumour suppressor that is commonly inactivated in a plethora of hereditary and sporadic human malignancies including malignant pleural mesothelioma. However, the biological function and impact of BAP1 disruption in cancer remains poorly defined. The goal of this thesis is to characterize the function of BAP1 in regulating BRCA1 protein stability and reveal the consequence of its loss in mitosis in mesothelioma cells. First, we reveal that BRCA1 regulates spindle assembly checkpoint proteins and controls mitotic progression in response to vinorelbine in mesothelioma cells. Second, we report that BAP1 controls BRCA1 stability in mesothelioma cells with depletion of BAP1 protein inducing proteasome-mediated degradation of BRCA1. Hence, BAP1 loss phenocopied the loss of BRCA1 not only in promoting spindle assembly checkpoint failure but also causing centrosome amplification and chromosome segregation errors. However, loss of BAP1 led to additional mitotic defects that were not observed upon BRCA1 loss, including an increase in spindle length and enhanced astral microtubules. Intriguingly, these consequences were explained by loss of expression of the KIF18A and KIF18B kinesin motors in response to BAP1 depletion. Finally, we showed a significant correlation in expression of BAP1 and BRCA1 in tumour samples taken from mesothelioma patients. Hence, we demonstrate both BRCA1-dependent and –independent roles for BAP1 in mitotic progression. Our findings also suggest that BAP1 may predict response to therapeutic agents such as vinorelbine and may be used as a novel biomarker for stratified treatment in mesothelioma.

Acknowledgements

First and foremost, I would like to thank my supervisor Prof. Andrew Fry for the support, encouragement and advice he has provided during these past four years. Andrew is one of the smartest people I know and hope that I could be as enthusiastic and energetic like him and to someday be able to command an audience as him. I would also like to thank my co-supervisor Dr. Raj Patel for his useful suggestion and discussions. Many thanks to my committee member Prof. Dean Fennell and Dr. Sue Shackleton for their advice and knowledge and many insightful discussions and suggestions.

A big thank you to Dr. Kees Straatman for all his support and guidance with the microscope imaging facility. A massive thank you to all past and present members of the Fry lab, who have been very supportive and helpful throughout my project. To my favourite lab girlfriends, Caroline, Alice, Georgina, Emily and Lia, who celebrated my every achievement big or small, they made lab life fun and entertaining and mostly for their big support. I will never forget the many wonderful lunches, dinners and fun activities we have done together. I would also like to thank Laura, Josephina, Tara and Rob who helped me get started in the lab and continue to offer advice.

To my family, Mum, Dad, my sister Neelam, my furbaby Scissor and my wonderful husband Shiv there is nothing I can say that would enunciate my feelings of appreciation. Your ongoing love and support is extraordinary. Truly, I am thankful to have you.

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ABBREVIATIONS

aa	amino acid
A	absorbance
AM	Alveolar macrophages
APC/C	Anaphase promoting complex/cyclosome
ASXL	Additional sex combs-like
APS	Ammonium persulfate
BSA	Bovine serum albumin
CO ₂	Carbon dioxide
DUB	Deubiquitinating enzymes
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
γ -TuRC	γ -tubulin ring complex
GFP	Green fluorescent protein

H ₂ O ₂	Hydrogen peroxide
HBM	HCF-binding motif
HCF-1	Host cell factor 1
HRP	Horseradish peroxidase
IF	Immunofluorescence microscopy
IHC	Immunohistochemistry
kDa	kilo Daltons
kMT	kinetochore microtubule
MAP	Microtubule associated protein
μg	micro-gram
μl	micro-litre
μM	micro-molar
μm	micro-meter
ml	milli-litre
mM	milli-molar
MPM	Malignant pleural mesothelioma

mRNA	messenger ribonucleic acid
MT	Microtubule
MTOC	Microtubule organising center
min	minutes
NLS	Nuclear localization signal
nm	nano-meter
nM	nano-molar
NP-40	Nonidet P-40
OD ₆₅₀	Optimal Density (absorbance) at 650 nm
PBS	Phosphate buffered saline
PCM	Pericentriolar material
Pen/Strep	penicillin/streptomycin
pH3	phospho-histone H3
PI	propidium iodide
PIC	Protease inhibitor cocktail
PR-DUB	Polycomb Repressive Deubiquitinase Complex
RIPA	RadiolImmuno Precipitation Assay Buffer

RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
rpm	revolutions per minute
RPMI	Rosewell Park Memorial Institute
SAC	Spindle assembly checkpoint
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
siRNA	small interfering RNA
STLC	S-trityl-L-cysteine
TEMED	N, N, N, N,-tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
v/v	volume per volume ratio
V	Volts
Ub	Ubiquitin C-terminal hydrolase
UCH	Ubiquitin
ULD	UCH-5 like domain

UPS	Ubiquitin proteasome system
IR	Irradiation
WT	Wild-type

CHAPTER 1

INTRODUCTION

1.1 Mesothelioma

1.1.1 Pathology, aetiology and epidemiology

Malignant mesothelioma is a rare aggressive neoplasm that originates from the mesothelial cells lining the body cavity of the pleura, pericardial, peritoneum and the tunica vaginalis (Kao *et al.*, 2010). Most commonly affected is the pleura, that originates in the parietal pleura and spreads to the visceral pleura. This specific form of cancer, also known as malignant pleural mesothelioma, accounts for 70% of all mesothelioma cases (Moore *et al.*, 2008). In this thesis, the focus is on malignant pleural mesothelioma (MPM).

The principal carcinogen associated with MPM is asbestos (Robinson & Lake, 2005). It was once known as the 'magic mineral' because of the outstanding properties of the substance. It is cheap, durable, electric and heat-resistant (Tweeddale, 2001). The term asbestos collectively refers to a group of naturally occurring fibrous minerals that have been commercially exploited (Sporn, 2014). These fibres belong to two mineral groups: serpentines and amphiboles (Robinson & Lake, 2005). Chrysotile belongs to the serpentine group whereas anthophyllite, amosite, crocidolite, tremolite and actinolite belong to the amphibole group (Virta, 2002). Mesothelioma is mostly associated with amphibole fibres (Virta, 2002; Churg & Wiggs, 1984; Roggli *et al.*, 2002; Hodgson & Darnton, 2000; Roggli, 2015).

The mesothelioma death rate in Britain is one of the highest in the world, with 2,496 deaths in 2016 (Cancer Research UK, 2019). However, the numbers are already decreasing in Western developed countries as result of the ban on the use of asbestos. By contrast it is expected that the rate of this disease will rise in countries that continually use or have increasing use of asbestos. It is still mined and exported by several countries including Canada, South Africa, Russia, China, India and Brazil without adequate or any precautions (Figure 1) (Frank & Joshi, 2014; Rudd, 2010).

The entire surface of the pleura is covered by a single layer of mesothelial cells that aid free movement of the pleural surfaces during respiration by expressing on their surface lubricating glycoproteins that are able to proliferate in response to injury and growth factors (Robinson *et al.*, 2005). Several mechanisms of damage to the pleural layer of mesothelial cells caused by asbestos fibres have been identified. Epidemiological evidence shows that the most dangerous fibres are thin and long and penetrate the lung, causing pleural irritation and leading to prolonged cycles of tissue damage, repair and local inflammation (Robinson *et al.*, 2005). This can lead to scarring (plaques) or initiate mesothelioma (Figure 2).

MPM is pathologically divided into the following subtypes (Zandwijk *et al.*, 2013):

- Epithelioid: this subtype accounts for 60% of all cases. These tumours have polygonal, oval or cuboidal cells that often mimic reactive mesothelial cells that occur in response to various types of injury.
- Sarcomatoid: this subtype represents 10-20% of mesotheliomas. These tumours consist of spindle shaped cells that may mimic malignant mesenchymal tumours.
- Biphasic: this subtype contains a mixture of epithelioid and sarcomatoid areas within the same tumour and accounts for 30% of mesothelioma cases.

The latency period between asbestos exposure and first clinical signs of this cancer is long and after at least 30 years. However, it has been shown that only a minority of asbestos exposed individuals develop MPM. In fact, the incidence was shown to vary between 0.5% and 18% amongst professionally exposed workers (Melaiu *et al.*, 2018). Alternative causes have also been suggested such as exposure to erionite, a zeolite mineral with some physical properties similar to asbestos. Exposure to erionite caused high rates of MPM in the rural village of Tierra Blanca de Abajo, Mexico, and in some Turkish villages (Demirer *et al.*, 2015; Carbone *et al.*, 2011; Ortega-Guerrero *et al.*, 2015).



Figure 1.1 Asbestos bans and regulation

Fifty-five countries have banned use of all types of asbestos including UK and Europe. Roughly around 140 countries including China, India, Mexico, Thailand, Indonesia, Brazil, Canada and many more, still permit full or partial use of asbestos, with little or no regulations at all. Many Asian-Pacific countries still allow import and export of asbestos. Major asbestos containing products that are still used are asbestos-cement sheets, textiles and insulation products. Taken from International Ban Asbestos Secretariat.

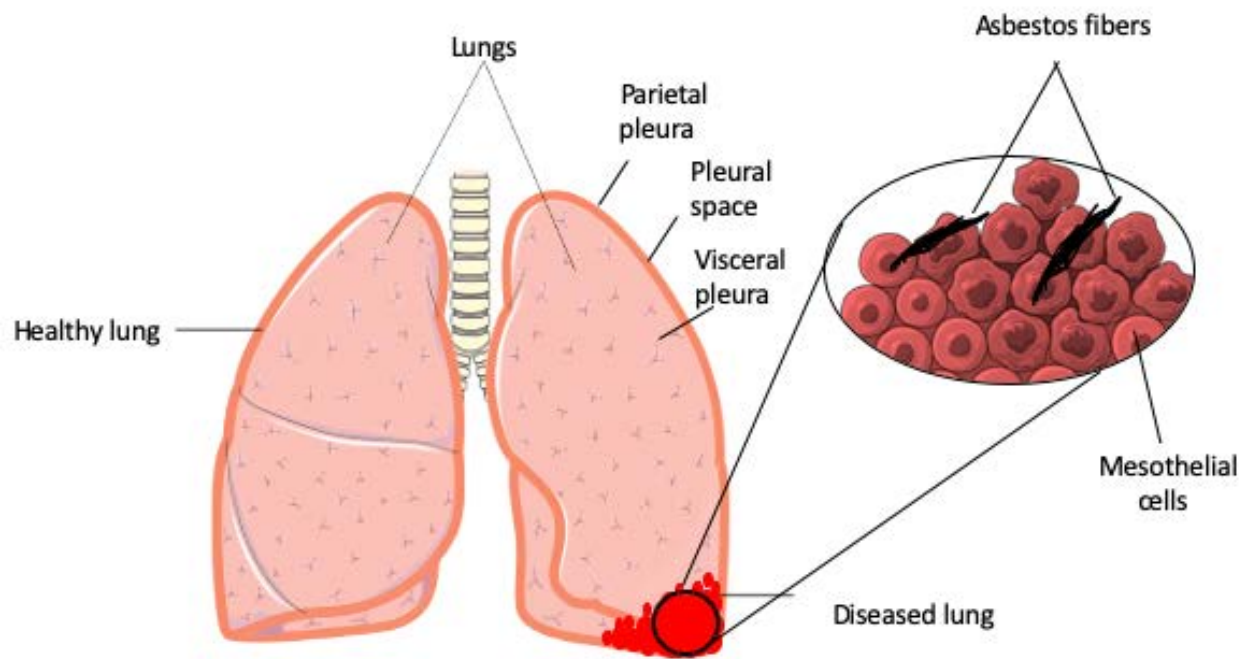


Figure 1.2 Parts of lung affected by pleural mesothelioma

Parietal and visceral pleura are membranes of the thoracic cavity. Parietal pleura line the inner surface whereas visceral pleura line the lungs. The pleural cavity is the space between the parietal and visceral pleura and is filled with fluid to reduce the friction caused by movement of the lungs during inhalation and exhalation. Asbestos fibres once inhaled accumulate in the lungs or the pleural lining leading to inflammation, scarring and genetic modifications within the cells.

1.1.2 Disease mechanisms

The primary mechanism of asbestos-related cancer is chronic inflammation with the ongoing generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Xu *et al.*, 1999; Robledo & Mossman, 1999). Asbestos fibers may also directly activate ROS producing enzymes, such as nicotinamide adenine dehydrogenase (NADPH) oxidase, and phospholipase C pathways with secondary activation of NADPH oxidase (Kamp & Weitzman, 1999). The fibers can also generate ROS in the presence of iron (present at approximately 26-30% in crocidolites and 1-3% in chrysotiles) leading to hydroxyl radical formation, lipid peroxidation and DNA breaks. The iron coating of these fibers serves as a catalyst for generation of hydroxyl radical formation. Treatment with desferroxamine (an iron chelator) inhibits the asbestos induced generation of hydroxyl radical formation suggesting that the iron content of asbestos fibres is critical to generation of ROS (Mossman & Marsh, 1989). Asbestos has also been reported to contribute to numerical and structural chromosomal aberrations, DNA breaks, impairment of cell division and aneuploidy (Huang *et al.*, 2011).

Fiber toxicology has been associated with three key factors: dose, dimension and durability. The dose of fibers is determined by physical characteristics/dimensions and exposure level. Fiber dimensions are important as only very thin fibers are respirable and penetrate into the deep alveolar region of the lung. Fiber length also impacts their toxicity as short fibers can be fully engulfed by macrophages, whereas the long fibers that macrophage cannot fully engulf are biopersistent and lead to disease (Mossman *et al.*, 2011). Pulmonary macrophages play a key role in asbestos induced chronic inflammation in the lungs (Rom *et al.*, 1991). Alveolar macrophages (AMs) are the first line of defence as increased numbers of activated AMs have been found in patients exposed to high concentrations of asbestos fibers (Rom *et al.*, 1987). AMs provide dual defence mechanisms for the lower respiratory tract by phagocytosis and by removal of inhaled fibers through promoting local immunological events. A phagocytic cell helps the AMs clear the lungs of inhaled particles. Humans and animals exposed to high concentrations of asbestos show phagocytized fibers in several AMs (Rosenthal *et al.*, 1998).

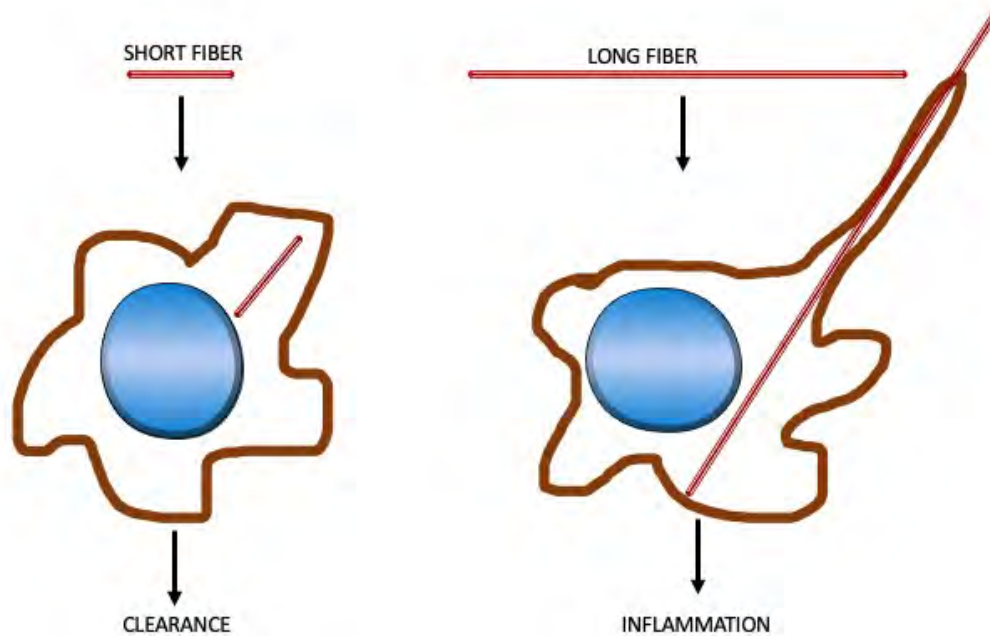


Figure 1.3 Diagrammatic representation of frustrated phagocytosis

When confronted by short asbestos fibres, the macrophage can enclose and clear them (left). However, the macrophage cannot extend itself sufficiently to enclose long asbestos fibres, resulting in incomplete or frustrated phagocytosis (right), which leads to inflammation. Adapted from Schinwald & Donaldson (2012) & Donaldson *et al.* (2010).

AMs are protected against toxic free radicals by several endogenous antioxidant defence systems, such as superoxide dismutase, catalase, glutathione and glutathione peroxidase. However, during inflammation prolonged production of ROS may occur. This may impair cellular antioxidants resulting in lipid peroxidation, enzyme inactivation, protein denaturation and DNA damage causing an injury to phagocytes and damage to surrounding tissues. Macrophages exposed to crocidolite fibers were stimulated to release ROS (Goodglick & Kane, 1986). Frustrated phagocytosis is not the only mechanism by which ROS are released since short fibers ($75\% \leq 1.0\mu\text{m}$), which can be completely engulfed by phagocytes caused similar H_2O_2 release to long fibers (Kamp & Weitzman, 1999) (Figure 3). ROS-dependent inflammasome (caspase activating complex) activation is an important sensor of asbestos and trigger of pulmonary inflammation (Dostert *et al.*, 2008; Cassel *et al.*, 2008).

Asbestos induced chronic inflammation activates multiple signalling cascades. Asbestos exposed cells show a rapid increase of mitogen-activated protein kinase (MAPK) signalling subsequently activating transcription factors, such as activator protein-1 (AP-1) and NF- κ B. These transcription factors are known to be involved in the activation of a number of downstream genes that regulate apoptosis, inflammation and proliferation. These genes might well therefore be associated with asbestos induced carcinogenesis (Huang *et al.*, 2011).

1.1.3 Genetics: acquired and inherited mutations

The Cancer Genome Atlas (TCGA) Research Network conducted a comprehensive integrated genomic study of MPM, to expand the understanding of MPM. Significantly mutated found in this study were *BAP1*, *NF2*, *TP3*, *LATS2* and *SETD2*. All the five genes showed high rates of mutations including nonsense, frameshift and splice-site alterations. TCGA tumours revealed somatic mutation in the DNA damage repair genes such as *BAP1*, *NF2* and *CDKN2A* that significantly associated with progression free and overall survival (TCGA, Pan cancer atlas; Hmeljak *et al.*, 2018; Ladanyi *et al.*, 2018; Sage *et al.*, 2018). As only a small fraction of individuals exposed to asbestos develop MPM, it is expected that both acquired and inherited genetic factors also play a role in predisposing to MPM (Cheung *et al.*, 2017).

Germline mutations identified *BAP1* as a predisposing gene in mesothelioma. Two families with a high incidence of mesothelioma despite no exposure to asbestos were identified. Six members from one family had *BAP1* germline mutations: four were diagnosed with mesothelioma, while two had breast or renal cancer. These individuals had identical *BAP1* mutations, whereas the unaffected family members did not (Testa *et al.*, 2011). Comparative genomic hybridization (CGH) analysis of two tumours (one per family) also identified alterations encompassing or adjacent to the *BAP1* locus at 3p21.1 (Testa *et al.*, 2011). This added to previous findings that 3p21.1 is a site of recurrent chromosomal loss in sporadic mesothelioma (Flejter *et al.*, 1989; Lu *et al.*, 1994; Murthy & Testa, 1999; Apostolou *et al.*, 2005; Neragi-Miandoab & Sugarbaker, 2009).

Several other germline mutations have been identified within the *BAP1* gene, with most mutations at the 3' end of the coding region (Ohar *et al.*, 2016; Cheung *et al.*, 2013). Recently, about 100 families with germline *BAP1* mutations have been described in the United States, Europe and New Zealand with genetic testing indicating that these mutations are transmitted across several generations (Carbone *et al.*, 2017). The majority of inherited mutations in *BAP1* are nonsense mutations or mutation that incur a premature stop codon, often resulting in the loss of nuclear localization signals and/or the C-terminal domain.

Besides the rare inherited *BAP1* mutations, 40%-60% of all mesothelioma have acquired somatic alteration of *BAP1* (Sneddon & Creaney, 2016). A variety of *BAP1* alterations were identified that were scattered throughout the gene including nonsense mutations, missense mutations, frameshift mutations and mutations at or near the splice sites (Zauderer *et al.*, 2013). Moreover, 25% of tumours do not express BAP1 protein without any genetic alteration in *BAP1*, suggesting post-translational dysregulation (Delsite *et al.*, 2011; Alakus *et al.*, 2015; Nasu *et al.*, 2015).

Cyclin dependent kinase inhibitor 2A (*CDKN2A*) deletion is seen in 72% of primary mesothelioma tumours and is mutated with an even higher frequency in mesothelioma cell lines (Prins *et al.*, 1998; Cheng *et al.*, 1993). Furthermore, *CDKN2A* promoter methylation has been described as an alternative mechanism for its inactivation (Hirao *et al.*, 2002).

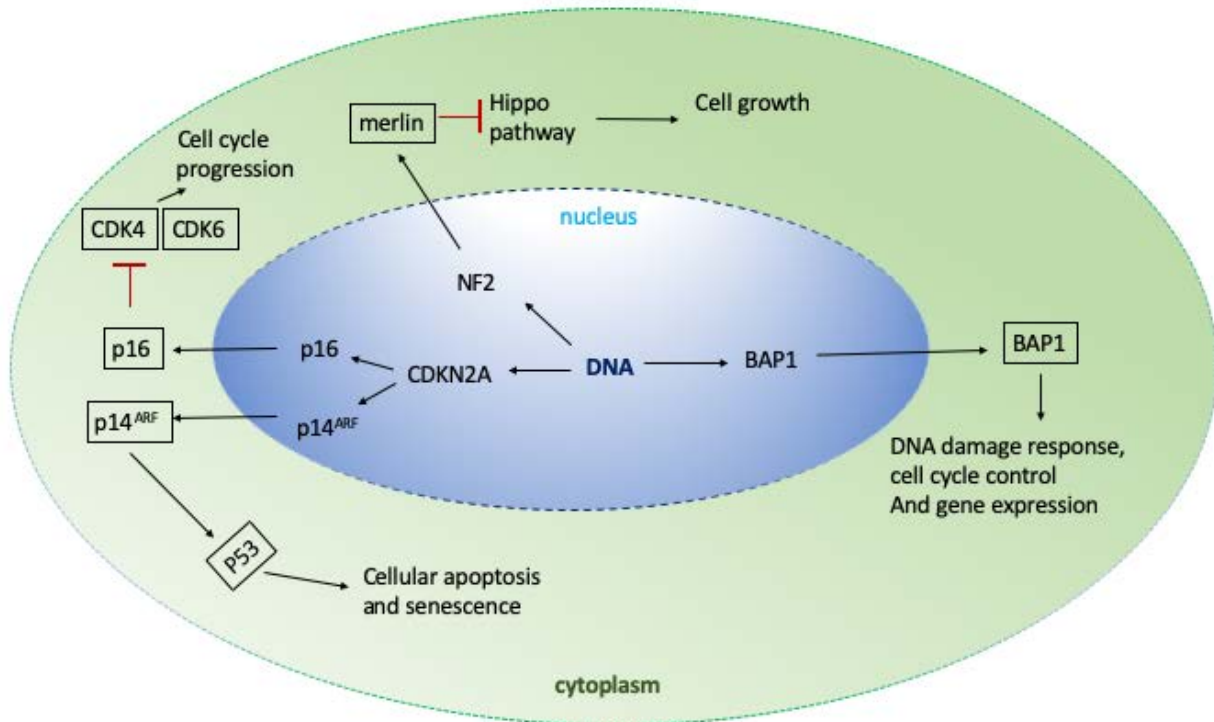


Figure 1.4 Genes and proteins involved in the development of MPM

Mutations in the *BAP1* gene dysregulate the DNA damage response, chromatin modification, cell cycle control and transcription. Inactivation of merlin activates the Hippo signalling pathway that plays a major role in the cell proliferation and tissue growth. *CDKN2A* encodes two important cell cycle regulatory proteins, p16 that inhibits CDK4/CDK6 and blocks phosphorylation of the RB protein, and p14^{ARF} which protects p53 from degradation. Adapted from Assis *et al.* (2014).

The *CDKN2A* gene encodes two cell cycle regulatory proteins: the p16 and p14ARF. Both proteins are tumour suppressors, p16 inhibits CDK4 and CDK6 which blocks transition from G1 to S phase whereas p14ARF activates p53. Forty percent of mesothelioma cases show mutations in the neurofibromatosis type 2 gene (*NF2*) that encodes the tumour suppressor merlin. These mutations include deletions and insertions that lead to truncations and inactivation of merlin. Merlin downregulates the expression of oncogenes through regulation of Hippo signalling (Figure 4). At the plasma membrane, merlin recruits LATS1/2 kinases that phosphorylate and inhibit the Yes-associated protein (YAP) transcription factor, a downstream component of the Hippo pathway. Mutations are also observed in the Hippo pathway components, including *LATS1/2*, *SAV1* and *AJUBA* (Sato & Sekido, 2018). While activating mutations in the YAP gene are also observed in mesothelioma. Together, these promote tumour growth, motility and invasion in mice (Kakiuchi *et al.*, 2016; Mizuno *et al.*, 2012). The progressive activation of YAP is also associated with remodelling of the extra-cellular matrix (ECM) (Rehrauer *et al.*, 2018). YAP-dependent transcriptional activation of cyclin D2 (*CCND2*), forkhead box M1 (*FOXM1*) and phospholipase C beta 4 (*PLCB4*) promote mesothelioma cell growth indicating that YAP regulates several cellular processes leading to malignant mesothelial cell transformation (Sato & Sekido, 2018). Merlin inactivation in mesothelioma also upregulates the mTOR pathway although no mutations in the mTOR genes have been identified so far.

TNF receptor associated factor 7 (*TRAF7*) which belongs to the tumour necrosis factor (TNF) is commonly mutated in mesothelioma (Bouwmeester *et al.*, 2004). TRAF7 is an E3 ubiquitin ligase that ubiquitinates and promotes degradation of an apoptosis inhibitor, FLIP (Rippo *et al.*, 2004). *NF2* and *TRAF7* mutations are mutually exclusive in mesothelioma suggesting that they may use a common signalling pathway (Bueno *et al.*, 2016).

1.1.4. Treatment options

As with most cancers, current treatment options for MPM include surgery, radiation therapy and chemotherapy (Haas & Serman, 2013). Surgery for MPM patients can be diagnostic, palliative or reductive, although potentially associated with significant

morbidity and mortality. Radiation therapy is limited by the large treatment volumes required and the radiation sensitivity of the surrounding organs (heart, lung, esophagus and spinal cord) (Haas & Stermann, 2013). However, as most patients diagnosed with MPM have unresectable disease, the first line of treatment is chemotherapy. The standard first line of treatment consists a combination of cisplatin and pemetrexed which increases the median overall survival (OS) from 9.3 to 12.1 months compared with cisplatin alone (Boyer *et al.*, 2018). Cisplatin can crosslink with the purine bases in the DNA. This interferes with DNA repair mechanisms causing DNA damage, which activates several signal transduction pathways, including ATR, P53, P73 and MAPK, and culminates in the activation of apoptosis (Siddik, 2003; Dasari & Tchounwou, 2014). Pemetrexed is a multi-targeted anti-folate compound that blocks several enzymes in the folate metabolism pathway. Pemetrexed is a potent inhibitor of thymidylate synthase (TS), the rate-limiting enzyme in the synthesis of thymidylate, which is required for DNA synthesis (Haas & Stermann, 2013). Unfortunately, nearly all patients progress during or after first-line of treatment (Zucali *et al.*, 2014).

1.2 Ubiquitin proteasome system

1.2.1 Ubiquitination

Ubiquitination is a common post-translational modification, that involves the covalent attachment of ubiquitin (Ub) to a target protein. This can regulate the stability, function or localization of the modified protein (Reyes-Turcu *et al.*, 2009). Proteomic analyses demonstrated that thousands of proteins can be modified with ubiquitin (Komander & Rape, 2012). Ub is a highly conserved 76 amino acid protein present from yeast to humans (Eletr & Wilkinson, 2014; Lauwers *et al.*, 2010). Ubiquitination of proteins is catalysed by the combined enzymatic actions of a Ub-activating E1 enzyme (2 human genes encode E1 enzymes) (Xu *et al.*, 2010), a Ub-conjugating E2 enzyme (40 human genes encode E2 enzymes) (Metzger *et al.*, 2014) and a E3 ligase enzyme (Reyes-Turcu *et al.*, 2009) (around 600 human genes encode for E3 enzymes) (Metzger *et al.*, 2012).

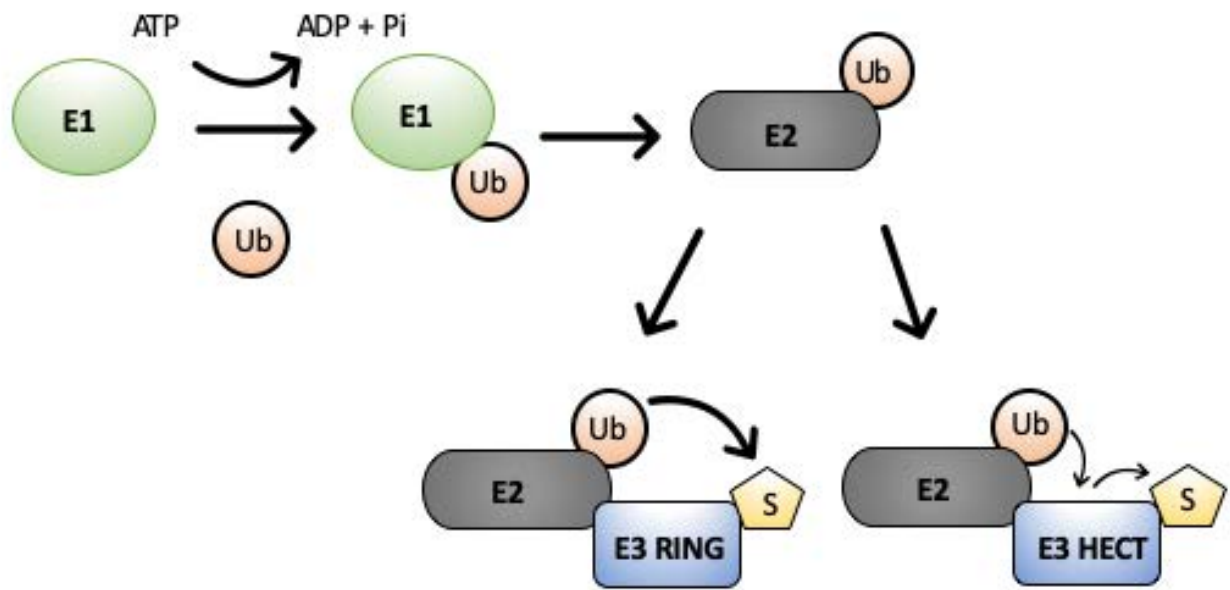


Figure 1.5 The enzymatic cascade for the ubiquitination of substrates

Free Ub is activated by a Ub-activating enzyme (E1), using ATP to form a complex with Ub. E1 transfers Ub to a Ub-conjugating enzyme (E2). E2 then binds to an E3 Ub ligase, which then facilitates ubiquitination of the substrate protein. There are two major classes of E3s: RING ligases, which chaperone the Ub-bound E2 to, and facilitate the ubiquitination of the, targeted substrate. HECT ligases, directly conjugate Ub before ligating it to the targeted substrate. Adapted from Perrett *et al.* (2011).

Ultimately, this results in the covalent attachment of Ub to the amino side-chain of a lysine or α -amino group of the protein substrate (Figure 5). Proteins can be modified with one Ub moiety (mono-ubiquitination) or with multiple Ub molecules that can form linear or branched chains (poly-ubiquitination) (Komander & Rape, 2012).

Ubiquitin has eight potential internal ubiquitination sites with seven lysine (K6, K11, K27, K29, K33, K48, K63) residues along with a primary amine at the N-terminus, MET-1, all of which can participate in polyubiquitin chain formation (Komander & Rape, 2012; Swatek & Komander, 2016; Yau *et al.*, 2016). Ubiquitin polymers can be homotypic with linear chains all connected through the same linkage or heterotypic, in which they contain several different types of linkages or one ubiquitin modified on multiple sites to form branched ubiquitin chains (Figure 6). Heterotypic chains provide different functionalities and can constitute independent signals in degradation and signalling (Mevissen & Komander, 2017).

1.2.2 Function of ubiquitin linkages

Mono-ubiquitination is the addition of a single ubiquitin moiety at one or multiple lysine residues of a target substrate. Whereas, addition of polymeric chains leads to poly-ubiquitination. Mono and poly-ubiquitination are implicated in endocytosis at the plasma membrane, the sorting of proteins to the multi-vesicular body, nuclear export, DNA repair, chromatin regulation and transcriptional regulation (Haglund *et al.*, 2003; Haglund *et al.*, 2003; Brooks *et al.*, 2004; Sun *et al.*, 2011; Hicke, 2001).

The classic function of Lysine 48 (K48) linked ubiquitin chains that usually consist of a minimum of four ubiquitin moieties is to enable high affinity interaction with the 26S proteasome. This generally leads to degradation of the targeted proteins by the proteasome (Li & Ye, 2008; Meyer & Rape, 2014). Lysine 11 (K11) linked ubiquitin chains also drive proteasomal degradation and mitotic exit. The abundance of K11 linkages strongly increase during mitosis when the anaphase promoting complex/cyclosome (APC/C) is active (Akutsu *et al.*, 2016).

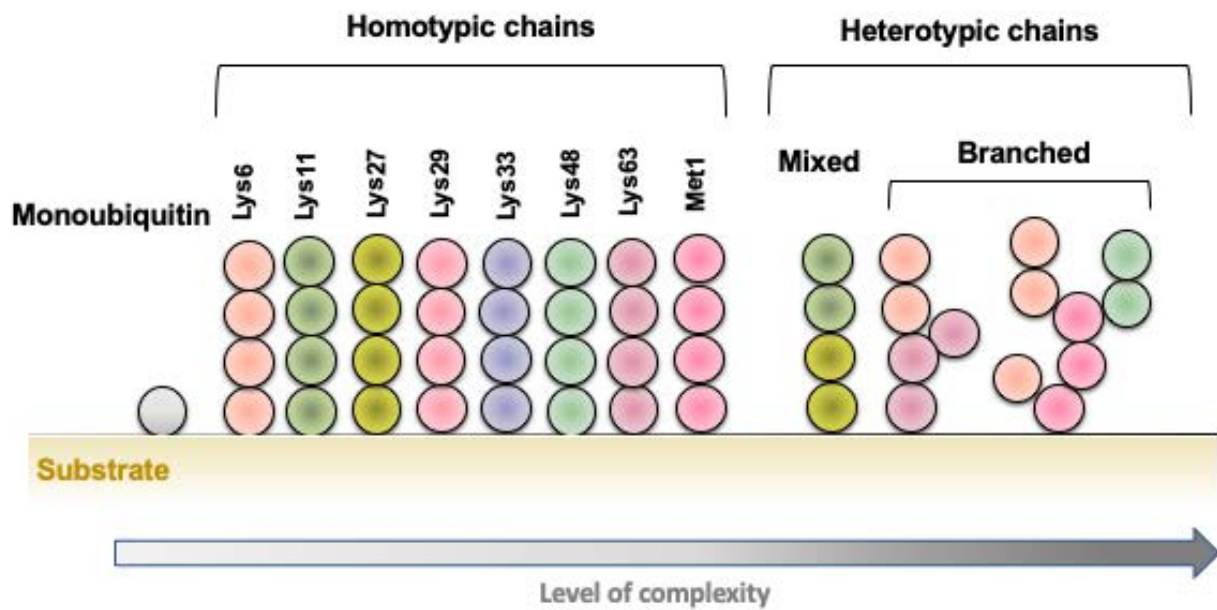


Figure 1.6 Schematic representation of diverse ubiquitin modifications

Mono-ubiquitination, the least complex modification, can be extended on the side-chains of lysine residues or the N-terminal MET-1 residue, resulting in eight homotypic poly-ubiquitin chains. Heterotypic chains have more than one linkage type in mixed or branched polymers. Adapted from Mevissen *et al.* (2017).

In contrast, Lysine 63 (K63) linked ubiquitination does not induce proteasome-mediated degradation but serves as a platform for protein-protein interaction. This is important for kinase signaling, receptor endocytosis, protein trafficking and DNA damage repair. Emerging evidence also suggests that K63-linked polyubiquitination plays a pivotal role in the regulation of the inflammatory response (Liu *et al.*, 2018; Baker & Ghosh, 2009; Wang *et al.*, 2012).

Lysine 27 (K27) linkages are essential for activation of the DNA damage response. K27-linked poly-ubiquitination is promoted by RNF168 on histone 2A (H2A) (Gatti *et al.*, 2015). K27 linkages also trigger host immune responses to microbial DNA (Akutsu *et al.*, 2016). Lysine 29 (K29) linkages are shown to inhibit Wnt signaling (Akutsu *et al.*, 2016), while Lysine 33 (K33) linkages play an important role in regulating T cell activation (Huang *et al.*, 2010). K29/K33 poly-ubiquitin linkages also regulate AMPK (AMP-activated protein kinase) regulated cell polarity (Al-Hakim *et al.*, 2008). Linear ubiquitin chains on MET1 (M1) play roles in both inflammatory and immune responses by regulating the activation of the transcription factor NF- κ B (Akutsu *et al.*, 2016). The tumour suppressor protein BRCA1 is reported to be modified with Lysine 6 (K6) poly-ubiquitinated linked chains, which do not result in destabilization of BRCA1. K6 linkages have also been identified on proteins on the mitochondrial outer membrane (MOM). K6 and K33 linkages increase in response to UV radiation, suggesting they also contribute to the DNA damage response (Akutsu *et al.*, 2016). While functions of several homotypic Ub chains have been characterized, the role of different heterotypic chains remain are only beginning to be understood. K11/K48 heterotypic linkages target mitotic regulators for degradation (Yau *et al.*, 2017).

K11/K63 linked chains are formed during endocytosis of the major histocompatibility complex class I (MHC 1) membrane proteins. K29/K48 linked chains are involved in the ubiquitin fusion degradation (UFD) pathway (Ohtake & Tsuchiya, 2016). Acetylation of ubiquitin at K6/K48 inhibits recognition by E2 enzymes to repress ubiquitination. K63/M1 heterotypic linked chains are important in NF- κ B signaling pathways. Determining how these combinations of heterotypic or branched chains alter the ubiquitin code, and associated biological outcomes, is an important future step towards understanding the complexity of ubiquitin signals.

1.2.3 De-ubiquitination

Deubiquitinating enzymes (DUBs) function to remove covalently attached ubiquitin from proteins, thereby controlling substrate activity and/or abundance (Sowa *et al.*, 2009). The main roles of DUBs can be summarized as follows: 1. Modulating E2 activity: DUBs can inhibit ubiquitination by interfering with the formation and reactivity of the E2-Ub intermediate. This opposes the activities of the ubiquitination machinery and indicates that DUBs can modulate the dynamic balance of the UPS both catalytically and non-catalytically (He *et al.*, 2016). 2. Counteracting E3s: several DUBs associate directly with E3 ligases in pairs or complexes. DUBs can also counteract the tendency of E3 ligases to auto-ubiquitinate in the absence of other substrates. This interaction may also target the DUB for degradation through the ligase-catalyzed ubiquitination of the associated DUB (Wilkinson, 2009). 3. Resisting proteasomal degradation: DUBs can antagonize protein degradation by trimming Ub chains leading to a decreased affinity of the protein for the proteasome (He *et al.*, 2016; Jacobson *et al.*, 2014). 4. DUBs in endocytic pathways: DUBs can localize to endosomes through interactions with the endosomal sorting complex. This is required for transport of components of the ESCRT machinery. 5. Ub homeostasis: this includes generation of Ub precursors, trimming of Ub precursors to free Ubs, and disassembling of poly-ubiquitination chains from proteins (He *et al.*, 2016) (Figure 7).

DUBs belong to the larger superfamily of proteases, of which there are an estimated 561 members in the human genome. There are two classes of Ub proteases: cysteine proteases and metalloproteases. Cysteine Ub proteases can be further distinguished into four classes based on their Ub-protease domains: ubiquitin specific protease (USP), ubiquitin C-terminal hydrolase (UCH), Otubain protease (OTU) and Machado-Joseph disease protease (MJD). The Ub metalloproteases have a Ub protease domain, called the JAMM (JAB1/MPN/Mov34 metalloenzyme) (Nijman *et al.*, 2005). A sixth DUB family was recently identified, the MINDYs, the main member of which is MINDY-1 that shows DUB activity and selectivity for Lys48-linkages cleaving polyubiquitin only from the distal end (Mevissen & Komander, 2017).

