MECHANISMS OF TRAIL RESISTANCE

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DECLARATION

I, Neelam Kumar, confirm that the work presented in this thesis is my own. Where information has been derived from other sources I confirm that this has been indicated and acknowledged in the thesis.

ABSTRACT

Malignant pleural mesothelioma (MPM) is a devastating disease for which limited effective therapies are currently available. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) and other death receptor (DR) agonists are pro-apoptotic agents that trigger the extrinsic apoptotic pathway selectively in cancer cells. Previous work has shown that supports that loss of function of the nuclear deubiquitinase BRCA associated protein-1 (BAP1) augments sensitivity to recombinant TRAIL (rTRAIL) in MPM cells. This study shows that BAP1 is a candidate biomarker for rTRAIL sensitivity in cell line, early passage culture and tumour explant models of MPM. In addition, BAP1 is a potential biomarker for sensitivity to other DR agonists and for additional cancer types with *BAP1* mutations. I have also identified other novel candidate biomarkers for DR agonist sensitivity, notably ASXL1/2, through exploration of the mechanism underlying the BAP1-rTRAIL association.

I present data supporting the clinical relevance and utility of the BAP1-TRAIL association. I have shown that loss of BAP1 function occurs in a significant proportion of MPM tumours in the UK and that loss of BAP1 function augments sensitivity to TRAIL in primary tumour tissue. I describe *in vitro* data supporting the hypothesis that loss of BAP1 function augments sensitivity to rTRAIL in MPM. I have shown that loss of BAP1 function augments sensitivity to other DR agonists and that BAP1 can act as a biomarker for DR agonist sensitivity in other cancers with *BAP1* mutations. Finally, I explore the mechanism underlying the BAP1-TRAIL association. I present data supporting the notion that BAP1 binds to the ASXL1/2 proteins to form the polycomb repressor deubiquitinase complex which underlies BAP1-induced TRAIL resistance. Loss of this function results in a change in the expression of proteins of the extrinsic apoptotic pathway, which may favour apoptosis upon DR agonist binding.

IMPACT STATEMENT

The findings presented in this thesis have significant potential clinical impact. I demonstrate the potential utility of loss of function of BAP1 and ASXL1/2 as biomarkers for sensitivity to DR agonists. Several DR agonists exist that have already completed phase I/II clinical testing in this context but so far have not demonstrated significant efficacy over standard therapy in several different malignancies when tested in unselected populations. Notably there have been no trials of DR agonists in MPM to date. Within completed trials however, a few patients were noted to exhibit partial or complete response, yet to date no means by which to identify such responders has been found. The data presented within this thesis supports the loss of function of BAP1, and potentially ASXL1/2, as candidate biomarkers for DR agonist sensitivity. BAP1 loss of function has been observed in 42-67% of MPM tumours, which would therefore be amenable to DR agonist therapy. *ASXL1* mutations are highly prevalent in myeloid malignancies (34-45%) and *ASXL2* mutations are present in 4% of pancreatic and 6% of prostate cancers. These malignancies would also be potentially amenable to targeted DR agonist therapy. Insights gained into the mechanism suggest that BAP1 loss of function alters expression of proteins of the extrinsic apoptotic pathway. This could be exploited to sensitise *BAP1* wild-type tumours to DR agonists either by inhibiting anti-apoptotic proteins or even by inhibiting BAP1 itself.

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TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I:

INTRODUCTION

1 INTRODUCTION

1.1 Malignant Pleural Mesothelioma

Malignant pleural mesothelioma (MPM) is a rare but highly aggressive tumour originating from the mesothelial cells of the pleura, the lining of the lungs. MPM is strongly associated with asbestos exposure and is characterised by a long latency period of 20-50 years between fibre exposure and disease presentation [1, 2]. The background incidence is relatively low; there were 2,717 new cases of MPM in the UK in 2014 (**Figure 1-1**) [3]. Owing to legislation banning the mining and use of asbestos, the incidence is expected to peak in the UK within the next 10-20 years. Globally however, industrialisation has led to an increase in asbestos use and the incidence is set to continue to increase for several decades. Prognosis is poor; the overall median survival of patients with MPM is 9-12 months, regardless of the stage of the disease [4]. The annual cost burden to the NHS is estimated to be approximately £16 million [5].

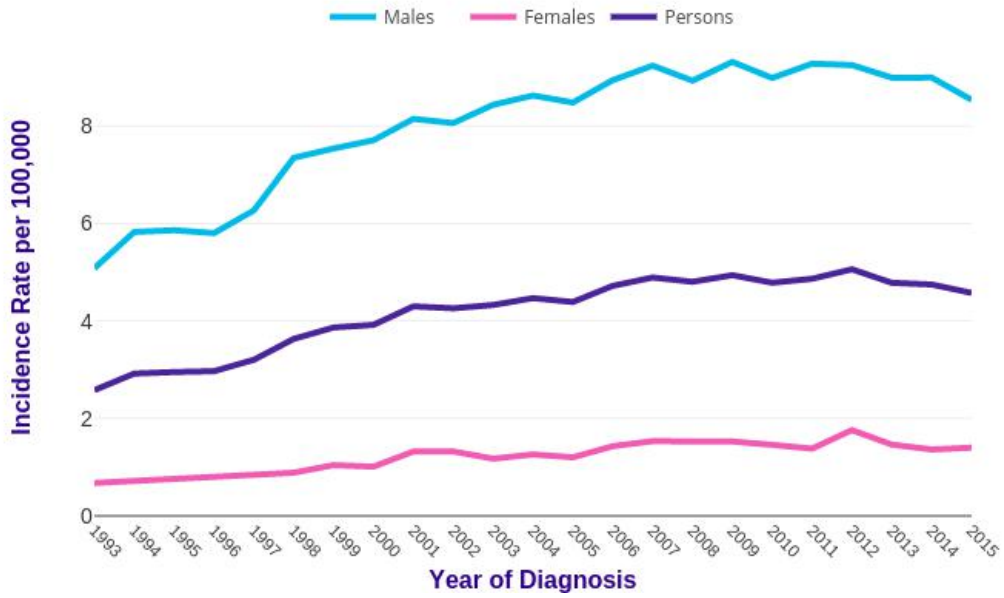


Figure 1-1: Mesothelioma incidence rates, UK, 1993-2015 [6]

There are three distinct subtypes of MPM: epithelioid (50-60% of MPM cases), sarcomatoid (10% of MPM cases) and biphasic (30-40% of MPM cases) [7]. Epithelial MPM can be further classified into subtypes including tubular, papillary, giant/large cell, small cell, myxoid and others that reflect morphological similarities to carcinomas of other origins [8]. Sarcomatoid MPM is characterised pathologically by spindle-shaped cells similar to those seen in fibrosarcomas and by poor prognosis compared with other types of MPM. Biphasic MPM has features of both the sarcomatoid and epithelial-types of MPM [8].

1.1.1 Pathogenesis of MPM

The vast majority of MPM (80%) is attributed to prior asbestos exposure [9]. There are two forms of asbestos (i) chyrosotile (white), consisting of curly fibres, and (ii) amphibole, consisting of needle-like fibres. Amphibole asbestos can be further classified as crocidolite (blue), amosite (brown), anthophyllite, actinolite and tremolite. The risk of developing MPM is related to the type of fibre, duration and burden of exposure; crocidolite is considered to be the most carcinogenic. As a result of its fire-resistant properties, industrialisation led to the mining and application of asbestos in constructions, heating arrangements, electrical works and plumbing. Those exposed as a result of working in such industries have been subjected to the occupational hazard of developing MPM. Para-occupational exposure can also occur, for example in family members exposed to fibres in workers clothes [1]. Additional known risk factors include simian virus 40 (SV40), chest wall radiation and erionite, an asbestos-like mineral found in Turkey.

Asbestos fibres are very long and thin allowing them to be inhaled and to migrate to the pleura where they cause local irritation and initiate a cycle of tissue damage and repair. The precise mechanism by which they cause MPM is unknown. Proposed mechanisms include the generation of reactive oxygen species which induce DNA damage, disruption of mitotic spindles, absorption of further hazardous molecules, macrophage accumulation and release of

cytokine and growth factors including tumour necrosis factor-α (TNF-α) [2]. TNF-α has been shown to activate nuclear factor-κB, which leads to mesothelial cell survival and inhibits asbestos-induced cytotoxicity [10]. The aberrantly activated signalling network may create a pool of mesothelial cells that harbour DNA damage and a tumour microenvironment that supports them.

1.1.2 Diagnosis and staging of MPM

The most common presenting symptoms of MPM are breathlessness and chest pain caused by a reactive pleural effusion, tumour encasement of the lung and/ or invasion into the chest wall. Fatigue, anorexia, weight loss, sweats and malaise are also often present as a result of circulating cytokines. Some patients however remain asymptomatic and the disease is identified incidentally.

Radiological imaging plays a key role in both diagnosis and staging; chest radiography, thoracic ultrasound and CT and PET-CT imaging are all routinely employed. MRI has been demonstrated to be superior to PET-CT for identifying locally invasive disease in cases where surgical resection is considered, though is not routinely used [11]. Definitive diagnosis however requires cytological or histological assessment of pleural fluid or tumour tissue. A number of diagnostic biomarkers including mesothelin, osteopontin and fibulin 3 have been evaluated but found to be of limited clinical use hence the urgent need to discover new candidate biomarkers [12, 13].

A number of staging classifications for MPM exist all of which have limitations for use in routine clinical practice largely due to limitations in current imaging techniques [14]. The consensus is a tumour, node, metastasis based system, using surgical or radiological information be employed. That proposed by the International Mesothelioma Interest Group is widely used (**Table 1-1**) [15].

Table 1-1. The International Mesothelioma Interest Group Staging System for malignant pleural mesothelioma [15]

T	
T1	Tumour of the ipsilateral parietal pleura, including diaphragm and mediastinal pleura
T1a	No visceral pleural involvement
T1b	With visceral pleural involvement
T2	Tumour affecting parietal, visceral, diaphragmatic and mediastinal pleura, with either involvement of diaphragmatic muscles or pulmonary parenchyma
T3	Involvement of the endothoracic fascia, extension into the mediastinal fat, non-transmural involvement of the pericardium or resectable focus of chest wall invasion
T4	Unresectable disease, diffuse chest wall or mediastinal involvement, direct transdiaphragmatic spread into the peritoneum, contralateral plural involvement, invasion of the spine, ribs or brachial plexus, trans-mural pericardial invasion or malignant pericardial effusion
N	
N0	No regional lymph node metastases
N1	Metastases in ipsilateral bronchopulmonary or hilar lymph nodes
N2	Metastases in subcarinal or ipsilateral mediastinal lymph nodes, including ipsilateral internal mammary chain
N3	Contralateral lymph node metastases, ipsilateral or contralateral supraclavicular lymph node involvement, and scalene nodes
M	
M0	No extrathoracic metastases
M1	Extrathoracic metastases present

1.1.3 Management of MPM

Surgery

Surgical management of MPM is controversial with a lack of robust randomised trial data. It is used only in early-stage disease and in patients with good functional status. Surgical procedures employed include radical and

debulking / tissue-sparing options. Extrapleural pneumonectomy (EPP) involves the removal of lung, pleura, pericardium and diaphragm with the aim of removing all disease [16]. A systematic review of early clinical studies concluded that it was impossible to determine whether EPP extended survival in MPM [17]. A feasibility study, the Mesothelioma and Radical Surgery trial (MARS) revealed potential harm associated with EPP with an adjusted hazard ratio for death of 2.75 (95% CI 1.21–6.26; p=0.016) [18]. EPP has since been largely abandoned in favour of less radical procedures such as pleurectomy/decortication (removal of the visceral and parietal pleura) or partial pleurectomy *via* video associated thoracic surgery (VATS). Such procedures do not aim for complete resection and they are usually employed alongside additional treatment modalities. A randomised trial is currently underway that aims to address if pleurectomy / decortication extends survival above non-surgical treatment [19]. Observational clinical studies have suggested that VATS partial pleurectomy controls symptoms in MPM however the only suitably powered randomised trial demonstrated no survival difference compared with talc pleurodesis *via* a chest drain [20].

Radiotherapy

Radiotherapy is predominantly employed as a palliative measure in MPM and has no impact on survival. It has been used as an adjuvant to surgery and chemotherapy in the context of trimodality treatment however evidence for this approach is lacking and it is not considered standard care [21].

Chemotherapy

Chemotherapy using third-generation anti-folate agents is the only treatment modality that has been shown to improve survival in MPM. A trial of 488 patients randomised to receive either pemetrexed and cisplatin or cisplatin alone demonstrated a median survival in the pemetrexed arm of 12.1 months, compared with 9.3 months with cisplatin alone (p=0.02) [22]. Toxicity rates were high initially, but fell after the addition of vitamin B12 and folic acid supplementation. A second trial comparing raltitrexed and cisplatin with

cisplatin alone in 250 patients demonstrated a median survival in the ratitrexed arm of 11.4 months compared with 8.8 months with cisplatin alone (p=0.048) [23]. The study appeared underpowered and consequently has had less impact on clinical care. Standard first line chemotherapy is therefore combination pemetrexed and cisplatin as the evidence base is stronger than that for ralitrexed and cisplatin, which is rarely used. Carboplatin can be substituted in older patients and patients with comorbidities, and has demonstrated similar efficacy to cisplatin [24]. Response rates to these regimes remain low however; an evaluation of over 1700 patients who received pemetrexed with either cisplatin or carboplatin demonstrated response rates of 26.3% and 21.7%, respectively [25].

Targeted Therapies

Bevacizumab, an anti-VEGF monoclonal antibody, has been shown to be effective in MPM. VEGF plays a key role in MPM by promoting angiogenesis and stimulating tumour growth. VEGF-targeted inhibitory therapies, such as bevacizumab, exert their effects through a number of potential mechanisms, including inhibition of new vessel growth, regression of newly formed tumor vasculature and alteration of tumour blood flow. The MAPS trial randomised 448 participants with MPM to receive cisplatin and pemetrexed chemotherapy with or without bevacizumab. Patients who received bevacizumab had significantly longer median (95% CI) overall survival at 18.8 (15.9–22.6) months compared with 16.1 (14.0–17.9) months in the chemotherapy alone arm (p=0.017). Patients given bevacizumab alongside chemotherapy also showed longer progression free survival of 9.2 (8.5–10.5) months *versus* 7.3 (6.7–8.0) months in those receiving standard care (p<0.0001) [26]. This combination is now included in the National Comprehensive Cancer Network guidelines as a possible first-line treatment in appropriately selected patients.

Immunotherapy

There have been promising results with immunotherapy in MPM. Checkpoint inhibitors such as tremelimumab and pembrolizumab have shown significant

disease control rates and prolonged disease stability; clinical trials assessing this further are underway [27].

1.1.4 Genomic alterations in MPM

A number of recent studies have shed significant light on the mutational landscape of MPM. Bueno *et al* sequenced transcriptomes and exomes from 216 MPM tumours and identified recurrent mutations with predicted functional impact in ten genes [28]. These are *BAP1*, *NF2*, *TP53*, *SETD2*, *DDX3X*, *ULK2*, *RYR2*, *CFAP45*, *SETDB1* and *DDX51*. The authors also found regions of recurrent copy loss that included *BAP1*, *NF2*, *CDKN2A*, *LATS2*, *LATS1* and *TP53* consistent with previous reports [29]. Copy number loss correlated with loss of expression in these genes. Previous studies involving smaller samples have also identified recurrent inactivation of *BAP1, NF2, CDKN2A* and *TP53* as a result of somatic mutations and/ or copy number alterations in MPM [30-33].

1.2 BRCA associated protein-1

BRCA associated protein-1 (BAP1) has been identified as a key tumour driver in the pathogenesis of MPM. Somatic loss-of-function (LOF) mutations were initially identified by Sanger sequencing in 20-25% of MPM tumours [34]. However integrated molecular approaches and immunohistochemical analysis suggest BAP1 loss of function is seen in as many as 42-67% of MPM tumours [35-37]. BAP1 is a nuclear deubiquitinase and transcriptional regulator with key roles in several important cellular processes (**Figure 1-2**).

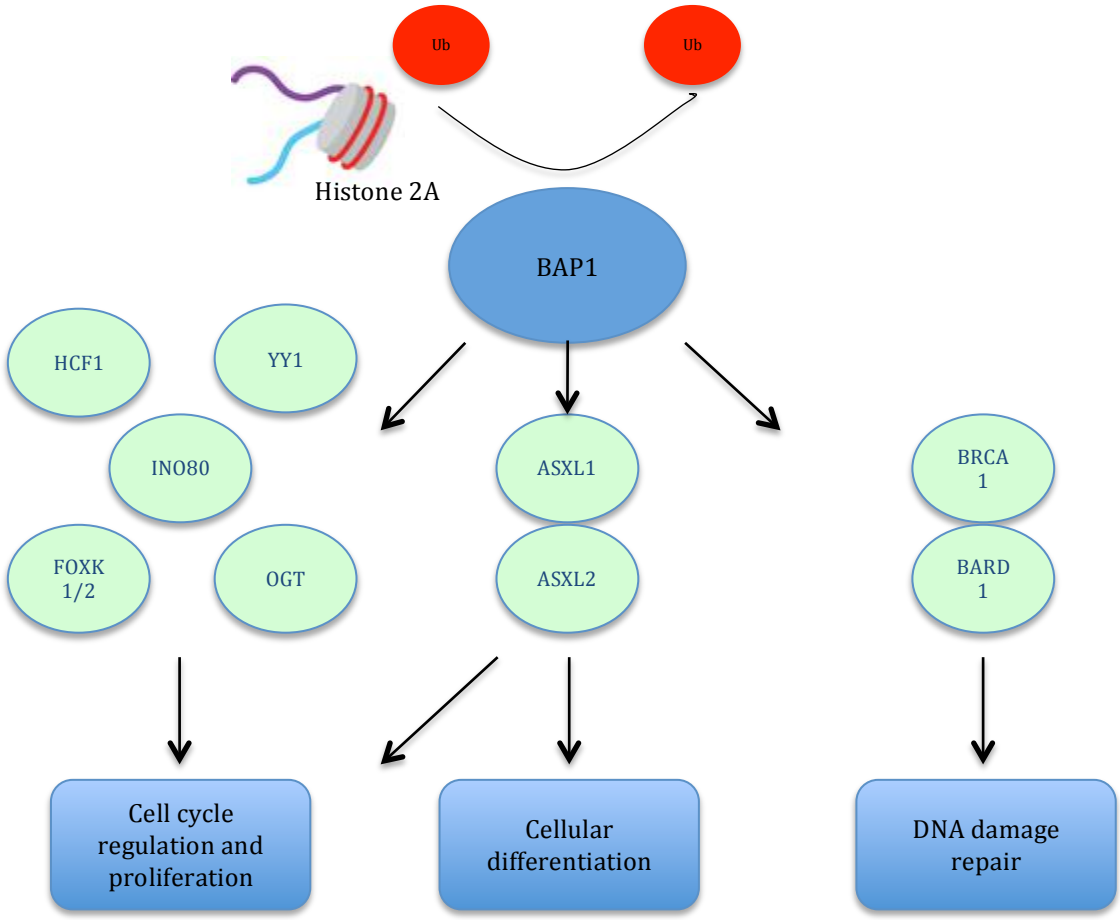


Figure 1-2 BRCA associated protein 1 – main binding partners and associated cellular functions [38]

BRCA associated protein 1 (BAP1) carries out its main functions in multi-protein complexes with several different protein-binding partners within which it acts to deubiquitinate protein substrates, the major substrate being histone 2A. Complexes with host cell factor 1 (HCF1), yin yang 1 (YY1), INO80 chromatin remodelling compex (INO80), forkhead transcription factors ½ (FoxK1/2) or O-linked N-acetylglucosamine transferase (OGT) mediate functions related to cell cycle regulation and proliferation. Complexes with additional sex combs like proteins 1/2 (ASXL1/2) mediate functions related to cellular differentiation. Complexes with BRCA1 and BRCA1-associated RING domain protein 1 (BARD1) mediate functions related to DNA damage repair. Adapted from Wang et al [38].

1.2.1 Deubiquitinases

Ubiquitin (Ub) is a 76 amino acid polypeptide covalently bound to other proteins by sequential action of Ub activation (E1), Ub conjugating (E2) and Ub ligase (E3) enzymes, forming an isopeptide bond between the C terminus of Ub and the lysine residues of the substrates [39]. It is a reversible process; deubiquitinases (DUBs) catalytically cleave monoubiquitin or polyubiquitin chains from proteins. Ubiquitination can mark a protein for degradation by the proteasome, alter cellular location, change its activity and modulate its protein interactions [39]. Ubiquitinating and deubiquitinating enzymes can therefore modulate several biological processes including cell cycle progression, signal transduction, plasma membrane transport, transcriptional regulation, immune response, apoptosis and oncogenesis [39].

At least 98 DUBs have been identified and classified into six families [40]. These are (1) ubiquitin-specific proteases (USPs), (2) ubiquitin carboxy-terminal hydrolases (UCHs) (3) ovarian-tumour proteases (OTUs) (4) Machado–Joseph disease protein domain proteases, (5) JAMM/MPN domain-associated metallopeptidases (JAMMs) (6) monocyte chemotactic protein-induced proteins (MCPIP). Apart from JAMMs, all DUBs are cysteine peptidases and the presence of cysteine at the active site is required for their activity. BAP1 is the largest member of the UCH family of DUBs that in humans also includes UCHL1, UCHL3, UCHL5/UCH37. UCH enzymes can only target small peptides from the C-terminus of ubiquitin as they have a confined loop that precludes the processing of polyubiquitin chains and large folded proteins [40].

1.2.2 BAP1 structure

BAP1 is a 90kDa protein consisting of 729 amino acids. It contains a number of identified key domains that mediate its functions. At the N- terminus it contains the ubiquitin carboxyl hydrolase (UCH) domain, a conserved catalytic domain of 230 amino acids that confers its deubiquitinase activity [41]. At the C- terminus it contains the C-terminal domain (CTD) including a nuclear

localisation signal (NLS) [41]. The ubiquitin-conjugating enzyme UBE20 induces BAP1 sequestration in the cytoplasm by multi-monoubiquitination of the NLS and BAP1 counteracts this by auto-deubiquitination of the same site thus regulating its own nuclear translocation [42]. The CTD is also proposed to interact electrostatically with the nucleosome core [43]. Between these termini a number of additional domains with key amino acids that mediate protein-protein interactions have been identified. These include a four amino acid (NHNY) host cell factor -1 (HCF-1) binding motif (HBM), [44], and a UCH-37 like domain (ULD) that contains interaction sites with a number of proteins including ASXL1/2 (R666-669) [45], YY-1 and BRCA-1 (lysine 691) [46]. Phosphorylation at threonine 493 enables interaction with FoxK1/2 [47] and at serine 592 facilitates its function in DNA repair [48]. BAP1 lacks a DNA binding domain and interacts with the genome through the assembly of multi-protein complexes. A schematic structure highlighting these key domains and amino acids is in **Figure 1-3**.

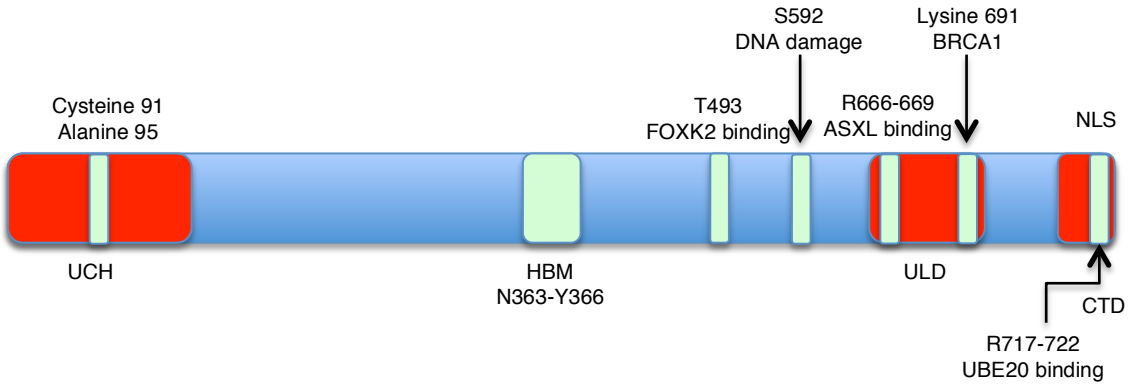


Figure 1-3 Functional architecture of BAP1

UCH – Ubiquitin hydroxyl carbolase domain, contains catalytic site for DUB activity, key amino acids cysteine 91 and alanine 95. HBM – HCF-1 binding motif. Threonine 493 (T493) is required for FOXK2 interaction. Serine 592 (S592) is phosphorylated during the DNA damage response. ULD – UCH-37 like domain, contains ASXL, BRCA1 and YY1 interaction sites. CTD – C terminal domain, contains nuclear localisation signal (NLS) and proposed to interact hydrostatically with nucleosome core. UBE20 monoubiquitinates the NLS.

1.2.3 BAP1 function

BAP1 has emerged as a master genetic regulator forming multi-protein regulatory complexes to modulate the transcription of thousands of genes and influence several biological processes, including development, DNA repair, chromatin remodelling and oncogenesis.

A significant interaction is with host cell factor 1 (HCF-1) with which BAP1 interacts through its HCF binding motif (HBM) and removes K48 ubiquitin linkages at lysine residues [49]. HCF-1 modulates chromatin architecture by recruiting histone-modifying complexes and activating transcription factors including the E2F family, which controls G1/S phase progression in the cell cycle. Loss of BAP1 function results in an increase in K48 linkages and accumulation of HCF-1, which the promotes transition from G1 to the S phase of the cell cycle[49].

BAP1 also interacts with yin yang 1 (YY1), a zinc finger protein capable of both transcriptional activation and repression, in an HCF1-dependent manner [44]. The ternary BAP1-HCF1-YY1 complex is recruited to the promoter of a number of genes including *COX7C* that encodes a component of the mitochondrial respiratory chain [44]. YY1 has been shown to act as an activator or suppressor of *COX7C*, depending on whether it is bound to the HCF1–BAP1 complex.

O-linked N-acetylglucosamine transferase (OGT) is a glycosyltransferase that catalyses the addition of a single N-acetylglucosamine by O-glycosidic linkage to serine or threonine residues. BAP1 deubiquitinates OGT which in turn modifies and activates HCF-1 [50]. OGT is more rapidly degraded and levels decreased in *BAP1*-knockout splenocytes, as are HCF-1 levels. HCF-1 levels however are unaltered when BAP1 is introduced in BAP1 deficient renal cancer cells [51]. As OGT is a positive regulator of HCF-1 it has been suggested BAP1 activity may indirectly modify HCF-1 levels through OGT. Indeed chromatin immunoprecipitation (ChIP) sequencing demonstrates the

majority of the ~6000 candidate binding sites for BAP1 are also associated with OGT and HCF-1 [51]. The BAP1-HCF-1-OGT complex also influences metabolism. HCF1 recruits OGT to O-glcNAcylate PGC-1α, a key regulator of gluconeogenesis. O-GlcNAcylation of PGC-1α enables BAP1 to deubiquitinate PGC-1α and protect it from degradation, thereby promoting gluconeogenesis [42].

BAP1 is required for the interaction between HCF-1 and the forkhead transcription factors FoxK1/K2. FoxK1/K2 directly bind to DNA via a forkhead winged helix-turn-helix DNA-binding domain. Phosphorylation of BAP1 at threonine 493 enables its interaction with FoxK2, which recruits BAP1 to chromatin, which in turn recruits HCF1, forming a ternary complex [52]. Depletion of BAP1 results in the upregulation of FoxK2 target genes such as *MCM3*, *CDC14* and *CDKN1B* [47]. Mutation of threonine 493 to alanine (T493A) on BAP1 abolishes this FoxK2 target gene regulation [47].

Evidence suggests BAP1 also has a role in DNA damage signalling and repair. It interacts with several homologous recombination (HR) proteins including the BRCA1/BARD1 complex that harbours E3 ligase activity and regulates the DNA damage response [53]. BAP1 binds and deubiquitinates BARD1, causing dissociation of the complex thus modulating the E3 ligase activity and response to DNA damage [53]. BAP1 is phosphorylated on serine 592 following UV irradiation as a result of which bound BAP1 dissociates from chromatin [53]. A model has been suggested whereby stress induced phosphorylation functions to displace BAP1 from specific promoters thus regulating the transcription of a subset of genes involved in the response to DNA damage. In response to DNA damage BAP1 is phosphorylated by the DNA repair protein ATM [54]. This recruits BAP1 to DNA damage sites together with ASXL1 to form the polycomb repressor deubiquitinase complex (PR-DUB; see below) which deubiquitinates H2AK119Ub in a PARP dependent manner [54]. Loss of BAP1 results in impaired homologous repair and increased sensitivity to radiation and PARP inhibitors.

BAP1 also interacts with INO80, a chromatin-remodelling complex that can alter chromatin structure by nucleosome sliding, histone eviction and histone exchange. INO80 is involved in various chromosomal processes, including DNA repair, telomere regulation, centromere stabilization and the transcription of genes. BAP1 stabilizes INO80 by deubiquitination and this contributes to DNA replication [55].

BAP1 may also play a role in inhibiting apoptosis caused by metabolic stress [56]. The unfolded protein response (UPR) protects cells from stress caused by misfolded proteins in the endoplasmic reticulum; if the stress is unresolved, this leads to induction of apoptosis by depleting ATP and generating reactive oxygen species (ROS). Under metabolic stress, BAP1 promotes the expression of genes essential for UPR by directly binding to promoters of the genes involved [56]. Studies performed with *BAP1*-null lung and renal cancer cell lines showed increased apoptotic induction following glucose deprivation.

1.3 The polycomb group proteins

A key interaction of BAP1 is with the ASXL 1/2 proteins to form the polycomb repressor deubiquitinase complex (PR-DUB). This regulatory complex interacts with other polycomb group (PcG) proteins to modulate diverse cellular functions including development, stem cell function, tissue homeostasis and oncogenesis

1.3.1 The polycomb repressor complexes

PcG proteins assemble multi-subunit complexes termed polycomb repressive complexes (PRCs), which regulate chromatin organisation and maintain it in a transcriptionally inactive state [57]. There are two main polycomb repressor complexes (PRC), PRC1 and PRC2. Both induce covalent post-translational histone modifications associated with transcriptional silencing [58].

PRC2

PRC2 has four core components: enhancer of zeste 2 (EZH2), or its homolog EZH1, suppressor of zest 12 (SUZ12), embryonic ectoderm development (EED) and retinoblastoma-associated protein 48 (RbAp48). EZH2/1 houses a catalytic subunit, the SET domain, which catalyses trimethylation of histone H3 at lysine 27 (H3K27Me3), a mark that strongly correlates with PcG silencing (**Figure 1-3**) [59]. SUZ12 regulates the histone methyltransferase activity while EED modulates the substrate specificity of EZH2 toward histone H3K27 or histone H1K26. Evidence supports a role for H3K27Me3 as a docking site for PRC1 and PRC2 may therefore act to recruit PRC1 to chromatin [60, 61]. However, many genomic sites accumulate PRC2 but not PRC1 [62] and few accumulate PRC1 without PRC2 [63]. Therefore while H3K27Me3 may contribute to PRC1 targeting it may be insufficient alone and the mechanism of targeting could vary at different sites. PRC2 also has a role in recruitment of the H3K4 demethylase retinol binding protein 2 (RBP2) a further epigenetic marker associated with the modulation of gene transcription [64].

PRC1

PRC1 comprises chromobox (CBX) proteins together with one member of the really interesting new gene family (RING1a or RING1b), one member of the polycomb group ring finger family (PCGF1-6) and one of the HPH family (HPH1-3) [58]. RING1a/b harbour E3 ligase activity and catalyse the monoubiquitination of histone H2 at lysine 119 (H2AK119Ub) (**Figure 1-3**). A diverse number of PRC1 complexes exist, however their distinct functional and physiological roles are not clear. Knockout studies suggest H2AK119Ub plays a key role in transcriptional repression however the mechanism of PRC1-mediated gene silencing is still debated. Studies suggest H2AK119Ub at bivalent promoters restrains RNA polymerase II activity [65] preventing the exposure of H2A/B dimers from nucleosomes necessary for transcriptional elongation [66].

1.3.2 The polycomb repressive deubiquitinase complex

The polycomb repressive deubiquitinase complex (PR-DUB) was initially identified in *Drosophila* as a complex of two proteins, Calypso and ASX, bound to PcG target genes, co-localising to a large extent with PRC1 [67]. The human homologues of Calypso and Asx are BAP1 and ASXL1/2. Both *Drosophila* and human PR-DUB deubiquitinate H2AK119Ub (**Figure 1-4**), and *Drosophila* mutants lacking PR-DUB show a strong increase in levels of H2AK119Ub and impaired HOX gene repression implicating a role for PR-DUB in PcG transcriptional regulation [67]. However, simultaneous depletion of both the catalytic subunit of PRC1 and the PR-DUB results in more rapid loss of HOX gene repression than depletion of either alone, therefore it does not appear that PR-DUB simply opposes PRC1 function [67]. It may be that the opposing complexes act locally in different sites or that ubiquitination/ deubiquitination must occur in a temporally regulated manner for HOX gene repression/ regulation.

Both BAP1 and ASXL1/2 are needed for deubiquitination of H2AK119Ub and mutations that disrupt this interaction lead to an increase in H2AK119Ub

levels in cancer cells [45]. Key sites required on both proteins have been identified. ASXL1/2 contains a N- terminus ASXH domain that has been shown to increase the affinity of BAP1 for ubiquitin and potentiate its deubiquitinase activity. The ASXH domain is hypothesised to interact with the UCH37-like domain (ULD) on BAP1 by anchoring it to the UCH domain [43]. As the ULD is close to the ubiquitin-binding site this conformational change optimises BAP1 ubiquitin binding. The ASXH domain corresponds to amino acids 253-391 and 253-411 in ASXL1 and ASXL2 respectively. Disruption of the amino acids R666-H669 within the ULD of BAP1 has been shown to abolish its ability to interact with ASXL1/2 and to deubiquitinate H2AK119Ub [45]. The BAP1 C- terminal domain (CTD) also appears to be important for the deubiquitination of H2AK119Ub by facilitating binding to the H2A nucleosome [43]. While the CTD houses the nuclear localisation signal and thus *in vivo* is needed for BAP1 to reach the nucleosome, *in vitro* studies demonstrate that PR-DUB with truncated BAP1 without the CTD exhibits low deubiquitinating activity towards H2AK119Ub [43]. The CTD is highly cationic and it has been suggested that these conserved positive charges interact electrostatically with anionic regions of the nucleosome core to tether it to BAP1. Indeed co-incubation of the PR-DUB with increasing concentrations of the CTD, or its scrambled peptide sequence, displace the PR-DUB from the nucleosome and inhibit its DUB activity towards H2AK119Ub [43].

ASXL1 and ASXL2 compete for interaction with BAP1 *in vitro* and form two distinct complexes both capable of deubiquitinating H2AK119Ub. BAP1 and ASXL1/2 expression also appears to be co-regulated. In vitro BAP1 levels increase with ASXL1/2 expression in a dose dependent manner and ASXL1/2 expression increases following BAP1 overexpression [45]. shRNA knockdown of ASXL1 or ASXL2 in human fibroblasts results in a significant reduction in BAP1 expression while knockdown of *BAP1* results in a significant reduction in ASXL2 expression [45]. ASXL2 is also downregulated in *BAP1*-deficient H28 and H226 MPM cells, and re-expression of BAP1 or the deubiquitinase

mutant BAP1C91S restores ASXL2 protein levels in these cells, without affecting its mRNA levels [45].

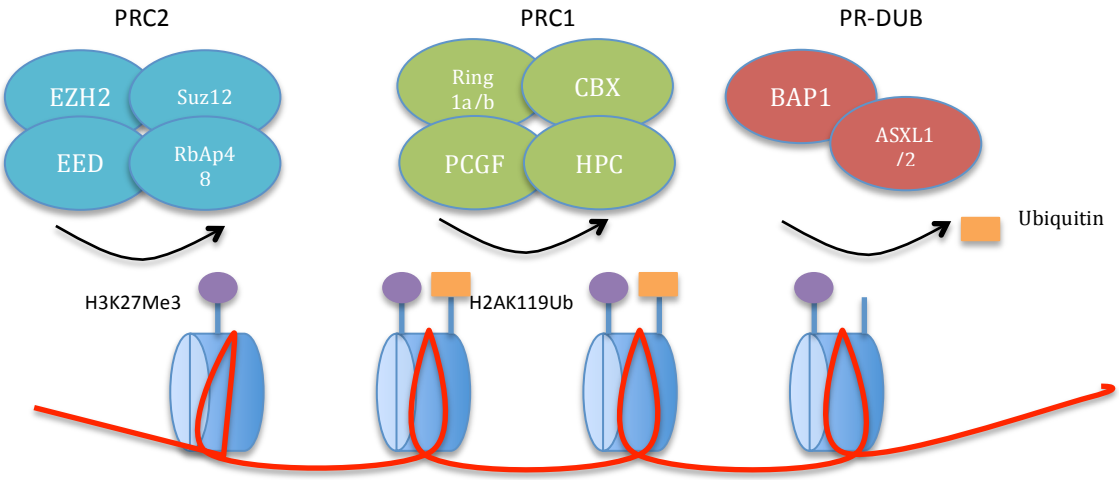


Figure 1-4 A simplified schematic of Polycomb group complex interactions

Polycomb repressive complex 2 (PRC2) consists of four subunits: enhancer of zeste homolog 2 (EZH2 – histone methyl transferase activity), suppressor of zeste 12 (Suz12 - zinc finger), embryonic ectoderm development (EED), and RbAp48. PRC2 catalyses trimethylation of histone 3 (H3) at lysine 27 (K27) facilitating binding of polycomb repressive complex 1 (PRC1). PRC1 consists of 4 core subunits: really interesting new gene 1a/b (Ring1a/b – E3 ligase), a chromobox family protein (CBX1-6), Polycomb group RING finger protein (PCGF) and a human polycomb protein (HPC1/2). PRC1 catalyses monoubiquitination of histone 2A (H2A) at lysine 119 (K119). The PR-DUB deubiquitinates H2AK119Ub.

1.3.3 The ASXL Proteins

The *additional sex combs-like* (*ASXL*) gene family consists of three members *ASXL1*, *ASXL2* and *ASXL3*.

ASXL Structure

ASXL proteins share a common architecture consisting of an ASXN domain in the N-terminal region, an ASXH domain in the N- terminal adjoining region, ASXM1 and ASXM2 domains in the middle region and a PHD domain in the C- terminal region (**Figure 1-5**). The PHD domain (C4HC3 originally

discovered in the plant homeodomain) is a histone- or DNA-binding module of chromatin regulators and transcription factors. The ASXN domain is structurally similar to the forkhead box (Fox) domain of the Fox family members responsible for DNA binding. The ASXH domain binds to BAP1 and KDM1A, ASXM1 to NCOA1 and ASXM2 to nuclear hormone receptors (NHRs) [68].

Figure 1- 5 Functional domains of the ASXL proteins

Protein structure of the ASXL proteins with the common functional domains and sites highlighted. The three ASXL proteins differ in size and site of domains. ASXN: DNA binding module; ASXH: protein-protein binding domain, binds to BAP1 and KDM1A; ASXM1 and ASXM2: nuclear hormone receptor binding; PHD: DNA binding module, likely binds to methylated histones.

ASXL1

Evidence supports a role for ASXL1 in transcriptional activation and repression *via* interaction with BAP1 and the polycomb group proteins. Constitutive adult *ASXL1* knockout mice show abnormalities of the axial skeleton with posterior and anterior transformations corresponding to the

drosophila polycomb group mutant (PcG) and trithorax group mutant (TrxG) phenotypes, respectively [69, 70]. ASXL1 is co-immunoprecipitated with components of PRC2, including EZH2 and SUZ12, and re-expression of ASXL1 promotes H3K27me2/3 to partially inhibit the aberrant expression of HOXA-cluster genes in ASXL1-null leukaemic cells [70]. ASXL1 loss is associated with loss of H3K27Me3 with minimal effect on H2AK119Ub [71]. It is unclear how ASXL1 interacts with PRC2 including whether it binds directly or indirectly or if it recruits PRC2 to specific genomic loci to deposit H3K27Me3. Interestingly there is evidence to suggest that aberrant truncated ASXL1 may complex with BAP1 to form a 'hyperactive' PR-DUB resulting in depletion of H2AK119Ub and subsequent failure of PRC2 recruitment [72].

ASXL2

Adult constitutive *ASXL2* knockout mice also exhibit posterior transformation (PcG phenotype) and anterior transformation (TrxG phenotype) axial skeletal abnormalities. Dilated cardiomyopathy is also observed and attributed to epigenetic aberrations associated with a decreased level of H3K27me3 in cardiac myocytes [73].

ASXL3

Less is known about ASXL3 than ASXL1/2. It is expressed in similar tissues to ASXL1 but at significantly lower levels. While all three ASXL proteins have been found to interact with BAP1 *in vitro,* ASXL3 has not been identified as a BAP1 binding partner in proteomic studies [74].

1.4 BAP1 and ASXL1/2 in cancer

Somatic mutations in *BAP1* and the *ASXL* genes are common in a number of cancers. Germline mutations in *BAP1* result in a tumour predisposition syndrome and those in *ASXL1/3* in developmental abnormalities. There is however little overlap between the phenotypes observed in *BAP1* and *ASXL* mutations suggesting some divergence of function between BAP1 and the ASXL proteins.

1.4.1 BAP1 in cancer

Somatic mutations in *BAP1* have been identified through Sanger sequencing of whole exomes at a high prevalence in a number of tumours including MPM (23-36%) [30, 33], metastatic uveal melanoma (UM) (84%) [75], intrahepatic cholangiocarcinoma (25%) [76] and clear cell renal carcinoma (RCC) (8-14%) [77, 78]. Recurrent chromosomal loss of 3p21.1, insertions, deletions, frameshift, nonsense and missense mutations, have all been reported resulting in loss of BAP1 expression or expression of mutant BAP1 with inactive DUB activity or lacking the NLS [79]. Immunohistochemistry has proven to be a rapid way to screen for *BAP1* alterations, which correlate with loss of nuclear staining with positive and negative predicative values of 100% and 98.6%, respectively [80] and a sensitivity and specificity of 88% and 97% [81]. A small number of missense mutations that inactivate the protein without epitope alteration may not be detected.

