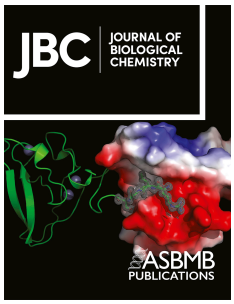

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BAP1 and YY1 regulate expression of death receptors in malignant pleural mesothelioma

Yuki Ishii[1]*, Krishna K. Kolluri[1]*, Adam Pennycuick[1], Xidan Zhang[1], Ersilia Nigro[1,2], Doraid Alrifai[1], Elaine Borg[3], Mary Falzon[3], Khalid Shah[4], Neelam Kumar[1#], Sam M. Janes[1#]

[1] Lungs for Living Research Centre, UCL Respiratory, University College London, London, United Kingdom

[2] Department of Environmental, Biological, and Pharmaceutical Sciences and Technologies, University of Campania Luigi Vanvitelli, Caserta, Italy

[3] Department of Histopathology, University College London Hospital, Euston Road, London, United Kingdom

[4] Center for Stem Cell Therapeutics and Imaging, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

* ; These authors contributed equally to this work.

; To whom correspondence should be addressed:

Neelam Kumar: neelam.kumar@alumni.ucl.ac.uk

Samuel M Janes: s.janes@ucl.ac.uk

Running title:

On the trail of BAP1 and YY1

Statement of significance:

We describe that YY1 interacts with BAP1, the most-frequently mutated tumour suppressor gene in mesothelioma, to regulate the response to TNF-related apoptosis-inducing ligand (TRAIL). These findings will accelerate a biomarker-driven cancer therapy.

Keywords:

BAP1, YY1, TRAIL, apoptosis, cancer therapy, tumor cell biology, receptor regulation

Abstract

Malignant pleural mesothelioma (MPM) is a rare, aggressive, and incurable cancer arising from the mesothelial lining of the pleura, with few available treatment options. We recently reported loss of function of the nuclear deubiquitinase BRCA1-associated protein 1 (BAP1), a frequent event in MPM, is associated with sensitivity to tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis. As a potential underlying mechanism, here we report that BAP1 negatively regulates the expression of TRAIL receptors: death receptors 4 (DR4) and 5 (DR5). Using tissue microarrays (TMAs) of tumour samples from MPM patients, we found a strong inverse correlation between BAP1 and TRAIL receptor expression. *BAP1* knockdown increased DR4 and DR5 expression, whereas overexpression of BAP1 had the opposite effect. Reporter assays confirmed wild-type *BAP1*, but not catalytically-inactive mutant *BAP1*, reduced promoter activities of *DR4* and *DR5*, suggesting deubiquitinase activity is required for the regulation of gene expression. Co-IP studies demonstrated direct binding of BAP1 to the transcription factor Ying Yang 1 (YY1), and ChIP assays revealed BAP1 and YY1 to be enriched in the promoter regions of *DR4* and *DR5*. Knockdown of *YY1* also increased DR4 and DR5 expression and sensitivity to TRAIL. These results suggest that BAP1 and YY1 cooperatively repress transcription of TRAIL receptors. Our finding that BAP1 directly regulates the extrinsic apoptotic pathway will provide new insights into the role of BAP1 in the development of MPM and other cancers with frequent BAP1 mutations.

Introduction

Malignant pleural mesothelioma (MPM) is a rare, aggressive cancer that arises from the mesothelial lining of the lungs and is commonly associated with occupational exposure to asbestos. There are currently no curative therapies. Standard first line treatment is combination chemotherapy consisting of an anti-folate and a platinum agent which offers only a modest survival benefit (1). Advances in the understanding of MPM tumour biology have led to the development of multiple novel targeted agents currently in preclinical and clinical development. Many of these therapies lack a biomarker for activity and results so far have not delivered an effective clinical therapy (2) .