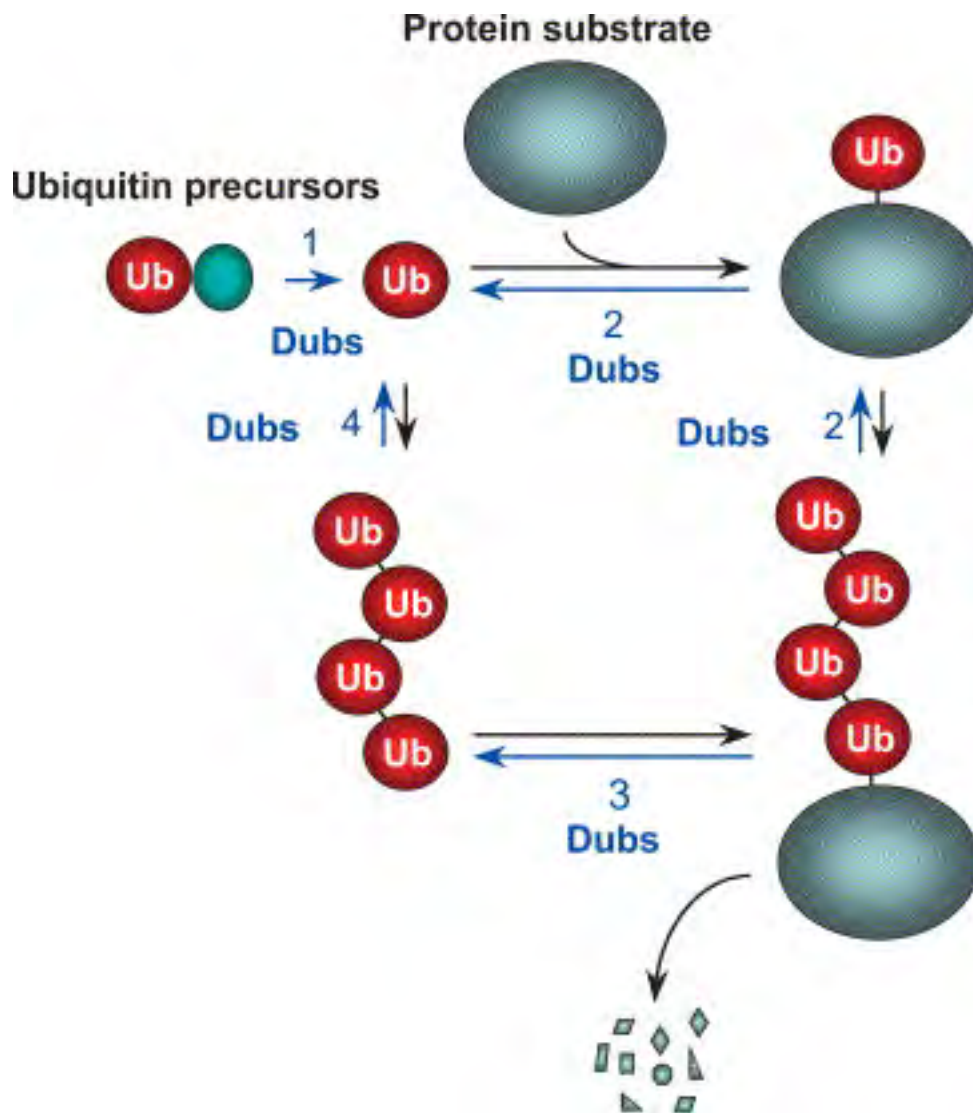


Figure 1.7 Functions of DUBs in the ubiquitin pathway

The schematic figure illustrates the processing of ubiquitin precursors (1) Editing or rescue of ubiquitin conjugates, which are generally adducts to other proteins in the cell but can also be ligated to abundant small nucleophiles such as glutathione. (2) Recycling of ubiquitin or ubiquitin oligomers from ubiquitin–protein conjugates targeted for degradation. (3) Disassembly of unanchored ubiquitin oligomers. (4) Release of ubiquitin from unanchored isopeptide-linked ubiquitin chains into ubiquitin pool. Taken from Amerik & Hochstrasser (2004).

1.2.4 The Ubiquitin C-terminal Hydrolase (UCH) family

DUBs of the UCH family are thiol proteases that contain an N-terminal UCH catalytic core consisting of approximately 230 amino acid. This is usually followed by C-terminal extensions that mediate protein-protein interactions. UCH DUBs consists of four family members, UCH-L1, UCH-L3, UCH37/UCH-L5 and BAP1. UCH-L1 and UCH-L3 prefer cleaving small chains from the C-terminus of Ub linked by ester, thioester and peptide bonds to the C-terminus of Ub, whereas UCH37 and BAP1 can disassemble di-ubiquitin and poly-ubiquitin chains (Eletr & Wilkinson, 2014; Reyes-Turcu *et al.*, 2009). The difference in specificity is due to the length of the active site crossover loop. The UCH domain with a long crossover loop (>14 residues), such as, present in UCH37 or BAP1, can cleave both small and large Ub chains (Zhou *et al.*, 2012).

1.3 BRCA1: an E3 ubiquitin ligase

1.3.1 BRCA1

E3 Ub ligases are the most heterogenous class of enzymes in the ubiquitination pathway with more than 600 E3 ligases in humans. E3 ligases mediate substrate specificity and can be classified into three main types depending on the presence of characteristic domains and on the mechanism of ubiquitin transfer to the substrate protein. These include the RING E3s, HECT E3s and the RBR E3s (Morreale & Walden, 2016).

RING E3s are characterized by the presence of a zinc-binding domain called the RING (Really Interesting New Gene) domain or by a U-box domain, which has the RING fold but does not bind zinc. RING E3s mediate direct transfer of ubiquitin to the substrate, functioning as a scaffold to orient the ubiquitin charged E2 with respect to the substrate protein (Morreale & Walden, 2016). RING E3s can function as monomers, homodimers or heterodimers (Metzger *et al.*, 2012).

BRCA1 DNA repair associated (*BRCA1*) is a RING E3 ubiquitin ligase that functions as a tumour suppressor protein in several cellular processes such as cell cycle regulation, homologous recombination, DNA repair and gene expression (Shabbeer *et al.*, 2013). *BRCA1*, hence the name (mutated in breast and ovarian cancer) (Brodie & Henderson, 2012). *BRCA1* maps to the long arm of chromosome 17 and encodes a protein of 1863 amino acids. The protein itself contains a number of domains that directly or indirectly interact with many proteins with diverse functions (Christou & Kyriacou, 2013).

The N-terminus comprises the zinc ring finger motif, common to many proteins with ubiquitin ligase activity (Christou & Kyriacou, 2013). The C-terminal region contains two BRCT domains with a functional role in DNA damage and repair. The BRCT domains interact with several cell cycle checkpoint proteins and signalling kinases (Deng, 2006). The zinc finger ring and BRCT domain are essential for the normal functioning of this gene as mutations in these domains are present in breast cancer (Christou & Kyriacou, 2013).

BRCA1 forms a functional heterodimer with another protein containing RING-BRCT domains, called *BARD1*, and this interaction stabilizes both proteins and enhances the E3 ubiquitin ligase activity (Shabbeer *et al.*, 2013) (Table 1). During auto-ubiquitination, *BRCA1* assembles K6 linked chains on itself but this does not result in degradation of the protein. Conversely, auto-ubiquitination of *BRCA1* may serve to stabilise the protein (Nishikawa *et al.*, 2004). The *BRCA1-BARD1* heterodimer can also potentially generate K48 and K63 linked polyubiquitin chains on substrate molecules (Wu-Baer *et al.*, 2003). It has been reported that ten E2 enzymes interact with the *BRCA1-BARD1* E3 ubiquitin ligase and they may influence the Ub linkages formed (Christensen *et al.*, 2007). The ability of *BRCA1* to synthesize different types of Ub chains depending on the E2 enzymes implies that it can target different substrates for different fates. *BRCA1* can either facilitate monoubiquitination or polyubiquitination through K6, K48 or K63, depending on the E2 present (Christensen *et al.*, 2007).

Target	Linkage	Effect	Function
Histone H2, H2AX, H2B	Monoubiquitination	Stabilization	Chromatin modification
RNA polymerase II	Polyubiquitination	Degradation	Inhibition of transcription
NPM/B23	Monoubiquitination	Stabilization	Apoptosis control
γ -tubulin	Polyubiquitination	Degradation	Microtubule nucleation, centrosome duplication
Aurora A	Polyubiquitination	Degradation	mitosis/anaphase
Aurora B	Polyubiquitination	Degradation	mitosis/cytokinesis
Oestrogen receptor α	Polyubiquitination	Degradation	ER response genes
Progesterone Receptor	Polyubiquitination	Degradation	PR response genes
BRCA1 BARD1	Monoubiquitination	Stabilization	Stability, increases activity

Table 1. Targets of the BARD1-BRCA1 ubiquitin ligase.

Adapted from Irminger-Finger *et al.* (2016).

The N-terminal 324 amino acid region of BRCA1 serves as a degron sequence in promoting proteasome mediated degradation of BRCA1. However, BARD1 is unlikely to be responsible for the ubiquitination and proteasome dependent degradation of BRCA1, suggesting that another E3 ligase targets BRCA1 for protein degradation (Lu *et al.*, 2007). Importantly, two HECT E3 ligases, HERC2 and HUWE1, were discovered that interact with the degron domain of BRCA1 (Figure 8). Furthermore, HERC2 and HUWE1 can ubiquitinate BRCA1 and target it for proteasomal degradation (Wang *et al.*, 2014; Wu *et al.*, 2010). Another E3 ligase, Skp1-Cul1-F-box-protein44 (SCF^{FBXO44}) also promotes the proteasome mediated degradation of BRCA1.

Moreover, the N-terminus of BRCA1 mediates interaction between BRCA1 and FBXO44 (Lu *et al.*, 2012). Cathepsin S (CTSS) a cysteine protease, induces proteolytic degradation of BRCA1 in response to irradiation (Kim *et al.*, 2018). Apart from E3 ligases and cysteine proteases, a few other proteins can regulate stability of the BRCA1 protein. Rak/Fyn-related kinase (FRK), a member of the Src tyrosine kinase family can regulate BRCA1 protein stability via tyrosine phosphorylation of BRCA1 at Tyr1552 within the BRCT domain; this is essential for interaction with Rak. However, *Rak* was also reported to stabilize BRCA1 through inhibiting its interaction with UBE2T, an E2 conjugating enzyme (Kim *et al.*, 2017). The tumour suppressor candidate 4 (TUSC4) interacts with HERC2 to prevent ubiquitination of BRCA1 (Peng *et al.*, 2015).

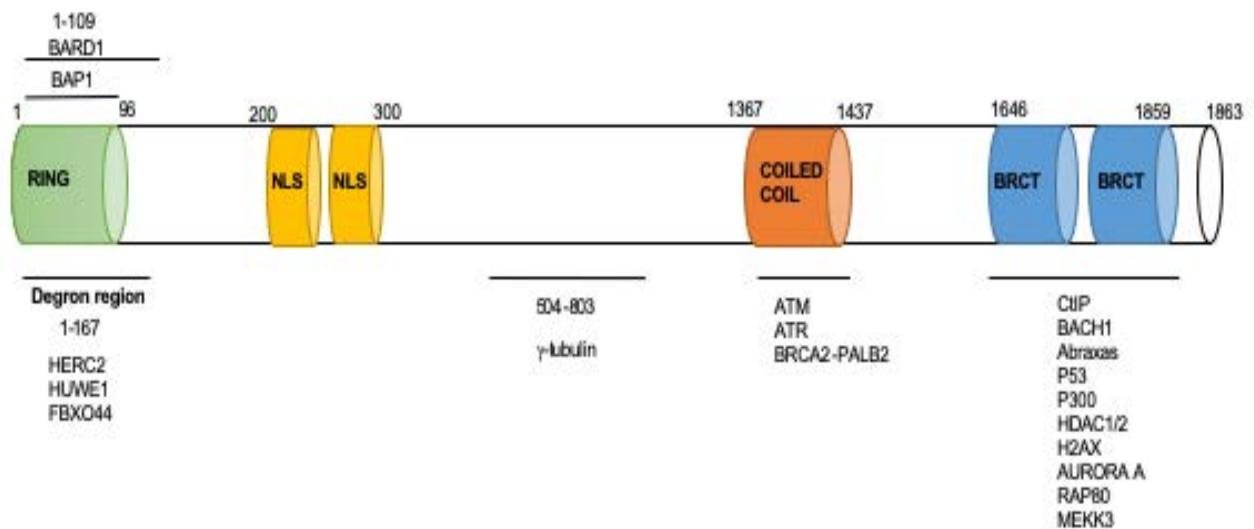


Figure 1.8 Schematic representation of *BRCA1* domains and interacting proteins

BRCA1 contains a RING domain at its N-terminus, two nuclear localization signals (NLS), two BRCT domains at the C-terminus and a coiled-coil domain upstream of the BRCT domains. The interacting proteins are shown above and below the region of *BRCA1* required for their association. *BRCA1* interacts with BARD1 via its RING domain that enhances the E3 Ub ligase activity of the complex. A deubiquitinating enzyme BAP1 also binds to the RING domain. The same region also acts as a degron domain from 1-167 that targets it for proteasome-mediated degradation. E3 ligases such as HERC2, HUWE1 and FBXO44 bind to *BRCA1* through its degron domain and targets *BRCA1* for degradation. PALB2, ATM and ATR bind to the coiled-coil domain of *BRCA1*. A large number of proteins have been demonstrated to bind to the BRCT domains of *BRCA1*. Adapted from Wang (2012a).

1.3.2 BRCA1 and DNA repair

Protein ubiquitination plays a central role in DNA repair and several E3 ubiquitin ligases have been identified with distinct functions in DNA damage repair, including BRCA1/BARD1, RNF8, RNF20/40, RNF168, RAD18, HERC2 and polycomb-repressive complex 1 (PRC1) (Ohta *et al.*, 2011). DNA damage arises as a consequence of multiple endogenous or exogenous events that cause different types of damage. The most hazardous type of DNA damage is double-stranded breaks (DSBs). DSBs are repaired through two major pathways, non-homologous end joining (NHEJ) and homologous recombination (HR) (Savage & Harkin, 2015). BRCA1 has a prominent role in the HR pathway. Upon DNA damage, BRCA1 is phosphorylated by several kinases including ATM, ATR and CHK2. This enhances function of a complex between BRCA1 and BCL-2 associated transcription factor (BCLAF1) that mediates the formation of a BRCA1-mRNA splicing complex after DNA damage. This results in enhanced pre-mRNA splicing of BRCA1/BCLAF1 target genes, which promotes their transcript stability and protein expression (Savage *et al.*, 2014).

The BRCA1 protein contains two putative nuclear localization sequences towards its C-terminus and forms distinctive small foci at nuclear sites of DNA damage (Scully *et al.*, 1997). BRCA1 colocalizes with BARD1 and RAD51 at DNA damage induced foci that are also marked by histone variant, H2AX, phosphorylated on Ser139 (γ H2AX) (Huen *et al.*, 2010). BRCA1 plays a number of roles in HR-mediated DSB repair through participation in at least four different protein complexes. The BRCA1-C complex consists of BRCA1, the MRN complex (Mre11, RAD50, Nbs1) and CtIP and contributes to the initiation of DSB end resection (Cruz-García *et al.*, 2014; Greenberg *et al.*, 2006). The BRCA1-A complex contains BRCA1, RAP80, MERIT40, BRCC36/45 and Abraxas, and inhibits DNA end resection (Coleman & Greenberg, 2011; Hu *et al.*, 2011). This complex also stabilizes and maintain DNA damage signalling from the break site and promotes G2/M checkpoint arrest. The BRCA1-A complex is recruited to DSB sites by binding phosphorylated H2AX (γ H2AX) at break sites through MDC1 (Stucki *et al.*, 2005).

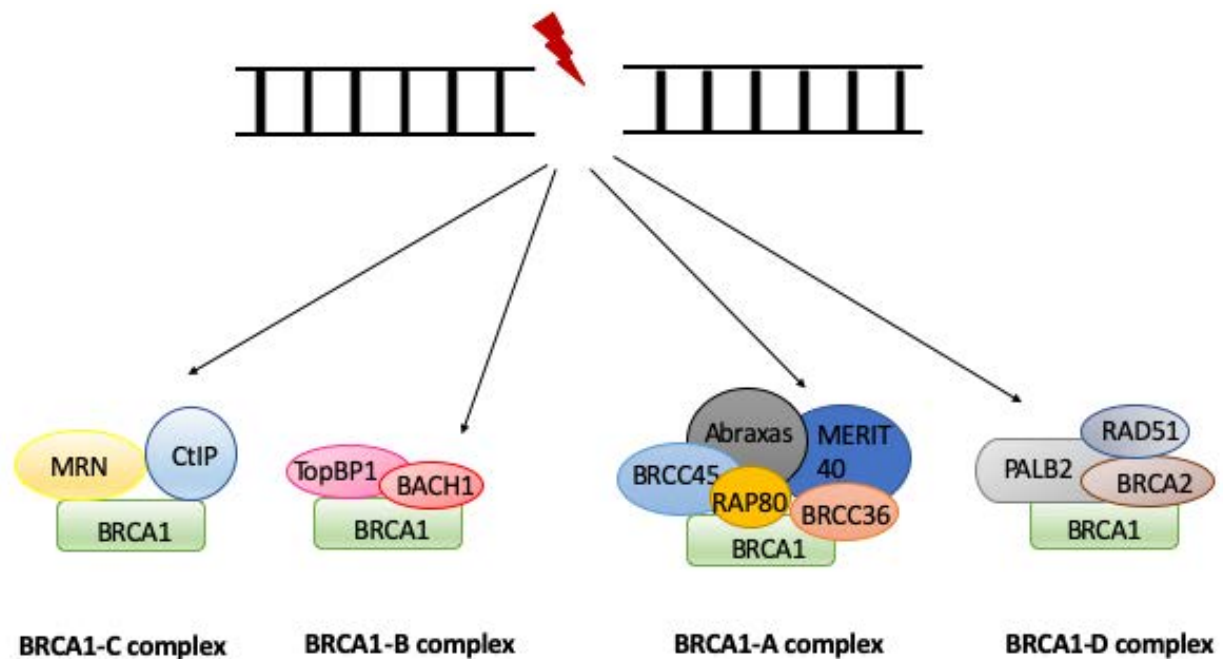


Figure 1.9 *BRCA1* complexes involved in HR-mediated DSB repair and cell cycle checkpoint control

During S and G2 phase, DSBs are faithfully repaired through HR. The BRCA1-C complex displaces 53BP1 from DSB ends, which initiates DSB end resection. Both BRCA1-C and B complexes are required to extend DNA end resection. BRCA1-C and B complexes are also required for the activation of the S phase checkpoint after DNA damage. The BRCA1-A complex is recruited to flanking chromatin and prevents over resection of DSB ends. The BRCA1-A complex is also required for the activation of the G2/M checkpoint. Finally, the BRCA1-D complex facilitates the exchange of RPA with RAD51. RAD51 then catalyses the invasion of the sister chromatid and alignment with the complementary DNA sequence, forming a Holiday junction. DNA polymerases restore the complementary DNA strand using the sister chromatid as a template. The Holiday junction then resolves, resulting in noncross-over or cross-over products, depending on the direction of junction resolution. Adapted from Savage & Harkin (2015).

Another BRCA1 complex, the BRCA1-B complex, contains BRCA1/TopBP1 and BACH1 and, as BACH1 is a DNA helicase, it is assumed that it contributes to end resection. This complex also plays a role in S-phase checkpoint activation in response to DNA damage. Finally, another BRCA1 complex, the BRCA1-D complex which contains BRCA1, BRCA1, PALB2 AND RAD51, promotes the exchange of RPA for RAD51 after DNA resection in order to facilitate sister chromatid invasion and completion of the HR process (Savage & Harkin, 2015) (Figure 9). Following DNA damage, BRCA1 also ubiquitinates two cell cycle proteins cyclin B and Cdc25C, that are critical for the transition of cells from the G2 to M. Ubiquitination of cyclin B and Cdc25C by BRCA1 leads to cell cycle arrest at G2/M (Shabbeer *et al.*, 2013).

1.3.3 BRCA1 and centrosome duplication

Centrosomes are microtubule organizing centres that regulate cell shape and polarity, cellular transport and cell division (Hinchcliffe & Sluder 2001). The centrosome consists of a pair of centrioles, embedded in the pericentriolar matrix (PCM). Centrioles are built from a cylindrical array of nine microtubules, each of which is attached to two partial microtubules (Azimzadeh *et al.*, 2010). To maintain genomic stability, centrosomes are duplicated only once per cell cycle to ensure formation of a bipolar spindle during mitosis, thus evenly segregating the pairs of sister chromatids (Hinchcliffe & Sluder 2001). BRCA1 localizes to the centrosome during interphase and mitosis and plays an important in regulation of centrosome number (Xu *et al.*, 1999; Hsu *et al.*, 2001; Tarapore *et al.*, 2012). Loss of BRCA1 results in centrosome reduplication without passing through mitosis (Ko *et al.*, 2006). While the underlying mechanism for this is not clear, BRCA1 has been shown to ubiquitinate γ -tubulin, a core component of the centrosome, at Lys 48 and 344. Moreover, mutation of these lysines increases MT nucleation suggesting that BRCA1 suppresses MT nucleation from the centrosome by ubiquitination of γ -tubulin (Sankaran *et al.*, 2005; Starita *et al.*, 2004). Furthermore, the Obg-like ATPase 1 (OLA1) binds to the N-terminus of BRCA1 and γ -tubulin binds to BRCA1 in the presence of OLA1. Depletion of BRCA1 did not suppress the expression of OLA1 but decreased centrosomal localization of OLA1. Depletion of either OLA or BRCA1 leads to centrosome amplification indicating that

these two proteins function in a shared pathway to regulate centrosome number (Matsuzawa *et al.*, 2014). BRCA1 also poly-ubiquitinates nucleophosmin/B23 (NPM), another centrosome component, reported to regulate centrosome duplication. This stabilizes NPM rather than targeting it for degradation (Sato *et al.*, 2004).

BRCA1 can regulate the centrosomal localization and stability of Ninein-like protein (Nlp), a protein involved in centrosome maturation and spindle formation. Depletion of Nlp or BRCA1 leads to aberrant spindle formation and chromosome missegregation (Jin *et al.*, 2009). In mitosis, BRCA1 is required for accumulation of TPX2, a MT associated protein that regulates spindle assembly. Upon BRCA1 depletion, TPX2 diffusely localized along the length of MTs and failed to concentrate on spindle poles indicating a role for BRCA1 in mitotic spindle pole organization (Joukov *et al.*, 2006).

1.3.4 BRCA1 and spindle assembly checkpoint control

Loss of BRCA1 inactivates the spindle assembly checkpoint (SAC) as it leads to downregulation of essential SAC component, including MAD2. BRCA1 regulates the expression of MAD2 by binding to the transcription factor OCT-1. Knockdown of BRCA1 in MCF-7 cells also downregulated the expression of another SAC component BUBR1 (Chabalier *et al.*, 2006). These studies reveal that BRCA1 deficient cells possess an ineffective SAC. These data are consistent with clinical studies that have shown that loss of BRCA1 renders cancer cells resistant to the microtubule poisons, vinorelbine and paclitaxel (Chabalier *et al.*, 2006), while re-expression of BRCA1 reverses this phenotype (Sung *et al.*, 2014).

1.4 BAP1 (BRCA1 associated protein 1)

BAP1 is a cysteine protease that belongs to the UCH family of DUBs. BAP1 is a tumour suppressor involved in several cellular processes including, DNA repair, cell cycle progression, transcriptional regulation and chromatin modification (Parrotta *et al.*, 2017). BAP1 shares 44% sequence homology with UCH-L5/UCH37 and has a UCH-L5 like domain (ULD) at its C-terminus that is important not only for regulating its catalytic activity but also for interacting with a range of binding partners. However,

BAP1 contains an additional 350 amino acid insert relative to UCH-L5 situated between the N-terminus UCH catalytic domain and C-terminus ULD domain (Hanpude *et al.*, 2017; Sahtoe, 2015). BAP1 can cleave the isopeptide and peptide-linked Ub extensions on substrate molecules as well as having specificity for K48 poly-ubiquitin chains (Hanpude *et al.*, 2017). The UCH domain of BAP1 is highly similar to that of UCH37, and both these proteins are thought to fold in a manner that allows interaction of their respective CTD (C-terminal domain) with the UCH domain. Indeed, the UCH flanking sequences of BAP1 have coiled-coil motifs that engage in intramolecular interactions (Mashtalir *et al.*, 2014).

Consistent with an intramolecular interaction, the N-terminal UCH domain of (BAP1N; 1-237 residues) exhibits a higher catalytic activity than full-length BAP1; this argues that the C-terminal ULD domain may act as an inhibitor of BAP1 (Bhattacharya *et al.*, 2015). Several mutants of the UCH domain of *BAP1* showed varying degrees of enzymatic activity impairment although few showed complete loss of activity (Hanpude *et al.*, 2017). Cancer derived mutations in the UCH domain abrogate auto-deubiquitination and promote cytoplasmic retention, demonstrating that BAP1 auto-deubiquitination activity is important for tumour suppression (Mashtalir *et al.*, 2014).

As the name suggests, BAP1 was first identified as an interacting partner of BRCA1 (Jensen *et al.*, 1998). This study showed that BAP1 interacts via its C-terminal region with the RING domain of BRCA1. An L691P mutation in the C-terminal region of BAP1 abolished BRCA1 interaction. This study also suggested that BAP1 was capable of removal of Ub or ubiquitin-like molecules from BRCA1 or a protein associated with BRCA1 thereby altering its function (Jensen *et al.*, 1998). Interestingly, BAP1 has also been reported to interact through its N-terminal region with the RING domain of BARD1. Furthermore, it is proposed that BAP1 deubiquitinates the Ub chains added to substrates by the BRCA1/BARD1 complex. The fact that BAP1 interacts with BRCA1 and BARD1 through distinct domains suggests that BAP1 could interact simultaneously with both proteins to form a trimeric complex *in vivo* (Figure 10). However, an inactive mutant of BAP1 (C91S) abolished the auto-ubiquitination of BRCA1 indicating that BAP1 inhibits the E3 ligase activity of BRCA1/BARD1 in a manner that is independent of its DUB activity (Nishikawa *et al.*, 2009).

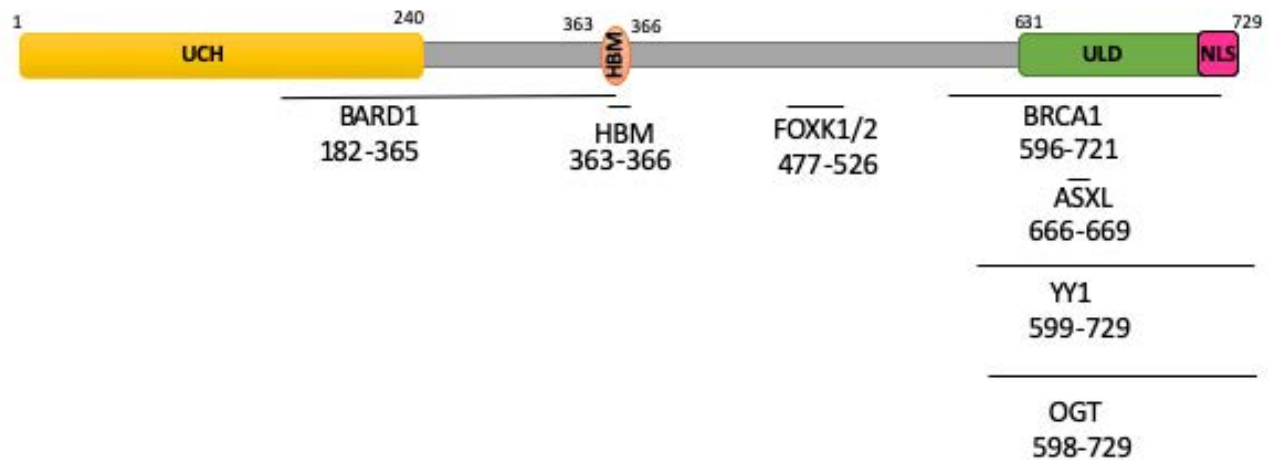


Figure 1.10 Schematic representation of BAP1 protein

The figure illustrates the BAP1 functional domains and mapped interacting proteins. BAP1 consists of 729 amino acids. UCH-ubiquitin carboxyl hydrolase domain (1-240), BARD1 binding domain (182-365), HBM-HCF1 (Host cell factor 1) binding domain (363-366), and domain that binds to the RING domain of BRCA1 (596-721). BAP1 interacts with ASXL1/2 to form a PR-DUB complex that de-ubiquitinates H2A. BAP1 interacts with several transcription factors including HCF-1, YY1 and OGT and plays an important role in transcriptional regulation. Adapted from Wang *et al.* (2016).

1.4.1 BAP1 mutations in human malignancies

The *BAP1* gene is located at the chromosome region 3p21.1 that is commonly deleted in various human cancers including 30-60% of mesothelioma patients (Bueno *et al.*, 2016; Comertpay *et al.*, 2011; Sneddon & Creaney, 2016; Murali *et al.*, 2013; Attanoos *et al.*, 2018; Testa *et al.*, 2011; Carbone *et al.*, 2013). Somatic *BAP1* mutations are seen in cutaneous melanocytic tumours, uveal melanoma, mesothelioma, clear cell renal carcinoma and other cancers (Murali *et al.*, 2013). Typical of tumour suppressor genes, germline mutations in *BAP1* predispose for hereditary cancers. *BAP1* tumour predisposition syndrome (*BAP1*-TPDS) is a newly recognized cancer syndrome that predisposes patients to uveal melanoma, mesothelioma, renal cell carcinoma and other cancers. Most of the *BAP1* related cancers are aggressive and triggered early in life (Masoomian *et al.*, 2018). The germline mutations found in *BAP1*-TPDS often lead to protein lacking the nuclear localization sequence. *BAP1* expression is therefore either absent due to loss of heterozygosity (LOH) and biallelic inactivation or *BAP1* protein is mis-localized to the cytoplasm (Carbone *et al.*, 2013). Gene fusions and splice alteration in *BAP1* lead to its inactivation in mesothelioma (Bueno *et al.*, 2016).

1.4.2 BAP1 and DNA repair

An RNAi screen for DUBs involved in HR-mediated DNA repair revealed that *BAP1* loss decreased RAD51 and BRCA1 nuclear foci after ionizing radiation (Yu *et al.*, 2014; Ismail *et al.*, 2014; Xiyu *et al.*, 2018). Moreover, constitutive BRCA1 foci that are distinct from ionizing radiation induced foci (IRIF) were also reduced in *BAP1* depleted cells indicating that *BAP1* contributes to BRCA1 association with chromatin under normal growth conditions (Yu *et al.*, 2014). Furthermore, *BAP1* knockout cells phenocopy BRCA1 and RAD51 knockout by being hypersensitive to DSB inducing agents (Yu *et al.*, 2014; Ismail *et al.*, 2014; Nishikawa *et al.*, 2009). As *BAP1* colocalized with γ H2AX at DNA damages sites after laser micro-irradiation and increased four-fold upon induction of DSBs by the FOK-1 endonuclease, this indicates that DSBs recruit *BAP1*. Interestingly, inhibition or depletion of poly (ADP-ribose) polymerase (PARP) enzymes abrogated *BAP1* accumulation at laser induced DNA damage sites suggesting that PARP1/2 are required for *BAP1* recruitment. Indeed,

loss of BAP1 or BRCA1 increases sensitivity to PARP inhibitors (Helleday, 2011; Cheung & Testa, 2017). RNF8, a RING E3 ligase that ubiquitinates histones at DSBs and promotes assembly of repair proteins is also required for BAP1 recruitment to the DNA damage sites (Mailand *et al.*, 2007). BAP1 does not appear to play a role in NHEJ repair as BAP1 depletion does not affect the localization, expression or activity of proteins involved in NHEJ, such as Chk1, pChk1, Chk2, pChk2, ATM and 53-BP1 (Xiyu *et al.*, 2018; Yu *et al.*, 2014; Ismail *et al.*, 2014).

1.4.3 BAP1 and gene expression control

The Polycomb Repressive Deubiquitinase complex (PR-DUB) comprises of the BAP1-ASXL1/2 complex with *BAP1* contributing to the catalytic activity that de-ubiquitinates H2A. The ASXL DEUBAD domain activates BAP1 on H2A that has been mono-ubiquitinated at K119 site, and all human paralogs such as ASXL1/2/3 can stimulate BAP1 DUB activity against H2A (Sahtoe *et al.*, 2016).

H2A is mono-ubiquitinated on the C-terminal tail at K119 and enriched at the satellite regions of the genome. So far three E3 Ub ligases have been identified that mono-ubiquitinate H2A, including RING1A/RING1B/BMI1, 2A-HUB and BRCA1/BARD1. This leads to repression of transcription correlating with gene silencing (Vissers *et al.*, 2008; Cao & Yan, 2012).

The C-terminus of BAP1 is required for H2A de-ubiquitination and recruited to the nucleosomes (Sahtoe *et al.*, 2016). De-ubiquitination of H2A by BAP1 is required for transcriptional activation (Zhou *et al.*, 2008; Zhu *et al.*, 2007; Joo *et al.*, 2007). Furthermore, BAP1 depletion significantly increased ubiquitination of H2A suggesting that BAP1 is a major DUB for this H2A modification (Campagne *et al.*, 2018). Depletion of ASXL1 or ASXL2 led to a decrease in BAP1 protein levels, while combined depletion of ASXL1 and ASXL2 caused a stronger decrease of BAP1 protein levels suggesting that ASXL1/2 are required for BAP1 stability (Campagne *et al.*, 2018; Daou *et al.*, 2015). Interestingly, phylogenetic analysis of partner proteins for BAP1 and UCH-L5 revealed frequent occurrence of a conserved deubiquitinase adaptor (DEUBAD) domain. Moreover, the DEUBAD domains of ASXL1, ASXL2 and ASXL3 can activate BAP1 by increasing its affinity for Ub (Sahtoe *et al.*, 2016) (Figure 11).

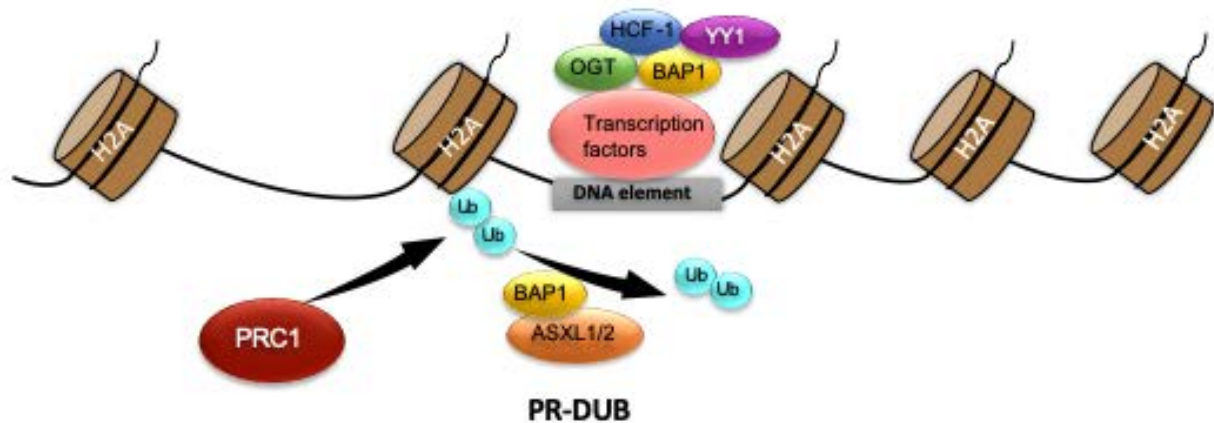


Figure 1.11 Diagrammatic representation of BAP1 function in regulating gene expression

H2A is ubiquitinated by the PRC1 complex on Lys 119 which represses transcription, while de-ubiquitination of H2A is mediated by the PR-DUB complex consisting of BAP1 and ASXL1/2. BAP1 also regulates gene expression via interacting with other protein partners such as HCF-1, YY1 and OGT. Adapted from Carbone *et al.* (2013).

BAP1 is also intimately involved in transcriptional regulation (Yu *et al.*, 2010; Ji *et al.*, 2014; Yu *et al.*, 2014; White & Harper, 2012; Machida *et al.*, 2009). Host cell factor1 (HCF-1) is a chromatin associated protein that regulates the expression of several of genes involved in diverse cellular processes (Tyagi *et al.*, 2007; Wysocka *et al.*, 2003; Zargar & Tyagi, 2012). BAP1 forms a ternary complex with HCF1 and YY1, another transcription factor (Yu *et al.*, 2010). HCF-1 potentially recruits this complex to the chromatin (Machida *et al.*, 2009) (Figure 10). Alternatively, the forkhead transcription factor FOXK2 can also recruit BAP1 to specific genomic regions. FOXK2 occupies thousands of genome loci, many found in open chromatin, which could explain how BAP1 functions across the genome (Ji *et al.*, 2014). Importantly, FOXK2 depleted cells, exhibited reduced BAP1 recruitment, to FOXK2 targets. However, a catalytically inactive BAP1 mutant (C91S) was also recruited to the FOXK2 binding regions, suggesting that BAP1 recruitment is not dependent on its DUB activity. However, phosphorylation of BAP1, by as yet unidentified kinases is required for its interaction with FOXK2 (Okino *et al.*, 2015).

BAP1 can control the cell cycle through influencing the expression of E2F1 target genes by binding to their promoters (Pan *et al.*, 2015). Several E2F target genes including *SKP2*, *p107*, *CDC2* and *CDC25A* were downregulated after BAP1 depletion (Yu *et al.*, 2010). This interaction between E2F1 and BAP1 is dependent on HCF-1 suggesting that HCF-1 acts as a scaffold to recruit BAP1 to specific gene loci to regulate gene transcription (Pan *et al.*, 2015) (Table 2).

Substrates	Function	References
HCF-1	Cell cycle progression, maintenance of pluripotency	(Misaghi <i>et al.</i> , 2009)
OGT	Mitochondrial biogenesis, cell cycle, metabolism and apoptosis	(Dey <i>et al.</i> , 2012)
γ -tubulin	Centrosomes; organizing centre of MT assembly	(Zarrizi <i>et al.</i> , 2014)
H2A	Transcriptional regulation and cell cycle regulation	(Sahtoe <i>et al.</i> , 2016)
β -catenin	Adherens junctions and Wnt signalling	(Wong, 2012)
IP3R3	Calcium signalling, apoptosis	(Bononi <i>et al.</i> , 2017)
MCRS1	Minus end MT dynamics and cell division	(Peng <i>et al.</i> , 2015)

Table 2. Target substrates for BAP1 de-ubiquitination activity

1.4.4 BAP1 and mitotic spindle regulation

The centrosome is the primary MT organizing center (MTOC) of the cell. Within the PCM are protein complexes of γ -tubulin and associated proteins that together have a ring like structure, called γ -tubulin ring complexes (γ -TuRCs), that mediate MT nucleation (Zheng *et al.*, 1995; Moritz *et al.*, 1995). During centrosome maturation at the onset of mitosis, additional γ -TuRCs as well as several other proteins are recruited that lead to an increase in the MT nucleation capacity of the centrosome (Blagden & Glover, 2003). As previously discussed, γ -tubulin is ubiquitinated by BRCA1 and contribute to regulation of MT nucleation and centrosome number.

An siRNA screen identified BAP1 as a DUB that regulates γ -tubulin de-ubiquitination (Zarrizi *et al.*, 2014). BAP1 and γ -tubulin co-localize at the centrosome during mitosis. BAP1 downregulation increased γ -tubulin ubiquitination and led to abnormal mitotic spindles with chromosomal abnormalities, including lagging chromosomes, multipolar spindles and telophase bridges. Together, these studies suggest that BAP1 de-ubiquitination activity is required for spindle pole organization and spindle assembly during mitosis and this is essential to maintain chromosomal stability (Zarrizi *et al.*, 2014).

Microspherule protein 1, MCRS1, is a centrosomal protein is involved in multiple cellular activities, including mitotic spindle assembly. Besides localizing to the centrosome, it localizes to the minus ends of chromosomal MTs and K-fibres, where it protects them from depolymerization (Meunier & Vernos, 2011; Petry & Vale, 2011). MCRS1 was identified as a substrate for BAP1 DUB activity, with its de-ubiquitination necessary for proper mitotic organization and chromosomal stability (Peng *et al.*, 2015).

1.4.5 BAP1 and cell death

BAP1 localizes at the endoplasmic reticulum (ER) where it binds, deubiquitinates and stabilizes the type 3 inositol-1,4,5-trisphosphate receptor (IP3R3). This modulates calcium (Ca^{2+}) release from the ER into the cytosol to promote apoptosis (Bononi *et*

al., 2017). BAP1 also induces cell death through interaction with the 14-3-3 protein. The association between BAP1 and 14-3-3 releases the apoptotic regulator BAX from 14-3-3 and promotes cell death through the intrinsic apoptosis pathway (Sime *et al.*, 2018). The solute carrier family 7 member (SLC7A11/xCT) is another key BAP1 target gene relevant to cell death. BAP1 decreases H2A ubiquitination on the SLC7A11 promoter and represses SLC7A11 expression in a DUB-dependent manner. As a consequence, BAP1 inhibits cysteine uptake by repressing SLC7A11 expression leading to elevated lipid peroxidation and ferroptosis, a non-apoptotic form of cell death (Zhang *et al.*, 2018).

1.5. Mitosis and microtubule organization

1.5.1 Mitosis

Mitosis is a critical and highly orchestrated event in the cell cycle, when the cell must accurately segregate its replicated chromosomes into two daughter cells. The task performed by the mitotic spindle, a cellular machine composed of MTs and associated proteins (Walczak & Heald, 2008; Schmit & Ahmad, 2007). MTs between the spindle poles are organized into an antiparallel array. The key interaction between each sister chromatid and the spindle MTs occurs at the centromere of the chromosome, where a macromolecular complex called the kinetochore assembles. A subset of spindle MTs called kinetochore microtubules (kMTs) attach directly to the kinetochores and enable them to align and segregate the chromosomes (Mayr, 2010).

1.5.2 Spindle assembly checkpoint

The spindle assembly checkpoint (SAC), also referred to as the mitotic checkpoint, is a complex mechanism, that maintains genomic stability by ensuring correct segregation of chromosomes during mitosis. The SAC becomes activated in the presence of unattached kinetochores and lack of tension between sister chromatid. Thus inhibits the APC/C an E3 ligase, thus blocking the metaphase to anaphase transition. Once all the kinetochores are attached and tension is exerted across the

sister chromatid pairs, the SAC becomes inactivated and the cell proceeds towards mitotic exit (Chabaliere *et al.*, 2006). A number of proteins are involved in SAC function, including MAD2, BUBR1, BUB3 and CDC20 which together form a mitotic checkpoint complex (MCC) that inhibits the APC/C (Lara-Gonzalez *et al.*, 2012).

1.5.3 Microtubule structure and dynamics

Microtubules (MTs) are a major component of the cytoskeleton with important functions in maintaining cell shape, cellular movement, cell signalling and in formation of the mitotic spindle. MTs are composed of α and β -tubulin heterodimer (Mandelkow & Mandelkow, 1994). Each α and β -tubulin monomer consists of three functional domains. The N-terminal domain is involved in nucleotide binding (GDP/GTP), the central domain is involved in the formation of protofilaments, and the C-terminus is involved in binding of microtubule associated proteins (MAPs) such as stathmin, tau, CENPE and Eg5 (McGrogan *et al.*, 2008). Each α/β tubulin heterodimer is 8 nm in length and interacts longitudinally with other heterodimers to form elongated protofilaments. Thirteen protofilaments then come together through the lateral interactions between protofilaments to form the cylindrical MT (Tuszynski *et al.*, 2003). The fashion in which these protofilaments are arranged gives polarity to the MTs, exposing the β -tubulin at the (+) end and α -tubulin at the (-) end. This property allows the MTs to be highly dynamic with the plus end capable of more rapid growth than the minus end. Plus ends of the MTs radiate freely towards the cell periphery, whereas the minus ends are often associated with the microtubule organizing center (MTOC) (Desai & Mitchison, 1997).

MTs exhibit dynamic instability and undergo periods of growth and shrinkage, as a result of association and disassociation of α and β -tubulin heterodimers. This transition from growth to shrinkage is known as 'catastrophe' whereas from shrinkage to growth is termed as 'rescue' (Caplow, 1992) (Figure 12). The exchange of tubulin dimers at both ends is termed as 'dynamicity', whereas 'treadmilling' is the net growth at the plus end associated with net shortening at the minus end (Wade & Hyman, 1997). Dynamic instability and treadmilling are regulated by various MAPs and critical for correct formation of mitotic spindle during mitosis (Jordan & Wilson, 2004).