Germline mutations of *BAP1* also predispose affected individuals to a tumour predisposition syndrome inherited in an autosomal dominant pattern [82, 83]. Affected individuals have a high risk of developing tumours, particularly MPM, atypical Spitz tumours (ASTs), UM and cutaneous melanoma (CM) and clear cell RCC [83]. The precise mechanism by which *BAP1* mutations predispose to tumour development is an area of active investigation. Evidence points to a role for BAP1 as a tumour suppressor dependent upon its DUB activity and nuclear localisation, functions typically lost by those mutations found in

cancer. *BAP1* null H226 MPM cells transduced with wild-type *BAP1* grow poorly in culture and when injected into athymic nude mice form 10-15 fold smaller tumours than H226 cells transduced with mutant BAP1 lacking DUB activity or a nuclear localisation signal [41]. Recent evidence suggests reduced levels of BAP1 in patients with a heterozygous germline *BAP1+/-* mutation decreases apoptosis in cells that accumulate DNA damage [84]. BAP1 was found to localise to the endoplasmic reticulum (ER) where it binds to, deubiquitinates and stabilises type 3 inositol-1,4,5-trisphosphate receptor (IP3R3), an ER channel that modulates calcium (Ca2+) release from the ER into the cytoplasm and mitochondria, a step that triggers apoptosis via mitochondrial Ca2+ overload. Reduced BAP1 was found to cause a reduction in IP3R3 levels and of Ca2+ flux preventing *BAP1+/-* fibroblasts from undergoing apoptosis in response to DNA damage.

BAP1 in MPM

Sanger sequencing initially revealed somatic *BAP1* point mutations in 23-36% of sporadic MPM samples [30, 33] however integrated molecular sequencing and immunohistochemistry suggest a significantly higher incidence (~60%) of *BAP1* alterations in MPMs [35]. BAP1 immunohistochemical analysis of 123 MPM samples indicated that loss of nuclear BAP1 expression (indicative of mutant *BAP1*) correlates with a longer survival time [34]. Loss of nuclear BAP1 expression has also been found to be of clinical utility in distinguishing malignant from benign mesothelial proliferations [85] and MPM from non-small cell lung carcinoma [86].

Owing to the high prevalence of *BAP1* mutations in MPM it has been the subject of interest as a novel target for treatment. As it has a role with BARD1/BRCA1 in homologous recombination repair, poly (ADP ribose) polymerase (PARP) inhibitors have been tested for their efficacy in *BAP1* mutant tumour cells with conflicting results. A recent study found that the PARP inhibitors niraparib and olaparib markedly decreased clonal survival in multiple MPM cell lines, irrespective of *BAP1* status [87]. However, increased

sensitivity to olaparib was observed in homozygous *BAP1*-null chicken DT40 cells compared to wild-type and heterozygous *BAP1*-null cells [48]. A further recent study has implicated the importance of the levels of an alternative splice variant of *BAP1* in conferring sensitivity to PARP inhibition [88]. This alternative splice isoform leads to the loss of 12 amino acids within the catalytic and BARD1 binding domains. Transfection of *BAP1*-deficient ZL55 MM cells with this isoform resulted in increased sensitivity to olaparib compared to cells transfected with wild-type *BAP1*.

BAP1 loss has been shown to reduce HDAC2 expression [89], and *BAP1* knockdown in MPM cells to increase cell death in response to HDAC inhibitors. However, in a phase III trial of 661 unselected MPM patients (VANTAGE 014) the HDAC inhibitor vorinostat did not improve overall survival compared with placebo [90]. Correlation of *BAP1* status from tumours in this study with response to HDAC inhibition may shed light on the role of BAP1 as a biomarker for HDAC inhibitor sensitivity.

BAP1 loss has also been found to increase EZH2 levels in MPM cell lines and *BAP1*-knockout mice [91]. Treatment of *BAP1* mutant MPM lines with an EZH2 inhibitor was subsequently found to decrease cell proliferation, invasion and clonogenicity. Further, treatment of *BAP1* mutant MPM mouse xenografts with EZH2 inhibitors significantly reduced tumour size compared to wild-type *BAP1* xenografts. The mechanism is thought to be through a decrease in H4K20 monomethylation (H4K20me1) in *BAP1* null cells, an epigenetic mark that plays a role in the transcriptional regulation of EZH2. Consistent with this, expression of the H4K20me1 methyltransferase SETD8 reduced EZH2 expression and the proliferation of *BAP1*-mutant cells [91]. Other studies however have not demonstrated a clear association between BAP1 loss and EZH2 upregulation in MPM or UM biopsies using immunohistochemistry [92, 93]. A phase 2 study of the EZH2 inhibitor Tazemetostat is underway (NCT02860286); unselected patients with MPM will be entered first followed by patients with a *BAP1* mutation.

1.4.2 ASXL proteins in cancer

ASXL1

Nonsense point mutations or frame-shift mutations of *ASXL1* are observed at a high rate in myeloid malignancies (**Table 1-2**) [94, 95]. These mutations mostly occur in exons 11 or 12 just before the PHD domain. It is not clear whether these truncating mutations result in loss of ASXL1 expression or expression of a stable truncated form of ASXL1. Initial studies failed to identify any ASXL1 expression in homozygous mutant cell lines and lower levels of full length ASXL1 in heterozygous mutants with reduced stability of the mutant form [71]. However use of a new N- terminus antibody has detected truncated ASXL1 in two cell lines with homozygous truncating mutations [96]. Further evidence suggests truncated ASXL1, which retains the N-terminal BAP1 binding site, may form a hyperactive PR-DUB resulting in depletion of H2AK119Ub and H3K27Me3 [72].

Consistent with a role for ASXL1 in haematopoeisis, adult *ASXL1* gene-trap mice exhibit splenomegaly and defects in B- and T- cell lymphopoeisis and myeloid skewing [97]. Myeloid malignancies however are not observed in these knockout mice. As mouse ASXL1/2 are co-expressed in embryonic and adult tissues the suppression of myeloid malignancies might stem from a functional redundancy between ASXL1 and ASXL2.

ASXL1 is less frequently mutated in solid malignancies but is observed in 55% of colorectal cancer (CRC) cell lines with microsatellite instability and ~1-2% of breast, prostate, liver and head and neck squamous cell carcinomas [74]. ASXL mutations are only sporadically observed in lymphoid malignancies [98, 99].

Germline mutations of human *ASXL1* occur in the Bohring-Opitz syndrome, characterised by intellectual disability, cranio- skeletal features and feeding problems [100].

ASXL2

ASXL2 mutations are observed in 23% of acute myeloid leukaemia (AML) t(8;21), a specific subset of AML with translocation between chromosomes 8 and 21, but is otherwise almost never observed in leukaemia [101, 102]. *ASXL1* mutations are also observed in AML t(8;21) however are mutually exclusive with *ASXL2* mutations suggesting there may be synthetic lethal effects between the two. *ASXL2* is mutated less frequently in solid malignancies including 6.0% of prostate cancers and 4.2% of pancreatic cancers (**Table 1-2**) [74].

ASXL3

ASXL3 mutations have been very infrequently reported in any malignancy, haematological or solid. Germline truncating mutations in *ASXL3* have been associated with a developmental syndrome with phenotypic overlap with Bohring-Opitz syndrome [103].

Table 1-2 Cancer genomics of the ASXL proteins

Gene	Genetic Alterations	Cancer	Mutation Rate
ASXL1	Gene amplification	Cervical cancer	5.1%
	Truncation	CRC with MSI	55%
		CMML	45.3%
		MPN	34.5%
		AML (secondary)	30%
		MDS	16.2%
		Liver cancer	10%
		AML (de novo)	6.5%
		CLL	2.9%
		Prostate cancer	2.0%
		HNSCC	1.4%
		Breast cancer	1.0%
ASXL2	Truncation	Prostate cancer	6.0%
		Pancreatic cancer	4.2%
		Breast cancer	1.0%
		ATLL	rare
ASXL3	Truncation	Melanoma	4.0%

AML acute myeloid leukaemia, secondary – evolving from prior myelodysplasia or myeloproliferative disorder, de novo – not evolving from prior myelodysplasia or myeloproliferative disorder; ATLL adult T-cell leukaemia/lymphoma; CLL chronic lymphocytic leukaemia; CMML chronic myelomonocytic leukaemia; CRC with MSI colorectal cancer with microsatellite instability; HNSCC head and neck squamous cell carcinoma; MDS myelodysplastic syndrome; MPN myeloproliferative neoplasm.
Adapted from Katoh, M 2013 [74]

1.5 TNF related apoptosis inducing ligand (TRAIL)

TRAIL, also known as APO2 ligand and TNFSF10, is a member of the TNF death ligand superfamily and is expressed as a type II transmembrane protein in both membrane-bound and soluble forms. The physiological function of TRAIL is not fully understood, but it is believed to play a role in the control of autoreactive immune cells and immune surveillance, particularly against transformed cells [104].

1.5.1 TRAIL receptors

TRAIL binds to four membrane bound death receptors (DR) (**Figure 1-6**). TRAIL-R1 (also known as DR4) and TRAIL-R2 (also known as DR5) contain an intracellular death domain (DD) required for cell death induction and can therefore induce apoptosis upon TRAIL binding and are widely expressed. TRAIL-R3 (also known as DCR1) and TRAIL-R4 (also known as DCR2) lack the DD and are incapable of inducing cell death. The DD lacking receptors are thought to act as 'decoy' receptors and negatively regulate apoptosis induction by TRAIL [105]. Evidence suggests DR4 and DR5 exist as preassembled multimers (dimers or trimers) activated by multimeric TRAIL ligands [106, 107]. These multimeric receptors can also in turn homo- and heterotrimerise to form higher order complexes [108, 109]. This clustering of DRs is thought to facilitate and stabilise assembly of the death inducing signaling complex (see below). TRAIL also binds to a soluble protein, osteoprotegerin, with low affinity [110].

1.5.2 TRAIL apoptotic pathway

TRAIL activates the extrinsic apoptotic pathway (**Figure 1-6**). On binding to TRAIL the intracellular DDs of three cross-linked receptors interact to recruit FAS-associated death domain protein (FADD) [111]. FADD in turn recruits the initiator caspases 8 and 10 to form the death-inducing signaling complex (DISC) [112]. The caspase 8 homologue FLICE like inhibitory protein (FLIP)

can compete with caspase 8 for FADD binding but has no catalytic activity. DISC formation induces cleavage and activation of caspases 8 and 10 that are subsequently released into the cytoplasm to cleave downstream effector caspases such as caspase 3, which induce apoptosis of the cell [113].

TRAIL can also indirectly activate the intrinsic apoptotic pathway (**Figure 1-6**) [114]. The intrinsic apoptotic pathway is primarily triggered by DNA damage recognised by proteins such as P53. P53, through BAX/BAK, permeabilises the mitochondrial membrane enabling release of cytochrome c (cyt-c), which in turn activates caspase 9 through apoptotic protease-activating factor-1 (APAF-1). Caspase 9 activates the effector caspases to induce apoptosis. Cross talk between the extrinsic and the intrinsic apoptotic pathways is mediated by BID. Upon activation, caspase 8 cleaves BID to truncated BID (t-BID), which induces BAX/BAK to permeabilise mitochondria.

1.5.3 TRAIL and cancer cells

TRAIL is expressed on monocytes, macrophages, dendritic cells (DCs) and natural killer (NK) cells. It is thought to be involved in the effector mechanisms of these cells and to regulate immune homeostasis in normal physiology. TRAIL-R1 (DR4) and TRAIL-R2 (DR5) are highly expressed in several different malignancies. Evidence from *in vitro* and mouse studies suggests the role of TRAIL in cancer biology is diverse and physiologically TRAIL may have a role in immune surveillance against primary tumours and metastasis [115].

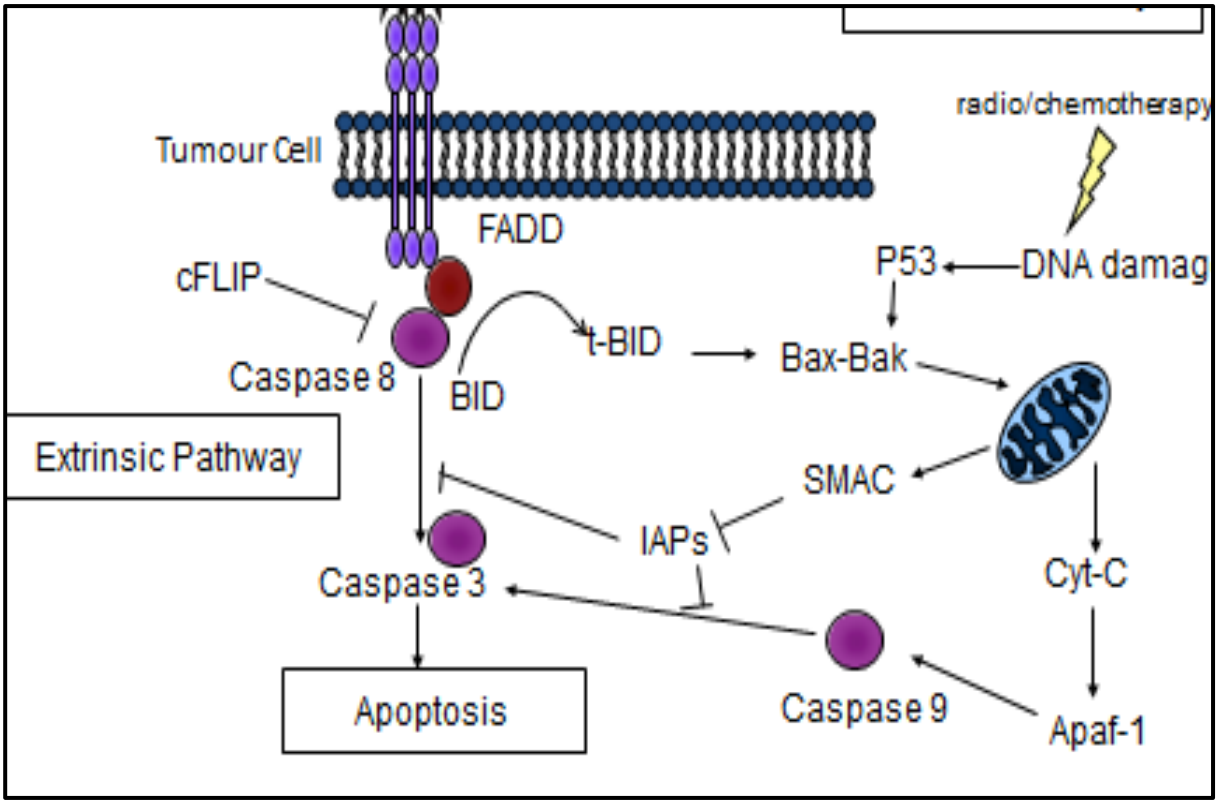


Figure 1-6 TRAIL activates the extrinsic apoptotic pathway

TRAIL triggers the extrinsic apoptotic pathway while conventional chemotherapeutics and radiotherapy trigger the intrinsic apoptotic pathway that is mediated by mitochondria. There is cross talk between the two pathways, which is mediated by cleavage of BID into t-BID by caspase-8. cFLIP and IAPs are potent inhibitors of apoptotic pathways and their inhibition could induce synergistic effects by simultaneously triggering both pathways. TRAIL: tumour necrosis factor apoptosis-inducing ligand, FADD: FAS activated death domain, cFLIP: cellular FLICE inhibitory protein, BID: BH3 interacting-domain death agonist, IAPs: inhibitors of apoptosis proteins, BAK: BCL-2 homologous antagonist, Cyt-C: cytochrome c, APAF-1: apoptotic protease-activating factor 1.

1.5.4 TRAIL and DR agonists as anti-cancer therapies

The ability of TRAIL to selectively induce apoptosis in transformed cells has been leveraged to develop anti-cancer agents in the form of TRAIL-R/ DR agonists. These include recombinant forms of TRAIL (rTRAIL) and monoclonal antibodies and small molecule agonists to DR4 and DR5. Unfortunately to date none of these agents have demonstrated a significant clinical benefit in clinical trials of unselected populations (**Table 1-3**) [116]. A number of factors contributing to this failure have been postulated. These

include unfavourable pharmacokinetics and weak agonistic activity of DR agonists developed to date, intrinsic resistance mechanisms of primary cancer cells to DR agonist monotherapy, and the lack of suitable biomarkers by which to identify patients most likely to respond. Attempts have been made to understand and overcome each of these barriers.

Table 1-3. Selected results of TRAIL-R/ DR agonists in clinical trials.

Phase	N	Cancer	Combination	Safety	Efficacy	Ref
Dulanermin						
I	71	Advanced cancers	-	Safe	2PR	[117]
I	27	Colorectal	Chemo + BV	Safe	6PR	[118]
II (RCT)	213	Lung	Chemo + BV		None	[119]
Mapatumumab						
I	49	Advanced cancers	-	Safe	None	[120]
I	49	Advanced cancers	Chemo	Safe	12PR	[121]
II (RCT)	109	Lung	Chemo	Safe	None	[122]
Conatumumab						
I	37	Advanced cancers	-	Safe	1PR	[123]
II (RCT)	83	Pancreatic	Chemo	Safe	None	[124]
II (RCT)	190	Colorectal	Chemo + BV	Safe	None	[125]
Lexatumumab						
I	37	Advanced cancers	-	Safe	None	[126]
Tigatuzumab						
II	61	Pancreatic	Chemo	Safe	8PR	[127]

PR, partial response. Chemo – combination chemotherapy. BV – bevacizumab. Dulanermin – recombinant TRAIL. Mapatumumab – agonist antibody to DR4. Conatumumab, lexatumumab, tigatuzumab – agonist antibodies to DR5.

Adapted from Lemke et al, 2014 [116]

Dulanermin is the first and only recombinant form of TRAIL developed for clinical application to date however is limited by a short half-life (approximately 30 minute *in vivo*) and an inability to induce higher order clustering of TRAIL-R/ DRs [128]. Agonistic antibodies have increased stability and a longer half-life than dulanermin however remain limited by their bivalent mode of receptor binding. Novel multivalent TRAIL-R/ DR agonists have been developed with

the aim of replicating the higher order clustering observed physiologically. TAS266 is a tetravalent nanobody consisting of four high affinity heavy chain domain (VHH) antibody fragments each of which bind a single DR5 potentially clustering four receptors [129]. Unfortunately a phase I study was terminated early due to acute hepatotoxicity thought to be secondary to an anti-drug antibody response in immunosensitised individuals [130]. Medi3039 is a preclinical multivalent DR5 superagonist. It consists of multivalent fibronectin type III domains engineered for high affinity DR5 binding [131]. Preclinical testing has demonstrated potent apoptosis induction in cell lines at subpicomolar concentrations [132]. A first in human adaptive phase I trial is currently being planned in colorectal cancer [133].

Several TRAIL-sensitising strategies have been tested, such as the combination of TRAIL-R/DR agonists with proteasome inhibitors, standard chemotherapeutic agents, SMAC mimetics and BH3 mimetics to antagonise anti-apoptotic BCL-2 family members, or various kinase inhibitors (e.g. AKT or PI3K inhibitors) [116]. Unfortunately, many of these studies show only limited therapeutic activity *in vivo* and likely underestimate potential *in vivo* toxicity. Recently, inhibition of cyclin-dependent kinase 9 (CDK9) was described as a potent TRAIL sensitisation strategy [134]. CDK9 inhibitors were shown to downregulate the anti-apoptotic factors, MCL1 and FLIP, simultaneously increasing DISC-generated caspase 8 activity and removing a mitochondrial block to maximal apoptosis induction rendering many TRAIL-resistant cancer cells sensitive. Thus the removal of multiple roadblocks to apoptosis may be key to TRAIL sensitisation.

Notable within a number of Phase I/II trials is the observation that a subset of patients displays partial or complete response to TRAIL/ DR agonists without additional sensitisation strategies (**Table 1-3**). To date however no biomarker has been identified by which to identify such responders. High expression of the O-glycosylation enzyme GALNT14 was proposed as a signature of TRAIL sensitivity [135], however increased expression did not appear to significantly

correlate with clinical response to dulanermin in a phase II study of NSCLC [119]. An alternative approach proposed the expression of a panel of apoptosis pathway factors could predict TRAIL response however this is cumbersome and is yet to be validated clinically [136].

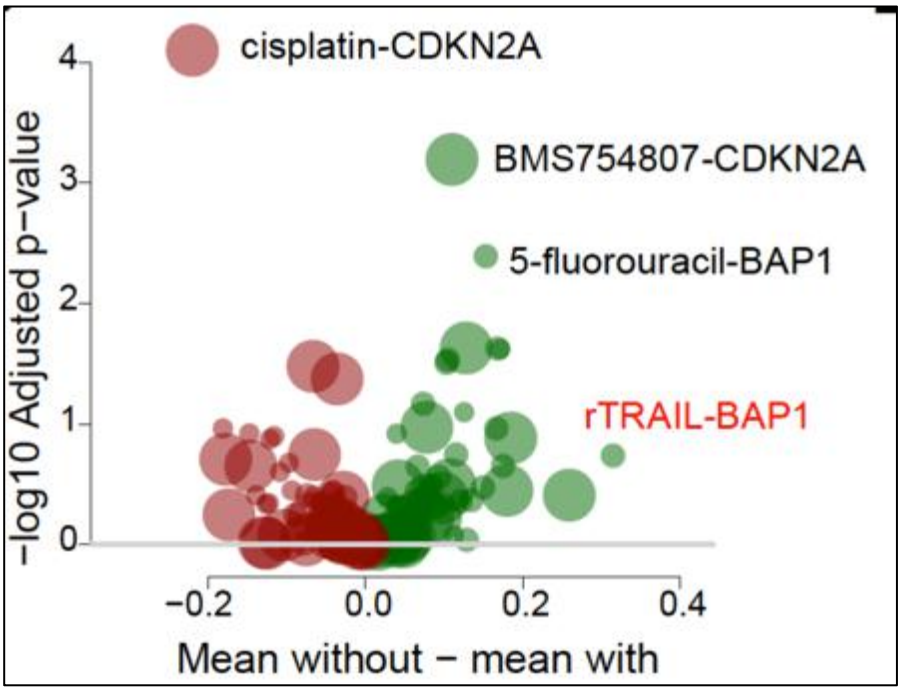
1.6 BRCA associated protein-1 and TRAIL

Much of this thesis is based upon and an extension of as yet unpublished data from the Janes lab presented below [137].

1.6.1 Loss of function of BAP1 as biomarker for TRAIL sensitivity

The WTSI characterised the genomic aberrations present in 15 MPM cell lines using whole-exome sequencing, copy number analysis and gene expression arrays. These cell lines were then treated with 94 drugs including small molecule inhibitors and cytotoxic chemotherapeutics. A 6-day cell viability assay was performed to assess response. 1,425 single agent activity data profiles across the 15 cell lines were generated. To detect novel markers of drug sensitivity, statistical associations were sought between drug response and the mutational status of the cell lines based on five genes identified as candidate drivers of tumourigenesis in MPM [30]. There were 24 significant associations (false discovery rate (FDR) <0.2) between single agent response and the presence of a genomic alteration. The most statistically significant sensitising association seen was between *BAP1* LOF mutations (mt BAP1) and treatment with recombinant TRAIL (rTRAIL; FDR = 0.18, effect size -0.48) (**Figure 1-7A and 1-7B**) No significant effect on cell viability as measured by XTT assay was observed in *BAP1-wild-type* (wt BAP1) lines when treated with rTRAIL. This association was subsequently confirmed in a larger panel of MPM cell lines (**Figure 1-8A and 1-8B**). 6 of the 8 cell lines (75%) harbouring a *BAP1* LOF mutation were sensitive or partially sensitive to a dose range of rTRAIL, while 7 of the 9 cell lines (78%) harbouring wild-type *BAP1* were resistant. *BAP1* LOF mutations correlated with a loss of BAP1 protein expression in the majority of cell lines.

A



B

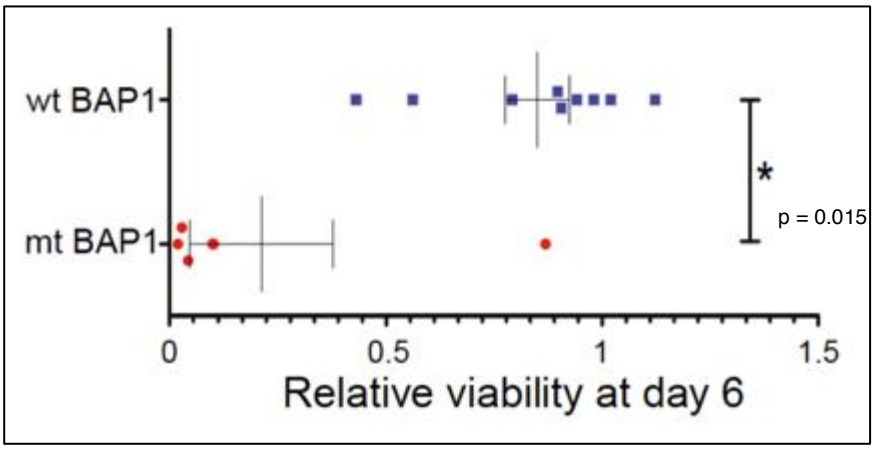
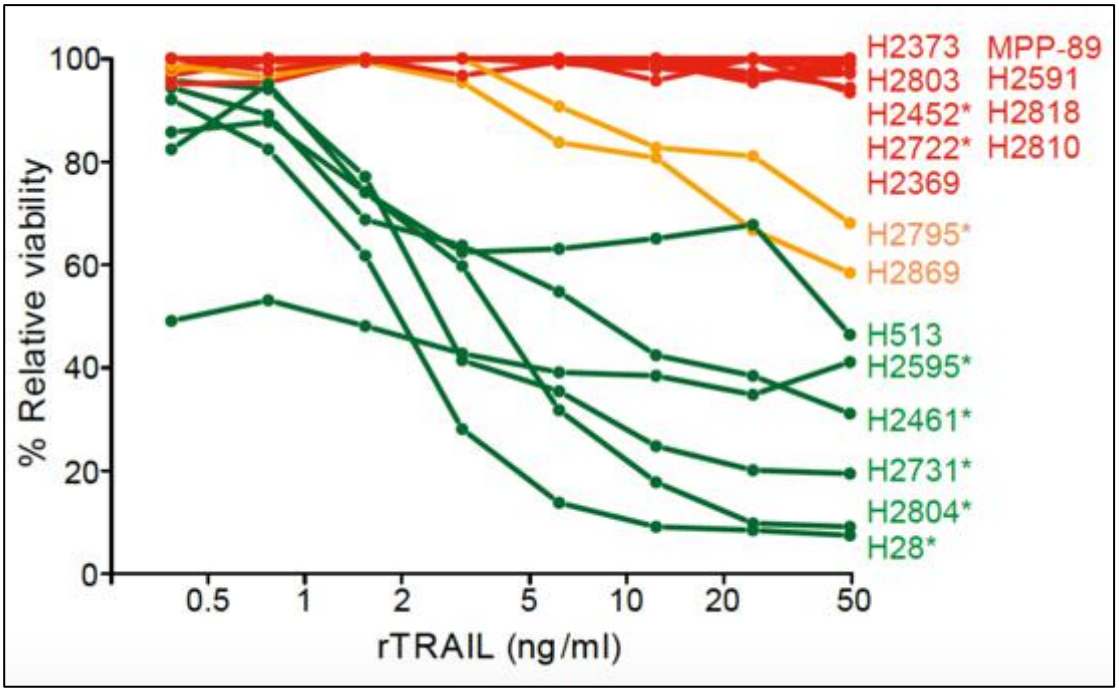


Figure 1-7 A chemical screen of molecularly characterised MPM cells [137]

(A) *A Welch t-test was used to test for significant pharmacogenomic interactions between 94 compounds and the presence of driver mutations in 5 MPM cancer genes. Each volcano plot circle corresponds to a significant gene–drug interaction; the position on the x-axis indicates the corresponding effect size. Both half-axes are positive; the right side (green circles) indicates sensitivity associations, whereas the left side (red circles) corresponds with resistance associations. The y-axis indicates the statistical significance of the identified interaction. The size of a given circle is proportional to the number of samples in which the selected mutational event occurs. Specific examples of associations are indicated where the effect size is large (rTRAIL and BAP1 mutations) or highly significant (cisplatin and CDKN2A mutations).* ***(B)*** *6-day cell viability of wild-type (wt) BAP1 (n=10) and mutant (mt) BAP1 (n=5) MPM cell lines following rTRAIL treatment (t-test; *p=0.015).*

A



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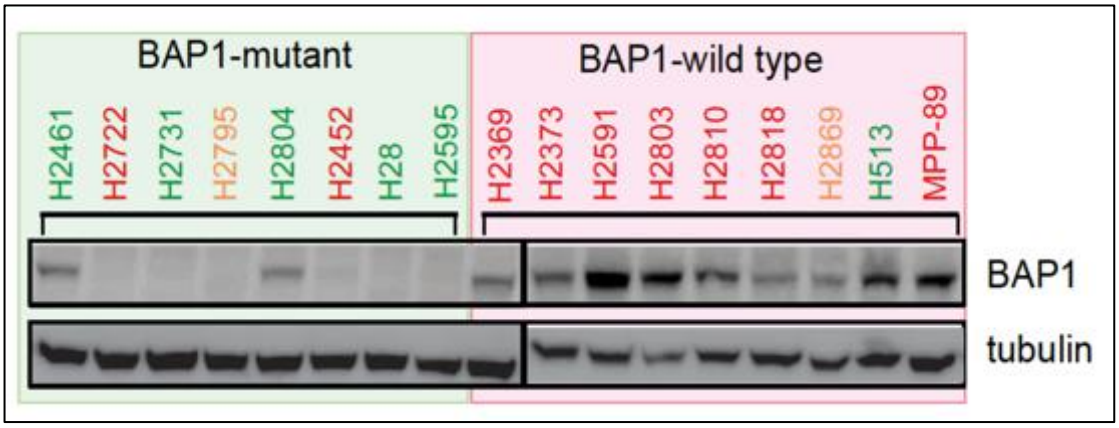


Figure 1-8 Initial validation of the identified BAP1-rTRAIL association [137]

(A) *6-day cell viability data for 17 malignant pleural mesothelioma (MPM) cell lines treated for 6 days with rTRAIL (0.4–50 ng/ml). Green = sensitive (S); orange = partially sensitive (PS); red = resistant (R). *Indicates lines harbouring BAP1 mutations. Those cell lines with BAP1 mutations are more sensitive to rTRAIL* ***(B)*** *Immunoblot of BAP1 expression in BAP1-mt versus BAP1-wt MPM cell lines. Sensitivity to rTRAIL treatment is indicated as font colour: green (S); orange (PS); red (R). Cell lines with BAP1 mutations mostly lose BAP1 expression and are more sensitive to rTRAIL than wild-type cell lines.*

BAP1 wild-type MPM lines – H2373, H2803, H2369, MPP-89, H2591, H2818, H2810, H2869, H513. BAP1 mutant MPM lines – H2452, H2722, H2795, H2595, H2461, H2731, H2804, H28.

1.6.2 The deubiquitinase function of BAP1 mediates TRAIL resistance

shRNA knockdown of *BAP1* in the *BAP1*-WT MPM cell line H2818 resulted in increased cell death following rTRAIL treatment compared with empty vector (EV) control shRNA and the parental cell line further supporting the observed BAP1-rTRAIL association (**Figure 1-9**).

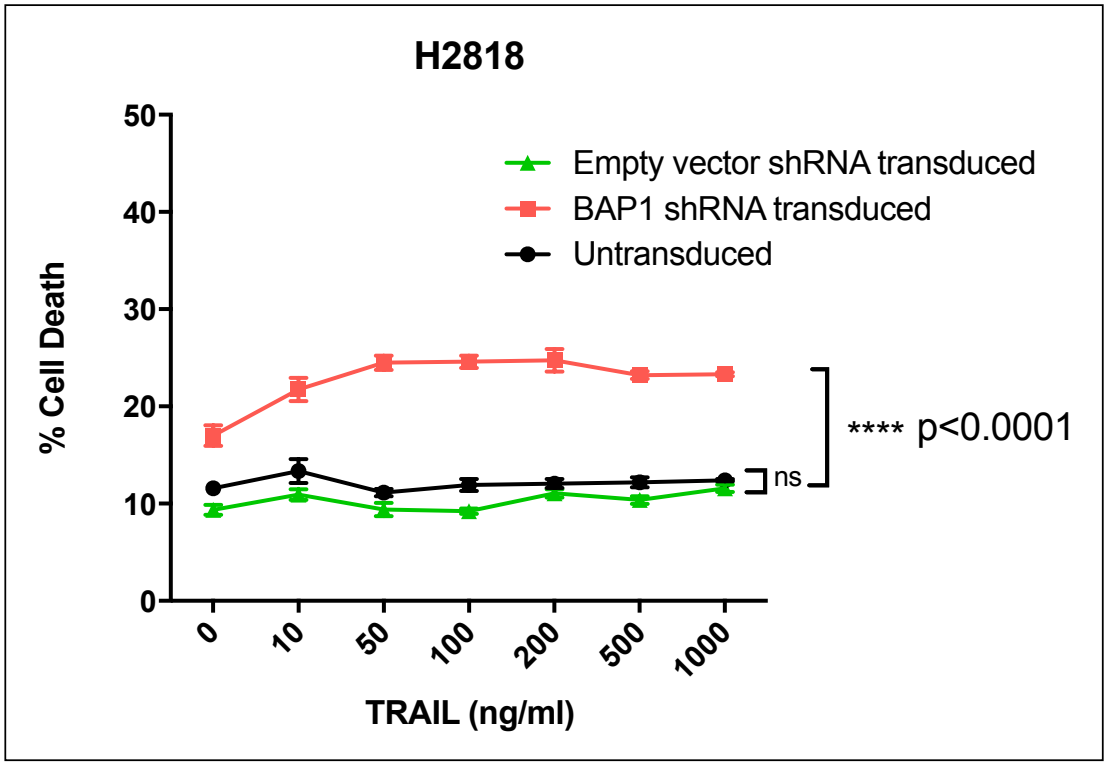


Figure 1-9 Knockdown of BAP1 in H2818 MPM cells sensitises them to rTRAIL [137]

BAP1 wild-type H2818 cells were transduced with BAP1 shRNA-expressing lentivirus and treated with rTRAIL (dose range 0-500 ng/ml). Cell death was measured after 24 hours with an AnnexinV/ DAPI flow cytometry assay. shRNA knockdown of BAP1 expression resulted in an increased sensitivity to rTRAIL. H2818 untransduced vs H2818 EV transduced p = 0.739; H2818 untransduced vs H2818 BAP shRNA transduced p < 0.0001; H2818 EV transduced vs H2818 BAP shRNA transduced p < 0.0001.

To elucidate the mechanism by which BAP1 modulates sensitivity to TRAIL expression vectors containing wild-type or mutant *BAP1* were generated, each with an inactive functional site or protein-binding domain. These included C91A (mutation in the deubiquitination catalytic site), ΔHBM (deletion of the HCF-1-binding site), and ΔNLS (deletion of the nuclear localisation signal).

H226 MPM cells, which harbour a homozygous deletion of *BAP1* and demonstrate complete loss of BAP1 expression, were transduced with a GFP expressing control vector, a wild-type *BAP1* expression vector or one of these three mutant *BAP1* expression vectors. rTRAIL sensitivity of the parental *BAP1*-null H226 MPM line was significantly diminished following expression of wild-type *BAP1* and each of the mutant constructs except the C91A deubiquitinase mutant (**Figure 1-10**), implicating the deubiquitinase activity of BAP1 in TRAIL resistance.

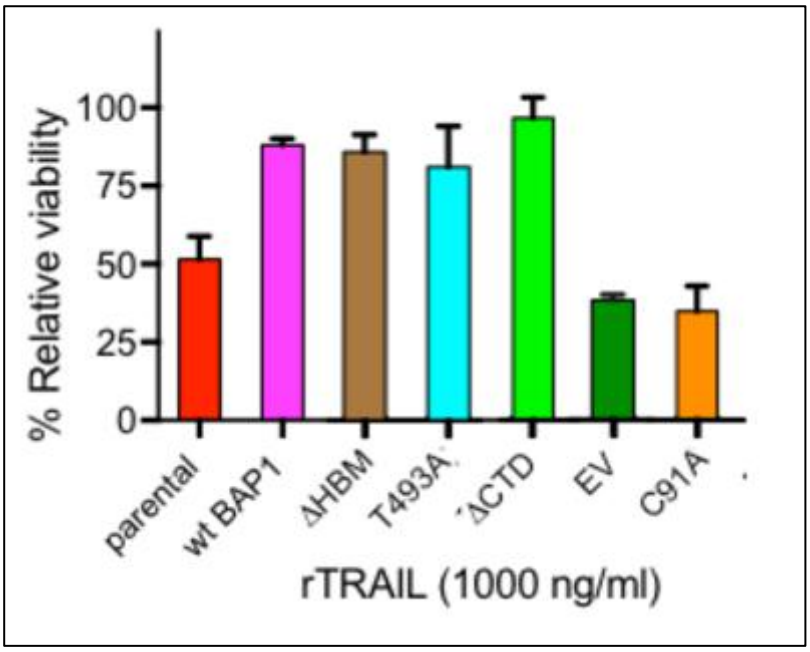


Figure 1-10 Cell viability of mutant BAP1 transduced H226 MPM cells treated with rTRAIL [137]

*The rTRAIL-sensitive H226 MPM cell line, which harbours a homozygous deletion of BAP1, was transduced with either a GFP (GFP) control, wild-type BAP1 or a mutant BAP1 containing an inactive functional domain: C91A — mutation in deubiquitinase catalytic site; ΔHBM — deletion of HCF-1-binding motif; ΔNLS — deletion of nuclear localisation signal. These transduced cell lines were treated with 50ng/ml rTRAIL and cell death was assessed with an XTT assay (One-way ANOVA; **p<0.01). Transduction with all constructs except the EV and deubiquitinase mutant constructs resulted in an increase in rTRAIL resistance implicating this function in the mechanism of rTRAIL resistance.*

1.6.3 BAP1 function affects transcription of extrinsic apoptotic proteins

As one of the main roles of BAP1 is as a transcriptional regulator, differential gene expression data from *BAP1*-null H226 cells expressing the sensitising C91A *BAP1* mutation or wild-type *BAP1* were compared (through collaboration with the WTSI) and a signalling pathway impact analysis SPIA was carried out. Among those pathways significantly altered when comparing wild-type versus C91A *BAP1* (FDR<0.2) was that of apoptosis. In particular, there was altered expression of components of the extrinsic apoptotic pathway. This manifested as an imbalance in levels of pro- and anti-apoptotic members with, for example, significantly decreased levels of the anti-apoptotic protein cIAP2 (p=2.32E-10) and increased levels of the pro-apoptotic death receptor 5 (p=7.79E-10) in the C91A *BAP1*-transduced cells (**Figure 1-11**).

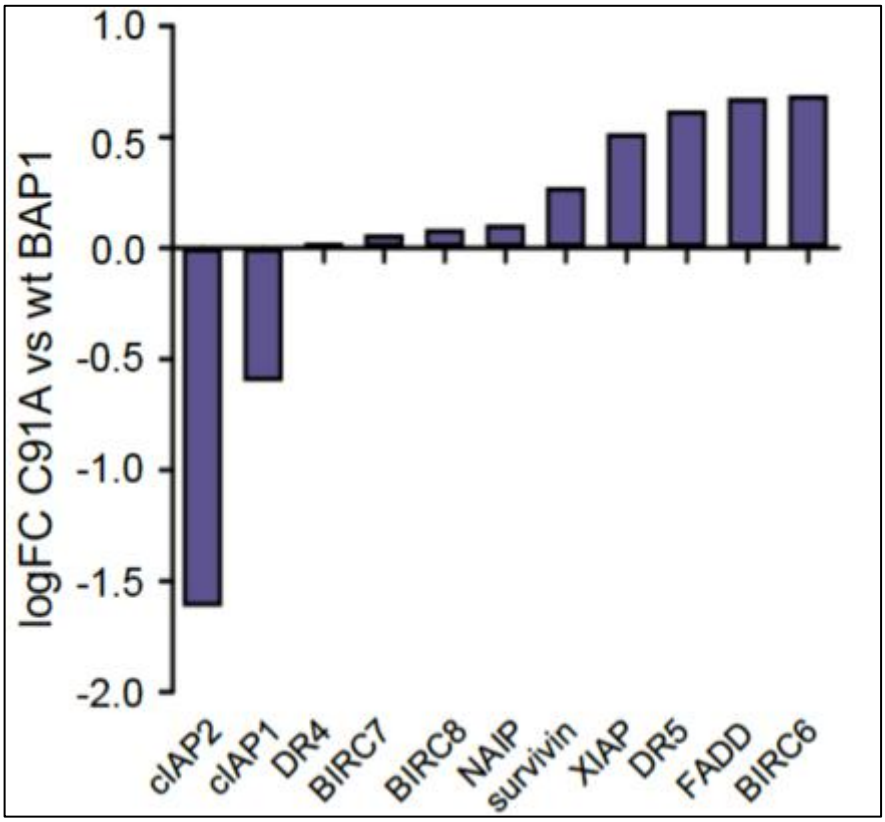


Figure 1-11 Differential gene expression of extrinsic apoptotic pathway genes in BAP1 mutant relative to BAP1 wild-type transduced H226 MPM cells

cIAP 1/2 - cellular inhibitor of apoptosis protein 1/2, DR4/5 - death receptor 4/5, BIRC 6/7 - baculoviral IAP repeat-containing protein 6/7, NAIP - NLR family, apoptosis inhibitory protein, XIAP - X-linked inhibitor of apoptosis protein, FADD - Fas-associated protein with death domain. The anti-apoptotic genes and cIAP2, cIAP1 are significantly downregulated and the pro-apoptotic genes DR5 and FADD upregulated in the BAP1 mutant transduced MPM line.

1.6.4 Loss of BAP1 function augments sensitivity to rTRAIL in mouse MPM xenograft models

To test the *in vivo* efficacy of TRAIL in inducing apoptosis in *BAP1*-mutant MPM cells, H226 *BAP1*-wild-type and the H226 C91A *BAP1*-mutant cell lines were transduced with luciferase and equal numbers of wild-type and mutant cells injected into the opposite flanks of mice. On day 14 after injection, the mice were divided into two groups and injected intraperitoneally with rTRAIL or vehicle for 6 days per week until day 40. Tumour growth was monitored longitudinally with bioluminescent imaging. *BAP1*-wild-type tumours showed no response to rTRAIL compared with vehicle. The growth rate of rTRAIL-treated *BAP1*-mutant tumours was significantly suppressed compared with rTRAIL-treated *BAP1*-wild type and vehicle-treated tumours ($p<0.05$) (**Figure 1-12**).