A molecular target of significant interest in MPM is *BRCA1-associated protein 1* (*BAP1)*(3–5). *BAP1* mutations are frequent in MPM (23-67%) and in other tumour types including uveal melanoma (31-50%), cholangiocarcinoma (20-25%) and clear cell renal cell carcinoma (CCRCC) (8-14%) (6, 7, 16–19, 8–15). BAP1 is a deubiquitinase (DUB) that binds to a number of transcription factors through which it regulates gene transcription and modulates cellular pathways such as DNA repair, cell cycle and cell death (4, 5) The response to drugs that act upon these pathways, including PARP and EZH2 inhibitors, has been shown to be increased in the absence of BAP1 function (20). Clinical trials of these drugs in *BAP1* mutant MPM are underway (21). In addition to its function as a nuclear deubiquitinase, a recent report suggests BAP1 also has cytoplasmic functions involving the regulation of cell death and mitochondrial metabolism (22).

We have previously demonstrated that loss of BAP1 function results in sensitivity to the death receptor (DR) agonist recombinant tumour necrosis factor-related apoptosis-inducing ligand (rTRAIL) (23). TRAIL is a member of the tumour necrosis factor (TNF) cytokine superfamily. It activates the extrinsic apoptotic pathway by binding to either of two death receptors, DR4 or DR5, which leads to the recruitment of the adaptor protein FADD and caspase-8 to form the death-inducing signalling complex (DISC) (24). Once formed, catalytic subunits of caspase-8 are cleaved and activate downstream effector caspases triggering apoptosis (25, 26). Activation of this pathway by TRAIL is specific to cancer cells, however the mechanism of this

selectivity is poorly understood (27, 28). Several therapeutic DR agonists including rTRAIL and agonistic DR4/5 antibodies have been developed (29–31). Clinical trials of such agents to date have demonstrated broad tolerability, but unfortunately limited therapeutic benefit (32). Potential reasons include the suboptimal pharmacokinetics of compounds, resistant cell populations and the lack of a targeting biomarker (33). Novel DR agonists with improved pharmacokinetics are in development and potential biomarkers such as BAP1 are emerging (34, 35).

We have extensively validated the association between loss of BAP1 function and increased sensitivity to rTRAIL in *in vitro*, *in vivo* and *ex vivo* models(23). Here we set out to delineate the mechanisms underlying this association. We hypothesise that BAP1 activity modulates expression of proteins of the extrinsic and intrinsic apoptosis pathways with an increase in pro-apoptotic protein expression in the absence of BAP1 activity. We demonstrate both BAP1 activity and rTRAIL sensitivity correlate with expression of the death receptors DR4 and DR5 at the transcriptional level. As BAP1 lacks DNA binding sites, we searched for the transcriptional factor that cooperates with BAP1 to modulate expression of DR4 and DR5 identifying the polycomb group (PcG) protein YY1.

Results

Loss of BAP1 activity correlates with increased DR4 and DR5 expression and increased rTRAIL sensitivity

We have previously shown that MPM cells with loss of BAP1 function are more sensitive to treatment with rTRAIL (23). To determine the mechanism underlying this, we investigated the expression of death receptors DR4 and DR5, the levels of which are known to significantly contribute to TRAIL response (36, 37), and nuclear BAP1 expression, a surrogate for *BAP1* wild-type status (7). Immunohistochemical analysis of human tissue micro arrays (TMAs) (88 cores from 32 patients) (Fig. 1A), demonstrated a significant correlation between loss of nuclear BAP1 expression and higher DR4 and DR5 expression (Fig. 1B and Fig. S1). This correlation was further supported by immunohistochemistry in primary MPM tissue samples collected as

part of the MSO1 clinical trial (NCT00075699) (38); samples that lacked nuclear BAP1 also showed elevated levels of DR4 and DR5 (Fig. S2B and S2C). Interestingly, when we used antibodies against cytokeratin 5 (CK5) and calretinin to confirm the areas of mesothelioma, we observed higher expression of DR4 and DR5 where CK5 or calretinin are expressed. This suggests that DR4 and DR5 are expressed in mesothelioma cells but not in surrounding stromal tissue (Fig. S2A). The expression of death receptors on cancer cells but not non-transformed cells, including stromal tissue, is an existing theory for the selectivity of rTRAIL and other death receptor agonists for cancer cells which our data supports (36).