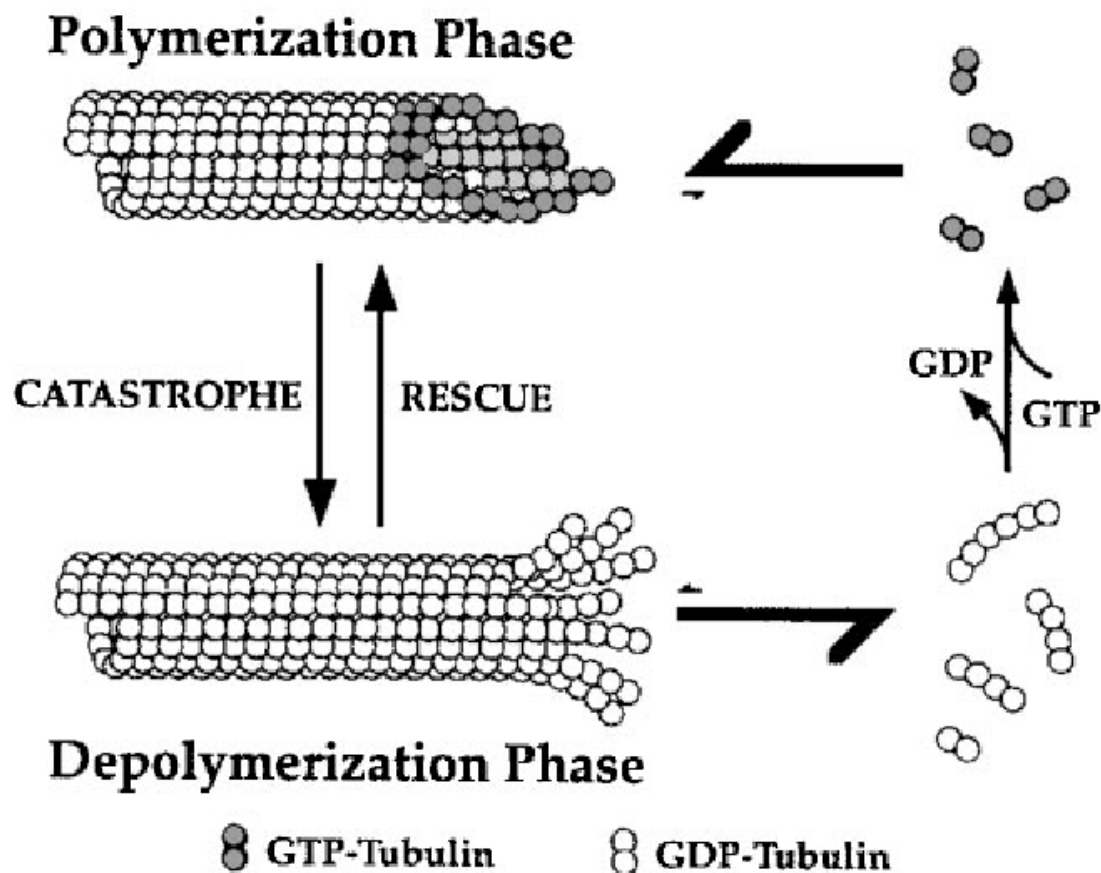


Figure 1.12 Microtubule dynamics

Microtubules polymerize by the addition of GTP-bound tubulin dimers, with the β -tubulin bound GTP hydrolyzed during or soon after polymerization. Thus, the β -tubulin within the MT is predominantly composed of GDP-tubulin. MTs switch between stages of polymerization and depolymerization. Transition from growth to shrinkage is termed catastrophe; transition from shortening to growth is called rescue. Taken from Desai & Mitchison (1997).

1.5.4 The Kinesin-8 family of motor proteins

There are three major classes of molecular motors: kinesins, dyneins and myosins and all three classes use energy derived from ATP hydrolysis for force generations (Wickstead & Gull, 2006). Kinesins and dyneins interact with MTs whereas myosins move along actin microfilaments. The actions of kinesins support many cellular functions, including mitosis, meiosis, transport of cargoes and cell movement. During mitosis, kinesins participate in spindle formation, chromosome congression and cytokinesis (Yu & Feng, 2010).

Kinesin-8 proteins including KIF18A and KIF18B are MT plus-end directed motors and plus-end MT destabilizing enzymes (Shrestha *et al.*, 2019). Kinesin-8 family members from diverse species regulate the length of cellular MTs and thereby influence the structure of the mitotic spindle and cilia (Dave *et al.*, 2018). During prometaphase, KIF18A localizes along spindle MTs near the outer kinetochore, while in metaphase cells, KIF18A localizes along the kMTs. During telophase and cytokinesis KIF18A concentrates at the midbody. Depletion of KIF18A leads to elongated spindles in mitosis (Mayr *et al.*, 2011). To constrain spindle MT length, KIF18A promotes catastrophe and slows MT growth rate (Du *et al.*, 2010; Tischer *et al.*, 2009). However, KIF18A also has stabilizing effects on MTs, as it slows MT shrinkage rate and increases the frequency of rescue (Su *et al.*, 2011). KIF18A also localizes to the plus ends of (kMTs), where it regulates kinetochore MTs dynamics to promote chromosome alignment. Cells depleted of KIF18A fail to form a stable metaphase plate and displayed prolonged prometaphase as a result of the spindle assembly checkpoint (SAC).

KIF18A is glycosylated on serine and threonine residues O-GlcNAc transferase (OGT) enzyme. Glycosylation generally can alter proteolytic resistance, protein solubility and stability, local structure and immunogenicity (Zusev & Benayahu, 2008). O-GlcNAcylation, and phosphorylation can compete for similar residues to determine its function (Hart *et al.*, 2007). This modification could therefore regulate the cellular distribution and function of KIF18A (Zusev & Benayahu, 2008). Interestingly, BAP1

de-ubiquitinates and stabilizes OGT, the enzyme responsible for glycosylation of KIF18A (Dey *et al.*, 2012).

A second member of the kinesin-8 family, KIF18B, is another MT plus end binding protein that localizes on astral MTs during prometaphase. Depletion of KIF18B did not affect mitotic progression and no change in spindle morphology was seen. However, there was an increase in astral MT length during metaphase (Stout *et al.*, 2011). The overly long astral MTs lead to spindle mispositioning and rocking during mitosis (McHugh *et al.*, 2018). KIF18B interacts with EB1 to localize to plus ends of the astral MTs (Stout *et al.*, 2011). KIF18B depletion alters the forces on chromosome arms but does not cause errors in kMT attachment or length and neither does it affect the fidelity of chromosome segregation (Walczak *et al.*, 2016).

KIF18B binds to the kinesin-13, MCAK, and forms a complex with MT plus-end depolymerase activity during mitosis. It is possible that KIF18B is initially recruited by EB1 at the plus ends to allow for selective depolymerization of longer MTs (Tanenbaum *et al.*, 2011). As KIF18B promotes catastrophe, this leads to a net reduction of MT length (McHugh *et al.*, 2018).

1.5.5 β -tubulin isotypes in cancer

Tubulin isotype expression can modulate resistance of cancer cells to MT poisons. Mammalian cells have six to seven isotypes of both α - and β -tubulin encoded by different genes. The α -tubulin proteins differ by only a few residues whereas the β -tubulin proteins differ significantly in their C-terminal region (Wells *et al.*, 2005). The β -tubulin isotypes differ from each other in normal tissue distribution (Ludueña, 1997). Based on their C-terminal sequences, β -tubulin isotypes can be assigned to seven distinct classes. In the normal tissues, β -1 (TUBB 1), β -IVb (TUBB2c), and β -V (TUBB6) are ubiquitously expressed; β -VI (TUBB1) is hematopoietic cell-specific, while β -IIa (TUBB2a), β -IIb (TUBB2b), β -III (TUBB3), and β -IVa (TUBB4) exhibit high expression in the brain (Leandro-García *et al.*, 2010). Expression of β -tubulin isotypes are often altered in tumour cells and some isotypes seem to be overexpressed as compared to normal cells. These can be associated not only with resistance to drugs but also poor prognosis and disease progression (Kavallaris, 2010).

β -III tubulin is overexpressed in breast, non-small cell lung, gastric and ovarian cancer suggesting that regardless of it being neuron and testis specific in normal cells, it possibly plays a specific role in progression and resistance of certain tumours (Katsetos *et al.*, 2003; Sève *et al.*, 2005). NSCLC cells with high expression of β -III tubulin had a lower response rate and shorter survival following treatment with the MT poisons, paclitaxel or vinorelbine than those with a lower expression of β -III tubulin (Sève *et al.*, 2005). As this indicates, β -tubulin isotypes influence the interaction with MT poisons (Huzil *et al.*, 2006). Nocodazole and colchicines differ structurally but have the highest binding affinity for MTs composed of $\alpha\beta$ -IV tubulin in comparison to $\alpha\beta$ -III tubulin. Meanwhile, paclitaxel is more effective in inhibiting the dynamics of MTs made up of $\alpha\beta$ -II and $\alpha\beta$ -IV tubulin than of $\alpha\beta$ -III tubulin (Huzil *et al.*, 2006). Similar results were obtained for vinblastine (Khan & Ludueña, 2003). MTs composed of $\alpha\beta$ -IV tubulin seems to have a less rigid conformation than those composed of $\alpha\beta$ -III tubulin (Sharma & Ludueña, 1994). This could be one reason for resistance to MT poisons associated with overexpression of β -III tubulin.

1.5.6 Microtubule poisons

MT poisons, a term collectively used for drugs that target MT and disrupt mitotic progression, have long been used in the treatment of cancer. These drugs can be broadly classified into two groups, the MT stabilizers and MT de-stabilizers, as the difference lies in their mechanism of action. The MT stabilizers, such as taxanes, enhance the polymerization of MTs, whereas the de-stabilizers, such as Vinca alkaloids, inhibit the polymerization of MTs (Murray *et al.*, 2007). Both types of MT poisons affect the MT dynamics and lead to cells undergoing mitotic arrest and eventually cell death through either apoptosis or mitotic catastrophe (Kennedy *et al.*, 2004).

Vinca alkaloids are a specific class of drugs that have shown clinical efficacy both as single agents and in combination in several cancers including MPM. Vinca alkaloids, such as vinorelbine, were first isolated from *Catharanthus roseus*, the Madagascar periwinkle. The compounds bind to the vinca binding site on the β -tubulin subunit of microtubules (Jordan & Wilson, 2004). Vinorelbine seems to alter microtubule

dynamics in a different fashion compared to other drugs of the vinca alkaloid family. Vinorelbine slows down the microtubule growth rate, suppressing the rate of treadmilling and reducing the polymer mass of the microtubules with no effect on the shortening rate (Ngan *et al.*, 2000). Vinorelbine has been studied in the first-line setting as a single agent and in combination with platinum compounds in MPM (Ceresoli & Zucali, 2015). However, 59% of MPM tumours exhibited extreme or intermediate resistance to vinorelbine as shown in a cohort of 203 resection specimens (Mujoomdar *et al.*, 2010).

1.6 Aims and Objectives

BRCA1 protein expression is reported to be lost in 39% of mesothelioma cases and this modifies the response to chemotherapeutic drugs; yet genetic mutation or loss of the *BRCA1* gene is not common (Busacca *et al.*, 2012). We therefore wished to explore the pathways that lead to loss of BRCA1 protein in mesothelioma (Kennedy *et al.*, 2004; Quinn *et al.*, 2003; Zhou *et al.*, 2003).

Here, we hypothesised that BAP1 regulates BRCA1 protein stability and also has functions in mitosis that are independent of BRCA1.

The aims of this project were to (1) determine whether BRCA1 modulates the spindle assembly checkpoint in response to vinorelbine; (2) explore whether BAP1 regulates BRCA1 stability and phenocopies BRCA1 function; and (3) determine whether BAP1 has BRCA1-independent roles during mitosis.

The experimental objectives of each of the result chapters are outlined below:

1. To examine the role of BRCA1 in regulating centrosome number and the SAC in MPM cells using fixed and live cell imaging, flow cytometry and western blotting. Determine whether BRCA1 loss affects MT dynamics by regulating β -tubulin expression using western blot. Analyze the effect of vinorelbine treatment on MTs in BRCA1 depleted cells.
2. To understand how BAP1 regulates BRCA1 stability and whether it is proteasome dependent. Undertake functional studies using RNAi interference and examine which functions of BRCA1 are phenocopied by BAP1 including centrosome number, regulation of SAC proteins, MAD2 and BUBR1 and vinorelbine resistance using western blotting, flow cytometry and fixed cell imaging. Analyze whether wild-type and BAP1 mutants can restore BRCA1 expression. Examine the effect of BAP1 loss in DNA repair by analyzing BRCA1 foci after DNA damage and whether it can be rescued by wild-type and cancer-associated BAP1 mutants.

3. To determine whether BAP1 has BRCA1 independent functions during mitosis using western blotting and fixed cell imaging. Examine and quantify any change in mitotic spindle organization and centrosome volume after BAP1 depletion. Identify whether BAP1 depletion perturbs the expression of any motor proteins involved in mitotic spindle assembly. Perform rescue studies to test whether the mitotic phenotypes observed upon BAP1 depletion are due to loss of specific motor proteins.

Chapter 2

Materials & Methods

2.1 MATERIALS

2.1.1 Reagents

Reagent	Supplier
Precision Plus all blue protein standards	Bio-Rad
BCA protein assay reagent	
Hoechst 33258	Calbiochem
Bovine serum albumin (BSA) fraction V	Fisher Scientific
EDTA; EGTA; KCL, MgCl ₂ , NaCl	
Ethanol	
Glycerol	
Methanol	
Tween-20	
EGF Recombinant Human Protein	
ProtoFLOWgel (30% w/v acrylamide)	Flowgen Bioscience
X-ray film	Scientific Lab Supplies
Oligofectamine reagent	Invitrogen/Gibco
Lipofectamine 2000 reagent	
RNase A	
F-12 nutmix media	
RPMI 1640 with GlutaMAX™	
Medium 199	
Opti-MEM with GlutaMAX™	
Penicillin/streptomycin	
NuPAGE™ 3-8% Tris-Acetate Protein Gels	
NuPAGE™ LDS Sample Buffer	
NuPAGE™ Tris-Acetate SDS Running Buffer (20X)	
NuPAGE™ Transfer Buffer (20X)	
NuPAGE™ Sample Reducing Agent (10X)	
Heat-inactivated Foetal Bovine serum	
Hydrocortisone	Sigma-Aldrich
Insulin solution from bovine pancreas	
HEPES	
SDS	Melford
ECL western blotting reagent	Pierce
WesternBright ECL HRP substrate	Advansta
Nitrocellulose transfer membrane	GE Healthcare Life Sciences
Coverslips 22 mm, No. 1.5 Glass slides	VWR International

2.1.2 Drugs

The following drugs were dissolved in DMSO, unless stated below. The final concentration of each drug was prepared in fresh pre-warmed culture media and mixed well before adding to cells.

Drug	Concentration	Supplier
MG132 (Proteasome inhibitor)	20 μ M	Sigma-Aldrich
Vinorelbine	20 nM	Sigma-Aldrich
STLC (EG5 inhibitor)	10 μ M	Sigma-Aldrich

2.1.3 Antibodies

2.1.3.1 Primary antibodies

A list of primary antibodies used for Western blotting (WB), indirect immunofluorescence (IF). Each antibody is represented with its working dilution and in brackets the final concentration where known. The product code for each antibody is shown in brackets in the supplier column.

Antibodies	Dilution	Supplier
α -tubulin (mouse)	1:250 (IF)	Fisher Scientific (DM1A)
α -tubulin (rabbit)	1:500 (IF); 1:1000 (WB)	Fisher Scientific (PA5-29444)
γ -tubulin (mouse)	1:500 (IF)	Abcam (ab27074)
γ -tubulin (mouse)	1:500 (IF)	Abcam (ab11321)
Pericentrin	1:500 (IF)	Abcam (ab28144)
GADPH	1:1000 (WB)	Cell Signalling (2118)
BRCA1 (Rabbit)	1:500 (WB)	Santa Cruz (C-20)
BRCA1 (mouse)	1:100 (IF)	Santa Cruz (sc-6954)
β -tubulin I (mouse)	1:500 (WB)	Sigma-Aldrich (T7816)
β -tubulin III (mouse)	1:500 (WB)	Sigma-Aldrich (T8660)
CENP-A	1:500 (IF)	Abcam (ab13939)
BUBR1	1:500 (IF)	Abcam (ab28193)
MAD2	1:500 (WB)	Fisher Scientific (PA5-21594)
CDK5RAP2	1:500 (IF)	Bethyl Laboratories (IHC-00063)
C-NAP1	1:500 (IF)	Proteintech (14498-1-AP)
KIF18A	1:200 (WB)	Bethyl Laboratories (A301-080A)
KIF18A	1:200 (IF)	Bethyl Laboratories (A301-079A)
KIF18B	1:500 (WB)	Bethyl Laboratories (A303-982A)

2.1.3.2 Secondary antibodies

Secondary Antibody	Dilution	Supplier
Goat anti-mouse Alexa 488	1:200 (IF)	Molecular Probes-Life Technologies
Goat anti-rabbit Alexa 488	1:200 (IF)	
Goat anti-mouse Alexa 594	1:200 (IF)	
Goat anti-rabbit Alexa 594	1:200 (IF)	
Goat anti-mouse IgG horseradish peroxidase conjugate	1:5000 (WB)	Bethyl Laboratories
Goat anti-rabbit IgG horseradish peroxidase conjugate	1:1000 (WB)	Sigma-Aldrich

2.2 Cell Culture

2.2.1 Cell line maintenance

REN and RVR cell lines were cultured in F12 nutmix media supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (1% Pen/strep). MSTO and MVR cells were cultured in RPMI 1640 supplemented with 10% v/v FBS and 1% Pen/strep. MSTO:shBRCA1 cells were cultured in RPMI 1640 supplemented with 10% v/v FBS, 1% Pen/strep and 0.3% puromycin for selection. H2452, H226 parental and stable cell lines were cultured in RPMI 1640 supplemented with 10% v/v FBS, 1% Pen/strep. MET-5A cells were cultured with Medium 199 with 10% v/v FBS, 1% Pen/strep, 3.3 nM epidermal growth factor (EGF), 400 nM hydrocortisone, 870 nM bovine insulin and 20 nM HEPES. MPM cell lines (REN, RVR, MSTO and MVR) used in this research study were a gift from Prof. Dean Fennell, Cancer Research UK Centre Leicester. Parental REN, MSTO and vinorelbine resistant cells RVR and MVR were generated by long-time exposure to vinorelbine. The vinorelbine resistant cells show low BRCA1 expression. MSTO:shBRCA1 cell

line, has shBRCA1 construct that downregulates the expression of BRCA1 through doxycycline induction. H226 parental, wild-type and mutant BAP1 cell lines were a gift from Dr. Krishna Kolluri, UCL, London.

Cell lines were maintained at 37°C in a humidified 5% CO₂ atmosphere and passaged upon reaching 80-90% confluency. Cells were washed with 1x PBS (phosphate buffer saline; 137 mM NaCl, 8.1 mM Na₂HPO₄, 2.7 mM KCl, 1.4 mM KH₂PO₂, pH 7.4) and detached with 1x PBS containing 0.5 mM EDTA for 10 minutes. Cells were seeded into appropriate dishes containing pre-warmed growth media. No more than 10 passages were used for all experiments.

Cell line	Disease	Cell morphology	BAP1 mutation
MET-5A	non-malignant	Epithelial	None
REN	MPM	Epithelioid	None
RVR	MPM	Epithelioid	None
MSTO	MPM	Biphasic	None
MVR	MPM	Biphasic	None
MSTO:shBRCA1	MPM	Biphasic	None
H2452	MPM	Epithelioid	A95D
H226-parental	MPM	Epithelioid	Deletion
H226-wildtype	MPM	Epithelioid	Wildtype
H226-C91A	MPM	Epithelioid	C91A
H226-NLS	MPM	Epithelioid	Stop codon at 719
H226-HBM	MPM	Epithelioid	Δ366-369
H226-T493A	MPM	Epithelioid	T493A
H226-ASXL1/2	MPM	Epithelioid	Δ666-669
MDA-MB-231	Breast cancer	Mesenchymal	Wildtype

Table 2.1. Cell lines categorized based on their cell morphology, disease and BAP1 mutation in the cell lines.

2.2.2 Storage of cell lines

Cells lines were washed with 1x PBS, detached using PBS-EDTA and pelleted by centrifugation at 1100 rpm for 5 minutes. The supernatant was removed and the pellet suspended in 10% v/v DMSO in FBS (HeLa and HBL-100) and transferred to cryotubes (TPP Helena Biosciences). The cryotubes were then incubated in an isopropanol filled cryo freezing container (Nalgene) and stored at -80°C for at least 16 hours. The cryotubes were then transferred to liquid nitrogen for long-term storage. To thaw cells, a cryotube was removed from liquid nitrogen and immediately thawed in a 37°C waterbath. Cells were then washed once with the appropriate pre-warmed media, centrifuged (1100 rpm, 5 minutes) at room temperature, resuspended in fresh media and transferred to a culture dish or flask.

2.2.3 Drug treatment of cell lines

Asynchronously growing cells were seeded to give 1×10^5 cells/ml either on a 6-well plate or 6 cm dish the day before treatment. The next day, cells were incubated with the appropriate dilutions of each drug shown in Table 2.1.2. For MG132 or monastrol treatment, cells were treated for 6 hours after BAP1 or BRCA1 depletion, whereas treatment with vinorelbine was performed for 24 hours. Control cells were treated with the same volume of DMSO.

2.2.4 RNA interference

Cells were seeded in 6-well plates at approximately 30% confluency in Opti-MEM Reduced Serum Medium supplemented with 10% FBS containing no antibiotics and cultured overnight. The following day, cells were washed once with 1x PBS and incubated with 40 nM siRNA oligonucleotides (BAP1_1; BAP1_3; BRCA1_14 Flexitube, Qiagen). siRNA duplexes were transfected using Oligofectamine (Invitrogen, UK) according to manufacturer's instructions. Cells were incubated with the siRNA mixture and Opti-MEM media for 5 hours (37°C, 5% CO₂). Opti-MEM with 30% v/v FBS (no antibiotic) was then added to cells and incubated for 72 hours before analysis by immunofluorescence microscopy or Western blotting.

2.2.5 Transient transfections

Cells were transfected with plasmids, 48 hours after BAP1 depletion. Briefly, plasmid DNA and lipofectamine 2000 were mixed at a ratio of 1 µg: 4 µl in Opti-MEM reduced serum medium (Invitrogen), according to the manufacturer's instructions. Transfection mixture was added drop-wise to cells on which the growth media had been replaced with Opti-MEM media. The plates were incubated for 5 hours after which the media was replaced with pre-warmed growth media and incubated for a further 24 hours before being processed as required.

2.2.6 Flow cytometry

To determine cell cycle profiles, cells were harvested, centrifuged at 1100 rpm for 5 minutes at room temperature and pellets re-suspended in 150 µl PBS. Cells were then fixed with 2 ml of ice-cold 70% ethanol in a drop-wise manner, whilst gently vortexing and incubated for 30 minutes at 4°C or kept at -20°C for a week. Briefly, cells were washed twice in 1x PBS, spun at 3000 rpm for 5 minutes at 4°C and re-suspended in 1x PBS containing 100 µg/ml RNase A and 50 µg/ml propidium iodide (PI). Samples were transferred into FACS tubes and incubated overnight at 4°C in the dark and analysed for flow cytometry using a BD FACScanto™ II instrument and analysed using FACSDiva™ 6.0 software (Becton Dickinson). 10,000 events were recorded in each sample to analyse their DNA content.

2.2.7 Ionizing radiation

Asynchronously growing cells were seeded in a 6-well plate, 24 hours prior to reach 70-80% confluency on the day of irradiation. Cells were irradiated using an Xstrahl RS320 X-Ray irradiator. Cells were exposed to 0 Gy or 5 Gy of irradiation. Cells were grown for an additional 8 hours and then processed for immunofluorescence staining.

2.3 MICROSCOPY

2.3.1 Indirect immunofluorescence microscopy

Cells were plated on acid-etched glass coverslips and treated as appropriate. Cells were washed in 1x PBS, fixed and permeabilized in ice-cold methanol at -20°C for 30 minutes. Coverslips were then washed three times with 1x PBS for 5 minutes each and blocked in 1x PBS supplemented with 1% w/v BSA and 0.2% Triton X-100 for 60 minutes. In the meantime, primary antibodies (Table 2.1.3.1) were diluted in 1x PBS with 3% w/v BSA and spun at 10,000 rpm for 3 minutes to remove any insoluble aggregates. Each coverslip was incubated with 150 µl of primary antibody solution for 2 hours. After incubation, coverslips were washed three times in 1x PBS for 5 minutes each. The secondary antibodies (Table 2.1.3.2) were diluted in 1x PBS supplemented with 1% w/v BSA and spun as before. Coverslips were incubated with the secondary antibody solution and Hoechst 33528 for 1 hour in the dark at room temperature. After incubation, coverslips were washed again three times in 1x PBS for 5 minutes each and mounted on a glass slide with a drop of mountant solution (80% v/v glycerol, 3% w/v n-propyl-gallate in 1x PBS). Coverslips edges were sealed with clear nail varnish and kept in the dark at 4°C.

2.3.2 Image quantifications

Quantification of centrosome volume of cells in interphase and mitosis following staining with γ -tubulin and CDK5RAP2 antibodies were done using Imaris3D in Easy 3D view using object surface selection. Spindle length was measured as pole-to-pole distance using Imaris3D using slice selection and measured the length by setting two measurements points. Microtubule length in monopolar spindles was measured using Imaris3D from the centre of the nucleus to the microtubule length using slice selection toolbar. KIF18A intensity and aster volume was measured using ImageJ 3D object counter by selecting appropriate measurement parameters by selecting the region of interest using the freehand tool. BUBR1 intensity was quantified as individual BubR1 antibody spots using the plugin designed for the measurement in ImageJ. Centrosome numbers and multipolar spindles were counted manually.

2.3.3 Live cell imaging

Time-lapse imaging was performed on a Nikon Eclipse Ti microscope equipped with an Andor iXonEM+ EMCCD DU 885 Camera using a 10× phase objective. Cells were cultured in multi-well plates and maintained on a stage with a heated incubator at 37°C supplemented with 5% CO₂ using a microscope temperature control system (Life Imaging Services). Images were acquired every 10 minutes for 24 hours using NIS-elements software. Videos were prepared using ImageJ (National Institutes of Health).

2.4 PROTEIN ANALYSIS

2.4.1 Preparation of cell extracts

Cells were washed with 1x PBS and harvested as normal. The cell suspension was spun at 1,200 rpm for 5 minutes. The supernatant was discarded and pellet re-suspended in 250-500 µl of ice-cold RIPA lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% w/v SDS, 0.5% v/v NP40 (Igepal), 0.5% w/v sodium deoxycholate, 0.5% Triton, 1x Protease inhibitor cocktail (PIC), 5 mM Sodium fluoride (NaF), 5 mM β-glycerolphosphate, 30 µg/ml RNase, 30µg/ml DNase I. Cell extracts were incubated on ice for 30 minutes and lysates passed through a 27G needle for shearing DNA and releasing proteins. Lysates were then centrifuged at 13,000 rpm for 10 minutes at 4°C to remove insoluble material. Supernatants were either analyzed directly by SDS-PAGE or snap frozen and stored at -80°C.

2.4.2 BCA protein assay

Protein concentration of cell lysates was determined using the BCA protein assay. BCA working reagent was prepared according to the manufacturer's instructions. The following reaction: 800 µl Reagent B with 100 µl Reagent A plus 20 µl of cell lysate was mixed in a cuvette. The assay mixture was then incubated for 15 minutes at room

temperature and the absorbance at 650 nm measured. A serial dilution of BSA standards was prepared and assayed in parallel to generate a standard curve from which the protein concentration of the samples could be calculated.

2.4.3 SDS-PAGE

Protein samples were resolved on 10 or 12% polyacrylamide gels by electrophoresis. Gels were cast and resolved using the Mini-PROTEAN 3 polyacrylamide gel electrophoresis (PAGE) system (Bio-Rad). Resolving gel (26.7-40% ProtoFlowgel (30% w/v acrylamide), 126 mM Tris-HCl pH 8.8, 0.1% w/v SDS, 0.13% w/v APS, 0.08% v/v TEMED) was overlaid with stacking gel (13% ProtoFlowgel (39% w/v acrylamide), 126 mM Tris-HCl pH 6.8, 0.1% w/v SDS, 0.15% w/v APS, 0.1% v/v TEMED). Protein samples were mixed with an appropriate volume of 3x Leammli buffer (62.5 mM Tris-HCl pH 6.8, 10% v/v glycerol, 2% w/v SDS, 5% v/v β -mercaptoethanol, 0.01% w/v bromophenol blue) and denatured at 95°C for 5 minutes. Precision Plus Protein TM Dual Color Standards (Bio-Rad) were loaded on the same gel. Electrophoresis was performed at 180 V for ~1 hour using SDS-running buffer (25 mM Tris-base, 192 mM glycine, 0.1% w/v SDS). For BRCA1 protein expression Nupage™ 3-8% Tris-acetate gel (ThermoFisher) were used. Lysates were denatured at 70°C for 10 minutes after addition of Nupage™ lithium dodecyl sulfate buffer (LDS) and Nupage™ reducing agent. 50-70 μ g of sample were loaded into the wells. Nupage™ 1x Tris-Acetate SDS running buffer was filled in the Invitrogen western blot tank running at 100V for approximately 60 min.

2.4.4 Western blotting

For BRCA1 protein, upon completion of the gel electrophoresis, the nitrocellulose membrane (0.45 μ m pore size), sponges and filter paper were soaked in the Nupage™ transfer buffer for 15 min. Membrane and gel were sandwiched between sponge and filter paper. It was ensured that no air bubbles were trapped between the membrane and the gel. The cassette was placed in an Invitrogen transfer system and transfer buffer was only used in sandwiched area, water was filled around the cassette in the tank. The proteins were transferred at 100 V for 60 min.

All other proteins were transferred from SDS-polyacrylamide gels to nitrocellulose membrane for immunodetection using semi-dry blotting. Transfer was carried out by soaking (0.45 µm pore size) nitrocellulose membrane in blotting buffer (25 mM Tris, 192 mM glycine, 10% v/v methanol) along with 6 pieces of Whatman 3 MM chromatography paper. The gel was then placed on the membrane and sandwiched between 3 pieces of blotting paper on either side. This gel sandwich was then transferred in a TE 77 semi-dry transfer unit (Amersham) for 1 hour at 1 mA/cm² membrane. Ponceau red stain solution (0.1% w/v Ponceau S, 5% v/v acetic acid) was used to visualize that the protein had transferred successfully. Blots were then blocked in 5% w/v non-fat milk powder in 0.1% v/v Tween-20 in 1x PBS for 1 hour at room temperature on a rocking platform. For Western blotting, the antibodies were diluted in PBS supplemented with 0.1% v/v Tween-20 and 5% nonfat milk powder. The membrane was then incubated with primary antibody at the appropriate dilution in 5% non-fat milk powder/1x PBST overnight at 4°C. Membranes were washed 3 times in 1x PBST for 10 minutes each and incubated with the horseradish peroxidase-conjugated secondary antibody in 5% non-fat milk powder/PBST for an extra 1 hour at room temperature. Secondary antibodies were diluted in PBS supplemented with 0.1% v/v Tween-20 and 5% non-fat milk powder. Membranes were then washed again 3 times in 1x PBST to remove unbound secondary antibody and developed in enhanced chemiluminescence (ECL) Western blotting detection solution (Pierce) according to the manufacturer's instructions. BRCA1 blots were developed using WesternBright ECL (Advansta). The proteins were visualised on X-ray film developed using a compact X4 X-ray film processor (Xograph imaging system).

2.5 Immunohistochemistry

Patient tissue samples were obtained as part of the MEDUSA study (Prof. Dean Fennell). Stained by Aarti Gaba at NHS using the Dakolink 48 autostainer. Fresh sections from patient tissues were cut 4 µm thick a day prior to staining, the slides were heat blasted at 60°C for one hour to remove excess wax. The slides were deparaffinized and pretreated in Dako PT Link (pre-treatment) with target retrieval solution, high or low pH for an hour. Slides were then transferred to the Dakolink 48

autostainer and queued using barcodes with their respective antibodies for four hours. Slides were scanned using a Hamamatsu Nanozoomer Digital slide scanner. The following antibodies were used for immunohistochemistry:

BRCA1 – Abcam ab213929, High pH antigen retrieval, heat blasting required, 1:100 + 3% albumin.

BAP1 – Santa Cruz sc28383, High pH antigen retrieval, no heat blasting required, 1:50 + 3% albumin.

All IHC slides were scored by pathologist Dr. Micheal Sheaff.

2.6 RT-PCR

Total RNA was extracted using the phenol-chloroform method (Doyle, 1996) and converted to cDNA using reverse transcriptase (Qiagen), according to the manufacturer's protocol. PCR for *BRCA1* expression was performed with forward and reverse primers, 5'-GCAGTTCTCAAATGTTGGAGTGGA-3' and 5'-CCATGCCCAGGTTTCAAGTTTC-3', respectively. Reaction volumes were: Template cDNA - 0.02µM, 2x SYBR green - 25µl, forward and reverse primer- 0.4µM, RNase-free water - variable (up to a final volume of 50µl). Reactions were performed on a BIO-RAD DNA engine Dyad peltier thermal cycler. Cycling conditions were: 95°C for 5 min, followed by 45 cycles of 95°C for 30 sec, 60°C for 30 sec. β-actin was used as a control. PCR products were visualised on an agarose gel, stained with ethidium bromide and quantified with ImageJ.

2.7 Statistical analysis

For data analysis, GraphPad Prism version 6.0 was used to calculate the mean of three independent experiments, unless otherwise stated. Errors bars show standard deviation of the mean (S.D., n=3). One-way ANOVA analysis was used to compare the means of three or more unrelated groups. The one-way ANOVA analysis determined whether any of the means are significantly different from each group. Two-way ANOVA compared multiple groups of two factors. One-tailed unpaired Student's t-test was used to compare the means of two unrelated groups and calculate the confidence interval, which must be 95%, for the difference between the means to be accurate. All *p* values less than 0.05 were considered significant. * *p*<0.05; ** *p*<0.01; *** *p*<0.001; **** *p*<0.0001.

CHAPTER 3

BRCA1 regulates response to vinorelbine

3.1 INTRODUCTION

Mesothelioma is an aggressive cancer with a dismal prognosis. It is mainly caused due to exposure to asbestos and due to its long latency period; its incidence is increasing worldwide (Odgerel *et al.*, 2017) Mesothelioma is generally resistant to therapy. Most of the patients are treated with chemotherapy during their course of the disease but no drug has shown a response rate greater than 20% (Steele *et al.*, 2000). The therapeutic options for patients are scarce.

Vinca alkaloid are a group of chemotherapeutic drugs with potent anti-tumour activity, related to their interaction with tubulin and disruption of MT function. This particularly effect the mitotic spindle MTs leading to mitotic arrest and activating the SAC (Moudi *et al.*, 2013) These drugs have demonstrated clinical efficacy in a variety of childhood and adult cancers (Verrills *et al.*, 2003). Vinorelbine is a representative agent of this (Moudi *et al.*, 2013) family and has shown promising clinical activity in mesothelioma with a response rate of 24% (Steele *et al.*, 2000). However about 59% of mesothelioma tumours exhibit drug resistance to vinorelbine (Mujoomdar *et al.*, 2010) As vinorelbine binds at the typical vinca alkaloid site of the β -tubulin, a possible mechanism of vinorelbine resistance is the altered expression of β -tubulin isotypes (Jordan & Wilson, 2004). Baseline expression of β -tubulin III was measured in a cohort of mesothelioma patients treated with vinorelbine showed that β -tubulin III positive expression associated with treatment resistant and significantly associated with prolonged progression free survival (PFS) and overall survival (OS) (Zimling *et al.*, 2012). Vinorelbine can also enhance the expression of β -tubulin III in breast cancer cells through an AP1 binding site on the β -tubulin III promoter (Saussede-Aim *et al.*, 2009), suggesting that vinorelbine could alter the microtubule dynamics in tumour cells and the elevated expression of β -tubulin III could possibly be associated with resistance to microtubule poisons (Parker *et al.*, 2014).

BRCA1 is another gene that has been reported as a potential predictive biomarker in response to microtubule poisons including vinorelbine, vincristine, paclitaxel and docetaxol (Kennedy *et al.*, 2004; Busacca *et al.*, 2012; Tassone *et al.*, 2003; Quinn *et al.*, 2003; Mullan *et al.*, 2001). Loss of *BRCA1* protein expression was identified by

immunohistochemistry in 39% in mesothelioma samples. A statistically significant correlation was identified between BRCA1 expression and vinorelbine efficacy (Busacca *et al.*, 2012).

BRCA1 is a tumour suppressor gene that was originally found to be mutated in breast and ovarian cancer (Brodie & Henderson, 2012). Deletion of Exon 11 of *BRCA1* in mouse embryonic fibroblasts led to centrosome amplification and a defective SAC suggesting that mutations in *BRCA1* cause genomic instability, while full length BRCA1 is important for normal cell function.

To maintain genomic stability, centrosomes are duplicated only once per cell cycle to ensure formation of a bipolar spindle during mitosis, thus segregating the sister chromatids correctly (Hinchcliffe & Sluder, 2001). The tumour suppressor role of BRCA1 can partly be attributed to its role in controlling the centrosome duplication cycle. BRCA1 deficient cells exhibit centrosomal amplification, as well as hyperactive centrosomal nucleation suggesting that BRCA1 might play role in regulating microtubule dynamics (Sankaran *et al.*, 2005). It has also been demonstrated that loss of BRCA1 inactivates the SAC, as it leads to downregulation of SAC proteins such as MAD2 and BUBR1 (Chabalier *et al.*, 2006). Loss of BRCA1 in MCF-7 cells inactivates the SAC and thus promotes premature sister chromatid separation (Sung & Giannakakou, 2014). These studies reveal that BRCA1 deficient cells clearly show an ineffective SAC.

Furthermore, knockdown of BRCA1 increased cell migration and anchorage-independent growth in breast cancer cells (Promkan *et al.*, 2009), which could potentially lead to metastasis. It can be concluded that loss of BRCA1 can dysregulate several pathways associated with malignancy. In summary, BRCA1 not only plays a major role in the DNA damage response but also functions as a negative regulator of multiple proteins involved in carcinogenesis, suggesting that the status of BRCA1 could play an important role in predicting the response to microtubule poisons.

Here, we used Parental REN and MSTO cells and their equivalent vinorelbine resistant cells, RVR and MVR, generated by long term exposure to vinorelbine. The vinorelbine

resistant cells show low BRCA1 expression. We investigated the mitotic phenotypes upon BRCA1 loss in these cell lines.

3.2. Results

3.2.1 Vinorelbine resistance in MPM cells downregulates expression of BRCA1 and impairs vinorelbine induced microtubule depolymerisation

To understand the key role of BRCA1 in resistance to microtubule poisons, we examined the association between BRCA1 expression and response to vinorelbine. REN and MSTO cell lines have been exposed to increasing concentrations of vinorelbine for long term, leading to vinorelbine resistant cell lines RVR and MVR (Busacca *et al.*, 2012). To eliminate any off targets caused by long term vinorelbine dosage, we used another cell line MSTO:shBRCA1 that has an integrated shBRCA1 plasmid that can knockdown BRCA1 by doxycycline induction. Parental REN and MSTO show a higher expression of BRCA1 as compared to the vinorelbine resistant, RVR and MSTO. Meanwhile BRCA1 protein was significantly reduced in the MSTO:shBRCA1 cell line after doxycycline induction for 48 hours (Fig. 3.1A, B).

As, vinorelbine is a microtubule destabilizing agent that inhibits MT dynamics by depolymerizing microtubules, we then examined whether loss of BRCA1 expression had an impact on the microtubule network after vinorelbine treatment. Cells were treated with 20 nM vinorelbine for 24 hours and stained with α -tubulin antibody. Vinorelbine disrupted the MT network in REN cells whereas with the same concentration of vinorelbine, RVR cells displayed an intact MT network. Hence, the ability of vinorelbine to induce microtubule depolymerisation is compromised in RVR cells. At the same concentration, MTs were depolymerized in MSTO:shBRCA1 without doxycycline whereas a mixed population of cells with intact MTs and depolymerized MTs were seen (Fig. 3.2A). Interestingly, MT intensity was higher after BRCA1 knockdown in MSTO:shBRCA1 cells, although no significant difference was seen after vinorelbine treatment (Fig. 3.2B).

We hypothesized that loss of BRCA1 alters MT dynamics by modifying the β -tubulin isotype composition. Significant differences in the protein expression of β -tubulin III were seen between the parental REN and MSTO cells and their equivalent and resistant RVR and MVR cells. However, there was no difference in MSTO:shBRCA1 cells with and without doxycycline (Fig. 3.3A, B). Thus, suggesting that long term

exposure to vinorelbine might have altered β -tubulin III expression, leading to vinorelbine resistance, despite exhibiting vinorelbine resistance whereas the knockdown of BRCA1 seems to have no direct impact on the levels of β -tubulin III.