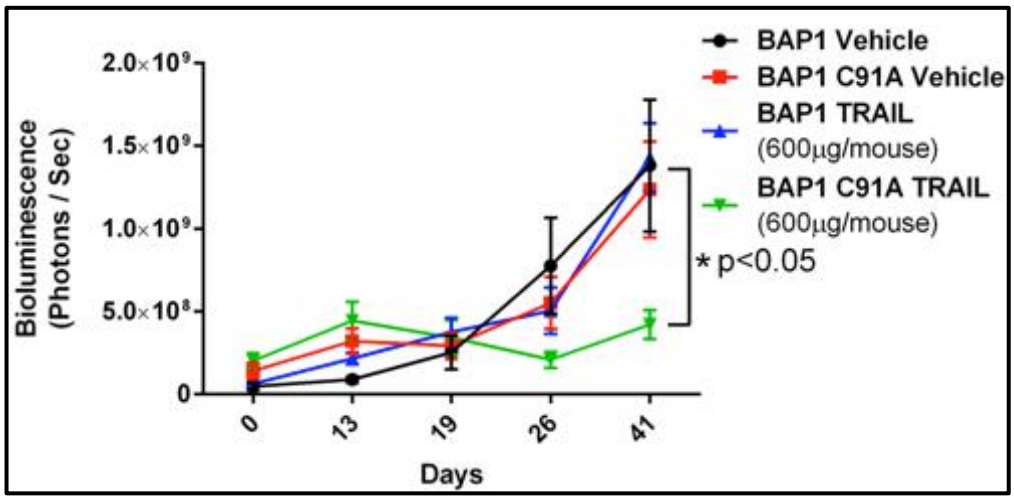


Figure 1-12 Growth of BAP1 wild-type vs. BAP1 mutant MPM xenografts treated with rTRAIL

Mice were treated with vehicle or rTRAIL 6 days per week from days 14-40 after tumour engraftment. Bioluminescence was measured on days 0, 13, 19, 26 and 41, 15 minutes after injecting the mice with 0.2 ml luciferin intraperitoneally. The number of photons emitted per second indicates the tumour burden. Values shown are the average recorded from the 6 mice used in the experiment in each group. BAP1 – wild-type xenografts, C91A BAP1 – mutant xengrafts. Growth of the BAP1 mutant transduced cell line xenograft was significantly reduced compared with the wild-type transduced cell line xenograft when treated with rTRAIL and compared with vehicle treatment.

1.7 Hypothesis

The above preliminary data suggests loss of BAP1 function augments sensitivity to rTRAIL in MPM. I hypothesise BAP1 can therefore act as a clinical biomarker for sensitivity to rTRAIL and the association will extend to other death receptor agonist drugs and other cancers with *BAP1* mutations. I further hypothesise the mechanism underlying this involves modulation of expression of proteins of the extrinsic apoptotic pathway to favour apoptosis when this pathway is activated.

1.8 Aims

1. To explore the clinical relevance of the BAP1-TRAIL association in MPM
2. To validate loss of function of BAP1 as a biomarker for sensitivity to rTRAIL and other death receptor agonists in MPM and other cancers
3. To further elucidate the mechanism of BAP1 induced TRAIL resistance

CHAPTER II:
METHODS

2 METHODS

2.1 General chemicals, solvents and plastic ware

All chemicals used were of analytical grade or above and obtained from Sigma Aldrich (Poole, UK) unless otherwise stated. Water used for preparation of buffers was distilled and deionised (ddH2O) using a Millipore water purification system (Millipore R010 followed by Millipore Q plus; Millipore Ltd., MA, US). Polypropylene centrifuge tubes and pipettes were obtained from Becton Dickenson (Oxford, UK).

2.2 Immunohistochemistry

2.2.1 Patients and tissue samples

Tumour samples were collected prospectively as part of the MSO1 trial [138]. The MSO1 trial is a phase III, three-arm randomized controlled trial in which active symptom control (ASC) was compared to ASC plus combination mitomycin, vinblastine and cisplatin (MVP) and ASC plus single agent vinorelbine. The two chemotherapy arms were combined in view of slow accrual and survival analysis revealed a small, non-significant benefit for ASC plus chemotherapy versus ASC alone in 409 patients, driven by a small, non-significant survival benefit in the ASC plus vinorelbine arm. Diagnoses of MPM had previously been confirmed by clinical, immunohistochemical and morphological evaluation. The tissue samples were taken from resected surgical specimens or following videothoracoscopic biopsy, fixed in neutral formalin and embedded in paraffin. Clinical data relating to each of the cases was recorded as part of the original trial.

2.2.2 Cell pellets

Early passage cell cultures were grown in T175 flasks until 80-90% confluent, trypsinised, washed twice with phosphate-buffered saline (PBS), centrifuged at 300g for 5 minutes and the supernatant removed. HistoGel specimen processing gel (American Mastertec) was liquefied by heating to 60°C and 4-6 drops added to each cell pellet. Each specimen was vortexed for 5 seconds and allowed to cool to room temperature. The specimens were then transferred to a tissue cassette and processed using an automated tissue processor (Leica TP1050) to generate the FFPE tissue blocks. The blocks were manually sectioned and mounted onto slides.

2.2.3 Staining protocol

Immunohistochemical parameters for the BAP1 antibody were initially optimised using a tissue micro-array consisting of lung cancer and malignant melanoma, tissues which express BAP1 and acted as positive controls. The IHC analysis for each antigen was performed using a Bond III Automated IHC Stainer (Leica Microsystems, Wetzlar, Germany). Slides were treated for 30 min with Leica BondMax Epitope Retrieval Solution 2 (ER2) to achieve post-sectioning antigen retrieval. The specific BAP1 primary antibody (SC-28383 (C4), mouse polyclonal antibody, 1:150; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was applied for 15 min and revealed using the Leica Bond Polymer Refine detection kit (Leica Microsystems). The signal was enhanced using the Leica BondMax DAB enhancer kit (Leica Microsystems). Slides were counterstained with haematoxylin before mounting and microscopic visualisation.

2.2.4 Scoring system

Slides were scored independently by two consultant histopathologists as BAP1 positive or BAP1 negative. Those samples with consistent strong nuclear staining were scored as positive and any other pattern of staining as negative. Slides were also assessed for the presence of an internal positive

control in the form of strong lymphocyte staining. Only those samples with a positive internal control and concordance between the two reviewers were included in subsequent analyses.

2.3 Cell culture

2.3.1 Cell lines

Cell lines were cultured in RPMI-1640 (H2461, H2722, H2731, H2795, H2803, CRL-2081, H2052, H28, H2804, H226, MPP-89, H2869, MDA-MB-231, H513) or Dulbecco's modified Eagle's medium and nutrient mix 12 medium (DMEM:F12) (H2818, H2810) supplemented with 10% fetal bovine serum (FBS), penicillin/ streptavidin and sodium pyruvate. 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. All cells were maintained in a humidified environment at 37°C and 5% CO2. Culture media was changed every 3 days. Cells were grown until approximately 80% confluent and mobilised by washing with sterile phosphate-buffered saline (PBS) followed by 0.05% trypsin in EDTA. After detachment cells were pelleted by centrifugation at 300g for 5 minutes and plated into 75 or 175 cm^2 tissue culture flasks at ratios of 1:3 to 1:10 every 5-10 days depending on rate of proliferation.

2.3.2 Early passage MPM cultures

The early passage MPM cultures were purchased from Mesobank (15,30, 52, 43,24, 34, 38, 33T, 50T, 53T, 3T, 7T, 8T, 12T, 14T, 23T, 19, 26, 35, 40, 45, 12, 2, 36, 18) [139]. The MPM cultures were cultured in RPMI-1640 medium supplemented with 5% FBS, 25 mM HEPES, penicillin/ streptavidin and sodium pyruvate. All cells were maintained in a humidified environment at 37°C and 5% CO2. Culture media was changed every 3 days. Cells were grown until approximately 80% confluent and mobilised by washing with sterile phosphate-buffered saline (PBS) followed by 0.05% trypsin in EDTA.

After detachment cells were pelleted by centrifugation at 300g for 5 minutes and plated into 75 or 175 cm^2 tissue culture flasks at ratios of 1:3 to 1:10 every 5-10 days depending on rate of proliferation.

2.4 Stock solutions and additives

All drugs and solutions used in tissue culture were sterile filtered through a 0.22µm filter unless otherwise stated. All solvents were tissue-culture grade. The drugs and solutions were stored as per manufacturer's instructions. The list of additives and drugs used in this study are listed in **Table 2-1**.

Table 2-1 Additives and drugs used in this study

Drug / Additive	Solvent	Stock Concentration	Supplier
Polybrene	Water	4mg/ ml	Sigma Aldrich
Ampicillin	Water	100µg/ ml	Sigma Aldrich
Puromycin	Media	100µg/ ml	Invitrogen
rTRAIL	Water	1000µg/ ml	Peprotech
Medi3039	DMSO	0.1µM	Medimmune

2.5 Lentiviral vectors and transduction

2.5.1 Cloning and mutagenesis of BAP1 expressing lentiviral vectors

The BAP1 vector pCCL-CMV-BAP1 had previously been generated by K Kolluri by PCR amplification of *BAP1* coding cDNA using a pCMV6-AC *BAP1* plasmid (Origene-SC117256) as a template and subsequent insertion of the *BAP1* cDNA into a lentiviral vector previously used in the laboratory, pCCL-CMV-flT, in place of flT via BamHI and SalI restriction sites (**Figure 2-1**) [140]. The ΔASXL mutation was performed on this pCCL-CMV-BAP1 vector using

the Q5 site directed mutagenesis kit (New England Biolabs-E0554) as per manufacturers instructions. The primers used are listed in **Table 2-2**.

Table 2-2 Primers used in BAP1 cloning

Primers	Sequence (5'-3')
BAP1 forward	CGTGGATCCGCCACCATGAATAAGGGCTGGCTGGA
BAP1 reverse	GTCGGTCGACTCACTGGCGCTTGGCCTTGTA
BAP1 ASXL mutation forward	AACTACGATGAGTTCATCTGCACCT
BAP1 ASXL mutation reverse	CTGGTCATCAATCTTGAACTTCTTCCTC

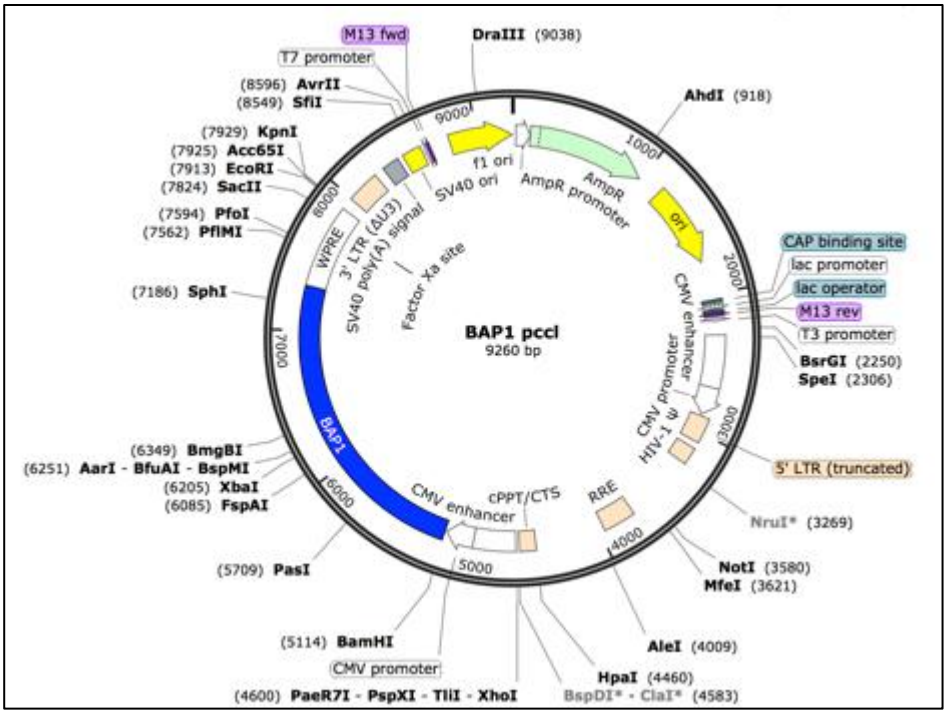


Figure 2-1 pCCL-CMV-BAP vector map [141]

The pCCL.CMV.BAP1 expresses wild-type BAP1 through a CMV promoter. The lentiviral vector contains an ampicillin resistance gene to facilitate selection of plasmid expressing bacteria. The original full length TRAIL coding region in the pCCL.CMV.flT was substituted with the BAP1 coding sequence using BAMHI and SALI restriction sites to generate this vector.

2.5.2 Propagation of lentiviral vector plasmids using *Escherichia. coli*

The large-scale production of lentiviral vectors requires significant quantities of plasmid DNA. Each plasmid contains a replication sequence to allow

replication of the plasmids in Escherichia. coli (*E. coli*) and an ampicillin resistance gene to allow selection of those bacteria that contain the plasmid [142].

Bacterial transformation of E. coli with plasmid DNA

Plasmids were expanded using chemically competent *E. coli* (C2987H, New England Biolabs, UK). 1*µ*l plasmid was added to a vial of competent cells and left on ice for 30 minutes before being heat shocked at 42^{o}C for 30 seconds and returned to ice for a further 5 minutes. 950*µ*l of SOC medium was added to the bacteria and incubated in an orbital incubator at 37^{0}C for 1 hour at 220rpm (Innova44, Eppendorf/ New Brunswick).

Production of single plasmid-transformed bacterial colonies and generation of starter cultures

35g LB agar was dissolved in 1L of dd$H_2$0, autoclaved at 121^{0}C for 15 minutes and cooled to approximately 50^{0}C prior to the addition of 50*µ*g/ml ampicillin. The LB agar was then poured into 90mm sterile petri dishes (Fisher) and cooled at 4^{0}C until the agar was set. Prior to use LB agar plates were pre-warmed at 37^{0}C. Different volumes of SOC medium containing transformed bacteria (from 50-200*µ*l) were spread onto the agar plates and incubated overnight at 37^{o}C. The following day single bacterial colonies were selected using a sterile loop and used to inoculate 5ml LB broth (Fisher Scientific, Loughborough, UK) containing 50*µ*g/ml ampicillin in a 50ml falcon tube. LB broth was prepared by dissolving LB broth powder in dd$H_2$0 at 20g/L, autoclaving at 121^{0}C for 15 minutes and cooling to approximately 50^{0}C prior to adding 50*µ*g/ml ampicillin. Falcon tubes containing single bacterial colonies were incubated overnight in an orbital incubator at 37^{o}C and 220rpm (Innova44, Eppendorf/ New Brunswick).

Miniprep – Extraction of plasmid from starter cultures

To confirm the bacteria had been successfully transformed with plasmid DNA, extraction was performed using a plasmid miniprep kit (QIAgen 27104/6) as per manufacturer's instructions.

Restriction digests

All restriction digests were performed using enzymes and buffers from New England Biolabs (Hitchin, UK) as per manufacturer's instructions. DNA was purified using the QIAquick PCR Purification Kit (QIAgen, 28104/6) according to the manufacturer's protocol. The purified product was run on a 1% (w/v) agarose gel using a HyperLadder I molecular weight marker (Bioline). An ImageQuant LAS 4000 (GE Healthcare) biomolecular imager was used to visualise DNA fragments.

Maxiprep - Large-scale production and extraction of plasmid DNA

To multiply the plasmid 2ml of the starter culture was added to 200ml of LB broth containing 50μg/ml ampicillin and incubated overnight in an orbital incubator at 220rpm and 37^{o}C (Innova44, Eppendorf/ New Brunswick). DNA was purified using the HiSpeed plasmid maxi kit (QIAgen, 12662/3) as per manufacturer's instructions.

DNA Quantification

The DNA was quantified using a NanoDrop 8000 spectrophotometer. Nucleic acids absorb ultraviolet light at specific spectra and the amount of light absorbed at different wavelengths is an indication of their purity. The ratio of absorbance at 260nm and 280nm (A260/A280 ratio) gives a measure of purity and a ratio of >1.8 is expected for pure DNA.

2.5.3 Lentivirus production

Lentivirus was produced by transfecting 293T cells with transfer and packaging plasmids using JetPEI [143]. On day one, 293T cells were seeded into 6 x T175 flasks to reach 80-90% confluence the following day for

transfection. On day two, 20μg transfer plasmid, 7μg pMD.G2 and 13 μg pCMV-dR8.74 were added to 1ml of 150mM sodium chloride solution per T175 flask to be transfected and vortexed for 10 seconds and passed through 0.2 μl filter. 80μl JetPEI was added to 1ml 150mM NaCl per T175 flask to be transfected and vortexed for 10 seconds. The NaCl/PEI solution was added to the NaCl/DNA solution, vortexed for 10 seconds and incubated at room temperature for 15-30 minutes. The medium in each T175 flask of 293T cells was then replaced with 2ml of the NaCl/DNA/PEI solution and 13ml fresh DMEM. At 4 hours this medium was replaced with 20ml DMEM. The media in the T175 flasks was collected in a sterile container at 24 and 40 hours and each time replaced with fresh media. The lentivirus in these collected supernatants were subsequently concentrated by ultracentrifugation at 17,000 rpm (SW28 rotor, Optima LE80K Ultracentrifuge, Beckman) for 2 hours at 4 °C. The virus was resuspended in DMEM, aliquoted and stored at -80˚C until further use.

2.5.4 Titration of lentivirus

The virus generated above was quantified by titration of different dilutions of the virus with 293T cells [144]. In a 6-well plate, 50,000 293T cells were seeded into each well and the following day, at 30-40% cell confluence, the medium was exchanged for medium containing virus dilutions of 1/100, 1/1000, 1/10000, and 1/100000 and 4μg/ml of polybrene, a cationic polymer enabling efficient cellular uptake of the virus. After 48 hours, the cells were trypsinised and the percentage of cells transduced measured by flow cytometry for the protein expressed by the virus. For detection of BAP1 expression, cells were stained with a primary BAP1 antibody (Santa Cruz SC28383, 2:100) and a fluorescent secondary antibody (AF-488, Invitrogen A32723,1:200). The percentage of positive cells at each viral dilution was assessed by flow cytometry for AF-488 on a FACS LSRII flow cytometer and the viral titre calculated in virus particles/ml using the equation below:

$$\text{Viral titre} = \frac{\text{number of cells transduced X proportion of positive cells}}{\text{volume of virus in ml}}$$

2.5.5 Transduction

Cells were transduced with viral particles at the required multiplicity of infection (MOI). Approximately 50,000 cells were seeded into each well of a 6-well plate [145]. The following day the medium was exchanged for medium containing a volume of virus solution for the required MOI based on the number of cells, and 4μg/ml of polybrene. Cells from one well were trypsinised and counted for accurate determination of the number of virus particles needed for a particular MOI. 4 hours after transfection the media was exchanged with fresh culture media. The next day transduction efficacy was measured by flow cytometry as above.

2.6 RNA interference

Short hairpin RNAs (shRNAs) were used to knock down protein expression in tumour cell lines [146]. The shRNAs were expressed as part of a mir30-based GIPZ lentiviral vector (Dharmacon) (**Figure 2-2**). By mimicking an endogenous RNA, the GIPZ hairpin is efficiently processed in the cells allowing for an effective knockdown of the gene of interest. Bacteria expressing lentiviral vector plasmids with GIPZ hairpins were obtained from Dharmacon through the UCL RNAi library. They were expanded in LB broth, plasmids extracted and virus made by the procedures described above. The tumour cell lines were transduced with lentivirus, and treated with puromycin to select the pure population expressing shRNA. The knockdown was confirmed by immunoblotting. The clones used in this study include BAP1 (V2LHS_4147) and ASXL1 (V2LHS_171023), ASXL2 (V2LHS_55252).

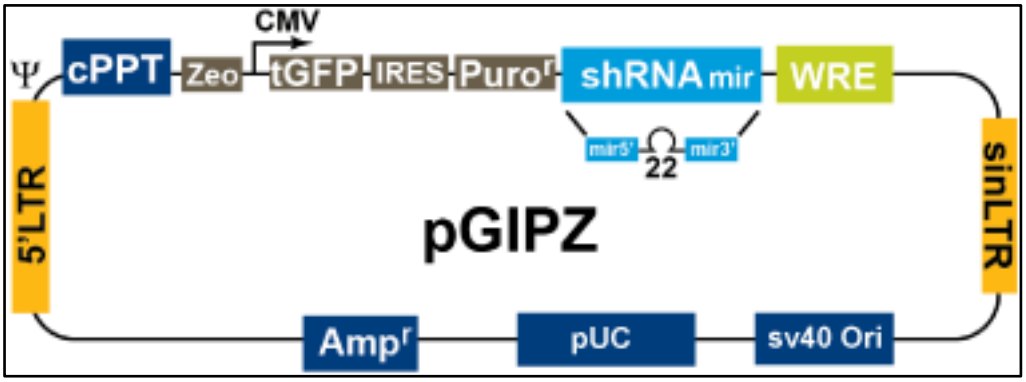


Figure 2-2 GIPZ shRNA vector map

The GIPZ shRNA expresses a 22 nucleotide shRNA mir via a CMV promoter. The lentiviral vector contains a puromycin resistance gene to facilitate selection of plasmid expressing bacteria and cells and GFP to aid in selection of transduced cells.

2.7 Immunoblotting

2.7.1 Sample collection and preparation

Protein lysates were obtained as follows [147]. Cells were trypsinised and collected by centrifugation at 300g for 5 minutes. The cells were then washed twice with PBS and an appropriate volume of 1% SDS in TBS containing protease and phosphatase inhibitor added. This suspension was passed through a 25G needle 5 times to lyse cells and the proteins then denatured by incubation at 95 degrees for 10 minutes. The suspension was then centrifuged at 13000rpm (Eppendorf 5415R Microcentrifuge) for 10 minutes, the supernatant collected and protein concentration measured by BCA assay as detailed below. Protein samples were then separated by SDS–PAGE and transferred onto nitrocellulose membranes. Membranes were incubated with specific primary antibodies, washed, incubated with secondary antibodies and visualised using an ImageQuant LAS 4000 imaging system (GE Healthcare). Antibodies used are detailed in **Table 2-3**.

2.7.2 BCA protein assay

To ensure equivalent amounts of protein were loaded for different samples the protein concentration of cell lysates was measured using the bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, IL, US) [148]. The BCA assay relies on two reactions. Firstly the peptide bonds in proteins reduce Cu2+ to Cu+, a reaction directly proportional to the amount of protein present. Secondly the bicinchoninic acid chelates with the reduced Cu+ ions to produce a purple coloured solution that strongly absorbs light at 562nm. The absorbance thus correlates with protein concentration. Known protein concentration standards were made by dissolving bovine serum albumin (BSA) in PBS at concentrations from 20µg/ml to 2000µg/ml. 20µl of each protein lysate sample along with 20µl of each standard concentration were added to individual wells of a 96-well plate and 180µl of BCA working solution added to each well. The plate was then incubated at 37^{0}C for 30 minutes and the absorbance read at 562nm. The absorbance of the samples was compared to those of the known protein standards to determine the protein concentration.

Table 2-3 Antibodies

Antibody	Manufacturer	Catalogue number	Source	Dilution
BAP1	Santa Cruz	sc-28383	Mouse	1:500
Caspase-8	Cell Signaling	9746	Mouse	1:1000
c-FLIP	Enzo Life Sciences	ALX-804-961-0100	Mouse	1:1000
c-IAP1	Cell Signaling	7065	Rabbit	1:1000
c-IAP2	Cell Signaling	3130	Rabbit	1:1000
FADD	Cell Signaling	2782	Rabbit	1:1000
XIAP	Cell Signaling	2045	Rabbit	1:1000
Survivin	Cell Signaling	2803	Rabbit	1:1000
ASXL1	Santa Cruz	sc-293204	Mouse	1:1000
ASXL2	Genetex	GTX44956	Rabbit	1:2000
H3K27Me3	Cell Signaling	9733	Rabbit	1:1000

H3	Cell Signaling	4499	Rabbit	1:1000
H2AK119Ub	Cell Signaling	8240	Rabbit	1:2000
H2A	Cell Signaling	12349	Rabbit	1:1000
α-tubulin	Cell Signaling	#2125	Rabbit	1:2000
Anti-mouse HRP	Cell Signaling	#7076	Rabbit	1:2000
Anti-rabbit HRP	Cell Signaling	#7074	Mouse	1:2000

2.7.3 Immunoblotting procedures

Samples were diluted in dH2O to equivalent protein concentrations and mixed with 5x Laemmli Buffer (3.125mM Tris-base pH 6.8, 10% (w/v) SDS, 20% (v/v) glycerol, 50mM Dithiothreitol (DTT), in dH2O with bromophenol blue). The samples were then incubated for 10 minutes at 70^{0}C and placed on ice prior to loading onto a precast 4-12% Bolt Bis-Tris Plus polyacrylamide gel (NW04122BOX). 25µl of each sample was added to each well. 5µl of a PageRuler pre-stained protein ladder (Thermo scientific) was also loaded. The gel was run at 150V in Tris/Glycine/SDS running buffer (0.25M Tris-base, 1.92M Glycine, 1% SDS, in dH2O). Following separation, the gel was removed from the cassette and the proteins were transferred onto a nitrocellulose membrane using an iBlot transfer system (Invitrogen) on program 3 for 7 minutes. The quality of protein transfer was assessed by briefly staining the membrane with 0.1% (w/v) Ponceau solution and the blot then placed in Tris-buffered saline (TBS) (20mM Tris-base, 150mM NaCl, pH7.4) containing 0.1% (v/v) Tween20 (TBST). Blots were incubated with blocking buffer containing 5% (w/v) non-fat dry milk in TBST for 1 hour. Blots were then incubated with primary antibodies in 5% (w/v) BSA or 5% milk in TBST overnight at 4°C. All blots were then washed in TBST 3 times for 5 minutes and incubated for 1 hour at room temperature with HRP-conjugated secondary antibody in 5% milk in TBST. After further washing 3 times in TBST for 5 minutes, 1 ml of Luminata western HRP chemiluminescence reagent

(Millipore) was applied to the membrane and incubated for 3 minutes. Excess reagent was drained off and immune-reactive bands visualised with an ImageQuant LAS 4000 biomolecular imager (GE Healthcare).

2.8 Immunofluorescence

Cells were seeded at $2.5x10^3$ cells per well into 96 well Greiner *μ*clear imaging plates in DMEM 10% FBS [149]. After 48 hours, cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes at room temperature and permeabilised in 0.3% NP-40 in PBS for 10 minutes. Cells were blocked in 1% BSA in 0.1% PBS tween for 1 hour at room temperature. Cells were incubated with H2AK119Ub primary antibody (cell signalling, #8240) overnight at 4^oC, before incubating for 1 hour at room temperature with Alexafluor-488 anti-rabbit secondary antibody. Nuclei were stained with Hoechst 33342 (Thermo, #62249). Images were acquired (n=3) using a BioTek Cytation3 Multimode reader, using a 10X objective. 4 fields of view were acquired per well (n=3), and the level of nuclear H2AK119Ub intensity was determined within the primary nuclear mask and normalised to total cell number.

2.9 Cell viability assay

An XTT assay was used to measure cell viability. XTT is a tetrazolium derivative (Applichem-A8088) that measures cell viability based on the activity of mitochondrial enzymes in live cells that reduce XTT and are inactivated shortly after cell death [150]. The amount of water-soluble product generated from XTT is proportional to the number of living cells in the sample and can be quantified by measuring absorbance at a wavelength of 475 nm.

Cells were seeded in 96-well plates in 100µl media per well at a density of 40,000 cells/ml one day prior to treatment with soluble recombinant TRAIL (rTRAIL; Peprotech-310-04) or Medi3039 (Medimmune). 25µL of activated XTT reagent (Applichem #A8088) was added at 72h following treatment, incubated for 2 hours and the fluorescent signal intensity quantified using a fluorescent plate reader to measure excitation and emission wavelengths of 490/650 nm. Relative cell viability was calculated as a fraction of viable cells relative to untreated cells.

2.10 Flow cytometry

2.10.1 Cell death assay

An Annexin V-based flow cytometry assay was used to measure cell apoptosis and death [151]. Cells were seeded in 96-well plates in 100µl media per well at a density of 100,000 cells/ml one day prior to treatment with soluble recombinant TRAIL (rTRAIL; Peprotech-310-04) or Medi3039 (Medimmune). 24 hours after treatment media, including all floating cells, was collected from each well and transferred to another 96-well plate. The adherent cells were washed with PBS, mobilised with 0.05% trypsin in EDTA and transferred to the second 96-well plate. Cells were then pelleted by centrifugation (300g, 5 minutes). The media was then discarded and cells pellets re-suspended in Annexin V binding buffer with Annexin V-647 antibody (Invitrogen) in a 1:100 ratio for 40 minutes on ice or 10 minutes at room temperature. 2µg/ml DAPI or PI was then added to each sample before flow cytometry analysis. Annexin V is a 35–36 kDa calcium-dependent phospholipid binding protein that has a high affinity for phosphatidlserine. Phosphatidlserine is located on the cytoplasmic side of the cell membrane, inaccessible to cell surface binding proteins, in normal viable cells. In apoptotic cells, it is translocated to the outer plasma membrane, thus exposing it to the external cellular environment and allowing binding of

Annexin V. The Annexin V is also able to pass through the membrane of dead cells that have lost their membrane integrity and bind to phosphatidlserine in the interior of the cell. These dead cells however will also stain with the nuclear stains DAPI or PI. Consequently, Annexin V-/DAPI- cells were judged to be viable, AnnexinV+/DAPI- cells were considered to be undergoing apoptosis (early apoptotic phase), and Annexin V+/DAPI+ cells were considered late apoptotic or necrotic, and recorded as dead. FlowJo® software was used to analyse all data.

2.10.2 Death receptor expression

For analysis of DR4 and DR5 expression on cell surface cells were stained with PE-conjugated antibody (DR4 - BioLegend Cat# 307205, DR5 - BioLegend Cat# 307405, Isotype control - Biolegend #400112). FlowJo® software was used to analyse all data.

2.11 Human tumour explants

2.11.1 Mesothelioma tumour explants

Appropriate ethical approval was obtained from the local Research Ethics Committee at the University of Leicester to carry out this work. The diagnosis of MPM was confirmed histologically for all patients prior to consent and surgery. Patients underwent pleurectomy, following which primary pleural tissue was sectioned into fragments measuring approximately 2 mm^3. These tissue explants were cultured in 50% neurobasal and 50% DMEM:F12, supplemented with B27 (2%), EGF (20 ng/ml) and FGF (10 ng/ml). After 24 hours the explants were treated with rTRAIL (vehicle, 50 ng/ml, 100 ng/ml or 200 ng/ml) for a further 24 hours, following which explants were fixed for immunohistochemistry (**Figure 2-4**). The explants were fixed in 10% neutral-buffered formalin (NBF) for 24 hours and then transferred into 70% ethanol

followed by paraffin embedding. Subsequently, 5µm sections were used for immunohistochemistry, as described below.

2.11.2 Explant immunohistochemistry

Cleaved PARP primary antibody (Abcam) was used at a 1:6000 dilution and the rabbit-specific HRP/DAB (ABC) detection IHC kit (Abcam) was used for immunohistochemistry, according to the manufacturer's instructions. Sections were counterstained with haematoxylin and mounted using Vectamount permanent mounting media (Vector Labs, Peterborough, United Kingdom). Images were taken at 40x magnification on a Hamamatsu Nanozoomer Digital slide scanner. Cleaved PARP-positive cells were scored as the percentage of cells with nuclear staining (**Figure 2-4**). Sections were also stained for BAP1 as described in 2.2.3.

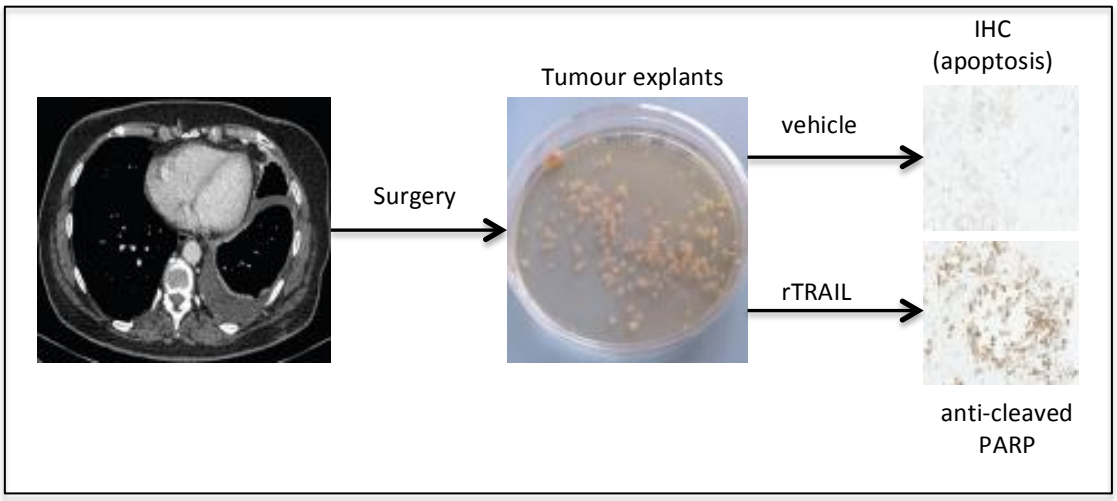


Figure 2-3 Generation of human MPM explants

Tumour explants were obtained by cutting primary pleural tissue from patients with MPM who underwent pleurectomy into fragments of approximately 2 mm3. The explants were treated with vehicle or rTRAIL (50 ng/ml, 100 ng/ml or 200 ng/ml) for 24 hours, following which time explants were fixed and stained for cleaved-PARP (a marker of apoptosis).

2.12 Statistics

Statistical analysis was performed using GraphPad Prism (GraphPad Software, CA, USA) and Microsoft Excel. Student's t-test was used to analyse differences between two groups whilst the analysis of variance (ANOVA) test with a Tukey post-hoc analysis was used to analyse differences between three groups. For multiple groups measured over multiple time points repeated measures ANOVA was used. Results were considered statistically significant for $p \leq 0.05$. All *in vitro* tests were performed in triplicate and all data are represented as mean values ± standard error of mean unless otherwise stated.

CHAPTER III:

RESULTS I

3 RESULTS I: BAP1 IN PRIMARY TUMOUR TISSUE

The work completed by K. Kolluri and C. Alifrangis presented in the introduction is highly supportive of loss of BAP1 function as a potential biomarker for rTRAIL sensitivity. This work was conducted entirely on established MPM cell lines [ref]. While a pragmatic model for initial validation and mechanistic work, cell lines suffer from a number of limitations but chiefly that the molecular makeup may differ significantly from the tissue of origin. In view of this, I aimed to assess loss of BAP1 function and its impact on rTRAIL sensitivity in primary tumour tissue. I secured access to three types: (1) formalin fixed, paraffin embedded (FFPE) tumour blocks and tissue sections collected as part of a prospective UK clinical trial (the MSO1 trial) [138], (2) early passage cell cultures from Mesobank [139], a UK based mesothelioma biobank and (3) FFPE tissue sections from tumour explants generated at the University of Leicester from tumour resected at pleurectomy [152].

In this chapter I use immunohistochemical assessment of nuclear BAP1 expression as a surrogate for *BAP1* molecular status to determine the prevalence of loss of BAP1 function in MPM and if there is an associated clinical phenotype in these primary tumour tissue samples. I treat early passage cell cultures and tumour explants (in collaboration with Professor Dean Fennell's laboratory at the University of Leicester) with rTRAIL to validate loss of BAP1 function as a clinical biomarker for rTRAIL sensitivity. I also aim to determine if loss of BAP1 function also predicts sensitivity to systemic cytotoxic chemotherapy. Patients in the MSO1 trial were treated with single agent vinorelbine (V) or combination mitomycin, vinblastine and cisplatin (MVP), which allowed me to determine if there was any correlation between loss of BAP1 function in tumours and outcomes of patients treated with these regimes. I also assess the response of MPM cell lines to treatment with the current first line agents for MPM, cisplatin and pemetrexed to determine if there is an association with loss of BAP1 function.

3.1 Nuclear BAP1 expression as a surrogate for *BAP1* molecular status in primary MPM tissue in the UK

Using Sanger sequencing, somatic *BAP1* mutations were initially identified in 23-36% of MPM biopsies in the US [30, 33]. The mutations identified were predicted to result in loss of BAP1 expression or in loss or inactivation of the nuclear localisation signal (NLS) or deubiquitinase catalytic site that also prevents the auto-deubiquitination of BAP1 necessary for nuclear localisation [42]. Immunohistochemical analysis of a cohort of 123 MPM biopsies from Japan however revealed a loss of BAP1 nuclear staining in 60% suggesting a higher proportion of MPM tumours with loss of BAP1 function than that identified by Sanger sequencing (as its functions are predominantly nuclear) [153]. Using an integrated molecular approach (including Sanger sequencing, multiplex ligation-dependent probe amplification (MLPA), TaqMan copy number analysis, messenger RNA sequencing and promotor and whole gene methylation analysis) [35] in a cohort of 22 tumour samples, Carbone *et al* demonstrated that the rate of *BAP1* mutations was indeed significantly higher than that identified by Sanger sequencing alone, and much closer to that implied by immunohistochemistry (IHC). While able to identify point mutations and small deletions, Sanger sequencing failed to identify larger exon losses and gains, reliably detected by MLPA. Both Sanger sequencing and MLPA failed to identify abnormal splicing forms, reliably detected by RNA sequencing. Sanger sequencing alone only identified *BAP1* mutations in 6/22 (27.3%) samples, while the integrated approach identified mutations in 14/22 (63.6%). Carbone *et al* found that all gene alterations identified by the molecular analyses resulted in loss of nuclear BAP1 expression while strong nuclear expression reliably identified all those MPM biopsies containing wild-type *BAP1*. A similar study using an integrated molecular approach by Koopmans *et al* also identified a strong association between loss of BAP1 nuclear staining and *BAP1* mutations with a sensitivity of 88% and a specificity of 97% in uveal melanoma [154]. Thus while a single molecular method is unable to identify all molecular *BAP1* aberrancies there is a strong

association with loss of nuclear BAP1 expression as assessed by IHC. Loss of nuclear BAP1 expression has subsequently been identified in 42-67% of cohorts of MPM tumour biopsies [36, 37, 85] supporting a significantly higher frequency of *BAP1* alterations in MPM than initially suggested by Sanger sequencing alone.

To date there has not been any study on BAP1 nuclear expression in primary tumour tissue derived from patients in the UK. I aimed to conduct IHC analysis of the above primary tumour tissue samples to determine the prevalence of loss of nuclear BAP1 expression, as a surrogate for *BAP1* molecular alterations.

3.1.1 Nuclear BAP1 expression in primary MPM tumours

79 tumour samples in the form of FFPE tissue blocks or mounted FFPE tissue sections from the MSO1 trial were available and suitable for IHC analysis. Tissue sections were mounted from the blocks and 79 sections were then stained for BAP1 using an automated staining protocol. Each tissue section was independently reviewed by two consultant histopathologists for the presence or absence of nuclear staining for BAP1. As these tumour biopsies were originally taken in 2003-2006 and stored at a facility outside our laboratory they were also assessed for the presence of a positive internal control in the form of lymphocyte staining to minimise confounding from false negatives owing to poor sample quality. Of the 79 samples 60 harboured a positive internal control and there was 100% concordance between the 2 scorers for these 60 samples that were carried forward for further analysis.

47 of the 60 samples were scored as lacking nuclear staining for BAP1, indicating a BAP1 LOF frequency of 78% in this cohort of tumour biopsies taken from patients in the UK. This is somewhat higher than the proportion reported in published studies outside the UK (**Figure 3-1**).

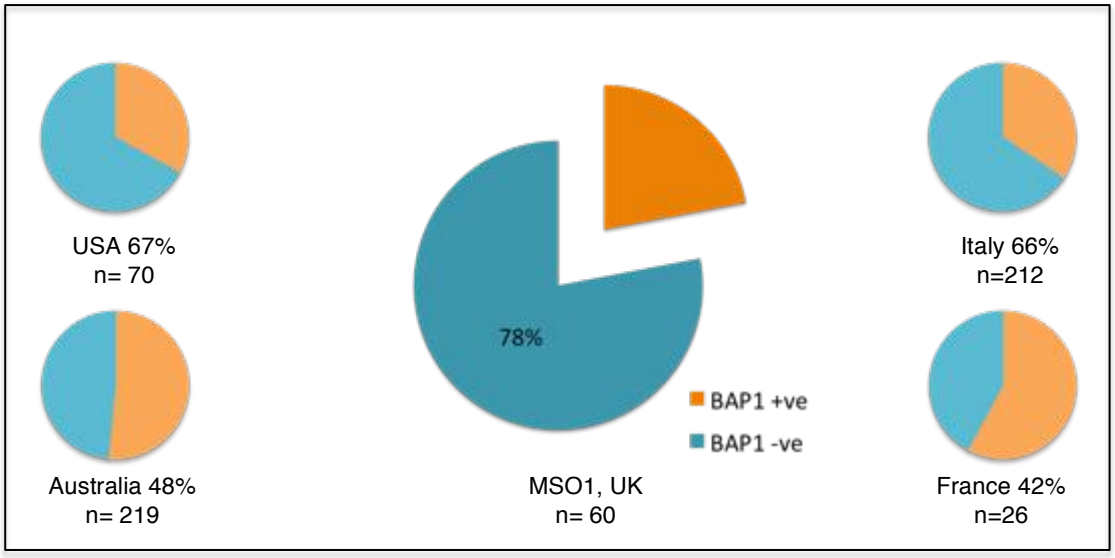


Figure 3-1 BAP1 nuclear staining in human MPM tumours

Pie charts of the proportion of MPM tumours with positive or negative nuclear BAP1 expression in the MSO1 cohort compared to cohorts previously assessed in studies in other countries. [35-37, 85]. The proportion of BAP1 negative in this study is higher than that identified in previous studies.

3.1.2 Nuclear BAP1 expression in early passage MPM cultures

Cell pellets from 25 early passage MPM cultures from the UK biobank Mesobank were generated, formalin fixed and paraffin embedded, sectioned and mounted onto slides. These cell pellet sections were assessed for nuclear BAP1 expression as above. In addition to this method of analysis for BAP1 expression, protein lysates were extracted from these cell cultures and immunoblot analysis for BAP1 was conducted to determine if there was concordance between immunoblot and IHC analysis for BAP1 expression. I hypothesised there may be a discrepancy between the two as immunoblot analysis would still identify BAP1 expression in the case of LOF mutations that maintained full length *BAP1* (e.g. point mutations, splice variants) whereas loss of BAP1 nuclear expression is seen in all *BAP1* LOF mutations in MPM [35].