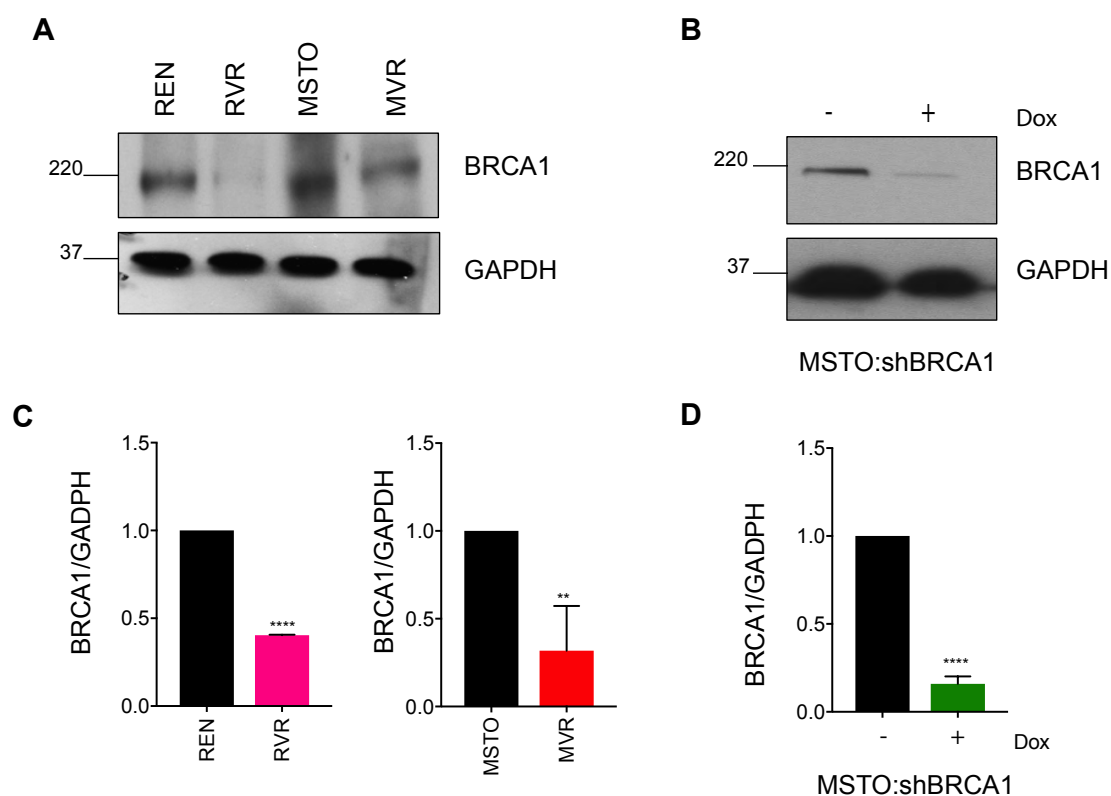
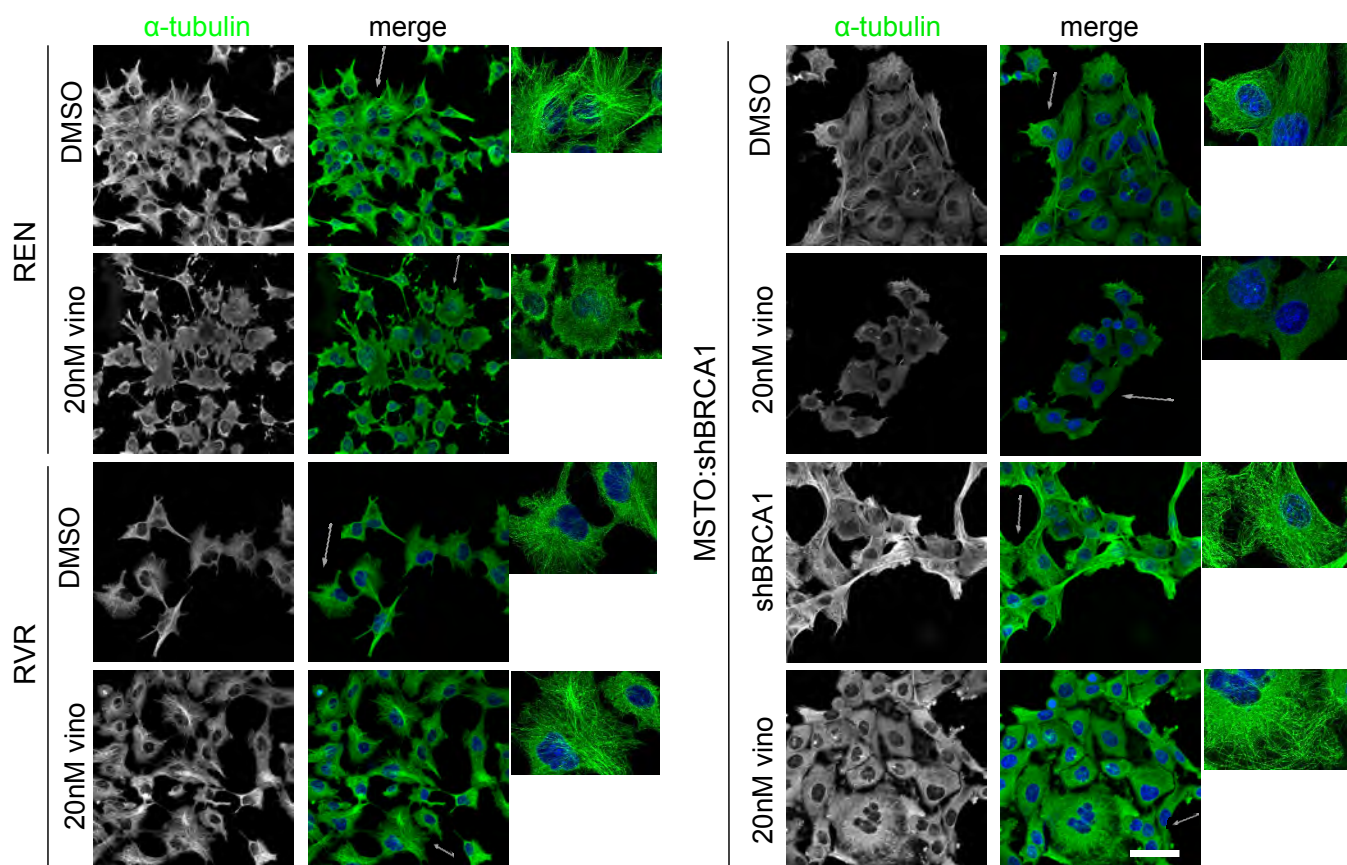


Figure 3.1 Protein expression of BRCA1 in mesothelioma cell lines

A. Cell extracts were prepared from REN, RVR, MSTO and MVR cells and western blotted with the antibodies indicated. **B.** Doxycycline was induced in MSTO:shBRCA1 cells to deplete BRCA1 for 48 hours. Lysates were analysed by SDS-PAGE and immunoblotted for BRCA1 and GAPDH antibodies. Molecular weights are indicated (kDa). **C.&D.** Immunoblots show the densitometric quantification of the immunoblots. Students t-test ** $p < 0.01$, **** $p < 0.0001$. Data show means $\pm$ S.D. of three independent experiments.

A



B

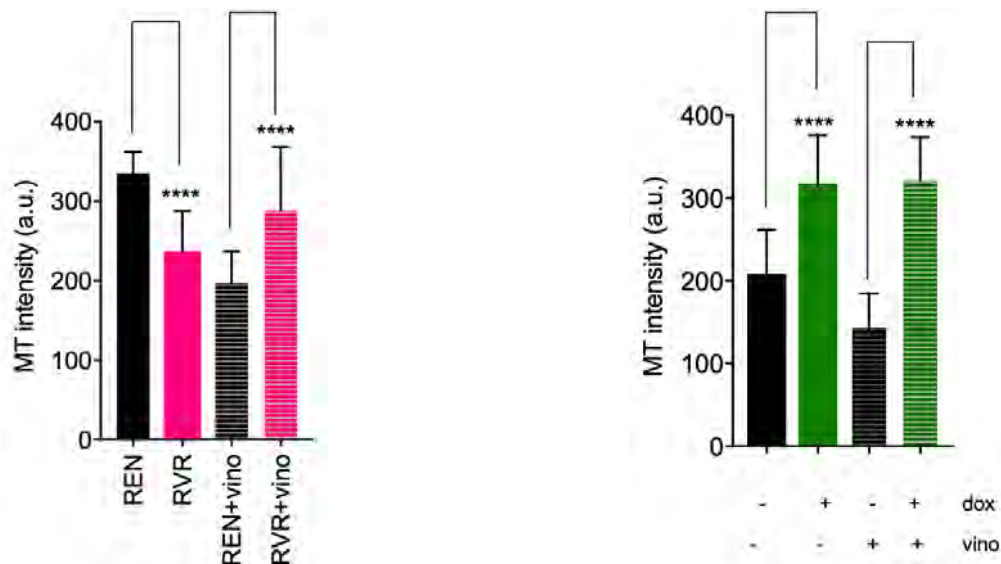


Figure 3.2 Vinorelbine treatment increases MT intensity

A. REN or RVR and MSTO:shBRCA1 cells with or without doxycycline, treated with 20 nM vinorelbine for 24 hours, then fixed and stained with α -tubulin (green) and DNA (blue) with Hoechst 33258. **B.** Histogram shows the quantification of MT intensity. Data show means $\pm$ S.D. of three independent experiments. One-way Anova **** $p < 0.0001$. Scale bar, 5 μ m.

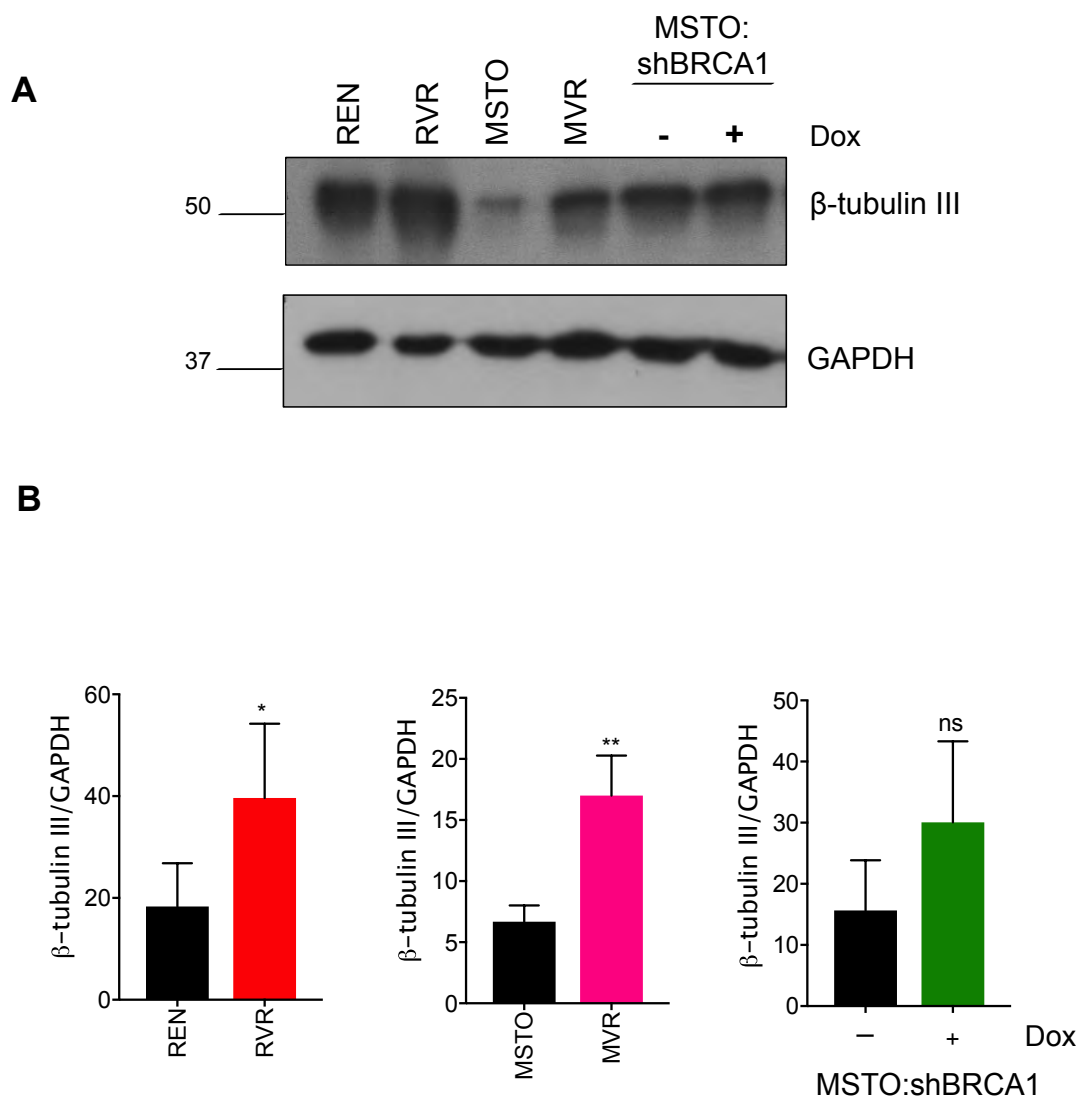


Figure 3.3 β -tubulin III is overexpressed in vinorelbine resistant cell lines

A. Parental REN or MSTO, their equivalent vinorelbine resistant RVR and MVR or MSTO:shBRCA1 cells were lysed and analysed by SDS-PAGE and immunoblotted with β -tubulin III and GAPDH antibodies. BRCA1 was depleted in MSTO:shBRCA1 cells by doxycycline induction for 48hours. **B.** Densitometric quantification of the immunoblots were performed using ImageJ. Data show means $\pm$ S.D. of three independent experiments. One-way Anova * $p < 0.05$, ** $p < 0.01$.

3.2.2 Vinorelbine resistance correlates with loss of the SAC

Microtubule poisons such as vinorelbine inhibit the MT dynamics leading to failure to align the chromosomes at the metaphase plate and the activation of the spindle assembly checkpoint (SAC) (Balachandran & Kipreos, 2017). Under some circumstances, such as BRCA1 loss, the SAC can become inactive allowing the cells to exit mitotic arrest.

To determine whether loss of BRCA1 might contribute to vinorelbine resistance via SAC abrogation in mesothelioma cells, flow cytometry was performed on parental REN and MSTO cells, drug-resistant RVR and MVR cells, REN and MSTO cells in which BRCA1 had been depleted for 48 hours by siRNA, and MSTO cells in which BRCA1 depletion was induced by addition of doxycycline for 48 hours (MSTO:shBRCA1). Cells were treated with 20 nM of vinorelbine for 0, 8 or 16 hours. The parental REN and MSTO cells, as well as the MSTO:shBRCA1 cells without doxycycline induction, showed a significant increase in the percentage of cells in G2/M indicating that these cells had an active SAC (Fig. 3.4A). In contrast, the percentage of cells in G2/M phase did not increase after 16 hours of vinorelbine treatment in RVR or REN cells depleted of BRCA1 by siRNA, indicating a loss of the SAC-induced mitotic arrest. MVR, and MSTO cells in which BRCA1 was depleted either using siRNAs or by doxycycline in the inducible cell line (MSTO:shBRCA1), showed an intermediate percentage of cells in G2/M after 16 hours of vinorelbine addition suggesting a transient mitotic arrest due to a compromised but not completely inactive SAC.

To confirm that cells accumulated in G2/M phase are under mitotic arrest, we stained REN, RVR and MSTO:shBRCA1 cells with the mitotic marker phospho-histone 3. REN and MSTO:shBRCA1 cells that were not induced with doxycycline show an increase in mitotic index as compared to RVR cells and after doxycycline induced MSTO:shBRCA1 cells (Fig. 3.5A,B; Fig. 3.6A,B). RVR cells did not show significant accumulation in the G2/M phase. MVR cells accumulated in the G2/M phase at 8 hours but at 16 hours the percentage of cells significantly decreased. This is consistent with result for time-lapse microscopy that reveal that RVR cells do not undergo mitotic arrest and continue with cell cycle progression even after addition of vinorelbine,

whereas MVR undergo transient mitotic arrest but soon override the arrest and continue with the normal cell cycle progression (data not shown, unpublished data, experiments done by Laura O'Regan, Fry lab). Time-lapse imaging of MSTO:shBRCA1 cells that had been treated with or without doxycycline for 24 hours to induce BRCA1 depletion and then incubated in the presence or absence of vinorelbine, confirmed that these cells underwent only a transient mitotic arrest indicative of a defective SAC (Fig. 3.7A,B). Furthermore, staining of these cells with antibodies against BUBR1, a SAC component that localises to the kinetochore, revealed significant reduction of BUBR1 intensity upon induction of BRCA1 depletion (Fig. 3.8A, B).

Cell cycle deregulation is an important molecular event in the acquisition of drug resistance (Kang *et al.*, 2004). We therefore looked at the cell cycle profiles of the parental and vinorelbine resistant cell lines without vinorelbine. Both the vinorelbine resistant cell lines RVR and MVR, showed accumulation of the cells in G1 phase (Fig. 3.9A). To test whether cell cycle deregulation is due to loss of BRCA1, we looked at the cell cycle profiles after BRCA1 knockdown. No difference was seen in the cell cycle profile after BRCA1 knockdown in all three cell lines REN, MSTO and MSTO:shBRCA1 (Fig. 3.9B). These data suggest that the deregulation of cell cycle in vinorelbine resistant RVR and MVR cell lines may play an additional role in the vinorelbine resistant phenotype.

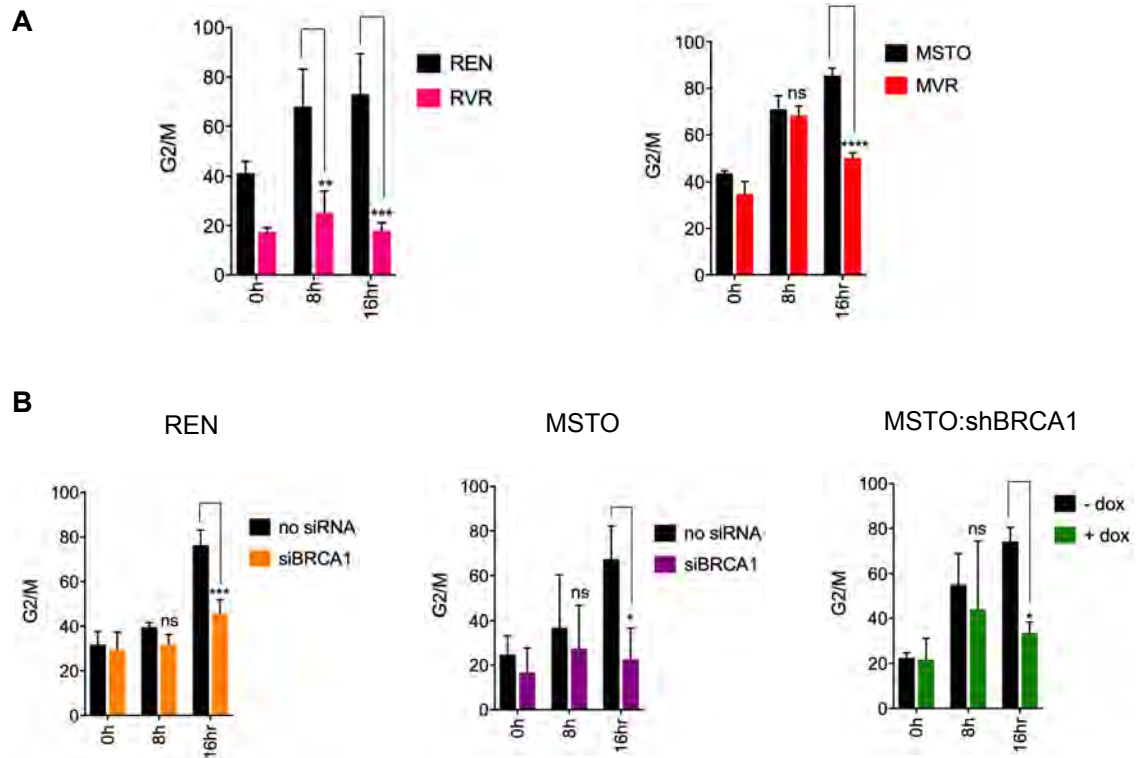


Figure 3.4 BRCA1 deficient cells override vinorelbine induced mitotic arrest

A. Parental REN and MSTO and vinorelbine resistant RVR and MVR cells were treated with 20 nM vinorelbine for 0, 8 and 16 hours and then analysed by flow cytometry. **B.** BRCA1 depleted using siRNAs in REN and MSTO or by doxycycline induction in MSTO:shBRCA1 before treatment with 20 nM vinorelbine for 0, 8 or 16 hours and the cell cycle profile analysed by flow cytometry. No siRNA indicates that cells were incubated with oligofectamine only. Data show means $\pm$ S.D. of three independent experiments. Two-way Anova, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

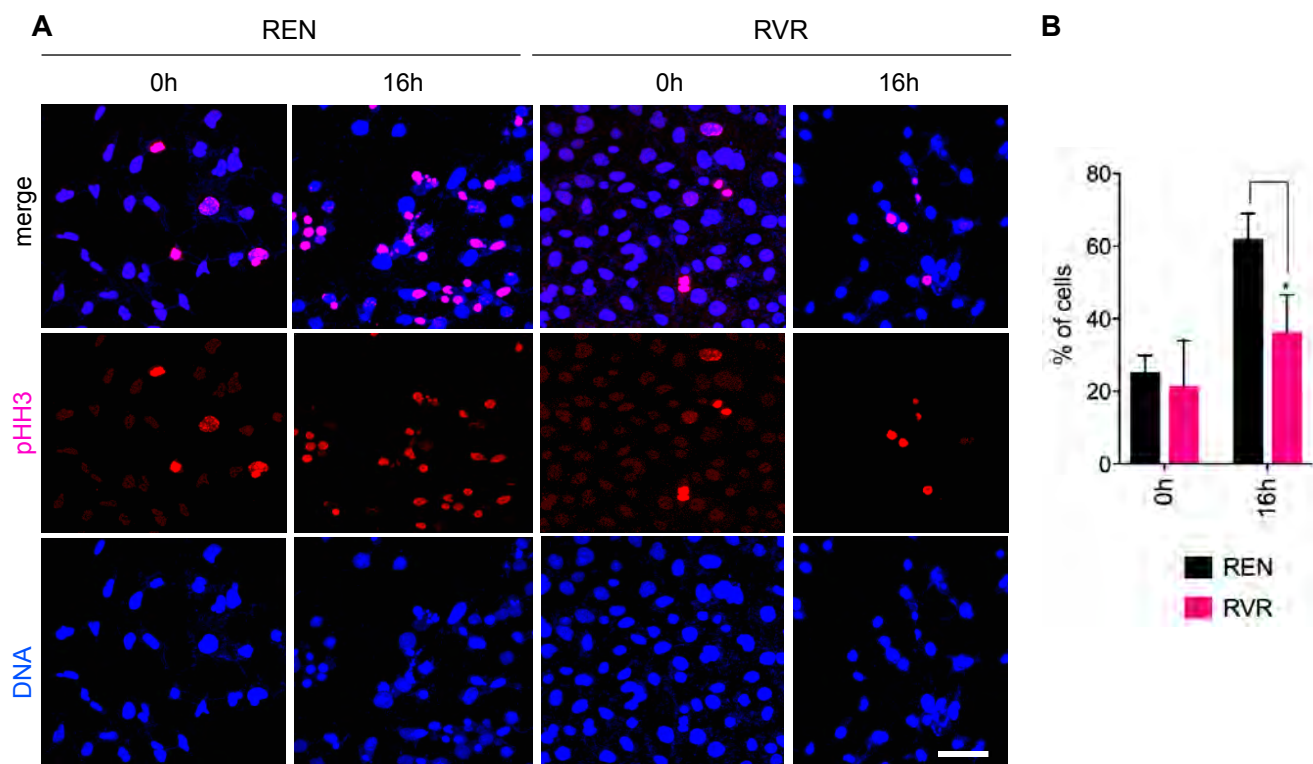


Figure3.5: Vinorelbine does not increase mitotic index in vinorelbine resistant RVR cells

A. REN and RVR cells were treated with 20 nM vinorelbine for 16 hours, fixed and stained with phospho-histone 3 antibody and DNA with Hoechst 33258. **B.** Mitotic index (percentage of mitotic cells/ total cell number) was counted under a confocal microscope. Data show means $\pm$ S.D. of three independent experiments. Scale bar, 5 μ m. Two-way Anova * $p < 0.05$.

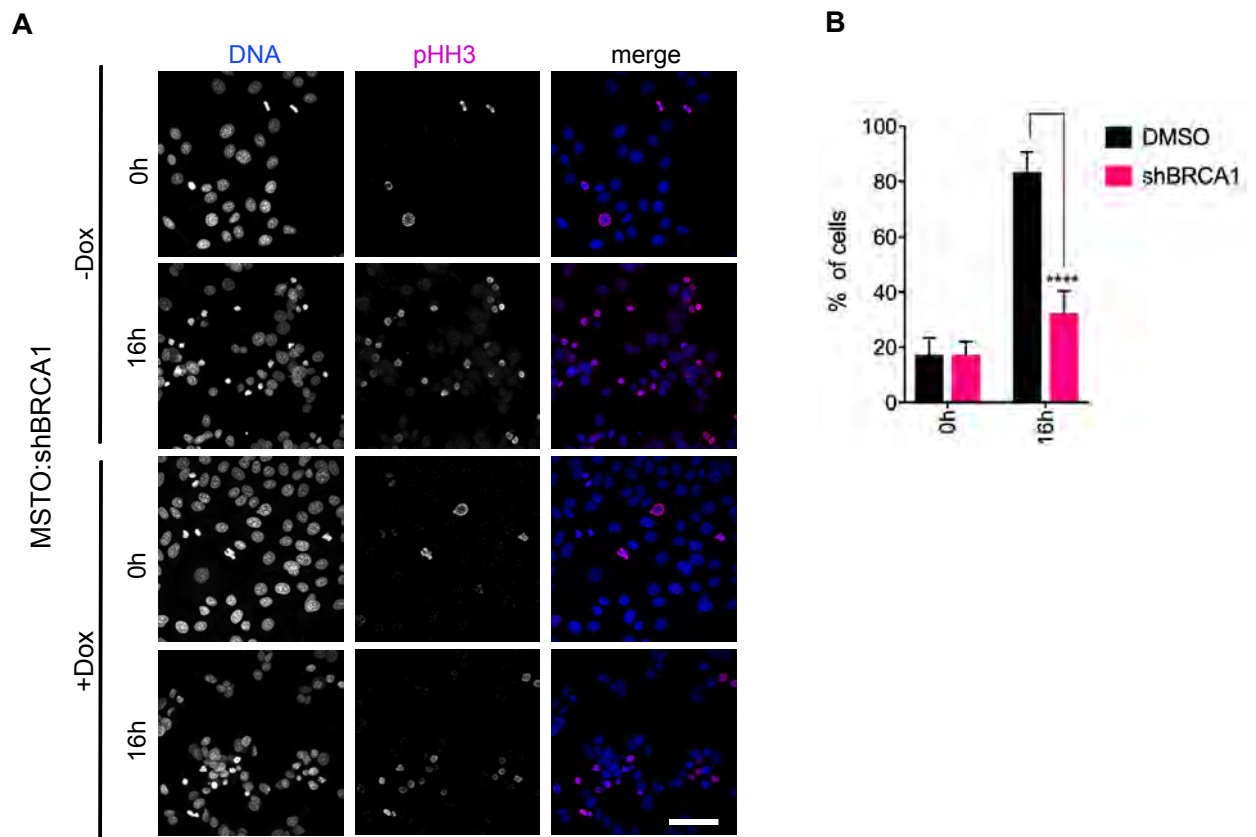


Figure 3.6 Vinorelbine does not increase mitotic index upon BRCA1 depletion

A. MSTO:shBRCA1 cells with or without doxycycline induction for 48 hours, treated with 20 nM vinorelbine for 16 hours, fixed and stained with phosphor-histone 3 antibody and DNA with Hoechst 33258. **B.** Mitotic index (percentage of mitotic cells/ total cell number) was counted under a confocal microscope. Two-way Anova, **** $p < 0.0001$. Data show means $\pm$ S.D. of three independent experiments. Scale bar, 5 μ m.

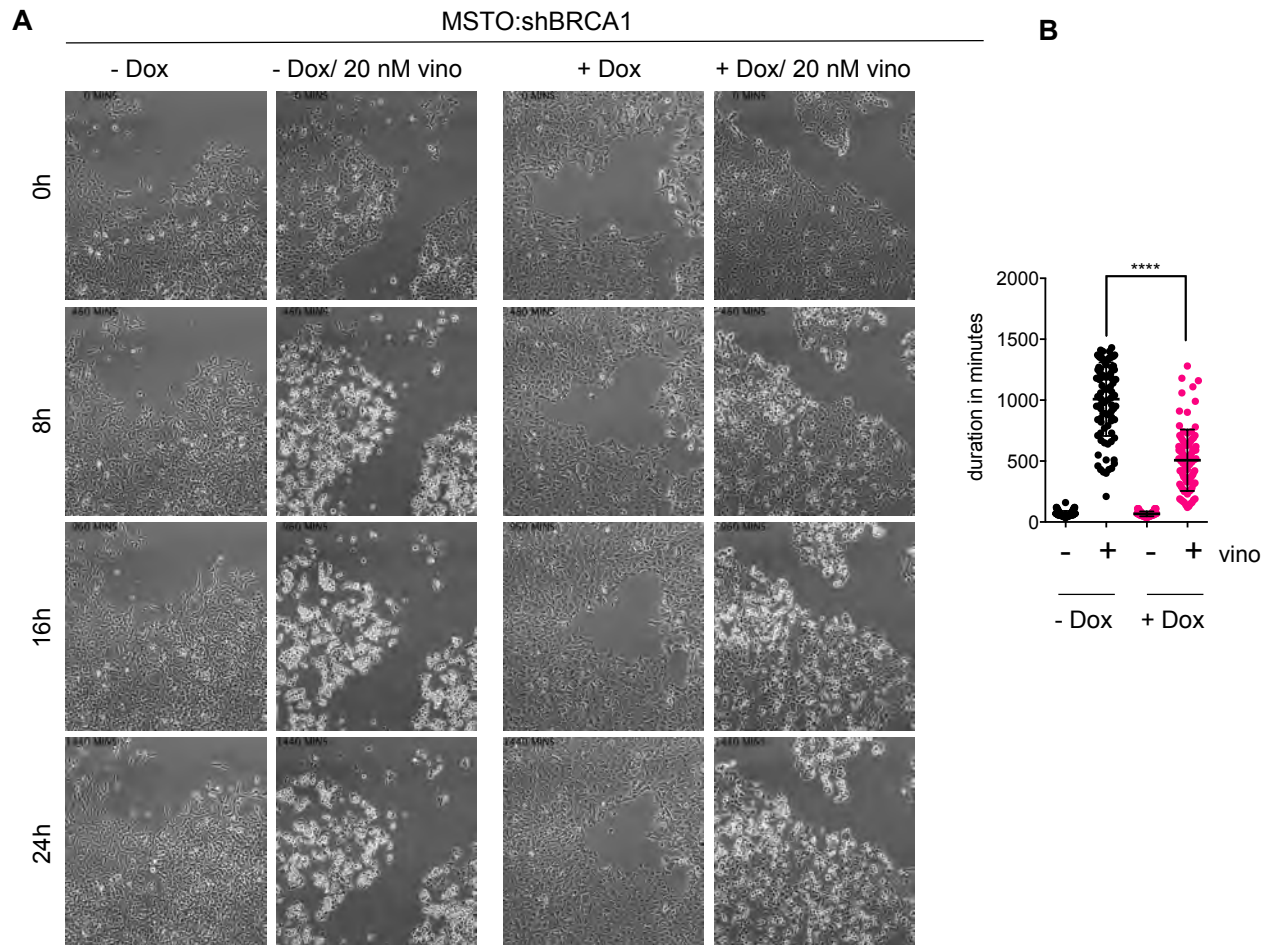


Figure 3.7 BRCA1 loss allows cells to override vinorelbine induced mitotic arrest

A. MSTO:shBRCA1 cells were uninduced (- Dox) or doxycycline induced (+ Dox) for 48 hours to deplete BRCA1. Cells were then treated with 20 nM vinorelbine before time lapse imaging using phase contrast microscopy. Cells were imaged every 10 minutes for 24 hours following the addition of drug. Stills from representative movies are shown with time (h) from addition of the drug indicated. **B.** The histogram shows the duration of the mitotic arrest for each condition. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, **** $p < 0.0001$.

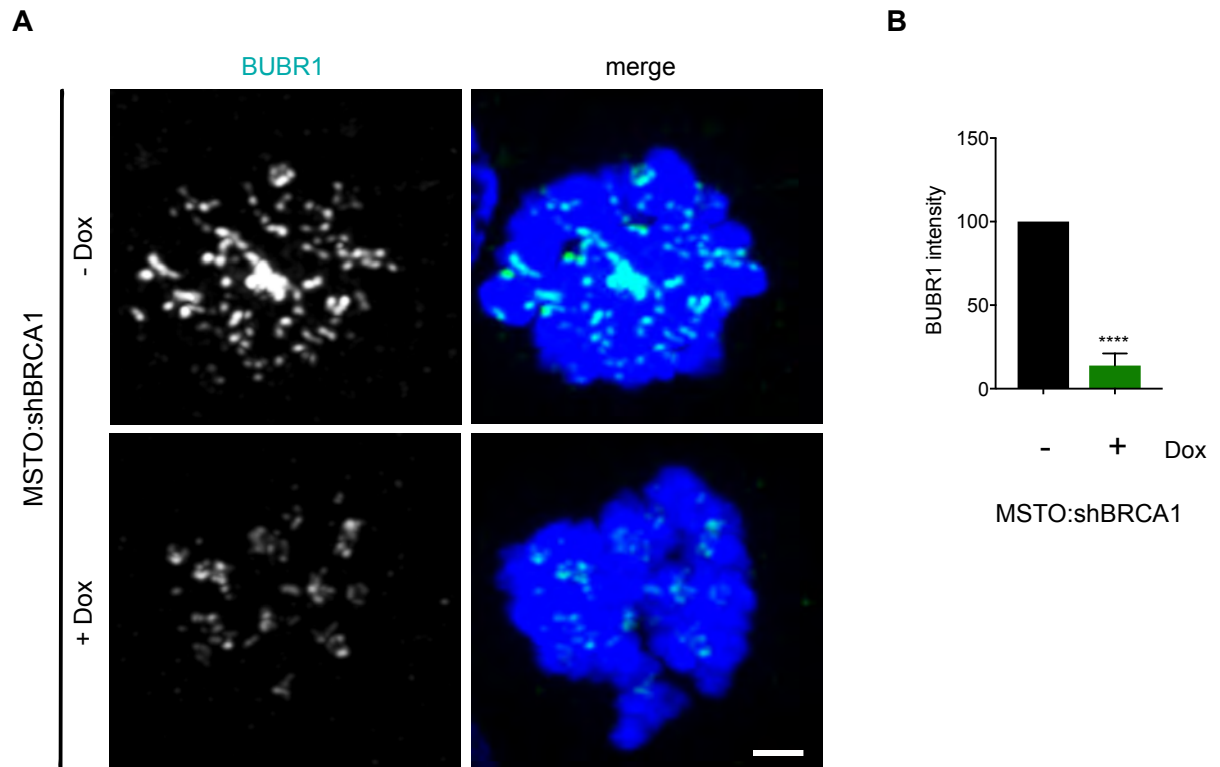


Figure 3.8 BRCA1 loss leads to reduced BUBR1 intensity at the kinetochores

A. MSTO:shBRCA1 cells were uninduced (- Dox) or doxycycline induced (+ Dox) for 48 hours to deplete BRCA1. Cells were then treated with 20 nM for another 24 hours. Cells were fixed and processed for immunofluorescence microscopy with BUBR1 antibody and DNA stained with Hoechst 33258. **B.** The graph shows the mean intensity of BUBR1 from three independent experiments. Values for mock cells were set to 100. The errors bars represent the SD of the data. One-way Anova, **** $p < 0.0001$. Scale bar, 5 μm .

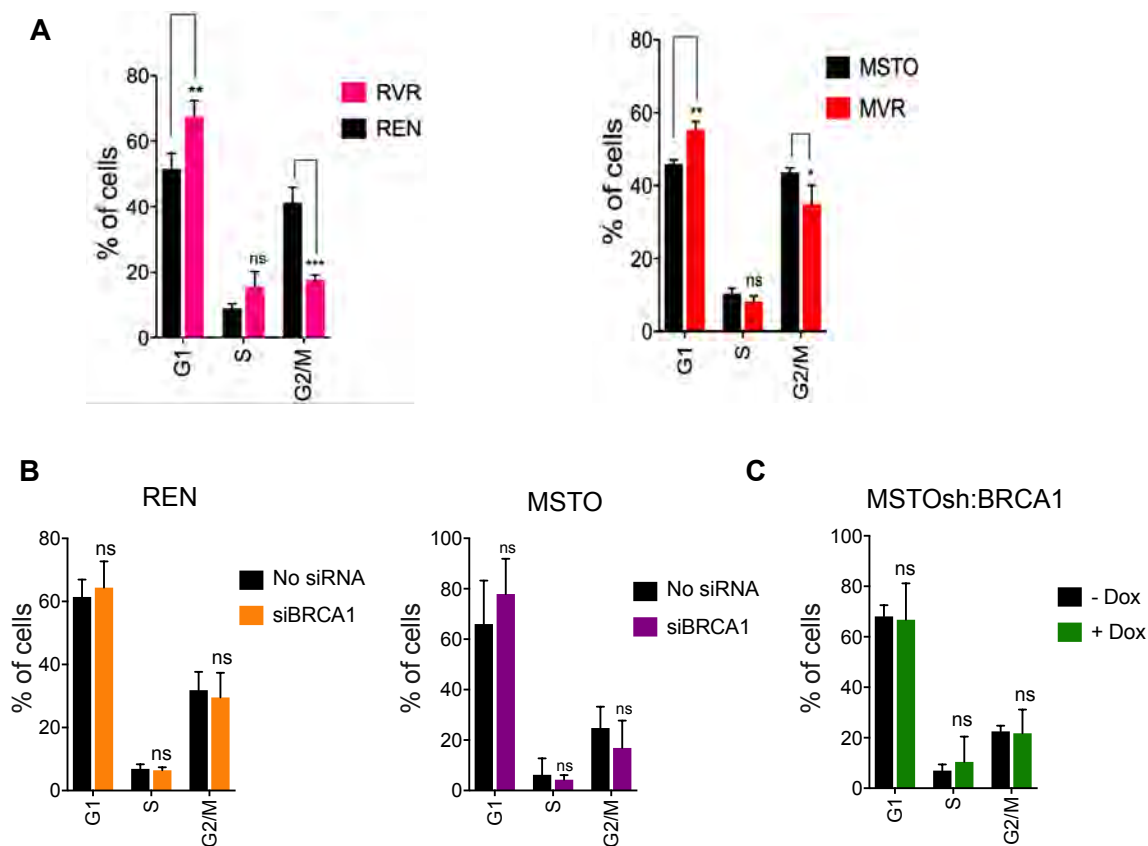


Figure 3.9 Cell-cycle progression is deregulated in vinorelbine resistant cells

A. Parental REN or MSTO and vinorelbine resistant RVR and MVR cells were analysed by flow cytometry. **B.** BRCA1 was depleted using an siRNA in REN or MSTO cells. **C.** Doxycycline was induced in MSTO:shBRCA1 cells to deplete BRCA1 for 48 hours. No siRNA indicates that cells were incubated with oligofectamine only. After BRCA1 depletion, cells were analysed by flow cytometry. Histogram indicate % of cells in each cell cycle stage, G1, S and G2/M. Data show means $\pm$ S.D. of three independent experiments. Two-way Anova, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.2.3 BRCA1 regulates centrosome number

BRCA1 has been shown to localize to the centrosome throughout the cell cycle, although at lower concentrations during mitosis (Hsu & White, 1998; Lotti *et al.*, 2002; Sankaran *et al.*, 2006). Disruption of BRCA1 in mice led to amplified centrosomes (Kais & Parvin, 2008). Later it was shown that γ -tubulin is a substrate of BRCA1 E3 ligase activity and that regulates centrosome number (Starita *et al.*, 2004). Therefore, we examined whether depletion of BRCA1 leads to altered centrosome number in mesothelioma cells. REN and MSTO cells and MSTO:shBRCA1 with uninduced doxycycline showed a lower number of cells with more than two centrosomes as compared to the vinorelbine resistant RVR and MVR cells and MSTO:shBRCA1 cells induced with doxycycline (Figure 3A). Centrosome amplification was observed by immunofluorescence staining with both pericentrin and γ -tubulin antibodies (Fig. 3.10A, B).

Thus, we next examined whether loss of BRCA1 leads to accumulation of γ -tubulin or pericentrin at the centrosomes as measured fluorescence intensity (Fig. 3.11A). γ -tubulin and pericentrin fluorescence intensity increased in MSTO:shBRCA1 after 48 hours induction cells as compared to without induction (Fig. 3.11B). The accumulation of γ -tubulin and pericentrin at the centrosomes suggests that loss of BRCA1 in these cells generally affects pericentriolar material recruitment.

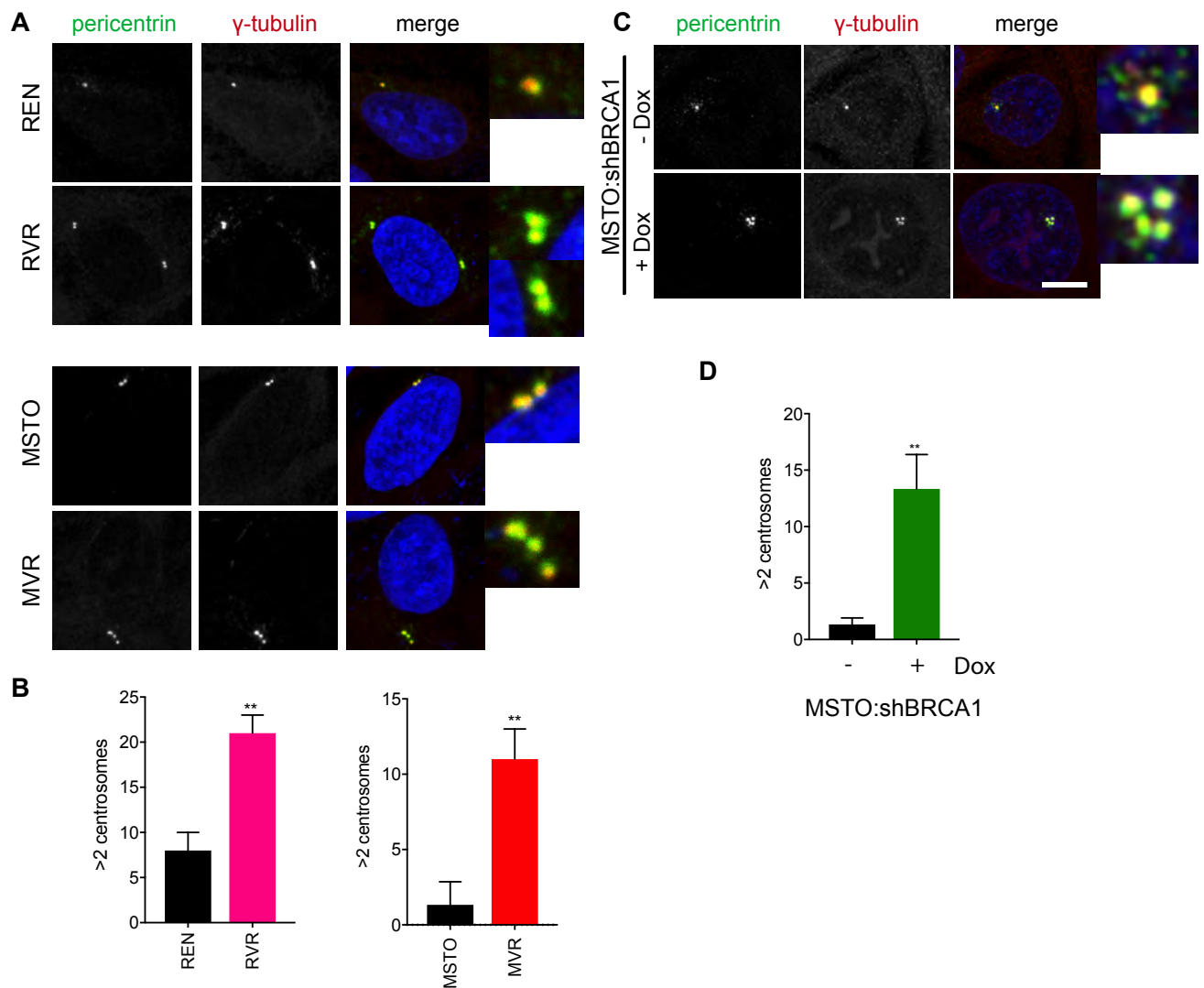


Figure 3.10 BRCA1 loss leads to centrosome amplification

A. REN, RVR, MSTO and MVR cells were stained with pericentrin (green), γ -tubulin (red) and DNA with Hoechst 33258. **B.** The histogram shows the percentage of cells with more than two centrosomes based on staining shown in A. 100 interphase cells were scored per cell line. **C.** MSTO:shBRCA1 cells were uninduced (- Dox) or doxycycline induced (+ Dox) for 48 hours to deplete BRCA1 as stained as mentioned in A. **D.** The histogram shows the percentage of cells in MSTO:shBRCA1 with or without doxycycline with more than two centrosomes. Data show means $\pm$ S.D. of three independent experiments. Students t-test, ** $p < 0.01$. Scale bar, 5 μ m.

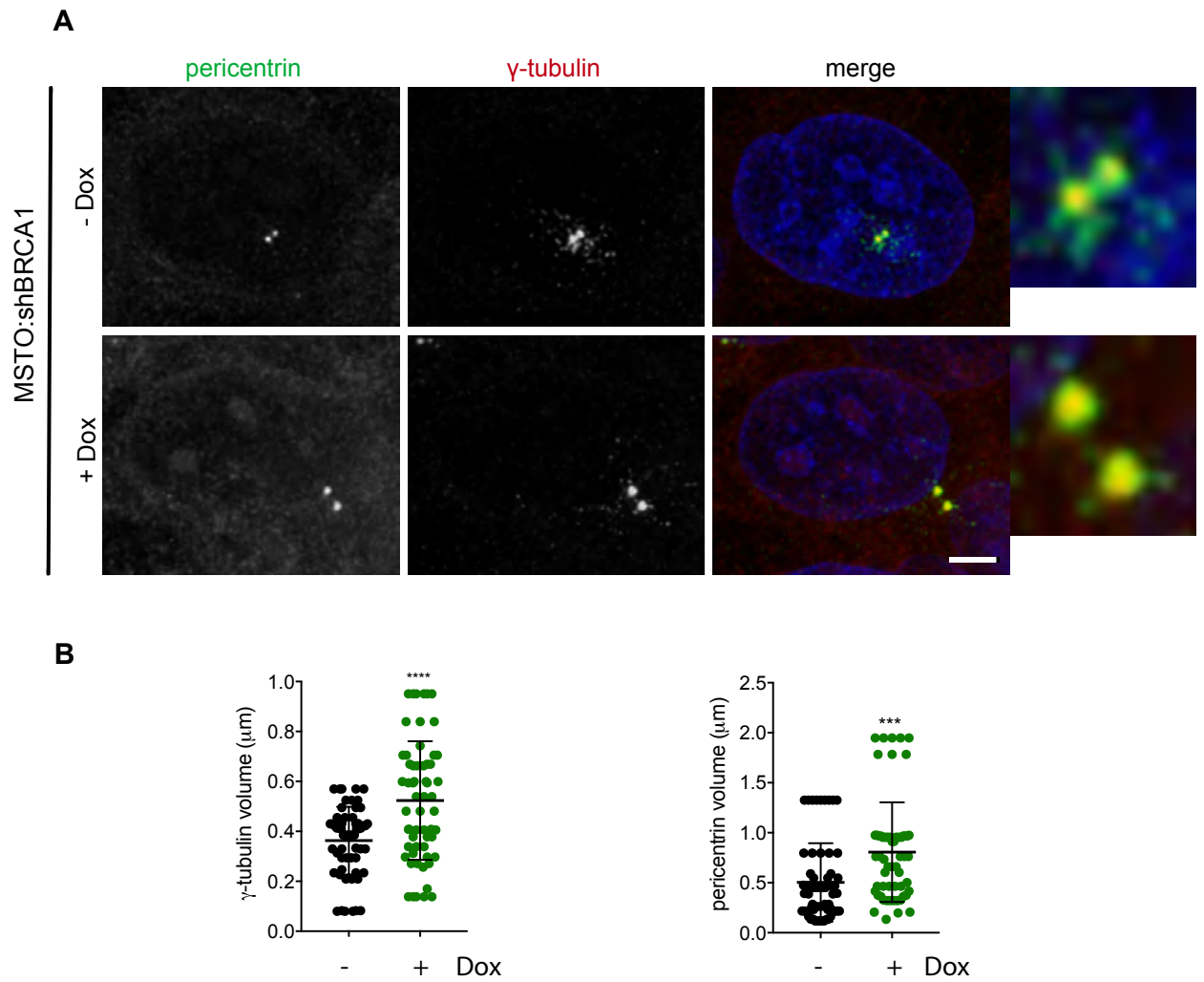


Figure 3.11 BRCA1 depletion increase centrosome size

A. MSTO:shBRCA1 cells were uninduced (- Dox) or doxycycline induced (+ Dox) for 48 hours. Cells were then stained with pericentrin (green) and γ -tubulin (red) antibodies, DNA (blue) was stained with Hoechst 33258. **B.** Dot plots the γ -tubulin and pericentrin as measured using Imaris 3D. Data show means $\pm$ S.D. of three independent experiments from 50 interphase cells for each experiment. Students t-test, *** p <0.001, **** p <0.0001. Scale bar, 5 μm .

3.3 Discussion

BRCA1 is a tumour suppressor gene, which was initially associated with breast and ovarian cancer. However, now BRCA1 has been identified as predictive biomarker of response to anti-mitotic agents and DNA damaging agents in many other cancers including MPM (Busacca *et al.*, 2012). BRCA1 is also reported as a critical regulator of mitosis by keeping centrosome duplication in check, ensuring normal mitotic spindle assembly and chromosome segregation, thereby maintaining genomic stability (Deng, 2006).

Our results demonstrate that protein expression of BRCA1 is important in determining the efficacy of microtubule poisons such as vinorelbine, which is critical in personalizing treatment options and enhancing clinical response. Here, we demonstrate how BRCA1 plays a role in vinorelbine resistance in MPM cells. The expression of BRCA1 is reduced upon selection of vinorelbine resistant cell lines. To observe a more direct effect of upon BRCA1 loss, the MSTO:shBRCA1 doxycycline inducible cell line was generated, whereby induction depletes BRCA1. Several studies have showed that loss of BRCA1 increases MT assembly rates, however the exact mechanism remains unknown (Sung & Giannakakou, 2014; Ertych *et al.*, 2016; Ertych *et al.*, 2014). Since MT dynamics can be modified by the altered expression of β -tubulin isotypes (Panda *et al.*, 1994), we checked the expression of β -III tubulin, as expression of β -III tubulin is related to lower progression free and overall survival in MPM (Zimling *et al.*, 2012). Both the vinorelbine resistant cell lines, RVR and MVR also showed significantly increase in expression of β -III tubulin as compared to the parental REN and MSTO cells. On the other hand, MSTO:shBRCA1 cells did not show any difference, in β -III tubulin with or without doxycycline suggesting that this is not an immediate consequence of BRCA1 loss.

We hypothesized that loss of BRCA1 would lead to centrosome amplification and accumulation of γ -tubulin at the centrosomes. Indeed, our results demonstrates that loss of BRCA1 leads to centrosome amplification in RVR, MVR and MSTO:shBRCA1

after doxycycline induction. We also observed γ -tubulin and pericentrin accumulation at the centrosome in MSTO:shBRCA1 after 48 hours doxycycline induction as compared to controls. Similarly, it has been shown that proteasome inhibition leads to accumulation of several centrosomal proteins including γ -tubulin (Didier *et al.*, 2008). We speculate that the accumulation of centrosomal proteins after BRCA1 loss is the failure of degradation of polyubiquitinated proteins such as γ -tubulin which is ubiquitinated by BRCA1 (Sankaran *et al.*, 2007).

Microtubule poisons such as vinca alkaloids (vinorelbine, vincristine) and taxanes (taxol, docetaxol) are widely used as potent chemotherapeutic drugs. These drugs induce SAC-mediated mitotic arrest (Foley & Kapoor, 2013; Yamada & Gorbsky, 2006). We examined the effect of BRCA1 loss on SAC integrity, by using brightfield microscopy, flow cytometry and BUBR1 intensity. For the parental cell lines, REN, MSTO and MSTO:shBRCA1 cells without induction went under a mitotic arrest upon vinorelbine treatment, whereas the RVR progressed through mitosis without delay in the presence of vinorelbine. The MVR and MSTO:shBRCA1 (induced with doxycycline) cell lines underwent a transient mitotic arrest upon vinorelbine treatment. The reason for the difference in cell cycle progression after vinorelbine treatment of the cell lines, suggest that the SAC is inactive in RVR and partially active in MVR and MSTO:shBRCA1. This was consistent with our analysis of BUBR1 intensity at kinetochores. Our results are consistent with those of by previous studies reporting that BRCA1 regulates the expression of the SAC regulators, MAD2L1 and BUBR1 (Wang *et al.*, 2004; Chabalier *et al.*, 2006; Shabbeer *et al.*, 2013).

The findings presented here strongly suggest that BRCA1 deficiency mediates vinorelbine resistance, partly through downregulation of SAC proteins resulting in premature inactivation of SAC checkpoint. Although somatic BRCA1 mutation is not reported, reduced expression of BRCA1 protein has been detected in 39% of MPM patients. Therefore, the status of BRCA1 might be a determinant of the efficacy of vinorelbine response in MPM.

CHAPTER 4

BAP1 regulates BRCA1 protein stability

4.1 Introduction

Ubiquitination is a reversible post-translational modification vital for regulating complex cellular processes, such as cell cycle progression, stress and immune response, signal transduction, DNA repair and programmed cell death (Hershko & Ciechanover, 1998; Katoh, 2013; Pickart & Eddins, 2004; Sowa *et al.*, 2009). Ubiquitin-mediated modification of many cellular proteins may target them for proteasomal degradation, or by alter their activity, localization or complex assembly (Pickart & Eddins, 2004; Sowa *et al.*, 2009; Komander & Rape, 2012). The Ubiquitin-proteasome system (UPS) is made mainly of six components, 1. Ubiquitin, 2. ubiquitin activating enzymes (E1), 3. ubiquitin conjugating enzymes (E2), 4. ubiquitin ligases (E3), 5. Proteasome, 6. DUBs (Hussain *et al.*, 2009; Shen *et al.*, 2013). Ubiquitin can generate different types of covalent modifications such as mono-ubiquitination, in which a single ubiquitin molecule attached to the target can regulate DNA repair or gene expression, or polyubiquitination where the target protein is modified with poly-ubiquitin chains (Singh, 2016).

Deubiquitinating enzymes (DUBs) are a family of proteases that remove ubiquitin from target proteins and disassemble poly-ubiquitin chains (Clague *et al.*, 2012). DUBs can catalytically deubiquitinate the substrate and can alter the stability or composition of the E3 complex by non-catalytic effects (Eletr & Wilkinson, 2014; Mevissen & Komander, 2017). A hallmark of E3 ligases is autoubiquitination. In the absence of substrates E3 ligases ubiquitinate themselves or another E3 ligase can ubiquitinate them to target them for proteasomal degradation. DUBs present in the same complex can also be ubiquitinated and degraded (Eletr & Wilkinson, 2014).

BAP1 is a DUB that belongs to the of Ubiquitin C-terminal Hydrolase (UCH) family, capable of disassembly of di-ubiquitin and poly-ubiquitin chains (Eletr & Wilkinson, 2014). BAP1N (1-240) exhibits a higher catalytic activity than full length BAP1 and also has specificity for cleavage of K48 poly-ubiquitin chains (Hanpude *et al.*, 2017). BAP1 is a tumour suppressor that was identified as an interacting protein of BRCA1 which is an E3 ligase (Jensen *et al.*, 1998). Subsequent work suggested that BAP1 rather interacts directly with BARD1 (BRCA1-associated RING domain 1), a partner of BRCA1 and not BRCA1 itself. Interestingly, BAP1 inhibits the E3 ligase activity of

the BRCA1/BARD1 complex independent of its UCH domain and together with BAP1 this complex regulates ubiquitination of cell cycle control proteins and proteins in the DNA damage response pathway (Nishikawa *et al.*, 2009). However, other studies show that loss of BAP1 leads to an increase in BRCA1 ubiquitination and reduced BRCA1 protein levels (Dkhissi *et al.*, 2015). In MPM cells, transduction of wild-type and mutant BAP1 increased BRCA1 expression (Hakiri *et al.*, 2015), while BAP1 depletion led to reduced BRCA1 foci formation after irradiation (Yu *et al.*, 2014; Xiyu *et al.*, 2018). The tumour suppressor function of BAP1 requires both its deubiquitinase activity and nuclear localization (Ventii *et al.*, 2008). BAP1 is involved in numerous cellular processes including cell cycle progression, DNA repair, histone modification, gluconeogenesis, myeloid transformation and stem cell pluripotency. A variety of proteins interact that with BAP1 have been described, including the cell cycle regulator HCF-1, transcription factors (YY1 and FOXK1/2), chromatin binding and modifying proteins (ASXL1/2/3, KDM1B, OGT1) and DNA repair proteins.

Germline BAP1 mutations can predispose to malignant pleural mesothelioma (MPM), uveal melanoma (UV), cutaneous melanoma (CM), renal cell carcinoma (RCC), bladder tumours and other cancers. Somatic mutations in BAP1 are seen in MPM, UV, RCC and other cancers (Murali *et al.*, 2013). BAP1 is mutated or deleted in 30-60% of all mesotheliomas (Carbone *et al.*, 2013; Comertpay *et al.*, 2011).

BAP1 enzymatic activity regulates the de-ubiquitination of γ -tubulin (Zarrizi *et al.*, 2014), MCRS1 (Peng *et al.*, 2015), INO80 and H2A (Loew *et al.*, 2018). Some of these proteins are ubiquitinated by the BRCA1/BARD1 complex including γ -tubulin (Starita *et al.*, 2004) and H2A (Kalb *et al.*, 2014). MCRS1 binds to BRCA1 but it is unknown whether MCRS1 is a substrate of BRCA1 E3 ligase activity (Hill, 2014).

BRCA1 was reported to be lost in 38% of MPM cases. Herein, we investigated the functional relationship between BAP1 and BRCA1 in MPM. In this chapter we clarify the role of BAP1 as a modulator of BRCA1 stability. Specifically, we demonstrate that loss of BAP1 not only destabilizes BRCA1 but also phenocopies the loss of BRCA1, including the response to the microtubule poison vinorelbine, regulates SAC integrity

and centrosome amplification. Thus, it appears clear that from these studies that BAP1 has a relevant role in BRCA1 dependent and independent processes.

4.2 Results

4.2.1 BAP1 positively regulates BRCA1 protein stability

Previous studies suggested that BAP1 is involved in BRCA1 de-ubiquitination (Dkhissi *et al.*, 2015) and that BAP1 and BRCA1 protein are not present in upto 65% and 38% of MPM cases, respectively (Busacca *et al.*, 2012; Quinn *et al.*, 2012; Bott *et al.*, 2011; Cheung & Testa, 2017). Therefore, we examined the protein expression of BRCA1 after BAP1 depletion in mesothelioma cell lines, MSTO:shBRCA1 and H2452. BAP1 was depleted from the cell lines using two different siRNA oligos for 72 hours. MSTO:shBRCA1 cells have wild-type BAP1 whereas H2452 cells have an A95D mutation in the UCH domain, that renders the protein catalytically inactive (Yoshikawa *et al.*, 2012; Ventii *et al.*, 2008). BAP1 depletion significantly reduced the level of BRCA1 protein levels in both cell lines. As, BAP1 enzymatic function as a DUB is inactive in H2452 cells, this suggests that the role of BAP1 in stabilizing BRCA1 is independent of its UCH domain (Fig. 4.1 A, B, C, D). As BAP1 being involved in transcriptional regulation (Loew *et al.*, 2018), we examined whether loss of BRCA1 protein levels is due to downregulation of gene expression after BAP1 depletion. However, there was no change in BRCA1 mRNA levels as determined by RT-PCR analysis after BAP1 depletion (Fig.4.1E).

Interestingly, BAP1 protein levels remains unchanged after BRCA1 depletion by doxycycline in MSTO:shBRCA1 cells suggesting BRCA1 does not regulate BAP1 expression or function. To confirm whether this relationship is present in normal mesothelial cells or in a breast cancer cell line, MDA-MB-231, BAP1 was depleted in MET-5A, a non-malignant human mesothelial cell line, and in MDA-MB-231 (Fig. 4.2A, B). BRCA1 protein expression was reduced in both the non-cancerous MET-5A cell line and in the breast cancer MDA-MB-231 cell line after BAP1 depletion. This suggests that BRCA1 requires BAP1 for its protein stabilization not only in mesothelioma cancer cells, but also in other

cancer cell type and in normal cells.

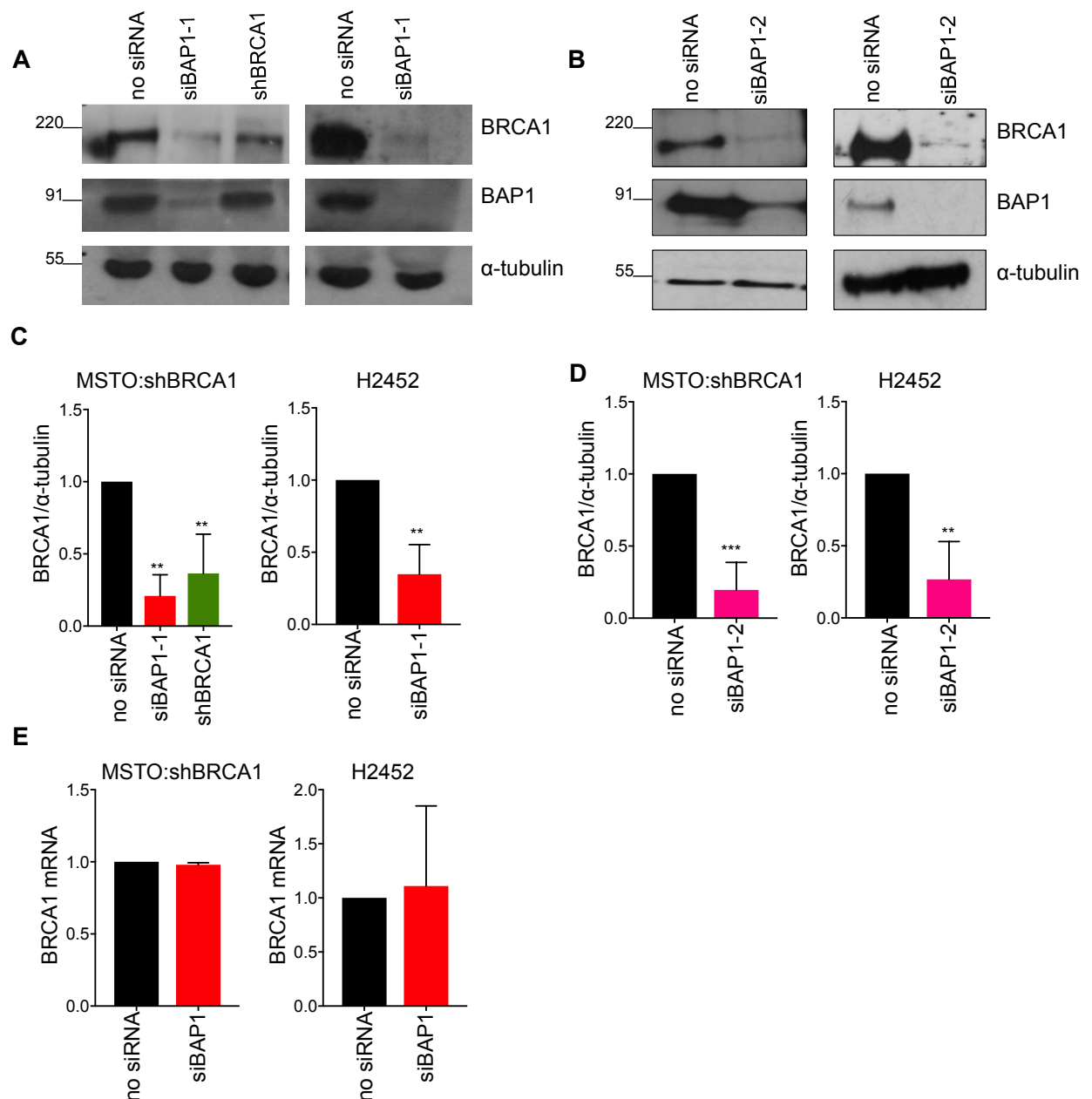


Figure 4.1 BAP1 depletion leads to loss of BRCA1 protein stability

A. BRCA1 protein expression was quantified after BAP1 knockdown with siBAP1-1 in MSTO:shBRCA1 and H2452 cells for 72 hours. Depletion of BRCA1 in MSTO:shBRCA1 was induced by incubating cells with doxycycline for 48 hours. **B.** MSTO:shBRCA1 and H2452 cells were depleted of BAP1 with siBAP1-2. No siRNA means cells were incubated with oligofectamine only. Cell lysates were analysed by western blotting with antibodies indicated. Molecular weights are indicated (kDa). **C.&D.** Histograms show the densitometric quantification of the immunoblots from A and B. **E.** mRNA expression of BRCA1, after BAP1 depletion was analysed by RT-PCR, beta-actin was used as a control. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, ** $p < 0.01$, *** $p < 0.001$.

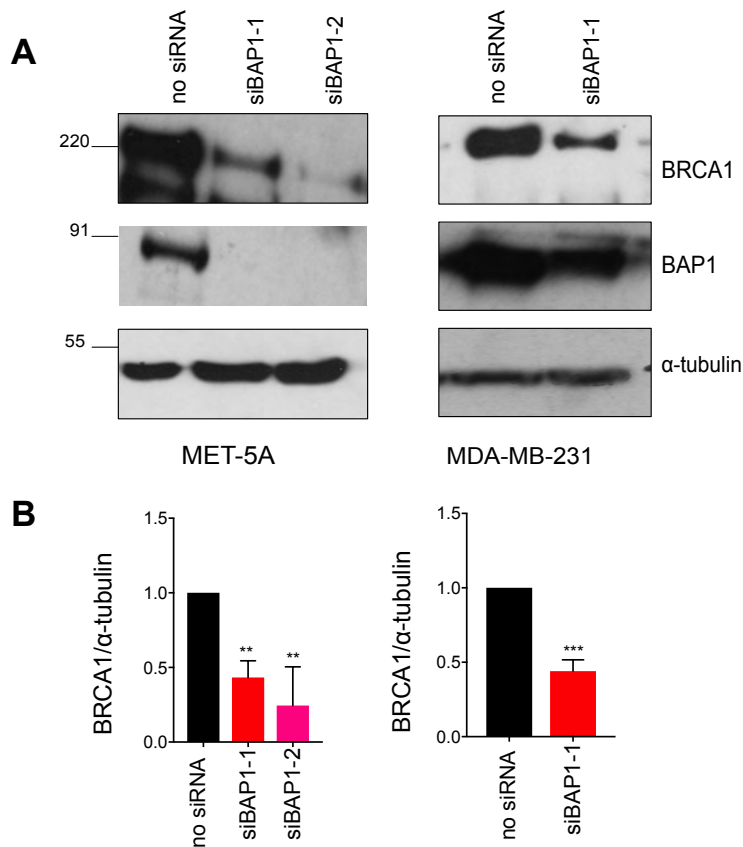


Figure 4.2 BAP1 depletion reduces BRCA1 protein in normal mesothelial and breast cancer cell line

A. BAP1 was depleted using two independent siRNA oligos in MET-5A and with siBAP1-1 in MDA-MB-231 for 72 hours. No siRNA means cells were incubated with oligofectamine only. Samples were analysed by western blotting using antibodies indicated. Molecular weights are indicated (kDa). **B.** Histograms show the densitometric quantification of the immunoblots from three independent experiments. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, **p<0.01, ***p<0.001.

4.2.2 BAP1 stabilizes BRCA1 independent of cell cycle progression

As BRCA1 is a cell cycle regulated protein, we analysed whether the BAP1 loss induced reduction of BRCA1 protein may be due to alterations in the cell cycle. However, using flow cytometry, we found no difference in the cell cycle distribution between untreated BAP1 depleted, and MSTO:shBRCA1 and H2452 cells or between MSTO:shBRCA1 that were uninduced or induced with doxycycline to deplete BRCA1 (Fig. 4.3A, B). Although statistical significance was not reached, there was a trend towards cells remaining in G1 of the cell cycle after BAP1 depletion (Fig. 4.3C). This suggests that reduced BRCA1 protein levels after BAP1 depletion are not due to alterations in cell cycle progression.

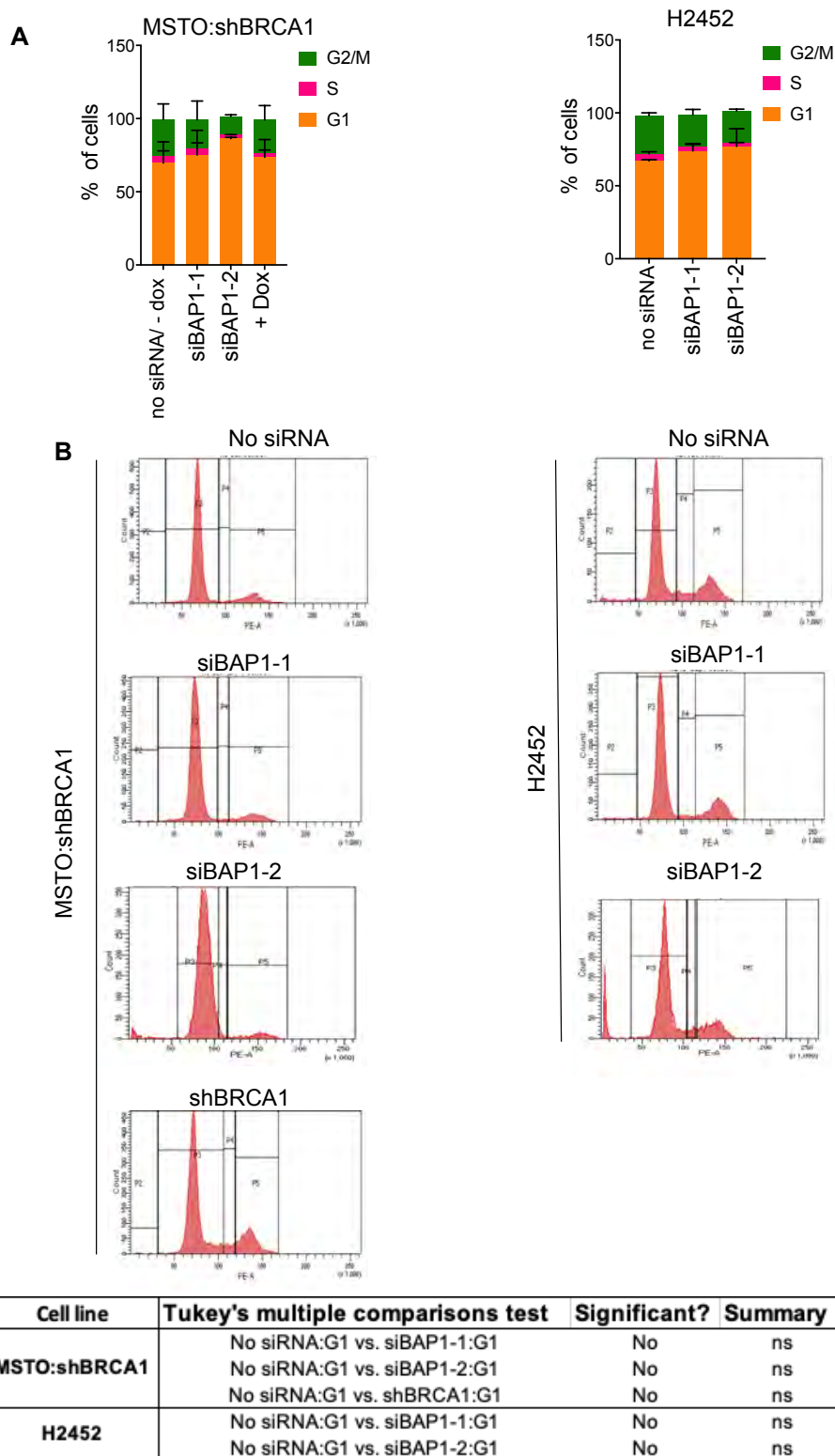


Figure 4.3 Cell cycle distribution of MSTO:shBRCA1 and H2452 cells following BAP1 depletion.

A. Histogram represents percentage of MSTO:shBRCA1 and H2452 of cells in each stage G1, S and G2/M as analysed by flow cytometry following BAP1 and BRCA1 depletion as indicated. **B.** Flow cytometry profile of cells indicated after No siRNA (cells were incubated with oligofectamine only) or depleted of BAP1 or BRCA1 as indicated. DNA content was determined using PI staining. **C.** Table shows the Tukey's multiple comparisons test for no siRNA controls and depletions in MSTO:shBRCA1 and H2452 cell lines. Data show means $\pm$ S.D. of three independent experiments.

4.2.3 BAP1 depletion enhances proteasomal degradation of BRCA1

To test whether BAP1 regulates BRCA1 protein stability through the 26S proteasomal pathway, we used siRNA interference to deplete BAP1 in MSTO:shBRCA1 and H2452 cells before cells were treated with proteasome inhibitor, 20 μ M MG132 for 6 hours. MG132 treatment rescued BRCA1 expression in BAP1 depleted cells indicating that BAP1 normally prevents the proteasome mediated degradation of BRCA1 (Fig. 4.4 A, B). It is important note that an enzymatically-inactive BAP1 in H2452 cells was able to rescue BRCA1 from proteasomal degradation supporting the hypothesis that BAP1 mediates BRCA1 protein stability in a manner independent of its DUB activity.

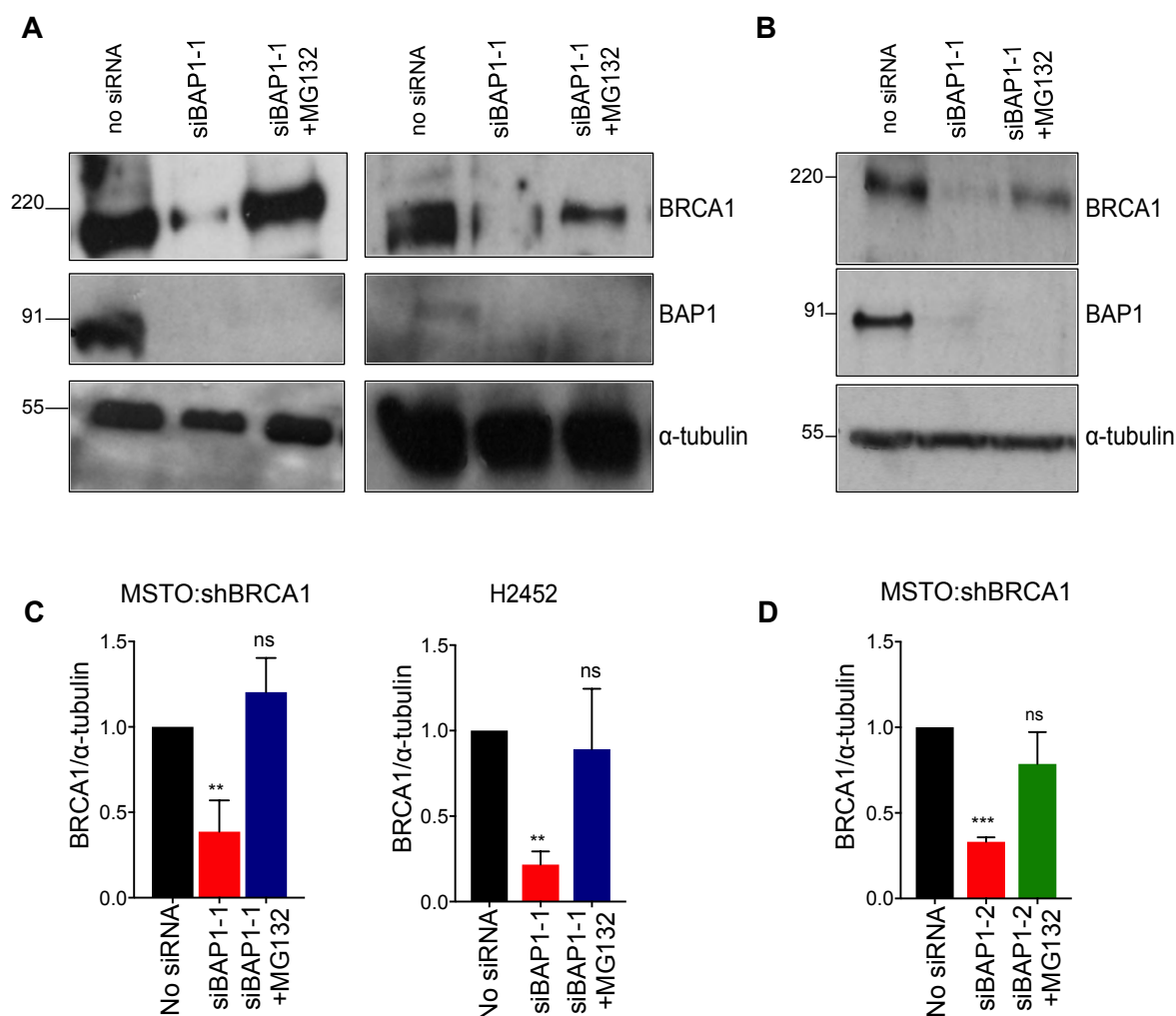


Figure 4.4 Proteasome inhibition restores BRCA1 expression after BAP1 depletion

A. MSTO:shBRCA1 and H2452 cells were transfected with BAP1 siRNA oligo for 72 hours. Cells were then untreated or treated with 20 μ M MG132 during the last 6 hours of transfection. **B.** MSTO:shBRCA1 cells were depleted of BAP1 with siRNA oligo 2 for 72 hours. No siRNA means cells were incubated with oligofectamine only. Cell lysates were analysed by Western blotting using the antibodies indicated. Molecular weights are indicated (kDa). **C.&D.** Quantification of the levels of BRCA1 protein from the western blot from A and B were done using ImageJ. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, ** $p < 0.01$, *** $p < 0.001$.

4.2.4 Rescue of BRCA1 expression by different of BAP1 constructs

To identify whether wild-type or mutant forms of BAP1 can rescue BRCA1 expression, we used the H226 cell line that harbours a homozygous deletion of BAP1 and exhibits complete loss of BAP1 expression. Stable H226 cell lines had been generated with a wild-type BAP1 expression vector or a series of mutant BAP1 constructs that included an inactivating C91A mutation in the UCH domain, a stop codon after 716 amino acids that removes the C-terminal NLS, T492A mutation in the FOXK2 binding site, a deletion from 363-366 that removes the HCF-1 binding site, and a deletion from 666-669 that removes the ASXL1/2 binding site (Fig. 4.5A).

Western blot analysis of lysates prepared from the stable H226 cell lines revealed that wild-type and all BAP1 mutant constructs rescued BRCA1 expression whereas ASXL binding mutant rescued BRCA1 expression to a lesser extent as compared to other mutants (Fig. 4.6A, B). The observation that the C91A mutant and Δ NLS mutant rescued BRCA1 expression suggested that BRCA1 stabilization by BAP1 is independent of its UCH and Nuclear localization signal (Δ NLS). However, ASXL1/2 binding is required to stabilise BRCA1.

A functional RNAi screen had identified BAP1 necessary for assembly of the HR factors, BRCA1 and RAD51, at ionizing radiation induced nuclear foci (IRIF). BAP1 knockdown decreased the foci formation at the IRIF (Yu *et al.*, 2014). We therefore also sought to examine whether wild-type or BAP1 mutants could rescue the expression of BRCA1 foci after 0 Gy or 5 Gy irradiation of H226 cells. An 8 hours, time point post-IR was selected for our study, time at which maximum number of cells exhibit DSB foci. The assembly of constitutive BRCA1 foci was increased in wild-type and all mutant stable cell lines at 8 hours post-IR except in the parental cell line and the ASXL1/2 binding mutant, neither of which rescued BRCA1 protein foci (Fig. 4.7A, B). These results suggest that rescue of BRCA1 expression and localization to IR-induced DNA damage foci are independent of DUB activity, HBM binding and nuclear localization of BAP1, but do depend on binding to ASXL1/2.

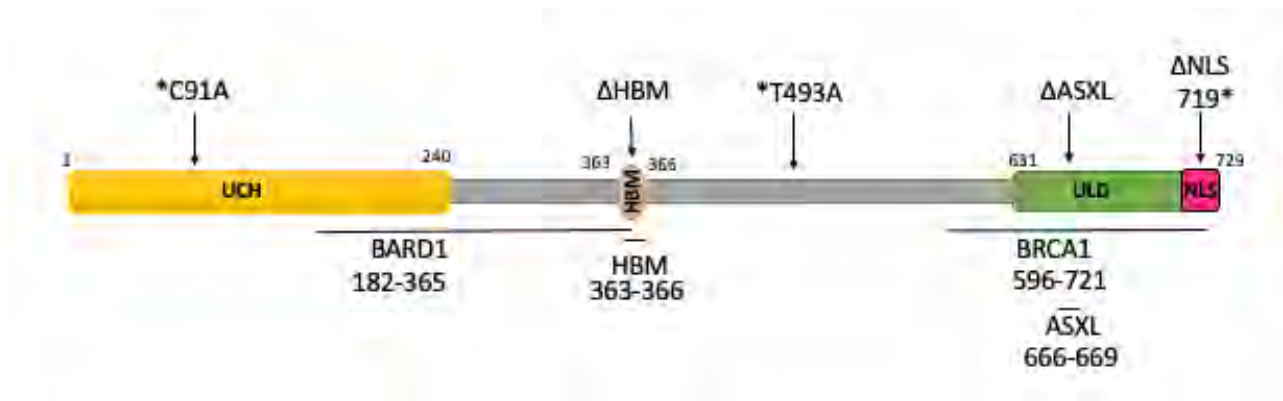


Figure 4.5 Schematic representation of BAP1 of stable cell lines used in this study

Schematic representation of BAP1 constructs used in this study. C91A mutation is in the UCH (ubiquitin COOH-terminal hydrolase) domain of BAP1. Domain comprised by residues 182-365 of BAP1 interacts with the RING finger domain of BARD1. HCF-1 interacts BAP1 through the HCF-binding motif (HBM). T493A is a mutation in the FOXK2 binding site. BAP1 binds to the RING finger domain of BRCA1 through its carboxyl-terminal region (596-721). ASXL (Δ 666-669) results in a selective loss of interaction with ASXL1/2.