Immunoblot analysis identified BAP1 expression in 17 of the 25 cell culture lysates (68%)(**Figure 3-2**) while IHC analysis identified nuclear BAP1 expression in 13 of the 25 cell cultures (52%)(**Figure 3-3**). The cell cultures

can therefore be place in one of three groups – (1) +ve immunoblot expression/ +ve nuclear expression, (2) +ve immunoblot expression/ -ve nuclear expression and (3) –ve immunoblot expression/ -ve nuclear expression. There was concordance between immunoblot and IHC expression for all cell cultures except 8T, 7T, 23T and 36, all of which expressed BAP1 on immunoblot but not on nuclear staining.

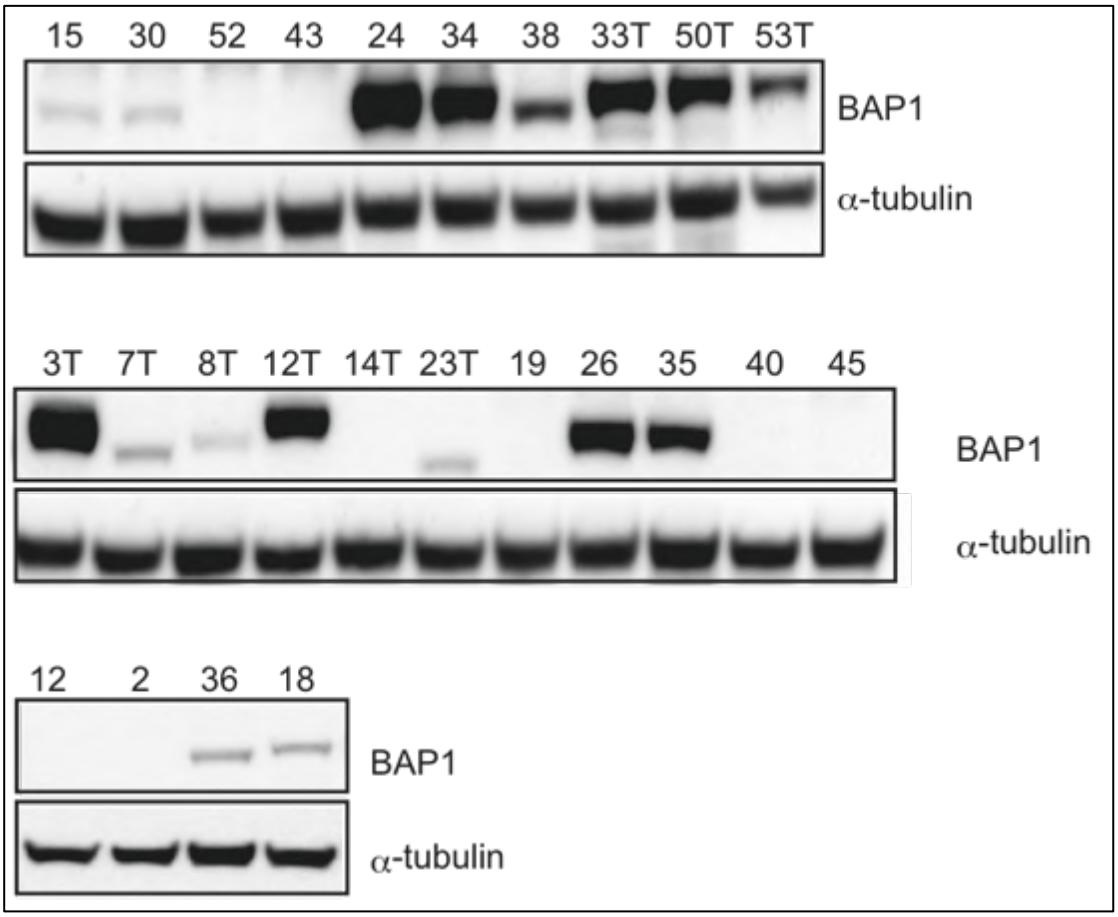
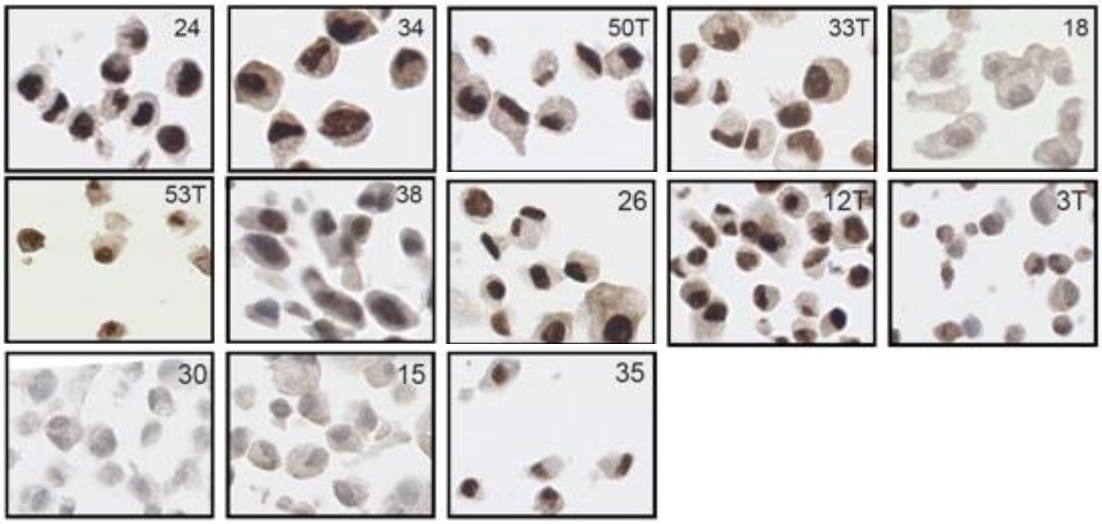


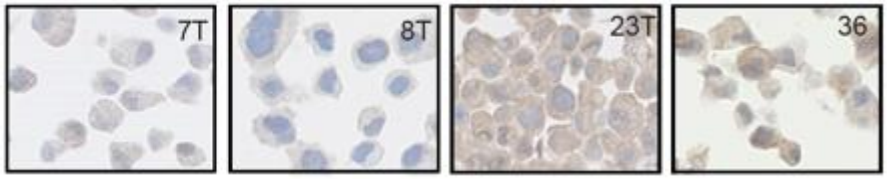
Figure 3-2 Immunoblot for BAP1 in protein lysates from early passage MPM cultures

*Protein lysates from 25 early passage MPM cultures were subjected to immunoblotting for BAP1. BAP1 expression was identified in 17 of the 25 cell culture lysates (68%). IHC analysis identified nuclear BAP1 expression in 13 of the 25 cell cultures (52%) (**Figure 3-3**). There was concordance between immunoblot and IHC expression for all cell cultures except 8T, 7T, 23T and 36, all of which expressed BAP1 on immunoblot but not on nuclear staining.*

Immunoblot +ve/ Nuclear BAP1 +ve



Immunoblot +ve/ nuclear expression –ve



Immunoblot –ve/ nuclear expression -ve

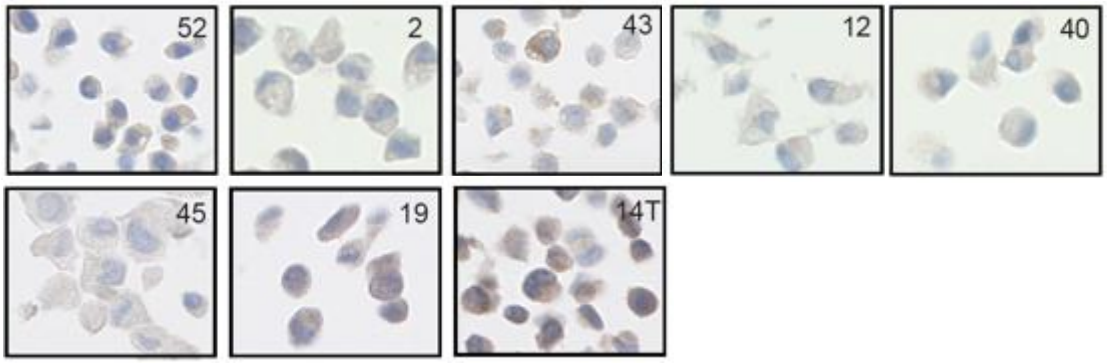


Figure 3-3 BAP1 immunohistochemistry in early passage MPM cultures

*Immunoblot analysis identified BAP1 expression in 17 of the 25 cell culture lysates (68%)(**Figure 3-2**) while IHC analysis identified nuclear BAP1 expression in 13 of the 25 cell cultures (52%)(**Figure 3-3**). The cell cultures can therefore be place in one of three groups – (1) +ve immunoblot expression/ +ve nuclear expression, (2) +ve immunoblot expression/ -ve nuclear expression and (3) –ve immunoblot expression/ -ve nuclear expression. There was concordance between immunoblot and IHC expression for all cell cultures except 8T, 7T, 23T and 36, all of which expressed BAP1 on immunoblot but not on nuclear staining.*

3.1.3 Nuclear BAP1 expression in human MPM explants

I collaborated with Dr S. Busacca in Professor Dean Fennell's laboratory at the University of Leicester to assess nuclear BAP1 expression in human tumour explants. S. Busacca generated the tissue explants at the University of Leicester from tissue resected at pleurectomy. Tumour tissue was cut into fragments and cultured as tissue explants. These explants were then formalin fixed, paraffin embedded, sectioned and mounted onto slides. I then conducted immunohistochemical analysis of nuclear BAP1 expression as above (**Figure 3-4**).

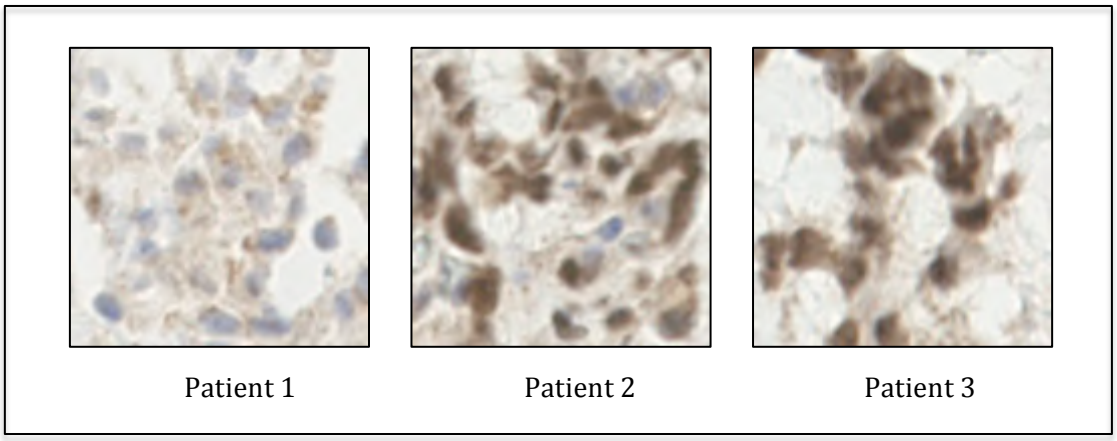


Figure 3-4 BAP1 immunohistochemistry of human MPM explants

Three human tumour explants were generated and stained for BAP1 expression. One of the three explants (patient 1) lacked nuclear expression of BAP1.

Three human tumour explants were generated one of which (patient 1) lacked nuclear expression of BAP1.

3.2 BAP1 and clinical characteristics in MPM

Using BAP1 nuclear expression as a surrogate for *BAP1* molecular status I aimed to determine if this correlated with any clinical characteristics in MPM in the MSO1 cohort and Mesobank cell cultures. As I had access to data for only three tumour explants this analysis was not conducted on the explants, as the sample size was not large enough to infer statistical significance.

3.2.1 Nuclear BAP1 expression and clinical characteristics in MPM from the MSO1 trial

The clinical data collected as part of the MSO1 trial included the gender and age at diagnosis of the patients and the histological subtype of the tumour. I used this data to determine if there was any correlation with BAP1 nuclear expression.

Table 3-1. Clinical characteristics in tumours with and without nuclear BAP1 expression

	Nuclear BAP1 IHC positive (N=13)	**Nuclear BAP1 IHC negative (N=47)**	**p-value**
Gender (M=male)	M: 100%	M: 91%	0.22
Median age at diagnosis (years)	68.8	66.0	0.80
Histology			0.31
Epithelioid	77% (10)	89% (42)	
Biphasic	23% (3)	9% (4)	
Sarcomatoid	0% (0)	2% (1)	
Treatment			0.81
Active symptom control (ASC)	31% (4)	40% (19)	
ASC + vinorelbine	31% (4)	26% (12)	
ASC + mitomycin, vinblastine, cisplatin	38% (5)	34% (16)	

A chi-squared test was used to determine if there was a difference in gender, histological subtypes and treatment received between the two groups and a 2-sided t-test for comparison of the age at diagnosis. There was no statistically significant difference in gender, age at diagnosis, histological subtype or

treatment received between those tumours with and without nuclear BAP1 expression (**Table 3-1**).

3.2.2 BAP1 and clinical characteristics in early passage MPM cultures

Clinical data regarding the sex and age at diagnosis of the patients and the histology of the tumours from which the early passage cell cultures were derived was available from Mesobank. I used this data to determine if nuclear BAP1 expression correlated with any of these characteristics.

A chi-squared test was used to determine if there was a difference in gender and histological subtypes between the two groups and a 2-sided t-test for comparison of age at diagnosis. There was no statistically significant difference in gender, age at diagnosis and histological subtype between those tumours with and without nuclear BAP1 expression (**Table 3-2**).

Table 3-2. Clinical characteristics in early passage cultures with and without nuclear BAP1 expression.

	Nuclear BAP1 IHC positive (N=13)	**Nuclear BAP1 IHC negative (N=12)**	**p-value**
Gender (M=male)	M: 85%	M: 83%	0.93
Median age at diagnosis (years)	64.0	64.0	0.82
Histology			0.35
Epithelioid	54% (7)	58% (7)	
Biphasic	31% (4)	42% (5)	
Sarcomatoid	15% (2)	0% (0)	

3.3 BAP1 as a biomarker in primary MPM tissue

As noted above, the data presented in the introduction from K. Kolluri and C. Alifrangis that initially identified loss of BAP1 function as a biomarker for rTRAIL sensitivity was conducted entirely on established cell lines. I therefore aimed to further validate loss of BAP1 function as a biomarker for rTRAIL sensitivity in primary tumour tissue to strengthen the translational applicability of this finding. As noted in the introduction, loss of BAP1 function has also been identified as a possible biomarker for sensitivity to a number of drugs in MPM including PARP, HDAC and EZH2 inhibitors. I therefore also aimed to determine if loss of BAP1 function/ nuclear expression correlated with response to systemic cytotoxic chemotherapy using the data collected prospectively as part of the MSO1 trial.

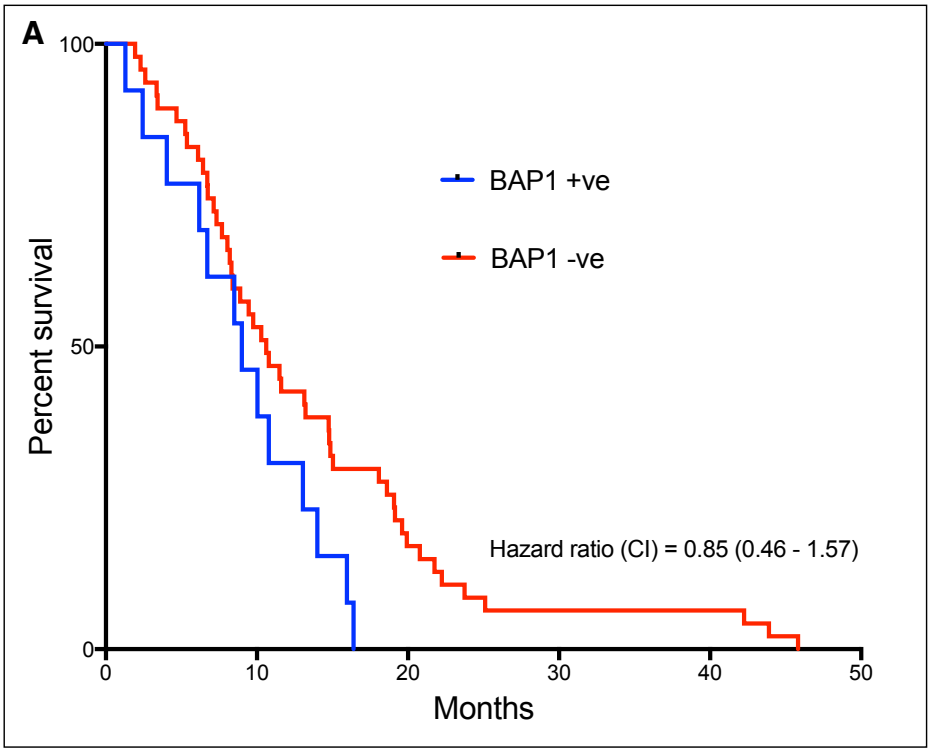
3.3.1 BAP1 as a biomarker for systemic cytotoxic chemotherapy

3.3.1.1 BAP1 and response to treatment with vinorelbine or mitomycin, vinblastine and cisplatin

The MSO1 cohort was taken from a prospective three-armed randomised clinical trial in which patients were treated with active symptom control (ASC), single agent vinorelbine (V) and ASC, or combination chemotherapy with mitomycin, vinblastine and cisplatin (MVP) and ASC [138]. Data was prospectively collected on overall survival. I therefore aimed to determine if loss of nuclear BAP1 expression was predictive of response to these two systemic cytotoxic chemotherapeutic regimens.

The 60 tumour samples that had been stained for BAP1 expression in **3.3.1** were used for this analysis. Kaplan Meier analysis of all patients, irrespective of treatment arm, revealed no significant difference in survival between patients with tumours with positive or negative nuclear BAP1 expression overall (**Figure 3-5A**). When stratified by treatment arm – ASC, ASC + V and ASC + MVP – no significant difference in median survival was identified

between those patients with BAP1 positive or BAP1 negative tumours within any treatment arm (**Figure 3-5B**).



B

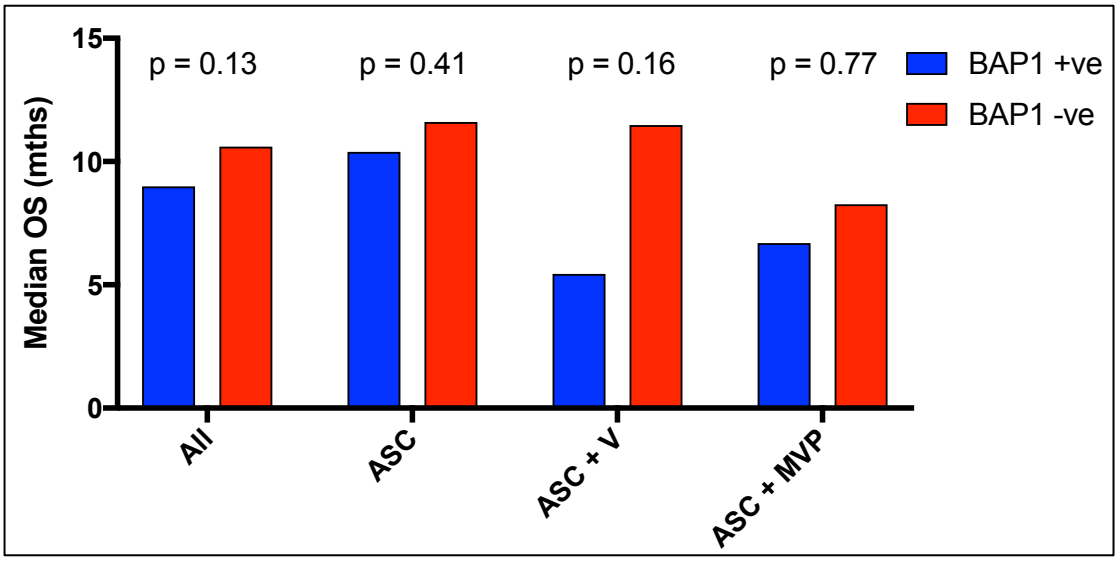


Figure 3-5 Nuclear BAP1 expression and survival in patients with MPM from the MSO1 trial

(A) *Kaplan Meier survival curve of all patients in the MSO1 trial stratified by BAP1 nuclear expression.* ***(B)*** *Median survival in months of all patients and patients within each treatment arm stratified by nuclear BAP1 expression. ASC – active symptom control, V + ASC – vinorelbine plus active symptom control, MVP + ASC – mitomycin, vinblastine and cisplatin plus active symptom control.*

3.3.1.2 Loss of BAP1 function does not predict sensitivity to cisplatin or pemetrexed

The chemotherapy regimes used in the MSO1 trial are no longer first line treatments for MPM. I therefore assessed if loss of BAP1 function predicted response to the current first line chemotherapeutic agents for MPM, cisplatin and pemetrexed. I treated 4 *BAP1* mutant and 5 *BAP1* wild-type MPM lines with a dose range of cisplatin or pemetrexed and measured the cell viability at 72 hours with an XTT assay (**Figure 3-6**). There was no obvious correlation between *BAP1* status and sensitivity to these agents among the lines tested.

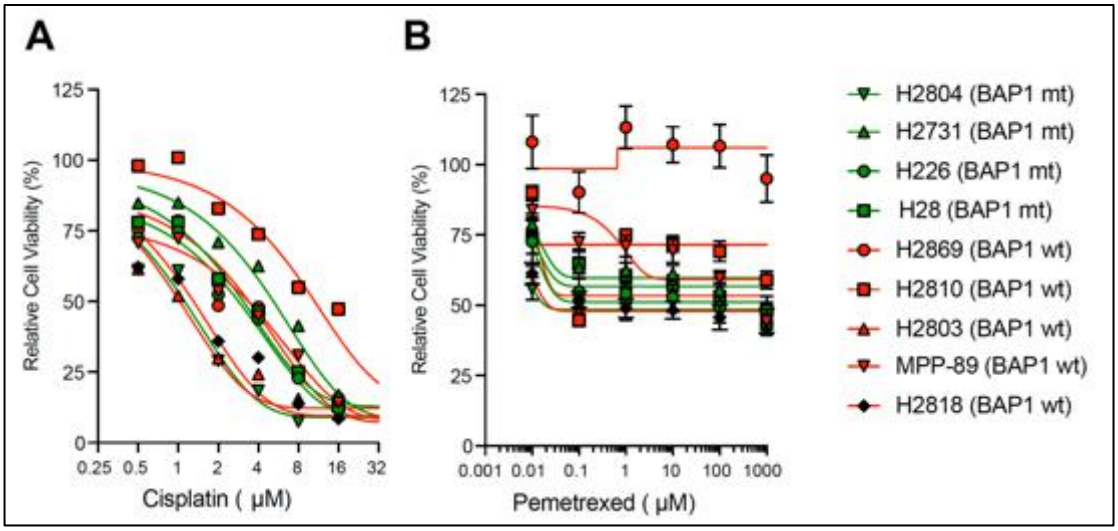


Figure 3-6 Relative cell viability of MPM cells treated with cisplatin and pemetrexed

*72-hour cell viability results for 9 MPM cell lines (4 BAP1 mutant - green and 5 BAP1 wild-type - red) treated with **(A)** cisplatin **(B)** pemetrexed*

3.3.2 BAP1 as a biomarker for rTRAIL in early passage MPM cultures

25 early passage MPM cultures were treated with a dose range of rTRAIL and cell viability at 72 hours measured to determine sensitivity (**Figure 3-7A**). The cells were categorised by their response as sensitive, partially sensitive or resistant to rTRAIL if treatment with rTRAIL resulted in <50%, 50-75% or > 75% cell viability at a dose of 50ng/ml rTRAIL respectively, above which dose minimal dose response was observed.

These MPM cultures had previously had their nuclear BAP1 expression status determined by immunohistochemistry in **3.1.2** thus allowing analysis of an association between BAP1 expression and rTRAIL sensitivity (**Figure 3-7B**). MPM cultures with negative nuclear BAP1 expression demonstrated significantly lower mean cell viability in response to 50ng/ml rTRAIL treatment than those with positive nuclear BAP1 expression.

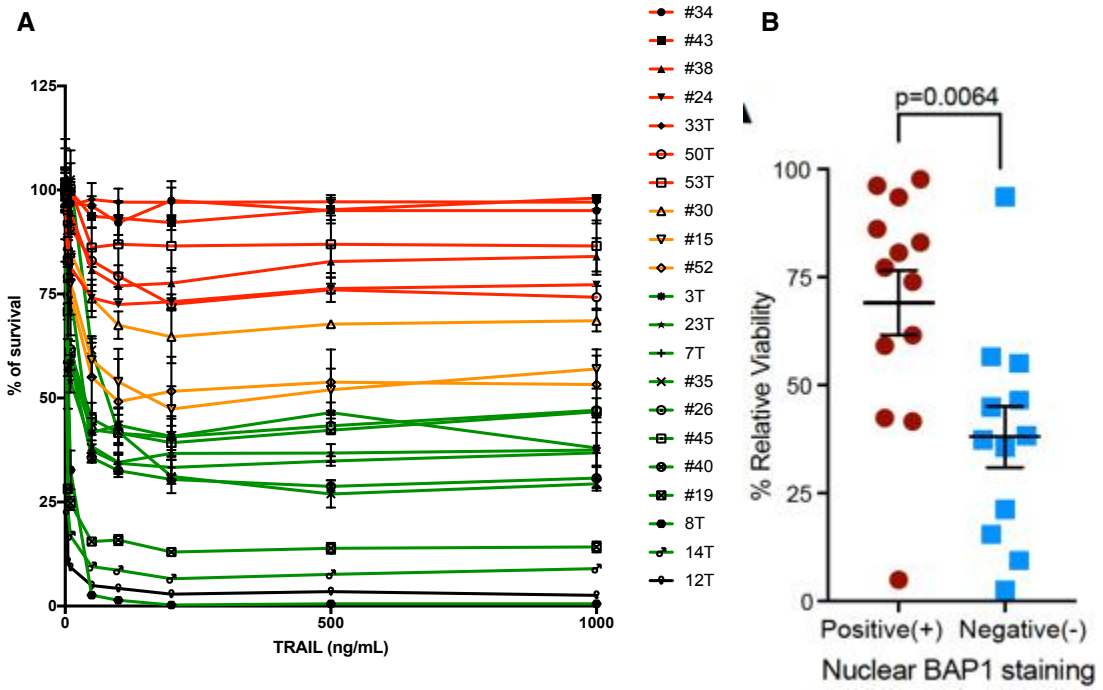


Figure 3-7 rTRAIL treatment of early passage MPM cultures

(A) *Early passage MPM cultures were treated with a dose range of rTRAIL and cell viability measured at 72h. Cells were categorised by response as sensitive (green - <50% survival at 50ng/ml), partially sensitive (orange – 50-75% survival at 50ng/ml) or resistant (red - >75% survival at 50ng/ml). 21 lines are shown.* ***(B)*** *72h cell viability of early MPM cultures treated with 50ng/ml rTRAIL stratified by positive nuclear BAP1 staining (n=13) or negative nuclear BAP1 staining (n=12) as assessed by immunohistochemistry.*

3.3.3 BAP1 as a biomarker for rTRAIL in human MPM explants

The tumour explants generated by S. Busacca at the University of Leicester were used to further validate loss of BAP1 function as a biomarker for rTRAIL sensitivity. Tumour resected at pleurectomy was cut into fragments and cultured as tissue explants. After 24 hours these explants were treated with a

dose range of rTRAIL or vehicle for a further 24 hours following which the explants were formalin fixed and paraffin embedded for immunohistochemistry. To quantify the apoptotic response to treatment sections of the treated explant were stained with anti- cleaved PARP antibody and the percentage of tumour cells with positive nuclear cleaved PARP staining calculated by a histopathologist. Sections of untreated explant were also stained for nuclear BAP1 expression as above (**Figure 3-8**).

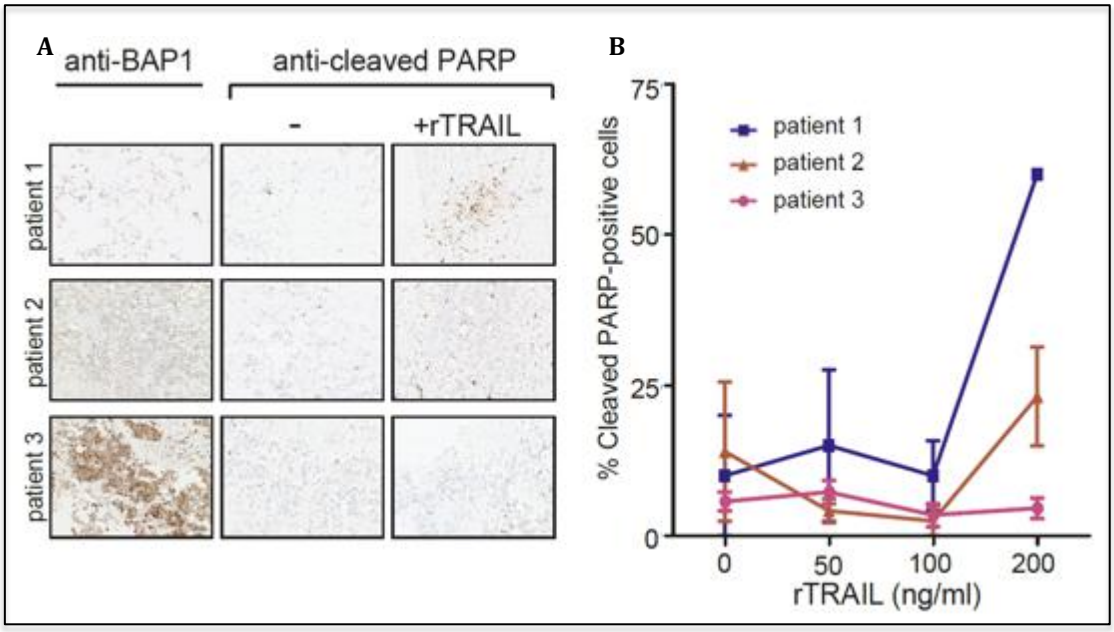


Figure 3-8 rTRAIL treatment of human MPM tumour explants

(A) *Tumour explants derived from three patients and treated with vehicle (-) or rTRAIL (+rTRAIL) for 24h. Explants were stained with anti-BAP1 and anti-cleaved PARP antibodies with and without rTRAIL treatment.* ***(B)*** *The percentage of cleaved PARP-positive cells in tumour explants treated with a dose range of rTRAIL for 24 hours was scored. These data were provided by Professor Dean Fennell's laboratory.*

Of the three tumour explants, those from patients 2 and 3 expressed nuclear BAP1, while the explant from patient 1 did not express BAP1. The explant from patient 1 demonstrated increased levels of apoptosis, as measured by percentage of cells that express cleaved PARP, compared to the levels of apoptosis seen in the explants from patients 2 and 3.

3.4 Discussion

3.4.1 There is a high prevalence of loss of BAP1 nuclear expression in MPM in the UK

This study has identified that 78% of MPM tumours resected from a cohort of patients in the UK harbour loss of nuclear BAP1 expression and would be targets for a BAP1 biomarker driven therapy. As highlighted in **Figure 3-1** this proportion is higher than the proportion identified by previous studies from cohorts outside the UK. A number of explanations are possible. The higher proportion of MPM tumours that harbour loss of nuclear BAP1 expression in this cohort may be due to the specifics of this population being from the UK. For example, the type of asbestos to which patients in the UK are more likely to have been exposed relative to populations in the US, Japan, Australia and mainland Europe may have had an effect. Evidence supports an association between asbestos exposure and *BAP1* mutations [155], however no study to date has investigated a possible association between the type of asbestos and *BAP1* mutations. Methodological issues can also not be excluded. As noted above the MPM tumours taken from the MSO1 trial were resected between 2003-2006 and degradation of tissue quality could have affected immunohistochemical analysis. I did however attempt to minimise confounding from this by only including those tissue specimens that harboured an internal positive control in the form of positive lymphocyte staining. Finally the specifics of IHC protocols vary between studies, which may affect results, however all studies in **Figure 3-1** used the same antibody as that used in this study.

48% of the early passage MPM cultures analysed were also found to have lost nuclear BAP1 expression. This is more in keeping with results from other studies (**Figure 3-1**). The advantage of the use of BAP1 IHC as the method to identify MPM tumours with loss of BAP1 function is highlighted by the results from IHC and immunoblot analysis of the MPM early passage cell cultures

(**Figure 3-3**). Four of the cultures (8T, 7T, 23T and 36) exhibited BAP1 expression on immunoblot however IHC revealed that this full length BAP1 was not expressed in the nucleus. The BAP1 antibody used (Santa Cruz C-4) is a monoclonal antibody raised against amino acids 430-729 of BAP1, i.e. the C- terminus and will therefore identify BAP1 that retains the NLS. It may be that these cell cultures harbour point mutations that inactivate the NLS or that inactivate the catalytic subunit that prevents BAP1 auto-deubiquitination (required for nuclear localisation) thus resulting in full length BAP1 (imaged on immunoblot) but sequestered in the cytoplasm. Integrated molecular sequencing of these cell cultures would resolve this issue but was prohibitively expensive for this thesis. As the functions of BAP1 are nuclear, this result emphasises the advantage of using BAP1 IHC as a method to identify MPM tumours with loss of BAP1 function. Determination of the molecular status of *BAP1* to identify MPM that harbour loss of BAP1 function can be reliably achieved by an integrated molecular approach to sequencing as described above [35]. This approach however is expensive and time consuming and IHC is an easy, cheap and validated surrogate [35]. As this study, and others, propose loss of BAP1 function to act as a biomarker for sensitivity to targeted agents it seems likely that BAP1 IHC will be the tool by which MPM tumours are stratified for a BAP1 biomarker driven therapy.

3.4.2 Nuclear BAP1 expression is not associated with a clinical phenotype

To date no distinct clinical phenotype has been associated with *BAP1* mutations in MPM. Somatic *BAP1* mutations have however been demonstrated to be associated with a higher risk of metastasis in uveal melanoma [156] and higher-grade tumours and shorter survival in clear cell renal carcinoma [157]. I found no significant correlation between age at diagnosis, sex and histological subtype and loss of BAP1 expression. This is consistent with results from Carbone *et al,* who in a cohort of 70 MPM tumours also did not find significant relationships between loss of nuclear BAP1 expression and sex, age at diagnosis, ethnicity and history of asbestos

exposure [35]. Zauderer *et al* also analysed 121 MPM tumours that had been tested for *BAP1* mutation by Sanger sequencing to determine if *BAP1* mutation status was associated with any of age, sex, histology, stage, smoking status, asbestos exposure and overall survival [34]. A history of smoking was significantly more common in patients whose tumours that harboured a *BAP1* mutation however no other clinical feature was significantly different among those with and without *BAP1* mutations. This study however suffers from the limitation of relying solely on Sanger sequencing to identify *BAP1* mutations and thus is likely to have misclassified a number of mutant tumours as wild-type as detailed above.

3.4.3 Nuclear BAP1 expression does not predict response to systemic cytotoxic chemotherapy

As highlighted in the introduction, preclinical data from the Janes' lab suggests loss of BAP1 function augments rTRAIL sensitivity. Published preclinical data also supports loss of BAP1 function as a potential therapeutic biomarker for EZH2, HDAC and PARP inhibitors [89, 91]. In view of this, and in view of the high prevalence of loss of BAP1 function in MPM, I aimed to first determine if loss of BAP1 function also sensitises to systemic cytotoxic chemotherapy. I first assessed this in primary tumours treated with single agent vinorelbine or combination chemotherapy with mitomycin, vinblastine and cisplatin (MVP) using samples and data from the MSO1 trial [138]. I found no significant difference in overall survival between those patients with tumours that did and did not express nuclear BAP1 in the cohort as a whole. I also found no significant difference in overall survival between those patients with tumours that did and did not express nuclear BAP1 when treated with either single agent vinorelbine or with combination MVP or in the untreated cohort. This data suggests that loss of nuclear BAP1 expression does not predict response to systemic cytotoxic chemotherapy in the form of vinorelbine or MVP. As I did not have access to primary tumour tissue and data from patients treated with the current first line chemotherapeutic agents, cisplatin and pemetrexed, I treated MPM lines with these agents. The results

support that there is no sensitising association between cisplatin or pemetrexed and loss of BAP1 function. There have been no published studies to date that have assessed BAP1 as a biomarker for sensitivity to systemic cytotoxic chemotherapy regimens.

3.4.4 Loss of nuclear BAP1 expression predicts sensitivity to rTRAIL

I also aimed to validate loss of BAP1 function as a biomarker for rTRAIL sensitivity in primary tumour tissue. Sensitivity to rTRAIL was found to be significantly higher in early passage MPM cultures that do not express nuclear BAP1 than in those that do express BAP1 in the nucleus consistent with data from established cell lines presented by K. Kolluri (**Figure 3-7**). I also collaborated with Dr S. Busacca in Prof Dean Fennell's laboratory in the University of Leicester who has expertise in the use of tumour explants as a preclinical model by which to assess novel therapies in MPM [158]. Of the three tumour explants successfully generated by S. Busacca, the explant that did not express nuclear BAP1 demonstrated higher levels of apoptosis in response to rTRAIL than the two explants that did express nuclear BAP1. While admittedly this experiment is underpowered for statistical inference, it remains an exciting finding supportive of loss of BAP1 function as a biomarker for sensitivity to rTRAIL and does not contradict the findings from the other preclinical models.

Thus data from primary tumour tissue presented in this thesis support the use of loss of BAP1 function as a biomarker for sensitivity to rTRAIL in MPM. The next step would be to run a clinical trial in patients to determine the true translational applicability of this finding. Review of tumour tissue from previous clinical trials of rTRAIL for BAP1 expression would also be a pertinent act.

3.5 Summary

- There is a high prevalence of loss of nuclear BAP1 expression in cohorts of tumour samples (78%) and early passage cell cultures (48%) from the UK.
- Loss of nuclear BAP1 expression is not associated with gender, age at diagnosis or histological subtype in MPM tumours or early passage cultures.
- Loss of BAP1 function does not predict response to systemic cytotoxic chemotherapy
- Loss of nuclear BAP1 expression predicts sensitivity to rTRAIL in early passage MPM cell cultures.
- Loss of nuclear BAP1 expression correlates with rTRAIL sensitivity in a limited sample of MPM tumour explants.

CHAPTER IV:

RESULTS II

4 RESULTS II: VALIDATION OF BAP1 AS A BIOMARKER FOR DR AGONIST SENSITIVITY

The second aim of this thesis was to further validate loss of BAP1 function as a biomarker for sensitivity to rTRAIL and other DR agonists in MPM and other cancers. The initial validation data conducted by K. Kolluri was conducted on only two MPM cell lines (one BAP1 mutant and one wild-type), as such I aimed to further validate loss of BAP1 function as a biomarker for rTRAIL sensitivity via manipulation of BAP1 expression in further MPM lines. Furthermore the original drug screen of 15 MPM lines carried out at the WTSI included recombinant TRAIL (rTRAIL) as the only DR agonist compound. As several additional DR agonists exist I hypothesised that loss of BAP1 function would extend as a biomarker for sensitivity to other DR agonists. Loss-of-function mutations in *BAP1* are also prevalent in additional cancers including uveal melanoma, clear cell renal carcinoma and intrahepatic cholangiocarcinoma [75, 159, 160]. I therefore also aimed to determine if loss of BAP1 function sensitises additional cancer types to DR agonists.

The observation that loss of BAP1 function sensitises MPM to TRAIL also has interesting implications for sensitising *BAP1* wild-type tumours; two potential strategies emerge. Firstly pharmacological replication of the mechanism by which loss of BAP1 function induces TRAIL sensitivity could be used in a combination therapy with TRAIL to treat *BAP1* wild-type (WT) tumours. However, elucidation of the precise mechanism by which biomarkers confer their effect is often difficult and in this case unlikely to involve a single pharmacological target. An alternative strategy would be to inhibit BAP1 itself through a BAP1 inhibitor. No clinical or preclinical compounds currently exist that are known to specifically inhibit BAP1 function. A valid concern however is the effect of loss of BAP1 function on rTRAIL sensitivity in non-transformed cells. TRAIL is known to induce apoptosis in transformed but not non-transformed cells, although the mechanism is unknown. However the effect of

BAP1 inhibition on rTRAIL sensitivity in non-transformed cells is unknown. I therefore also aimed to determine the effect of BAP1 loss of function on rTRAIL sensitivity in non-transformed cells.

4.1 Expression of wild-type *BAP1* in mutant *BAP1* MPM cells decreases rTRAIL sensitivity

The H28 MPM line harbours a homozygous deletion of *BAP1* and therefore expresses no BAP1 protein, while H2804 harbours a heterozygous splice mutation predictive of loss of function and expresses mutant *BAP1* (**Figure 1-6B**). Both cell lines were found to be sensitive to rTRAIL in the WTSI MPM line drug screen (**Figure 1-6A**). I aimed to manipulate these cell lines to further validate loss of BAP1 function as a biomarker for rTRAIL sensitivity.

4.1.1 Transduction of mutant *BAP1* MPM cells with BAP1 expressing lentivirus

I transduced H28 and H2804 cells with wild-type *BAP1* (BAP1 WT) and deubiquitinase mutant *BAP1* (BAP1 C91A) expressing lentiviral constructs that had previously been generated and titrated by K. Kolluri. As H28 expresses no BAP1 protein I was able to quantify the percentage of cells successfully transduced by flow cytometry using a primary BAP1 antibody and a secondary fluorescent antibody (AlexaFluor 488). 87.1% and 91.9% of H28 cells transduced at multiplicity of infection (MOI) 3 and MOI 5 respectively were found to express BAP1 WT. 94.6% and 94.8% of H28 cells transduced at MOI 3 and MOI 5 respectively were found to express BAP1 C91A (**Figure 4-1**). H2804 however expresses a mutant BAP1 protein and therefore flow cytometry could not be conducted to determine transduction success. I therefore performed immunoblot analysis to verify successful overexpression of BAP1 WT and BAP1 C91A (**Figure 4-2**).