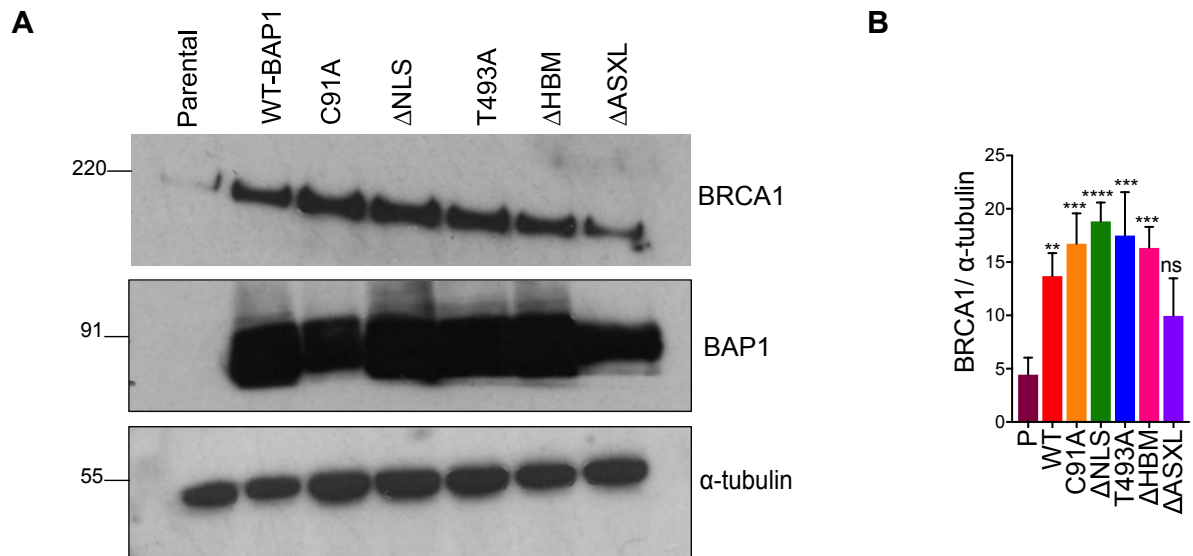


Figure 4.6 Different BAP1 constructs stabilize BRCA1 protein expression in H226 cells

A. Western blot analysis show the protein expression of BAP1 and BRCA1 in H226 cells stably expressing WT or BAP1 mutants. **B.** Densitometric quantification of the BRCA1 immunoblot was analysed using ImageJ. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

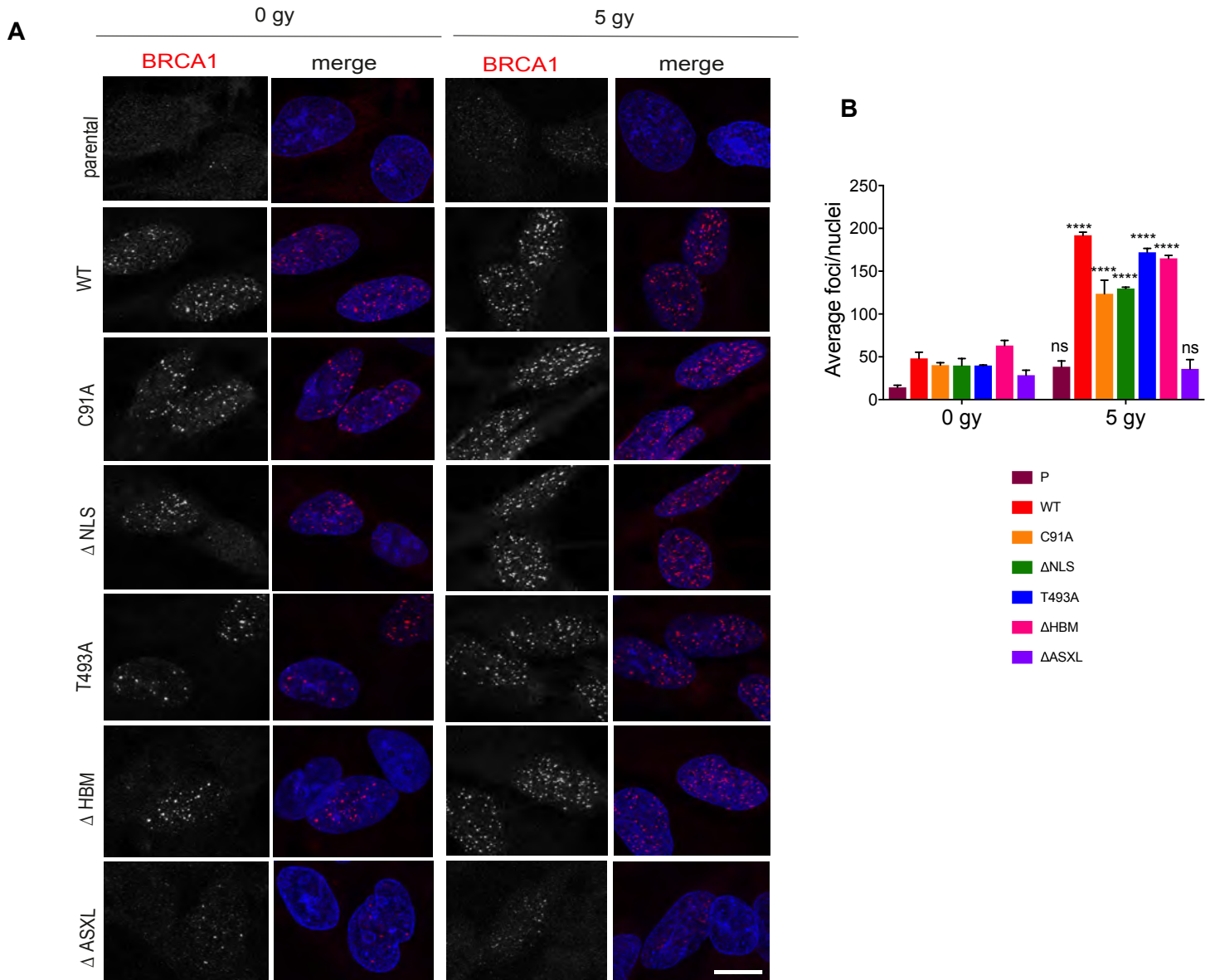


Figure 4.7 BAP1 constructs promote formation of BRCA1 foci at sites of DNA damage

A. H226 cells as indicated were fixed after irradiation with 0 gy or 5 gy and analysed by immunofluorescence microscopy for BRCA1 foci. Representative images of cells at 8 hours post-IR treatment. **B.** Histogram shows the mean BRCA1 foci per nuclei in parental H226 and stable cell lines expressing the BAP1 constructs as indicated. Data show means $\pm$ S.D. of three independent experiments. Scale bar, 8 μ m. One-way Anova, * $p < 0.05$, **** $p < 0.001$.

4.2.5 BAP1 loss correlates with BRCA1 loss in mesothelioma in tumour samples

Loss of BAP1, independent of whether the underlying cause is through gene deletion, or insertion or point mutation, translates into nuclear negativity for BAP1 upon immunohistochemical staining (IHC). Mutations in BAP1 leads to cytoplasmic staining as identified by IHC for BAP1 (Righi *et al.*, 2016).

Since BRCA1 is destabilized in the absence of BAP1 in cell lines, we evaluated whether a correlation is present between BAP1 and BRCA1 expression in mesothelioma patient tissue by immunohistochemistry. Patient samples were obtained from the MEDUSA study (Samples were stained by Aarti Gaba at NHS and scored by pathologist Dr. Michael Sheaff). We evaluated the expression of BAP1 and BRCA1 in tumour samples taken from chemo-naïve mesothelioma patients undergoing extended pleurectomy decortication (n=25). Although the exact mutation status of BAP1 and BRCA1 was unknown, we found that there is a moderate correlation between BAP1 and BRCA1 expression ($r= 0.403$, $p<0.05$). These findings together suggest that the loss of BAP1 expression heavily contributes to the inactivation of BRCA1 as analysed by IHC in MPM patient samples (Fig 4.8 A, B).

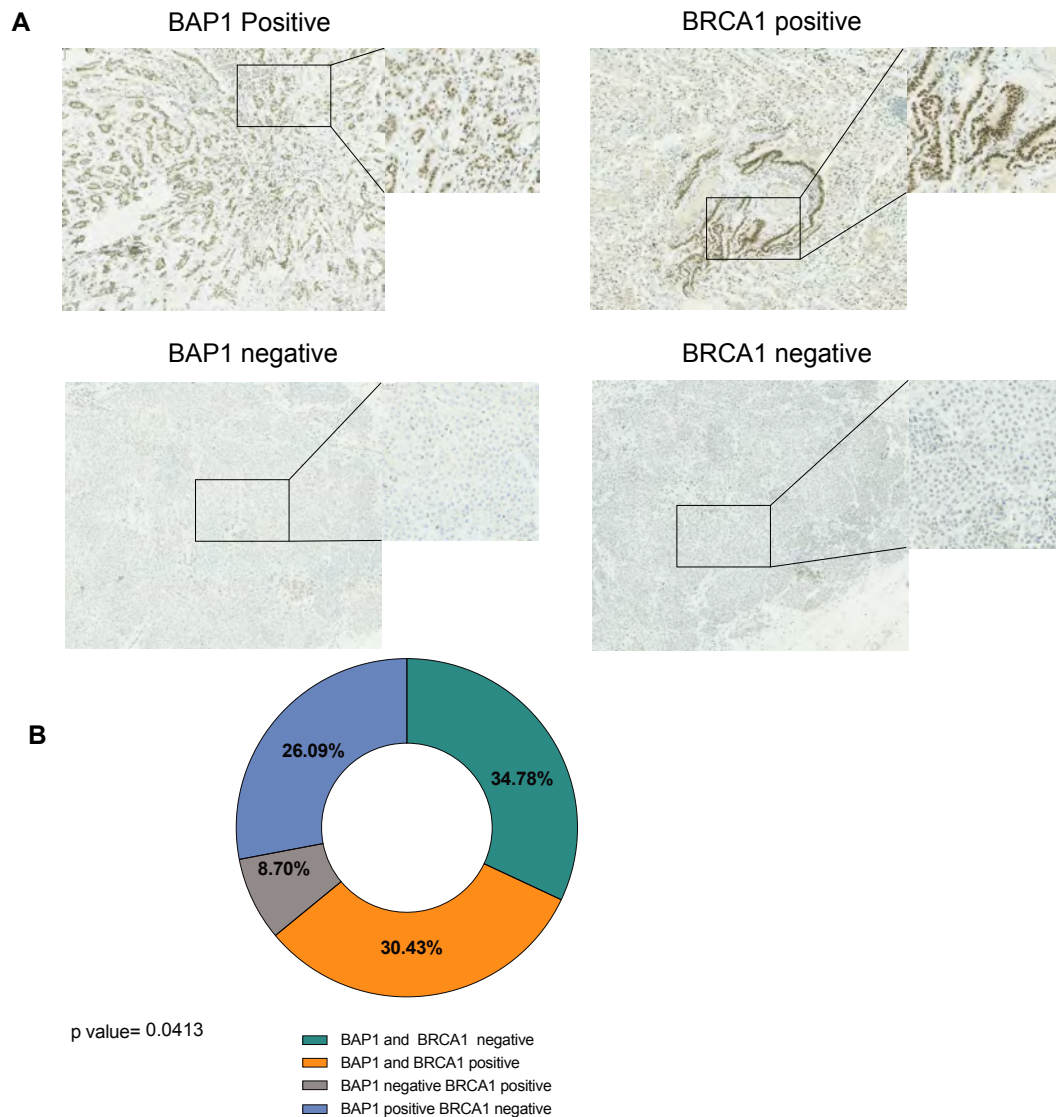


Figure 4.8 Positive correlation exists between BAP1 and BRCA1 in MPM patients

A. Representative immunohistochemical staining of BAP1 and BRCA1 in MPM patient tissues. Top panel shows BAP1 and BRCA1 samples show positive nuclear staining; lower panel shows negative nuclear staining for BAP1 and BRCA1. BAP1 was scored as positive if slides showed nuclear staining and negative if BAP1 staining was either absent or cells showed cytoplasmic localisation. **B.** Samples were scored based on the nuclear staining of BAP1 and BRCA1. A moderate correlation was identified between BAP1 and BRCA1 using spearman's correlation.

4.2.6 BAP1 is required for SAC integrity and chromosome stability in mesothelioma cells

MPM is a rapidly progressive lethal cancer. Standard treatment options are limited and new therapies are crucial for improved outcomes for mesothelioma patients. Vinorelbine, a microtubule poison, inhibits microtubule dynamics and causes mitotic arrest due to activation of the SAC (Ngan *et al.*, 2001). Previously it has been shown that BRCA1 loss mediates resistance to vinorelbine through abrogation of the SAC (Busacca *et al.*, 2012). We therefore, investigated whether loss of BAP1 phenocopies BRCA1 loss by also including resistance to vinorelbine.

Using siRNA transfection we determined whether depletion of BAP1 could modulate sensitivity to vinorelbine in MSTO:shBRCA1 and H2452 cells. Using flow cytometry, we first investigated the cell cycle distribution of cells depleted of BAP1 and BRCA1 depleted in MSTO:shBRCA1 cells by induction of doxycycline. No difference was observed in the cell cycle distribution following BAP1 or BRCA1 depletion at 72 or 48 hours respectively, in the absence of vinorelbine. After vinorelbine treatment, the G2/M fraction of cells increased dramatically in mock-transfected cell, but not in BAP1 or BRCA1 depleted cells (Fig. 4.9 A, B). It is noteworthy that H2452 cells with enzymatically inactive BAP1 were still sensitive to vinorelbine and BAP1 depletion in these cells mediated resistance to vinorelbine.

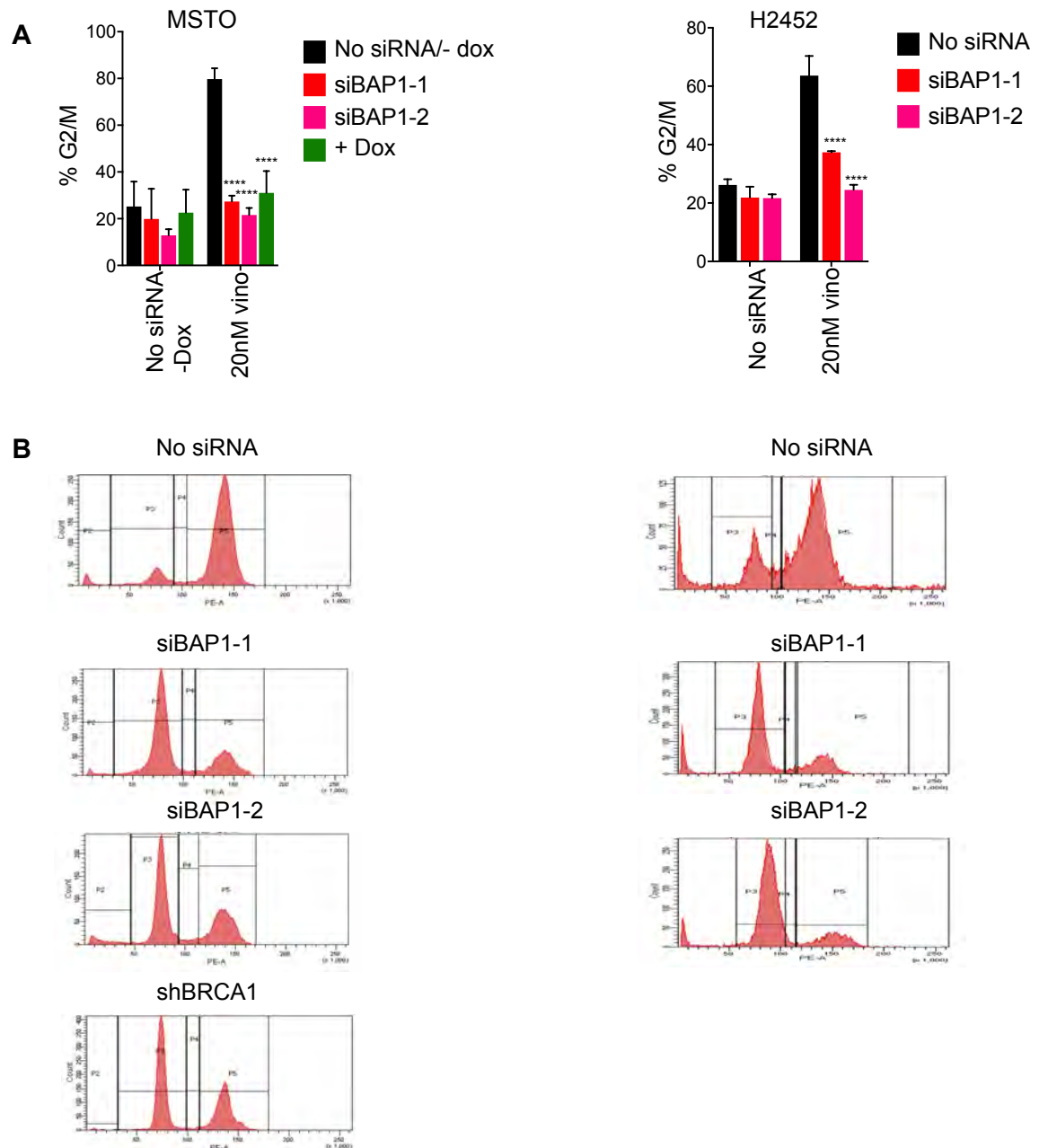


Figure 4.9 Loss of BAP1 leads to vinorelbine resistance

A. MSTO:shBRCA1 or H2452 cells, that had been mock depleted or depleted of BAP1 or BRCA1 as indicated, were treated with 20 nM vinorelbine for 24 hours. No siRNA means cells were incubated with oligofectamine only. The proportion of cells in G2/M phase was detected by flow cytometry using propidium iodide staining and the percentages of cells in this phase are shown in the histogram. **B.** Graphs represent the flow cytometry profile from cells treated as in A. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, **** $p < 0.0001$.

4.2.7 BAP1 depletion leads to loss of MAD2 expression

Microtubule poisons induce mitotic arrest through activating the SAC. As the SAC detects loss of functional connection of kinetochores to the mitotic spindle. This lack of attachment disseminates signals through SAC components such as MAD2 and BUBR1, that inhibit the APC/C.

As it has been previously reported that BRCA1 regulates MAD2 expression (Wang *et al.*, 2004; Busacca *et al.*, 2012), we therefore examined the protein expression of MAD2, in BAP1 depleted cells. MSTO:shBRCA1 and H2452 cells were mock-depleted or depleted with two independent siRNA oligos against BAP1; BRCA1 was depleted by induction in the MSTO:shBRCA1 cells using doxycycline. Mock depleted cells in both cell lines showed significantly higher expression of MAD2 than BAP1 or BRCA1 depleted cells show reduced expression of MAD2 (Fig. 4.10 A, B). In fact the loss of MAD2 upon BAP1 depletion was similar to if not greater than upon BRCA1 depletion. To confirm BAP1 inactivates the SAC, we analysed the kinetochore localization of BUBR1. Kinetochore-associated BUBR1 levels were significantly higher in the mock depleted cells as compared to the BAP1 or BRCA1 depleted cells (Fig.4.11A, B). However, this was not due to altered expression of BUBR1 as there was no difference in the protein expression of BUBR1 as determined by Western blot.

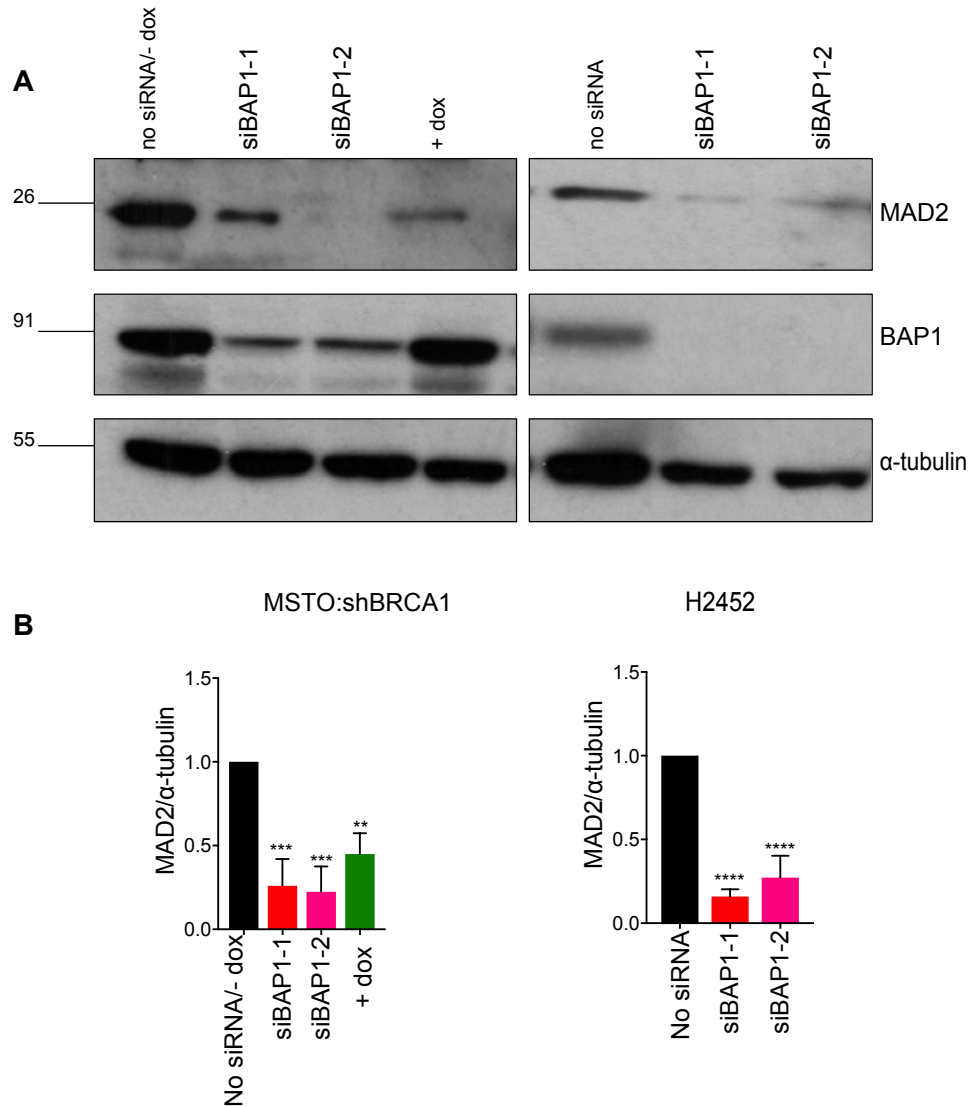


Figure 4.10 BAP1 depletion leads to loss of MAD2 protein expression

A. BAP1 was depleted in MSTO:shBRCA1 and H2452 using two siRNAs against BAP1 for 72 hours while BRCA1 was depleted in MSTO:shBRCA1 cells for 48 hours using doxycycline induction. No siRNA means cells were incubated with oligofectamine only. Western blot analysis of lysates showing expression of MAD2, BAP1 and α -tubulin. **B.** Histograms show the densitometric quantification of MAD2 immunoblots. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, **p<0.01, ***p<0.001, ****p<0.0001.

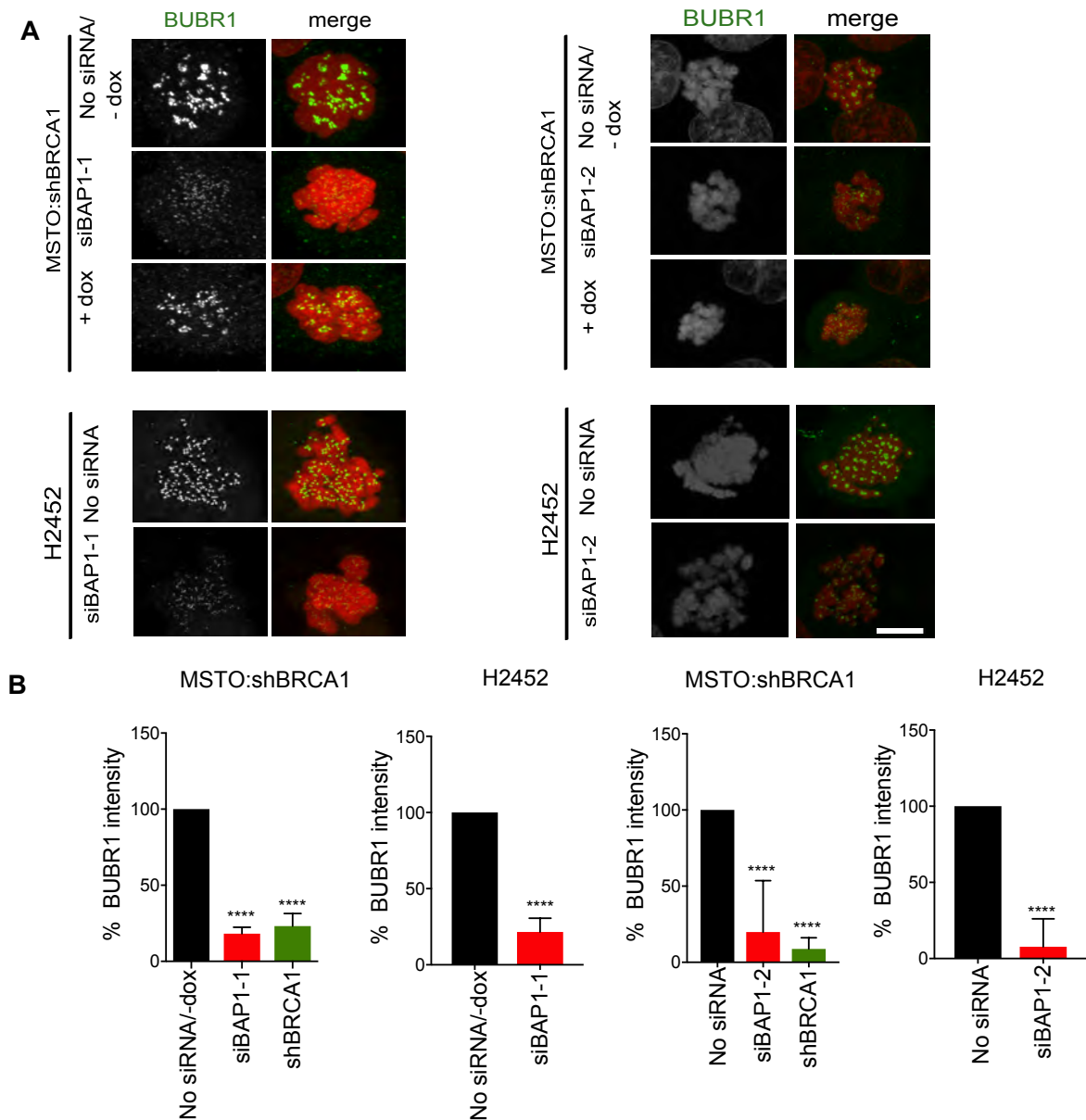


Figure 4.11 BAP1 depletion causes loss of BUBR1 levels at the kinetochores

A. BAP1 was depleted in MSTO:shBRCA1 and H2452 cells using two siRNA for 72 hours; BRCA1 was depleted in MSTO:shBRCA1 cells by doxycycline induction for 48 hours. No siRNA means cells were incubated with oligofectamine only. After 48 hours of transfection, cells were treated with 20 nM vinorelbine for another 24 hours. Cells were then fixed and processed for immunofluorescence microscopy with BUBR1 antibody, DNA was stained with Hoechst 33258. **B.** The histograms show the mean intensity of BUBR1 at kinetochores. Values for mock cells were set to 100. Data show means $\pm$ S.D. of three independent experiments. One-way Anova **** $p < 0.0001$. Scale bar, 5 μ m.

4.2.8 Inactive SAC after BAP1 depletion causes aneuploidy

Defects in SAC integrity lead to chromosomal instability (CIN) (Khodjakov & Rieder, 2009; Lara-Gonzalez *et al.*, 2012; Schuyler *et al.*, 2012). Therefore, we identified and quantified mitotic cells with misaligned chromosomes in both the MSTO:shBRCA1 and H2452 cell line. As visualized by centromere staining with CENPA antibodies, there was a significant increase in the number of mitotic cells after BAP1 and BRCA1 depletion that had chromosomes that were unaligned at the metaphase plate as compared to the mock depleted cells (Fig. 4.12 A, B). A consequence of chromosome missegregation is the formation of micronuclei and multinucleated cells (Park, 2010), while the presence of micronuclei and multinucleated cells is a hallmark of CIN (He *et al.*, 2018; Soto *et al.*, 2018). We therefore quantified the frequency of interphase MSTO:shBRCA1 and H2452 cells that had micronuclei or were multinucleated after BAP1 or BRCA1 depletion. This revealed a significantly higher number of micronuclei and multinucleated cells after BAP1 or BRCA1 depletion as compared to mock depleted cells (Fig. 4.13 A, B). Taken together, our data strongly suggest that BAP1, as well as BRCA1 is required to maintain chromosome stability.

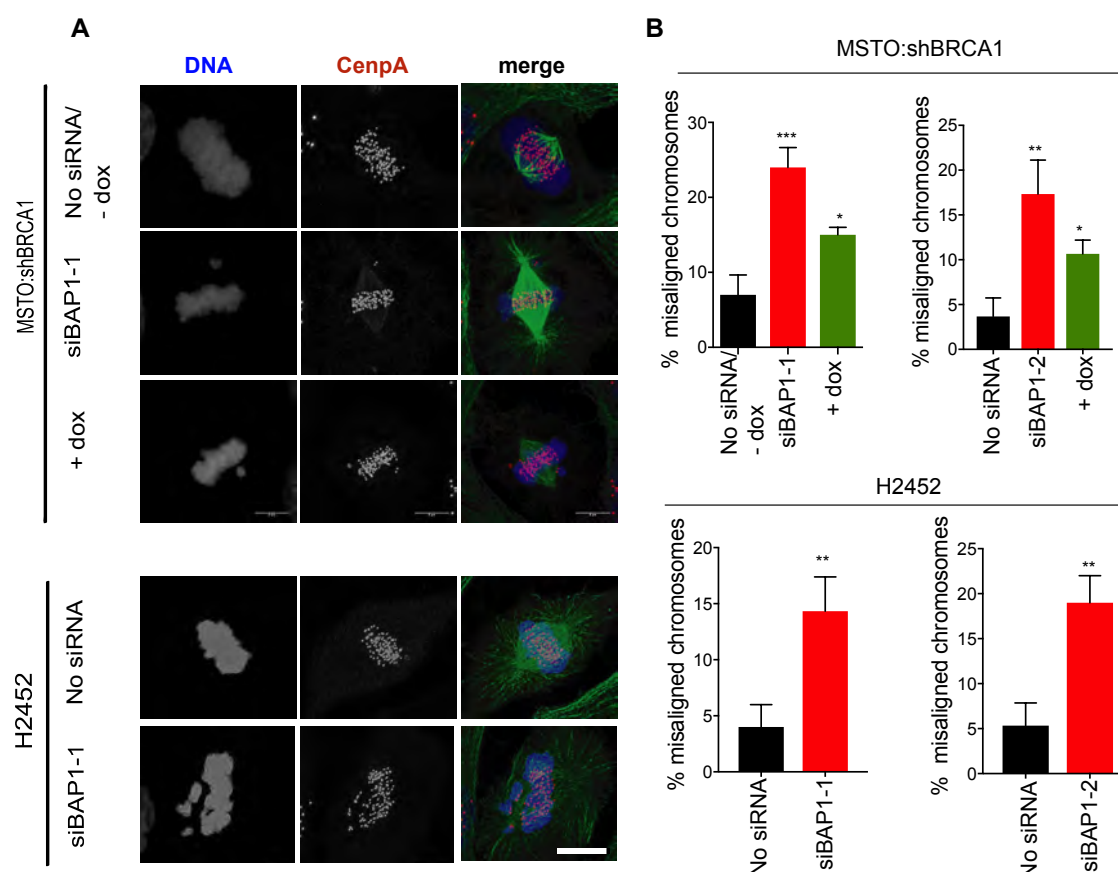


Figure 4.12 BAP1 depletion leads to chromosome misalignment defects

A. Representative images show metaphase in MSTO:shBRCA1 and H2452 cells following mock or BAP1 or BRCA1 depletion. No siRNA means cells were incubated with oligofectamine only. Cells were fixed and stained with CenpA (red) to analyse the kinetochores, α -tubulin (green) and DNA (blue) was stained with Hoechst 33258.

B. Histograms show % of cells with misaligned chromosomes. Three independent experiments were carried out with 50 mitotic cells counted for each experiment. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar, 5 μ m.

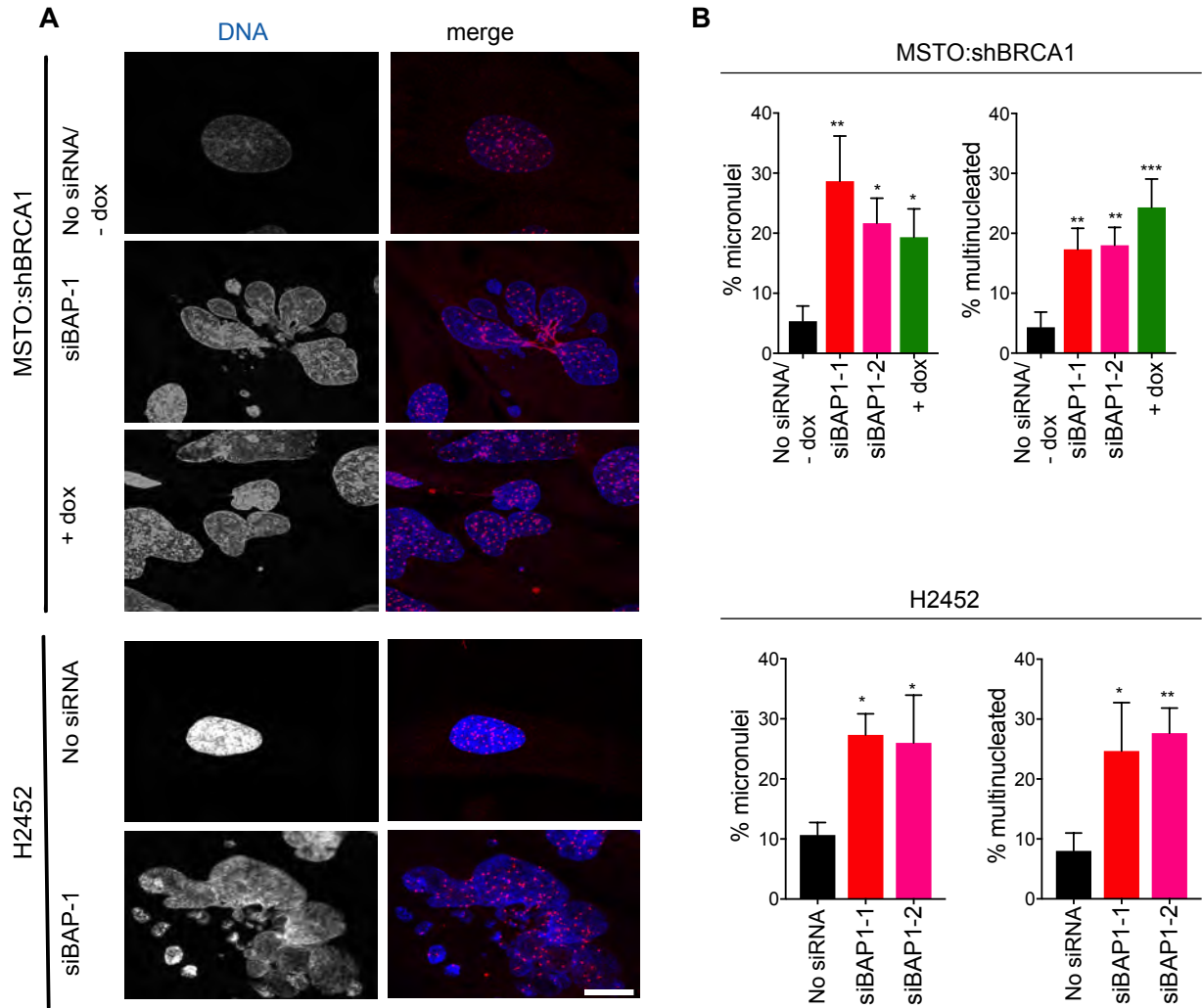


Figure 4.13 BAP1 depletion promotes aneuploidy

A. MSTO:shBRCA1 and H2452 cells were mock-depleted or depleted of BAP1 for 72 hours or doxycycline treated to induce BRCA1 depletion in the MSTO:shBRCA1 cells for 48 hours. No siRNA means cells were incubated with oligofectamine only. Cells were fixed and stained with antibody against CenpA (red) to identify the kinetochores and DNA was stained with Hoechst 33258 (blue in merge). **B.** Histograms show the % of multinucleated and micronucleated cells. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar, 5 μ m.

4.2.9 BAP1 and BRCA1 loss leads to centrosome amplification in MPM

BRCA1 has an established role in inhibiting centrosome amplification in other cancer types (Xu *et al.*, 1999; Starita *et al.*, 2004). We therefore first assessed whether BRCA1 depletion lead to centrosome amplification in MSTO:shBRCA1 (Fig. 3.11A, B). To determine whether centrosome amplification is also enhanced after BAP1 depletion, we analysed centrosomes in MSTO:shBRCA1 and H2452 cells that were depleted of BAP1. Quantitative analysis indicated a significant increase in cells with more than two centrosomes per nucleus after BAP1 depletion as compared to the controls (Fig. 4.14A, B).

As centrosome amplification may lead to multipolar spindles, herein we examined whether BAP1 and BRCA1 depletion also lead to multipolar spindles. Upon either BAP1 or BRCA1 depletion we noted an increased frequency of multipolar spindles in both MSTO:shBRCA1 and H2452 cells (Fig. 4.15A, B). We conclude that BAP1 phenocopies BRCA1 function to regulate centrosome number in MPM cells.

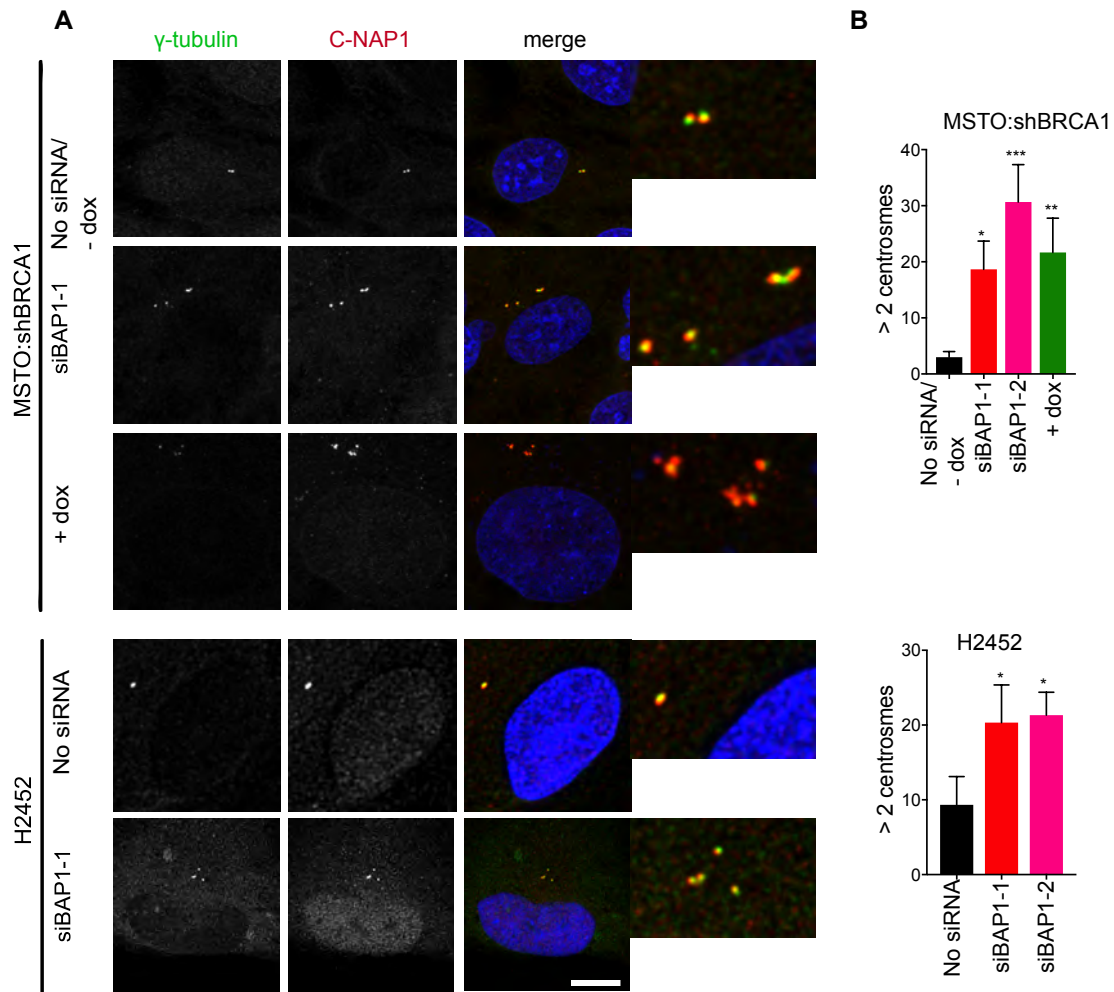


Figure 4.14 BAP1 loss phenocopies BRCA1 to cause centrosome amplification

A. MSTO:shBRCA1 and H2452 cells were mock depleted or depleted of BAP1 for 72 hours, or doxycycline induced in MSTO:shBRCA1 to deplete BRCA1. No siRNA means cells were incubated with oligofectamine only. Cells were fixed and analysed by immunofluorescence microscopy with antibodies against α -tubulin (green), C-NAP1 (red) and DNA stained with Hoechst 33258. Confocal micrographs show representative images of centrosome amplification in mock, siBAP1 or shBRCA1 depleted cells. 100 cells were counted for each experiment. **B.** Histograms show the % of cells with centrosome amplification. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar, 5 μ m.

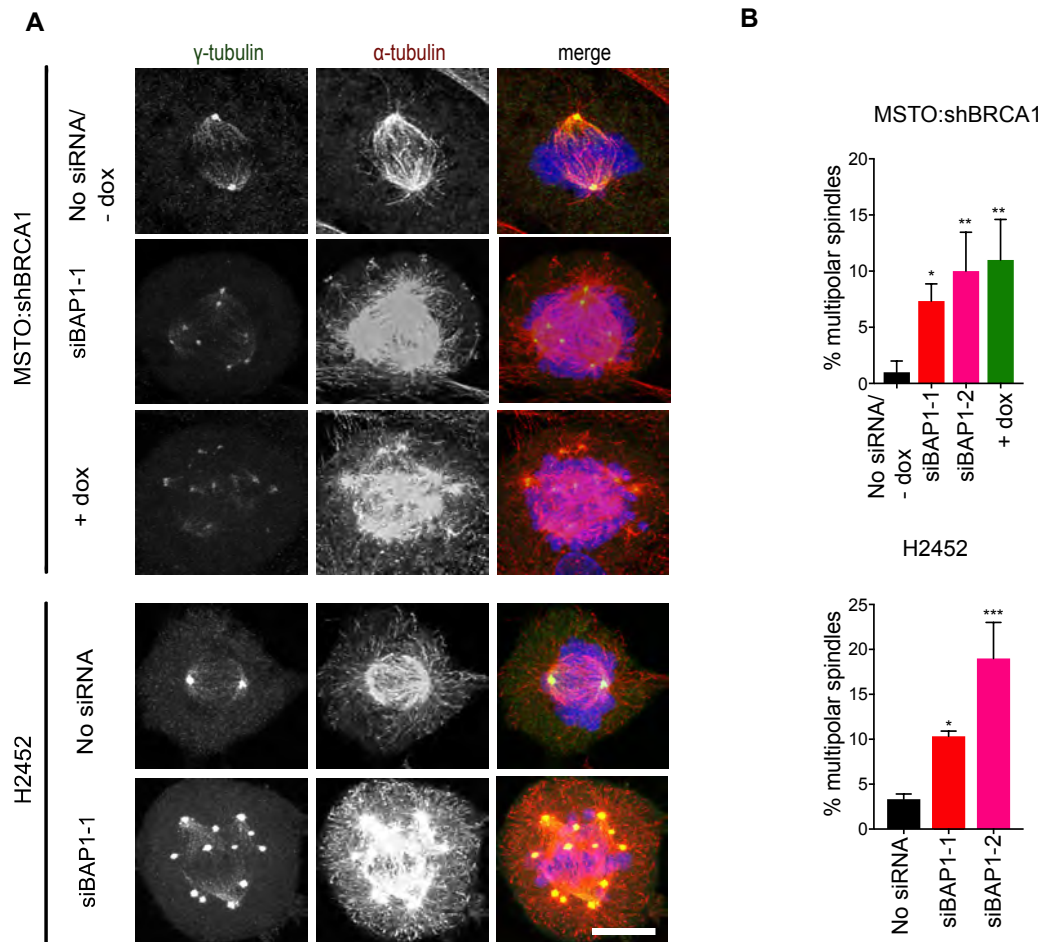


Figure 4.15 Depletion of BAP1 or BRCA1 promotes formation of multipolar spindles

A. MSTO:shBRCA1 and H2452 cells were mock-depleted or depleted of BAP1 for 72 hours, or doxycycline induced in MSTO:shBRCA1 to deplete BRCA1. No siRNA means cells were incubated with oligofectamine only. Cells were fixed and analysed by immunofluorescence microscopy with antibodies against α -tubulin (red), γ -tubulin (green), and DNA stained with Hoechst 33258. **B.** Histograms show the % of cells with multipolar spindles. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Scale bar, 6 μ m.

4.3 DISCUSSION

Ubiquitination and de-ubiquitination play a critical role in several biochemical pathways. This process regulates in a timely and controlled manner the proteolysis of a multitude of substrates for the regulation of gene transcription, removal of misfolded or damaged proteins, immune processes, recognition and surveillance. It also plays role in non-proteolytic events such as membrane trafficking, protein kinase activation, DNA repair and chromatin dynamics (Chen & Sun, 2009). With a myriad of processes involved, it is not surprising that aberrations in this pathway are implicated in the pathogenesis of a variety of diseases including, cancer (Glickman, 2004).

BAP1 is a DUB that is mutated approximately in 40-60% of MM tumours (Sneddon & Creaney, 2016). As the name implies, BAP1 binds to BRCA1, an E3 ubiquitin ligase that plays major roles in DNA repair and gene expression (Metzger *et al.*, 2014). Earlier studies show that the interaction between BAP1 and BRCA1 is highly specific and prevents ubiquitination of BRCA1 (Jensen *et al.*, 1998). BAP1 interacts with BARD1 and inhibits the autoubiquitination of BRCA1 by interfering with BRCA1/BARD1 association. BAP1 has a BARD1 binding domain comprised of residues 182-365. It seems plausible that BAP1 forms a trimeric complex with BRCA1 BARD1 and BAP1 binds to BRCA1 through a domain distinct from the BARD1 binding site (Nishikawa *et al.*, 2009). Data from a number of studies this dual interaction between BAP1 and BRCA1 (Dkhissi *et al.*, 2015; Hakiri *et al.*, 2015).

Excitingly, we found that depletion of BAP1 leads to reduced BRCA1 protein levels not only in cancer cells but also in healthy mesothelial cells without altering BRCA1 gene expression or cell cycle progression. A previous study has shown that enzymatic activity of BAP1 is critical for its tumour suppressor activity and inactivating mutations in the UCH domain, such as A95D, retain nuclear localization but show reduced catalytic activity (Ventii *et al.*, 2008). H2452 cells have an A95D mutation in the UCH domain but still express high BRCA1 protein levels. Depletion of full-length BAP1 reduced BRCA1 levels. Consistent with our results, it was reported that wild-type BAP1 protein or UCH mutant abolishes the auto-ubiquitination of BRCA1/BARD1 complex suggesting DUB- independent mechanism of action (Nishikawa *et al.*, 2009).

BRCA1 expression was rescued in stable cell lines expressing wild-type or mutants of BAP1 apart from a construct lacking the binding site for ASXL1/2. Similar to the above results wild-type and mutants of BAP1 except the construct lacking the binding site for ASXL1/2 (Δ ASXL) significantly increased BRCA1 nuclear foci after irradiation. These results suggest that BRCA1 protein stabilization is independent of BAP1 catalytic activity but dependent on the ASXL1/2 binding site and that BAP1 is required in DNA damage repair HR pathway. Moreover, we speculate that BAP1/ASXL1/2 complex is required for BRCA1 protein stabilization in mesothelioma.

We have confirmed here that BAP1 regulates BRCA1 stability in a proteasome-dependent manner. Cellular BRCA1 protein levels are tightly regulated by transcriptional and post-translational mechanisms (PTMs). Firstly, BRCA1 can be transcriptionally regulated which is inhibited by hypermethylation of its promoter. BAP1 forms a ternary complex with HCF-1 and Yin Yang 1 (YY1), a zinc finger multifunctional protein that possesses dual functionality by either repression and activation of many genes involved in several biological processes (Yu *et al.*, 2010). Interestingly, YY1 positively regulates BRCA1 expression by binding to its promoter (Lee *et al.*, 2012). Thus, it seems plausible that BAP1 depletion might affect the expression of the genes involved in BRCA1 protein stability. Secondly, BRCA1 is regulated by PTMs such as ubiquitination. A number of E3 ligases have recently identified been including HERC2, SCF^{FBX044} and HUWE1 that are responsible for BRCA1 ubiquitination and proteolysis (Wang *et al.*, 2014). Other proteins such as RAK, a tyrosine kinase that binds to HERC2, and TUSC4 which is involved in regulating the mTOR pathway, also regulate the protein stability of BRCA1 (Kim *et al.*, 2017; Peng & Lin, 2014).

BRCA1 has been shown to mediate differential response to chemotherapeutic drugs. (Rottenberg *et al.*, 2008, Tutt *et al.*, 2010, Zhou *et al.*, 2003, Nishikawa *et al.*, 2009, Busacca *et al.*, 2012). Interestingly, BAP1 loss leads to resistance to same drugs as loss of BRCA1, including targeted PARP inhibitors as well as a similar level of radioresistance (Nishikawa *et al.*, 2009, Yu *et al.*, 2014, Peña-Llopis *et al.*, 2012). Irradiation and PARP inhibitors utilise DNA damage and repair pathways, however the mechanism of action of vinorelbine is different. Mitotic arrest induced by vinorelbine is

dependent on an active SAC. Previously it was shown that BRCA1 loss mediates resistance to vinorelbine (Busacca *et al.*, 2012). For the first time, we show here that BAP1 depletion phenocopies BRCA1 function in maintaining SAC integrity. Following depletion of BAP1 in MSTO:shBRCA1 and H2452 cells, flow cytometry revealed that cells failed to arrest upon vinorelbine treatment. The results were consistent with the loss of the G2/M population after BRCA1 depletion and vinorelbine treatment. As BAP1 is required for BRCA1 stability, this suggests that loss of BAP1 affects SAC integrity through BRCA1 loss. Previously it has been shown that BRCA1 regulates the SAC through control of MAD2 expression. We confirmed that inactivation of SAC upon BAP1 depletion was due to reduced MAD2 protein levels and this was accompanied by reduced BUBR1 intensity at the kinetochores after BAP1 depletion in both MSTO:shBRCA1 and H2452 cells.

Untimely initiation of anaphase due to SAC failure predisposes cells to chromosomal instability (CIN) and thus is likely to contribute significantly to tumour progression (Rao *et al.*, 2009). CIN represents the loss of fidelity of chromosome segregation during mitosis. Faithful chromosome segregation requires the timely removal of sister chromatid cohesion during mitosis as determined by the coordinated activities of cyclin-dependent kinases and the SAC (Thompson *et al.*, 2010). Previous studies show that MM cells have a high frequency of multinucleated cells (Ahmed *et al.*, 2013; Reale *et al.*, 1987; Hjerpe *et al.*, 2015; Nabeshima *et al.*, 2016). We observed a high number of mitotic cells with misaligned chromosomes after BAP1 and BRCA1 depletion together with an elevated frequency of cells with micronuclei and multinucleated cells.

For example, the centrosome protein MCRS1 has been identified as substrate of BAP1 DUB activity and plays a role in chromosomal stability (Peng *et al.*, 2015). Moreover, as depletion of BAP1 and BRCA1 is expected to decrease HR repair, cells might become more reliant on NHEJ, an error-prone repair mechanism, resulting in the net accumulation of mutations and chromosome aberrations that interfere with chromosome segregation and cause genomic instability (Isono *et al.*, 2017; Srinivasan *et al.*, 2017; Yu *et al.*, 2014).

Centrosome amplification directly promote chromosome missegregation and this can facilitate evolution of more malignant phenotypes (Ganem *et al.*, 2009). Previous studies have shown that BRCA1 loss leads to centrosome amplification (Starita *et al.*, 2004; Xu *et al.*, 1999; Deng, 2002; Kais & Parvin, 2008) and we show here that BAP1 depletion also lead to amplified centrosomes in MPM cells. However, it remains unclear whether this phenotype is solely due to BAP1 depletion or due to reduced BRCA1 protein levels. Taken together, our data shows that BAP1 is required for BRCA1 protein stability and that loss of BAP1 phenocopies many of the defects observed upon loss of BRCA1 function, including mitotic progression and chromosome stability.

CHAPTER 5

Role of BAP1 in Mitosis

5.1 Introduction

In the previous chapter, we have shown that loss of BAP1 leads to chromosome segregation errors and here we intend to explain this further. The genetic integrity of each cell is intrinsically tied to the faithful segregation of chromosomes during mitosis, a task performed by the mitotic spindle, a microtubule based macromolecular machine composed of an array of dynamic MTs and associated proteins (Karsenti & Vernos, 2001). The slow-growing minus ends of the MTs are focused into the two poles of the bipolar spindle forward by the duplicated centrosomes. The faster growing plus ends of MTs extend towards the spindle equator to form interpolar and kinetochore MTs, responsible for aligning and segregating the chromosomes (Walczak & Heald, 2008). A third population of MTs, the astral MTs extend towards the cell cortex. These MTs position and orient the spindle within the cell. The mitotic spindle determines the plane of cytokinesis and is critical for tissue morphogenesis (Pease & Tirnauer, 2011). During early mitosis, the spindle maintains a constant length, even though the spindle MTs are constantly undergoing polymerization and depolymerization (Weaver *et al*, 2011). Changes in the length of spindle or astral MTs could be deleterious for the cell resulting in misaligned chromosomes and aneuploidy. Spindle length is therefore tightly controlled by a series MT end and lattice binding proteins, including kinesin and dynein motors.

The centrosome is a small organelle that consists of a pair of orthogonally positioned centrioles embedded in a pericentriolar matrix of proteins (PCM). Centrosomes function as the main MT organizing centres (MTOC) (Arquint *et al.*, 2014). During the cell cycle, the centrosome duplicates once during S-phase so that their number remains stable like the genetic material of the cell. Apart from their function in mitosis, centrosomes regulate cell shape, polarity, adhesion and motility along with intracellular transport and positioning of organelles (Glover & Bettencourt-Dias, 2007). The PCM comprises of γ -tubulin ring complexes (γ TuRCs) that are important for MT nucleation along with a large number of scaffolding proteins. Several of these proteins play key roles in regulating MT dynamics, nucleation and kinetochore attachment to enable correct chromosome alignment and segregation. Abnormal regulation of centrosome proteins can lead to structural (shape, size and composition), numerical

(amplified centrosomes) and functional (abnormal MT nucleation and unorganized spindles) changes in centrosome behaviour (Rivera-Rivera *et al.*, 2016).

The UPS pathway is responsible for the constitutive degradation of many of cellular proteins. It plays a key role in maintaining a constant balance between *de novo protein* synthesis and proteolysis (Didier *et al.*, 2008). Although proteasome activity is present in different subcellular locations, proteasome components, such as ubiquitin and the 20S and 19S sub-units of proteasome are concentrated in the centrosome (Livneh *et al.*, 2016; Wigley *et al.*, 1999; Badano *et al.*, 2005). Furthermore, there is increasing evidence that the centrosome duplication cycle is regulated by the UPS (Badano *et al.*, 2005). Indeed, proteasome inhibition in HeLa cells leads to an increase in centrosome size and accumulation of centrosome proteins including γ -tubulin, PCM1, NEDD1 and pericentrin along the with MT anchoring protein ninein and dynactin (Didier *et al.*, 2008).

BRCA1 an E3 ligase is also located at the centrosome, and BRCA1 dependent ubiquitination of γ -tubulin plays a key role in regulating centrosome duplication and MT nucleation (Starita *et al.*, 2004; Sankaran *et al.*, 2005). Consequently, loss of BRCA1 leads to centrosome amplification and an increase in centrosome size (Sung & Giannakakou, 2014). BAP1 also interacts with γ -tubulin during mitosis and cells expressing low BAP1 show mitotic defects (Zarrizi *et al.*, 2014).

BAP1 de-ubiquitinates MCRS1, a multifunctional protein that localizes to the centrosome, minus ends of K-fibres, associates with cytoplasmic dynein mediating pericentrosomal recruitment of proteins and mTOR signalling (Lee *et al.*, 2016; Petry & Vale, 2011; Meunier & Vernos, 2011; Peng *et al.*, 2015). MCRS1 binds to BRCA1 but it unknown whether MCRS1 is a substrate of BRCA1 E3 ligase activity (Hill, 2014).

In this chapter, we explored what role BAP1 might play in spindle organization, besides its control of centrosome number and size. We also examined whether BAP1 and BRCA1 have independent functions in centrosome activity and spindle assembly during mitosis.

5.2 Results

5.2.1 BAP1 but not BRCA1 is required to control centrosome size in interphase cells.

BRCA1 ubiquitinates γ -tubulin a key centrosomal protein component whereas BAP1 can de-ubiquitinate γ -tubulin (Sankaran *et al.*, 2007; Zarrizi *et al.*, 2014). Meanwhile, BRCA1 loss is associated with accumulation of γ -tubulin at the centrosomes (Sung & Giannakakou, 2014). Therefore, we examined the consequence of loss of BAP1 on γ -tubulin recruitment to the centrosomes, as well another PCM protein CDK5RAP2. Moreover, CDK5RAP2 is relevant as it is required for γ -tubulin complex localization to spindle poles during mitosis (Barr *et al.*, 2010). Strikingly, quantification of immunofluorescence microscopy data showed a significant decrease in both γ -tubulin and CDK5RAP2 volume at centrosomes after BAP1 depletion in interphase whereas BRCA1 depletion lead to an accumulation of γ -tubulin and CDK5RAP2 (Fig. 5.1A, B). We then analysed how BAP1 or BRCA1 depletion effect the recruitment of the proteins in mitosis when the centrosome matures with an expansion of the PCM (Kim *et al.*, 2014). Upon analysis of mitotic cells, we found that γ -tubulin and CDK5RAP2 volume were significantly reduced at mitotic centrosomes after BAP1 depletion, whereas in BRCA1 depleted cells there was an increase in accumulation of both proteins (Fig. 5.2A, B). H2452 cells have an enzymatically inactive BAP1 mutant; however, BAP1 depletion still led to a decrease in centrosome volume as measured through γ -tubulin and CDK5RAP2 recruitment in these cells. This suggests that this phenotype is independent of BAP1 catalytic activity. It is nevertheless possible that an increase in ubiquitination of γ -tubulin, and quite possibly of CDK5RAP2, after BAP1 depletion leads to reduced centrosome volume.

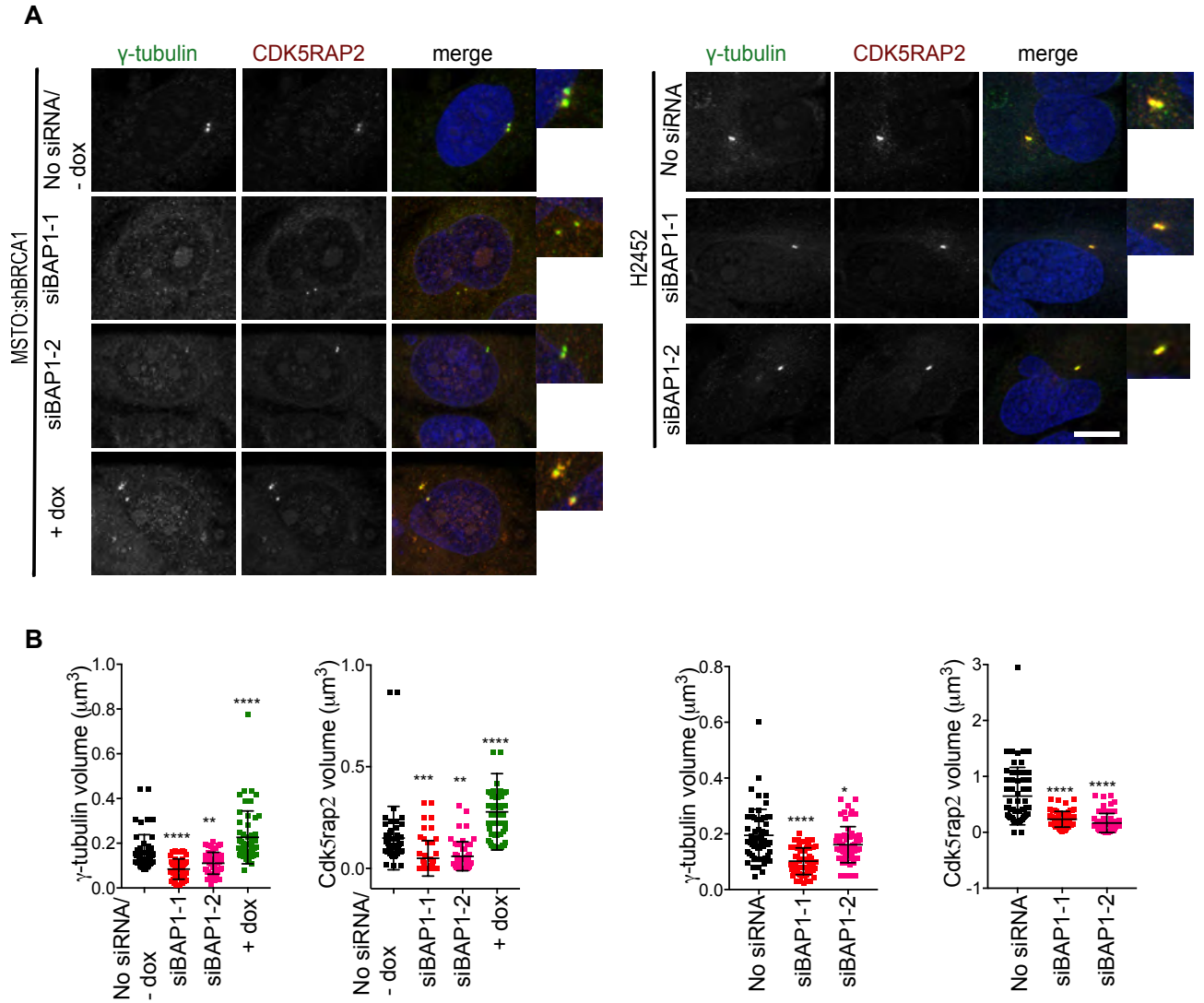


Figure 5.1 Loss of BAP1 reduces centrosome volume in interphase cells

A. MSTO:shBRCA1 and H2452 cells were mock-depleted or depleted with siRNA against BAP1 for 72 hours; BRCA1 was depleted in MSTO:shBRCA1 cells by doxycycline induction for 48 hours. No siRNA means cells were incubated with oligofectamine only. Cells were analysed by immunofluorescence microscopy with antibodies against γ -tubulin (green) and CDK5RAP2 (red), DNA was stained with Hoechst 33258. **B.** Histograms show the γ -tubulin and CDK5RAP2 volume in cells transfected as in A. Measurement of volume was done using Imaris 3D. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Scale bar, 5 μm .

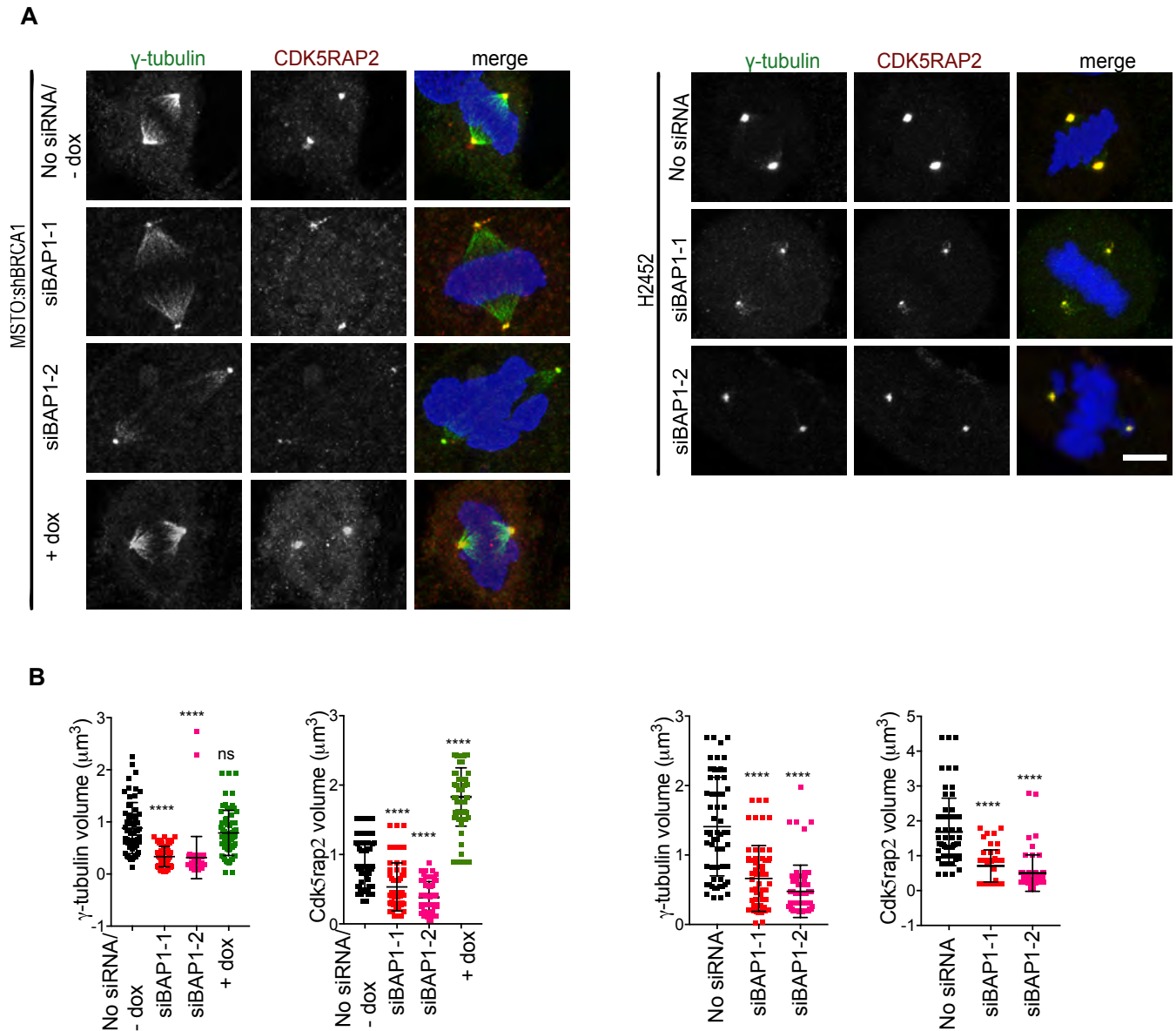


Figure 5.2 BAP1 depletion causes reduced PCM volume in mitotic cells

A. MSTO:shBRCA1 and H2452 cells were mock-depleted or depleted with siRNA against BAP1 for 72 hours; BRCA1 was depleted in MSTO:shBRCA1 cells by doxycycline induction for 48 hours. No siRNA means cells were incubated with oligofectamine only. Cells were analysed by immunofluorescence microscopy with antibodies against γ -tubulin (green) and CDK5RAP2 (red), DNA was stained with Hoechst 33258. **B.** Histograms show the γ -tubulin and CDK5RAP2 volume in cells transfected as in A. Measurement of volume was done using Imaris 3D. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, **** $p < 0.0001$. Scale bar, 5 μ m.

5.2.2 BAP1 depletion leads to an increase in spindle length

Analysis of mitotic cells following BAP1 depletion suggest the presence of additional defects as spindle poles appeared to be separated by a greater distance than in BRCA1 or mock depleted cells. We therefore examined spindle length in mitotic cells by immunofluorescence microscopy analysis of γ -tubulin and α -tubulin after BAP1 or BRCA1 depletion. This confirmed that spindle length was significantly increased in BAP1 depleted cells as compared to BRCA1 or mock (Fig. 5.3 A, B). Interestingly, it also revealed that 10% mitotic cells depleted of BAP1 also had spindle poles that were detached from the bulk of spindle MTs (Fig. 5.4 A, B). Impaired centrosome-spindle attachment can cause abnormal centrosome segregation, which in turn can lead to supernumerary centrosomes (Chavali *et al.*, 2016). Hence, this could explain the increase in amplified centrosomes and spindle multipolarity as previously described in BAP1-depleted cells.

When analysing the spindle morphology in BAP1 depleted cells, we also noticed that astral microtubule appeared overly long as compared to those in mock or BRCA1 depleted cells. To examine directly whether BAP1 depletion causes longer MTs, we depleted cells with BAP1 or BRCA1 and then treated cells with an Eg5 inhibitor, STLC to arrest mitotic cells in a monopolar mitotic state. This approach revealed that BAP1 depletion but not BRCA1 depletion resulted in a dramatic increase in length of the mitotic MTs (Fig. 5.5A, B). Taken together, these results suggest that BAP1 has a role that is distinct and independent of BRCA1 in mitosis, in maintaining spindle microtubule length and pole attachment and that loss of these functions is also likely to promote genomic stability.

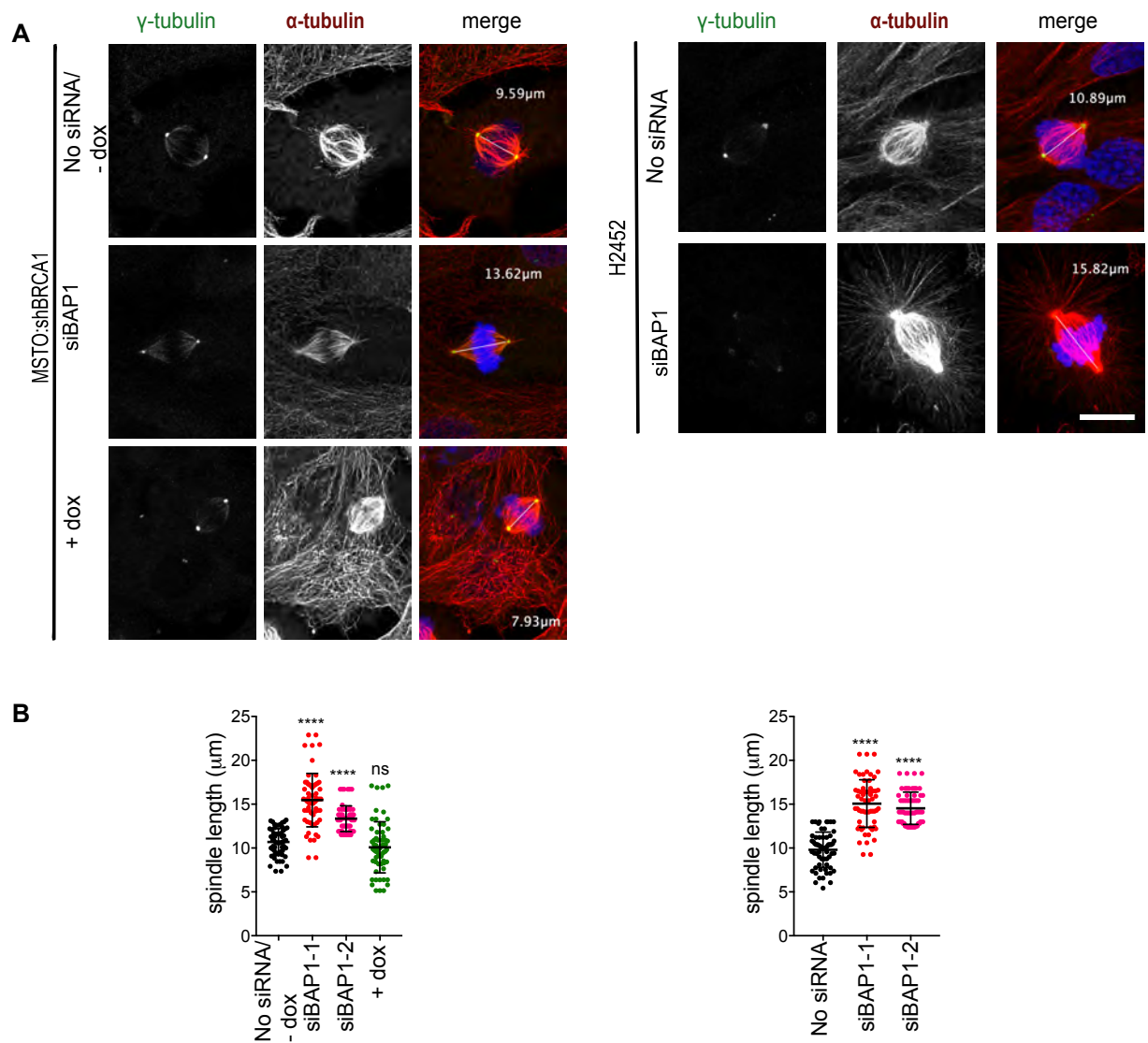


Figure 5.3 Loss of BAP1 but not BRCA1 leads to an increase in spindle length

A. Immunofluorescence microscopy analysis of mitotic spindles in MSTO:shBRCA1 and H2452 cells after depletion of BAP1 for 72 hours or doxycycline induced depletion of BRCA1 for 48 hours. No siRNA means cells were incubated with oligofectamine only. Cells were stained with α -tubulin (red), γ -tubulin (green) and DNA with Hoechst 33258. **B.** Scatter plot shows the spindle length from cells as shown in A as measured as pole to pole distance. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, **** p <0.0001. Scale bar, 6 μ m.

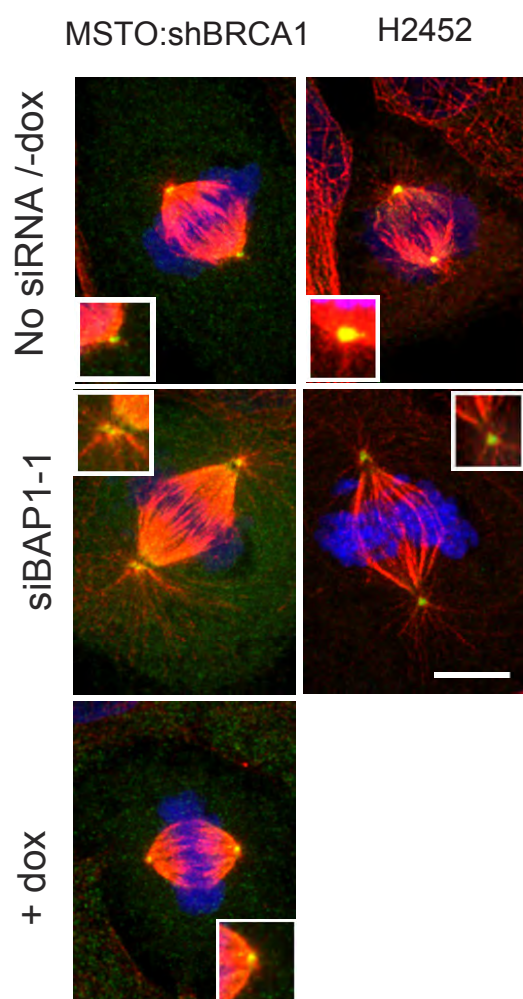


Figure 5.4 Loss of BAP1 leads to spindle pole detachment

Immunofluorescence microscopy analysis of mitotic spindles in MSTO:shBRCA1 and H2452 cells after depletion of BAP1 for 72 hours or doxycycline induced depletion of BRCA1 for 48 hours. No siRNA means cells were incubated with oligofectamine only. Cells were stained with α -tubulin (red), γ -tubulin (green) and DNA with Hoechst 33258. Scale bar, 8 μ m.

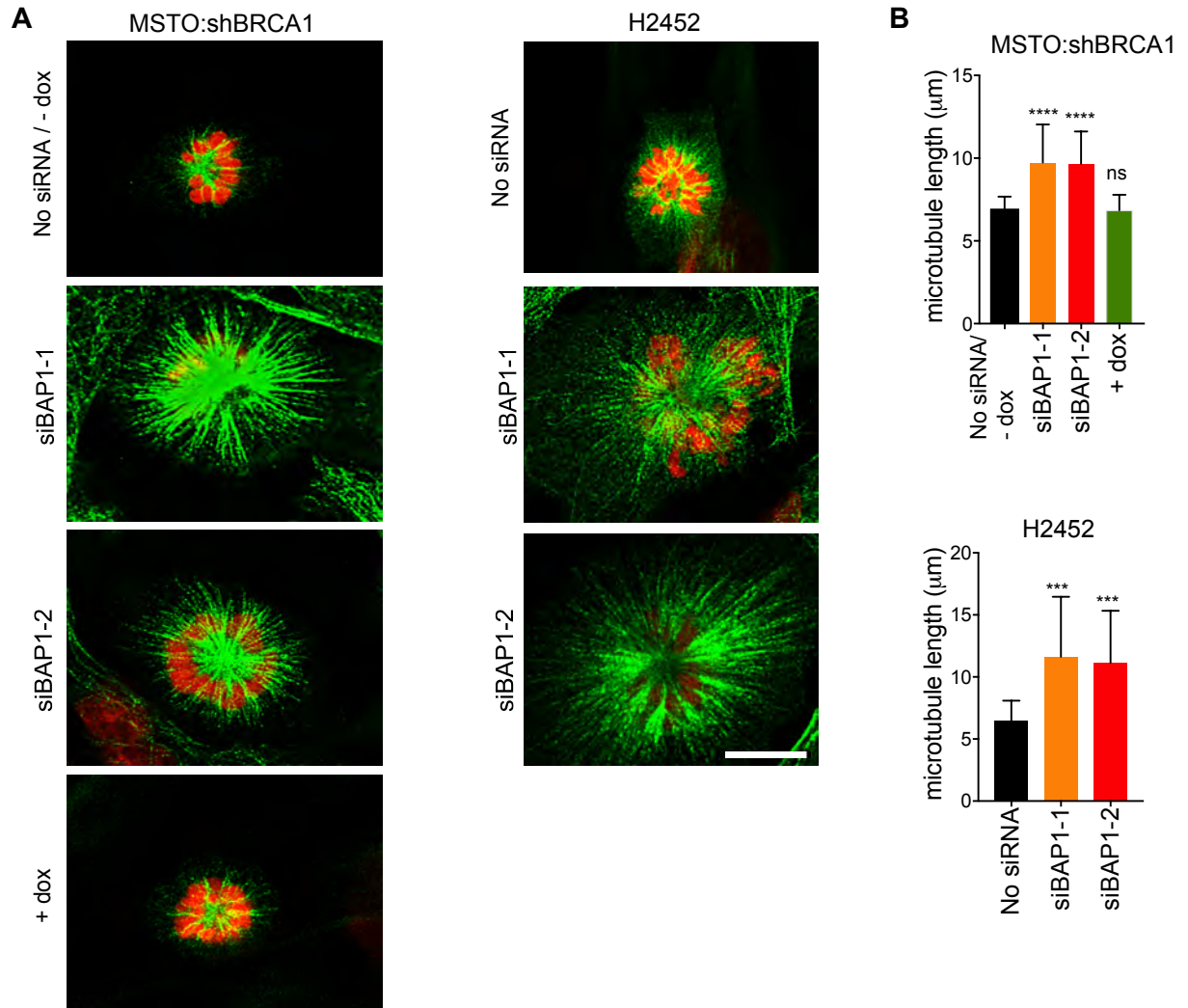


Figure 5.5 Loss of BAP1 but not BRCA1 leads to increase in astral MT length

A. Immunofluorescence microscopy analysis of mitotic spindles in MSTO:shBRCA1 and H2452 cells after depletion of BAP1 for 72 hours or doxycycline induced depletion of BRCA1 for 48 hours. No siRNA means cells were incubated with oligofectamine only. Cells were then treated with STLC for 6 hours to visualise monopolar spindles. Cells were stained with α -tubulin (green) and DNA with Hoechst 33258. **B.** Histograms show the length of MTs quantified from the monopolar spindles quantified using Imaris 3D. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, *** $p < 0.001$, **** $p < 0.0001$. Scale bar, 10 μm .

5.2.3 BAP1 controls spindle microtubule length through regulating Kif18A expression

Maintaining appropriate spindle length is critical during mitosis for faithful chromosome segregation. Previous studies have shown that the kinesin motor, Kif18A regulates mitotic spindle length through its microtubule destabilizing activity (Weaver *et al.*, 2011). KIF18A localizes to the kinetochore microtubules (K-fibers) where it suppresses microtubule dynamics to control chromosome congressional positioning (Stumpff *et al.*, 2008; Stumpff *et al.*, 2011). To determine whether the elongated spindles observed after BAP1 depletion might be due to perturbation of KIF18A, the localization of KIF18A at the plus ends of the microtubules was quantified by immunofluorescence microscopy. This revealed that KIF18A intensity was decreased at kinetochores after BAP1 depletion in both MSTO:shBRCA1 and H2452 cells (Fig. 5.6A, B). To confirm that it was loss of KIF18A after BAP1 depletion that caused the elongated spindles, we transfected BAP1 depleted cells with a GFP-KIF18A expression plasmid for 24 hours. Strikingly, the transgene KIF18A caused spindle length to reduce to that seen in mock-depleted cells (Fig. 5.7A, B). Hence, we conclude that the increased spindle length observed in BAP1 depleted cells is due to loss of KIF18A from microtubule plus ends.

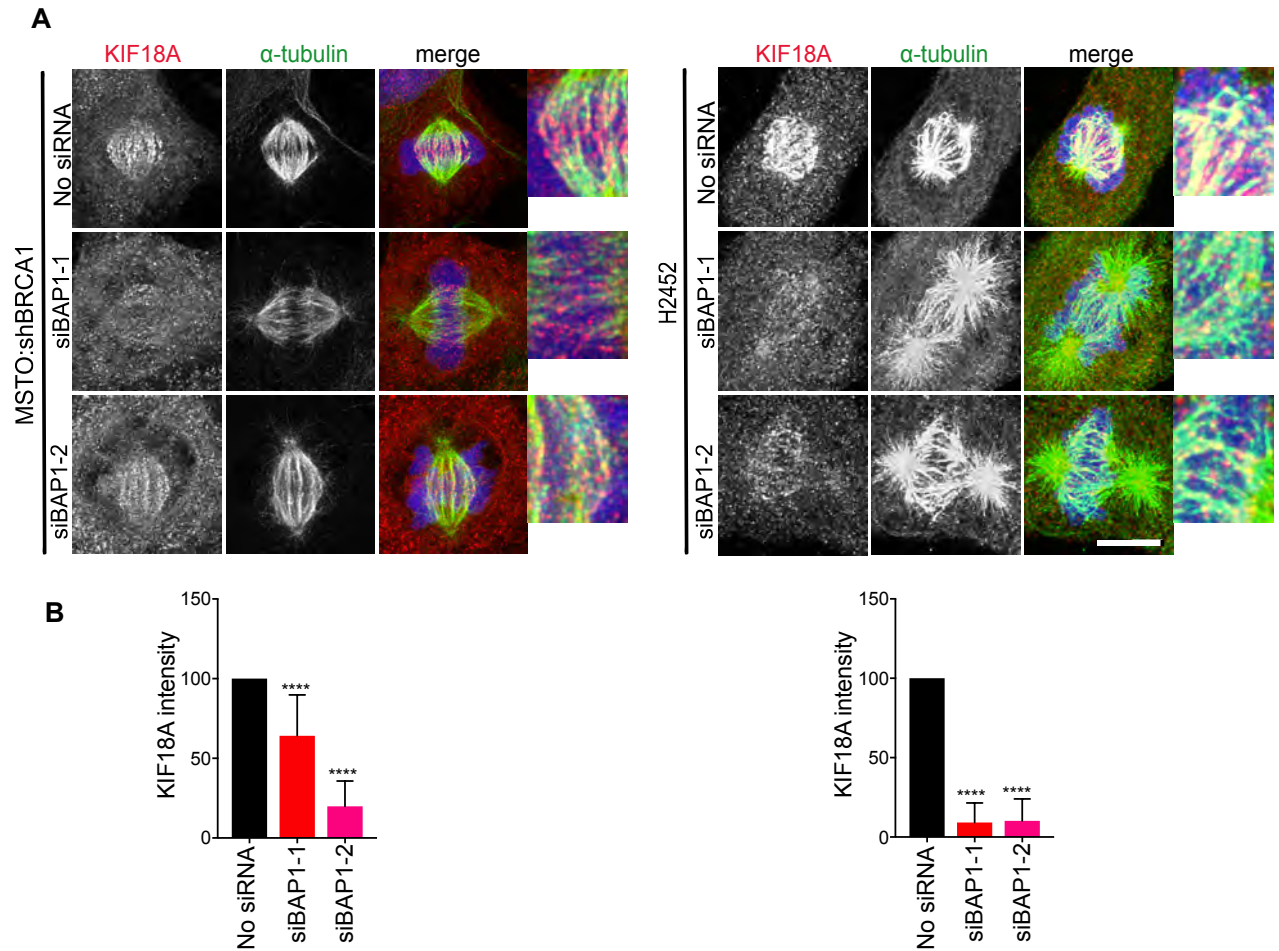


Figure 5.6 BAP1 depletion leads to loss of KIF18A from kinetochores

A. Immunofluorescence microscopy analysis of mitotic spindles in MSTO:shBRCA1 and H2452 cells after depletion of BAP1 for 72 hours. No siRNA means cells were incubated with oligofectamine only. Cells were stained with α -tubulin (green), KIF18A (red) and DNA with Hoechst 33258. Insets show higher magnification of the representative images **B.** Histograms show KIF18A intensity in mock and BAP1 depleted cells. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, **** $p < 0.0001$. Scale bar, 6 μ m.