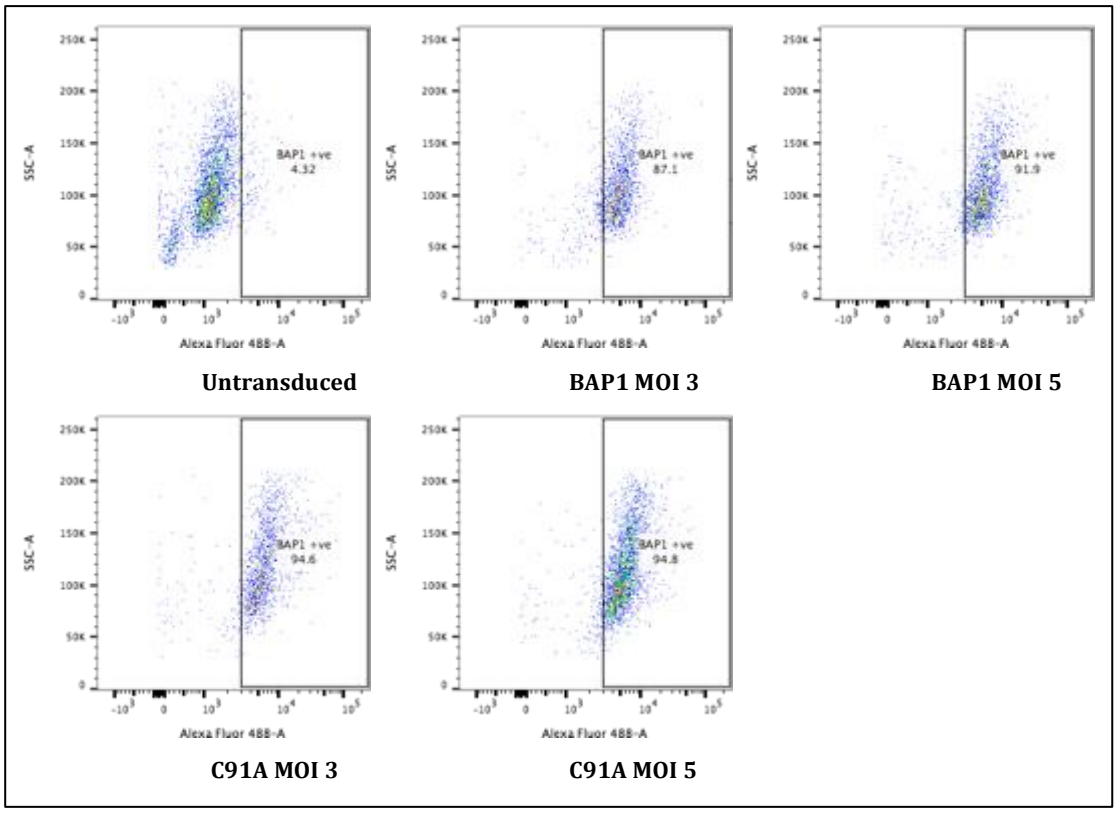


Figure 4-1 Flow cytometry of H28 MPM cells transduced with BAP1 lentiviral constructs

Untransduced, BAP1 WT (BAP1) and BAP1 C91A transduced H28 cells at MOIs 3 and 5 were stained for BAP1 with primary antibody and assessed by flow cytometry with a secondary Alexa Fluor 488 secondary antibody to determine the percentage of successfully transduced cells. As the MOI increased the percentage of successfully transduce cells increased. A Becton Dickinson LSRFortessa analyser was used.

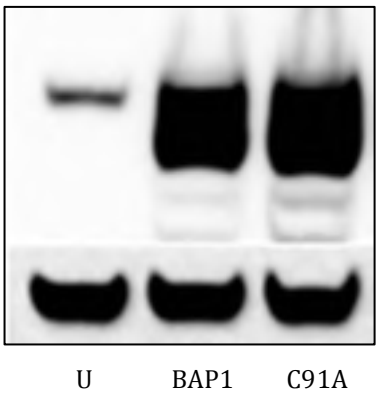


Figure 4-2 Immunblot of BAP1 in H2804 MPM cells tranduced with BAP1 lentiviral constructs

Protein lysates from untransduced, BAP1 WT and BAP1 C91A transduced H2804 cells at MOI 5 were immunoblotted for BAP1 expression. Cells transduced with BAP1 contructs express significantly higher amounts of BAP1 protein in light of an overexpression model being used.

4.1.2 Overexpression of wild-type BAP1 in mutant BAP1 MPM cells results in reduced sensitivity to rTRAIL

H28 and H2804 cells transduced with BAP1 WT and BAP1 C91A at MOI 5 were treated with a dose range of rTRAIL for 24 hours and an Annexin V/ DAPI cell death assay was performed.

Transduction of the parental H28 line with BAP1 WT resulted in a significant decrease in cell death in response to rTRAIL treatment ($p = 0.002$) while transduction with BAP1 C91A did not result in a significant change ($p=0.7375$) A significantly higher cell death response was observed in the BAP1 C91A transduced cells relative to the BAP1 WT transduced cells ($p=0.0145$) (**Figure 4-3A**).

Transduction of the parental H2804 line with BAP1 WT also resulted in a significant decrease in cell death in response to rTRAIL treatment ($p < 0.0001$) while transduction with BAP1 C91A resulted in no significant change ($p = 0.2407$). There was a significantly lower cell death response observed in the BAP1 WT transduced lines relative to the BAP1 C91A transduced lines ($p < 0.0001$) (**Figure 4-3B**).

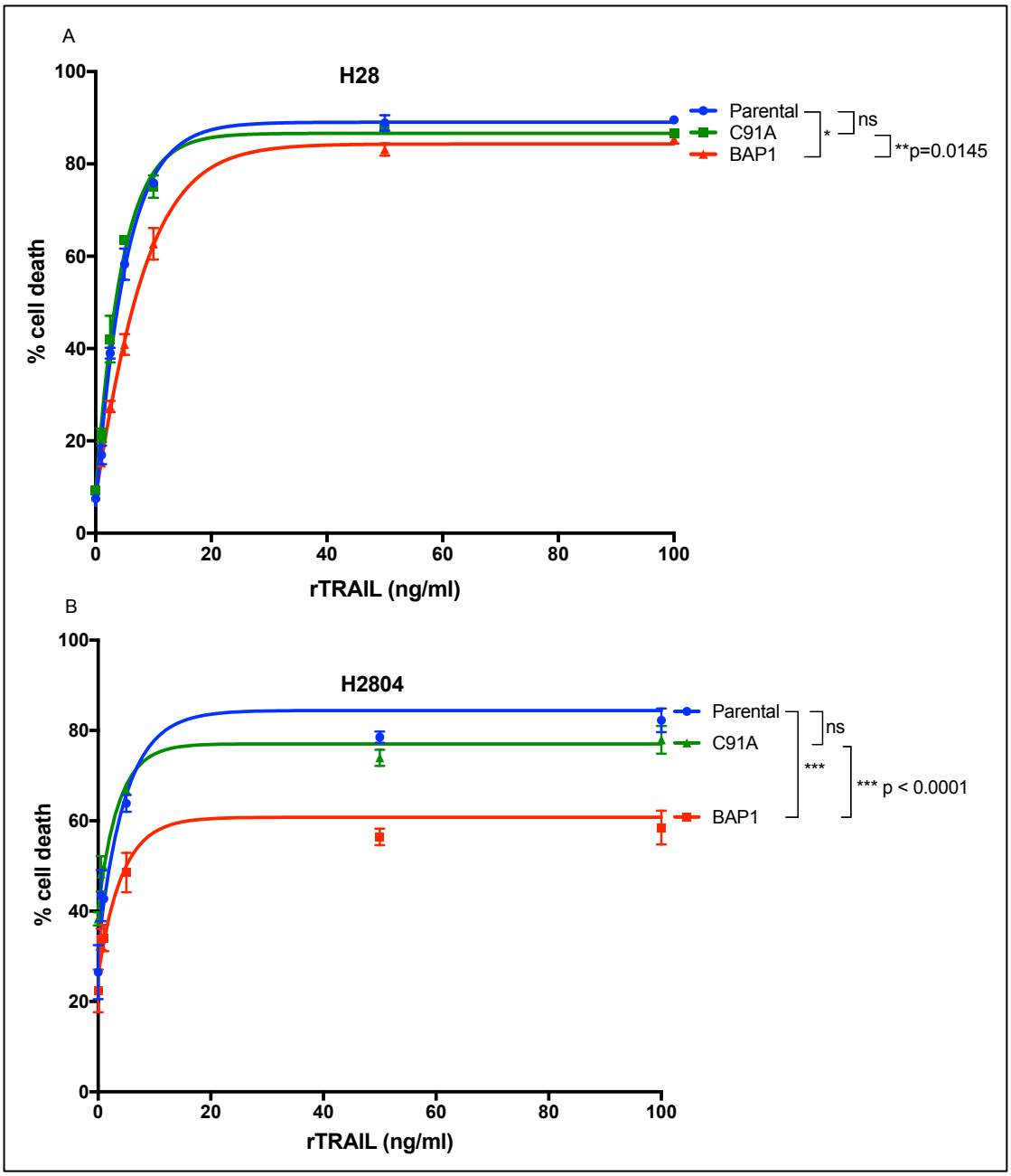


Figure 4-3 Loss of BAP1 function and response to rTRAIL treatment in BAP1 mutant MPM lines

Cell death in response to 24h treatment with rTRAIL assessed by an Annexin V/DAPI flow cytometry assay in ***(A)*** *H28 lines - at 100ng/ml rTRAIL a significant difference in parental vs BAP1 (*p= 0.002) and BAP1 vs C91A (**p = 0.0145) lines is observed* ***(B)*** *H2804 lines – parental vs C91A * p = 0.2407, parental vs BAP1 ** p = <0.0001, C91A vs BAP1 *** p = < 0.0001. Parental – untransduced, C91A – BAP1 DUB mutant transduced, BAP1 WT – wild-type BAP1 transduced.*

4.2 Knockdown of *BAP1* in wild-type *BAP1* MPM cells increases rTRAIL sensitivity

The MPP-89 and H2869 MPM lines express wild-type *BAP1* and were found to be resistant to rTRAIL treatment in the WTSI MPM line drug screen (**Figure 1-6**). I aimed to knock down *BAP1* in these cell lines as an additional model to validate loss of BAP1 function as a biomarker for rTRAIL sensitivity.

4.2.1 Titration of BAP1 shRNA lentivirus

BAP1 shRNA lentivirus was generated using a mir30-based GIPZ BAP1 shRNA plasmid (V2LHS_4147, Dharmacon) and the packaging plasmids as described in methods. The shRNA plasmid expresses a green fluorescent protein (GFP) marker to allow for selection and titration. 5 x 10^4 293T cells were plated in a 6-well plate and transduced with different dilutions of the BAP1 shRNA expressing lentivirus. The cells were grown for 48 hours, the culture media removed and the cells washed with PBS, trypsinised and transferred to FACS buffer to determine the proportion of transduced cells by flow cytometry for GFP. The virus quantity that transduced approximately 20% of cells was used to calculate the viral titre. The following equation was used:

$$\text{Viral titre} = \frac{\text{number of cells transduced X proportion of positive cells}}{\text{volume of virus in ml}}$$

0.125µL of virus transduced 22.7% of 293T cells and the viral titre was therefore determined to be 9.1 x 10^7 transduction units/ml (**Figure 4-4**).

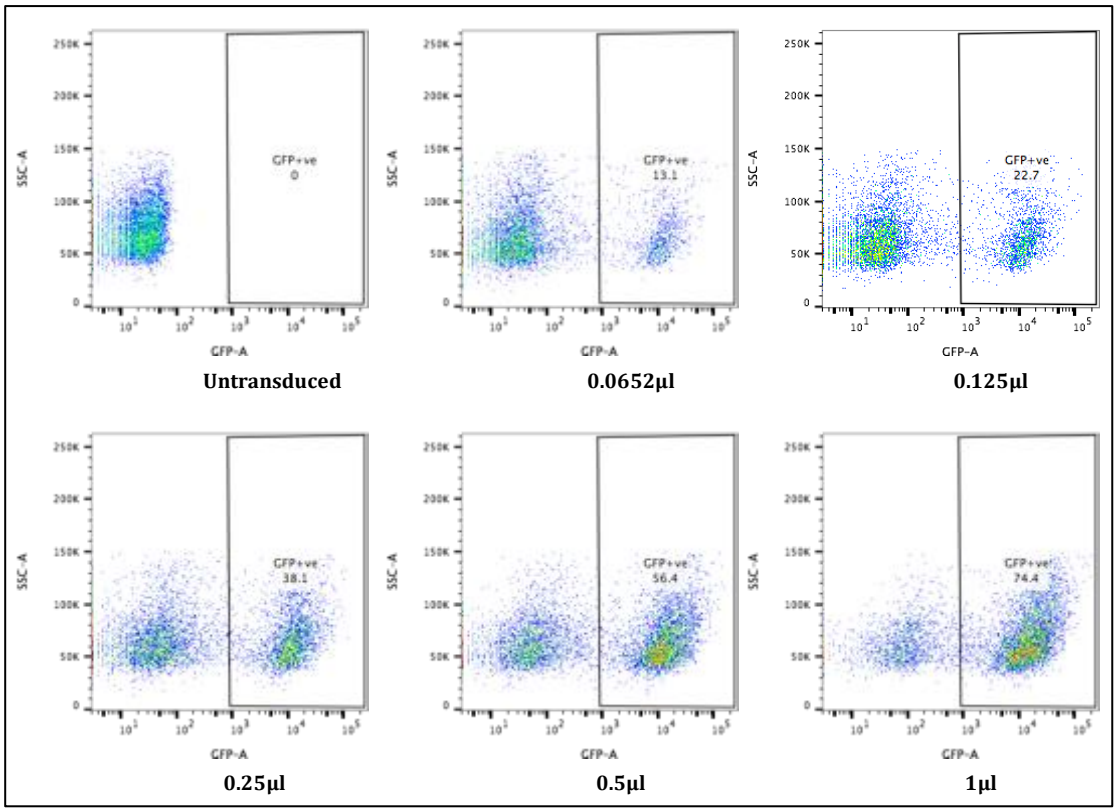


Figure 4-4 Titration of BAP1 shRNA expressing lentivirus

293T cells were transduced using a dilution series of concentrated lentiviral particles. GFP expression as determined by flow cytometry for a range of viral volumes are shown which reflects the percentage of transduced cells. As the volume of concentrated lentiviral particles added was increased the percentage of GFP +ve cells, reflective of successful transduction, also increased.

4.2.2 Titration of EV shRNA lentivirus

An empty vector (EV) shRNA expressing lentivirus was also generated using a clone from Dharmacon. The procedure for viral titration was as per that of the BAP1 shRNA expressing lentivirus as described above. 0.5µL of virus transduced 22.6% of 293T cells and the viral titre was therefore determined to be 2.3 x 10^7 transduction units/ml (**Figure 4-5**).

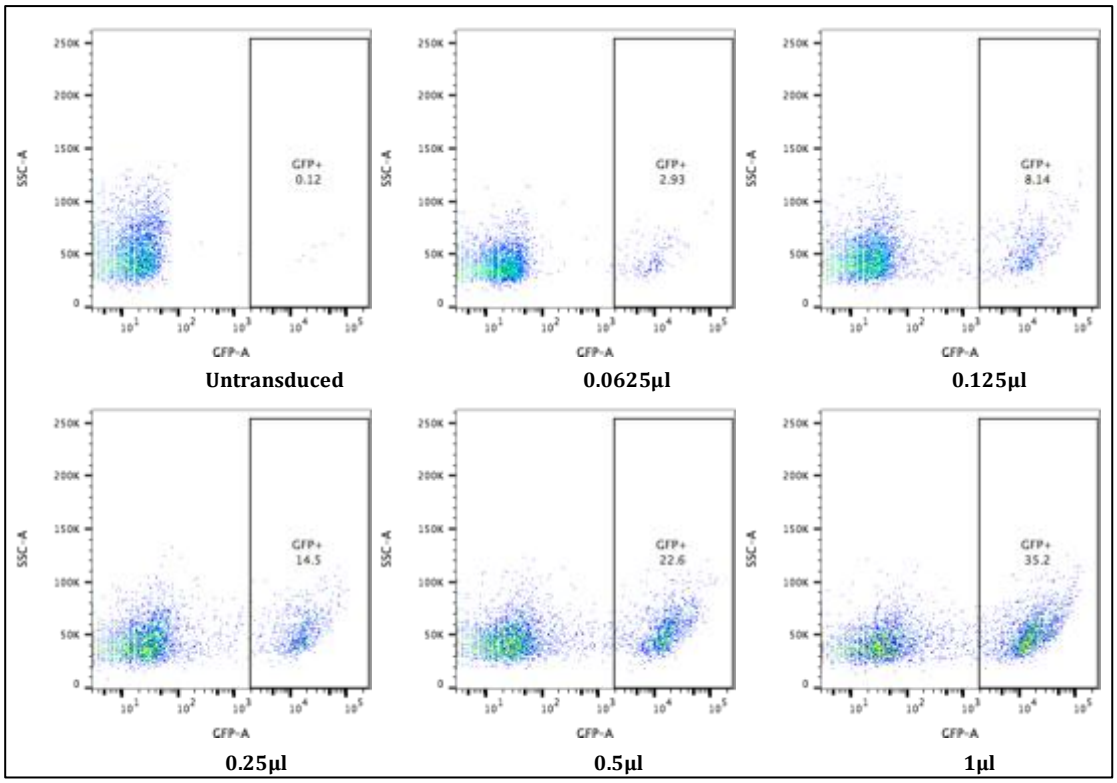


Figure 4-5 Titration of EV shRNA expressing lentivirus

293T cells were transduced using a dilution series of concentrated lentiviral particles. GFP expression as determined by flow cytometry for a range of viral volumes are shown which reflects the percentage of transduced cells. As the volume of concentrated lentiviral particles added was increased the percentage of GFP +ve cells, reflective of successful transduction, also increased.

4.2.3 Transduction of wild-type *BAP1* MPM cells with BAP1 shRNA lentivirus

MPP-89 and H2869 cells were transduced with BAP1 shRNA and EV shRNA expressing lentivirus at MOI 5. As the shRNA lentivirus expresses a puromycin resistance marker, treatment with 10µg/ml puromycin was used to select a pure population of BAP1 shRNA expressing cells. Immunoblot analysis confirmed reduced BAP1 expression in the BAP1 shRNA transduced relative to the EV transduced cells in both cell lines however BAP1 expression was still observed in the BAP1 shRNA transduced cells (**Figure 4-6**).

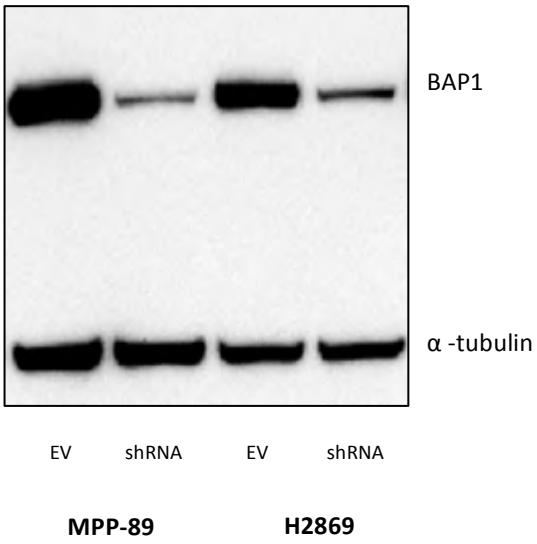


Figure 4-6 Immunoblot of BAP1 in BAP1 shRNA transduced MPM cells

Immunoblot of MPP-89 and H2869 cells transduced with MOI 5 of EV shRNA (EV) or BAP1 shRNA (shRNA) virus. Transduction with shRNA lentivrus successful resulted in knockdown of BAP1 expression in both cell lines.

4.2.4 Knockdown of *BAP1* in wild-type *BAP1* MPM cells results in increased rTRAIL sensitivity

Parental, BAP1 shRNA and EV shRNA transduced MPP-89 and H2869 cells were treated with a dose range of rTRAIL for 24 hours following which an Annexin V/ DAPI cell death assay was performed. Transduction of the parental line with the EV shRNA construct resulted in no significant change in cell death in response to rTRAIL treatment in either MPP-89 ($p=0.8768$) or H2869 ($p=0.6095$) cells. Transduction with BAP1 shRNA resulted in a significant increase in cell death in response to rTRAIL treatment in both lines relative to both the parental and EV shRNA transduced lines ($p = <0.0001$ for all comparisons) (**Figure 4-7**). Beyond 50ng/ml of rTRAIL however no dose response relationship was observed in all cell lines.

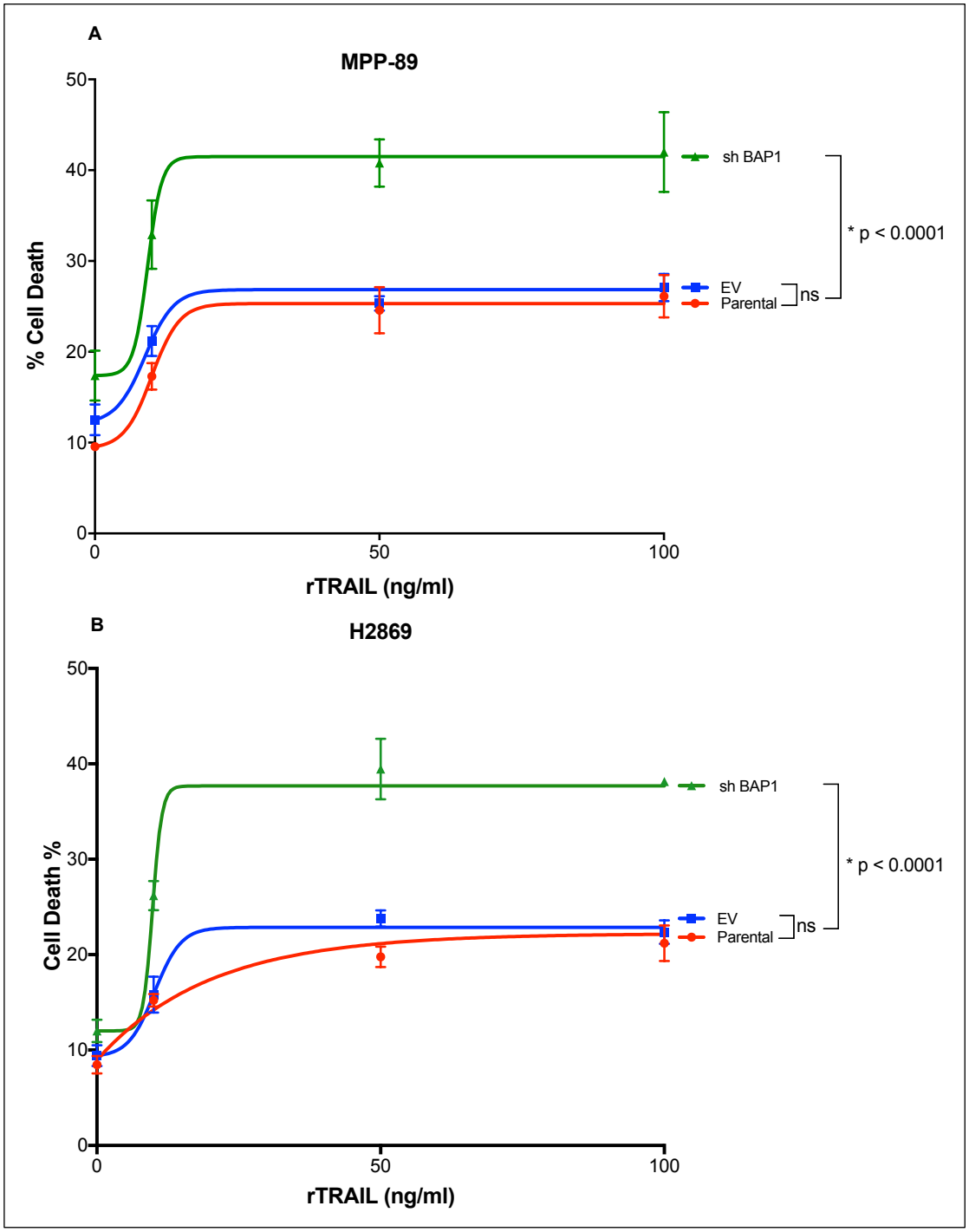


Figure 4-7 shRNA knockdown of BAP1 and response to rTRAIL treatment in BAP1 wild-type MPM lines

Cell death in response to 24h treatment with rTRAIL as assessed by an Annexin V/DAPI flow cytometry assay in ***(A)*** *MPP-89 lines - Parental vs C91A (p = 0.8768), Parental vs shBAP1 (p = <0.0001), EV vs shBAP1 (p = <0.0001)* ***(B)*** *H2869 MPM lines - Parental vs C91A (p = 0.6095), Parental vs shBAP1 (p = <0.0001), EV vs shBAP1 (p = <0.0001). Parental – untransduced, EV – empty vector shRNA transduced, shBAP1 – BAP1 shRNA transduced.*

4.3 BAP1 as a biomarker for sensitivity to other DR agonists

A number of clinical and preclinical death receptor (DR) agonist compounds exist in addition to recombinant TRAIL (rTRAIL) including antibodies and small molecule agonists to both DR4 and DR5. I hypothesised that as rTRAIL induces apoptosis through binding to DR4/5 and as the evidence presented by K. Kolluri suggests loss of BAP1 function alters expression of components of the apoptotic pathway, any compound that binds to and activates DRs would induce greater cell death in the context of loss of BAP1 function. Loss of BAP1 function would therefore be an effective biomarker for these DR agonists in addition to rTRAIL. Through collaboration with Medimmune LLC, I gained access to the preclinical DR5 agonist Medi3039 to test this hypothesis in *in vitro* models.

4.3.1 Loss of BAP1 function sensitises MPM cells to Medi3039

K. Kolluri generated the BAP1 WT expressing plasmid pCCL.CMV.BAP1 and site directed mutagenesis of this plasmid was used to generate lentiviruses each with a mutation in a key functional BAP1 site as previously described. The mutant vectors generated by K Kolluri were: C91A – inactive DUB catalytic site, NLS – deletion of the nuclear localization signal and ΔHBM – inactive HCF1 binding motif, in addition to a GFP expressing control construct. The H226 cell line harbours a homozygous deletion of *BAP1* and expresses no BAP1 protein. H226 cells were transduced with each of these constructs by K Kolluri to generate H226 cells that express either wild-type *BAP1*, mutant *BAP1* or *GFP* as a control. As highlighted in the introduction, transduction with BAP1 WT, NLS or ΔHBM conferred rTRAIL resistance while transduction with BAP1 C91A had no effect on rTRAIL sensitivity implicating DUB activity as key to BAP1 induced TRAIL resistance (**Figure 1-8**). I took these transduced H226 cells and treated them with rTRAIL and Medi3039 along with the parental line to determine if loss of BAP1 function and DUB activity conferred sensitivity to Medi3039 as for rTRAIL.

Parental, GFP, C91A, BAP1 WT, NLS and ΔHBM transduced H226 cells were treated with a dose range of rTRAIL for 72h following which an XTT viability assay was performed (**Figure 4-8A**). Transduction of the parental line with the GFP construct (control) resulted in no significant change in cell viability in response to rTRAIL treatment ($p=0.0863$). The GFP control was used for statistical comparison of the mutant constructs to account for any effect of transduction on response to rTRAIL. Transduction of the parental line with BAP1 WT resulted in a significant increase in cell viability in response to rTRAIL treatment relative to the GFP transduced line ($p<0.0001$). Similarly transduction with the NLS or ΔHBM constructs also resulted in a significant decrease in cell viability in response to rTRAIL treatment ($p<0.0001$) relative to the GFP transduced line. Transduction of the parental line with the C91A construct however resulted in no significant change in cell viability in response to rTRAIL treatment relative to the GFP transduced line ($p=0.4037$).

I next treated the cells with a dose range of Medi3039 for 72h following which an XTT viability assay was performed (**Figure 4-8B**). The same pattern of results was observed for Medi3039 as for rTRAIL. Transduction of the parental line with the GFP construct (control) resulted in no significant change in cell viability relative to the parental line ($p=0.2221$) and transduction with the C91A DUB mutant resulted in no significant change in cell viability in relative to the GFP transduced cells ($p=0.0572$) in response to Medi3039 treatment. Transduction with the BAP1 WT, NLS or HBM constructs resulted in a significant increase in cell viability in response to Medi3039 treatment relative to the GFP transduced line ($p<0.0001$ for all).

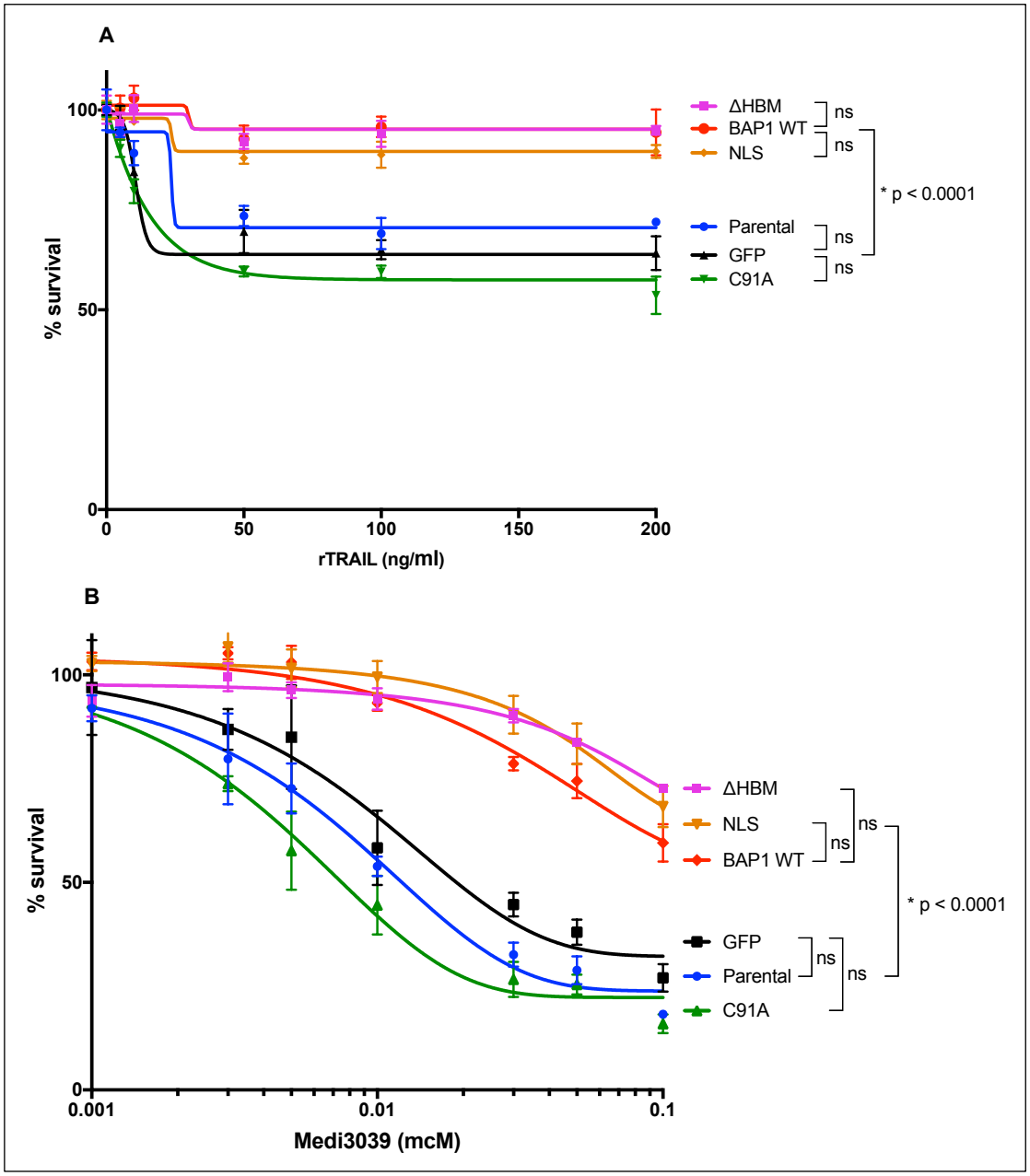


Figure 4-8 Response to DR agonist treatment in transduced H226 MPM cells

Parental and transduced H226 cell lines were treated with a dose range of a DR agonist and cell viability assessed at 72h with an XTT assay ***(A)*** *rTRAIL – parental vs GFP (p = 0.0863) GFP vs C91A (p = 0.4037), BAP1 WT vs NLS (p = 0.5778), BAP1 WT vs ΔHBM (p >0.9999), GFP vs BAP1 WT/ NLS/ ΔHBM (p = <0.0001)* ***(B)*** *Medi3039 – parental vs GFP (p = 0.2221), GFP vs C91A (p = 0.0572), BAP1 WT vs NLS (p = 0.2179), BAP1 WT vs ΔHBM (p = 0.1658) GFP vs BAP1 WT/ NLS/ ΔHBM (p= <0.0001). Parental – untransduced, GFP – GFP transduced control, C91A – inactive DUB mutant transduced, BAP1 – BAP1 WT transduced, NLS – deleted NLS transduced, ΔHBM – inactive HBM mutant transduced. Transduction of the parental line with the GFP construct (control) resulted in no significant change in cell viability relative to the parental line (p=0.2221) and transduction with the C91A DUB mutant*

resulted in no significant change in cell viability in relative to the GFP transduced cells (p=0.0572) in response to Medi3039 treatment. Transduction with the BAP1 WT, NLS or HBM constructs resulted in a significant increase in cell viability in response to Medi3039 treatment relative to the GFP transduced line (p<0.0001 for all). This is the same pattern of results as for rTRAIL which suggests the observations made for rTRAIL can be extended to other DR agonists.

4.4 BAP1 as a biomarker for sensitivity to DR agonists in additional cancer types

4.4.1 Loss of BAP1 function sensitises breast cancer cells to Medi3039

Using an shRNA knockdown model, loss of BAP1 function was also demonstrated to act as a biomarker for rTRAIL sensitivity in *BAP1* wild-type MDA-MB-231 breast cancer cells by K. Kolluri (**Figure 4-9A and B**). I aimed to determine if this association between loss of BAP1 function and DR agonist sensitivity extended to Medi3039 in a non-MPM cancer line. K. Kolluri previously transduced MDA-MB-231 breast cancer cells with BAP1 shRNA and EV shRNA lentivirus. I treated the parental, BAP1 shRNA and EV shRNA transduced cell lines with a dose range of Medi3039 for 72 hours following which response was assessed with an XTT cell viability assay (**Figure 4-9C**).

Transduction of the parental line with EV shRNA did not result in a significant change in cell viability in response to Medi3039 treatment (p=0.7798). Transduction of the parental line with BAP1 shRNA however resulted in a significant decrease in cell viability in response to Medi3039 treatment relative to both the parental (p<0.0001) and EV transduced (p<0.0001) lines. These results reflect those observed in response to rTRAIL treatment. Transduction of the parental line with EV shRNA however did result in an increase in cell death in response to rTRAIL treatment (p< 0.0001) yet a significant increase in cell death relative to this control was still seen in the BAP1 shRNA transduced cells (p< 0.0001).

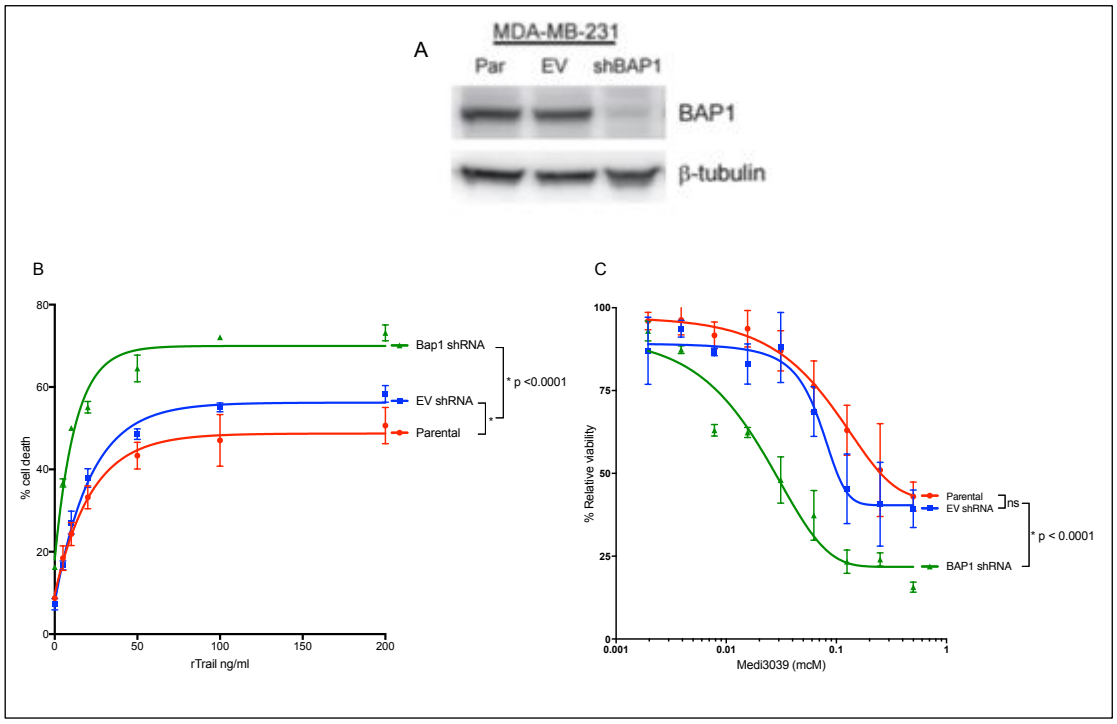


Figure 4-9 Loss of BAP1 function and response to DR agonist treatment in MDA-MD-231 breast cancer cells

(A) *Immunoblot of parental, EV shRNA and BAP1 shRNA transduced cell lines.* ***(B)*** *Cell death assay of MDA-MB-231 cells treated with rTRAIL - Parental vs EV (p <0.0001), Parental vs shBAP1 (p = <0.0001), EV vs shBAP1 (p = <0.0001) (data from K. Kolluri).* ***(C)*** *XTT cell viability assay of MDA-MB-231 cells treated with Medi3039 - Parental vs EV (p = 0.7798), Parental vs shBAP1 (p <0.0001), EV vs shBAP1 (p = 0.002). Transduction of the parental line with EV shRNA did not result in a significant change in cell viability in response to Medi3039 treatment (p=0.7798). Transduction of the parental line with BAP1 shRNA however resulted in a significant decrease in cell viability in response to Medi3039 treatment relative to both the parental (p<0.0001) and EV transduced (p<0.0001) lines. These results reflect those observed in response to rTRAIL treatment. Transduction of the parental line with EV shRNA however did result in an increase in cell death in response to rTRAIL treatment (p< 0.0001) yet a significant increase in cell death relative to this control was still seen in the BAP1 shRNA transduced cells (p< 0.0001).*

4.4.2 Loss of BAP1 function sensitises clear cell renal carcinoma cells to DR agonists

The *BAP1* wild-type clear cell renal carcinoma cell line Caki-1 was transduced with empty vector or BAP1 shRNA at MOI 5. Immunoblot confirmed a decrease in expression of BAP1 in the BAP1 shRNA but not EV shRNA transduced cells (**Figure 4-10A**). Parental, EV shRNA and BAP1 shRNA transduced cells were treated with a dose range of rTRAIL or Medi3039 and cell viability at 3 days was measured (**Figure 4-10B** and **C**). Transduction of the parental line with EV shRNA did not result in a significant change in cell viability in response to either rTRAIL (p=0.1922) or Medi3039 (p=0.0650) treatment. Transduction with BAP1 shRNA however resulted in a significant decrease in cell viability in response to both rTRAIL and Medi3039 relative to both the parental and EV shRNA transduced lines (p<0.0001 for all).

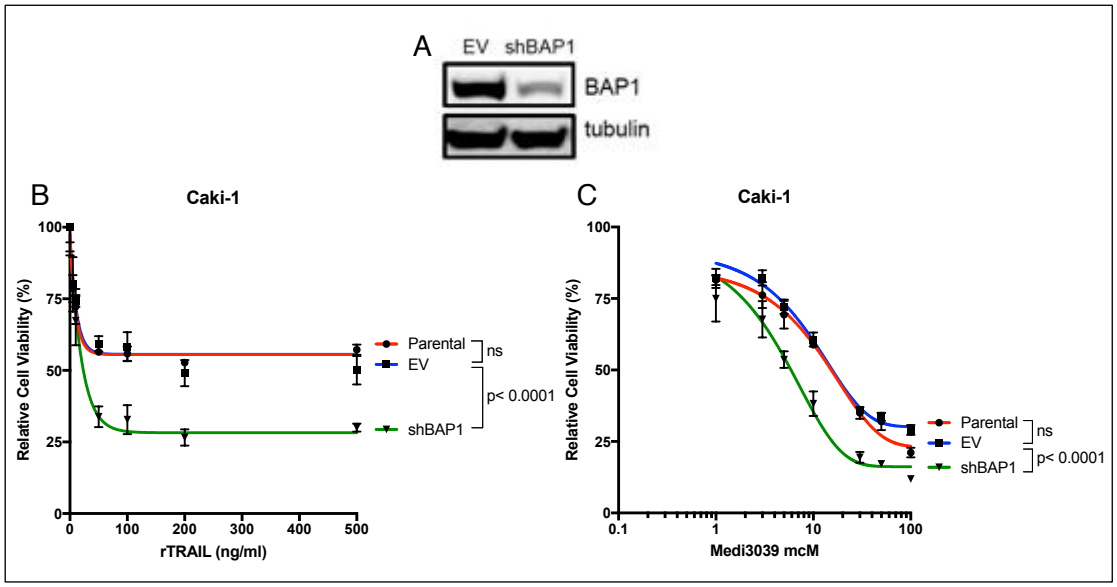


Figure 4-10 Loss of BAP1 function and response to DR agonist treatment in Caki-1 clear cell renal carcinoma cells

(A) *Immunoblot EV shRNA (EV) and BAP1 shRNA (shBAP1) transduced cell lines.* ***(B)*** *XTT cell viability assay of Caki-1 cells treated with rTRAIL* ***(C)*** *XTT cell viability assay of Caki-1 cells treated with Medi3039. Transduction of the parental line with EV shRNA did not result in a significant change in cell viability in response to either rTRAIL (p=0.1922) or Medi3039 (p=0.0650) treatment. Transduction with BAP1 shRNA however resulted in a significant decrease in cell viability in response to both rTRAIL and Medi3039 relative to both the parental and EV shRNA transduced lines (p<0.0001 for all).*

The *BAP1* wild-type clear cell renal carcinoma cell line BB65 was also transduced with empty vector or BAP1 shRNA at MOI 5. Immunoblot confirmed a decrease in expression of BAP1 in the BAP1 shRNA but not EV shRNA transduced cells (**Figure 4-11A**). Parental, EV shRNA and BAP1 shRNA transduced cells were treated with a dose range of rTRAIL or Medi3039 and cell viability at 3 days was measured (**Figure 4-11B** and **C**). Transduction of the parental line with EV shRNA did not result in a significant change in cell viability in response to either rTRAIL (p=0.5245) or Medi3039 (p=0.4670) treatment. Transduction with BAP1 shRNA however resulted in a significant decrease in cell viability in response to both rTRAIL and Medi3039 relative to both the parental and EV shRNA transduced lines (p<0.0001 for all).