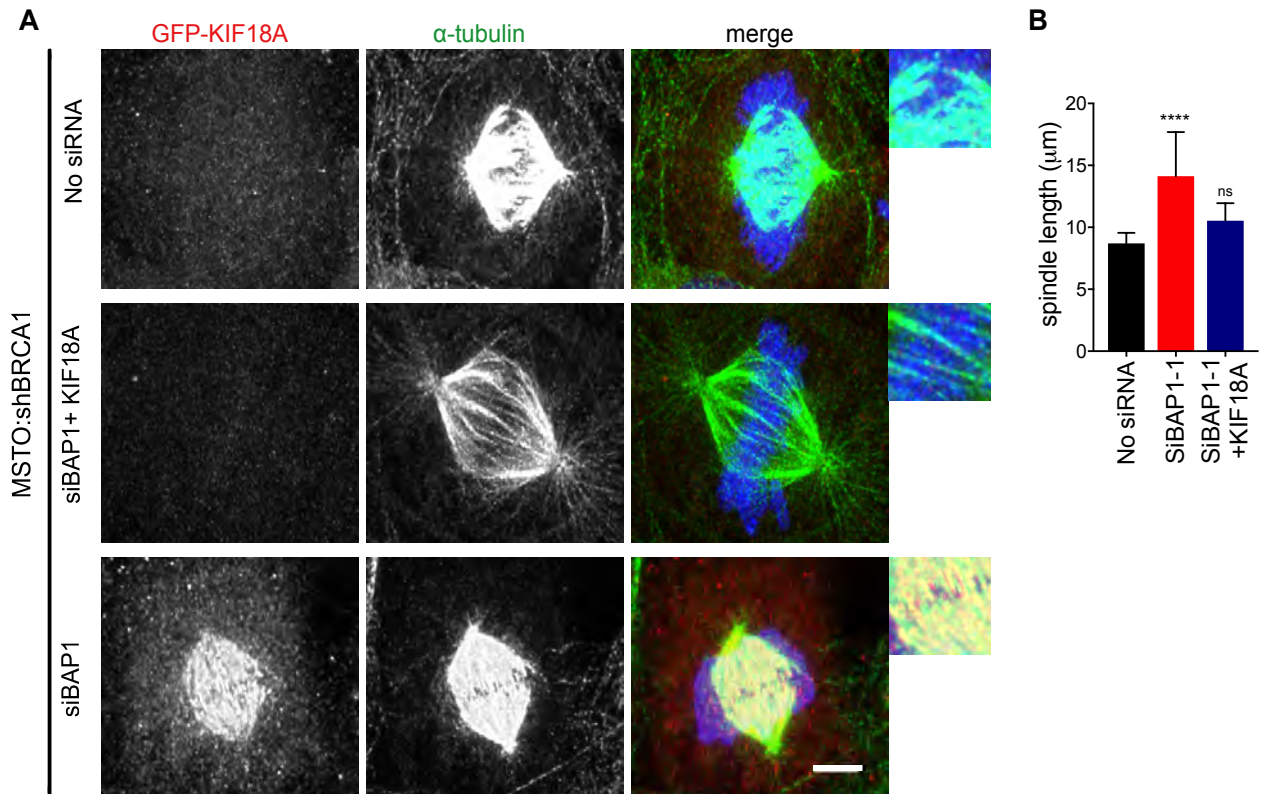


Figure 5.7 Expression of wild-type KIF18A rescues spindle length in BAP1 depleted cells

A. Immunofluorescence microscopy analysis of mitotic spindles in MSTO:shBRCA1 and H2452 cells after depletion of BAP1. No siRNA means cells were incubated with oligofectamine only. Cells were stained with α -tubulin (green), GFP (red) and DNA with Hoechst 33258. **B.** Histogram shows the spindle as quantified using Imaris 3D. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, **** $p < 0.0001$. Scale bar, 5 μ m.

5.2.4 BAP1 regulates aster volume through KIF18B

Another member of the kinesin 8 family, KIF18B has been shown to regulate the length of astral microtubules during mitosis to ensure spindle centering (Stout *et al.*, 2011; McHugh *et al.*, 2018). Furthermore, KIF18B and not KIF18A is required to regulate microtubule length in monopolar spindles (Walczak *et al.*, 2016; McHugh *et al.*, 2018). Due to lack of antibodies against KIF18B that gave a reliable signal by immunofluorescence microscopy, we were not able to look at the localization of KIF18B in cells. However, to test whether perturbation of KIF18B was responsible for the increase in aster volume in BAP1 depleted cells, we first depleted cells of BAP1 for 48 hours and then transfected the cells with an GFP-KIF18B expression plasmid for another 24 hours. This revealed that the aster volume phenotype was restored to its normal size by expression of GFP-KIF18B (Fig. 5.8A, B). Interestingly though, the spindle length did not change (Fig. 5.8C) indicating that KIF18B controls astral microtubule length but not the spindle microtubule length.

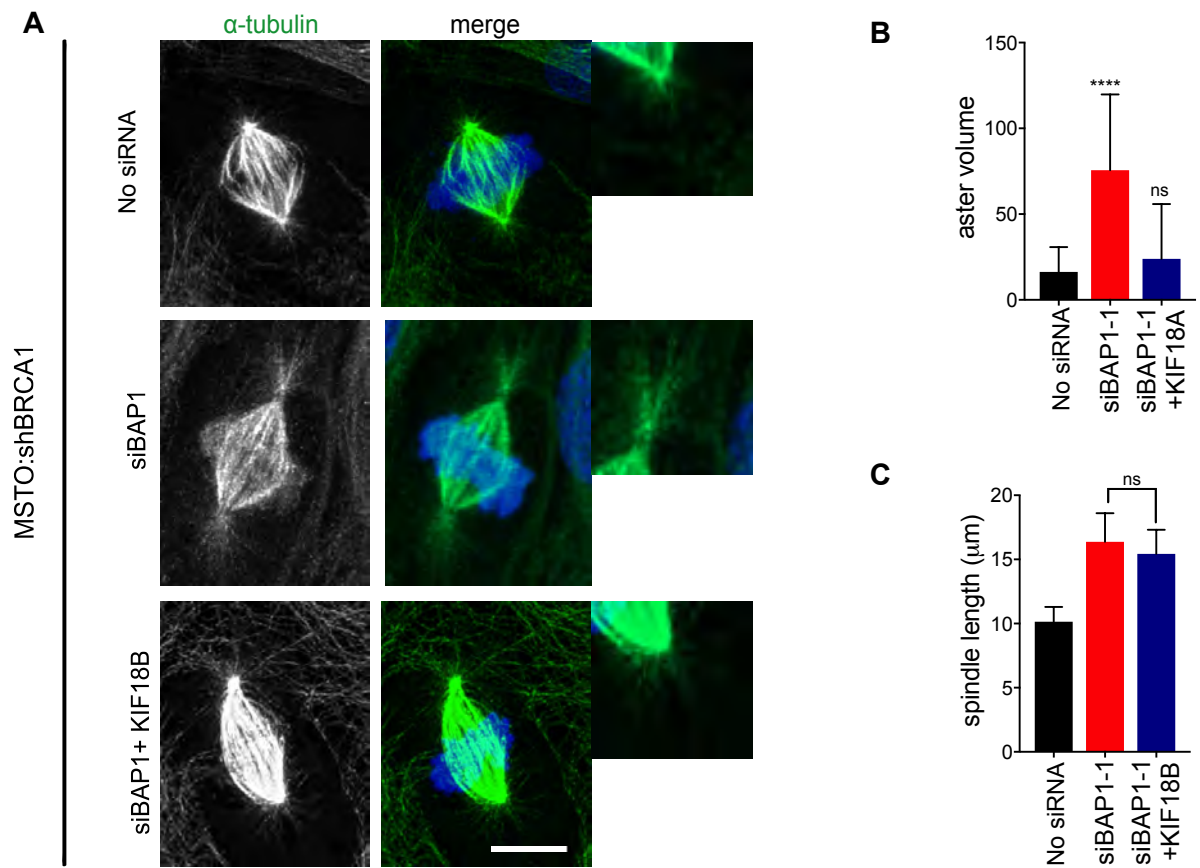


Figure 5.8 Expression of wild-type KIF18B rescues aster volume but not spindle length in BAP1 depleted cells

A. MSTO:shBRCA1 cells were mock-depleted or depleted with siRNA against BAP1 for 48 hours; GFP-KIF18B plasmid was transfected for 24 hours. No siRNA means cells were incubated with oligofectamine only. Cells were analysed by immunofluorescence microscopy with antibodies against α -tubulin (green) and DNA was stained with Hoechst 33258. Scale bar, 5 μ m. **B.** Histograms shows the aster volume as measured by drawing an area around the astral microtubules using free hand tool and quantified the volume using 3D object counter in ImageJ. **C.** Histograms shows the spindle length measured as pole to pole distance. Data show means $\pm$ S.D. of three independent experiments. One-way Anova, ****p<0.0001. Scale bar, 8 μ m.

5.2.5 BAP1 depletion leads to reduced protein expression of KIF18A and KIF18B

To confirm whether increased spindle length and aster volume after BAP1 depletion was due to reduced protein expression of KIF18A and KIF18B, we examined their expression by western blot. This showed reduced protein expression of KIF18A and KIF18B in both MSTO:shBRCA1 and H2452 cells after BAP1 depletion as compared to mock-depleted cells (Fig. 5.9 A, B). We then examined whether expression of wild-type or mutant versions of BAP1 could rescue KIF18A expression in the H226 stable cell lines. The western blots show that KIF18A expression was very low in the parental H226 cells which lack BAP1 but was increased in cells expressing wild-type BAP1 and all the mutants of BAP1 (Fig. 5.9 C, D). Unfortunately, we were not able to confirm expression of KIF18B due to the lack of specificity of this antibody in H226 cells. As H2452 cells have an enzymatically inactive BAP1 and the protein expression of KIF18A or KIF18B was reduced after BAP1 depletion, this suggests that the DUB activity of BAP1 is not required for regulating KIF18A or KIF18B expression. Interestingly, the fact that the Δ ASXL mutant BAP1 rescued KIF18A expression but not BRCA1 expression also suggest that the mechanism through which BAP1 controls expression of these genes is distinct. Taken together, we have identified new mechanisms for how BAP1 regulates mitotic spindle assembly and genetic stability in a manner that is independent of BRCA1 in MPM cells.

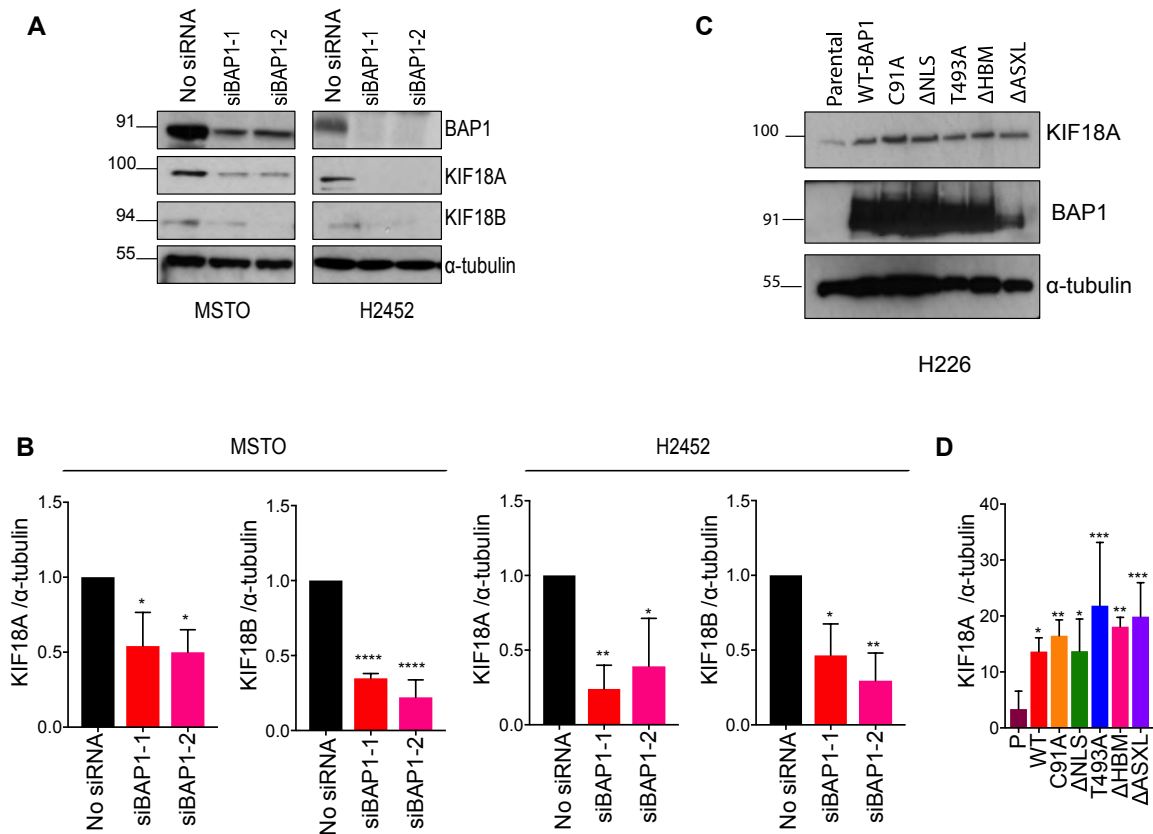


Figure 5.9 BAP1 depletion leads to loss of KIF18A and KIF18B protein expression

A. MSTO:shBRCA1 and H2452 cells after depletion of BAP1 for 72 hours. No siRNA means cells were incubated with oligofectamine only. Lysates were analysed by Western blotting with antibodies indicated. **B.** Histogram shows the densitometric quantification of the immunoblots. **C.** H226 stable cell lysates were analysed by Western blot using KIF18A antibodies. **D.** Histogram shows densitometric quantification of KIF18A in H226 wild-type and BAP1 mutants. Data show means $\pm$ s.d. of three independent experiments. One-way Anova, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.3 DISCUSSION

Many cancers display not only numerical but structural centrosome abnormalities, with enlarged centrosomes being most common. Although the enlarged centrosomes may appear superficially similar, their functional properties and protein composition might be altered which could have an impact on cell shape, polarity, adhesion and migration and may lead to genomic instability and loss of cellular architecture. These adverse changes could also affect spindle formation and chromosome segregation (Nigg, 2002; Kushner *et al.*, 2014).

Numerical centrosome defects have been studied extensively and shown to promote chromosome segregation defects and enhanced cell migration (Lingle *et al.*, 2002; Ganem *et al.*, 2009; Fukasawa, 2005; Kushner *et al.*, 2014; Mittal *et al.*, 2015; Ogden *et al.*, 2013); however, the consequence of structural centrosome defects has received comparatively little attention. We report for the first time that, whereas loss of BRCA1 increases centrosome volume, loss of BAP1 leads to decrease in centrosome volume.

We noticed smaller centrosome size in BAP1 depleted cells. Upon quantification of CDK5RAP2 and γ -tubulin volume at the centrosomes, it was noted that there was a significant reduction of centrosome volume in BAP1 depleted cells. CDK5RAP2 is essential for γ -tubulin recruitment at the centrosomes and is also essential for attachment of centrosomes to the mitotic spindle (Fong *et al.*, 2008; Barr *et al.*, 2010). Detachment of spindle poles was seen in cells with bipolar spindles after BAP1 depletion. It is possible that the low levels of CDK5RAP2 are unable to recruit sufficient γ -tubulin at the centrosomes and thus centrosomes detach from the spindle poles. However, further work is required to understand the loss of CDK5RAP2 after BAP1 depletion.

The consequence of such defect is unknown and further studies will be required to shed light on how this might affect cell polarity or migration. However, it is interesting to note that BAP1 loss is associated with cancer progression and metastasis in uveal melanoma (Harbour *et al.*, 2010; Matatall *et al.*, 2013). Moreover, mesothelioma tumours have a high rate of local and distant metastatic spread that leads overall

poor prognosis (Bussani *et al.*, 2007; Zardawi *et al.*, 2015; Tertemiz *et al.*, 2014). BAP1 depletion leads to a decrease in β -catenin and E-cadherin levels in MCF-7 and A549 cells that lead to partial loss of cell to cell contact. Loss of expression or reduction of E-cadherin and β -catenin is associated with invasion, metastasis and poor prognosis in several types of cancers (Yoshida *et al.*, 2001). Similar to our data on centrosome volume, the catalytic activity of BAP1 is dispensable for its regulation of β -catenin (Wong, 2012).

The PRC2 complex regulates genes that are linked to epithelial-mesenchymal transition (EMT) including E-cadherin (Cao *et al.*, 2008). Loss of BAP1 in mice lead to an increase in trimethylated histone H3 lysine 27 (H3K27me3) and EZH2 expression. Moreover, BRCA1 loss also leads to a substantial increase in (H3K27me3) and EZH2 expression and a interaction between the BRCA1 NTD (N-terminal domain) and EZH2 has been shown by coimmunoprecipitation and mass spectrometry analyses (Wang *et al.*, 2013; LaFave *et al.*, 2015). It is possible that BAP1 and BRCA1 might together work to suppress cell migration and invasion through multiple pathways. In this regard, it is interesting that an EZH2 inhibitor is in clinical trial in mesothelioma patients with BAP1 inactivation (Zauderer *et al.*, 2018).

Regulation of microtubule dynamics is critical for cell migration, cell signalling and trafficking and mitosis (Honore *et al.*, 2005). KIF18A and KIF18B belong to the family of kinesin 8 plus end directed microtubule-based motor proteins. Both these proteins play a critical role in regulating microtubule length in mitotic cells. They bind to the plus-end of microtubules and promote microtubule depolymerization (Su *et al.*, 2011). During interphase KIF18A shows colocalization with EB1 at microtubule plus ends, while depletion results in more dynamic microtubules at the leading edges of the cells suppressing cell migration and tumour development. Overexpression of KIF18A is reported in several cancers including breast cancer, hepatocellular carcinoma, renal cell carcinoma, colorectal cancer, lung cancer and cholangiocarcinoma (Chen *et al.*, 2016; Zhang *et al.*, 2010; Luo *et al.*, 2018). In mitosis, KIF18A depletion is linked to excessive chromosome oscillations, misalignment, segregation errors and elongated spindles, and more recently with SAC activation (Stumpff *et al.*, 2008; Weaver *et al.*,

2011b; Häfner *et al.*, 2014; Mayr *et al.*, 2007; Janssen *et al.*, 2018). Interestingly, KIF18A levels are significantly decreased in the sera of asbestos exposed individuals and in mesothelioma is found to be downregulated (Schmitz, 2017; Avila, 2012).

KIF18A expression is controlled during mitosis through ubiquitination by the APC/C-Cdc20, a E3 ubiquitin ligase complex (Sedgwick *et al.*, 2013). We can rule out that BAP1 DUB activity is directly involved in regulating KIF18A expression as H2452 cells have an enzymatically inactive BAP1 and yet depletion of BAP1 in these cells still leads to elongated mitotic spindles. Interestingly, BRCA1 depletion did not affect the length of the mitotic spindle demonstrating that this role of BAP1 is independent of BRCA1. Clearly, our work and others have shown that BAP1 depletion deregulates the expression of several proteins including in cell cycle control, DNA repair, cell survival or metabolism. Other proteins downregulated include SKP2, a component of the SCF E3 ubiquitin ligase, CCNE2 (cyclin E2) and CDC6 (cell division cycle 6) (Wang *et al.*, 2012b; Yu *et al.*, 2010). Meanwhile, proteomics analysis showed that KIF18A, CDC6 and EZH2 are functionally interconnected (Stangeland *et al.*, 2015). Thus, it seems plausible that BAP1 depletion affects the expression of genes that regulate KIF18A; alternatively, there might be an interaction between the two proteins which remains to be identified.

During cell division, the length of astral microtubule governs the probability that their plus-ends will be captured by the cortex and participate in spindle positioning (Su *et al.*, 2012). Regulation of astral microtubules is equally crucial to maintain the register between the mitotic spindle and the cleavage furrow during cytokinesis (Rankin & Wordeman, 2010). Depletion of KIF18B substantially increases astral microtubule length causing spindle mispositioning and rocking. KIF18B regulates microtubule plus-end dynamics by promoting catastrophe and its spatial regulation contributes to remodelling of the cytoskeleton during mitosis (McHugh *et al.*, 2018).

Our results show that BAP1 depletion results an increase in aster volume while ectopic expression of KIF18B reduces the aster volume without affecting the elongated

spindle length. Furthermore, BAP1 loss led to reduced protein expression of both KIF18A and KIF18B, explaining the functional consequence on microtubule dynamics.

Previous study demonstrates that loss of BAP1 leads to chromosome abnormalities by regulating mitotic spindle organization (Zarrizi *et al.*, 2014) . However, the detailed mechanisms through which BAP1 contributes to spindle organization were not clear. Herein we show that BAP1 regulates centrosome size, spindle microtubule length, aster volume and chromosome alignment independent of BRCA1. Future work will be required to better understand the mechanisms by which BAP1, specifically regulates KIF18A and KIF18B expression to control some of these events.

CHAPTER 6

DISCUSSION

6. Discussion

The regulation of protein stability is controlled in part through reversible post-translational ubiquitination (Devoy *et al.*, 2005). The ubiquitin pathway, which targets substrates to the proteasome, degrades thousands of short-lived proteins along with damaged and misfolded proteins. As a result, it contributes to many key biochemical pathways, including cell cycle progression, cell survival, apoptosis, cell signalling, DNA damage response, gene expression and metabolism (Hussain *et al.*, 2009; Rousseau & Bertolotti, 2018; Hoeller & Dikic, 2009; Senft *et al.*, 2018).

Dysfunction of the ubiquitin pathway could either enhance the effect of oncoproteins or reduce the level of tumour suppressor proteins as many of the key cancer relevant proteins are targets of ubiquitination and de-ubiquitination (Wilkinson, 2009; Darling *et al.*, 2017; Hershko & Ciechanover, 1998). Indeed, studies show that dysregulated E3 ligases themselves play a critical role in development, progression and response to therapy of cancers indicating that the E3 ligases can directly act as tumour suppressors or promoters (Qi & Ze'ev, 2015). DUBs also play fundamental roles through their ability to remove ubiquitin from targeted proteins. The activity of DUBs affects the turnover rate, activation, recycling and localization of multiple proteins and a range of signalling pathways, all of which are important processes that when altered can lead to malignant transformation (Fraile *et al.*, 2012; Pinto-Fernandez & Kessler, 2016) (Figure 6.1).

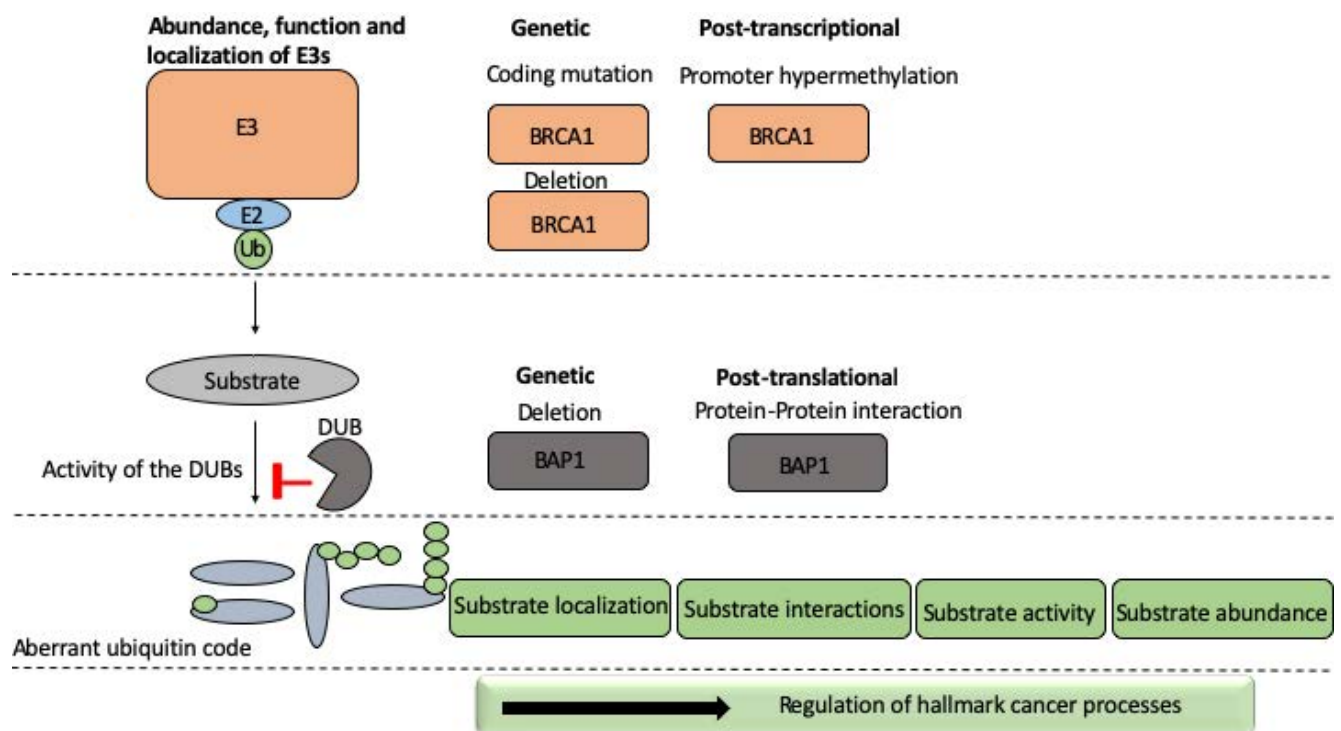


Figure 6.1 Mechanisms underlying deregulated ubiquitination in cancer.

Dysregulated ubiquitination in cancer can be attributed to genetic, post-transcriptional and translational mechanisms. Some E3 Ub ligases, such as BRCA1, confer susceptibility in breast and ovarian cancer when absent. The activity and abundance of DUBs are also regulated genetically and post-translationally, such as BAP1. In addition to deregulation of E3s and DUBs, the ubiquitin pathway is modulated by genetic alterations of the targeted substrates. Each individual alteration can have a profound effect on the regulation of cancer-associated pathways by modulating the localization, activity, signalling complex formation and abundance of major regulatory hubs. Adapted from Senft *et al.* (2018).

6.1 How does BRCA1 mediate resistance to vinorelbine?

Vinorelbine, a microtubule poison, has shown clinical efficacy in many cancers, including breast cancer, non-small lung cancer, colon cancer, oesophageal squamous cell carcinoma and mesothelioma (Joensuu *et al.*, 2006; Winton *et al.*, 2005; Conroy, 2002; Vecchione *et al.*, 2016; Stebbing *et al.*, 2009). However, the mechanisms underlying clinical vinorelbine resistance remains poorly understood. Therefore, a better understanding of the pathways responsible for drug resistance is important, to design improved therapeutic strategies.

BRCA1 has emerged as a predictive biomarker of chemotherapeutic drugs (Kennedy *et al.*, 2004). Indeed, several studies have reported that low BRCA1 expression or loss of function is associated with resistance to microtubule poisons including taxol and vinorelbine (Busacca *et al.*, 2012; Price & Monteiro, 2010; Sung & Giannakakou, 2014; Tassone *et al.*, 2005). BRCA1 is an E3 ligase with germline mutations in this gene conferring susceptibility to breast and ovarian cancer. Many mutations have been identified scattered throughout the gene, some which cause loss of protein expression through rapid turnover of the mRNA or protein (Miki *et al.*, 1994; Jhanwar-Uniyal, 2003). Meanwhile, mutations in the RING finger domain of BRCA1 diminishes its E3 ligase activity (Hashizume *et al.*, 2001; Morris *et al.*, 2006). Hypermethylation of the BRCA1 promoter is an alternate mechanism for reduced expression of BRCA1 in cancers (Esteller *et al.*, 2000; Catteau *et al.*, 1999).

To better understand the mechanism of BRCA1 mediated resistance in MPM, we investigated whether loss of BRCA1 affects MT depolymerization mediated by vinorelbine. We used vinorelbine resistant cell lines with low BRCA1 protein expression and a cell line carrying an inducible shBRCA1 plasmid. Vinorelbine treatment in these cell lines showed that BRCA1 status potentially modulates vinorelbine efficacy at least in part through reduced the stabilization of MTs as visualized by immunofluorescence microscopy. This is consistent with reports that BRCA1 modulates MT dynamics by increasing the MT assembly rates during mitosis (Lüddecke *et al.*, 2016). Another study showed that BRCA1 co-localized with MTs of the mitotic spindle and that BRCA1 co-precipitated with α -tubulin and β -tubulin (Lotti

et al., 2002). However, later work argued that BRCA1 did not co-sediment with MT polymers suggesting that BRCA1 does not bind MTs directly (Sung & Giannakakou, 2014). Nevertheless, BRCA1 associates with a number of microtubule-associated proteins, such as Nlp, TPX2, NuMA and XRHAMM (Sung & Giannakakou, 2014). Our results support the hypothesis that BRCA1 modulates MT dynamics as after loss of BRCA1, MTs become resistant to the depolymerization activity of vinorelbine, although β -tubulin levels were not altered after BRCA1 depletion. Furthermore, as BRCA1 regulates MAD2 and BUBR1 levels (Rui-Hong Wang *et al.*, 2004; Chabaliere *et al.*, 2006; Busacca *et al.*, 2012). Hence, we examined whether a defective SAC mediates resistance to vinorelbine. Flow cytometry and live cell imaging analysis revealed that BRCA1 deficient cells override the mitotic arrest after vinorelbine treatment. The recruitment of BUBR1 to the kinetochores was also diminished confirming that BRCA1 loss mediates resistance to vinorelbine by SAC inactivation.

The centrosomal localization and MT nucleation activity of γ -tubulin is dependent on BRCA1 activity as loss of BRCA1 enhances centrosomal accumulation of γ -tubulin and leads to increased MT nucleation (Hsu *et al.*, 2001; Sankaran *et al.*, 2005; Sankaran *et al.*, 2007; Sung & Giannakakou, 2014). Moreover, BRCA1 localizes to the centrosome and this play an important role in centrosome duplication as depletion of BRCA1 leads to premature centriole separation and reduplication (Hsu & White, 1998; Tarapore *et al.*, 2012; Sankaran *et al.*, 2006; Ko *et al.*, 2006). Indeed, our results show that depletion of BRCA1 in MPM cells led to both enlarged centrosome size and centrosome amplification.

6.2 How does BAP1 regulate BRCA1 protein expression?

In order to understand the mechanism for BRCA1 loss in MPM, we depleted BAP1 to analyse whether BAP1 regulates BRCA1 expression in MPM cells. As, BAP1 is a de-ubiquitinase that was initially identified as a BRCA1 interacting protein and mutated in 40-70% of all mesothelioma cases. A key finding of this work is the demonstration that BAP1 is required for BRCA1 protein stability in both normal mesothelial and different mesothelial cell lines. We also confirmed this is in a breast cancer cell line suggesting that BAP1 regulation of BRCA1 occurs in various cell types. We also confirmed that

BAP1 regulates BRCA1 protein through proteasomal mediated degradation by using a proteasome inhibitor MG132 that restored BRCA1 expression after BAP1 depletion. Previous study has shown that BAP1 inhibits the E3 ligase activity of BRCA1 in a manner independent of its DUB activity (Nishikawa *et al.*, 2009).

Indeed, our results confirmed that BAP1 stabilises BRCA1 independent of its enzymatic activity. To identify which domain of BAP1 is required for BRCA1 stabilisation, we used H226 stable cell lines with wild-type and various mutants of BAP1. Wild-type and all mutants including the mutant in the UCH domain restored BRCA1 expression except Δ ASXL mutant which is located in the BRCA1 binding region showed an intermediate BRCA1 expression. Several studies have shown that BAP1 depletion that reduces BRCA1 foci after irradiation (Liu *et al.*, 2018; Ismail *et al.*, 2014; Yu *et al.*, 2014). Therefore, we examined whether wild-type and mutant BAP1 can increase BRCA1 foci after DNA damage. Wild-type and all mutants of BAP1 in H226 stable cell lines increased nuclear BRCA1 foci except for parental and Δ ASXL mutant. These results suggest BAP1 regulates BRCA1 foci formation during DNA damage and might be crucial for efficient DNA repair. Interestingly, a binding partner of BAP1, transcriptional factor Yin Yang 1 (YY1), is a key regulator of BRCA1 expression (Lee *et al.*, 2012). YY1 binds BAP1 through a region of 559-729 whereas BRCA1 binds 596-721 region (Yu *et al.*, 2010; Jensen *et al.*, 1998). Although BAP1 depletion does not affect the stability of YY1, it seems plausible that BAP1 may form a ternary complex with BRCA1 and YY1 and thus regulate BRCA1 protein stability. Further studies will be required to test this hypothesis.

Our data also shows that BAP1 depletion leads to centrosome amplification and inactivation of the SAC proteins MAD2 and BUBR1 suggesting that BAP1 loss phenocopies BRCA1 function. Indeed BRCA1 plays key role in regulating centrosome duplication (Ko *et al.*, 2006; Starita *et al.*, 2004). BRCA1 also regulates SAC by controlling MAD2 and BUBR1 expression (Chabaliere *et al.*, 2006; Wang *et al.*, 2004). BAP1 loss also mediates resistance to vinorelbine similar to BRCA1 loss as reported previously (Busacca *et al.*, 2012).

We tested our hypothesis in clinical settings whether frequent loss of BAP1 in pleural mesothelioma patients correlates with loss of BRCA1 expression. We evaluated the

expression of BAP1 and BRCA1 in tumour samples taken from Medusa cohort. We identified a positive correlation between BAP1 and BRCA1 expression supporting our hypothesis. Our results suggest that BAP1 may be a potential biomarker for microtubule poison, vinorelbine. Previous studies have shown that depletion of BAP1 or BRCA1 increases sensitivity to DNA damaging agents and PARP inhibitors this suggests that mesothelioma patients with BAP1 loss may well benefit from PARP inhibitor therapies (Yu *et al.*, 2014; Ismail *et al.*, 2014; Xiyu *et al.*, 2018; Nishikawa *et al.*, 2009; Alli *et al.*, 2011; Kan & Zhang, 2015). The study of BAP1 regulating BRCA1 stability has far reaching implications as both proteins work together and independently in numerous pathways such as DNA repair, calcium signalling, transcription and cell cycle control.

6.3 Identification of BAP1 as a novel regulator of the mitotic spindle

Assembly of a bipolar mitotic spindle and efficient capture and congression of chromosomes is absolutely dependent on the dynamic properties of different microtubule population. Astral MTs emanate from the spindle pole and attach to the cell cortex, where they play a major role in spindle positioning. Interpolar MTs are key for the establishment of spindle bipolarity and spindle assembly, while the kMTs connect the kinetochores with the spindle poles and contribute to chromosome congression as sister chromatid separate during anaphase (Meunier & Vernos, 2012). Proper alignment of the mitotic spindle is essential to ensure genomic integrity is maintained and chromosome segregation errors that would lead to cancer are avoided (Dumont & Mitchison, 2009; Silkworth & Cimini, 2012).

Spindle length is one parameter that is dependent upon microtubule dynamics and the activity of microtubule-associated proteins (MAPS), including microtubule-based motors. The optimal length of the spindle during metaphase ensures that the poles are separated beyond the minimal spacing required for them to ensure complete sister chromatid separation and allow cytokinesis to effectively divide the cell faithfully into two genetically identical daughter cells. The metaphase spindle length is constant in a given cell type, which indicates that deviations from the characteristic length might cause defects that promote segregation defects.

Our results demonstrate that BAP1 is essential for proper spindle assembly and spindle integrity. Upon depletion of BAP1, centrosome volume dramatically reduced as measured by γ -tubulin and CDK5RAP2, while BRCA1 depletion led to accumulation of γ -tubulin and CDK5RAP2 at the centrosomes. It has been previously shown that BRCA1 ubiquitinates and BAP1 de-ubiquitinates γ -tubulin and localize at the centrosome during mitosis (Starita *et al.*, 2004; Zarrizi *et al.*, 2014; Hsu *et al.*, 1998). It is plausible that depletion of BAP1 increases ubiquitination of γ -tubulin at the centrosomes, whereas BRCA1 depletion decreases ubiquitination which leads to accumulation of centrosome proteins, such as γ -tubulin and CDK5RAP2.

We also observed an increase in spindle and aster length upon BAP1 depletion that was not observed upon BRCA1 depletion. Several studies have shown that KIF18A depletion leads to an increase in spindle size, while KIF18B depletion leads to an aster length. Thus, we examined whether BAP1 regulates the expression of KIF18A and KIF18B. Our results show that BAP1 depletion reduces the KIF18A intensity at the microtubule plus-ends leading to elongated spindles. We further confirmed that the increase in spindle length is due to perturbation of KIF18A by transfecting cells GFP-KIF18A which restored the spindle length to normal as seen in mock-depleted cells. Transfection of GFP-KIF18B in MPM cells reduced aster length as compared to BAP1 depleted cells but had no effect on spindle length. We show that BAP1 depletion reduces the protein expression of KIF18A and KIF18B. Our results show that BAP1 regulates mitotic spindle assembly in a manner independent of BRCA1.

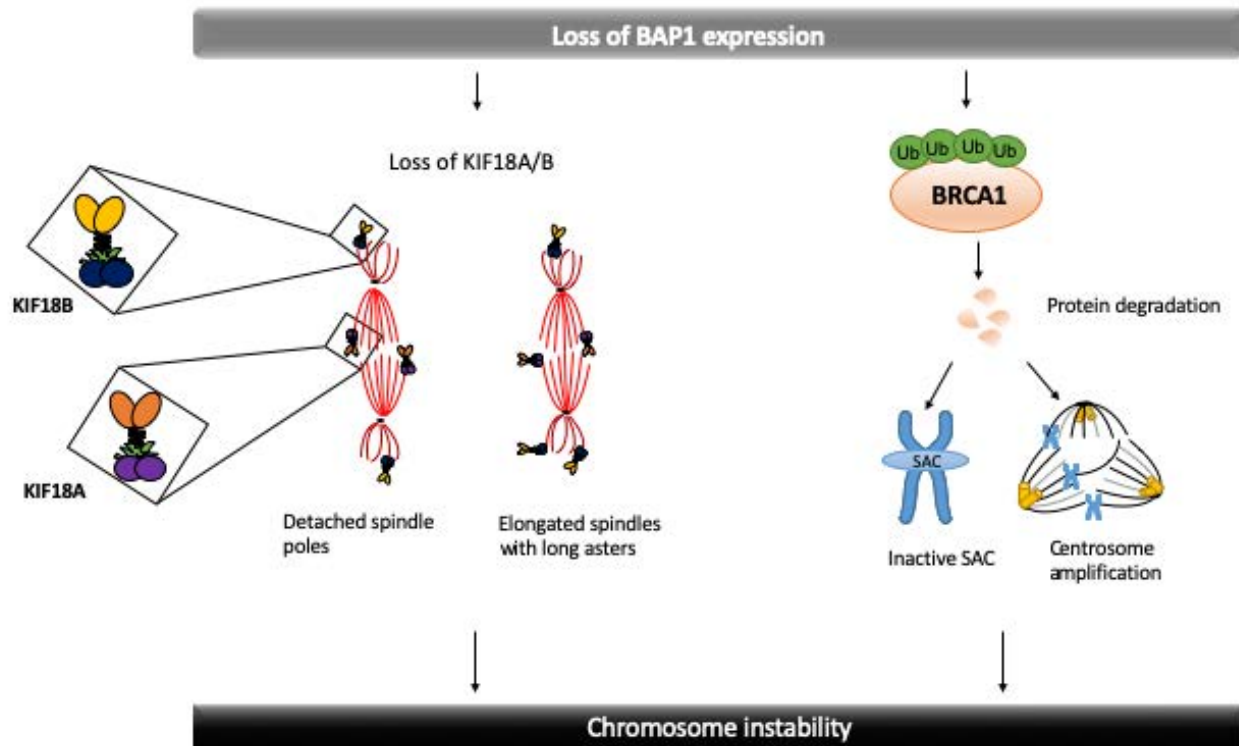


Figure 6.2. A model for roles of BAP1 in promoting genomic stability through both BRCA1-dependent and independent pathways

In this model, loss of BAP1 expression leads to destabilization of BRCA1 protein through proteasome mediated degradation, in a manner independent of BAP1 enzymatic activity. BAP1 loss then phenocopies BRCA1 loss and leads to centrosome amplification and inactive SAC causing chromosomal instability. BAP1 independently regulates the mitotic spindle assembly and dynamics. BAP1 loss leads to an increase in spindle and aster length by regulation of kinesin motor proteins, KIF18A and KIF18B. BAP1 depletion also leads to detached spindle poles, both mitotic phenotypes lead to chromosomal instability. BAP1 and BRCA1 co-operate to regulate genomic stability in MPM.

6.4 Future perspectives

This research has not only confirmed findings from previous studies but also provided new insights about the role of BAP1 in MPM. Although the primary hypothesis was addressed, several findings need further investigation.

We have identified BAP1 as a key regulator of BRCA1 protein stability in MPM. However, the mechanism still needs to be identified. We have shown that BAP1 depletion does not affect BRCA1 mRNA expression using RT-PCR; however, another study has shown that BAP1 depletion deregulates BRCA1 mRNA expression using microarrays. In future, a qPCR experiment would be appropriate to examine whether BAP1 transcriptionally regulates BRCA1. Our study also highlighted that Δ ASXL mutant of BAP1 did not fully restore BRCA1 protein expression or increase BRCA1 foci after DNA damage. There are two possibilities that can be investigated further: whether BAP1/ASXL act together to regulate BRCA1 protein expression or whether BAP1 exists in a ternary complex with YY1 and BRCA1 and is the complex required for BRCA1 protein stability as YY1 is a BAP1 binding partner and regulates BRCA1 expression (Yu *et al.*, 2010; Lee *et al.*, 2012).

Our results have shown that depletion of BAP1 leads to vinorelbine resistance in MPM cell lines. In future, this will need to be evaluated in multicentre randomised phase II trial. This could show that BAP1 can be used as a potential biomarker for vinorelbine in clinical settings to underpin chemotherapy stratification.

We also have shown that BAP1 regulates BRCA1 stability not only in malignant mesothelioma cell lines but also in normal mesothelial cells and in a breast cancer cell line. This could be tested in other cancers as BAP1 is mutated or deleted in several cancers including uveal melanoma, cutaneous melanoma and renal cell carcinoma (Murali *et al.*, 2013).

We show that BAP1 depletion reduces γ -tubulin and CDK5RAP2 recruitment at the centrosomes, although it was previously shown that γ -tubulin is de-ubiquitinated by BAP1 (Zarrizi *et al.*, 2015). The mechanism behind less recruitment of CDK5RAP2 at the centrosomes is not known. Future work should determine whether CDK5RAP2 is substrate of BAP1 or other centrosome proteins are involved in the recruitment of γ -tubulin and CDK5RAP2 to the centrosomes. We had also observed detached spindle poles upon BAP1 depletion however, how and when centrosomes get detached from the spindle poles. Live-cell imaging of the mitotic spindles can provide an insight at which stage of mitosis, centrosomes get detached from the spindle poles and whether it is a reversible process. Measuring microtubule dynamics using EB1 can show whether centrosome detachment is dependent on the forces generated by the microtubules.

BAP1 depletion led to an increase in spindle length by regulating KIF18A and KIF18B expression. However, the detailed molecular mechanisms by which BAP1 regulates their expression is unclear. Future studies should be focused on whether KIF18A and KIF18B are transcriptionally regulated or whether they are a substrate of BAP1 de-ubiquitination activity. Taken together, future research is needed to improve our understanding of the role of BAP1 in MPM carcinogenesis, as this may unravel new therapeutic targets.

6.5 Conclusions

Our results show that BAP1 is a key regulator of BRCA1 protein stability and this is independent of BAP1 de-ubiquitination activity. BAP1 loss phenocopies BRCA1 loss to regulate centrosome amplification and expression of the spindle assembly checkpoint proteins, MAD2 and BUBR1. BAP1 has a BRCA1-independent role in mitotic spindle assembly by controlling KIF18A and KIF18B expression. BAP1 and BRCA1 show a positive correlation in MPM patients suggesting that loss of BRCA1 protein expression as reported in previous studies is due to BAP1 loss of expression. BAP1 can potentially be used as a biomarker for vinorelbine in MPM.

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