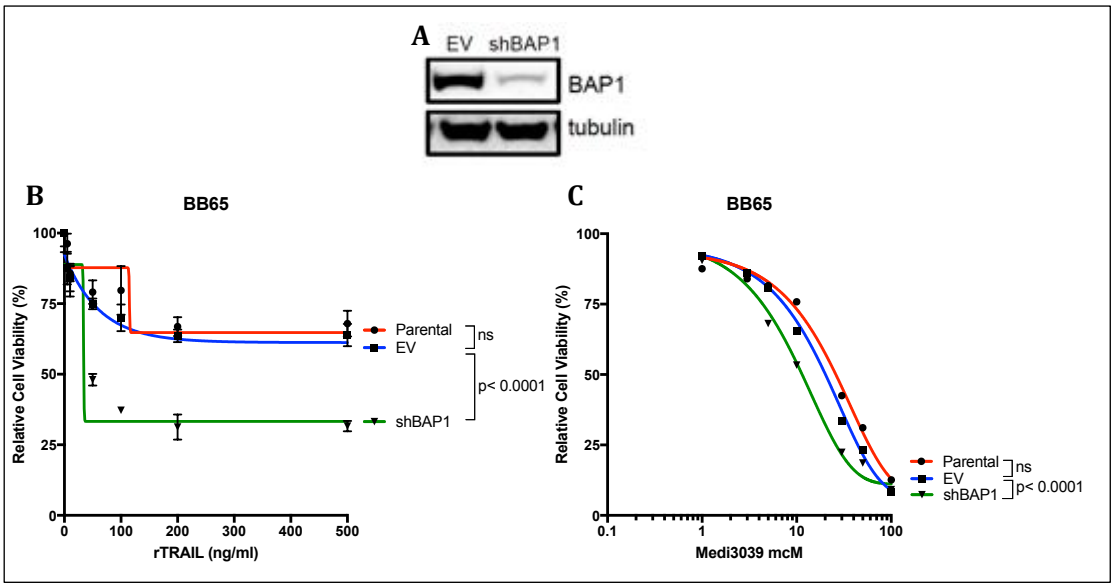


Figure 4-11 Loss of BAP1 function and response to DR agonist treatment in BB65 clear cell renal carcinoma cells

(A) *Immunoblot EV shRNA (EV) and BAP1 shRNA (shBAP1) transduced cell lines.* ***(B)*** *XTT cell viability assay of BB65 cells treated with rTRAIL* ***(C)*** *XTT cell viability assay of BB65 cells treated with Medi3039. Transduction of the parental line with EV shRNA did not result in a significant change in cell viability in response to either rTRAIL (p=0.5245) or Medi3039 (p=0.4670) treatment. Transduction with BAP1 shRNA however resulted in a significant decrease in cell viability in response to both rTRAIL and Medi3039 relative to both the parental and EV shRNA transduced lines (p<0.0001 for all).*

4.5 BAP1 and TRAIL sensitivity in benign cells

In view of the above data combined BAP1 inhibition and DR agonist therapy might be an effective treatment for *BAP1* wild-type tumours. I therefore aimed to determine the effect of loss of BAP1 function on rTRAIL sensitivity in non-transformed non-malignant lines to assess for potential off target effects of this treatment strategy. I also aimed to determine the effect of loss of BAP1 function on rTRAIL sensitivity in transformed non-malignant lines.

4.5.1 Human non-transformed primary fibroblasts

A human primary fibroblast culture derived from a patient that underwent bronchoscopy was transduced with BAP1 shRNA or EV shRNA lentivirus at MOI 5. As the lentivirus expresses a puromycin resistance marker treatment with 10µg/ml puromycin was used to select a pure population of BAP1 shRNA expressing cells. Immunoblot analysis confirmed reduced BAP1 expression following BAP1 shRNA transduction and puromycin treatment (**Figure 4-12A**). BAP1 expression in the EV transduced fibroblasts was unaffected. The parental, EV shRNA and BAP1 shRNA transduced fibroblasts were treated with a dose range of rTRAIL for 72 hours following which an XTT cell viability assay was performed. Transduction of parental fibroblasts with EV shRNA did not result in a significant change in response to rTRAIL treatment ($p=0.2655$). There was also no significant difference in response to rTRAIL treatment in the BAP1 shRNA transduced fibroblasts relative to the parental ($p=0.7828$) or EV shRNA transduced fibroblasts ($p=0.3602$) (**Figure 4-12B**).

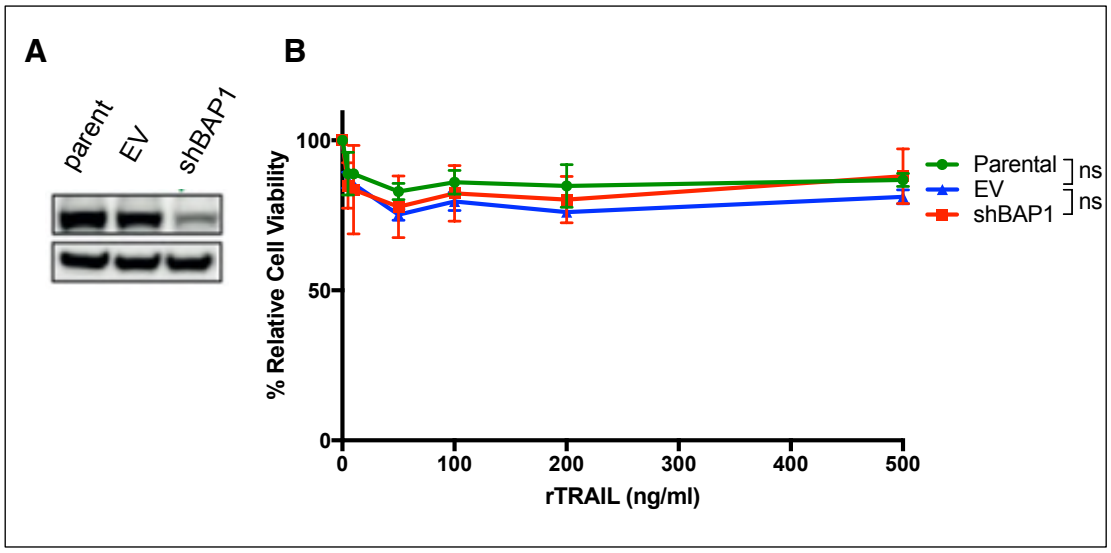


Figure 4-12 Loss of BAP1 function and response to rTRAIL treatment in non-transformed fibroblasts

(A) *Immunoblot of BAP1 expression in parent, EV shRNA (EV) and BAP1 shRNA (shBAP1) transduced fibroblasts* ***(B)*** *72h XTT cell viability assay to assess cell survival in response to rTRAIL treatment. Transduction of parental fibroblasts with EV shRNA did not result in a significant change in response to rTRAIL treatment (p=0.2655). There was also no significant difference in response to rTRAIL treatment in the BAP1 shRNA transduced fibroblasts relative to the parental (p=0.7828) or EV shRNA transduced fibroblasts (p=0.3602)*

4.5.2 Human non-transformed bronchoepithelial cell culture

A human primary bronchoepithelial cell (HBEC) culture derived from a patient that underwent a diagnostic bronchoscopy was transduced with BAP1 shRNA expressing or EV shRNA lentivirus at MOI 5. As the lentivirus expresses a puromycin resistance marker treatment with 10µg/ml puromycin was used to select a pure population of BAP1 shRNA expressing HBECs. Immunoblot analysis confirmed reduced BAP1 expression following BAP1 shRNA transduction and puromycin treatment (**Figure 4-13A**). BAP1 expression in the EV transduced HBECs was unaltered. The parental, EV shRNA and BAP1 shRNA transduced HBECs were treated with a dose range of rTRAIL for 72 hours following which an XTT cell viability assay was performed (**Figure 4-13B**). Transduction of parental HBECs with EV shRNA did not result in a significant change in response to rTRAIL treatment (p=0.2276). There was also no significant difference in response to rTRAIL treatment in the BAP1

shRNA transduced HBECs relative to the parental (p=0.1435) or EV shRNA transduced HBECs (p=0.9662) (**Figure 4-12B**).

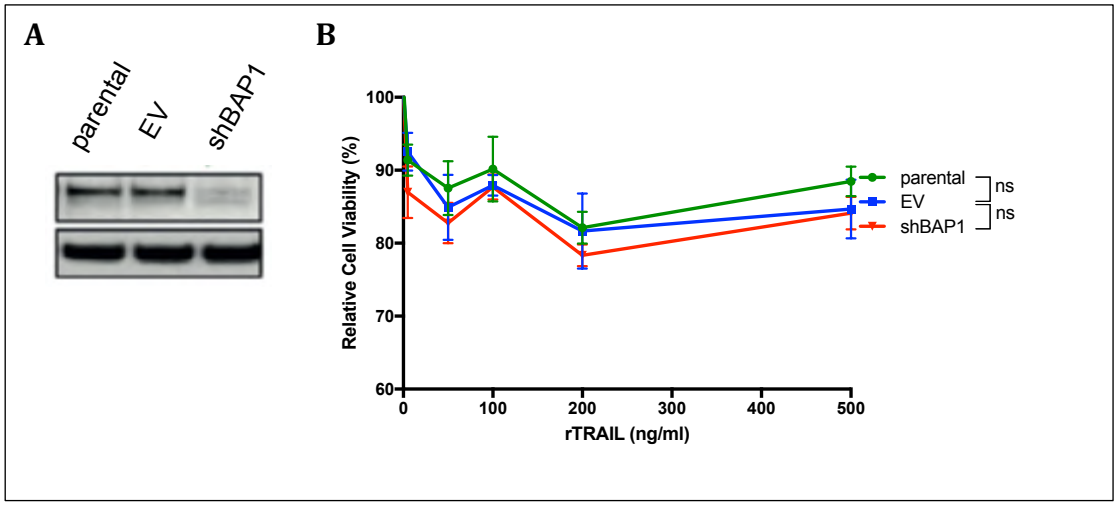


Figure 4-13 Loss of BAP1 function and response to rTRAIL treatment in non-transformed HBECs

(A) *Immunoblot of BAP1 expression in parent, EV shRNA (EV) and BAP1 shRNA (shBAP1) transduced HBEC at MOIs 1 and 5* ***(B)*** *72h XTT cell viability assay to assess cell survival in response to rTRAIL treatment. Transduction of parental HBECs with EV shRNA did not result in a significant change in response to rTRAIL treatment (p=0.2276). There was also no significant difference in response to rTRAIL treatment in the BAP1 shRNA transduced HBECs relative to the parental (p=0.1435) or EV shRNA transduced HBECs (p=0.9662)*

4.5.3 Human transformed bronchoepithelial cells

The NL-20 cell line is a non-malignant but immortalised, transformed bronchoepithelial line derived from normal bronchus taken from an accident victim at autopsy. The cell line was originally transformed by transfection with a SV40 containing construct. I transduced NL-20 cells with BAP1 shRNA or EV shRNA lentivirus at MOIs 1 and 5. As the lentivirus expresses a puromycin resistance marker treatment with 10µg/ml puromycin was used to select a pure population of BAP1 or EV shRNA expressing cells. Immunoblot analysis confirmed reduced BAP1 expression following BAP1 shRNA transduction and puromycin treatment at both MOIs (**Figure 4-14**). BAP1 expression in the EV transduced cells was unaffected. The EV shRNA and BAP1 shRNA

transduced lines transduced at MOI 5 were used for further analysis. The parental, EV shRNA and BAP1 shRNA transduced cells were treated with a dose range of rTRAIL for 72 hours following which an XTT cell viability assay was performed. A significant decrease in cell viability in response to rTRAIL treatment was seen in the EV shRNA transduced cells relative to the parental cells ($p < 0.0001$) suggesting that transduction may have an effect on rTRAIL sensitivity in this cell line. A significant decrease in cell viability however was seen in the BAP1 shRNA transduced relative to the EV shRNA transduced cells in response to rTRAIL treatment ($p = 0.0005$).

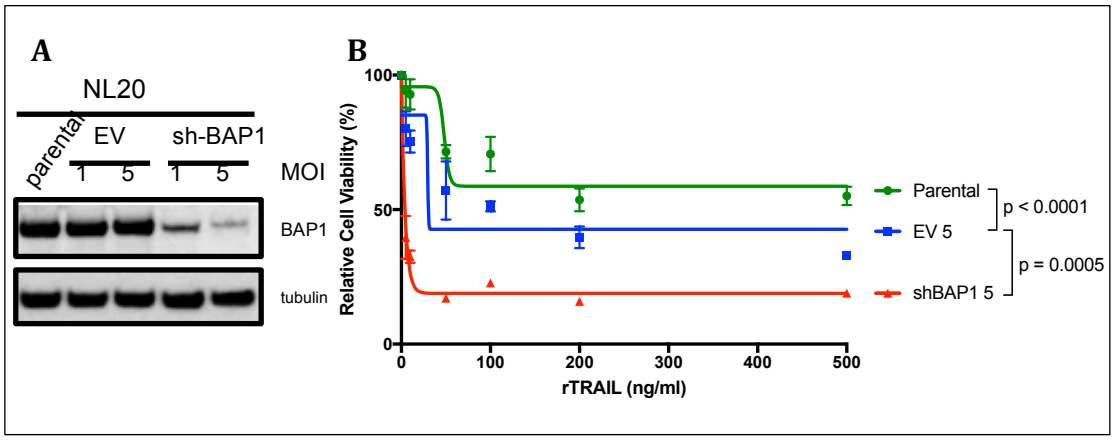


Figure 4-14 Loss of BAP1 function and response to rTRAIL treatment in a transformed HBEC

*(**A**) Immunoblot of BAP1 expression in parent, EV shRNA (EV) and BAP1 shRNA (shBAP1) transduced the transformed HBEC line NL20 at MOIs 1 and 5 (**B**) 72h XTT cell viability assay. A significant decrease in cell viability in response to rTRAIL treatment was seen in the EV shRNA transduced cells relative to the parental cells ($p < 0.0001$) suggesting that transduction may have an effect on rTRAIL sensitivity in this cell line. A significant decrease in cell viability however was seen in the BAP1 shRNA transduced relative to the EV shRNA transduced cells in response to rTRAIL treatment ($p = 0.0005$). Data generated with Yuki Ishii.*

4.6 Discussion

4.6.1 Expression of wild-type *BAP1* in mutant *BAP1* MPM lines increases resistance to rTRAIL

The original WTSI cell line-drug screen was a single experiment using a single dose of rTRAIL to assess cell viability in response to drug treatment. Therefore the identified association between *BAP1* LOF mutation and rTRAIL sensitivity must be validated in further models for confidence in the finding. K. Kolluri transduced the *BAP1* null H226 cell line with C91A mutant and BAP1 WT expressing vectors and demonstrated that only WT BAP1 conferred resistance to rTRAIL. However, the association in a single cell line could be attributed to the specific molecular alterations in that cell line. The above finding that transduction of two further *BAP1* null cell lines, H28 and H2804, with C91A BAP1 and BAP1 WT results in a reduction in cell death only in the case of BAP1 WT transduction significantly strengthens and further validates the original WTSI observation (**Figure 4-3**). These data also provide further evidence that the deubiquitinase function of BAP1 is key to mediating rTRAIL resistance. It is notable that the two cells lines differ in the degree to which rTRAIL sensitivity is modulated by transduction with wild-type *BAP1*. At higher doses the sensitivity of WT BAP1 transduced H28 cells to rTRAIL increases to approach that of the parental and C91A BAP1 transduced cell lines. It may be that differences in the endogenous expression of apoptosis pathway components between the two cell lines accounts for this difference. For example untransduced H28 cells might express higher levels of anti-apoptotic components modulated by BAP1 function than H2804 cells such that these higher levels persist despite introduction of functional BAP1 and are overcome to a lesser degree in response to rTRAIL treatment. Alternatively it may be that differences in the transduction between the two cell lines resulted in H28 cells having less functional BAP1 activity compared to H2804 cells for higher doses of rTRAIL to overcome the shRNA transduction. A method of determining BAP1 activity, through a deubiquitinase assay for example would resolve this.

4.6.2 Knock-down of *BAP1* in MPM cells results in increased rTRAIL sensitivity

Having used an overexpression model to further validate loss of BAP1 function as a biomarker for rTRAIL sensitivity in MPM, I have also successfully used an shRNA knockdown model to the same end. Both untransduced MPP-89 and H2869 cells are relatively resistant to rTRAIL however when BAP1 expression is reduced, but not completely silenced, using shRNA the sensitivity of both these cell lines significantly increases; the cell death in response to 50ng/ml rTRAIL treatment almost doubles in both (**Figure 4-7**). It is interesting to note that above this dose no dose-response is observed suggesting there is a limiting factor to rTRAIL response in the parental and transduced cell lines. One possible explanation is that the residual BAP1 expressed despite the knockdown limits the response seen. This observation, that an increase in cell death is observed in spite of residual BAP1 expression, also implies that the degree of BAP1 activity rather than absolute presence determines rTRAIL sensitivity. Use of CRISPR-Cas9 technology to edit out *BAP1* would allow determination of the effect of complete loss of BAP1 function rather than a reduction in expression.

4.6.3 Loss of BAP1 function also sensitises cancer cells to the death receptor 5 agonist Medi3039

A soluble recombinant form of TRAIL (rTRAIL), dulanermin (Roche), was the first TRAIL-R/DR agonist to be developed and assessed in a clinical trial [119, 161]. Since then numerous further TRAIL-R/DR agonist compounds have been developed as highlighted in the introduction (**Table 1-3**). Given the pharmacological limitations of rTRAIL it seems likely that the newer multivalent DR agonists will supersede it in clinical use. As such, from a translational perspective, it is important to determine if loss of BAP1 function extends to other DR agonists in addition to rTRAIL. Above I have demonstrated in overexpression and knockdown models of MPM that loss of BAP1 deubiquitinase function results in sensitivity to Medi3039, and it seems likely therefore that this association will extend to other DR agonists, although

not formally tested here. Medi3039 is significantly more potent than rTRAIL as can be seen in **Figure 4-8**. Higher doses of rTRAIL still result in >50% relative cell survival, even in cells with loss of BAP1 function, however micromolar doses of Medi3039 result in <25% survival in these cells and even in some decreased survival in BAP1 wild-type transduced cells. Medi3039 is a multivalent DR agonist while rTRAIL binds in a univalent manner which likely accounts for its increased potency as multivalent DR binding triggers greater DISC activation. The significant levels of reduced cell survival in the presence of loss of BAP1 function cells highlights the potential clinical potency of using BAP1 as a biomarker to stratify the use of such potent DR agonists.

4.6.4 Loss of BAP1 function sensitises other cancer types to DR agonists

As noted, *BAP1* loss-of-function mutations are observed in a number of additional cancers. The above data supports that loss of BAP1 function also sensitises breast cancer and clear cell renal carcinoma to DR agonists. 8-14% of clear cell renal carcinoma tumours harbor loss-of-function mutations in *BAP1* and these might therefore be amenable to DR agonist therapy [162]. It would be pertinent to assess this in other cancer with a high-observed frequency of BAP1 mutations including uveal melanoma and intrahepatic cholangiocarcinoma. Although BAP1 is not frequently mutated in breast cancer, the observation that loss of BAP1 function sensitises a breast cancer cell line to DR agonists suggests that this association may have widespread clinical relevance to any cancer with loss of BAP1 function.

4.6.5 Loss of BAP1 function does not sensitise non-transformed cells to rTRAIL

As evidence presented supports that loss of BAP1 function sensitises MPM tumours to DR agonists, combined BAP1 inhibition and DR agonist therapy might be an effective treatment for *BAP1* wild-type tumours. I therefore aimed to determine the effect of BAP1 inhibition on rTRAIL sensitivity in non-tumour

cells. The data above demonstrates that non-transformed human primary fibroblasts and HBECs are resistant to rTRAIL and this resistance cannot be overcome by shRNA inhibition of BAP1 expression, unlike established MPM lines and primary MPM cultures. This evidence suggests that the combination therapy proposed would not be toxic to non-malignant cells. The data also however demonstrates that non-malignant but transformed HBECs are sensitised to rTRAIL by BAP1 inhibition. Transformation of non-malignant TRAIL resistant cells has been shown to induce rTRAIL sensitivity however the mechanisms are poorly understood. A study of TRAIL resistance in non-transformed primary human fibroblasts suggests they rely upon multiple redundant mechanisms for resistance – both cFLIP regulation of DISC activation and high levels of anti-apoptotic Bcl-2 or XIAP expression downstream [163]. Removal of only one of these 'blocks' is insufficient to overcome TRAIL resistance. Oncogenic Ras transformation of human fibroblasts has been shown to confer TRAIL resistance and this was associated with an enhanced recruitment of pro-caspase 8 to the DISC [164] and induction of the pro-apoptotic protein Bak [165]. Similar findings have been observed in transformed keratinocytes where increased TRAIL sensitivity was associated with both reduced cFLIP and XIAP expression [166, 167]. A balance of pro- and anti- apoptotic factors expressed therefore ultimately determines whether a cell is TRAIL sensitive. It may be that non-transformed cells have multiple levels of TRAIL resistance that cannot be overcome by BAP1 inhibition whereas transformation already removes some 'roadblocks' such that BAP1 inhibition further sensitises to rTRAIL. Benign human tissue is non-transformed and therefore these results support that BAP1 inhibition will not increase their TRAIL sensitivity and thus the off target effects of DR agonists if used in conjunction with a BAP1 inhibitor.

4.7 Summary

- Loss of BAP1 function in both overexpression and shRNA knockdown cell line models of MPM result in increased sensitivity to rTRAIL.
- This association of loss of BAP1 function with DR agonist sensitivity extends to Medi3039, a preclinical multivalent DR agonist.
- Medi3039 is significantly more potent than rTRAIL.
- Loss of BAP1 function is associated with sensitivity to DR agonists in breast and clear cell renal carcinoma lines.
- Loss of BAP1 function does not sensitise non-transformed cells to rTRAIL.

CHAPTER V:

RESULTS III

5 RESULTS III: THE MECHANISM OF BAP1 MEDIATED DR AGONIST RESISTANCE

Delineation of the mechanism underlying BAP1 induced DR agonist resistance would potentially reveal targets for sensitisation of DR agonist resistant tumours. The data presented by K. Kolluri reveals that the deubiquitinase (DUB) function of BAP1 is necessary for DR agonist resistance and an inactive DUB domain results in sensitivity (**Figure 1-8**). The gene expression data from the Wellcome Trust Sanger Institute (WTSI) supports a significant change in the mRNA expression of components of the apoptosis pathway in response to loss of BAP1 DUB function as a potential underlying mechanism (**Figure 1-9**). In line with these findings, BAP1 is known to complex with transcription factors to modulate gene transcription via its DUB function. I therefore aimed to determine which transcriptional regulatory partners might be key to BAP1 induced DR agonist resistance as this might point to those genes most likely to be involved. Review of the literature highlights HCF-1, ASXL1/2, and FoxK1/2 as transcriptional regulatory partners for BAP1. The literature also identifies mutations of *BAP1* that disrupt the interaction with each of these proteins [43-45, 52, 168, 169]. K. Kolluri employed site directed mutagenesis of the pCCL.CMV.BAP1 plasmid to generate lentiviral constructs with mutations in the binding sites for HCF-1 and Fox K1/2 and demonstrated that H226 cells transduced with these mutants remained resistant to rTRAIL (**Figure 1-8**). This implies the transcriptional regulatory complexes that BAP1 forms with these transcription factors are not involved in mediating DR agonist sensitivity. I used this strategy to generate a lentiviral construct with mutations in the ASXL1/2 binding site to determine if the transcriptional regulatory complex that BAP1 forms with these proteins, the PR-DUB, is involved in mediating DR agonist sensitivity. This identified BAP1/ASXL binding as a key interaction implicating PR-DUB function. I therefore aimed to determine if activity of this complex also affects expression of components of the apoptotic pathway and if the PR-DUB target substrate

H2AK119Ub, which alters chromatin architecture and gene transcription, correlates with DR agonist sensitivity in MPM.

5.1 BAP1 and ASXL1/2 and TRAIL resistance

BAP1 binds to ASXL1/2 to form two mutually exclusive complexes both capable of deubiquitinating H2AK119Ub [43, 45]. These PR-DUB complexes regulate gene transcription along with the polycomb repressor complexes through epigenetic modification of chromatin structure [58]. Deletion of amino acids R666-H669 in BAP1 has been shown to abolish BAP1-ASXL1/2 binding and to significantly disrupt the ability of BAP1 to deubiquitinate H2AK119Ub [45]. I used site directed mutagenesis of the pCCL.CMV.BAP1 plasmid to generate a lentiviral construct that expresses *BAP1* with this mutation, and is therefore incapable of binding to ASXL1/2, to determine the impact of disruption of this complex on rTRAIL sensitivity.

5.1.1 Titration of the ΔASXL lentivirus

The pCCL.CMV.BAP1 plasmid was used as a template for site directed mutagenesis to generate a lentivirus that expresses a BAP1 protein with an inactive ASXL protein-binding site (ΔASXL). 5 x 10^4 293T cells were plated in a 6-well plate and transduced with different dilutions of this mutant *BAP1* expressing lentivirus. The cells were grown for 48 hours, the culture media removed and the cells washed with PBS, trypsinised and stained with a BAP1 primary and an AlexaFlour-488 secondary antibody for titration by flow cytometry. The virus quantity that transduced approximately 20% of cells was used to calculate the viral titre and the equation used in **3.2.1** used as before.

0.5µL of virus transduced 20.9% of 293T cells and the viral titre was therefore determined to be 2.1 x 10^7 transduction units/ml (**Figure 5-1**).

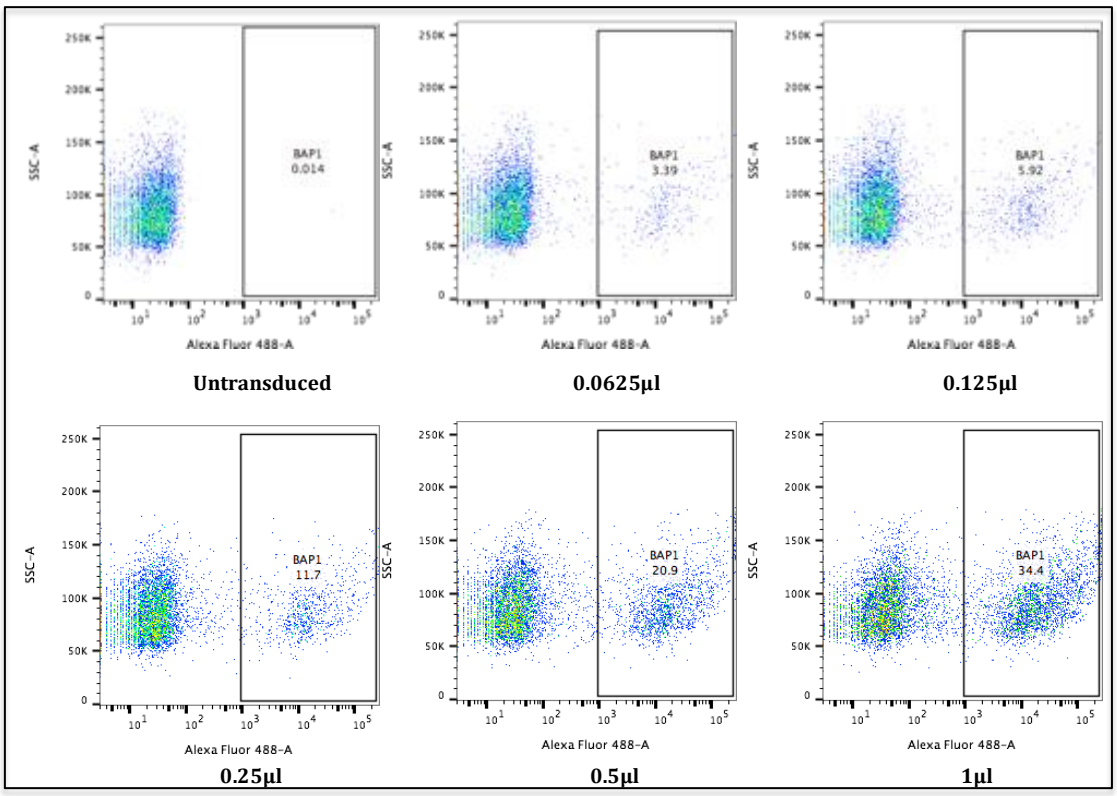


Figure 5-1 Titration of ΔASXL BAP1 expressing lentivirus

293T cells were transduced using a dilution series of concentrated lentiviral particles. BAP1 expression as determined by flow cytometry to AF-488 for a range of viral volumes are shown which reflects the percentage of transduced cells. As the volume of concentrated lentiviral particles added increased the percentage of BAP1 expressing cells also increased implying successful transduction.

5.1.2.2 Transduction of BAP1 null H226 MPM cells with ΔASXL BAP1 expressing lentivirus results in expression of BAP1

H226 cells were transduced with the ΔASXL lentivirus at MOI 3 and 5. Transduction efficacy was assessed by flow cytometry for BAP1 expression. 71.1% of H226 cells were transduced at MOI 3 and 86.1 at MOI 5 (**Figure 5-2**). The cells transduced at MOI 5 were therefore used for further experiments.

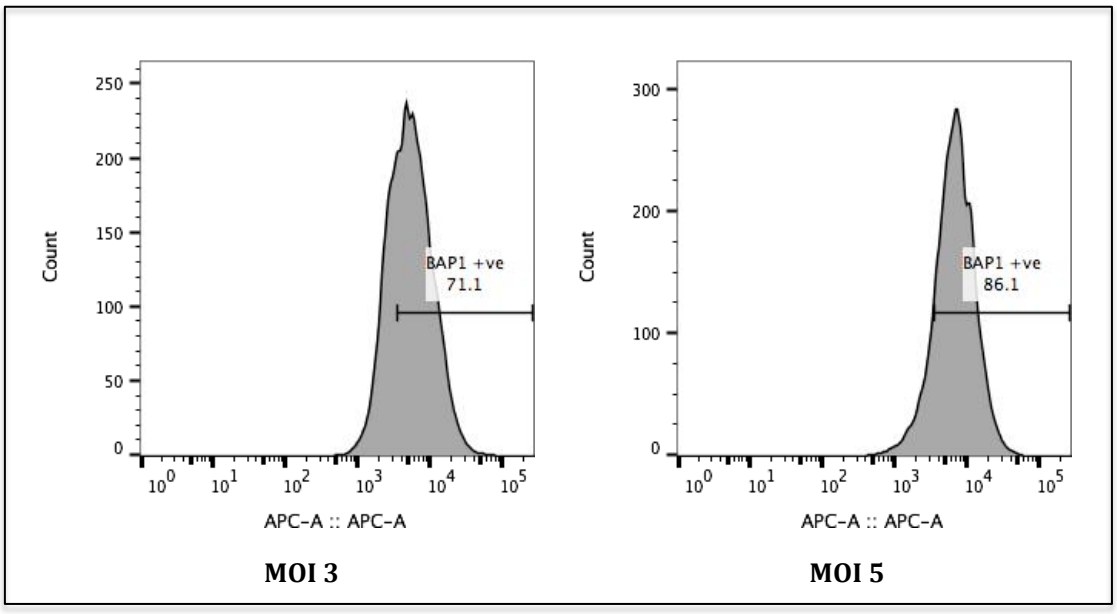


Figure 5-2 Transduction of H226 MPM cells with ASXL BAP1 expressing lentivirus

Flow cytometry assessment of H226 cells for BAP1 expression after transduction with ΔASXL lentivirus at MOIs 3 and 5. At both MOI 3 and 5 a significant proportion of cells were successfully transduced and expressed BAP1. As a higher proportion of the cells transduced at MOI 5 expressed BAP1 (86.1%), these cells were used in further experiments.

5.1.2 Loss of ASXL binding on BAP1 results in increased rTRAIL sensitivity

Parental, C91A (DUB mutant), BAP1 WT and ΔASXL transduced H226 cells were treated with a dose range of rTRAIL for 24 hours and an Annexin V/DAPI assay performed to determine response.

Transduction of the *BAP1* null parental line with wild-type BAP1 resulted in a significant decrease in cell death in response to rTRAIL treatment ($p < 0.0001$) (**Figure 5-3**). Interestingly transduction with the C91A and ΔASXL mutants resulted in an increase in cell death in response to rTRAIL treatment relative to the parental line ($p < 0.0001$), which could indicate that transduction itself has an effect on sensitivity to rTRAIL. However, a statistically significant difference in rTRAIL sensitivity between the BAP1 WT transduced line (control) and the C91A and ΔASXL transduced lines ($p < 0.0001$) was seen. Thus, loss of activity of these sites results in rTRAIL sensitivity implicating the

function of these sites in BAP1 mediated rTRAIL resistance. Notably the cell death response to rTRAIL seen in both the C91A and ΔASXL transduced cells was almost identical (p= 0.9874).

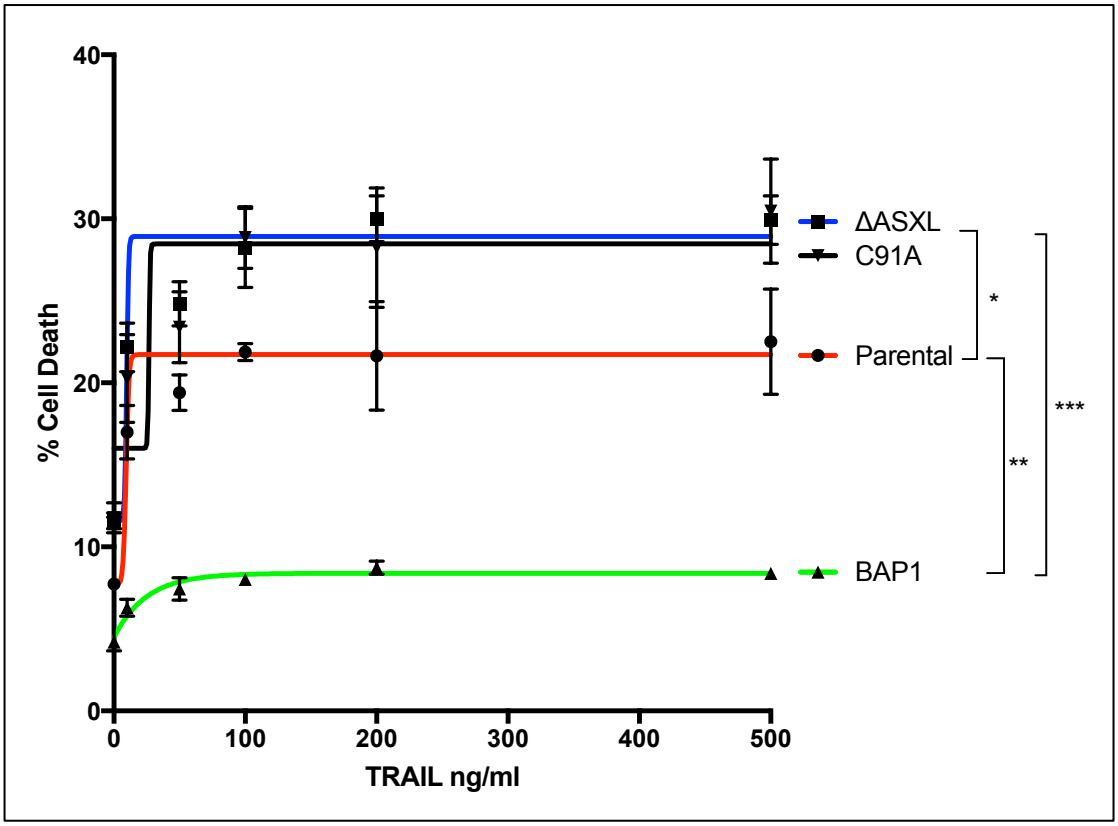


Figure 5-3 Loss of BAP1-ASXL binding and response to rTRAIL treatment in H226 MPM cells

*Parental, BAP1 WT, C91A and ΔASXL transduced H226 cells were treated with a dose range of rTRAIL and cell death measured with an Annexin V/DAPI assay. *p< 0.0001, **p< 0.0001, *** p< 0.0001. Transduction of the BAP1 null parental line with wild-type BAP1 resulted in a significant decrease in cell death in response to rTRAIL treatment (p< 0.0001). Interestingly transduction with the C91A and ΔASXL mutants resulted in an increase in cell death in response to rTRAIL treatment relative to the parental line (p< 0.0001), which could indicate that transduction itself has an effect on sensitivity to rTRAIL. However, a statistically significant difference in rTRAIL sensitivity between the BAP1 WT transduced line (control) and the C91A and ΔASXL transduced lines (p< 0.0001) was seen.*

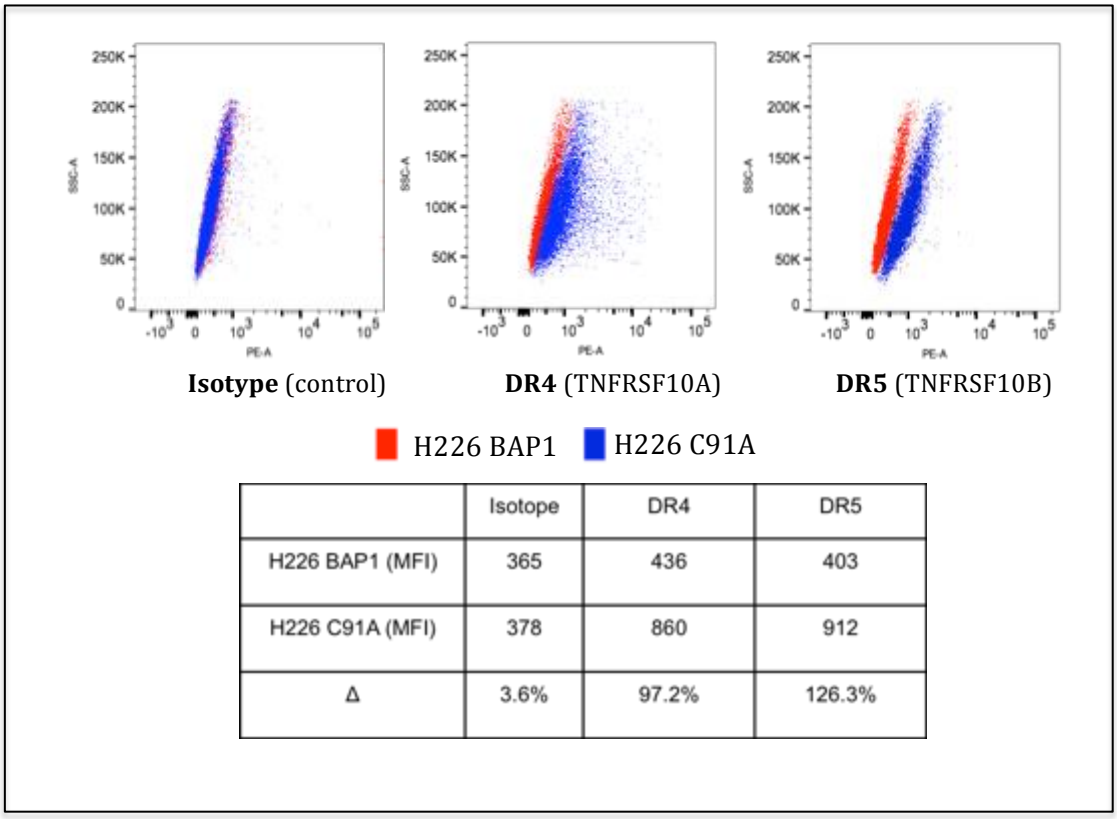
5.2 BAP1 and expression of extrinsic apoptotic pathway proteins

My hypothesis is that BAP1 modulates expression of the apoptotic pathway proteins to alter DR agonist sensitivity. The above finding that disruption of a known transcriptional regulatory complex supports this. Also consistent with this hypothesis are the previous findings of C. Alifrangis at the Wellcome Trust Sanger Institute (WTSI) who compared the mRNA levels of the apoptotic pathway proteins expressed in wild-type *BAP1* and DUB mutant *BAP1* transduced H226 MPM lines. This revealed a significant difference in mRNA expression of several extrinsic apoptotic pathway components (**Figure 1-9**). The mRNA expression of the pro-apoptotic proteins DR4, DR5 and FADD increased and that of the anti-apoptotic proteins cIAP1 and cIAP2 decreased in the rTRAIL sensitive DUB mutant transduced cells. This pattern is consistent with the increase in rTRAIL sensitivity observed in these cells. However, the mRNA expression of the anti-apoptotic proteins survivin, BIRC6, BIRC7, BIRC8, NAIP and XIAP was found to increase in these cells. One might expect mRNA expression of these anti-apoptotic proteins to decrease in cells that exhibit an increase in rTRAIL sensitivity, as is the case with the DUB mutant transduced cells. I therefore aimed to assess the protein expression of the extrinsic apoptotic pathway components in the DUB mutant transduced H226 cells compared to the wild-type *BAP1* transduced cells to determine if the protein, rather than mRNA, expression changes are more consistent with the increase in rTRAIL sensitivity observed in these cells.

5.2.1 Loss of BAP1 function results in increased DR expression on MPM cells

The mRNA microarray identified a significant increase in the expression of death receptors 4 and 5 (DR4/5) in the presence of DUB mutant versus wild-type *BAP1*. To determine if this difference was reflected in cell surface expression I conducted flow cytometry analysis of DR4 and DR5 expression in BAP1 WT and DUB mutant transduced H226 cells.

No significant difference in flow cytometry analysis of an isotype control was observed between BAP1 WT and C91A (DUB mutant) transduced H226 cells. Expression of both DR4 and DR5 however was much higher in C91A transduced cells relative to wild-type BAP transduced cells (**Figure 5-4**). The median fluorescent intensity recorded for DR4 and DR5 expression in C91A transduced cells was double that of the BAP1 WT transduced cells. This increase in DR4 and DR5 expression is consistent with the increase in mRNA expression of these proteins observed.



	Isotope	DR4	DR5
H226 BAP1 (MFI)	365	436	403
H226 C91A (MFI)	378	860	912
Δ	3.6%	97.2%	126.3%

Figure 5-4 Death receptor expression in BAP1 wild-type and mutant transduced H226 MPM cells

Flow cytometry analysis of an isotype control, DR4 and DR5 expression in BAP1 WT (H226 BAP1) and DUB mutant (H226 C91A) transduced cells. Expression was quantified using median fluorescent intensity (MFI) and the % relative difference in expression in the mutant vs wild-type BAP1 transduced cell lines is shown (Δ). No significant difference in flow cytometry analysis of an isotype control was observed between BAP1 WT and C91A (DUB mutant) transduced H226 cells. Expression of both DR4 and DR5 however was much higher in C91A transduced cells relative to wild-type BAP transduced cells. This increase in DR4 and DR5 expression is consistent with the increase in mRNA expression of these proteins observed.

I also conducted immunoblot analysis of DR4 and DR5 expression in the sequenced MPM cell lines to determine if there was any correlation with rTRAIL sensitivity or *BAP1* status (**Figure 5-5**). Interestingly for DR4 expression there was an almost exact correlation with protein expression and rTRAIL sensitivity in these cell lines. Those cell lines that were rTRAIL sensitive expressed higher levels of DR4 than those cell lines that were rTRAIL resistant. There was a less striking correlation with DR5 expression however the *BAP1* wild-type rTRAIL resistant cell lines also appeared to express less DR5 than *BAP1* mutant rTRAIL sensitive cell lines overall.

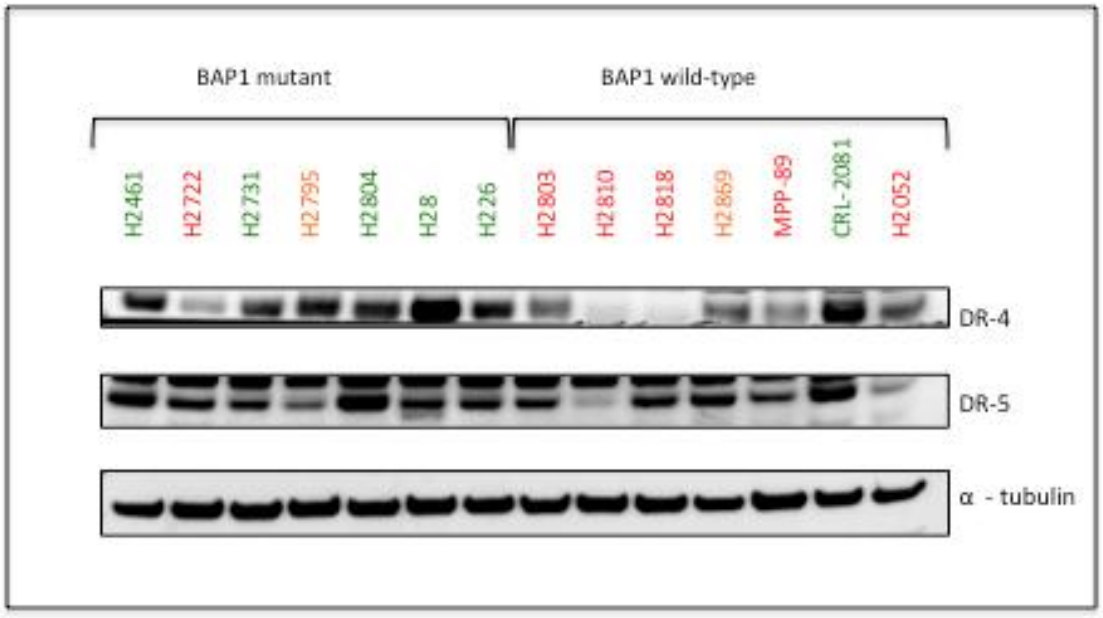


Figure 5-5 Death receptor 4 and 5 expression in MPM cell lines

The MPM cell lines are coded according to rTRAIL sensitivity (red – resistant, orange – partially sensitive, green – sensitive). Those cell lines that were rTRAIL sensitive expressed higher levels of DR4 than those cell lines that were rTRAIL resistant. There was a less striking correlation with DR5 expression however the BAP1 wild-type rTRAIL resistant cell lines also appeared to express less DR5 than BAP1 mutant rTRAIL sensitive cell lines overall.

5.2.2 Loss of BAP1 function results in decreased expression of inhibitors of apoptosis

I conducted immunoblot analysis of expression of cFLIP, cIAP1, cIAP2, FADD, survivin and XIAP in the DUB mutant and BAP1 WT transduced H226

cells. I also conducted this analysis in the ΔASXL mutant transduced cells to determine if the pattern of change was consistent with that of the DUB mutant, further implicating the PR-DUB (**Figure 5-6**). In the presence of both C91A and ΔASXL, expression of the anti-apoptotic proteins cIAP1 and cIAP2 decreased relative to WT BAP1 transduced cells while expression of the anti-apoptotic proteins XIAP and survivin and the pro-apoptotic protein FADD did not differ. This is more consistent with my hypothesis than the mRNA microarray data where the mRNA of the anti-apoptotic proteins XIAP and survivin were found to increase in the rTRAIL sensitive cells.

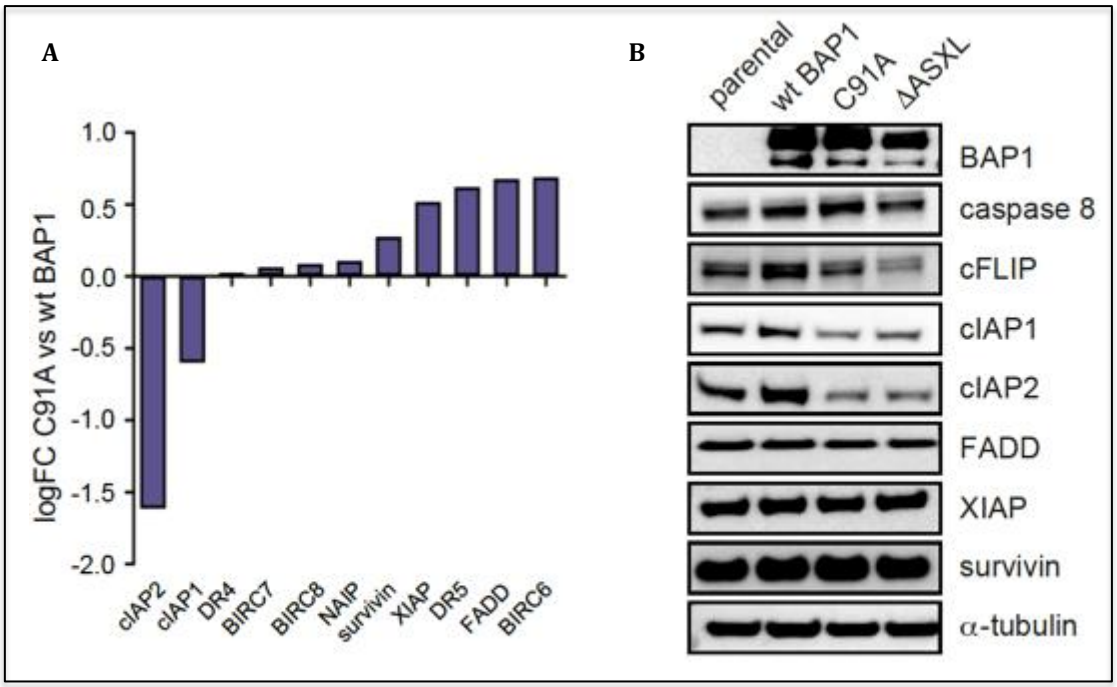
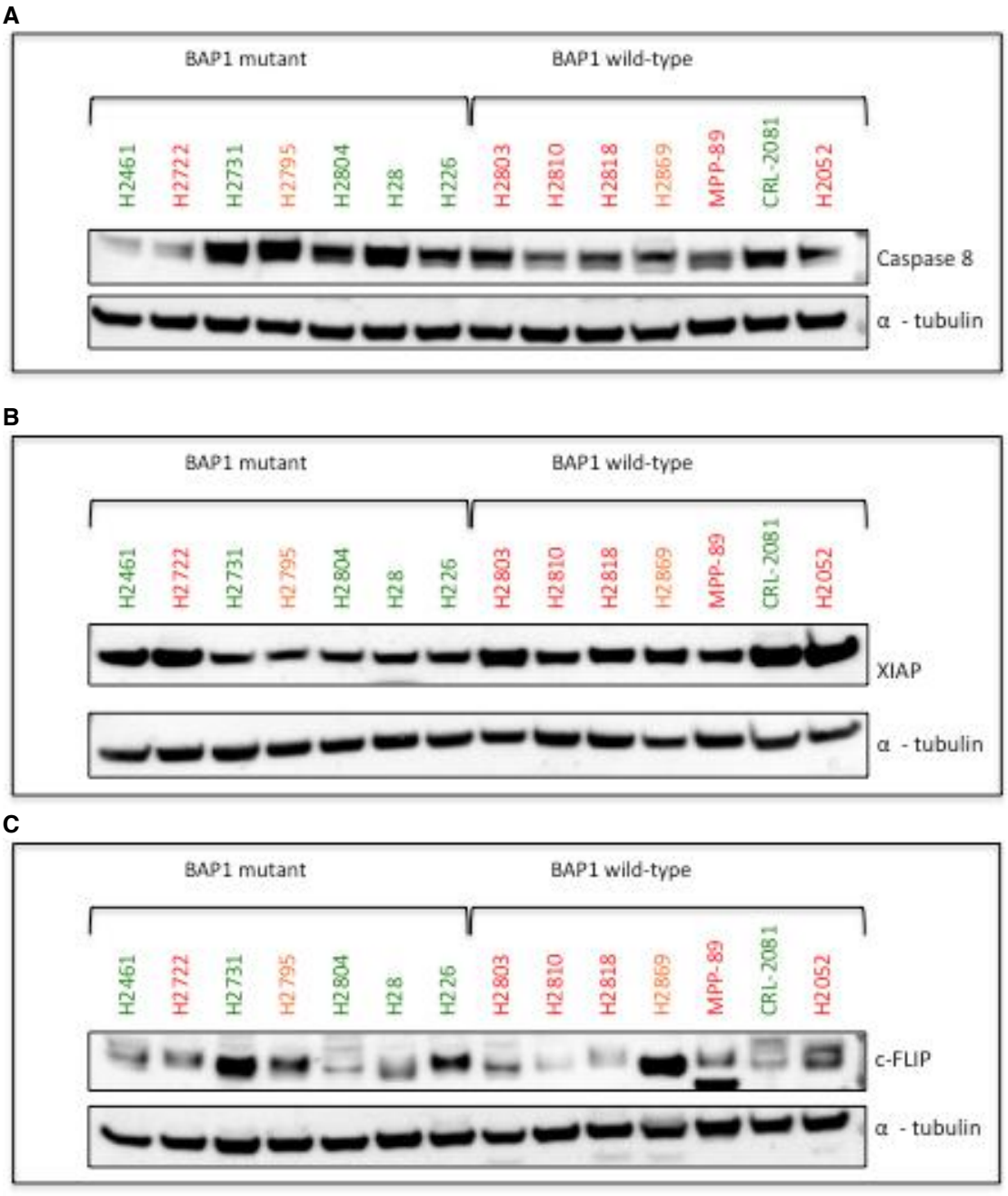


Figure 5-6 The extrinsic apoptotic pathway in BAP1 wild-type and mutant transduced H226 MPM cells

(A) *mRNA expression of extrinsic apoptotic pathway components in BAP1 DUB mutant transduced relative to BAP1 WT transduced H226 MPM cells (data generated by C Alifrangis for reference).* ***(B)*** *Immunoblot of extrinsic apoptotic pathway components in BAP1 C91A and ΔASXL mutant transduced relative to BAP1 WT transduced H226 MPM cells. In the presence of both C91A and ΔASXL, expression of the anti-apoptotic proteins cIAP1 and cIAP2 decreased relative to WT BAP1 transduced cells while expression of the anti-apoptotic proteins XIAP and survivin and the pro-apoptotic protein FADD did not differ.*

I also conducted immunoblot analysis of expression of these extrinsic apoptotic pathway proteins in the sequenced MPM cell lines to determine if there was any correlation with rTRAIL sensitivity or *BAP1* status.



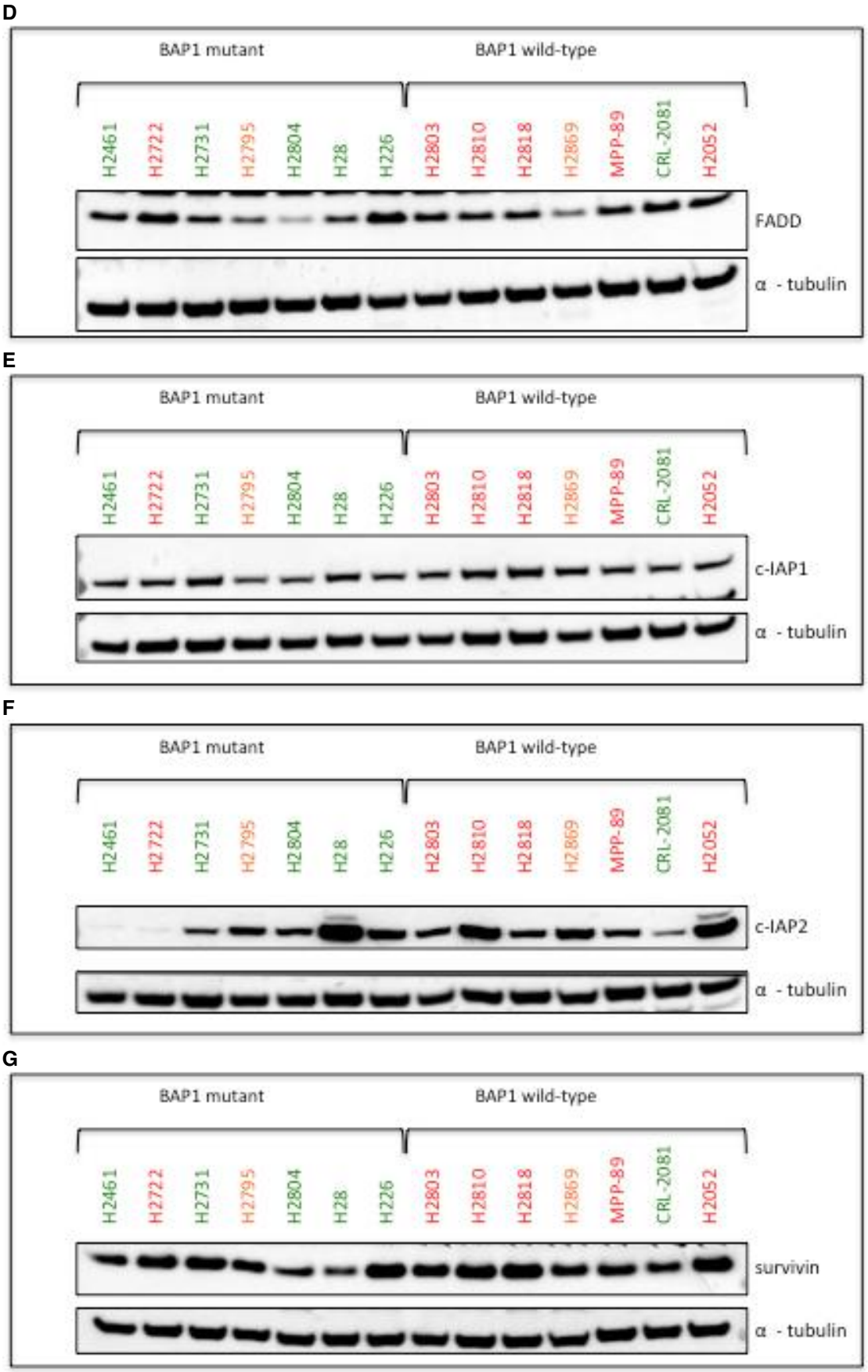

D
BAP1 mutant
BAP1 wild-type
H2461
H2722
H2731
H2795
H2804
H28
H226
H2803
H2810
H2818
H2869
MPP-89
CRL-2081
H2052
FADD
α - tubulin
E
BAP1 mutant
BAP1 wild-type
H2461
H2722
H2731
H2795
H2804
H28
H226
H2803
H2810
H2818
H2869
MPP-89
CRL-2081
H2052
c-IAP1
α - tubulin
F
BAP1 mutant
BAP1 wild-type
H2461
H2722
H2731
H2795
H2804
H28
H226
H2803
H2810
H2818
H2869
MPP-89
CRL-2081
H2052
c-IAP2
α - tubulin
G
BAP1 mutant
BAP1 wild-type
H2461
H2722
H2731
H2795
H2804
H28
H226
H2803
H2810
H2818
H2869
MPP-89
CRL-2081
H2052
survivin
α - tubulin

Figure 5-7 Immunoblot of extrinsic apoptotic pathway proteins in MPM lines

The MPM cell lines are coded according to rTRAIL sensitivity (red – resistant, orange – partially sensitive, green – sensitive). ***(A)*** *caspase 8* ***(B)*** *XIAP* ***(C)*** *cFLIP* ***(D)*** *FADD* ***(E)*** *c-IAP1* ***(F)*** *c-IAP2* ***(G)*** *surviving*

For the pro-apoptotic caspase 8 there appears to be higher expression levels in the rTRAIL sensitive *BAP1* mutant cell lines than the rTRAIL resistant *BAP1* wild-type lines (**Figure 5-7A**). Notably, the BAP1 mutant but rTRAIL resistant MPM line H2722 expresses a lower level of caspase 8 than the other *BAP1* mutant lines and the *BAP1* wild-type but rTRAIL sensitive MPM line CLR-2081 expresses a higher level of caspase 8 than the other *BAP1* wild-type lines. The anti-apoptotic protein XIAP appears to be expressed at lower levels in the rTRAIL sensitive *BAP1* mutant lines than the rTRAIL resistant *BAP1* wild-type lines overall (**Figure 5-8B**). For the pro-apoptotic protein FADD there is no obvious correlation between expression and rTRAIL sensitivity in either the *BAP1* mutant or wild-type lines (**Figure 5-7C** and **D**). For the anti-apoptotic proteins cFLIP, c-IAP1, c-IAP2 and survivin there also does not appear to be an obvious correlation between expression and rTRAIL sensitivity in either the *BAP1* mutant or wild-type lines (**Figure 5-7E, F** and **G**).

5.3 ASXL1/2 and DR agonist sensitivity in MPM

As disruption of the BAP1-ASXL1/2 complex sensitises MPM cells to DR agonists I hypothesised that ASXL1 and ASXL2 are also key to mediating DR agonist sensitivity and loss of function of ASXL1/2 would also result in increased sensitivity to DR agonists. I therefore aimed to assess the expression of these proteins in MPM lines to determine if there is any correlation with DR agonist sensitivity. I also aimed to determine the effect of loss of BAP1 function on ASXL1/2 expression to assess if there is any co-regulation of expression between the proteins of the PR-DUB complex. I subsequently conducted shRNA knockdown experiments in *BAP1/ASXL1/ASXL2* wild-type MPM lines to assess the effect of loss of ASXL1/2 function on rTRAIL sensitivity.

5.3.1 ASXL1/2 expression in MPM cell lines

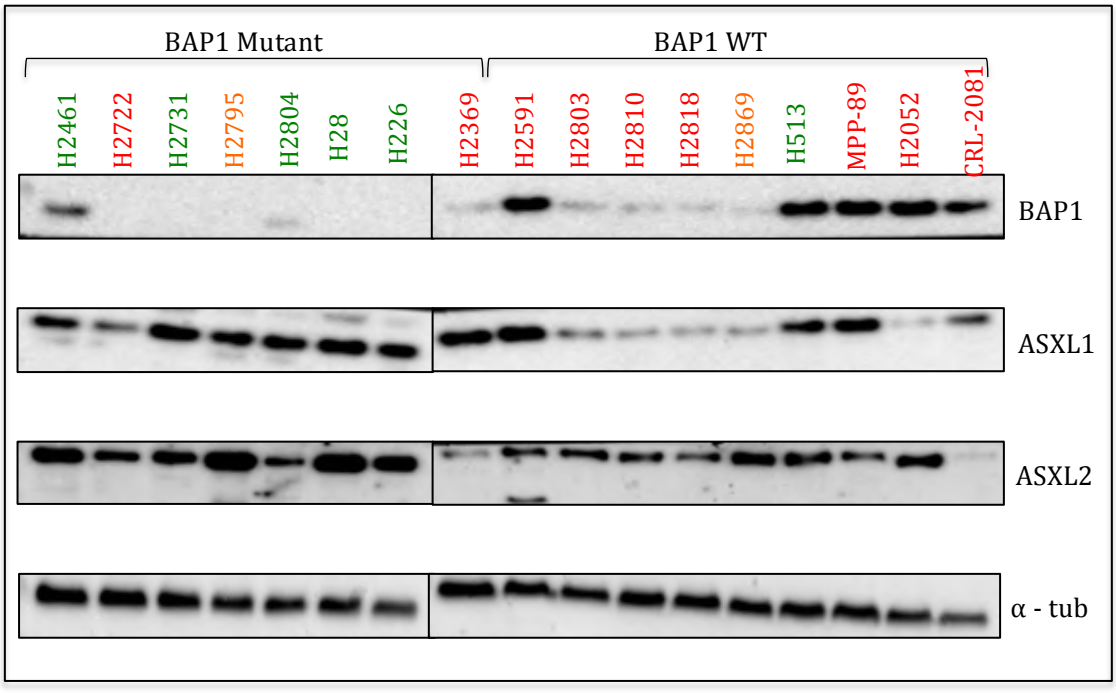


Figure 5-8 Immunoblot of PR-DUB components in MPM lines

Immunoblot analysis of BAP1, ASXL1 and ASXL2 expression in MPM lines stratified by mutant status and TRAIL sensitivity. Red – resistant, orange – partially sensitive, green – sensitive.

Immunoblot analysis of BAP1, ASXL1 and ASXL2 expression in MPM lines reveals that there is no association between rTRAIL sensitivity and ASXL1 and ASXL2 expression in these lines. There also does not appear to be an association between BAP1 expression and ASXL1/2 expression.

5.3.2 ASXL1/2 expression in BAP1 mutant transduced cell lines

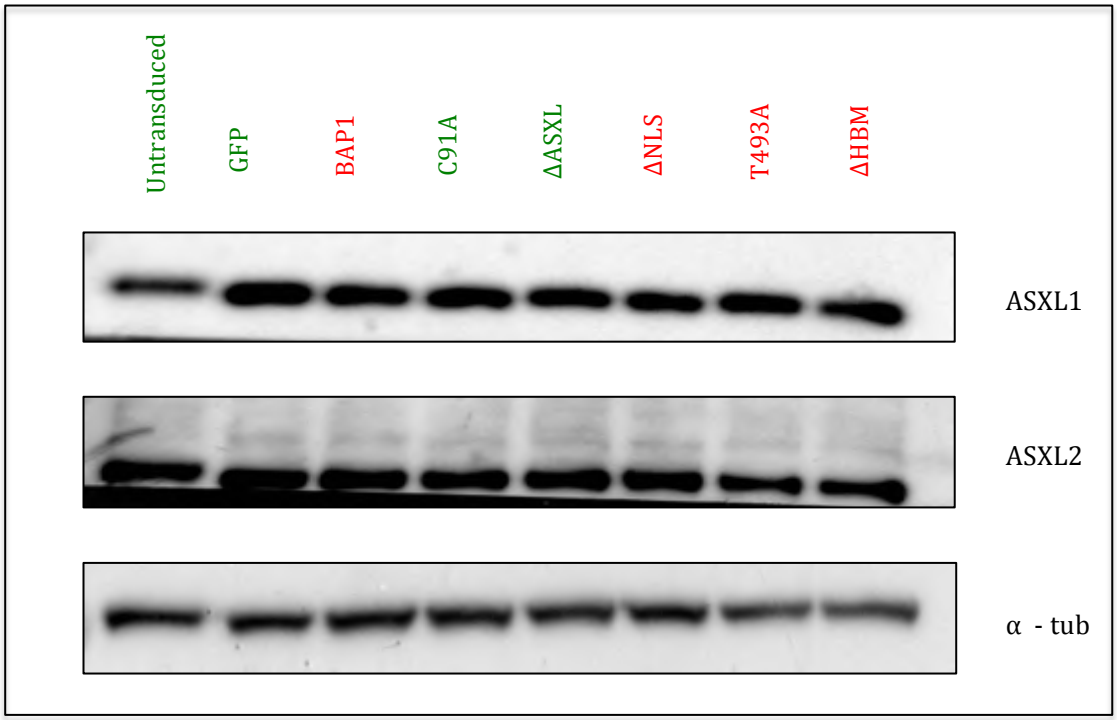


Figure 5-9 Immunoblot of ASXL1/2 in BAP1 mutant MPM lines

No significant change in expression of ASXL1/2 in the presence of any of the BAP1 mutations was identified implying no regulatory effect of BAP1 function on these proteins.

Immunoblot analysis of ASXL1 and ASXL2 expression in the mutant *BAP1* transduced cell lines revealed no significant change in expression of these proteins in the presence of any of the *BAP1* mutations identified (**Figure 5-9**).

5.3.3 Titration of ASXL1 and ASXL2 shRNA virus

ASXL1 and ASXL2 shRNA lentivirus was generated using a mir30-based GIPZ ASXL1/2 shRNA plasmid (ASXL1 - V3LHS_313251, ASXL2 –

V3LHS_313940, Dharmacon) and the packaging plasmids as described in methods. The shRNA plasmid expresses a green fluorescent protein (GFP) and a puromycin resistance marker to allow for titration and selection. 5 x 10^4 293T cells were plated in a 6-well plate and transduced with different dilutions of the ASXL1/2 shRNA expressing lentivirus. The cells were grown for 48 hours, the culture media removed and the cells washed with PBS, trypsinised and transferred to FACS buffer to determine the proportion of transduced cells by flow cytometry for GFP (**Fig 5-10** and **Fig 5-11**). The virus quantity that transduced approximately 20% of cells was used to calculate the viral titre and the equation used in **4.2.1** used as before.

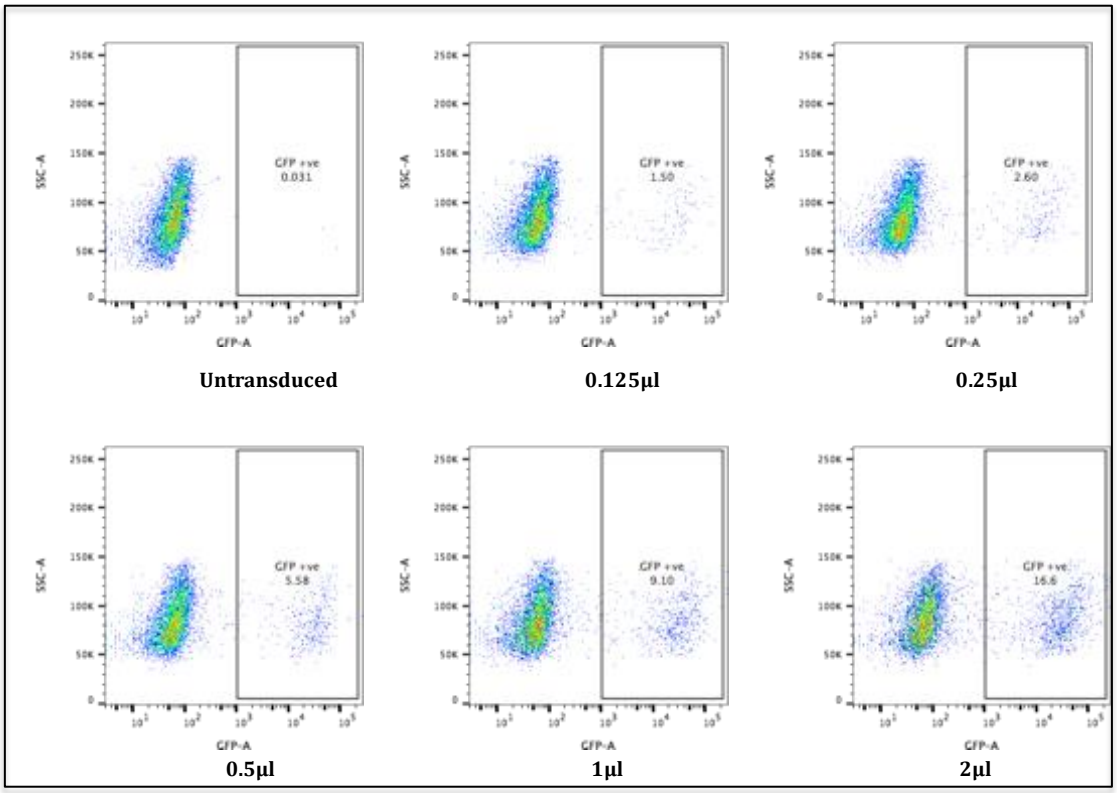


Figure 5-10 Titration of ASXL1 shRNA lentivirus

293T cells were transduced using a dilution series of concentrated lentiviral particles. The shRNA virus expresses GFP. Flow cytometry of GFP expression for a range of viral volumes are shown which reflects the percentage of transduced cells. As the volume of lentiviral particles added increased the percentage of successfully transduced cells also increased.

For the ASXL1 shRNA virus 2µL of virus transduced 16.6% of 293T cells and the viral titre was therefore determined to be 4.2 x 10^6 transduction units/ml (**Figure 5-10**).

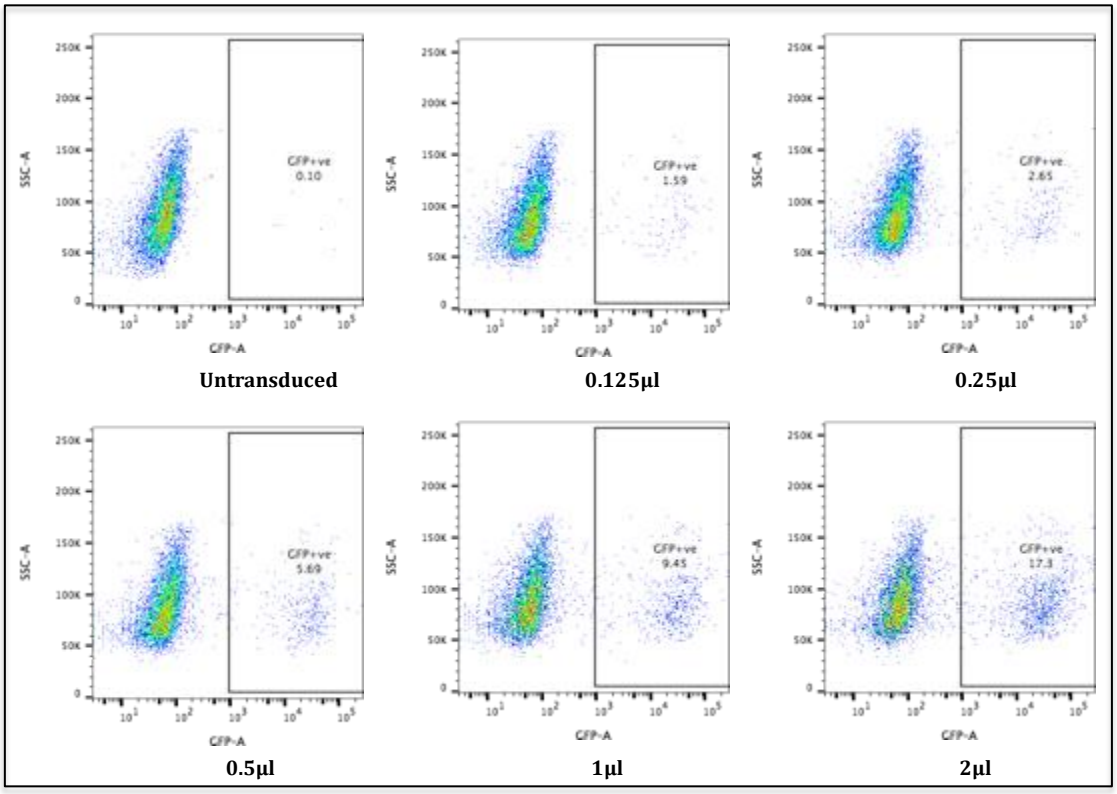


Figure 5-11 Titration of ASXL2 shRNA lentivirus

293T cells were transduced using a dilution series of concentrated lentiviral particles. The shRNA virus expresses GFP. Flow cytometry of GFP expression for a range of viral volumes are shown which reflects the percentage of transduced cells. As the volume of lentiviral particles added increased the percentage of successfully transduced cells also increased.

For the ASXL2 shRNA virus 2µL of virus transduced 17.3% of 293T cells and the viral titre was therefore determined to be 4.3 x 10^6 transduction units/ml (**Figure 5-11**).

5.3.4 Transduction of MPM cell lines with ASXL1 and ASXL2 shRNA lentivirus

Three BAP1/ASXL1/ASXL2 wild-type MPM lines H513, H2689 and MPP-89 were transduced with ASXL1 and ASXL2 virus at MOI 5. As the shRNA

lentiviruses express a puromycin resistance marker, treatment with 10µg/ml puromycin was used to select a pure population of shRNA expressing cells. To assess ASXL1 and ASXL2 expression in the transduced cell lines real time polymerase chain reaction (RT-PCR) was conducted (**Figure 5- 12**). This revealed a successful reduction in ASXL1 and ASXL2 mRNA expression in the transduced cell lines.

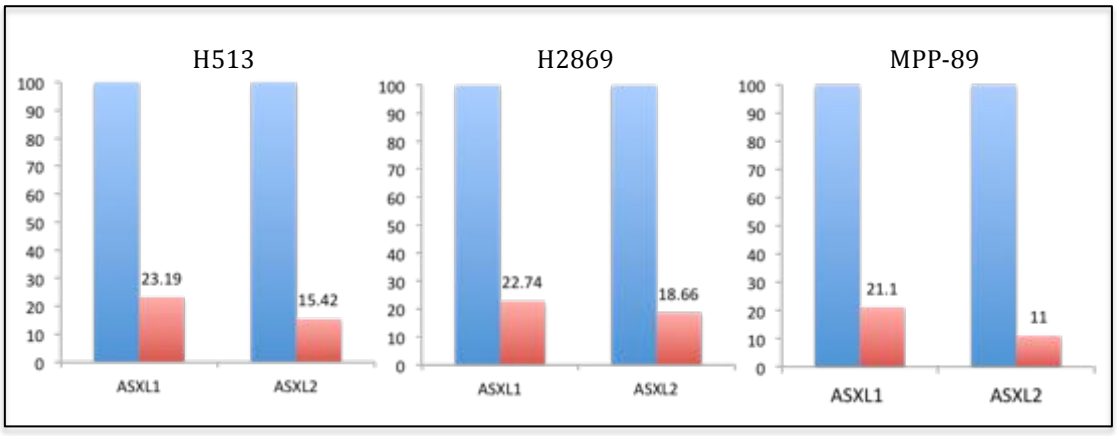


Figure 5-12 ASXL1/2 mRNA expression in ASXL1/2 shRNA transduced MPM lines

RT-PCR analysis of ASXL1/2 mRNA expression in MPM lines transduced with ASXL1/2 shRNA. Figures represent percentage mRNA expression relative to the untransduced line. Blue – untransduced cells, red – transduced cells. A successful reduction in ASXL1 and ASXL2 mRNA expression in the transduced cell lines is demonstrated.

5.3.5 Knockdown of *ASXL1/2* modulates DR agonist sensitivity in MPM cells

The parental, EV, ASXL1 and ASXL2 shRNA transduced MPM cells were treated with a dose range of rTRAIL for 24 hours and cell death was measured with an Annexin V/DAPI assay. These cells were also treated with a dose range of Medi3039 and cell viability measured at 72 hours with an XTT assay.

5.3.5.1 Knockdown of both ASXL1 and ASXL2 sensitises H513 MPM cells to rTRAIL and Medi3039.

Transduction of the parental H513 line with EV shRNA resulted in no significant difference in cell death in response to rTRAIL treatment ($p= 0.9254$) (**Figure 5-13**). Transduction with both ASXL1 and ASXL2 shRNA however resulted in a significant increase in cell death in response to rTRAIL treatment relative to both the EV shRNA transduced and parental cells ($p< 0.0001$). There was no significant difference in cell death in response to rTRAIL treatment between the ASXL1 and ASXL2 shRNA transduced cells ($p= 0.7739$).

This pattern was replicated in cell viability response to Medi3039 treatment. Transduction of the parental H513 line with EV shRNA resulted in no significant difference in cell viability in response to Medi3039 treatment ($p= 0.9770$). Transduction with both ASXL1 and ASXL2 shRNA however resulted in a significant decrease in cell viability in response to Medi3039 treatment relative to both the EV shRNA transduced and parental cells ($p< 0.0001$). There was no significant difference in cell viability in response to Medi3039 treatment between the ASXL1 and ASXL2 shRNA transduced cells ($p= 0.2925$).

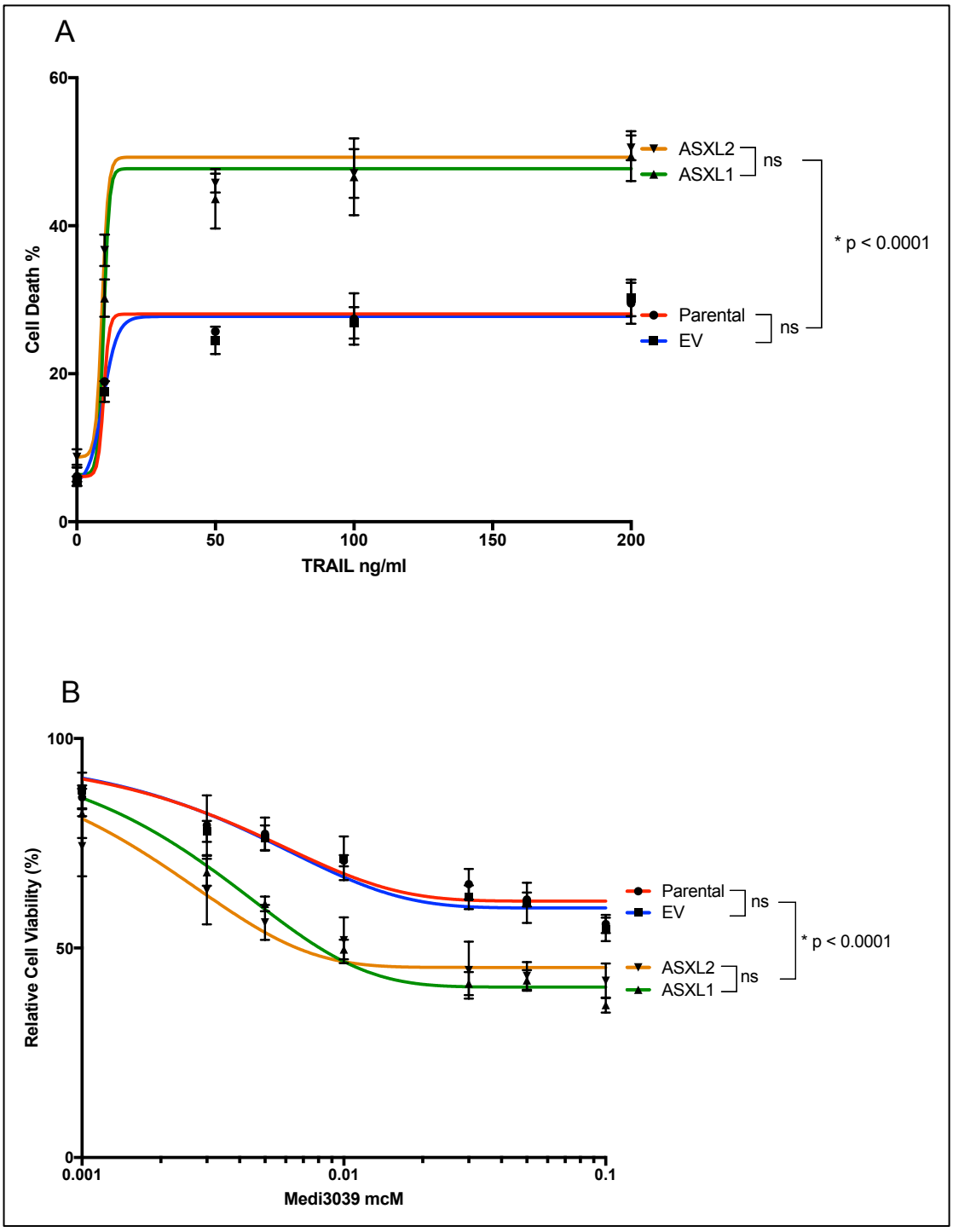


Figure 5-13 shRNA knockdown of ASXL1/2 and response to DR agonist treatment in H513 MPM cells

(A) *Cell death response to rTRAIL treatment – parental, EV, ASXL1 and ASXL2 shRNA transduced cells were treated with a dose range of rTRAIL and cell death measured with an Annexin V/DAPI assay at 24h. Parental vs EV – p = 0.9254. Parental/EV vs ASXL1, parental/EV vs ASXL2 - all p < 0.0001.* ***(B)*** *Cell viability response to Medi3039 treatment - parental, EV, ASXL1 and ASXL2 shRNA transduced cells were treated with a dose range of Medi3039 and cell viability measured with a XTT cell viability assay at 72h. Parental vs EV – p = 0.9770. Parental/EV vs ASXL1, parental/EV vs ASXL2 - all p < 0.0001. Results suggest ASXL1/2 knockdown results in increased sensitivity to DR agonists.*

5.3.5.2 Knockdown of both ASXL1 and ASXL2 sensitises H2869 MPM cells to rTRAIL and Medi3039.

Transduction of the parental H2869 line with EV shRNA resulted in a significant increase in cell death in response to rTRAIL treatment ($p=0.0001$) suggesting an effect of transduction on rTRAIL sensitivity in these cells (**Figure 5-14**). Transduction with both ASXL1 and ASXL2 shRNA however resulted in a significant increase in cell death in response to rTRAIL treatment when compared to both the parental and EV shRNA transduced line ($p<0.0001$). Notably the cell death seen in the ASXL2 transduced line was significantly greater than that seen in the ASXL1 transduced line ($p<0.0001$).

This pattern was replicated in cell viability response to Medi3039 treatment. Transduction of the parental H2869 line with EV shRNA resulted in a significant decrease in cell viability in response to Medi3039 treatment ($p<0.0001$). Transduction with both ASXL1 and ASXL2 shRNA resulted in a significant decrease in cell viability in response to Medi3039 treatment when compared to both the parental and EV shRNA transduced line ($p<0.0001$). Notably the cell viability seen in the ASXL2 transduced line was significantly lower than that seen in the ASXL1 transduced line ($p<0.0001$).

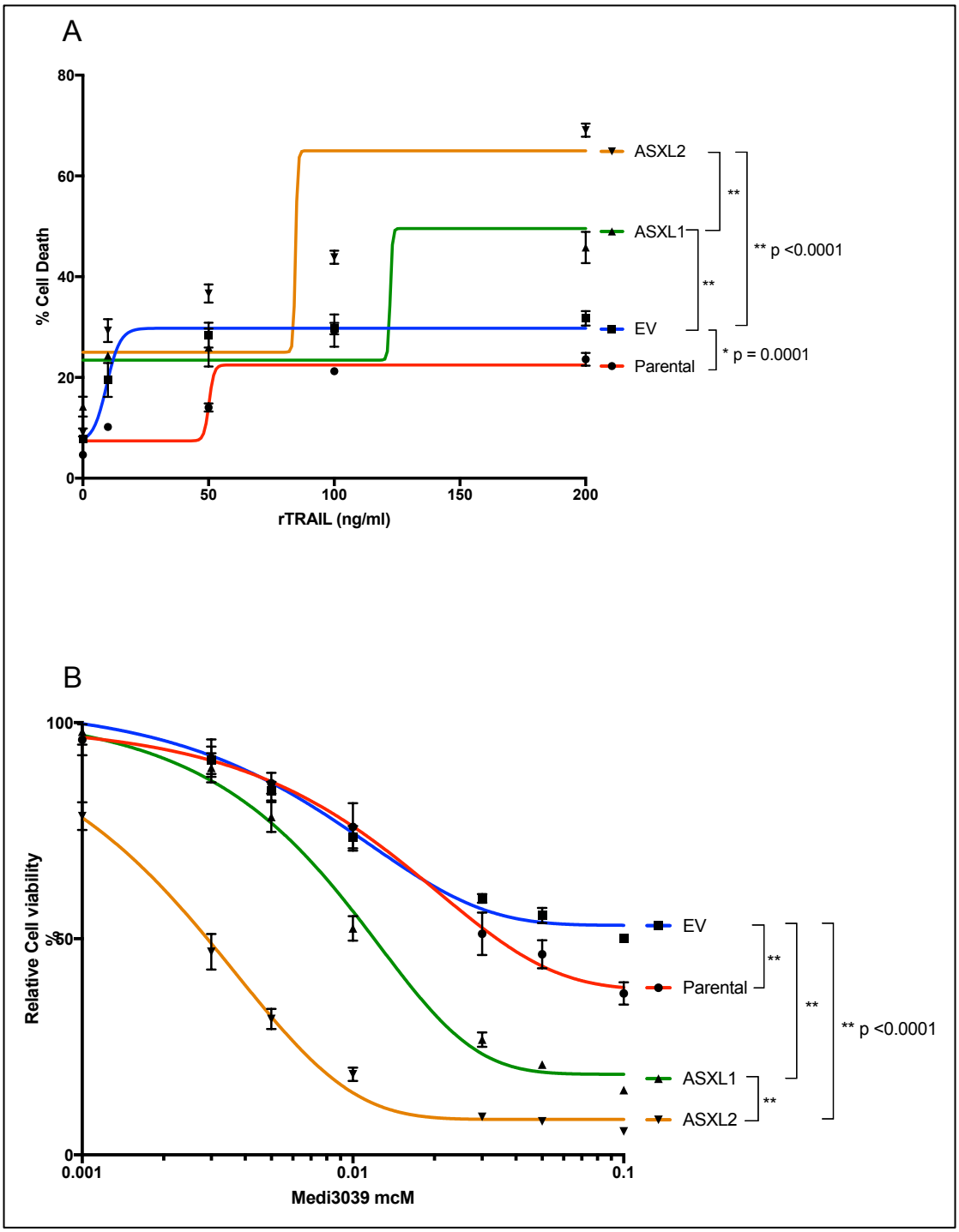


Figure 5-14 shRNA knockdown of ASXL1/2 and response to DR agonist treatment in H2869 MPM cells

(A) *Cell death response to rTRAIL treatment – parental, EV, ASXL1 and ASXL2 shRNA transduced cells were treated with a dose range of rTRAIL and cell death measured with an Annexin V/DAPI assay at 24h. Parental vs EV – p = 0.0001. EV vs ASXL1 and EV vs ASXL2 – p < 0.0001* ***(B)*** *Cell viability response to Medi3039 treatment - parental, EV, ASXL1 and ASXL2 shRNA transduced cells were treated with a dose range of Medi3039 and cell viability measured with a XTT cell viability assay at 72h. Parental vs EV, EV vs ASXL1, EV vs ASXL2 – all p < 0.0001. Results suggest ASXL1/2 knockdown results in increased sensitivity to DR agonists, with ASXL2 having a more significant effect.*

5.3.5.3 Knockdown of ASXL1 but not ASXL2 sensitises MPP-89 MPM cells to rTRAIL and Medi3039.

Transduction of the parental MPP-89 line with EV shRNA resulted in no significant change in cell death in response to rTRAIL treatment (p=0.6671) (**Figure 5-15**). Relative to the parental and EV shRNA transduced cells, transduction with ASXL2 shRNA resulted in no significant change in cell death in response to rTRAIL treatment (p=0.1281 and p=0.3218 respectively). Transduction with ASXL1 shRNA however resulted in a significant increase in cell death in response to rTRAIL treatment relative to both the EV shRNA transduced and parental cell line (p<0.0001).

This pattern was replicated in cell viability response to Medi3039 treatment. Transduction of the parental MPP-89 line with EV shRNA resulted in no significant change in cell viability in response to rTRAIL treatment (p=0.9283). Relative to the parental and EV shRNA transduced cells, transduction with ASXL2 shRNA resulted in no significant change in cell viability in response to rTRAIL treatment (p=0.7723 and p=0.4047 respectively). Transduction with ASXL1 shRNA however resulted in a significant decrease in cell viability in response to rTRAIL treatment relative to both the EV shRNA transduced and parental cell line (p<0.0001).

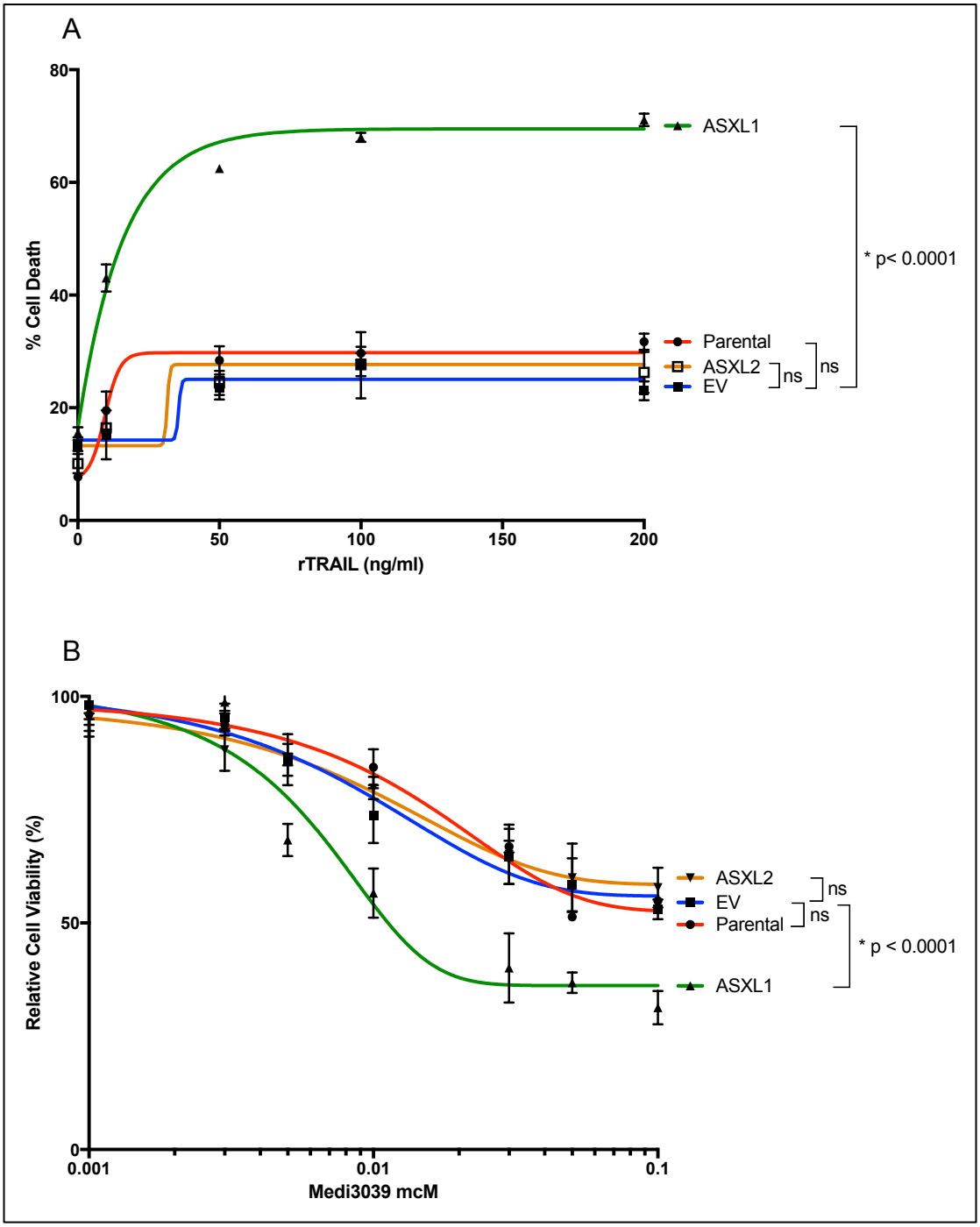


Figure 5-15 shRNA knockdown of ASXL1/2 and response to DR agonist treatment in MPP-89 MPM cells

(A) Cell death response to rTRAIL treatment – parental, EV, ASXL1 and ASXL2 shRNA transduced cells were treated with a dose range of rTRAIL and cell death measured with an Annexin V/DAPI assay at 24h. Parental vs EV – p = 0.6671, parental/EV vs ASXL1 – p < 0.0001, parental vs ASXL2 - p = 0.1281, parental vs ASXL2 - p = 0.3218. (B) Cell viability response to Medi3039 treatment - parental, EV, ASXL1 and ASXL2 shRNA transduced cells were treated with a dose range of Medi3039 and cell viability measured with a XTT cell viability assay at 72h. Results suggest ASXL1 but not ASXL2 knockdown results in increased sensitivity to DR agonists in this cell line. Parental vs EV – p = 0.9283, parental/EV vs ASXL1 – p < 0.0001, parental vs ASXL2 - p = 0.7723, parental vs ASXL2 - p = 0.4047.

5.4 Polycomb repressor complex 1 and 2 signatures

The observation that loss of any of BAP1 or ASXL1/2 function or their interaction sensitises MPM cells to DR agonists points to the role of the PR-DUB in mediating DR agonist sensitivity. The PR-DUB modulates gene transcription by modifying chromatin architecture through the deubiquitination of histone 2A at lysine 119 (H2AK119). This modulation can also be influenced by PRC1, the enzymatic activity of which results in the ubiquitination of H2AK119, but also indirectly by PRC2 that trimethylates histone 3 at lysine 27 (H3K27Me3) that in turn recruits PRC1. In view of this I hypothesised that there is a correlation between H2AK119Ub, or possibly H3K27Me3, and DR agonist sensitivity. If correct, this would lend further support to DR agonist sensitivity being mediated by regulation of gene transcription by the PR-DUB.

5.4.1 Loss of BAP1 function results in an increase in H2AK119 ubiquitination

First I aimed to determine if the mutations of BAP1 that disrupt the PR-DUB, C91A DUB and ΔASXL, increase H2AK119Ub levels in MPM cells. I also assessed if these mutations resulted in an increase in PRC2 activity, as measured by H3K27Me3, these levels might indirectly affect H2AK119Ub by recruiting the PRC1 complex. I therefore conducted immunoblot analysis of H2AK119Ub and H3K27Me3 expression in parental, GFP, BAP1 WT, C91A DUB and ΔASXL transduced H226 lines (**Figure 5-16A).** Following this, through collaboration with Alan Holmes in the UCL translational research office, I also performed quantitative assessment of H2AK119Ub levels in these cells using immunofluorescence.

Immunoblot analysis revealed that transduction of the *BAP1* null parental line with wild-type *BAP1* reduced expression of H2AK119Ub, consisted with increased PR-DUB activity. Transduction with control GFP, or those mutants that disrupt the PR-DUB - C91A or ΔASXL - did not result in any visible

change (**Figure 5-16B**). Transduction of the *BAP1* null H226 line with GFP, BAP1 WT, C91A or ΔASXL did not result in any change in H3K27Me3 levels suggesting that PR-DUB function does not affect PRC2 activity in these cells.

Immunofluorescence allowed quantitative assessment of H2AK119Ub in these cells lines (**Figure 5-16C** and **D**). Transduction of the *BAP1* null parental line with wild-type *BAP1* resulted in a significant decrease in H2AK119Ub levels, while transduction with the C91A mutant resulted in no significant difference. Transduction of the parental H226 line with the ΔASXL mutant resulted in a smaller but significant decrease in expression of H2AK119Ub, but this level was still significantly higher than that transduced with wild-type *BAP1*.

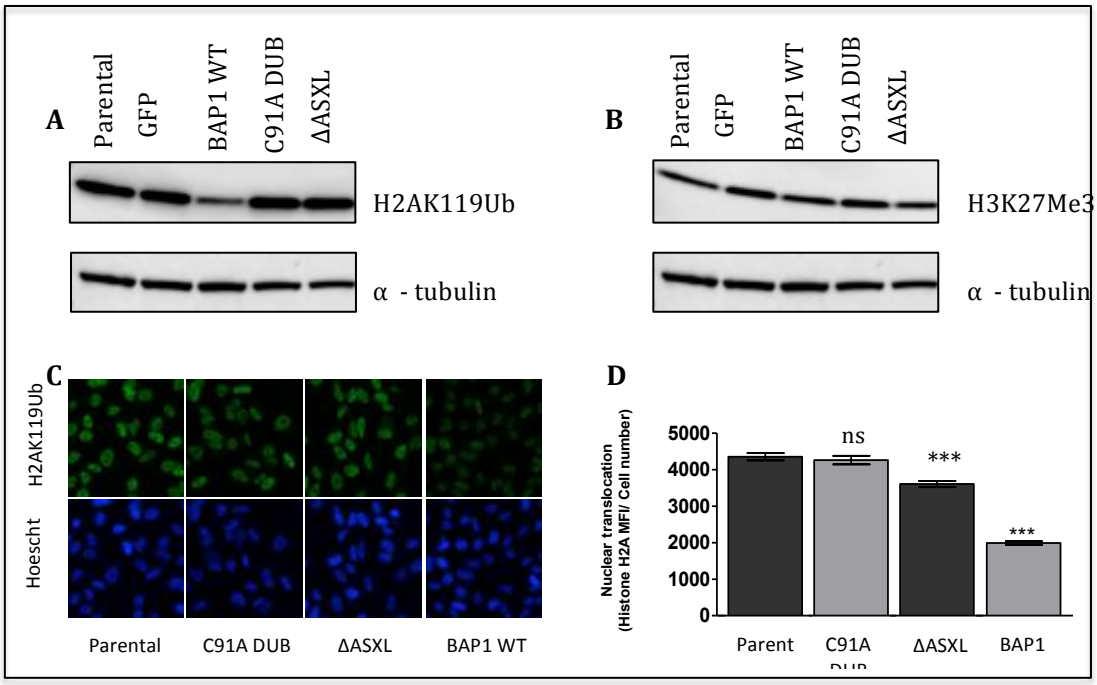


Figure 5-16 H2AK119Ub and H3K27Me3 expression in BAP1 transduced H226 MPM cells

(A) *Immunoblot analysis of H2AK119Ub expression* ***(B)*** *Immunoblot analysis of H3K27Me3 expression* ***(C)*** *Immunofluorescence images of H2AK119Ub expression* ***(D)*** *Median fluorescent intensity (MFI) of cells stained for H2AK119Ub. *** p < 0.0001.*

Parental – untransduced, C91A – DUB mutant transduced, ΔASXL – ASXL binding mutant transduced, BAP1 WT – wild type transduced.

5.4.2 PRC signatures and DR agonist sensitivity in MPM cells

The above data demonstrates that disruption of the PR-DUB in MPM results in an increase in H2AK119Ub levels and no effect on H3K27Me3 levels. This correlates with DR agonist sensitivity in H226 cells. I next aimed to determine if there is a correlation between H2AK119Ub and DR agonist sensitivity in the established MPM lines. A correlation would be supportive of PR-DUB activity upon H2AK119Ub levels as being the mechanism underlying DR agonist sensitivity.

Immunoblot analysis of the established MPM lines however did not reveal an obvious correlation between H2AK119Ub expression and DR agonist sensitivity (**Figure 5-17**). Interestingly, the *BAP1* mutant lines did not demonstrate significantly higher H2AK119Ub expression than the wild-type *BAP1* lines. I also assessed immunoblot expression of H3K27Me3 in these lines to determine if a change in this, and therefore PRC1 recruitment, might account for the discrepancy but no consistent correlation was identified.

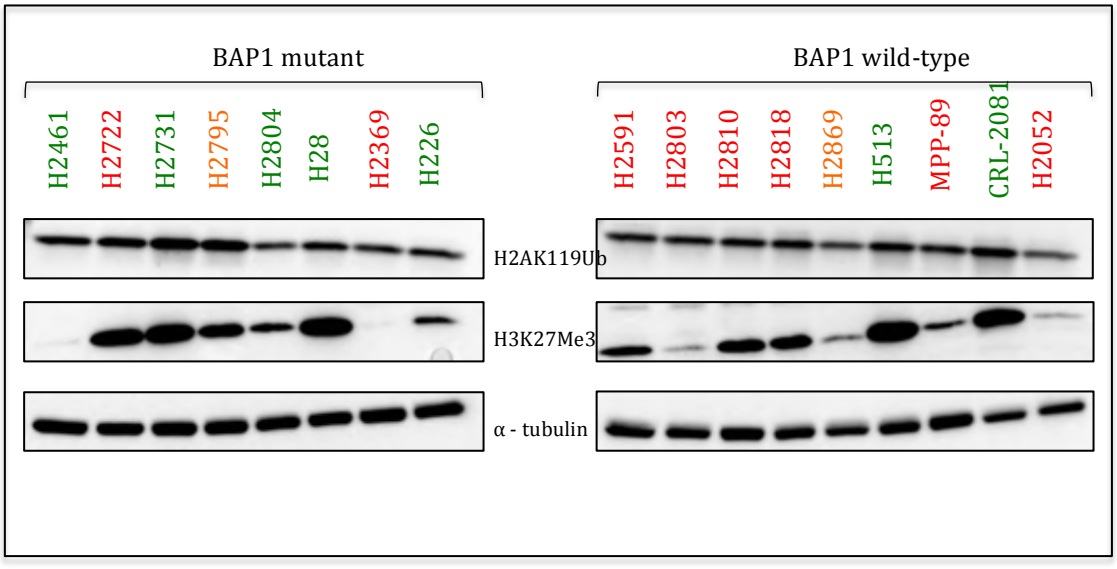


Figure 5-17 Immunoblot of H2AK119Ub and H3K27Me3 in MPM lines

Cell lines are stratified by BAP1 status and rTRAIL sensitivity. Green – sensitive, orange – partially sensitive, red – resistant. There is no correlation between H2AK119Ub or H3K27Me3 expression and DR agonist sensitivity

5.5 Discussion

5.5.1 Interaction of BAP1 with ASXL1/2 mediates rTRAIL resistance

The above data suggests that the interaction of BAP1 with ASXL1/2 is key in mediating rTRAIL sensitivity. This finding, together with those of K. Kolluri that binding of BAP1 to HCF-1 and FoxK1/2 does not affect TRAIL sensitivity, raises several interesting implications for the underlying mechanism. The different proteins to which BAP1 binds form different complexes that mediate different functions, predominantly the regulation of expression of different genes. Therefore those BAP1 interactions that are key to rTRAIL resistance may point to which regulatory complexes, and therefore which genes, might be involved.

BAP1 binds to ASXL1/2 to form the polycomb repressor deubiquitinase complex (PR-DUB), a known transcriptional regulatory complex [67]. While a number of deubiquitination substrates for BAP1 have been identified, including itself, the main substrate for the PR-DUB complex thus far identified is lysine 119 on histone 2A (H2AK119Ub) [67]. Deubiquitination at this site modifies chromatin architecture to regulate gene expression and is opposed by the ubiquitination activity of the Ring1A/B enzyme within the polycomb repressor complex 1 (PRC1). Thus as disruption of the PR-DUB results in increased rTRAIL sensitivity it is likely that those genes regulated by the PR-DUB complex are involved. While the number of genes regulated by the PR-DUB is in the order of thousands, the interaction of BAP1 with other proteins might further narrow down those genes involved in TRAIL sensitivity.

FoxK1/2 are transcription factors the functions of which have been linked to cell cycle regulation and cell proliferation [170, 171]. Less is known about the activities of FoxK1 and there have been no published studies looking at the effect of the BAP1/FoxK1 interaction on gene expression. FoxK2 however has been found to recruit BAP1 to FoxK2 target genes through the forkhead-

associated domain and BAP1 depletion results in an up-regulation of FoxK2 target genes [47]. As the finding of K. Kolluri that disruption of the BAP1/FoxK2 interaction does not affect rTRAIL sensitivity one can infer that FoxK2 target genes are not involved in mediating rTRAIL sensitivity. Interestingly, evidence suggests that this BAP1 dependent FoxK2 gene repression involves the activity of BAP1 as part of the PR-DUB complex [52] and that it is opposed by PRC1 dependent ubiquitination of H2AK119 which results in the activation of FoxK2 target genes. Thus it appears that the activity of the PR-DUB may be targeted towards specific genes by additional proteins that bind to BAP1 such as FoxK2. Evidence suggests that HCF-1 is also bound to BAP1 within this complex but it is not necessary for FoxK2 target gene repression [47]. K. Kolluri found that disruption of BAP1-HCF1 interaction does not affect rTRAIL sensitivity. Thus neither FoxK2 nor HCF-1 binding is involved in rTRAIL sensitivity, which suggests that the target genes to which these transcription factors direct the PR-DUB are not involved in rTRAIL sensitivity.

To further identify those genes involved in rTRAIL sensitivity it would be interesting to perform a mRNA microarray study of H226 cells transduced with the ASXL binding mutant and compare this analysis with that already obtained from the WTSI from the BAP1 WT and DUB mutant transduced cells. Those genes that have the same change in the DUB and ΔASXL mutants are likely to be regulated by PR-DUB activity and this would further narrow the potential genes down. Identification of FoxK2 and HCF-1 target genes through a ChIP assay might also help to exclude those genes less likely to be involved in rTRAIL sensitivity.

5.5.2 Loss of BAP1 PR-DUB function alters expression of apoptotic pathway proteins

I hypothesised that BAP1 activity modulates expression of components of the apoptotic pathway to influence DR agonist sensitivity. Microarray data from the WTSI confirmed that the mRNA expression of components of the

apoptotic pathway significantly differed between wild-type and DUB mutant *BAP1* transduced cells (**Figure 1-9**). One might expect a consistent pattern where the expression of anti-apoptotic proteins decreased and pro-apoptotic proteins increased in the rTRAIL sensitive DUB mutant transduced cells. However the mRNA expression of some anti-apoptotic components was found to increase in the presence of DUB mutant *BAP1*. The above flow cytometry and immunoblot data however reveals that protein expression of the levels of pro- and anti- apoptotic components differ from the mRNA expression. Cell surface expression of the pro-apoptotic receptors DR4 and DR5 were found to increase in the presence of DUB mutant *BAP1*, consistent with the mRNA expression, which was also found to increase. Protein expression of the inhibitory apoptotic proteins cFLIP, cIAP1 and cIAP2 however were found to decrease in the presence of the DUB and ΔASXL binding mutant while the mRNA expression was conversely found to be increased. Protein expression of FADD and survivin did not change in the presence of both DUB and ΔASXL, while the mRNA expression of these proteins was found to increase. It seems therefore that although BAP1 activity affects gene transcription of the apoptosis pathway, there are additional post-transcriptional events that occur that affect protein expression. Overall pro-apoptotic protein expression increased and anti-apoptotic protein expression decreased. It is however important to note that the expression of all apoptotic pathway proteins was not assessed. Therefore the conclusion that can be drawn is limited to expression of some of the apoptotic pathway components is altered by BAP1 activity at both an mRNA and protein level and that these two are not always consistent. The consistent pattern of change of protein expression in the presence of both the DUB and ASXL binding mutant however supports both these functions mediate TRAIL sensitivity and further implicates the PR-DUB.

5.5.3 MPM lines express different levels of extrinsic apoptotic pathway proteins

The correlation of DR4 expression, and to a lesser extent DR5 expression, with rTRAIL sensitivity observed in the established MPM lines is notable. This

is suggestive that an increase in DR expression contributes to rTRAIL sensitivity in MPM. Although the flow cytometry data confirms that loss of BAP1 DUB function results in an increase in the expression of DR4 and DR5, the observation that the *BAP1* wild-type but rTRAIL sensitive cell CRL-2081 has high DR4/5 expression and the *BAP1* mutant but rTRAIL resistant cell H2722 has low DR4 expression suggests that mechanisms other than BAP1 exist that can also regulate their expression.

On analysis of expression of the other extrinsic apoptotic pathway proteins in these cell lines there is a much less consistent correlation with TRAIL sensitivity and *BAP1* status. There is a trend towards increased expression of the pro-apoptotic caspase 8 and lower expression of the anti-apoptotic XIAP in the rTRAIL sensitive *BAP1* mutant cells but not an obvious trend in the other proteins assessed. While these are interesting observations, mechanistic conclusions cannot be drawn from these trends. One can however conclude that protein expression of components of the extrinsic apoptotic pathway vary between different MPM lines. The balance of pro- and anti- apoptotic proteins expressed may determine the degree of apoptosis seen in response to death receptor activation. Indeed this is believed to underlie much of the heterogeneity in response to DR agonists observed in cell lines from other malignancies.

5.5.4 Loss of ASXL1/2 function increases sensitivity to DR agonists

The above data suggests that ASXL1 and ASXL2 loss of function can also result in increased sensitivity to DR agonists in MPM. Initial assessment of ASXL1/2 expression in MPM lines did not reveal any obvious correlation with rTRAIL sensitivity. All the MPM lines expressed both ASXL1 and ASXL2 irrespective of *BAP1* status. Perhaps this is not surprising, as *ASXL1* and *ASXL2* mutations are not known to be associated with MPM. Evidence from the literature also suggests that there is a degree of co-regulation of expression between ASXL1/2 and BAP1. Daou *et al*. found that ASXL1/2 protein levels were increased following overexpression of BAP1 in 293T cells

while conversely BAP1 protein levels increased in a dose dependent manner with increased ASXL1/2 expression [45]. Knockdown of *BAP1* also reduced ASXL2 expression [45]. In view of this I assessed ASXL1/2 expression in the BAP1 mutant transduced H226 MPM cells to determine if any particular loss of BAP1 function resulted in a change in ASXL1/2 expression. Interestingly this did not reveal a change in ASXL1/2 expression with any of the *BAP1* mutants. It is possible that this is as none of the functional sites mutated in the above lines are involved in ASXL1/2 regulation, however this seems unlikely as these sites cover the major functional domains of BAP1. Alternatively the 293T transfection model used by Daou *et al*. might not replicate the complexity of a cancer cell line. In either case the data does not support a role for BAP1 in the regulation of ASXL1/2 expression in MPM.

The above data also supports that loss of ASXL1/2 function increases DR agonist sensitivity in MPM. It is notable however that the ability of ASXL1 and ASXL2 to modulate DR agonist sensitivity is not consistent across the cell lines. In H513 and H2869 cells, knockdown of both ASXL1 and ASXL2 increases the sensitivity of cells to DR agonists, however the effect of ASXL2 knock down is significantly greater than ASXL1 in H2869. In MPP-89 cells knock down of ASXL1, but not ASXL2, increases the sensitivity of cells to DR agonists. Both ASXL1 and ASXL2 are capable of binding to BAP1 to form the PR-DUB and deubiquitinate H2AK119Ub and these complexes are mutually exclusive. It is notable therefore that immunoblot analysis (**Figure 5-8**) of MPP-89, where ASXL1 but not ASXL2 knockdown increases DR agonist sensitivity, ASXL1 expression is higher than ASXL2 expression. In H2869, where ASXL2 knockdown has a significantly greater effect on DR agonist sensitivity than ASXL1, ASXL2 expression is higher and in H513 where both ASXL1 and ASXL2 knockdown has the same effect on DR agonist sensitivity the expression of both appears to be equal. It may be therefore that the overall amount of ASXL1 and ASXL2 expressed determines the activity of the PR-DUB, which could explain the different patterns seen in the different cell lines.

5.5.5 Loss of BAP1 PR-DUB function increases H2AK119Ub expression

The above data demonstrates that disruption of the PR-DUB in the H226 MPM line results in an increase in H2AK119Ub expression with no effect on H3K27Me3 levels. However assessment of H2AK119Ub and H3K27Me3 expression in MPM lines did not reveal an obvious correlation with DR agonist sensitivity. Although this data does not conclusively support my hypothesis that H2AK119Ub levels mediate DR agonist sensitivity, it does not conclusively refute it. To infer causation, rather than correlation, between H2AK119Ub and DR agonist sensitivity in MPM, an interesting experiment might be conducted whereby PRC1 enzymatic activity is inhibited which would decrease H2AK119Ub level. If this were to result in a corresponding decrease in DR agonist sensitivity this would demonstrate that modulation of H2AK119Ub affects DR agonist sensitivity. Ring1A/B inhibitors are available to inhibit PRC1 enzymatic activity and this experiment is part of my future work plan.

5.6 Summary

- The interaction of BAP1 with ASXL1/2 is key to mediating DR agonist sensitivity in MPM indicating a role for the PR-DUB
- Loss of BAP1 activity modulates expression of components of the apoptosis pathway at both an mRNA and protein level
- Loss of ASXL1 and ASXL2 function increases sensitivity to DR agonists in MPM
- The influence of ASXL1 and ASXL2 on DR agonist sensitivity varies across MPM lines and may be related to total protein expression
- Loss of PR-DUB activity increases H2AK119Ub expression in MPM lines

CHAPTER VI:

SUMMARY

6 SUMMARY AND FUTURE DIRECTIONS

The overall hypothesis for this thesis is that loss of BAP1 function can act as a biomarker for DR agonists in MPM and that the underlying mechanism involves the regulation of expression of components of the extrinsic apoptotic pathway by BAP1. The data presented within this thesis provides data that supports a loss of BAP1 function augments sensitivity to rTRAIL in *in vitro* models in the form of cell lines and early passage cell cultures and in an *ex vivo* model in the form of MPM tumour explants. The data presented also supports that loss of BAP1 function also augments sensitivity to other DR agonists such as Medi3039 as demonstrated in *in vitro* models. With regard to the underlying mechanism the data supports that loss of the capabilities of BAP1 that underlie its role in the polycomb repressor deubiquitinase (PR-DUB), deubiquitiniase and ASXL binding, mediate response to DR agonists. Loss of both the deubiquitinase and ASXL binding functions also result in modulation of expression of components of the extrinsic apoptotic pathway at both an mRNA and protein level. Consistent with a role for the PR-DUB in mediating DR agonist sensitivity, the data presented supports that loss of ASXL1 and/or ASXL2 function also augments sensitivity to DR agonists in MPM. ASXL1/2 therefore may act as further biomarkers for DR agonist sensitivity in relevant cancers. Here I discuss the clinical implications and future directions for the work.

6.1 BAP1 expression and mutations in mesothelioma

Data presented in chapter III supports that a significant proportion of MPM tumours taken from a cohort of UK patients lose nuclear expression of BAP1. Published research supports that this loss of expression is the result of mutations in *BAP1,* which supports the use of immunohistochemistry to

identify MPM tumours with *BAP1* mutations [35]. It would be interesting to sequence the MPM tumours from the MSO1 study to confirm that those with loss of BAP1 nuclear expression indeed harbour mutations in *BAP1*. This would need to comprise a comprehensive integrated molecular approach to identify all *BAP1* mutations rather than Sanger or next generation sequencing alone. Laser capture microdissection of single cells would also need to be conducted to obtain tissue suitable for analysis. It would be relatively easy however to perform comprehensive integrated molecular sequencing of the Mesobank early passage MPM cultures for *BAP1* mutational status to determine if this correlates with nuclear expression and rTRAIL sensitivity. I therefore intend to conduct this as part of my future work plan.

6.2 Further validation of BAP1 as a biomarker for TRAIL sensitivity

Data presented in chapters III and IV supports that loss of BAP1 function augments sensitivity to DR agonists as demonstrated in cell lines, early passage cell cultures, and a limited number of tumour explants. K. Kolluri also presented data validating the association between loss of BAP1 function and rTRAIL sensitivity in a mouse xenograft model (**Figure 1-10**). With regards to further validation I would propose concentrating on expanding the cohort of human tumour explants as the most convincing preclinical model. This however is dependent upon the number and quality of surgical biopsy specimens available. The tumour explants generated in this thesis were taken from patients undergoing therapeutic pleurectomy. We have also attempted to generate tumour explants from diagnostic biopsy specimens taken at video assisted thoracoscopic surgery. However, these specimens are significantly smaller than those obtained at pleurectomy and as such the explants generated were unsuitable for immunohistochemical analysis. Thus the expansion of the explant cohort is subject to the number of patients

undergoing pleurectomy at our local surgical centre. As few patients with MPM are suitable for this operation this is a relatively infrequent procedure and therefore it may take some time to develop a large cohort.

Further validation in a genetically engineered mouse model (GEMM) of mesothelioma would also be an advance on the mouse xenograft model used by K. Kolluri. A published *BAP1*$^{+/-}$ GEMM exists that develops peritoneal biphasic mesothelial tumours following intraperitoneal asbestos injections every 3 weeks for 4 cycles with a median survival time of 43 weeks in BAP1$^{+/-}$ vs 55 weeks in WT littermates [172]. However Professor Anton Berns at the Netherlands Cancer Institute has developed an as yet unpublished conditional knockout GEMM of mesothelioma that I believe is superior to which we will have access through collaboration [173]. These GEMMs have a background genotype of *CDKN2a*$^{-/-}$*/NF2*$^{f/f}$, which expedites tumour development, and alternate additional genotypes of *BAP1*$^{+/+}$, *BAP1*$^{f/+}$ or *BAP1*$^{f/f}$ thus enabling the assessment of the effect of *BAP1* status against a common genetic background. This model spontaneously develops pleural mesothelial tumours with a median survival time of 13 weeks in *BAP1* mutant vs 19 weeks in WT littermates, thus radically reducing experimental time, negating the use of hazardous asbestos administration and offering a model in keeping with the focus of this project on pleural not peritoneal mesothelioma. Furthermore the tumours in this model recapitulate several features of human pleural mesothelioma including an epithelioid phenotype, pleural effusions and mediastinal invasion. This model also offers locotemporal control over induction of the mutations thus facilitating experimental control over both the timing of tumour development and targeting of the mutation to the thoracic mesothelial lining, rather than the germline. Therefore I plan to use these mice in the future to further validate BAP1 as a biomarker for rTRAIL sensitivity *in vivo*.

With regard to further *in vitro* validation, CRISPR-Cas9 technology to edit out the *BAP1* gene would also be a more robust model than shRNA knockout

models in which to study the effect of BAP1 loss of function in cell lines and early passage cell cultures and to study the downstream mechanism. Conference data from other labs and the experience of our own however suggests that complete knockout of *BAP1* often results in cell death highlighting the ubiquitous nature of the functions of this protein.

BAP1 mutations are prevalent in a number of additional malignancies including uveal melanoma, clear cell renal carcinoma and intrahepatic cholangiocarcinoma [75, 78, 160, 174]. Although in this study we have demonstrated that loss of BAP1 function sensitises the breast cancer cell line MDAMB-231 and the clear cell renal carcinoma lines Caki-1 and BB65 to DR agonists, it would be pertinent to demonstrate this in additional cancer types with a high prevalence of *BAP1* mutations. I plan to perform shRNA knockdown of BAP1 in uveal melanoma and cholangiocarcinoma cells and treat them with DR agonists to this end.

6.3 Mechanism of BAP1 induced TRAIL resistance

Data presented in chapter V indicates that BAP1 might modulate DR agonist sensitivity in a complex with ASXL1 or 2 as part of the polycomb repressor deubiquitinase (PR-DUB). This is a known transcriptional regulatory complex that along with the polycomb repressor complexes 1 and 2 (PRC1/2) conducts epigenetic modifications to influence gene transcription [67]. Data presented also supports that disruption of the functions of BAP1 that underpin the activity of this complex alter expression of components of the extrinsic apoptotic pathway. This raises a number of further interesting questions to be explored.

Although the data within this thesis supports that loss of BAP1 deubiquitinase and ASXL binding activity mediates DR agonist sensitivity, further experiments will need to be conducted to directly implicate the PR-DUB. Loss of BAP1 function results in an increase in global H2AK119Ub levels and an

associated increase in DR agonist sensitivity. Therefore if treatment of MPM cells with a RING1A/B inhibitor, which would decrease global H2AK119Ub levels, results in a decrease in DR agonist sensitivity, this would directly implicate this epigenetic modification, and in turn the PR-DUB, in DR agonist sensitivity. Such combination experiments are part of my future work plan

I also plan to conduct further experiments to clarify the effect and significance of loss of ASXL1/2 function on DR agonist sensitivity. The data within this thesis suggests that loss of ASXL1 and/ or 2 function can increase sensitivity to DR agonists. The reason for the discrepancy in response to loss of ASXL1/2 between the cell lines tested in this thesis is however is not clear. Both ASXL1 and ASXL2 are capable of binding to BAP1 and the resulting complexes both of deubiquitinating H2AK119Ub. It might be that the total amount of ASXL1 and ASXL2 determines PR-DUB activity and thus response to DR agonist treatment. To this end it would be interesting to knockdown both ASXL1 and ASXL2 in MPM lines and assess the response to DR agonists.

Although the above experiments are interesting mechanistically, a more clinically relevant model would be to assess loss of function of ASXL1/2 in those cancers in which mutations are found. I plan to assess response to DR agonist therapy in *ASXL1* wild-type and mutant AML cell lines and also in knockdown *ASXL1* in wild type AML cell lines and assess response. Similar experiments could also be conducted on breast and prostate cancer cell lines to assess the clinical significance of ASXL2 on DR agonist sensitivity.

6.4 Sensitisation of BAP1 wild type tumours

If the mechanism by which loss of BAP1 function sensitises cancer cells to DR agonists can be determined then pharmacological replication of this could sensitise *BAP1* wild-type tumours to DR agonists. However the data within

this thesis suggests that the expression of several apoptotic pathway proteins is affected by BAP1 function and therefore an attempt to identify the key proteins is likely to be a challenge. An alternate strategy however could be to inhibit BAP1 itself. We have therefore partnered with the UCL drug discovery group to develop an inhibitor of BAP1 deubiquitinase activity. The successful development of such an inhibitor could not only be used to sensitise *BAP1* wild-type tumours to DR agonists, but also to other targeted agents such as EZH2, HDAC and PARP inhibitors sensitivity to which is associated with loss of BAP1 function [87, 89, 91].

6.5 Clinical implications

6.5.1 Clinical implications in haematological malignancies

ASXL1 mutations have been frequently observed in myeloid malignancies and *ASXL2* mutations in breast, prostate, bladder and pancreatic cancer [68]. The data within this thesis supports that loss of function of ASXL1 and/or 2 also leads to increased sensitivity to DR agonists in MPM lines. As noted above it would be pertinent to assess the effect of loss of ASXL1/2 function in clinically relevant cell lines. However should this also demonstrate that loss of ASXL1/2 function augments sensitivity to DR agonists in these models, the potential clinical relevance of this would remain unclear. All *BAP1* mutations identified in MPM are loss of function and therefore one would predict such tumours would be sensitive to DR agonist therapy. In the case of myeloid malignancies with *ASXL1* mutations however it is not clear whether these result in loss of function, gain of function or dominant negative effects [72]. The potential clinical utility would therefore depend on further clarity in this area as it is likely that only loss of function mutations would result in DR agonist sensitivity. Truncating mutations of *ASXL2* in breast and prostate cancers have been identified and are predicted to result in either a degraded protein or small N terminal proteins thus DR agonist therapy may be of more relevance in such

cases. However, this comprises a significantly smaller population of patients than those with *ASXL1* mutations.

6.5.2 Clinical implications in MPM

New therapies are desperately needed for MPM. Current first line therapy for MPM, pemetrexed/cisplatin, suffers from limited efficacy and a significant side effect profile. Alternative solutions for MPM treatment include checkpoint inhibitors and targeted agents against VEGF however both suffer from limitations. Phase 1b testing of the anti PD-1 antibody Pembrolizumab as second line therapy in PD-L1 positive MPM demonstrated a stable disease in 72% of a limited cohort (n = 25) but 64% of patients reported a treatment related adverse effect including fatigue, nausea and arthralgia [175]. Phase II results are pending, however evidence suggests this therapy is likely to only be effective in the 20% of patients with PD-L1 positive MPM [176]. The anti VEGF antibody Bevacizumab has demonstrated a modest improvement in overall survival over pemetrexed/cisplatin (18.8 vs. 16. months) but only when used in combination with pemetrexed/cisplatin [177]. Furthermore significant rates of hypertension, cardiovascular events, and arterial and venous thromboembolic events were noted. Therefore while it has received FDA approval for first line-treatment in MPM those at risk of the above side effects are excluded, a significant proportion given the demographic affected by MPM. Following cost-effectiveness analysis it has not been licenced by NICE.

An alternative treatment for the significant majority of MPM patients that harbour *BAP1* mutations in the form of DR agonist therapy is therefore a clinically significant finding. To date there have been no trials of any DR agonist in MPM or any other cancer with a high prevalence of *BAP1* mutations. Such a trial might have demonstrated clinical efficacy of DR agonists unlike those trials conducted to date. I believe the data presented supports a case for conducting a BAP1 stratified clinical trial for a DR agonist therapy in MPM. Indeed similar preclinical data demonstrating that loss of BAP1 function predicts sensitivity to EZH2 inhibitors in MPM has lead to a

phase II clinical trial of tazemetostat currently underway [91] (NCT02860286). Despite limited success in clinical trials to date new DR agonists continue to be developed by the pharmaceutical industry that aim to overcome the limitations of early DR agonists, in particular that of univalent binding. Novel DR agonists such as Medi3039 (Medimmune) and ABBV-621 (Abbvie) aim to maximise receptor clustering and activation of the death inducing signaling complex (DISC). Such compounds have currently yet to complete phase I testing however a shrewd strategy would be to trial these compounds in MPM and/or other malignancies with a high rate of *BAP1* mutations to assess the effect of this proposed biomarker.

CHAPTER VII:

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7 BIBLIOGRAPHY

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