We further confirmed the correlation of loss of BAP1 activity and high DR4 and DR5 expression in a panel of MPM cell lines. Immunoblot analysis of MPM cell lines (7 *BAP1* mutant, 7 *BAP1* wild-type) overall demonstrated a higher level of DR4 and DR5 expression in *BAP1* mutant *vs BAP1* wild-type cell lines (Fig. 1C and 1D). The *BAP1* wild-type cell line, CRL2081, however expressed a high level of DR4 and was found to be rTRAIL sensitive. The *BAP1* wild-type line H2803 expressed a high level of DR5 yet remained rTRAIL resistant. It cannot simply be inferred, therefore, that expression levels of DR4 or DR5 alone determine rTRAIL sensitivity in these *BAP1* wild-type cells. Indeed, the apoptotic pathway consists of dozens of proteins, many of which are mutated in cancer cells. We hypothesise it is the balance of pro- and anti- apoptotic factors that determine TRAIL sensitivity of which DR4 and DR5 are likely to be dominant but not fully determinant. Indeed we observed additional heterogeneous changes in expression of 20 other proteins involved in the extrinsic and intrinsic apoptosis pathways, however, they did not directly correlate with the mutational status of *BAP1* or rTRAIL sensitivity (Fig. S3).

We have previously shown that strong nuclear BAP1 expression is highly correlated with rTRAIL resistance in human early passage, unsequenced MPM cultures (MesobanK UK) (39–41)(23). Here, in further support of a correlation between loss of BAP1 activity and increased DR4 and DR5 expression and rTRAIL sensitivity, immunoblot analysis revealed that DR4 and DR5 expression was higher in MPM cultures with loss of nuclear BAP1 expression and these cells were more sensitive to rTRAIL treatment (Fig. 1E and Fig. S4). Flow cytometry analysis also showed higher surface expression of DR4 and DR5 in MPM cultures with loss of nuclear BAP1

expression (Fig. 1F). Taken together, our data demonstrate strong inverse correlations between BAP1 expression and DR4 and DR5 expression, which may underlie the ability of BAP1 to determine rTRAIL sensitivity.

TRAIL has been documented in some cells to induce anti-apoptotic, rather than pro-apoptotic, pathways. Therefore, we investigated expression of anti-apoptotic proteins following treatment with rTRAIL (42–46). We examined c-FLIP, a catalytically inactive caspase-8 homologue that competes with caspase-8, inhibitors of apoptosis proteins (cIAP1/2), MAPK and NFκB pathways that enhance proliferation and induce cIAPs (36). We saw no induction of these proteins, excluding this as a mechanism of TRAIL-resistance in BAP1- mutant cells (Fig. S5).

Loss of BAP1 function increases DR4 and DR5 expression in malignant but not in non-transformed cells

To further investigate the relationship between BAP1 and DR4 and DR5 expression we knocked down BAP1 expression in a *BAP1*-wild type MPM cell line using lentiviral shRNA constructs. *BAP1* knockdown significantly increased expression of both DR4 and DR5 (Fig. 2A). DR5 has two isoforms; the expression of both was found to increase with *BAP1* knockdown (Fig 2A). It is not understood if there is a difference in function between these isoforms (47, 48). We confirmed BAP1 knockdown resulted in increased sensitivity to rTRAIL in these cells (Fig. 2B). Induction of cleaved caspase-8 and cleaved PARP was observed only in BAP1 knockdown cells indicating apoptosis activation only in the absence of BAP1. To examine the effect across additional tumour types we next knocked down *BAP1* in two *BAP1* wild-type clear cell renal cell carcinoma (CCRCC) cell lines. This also resulted in increased expression of DR4 and DR5 and increased sensitivity to rTRAIL (Fig. 2C and D). An additional shRNA clone confirmed these results in these two CCRCC lines and in a *BAP1* wild-type MPM cell line (Fig.2C and Fig.S6). BAP1 knockdown also lead to increased DR4 and DR5 mRNA levels (Fig. 2E) indicating the effect of BAP1 on DR4 and DR5 expression is at the transcriptional level. Significantly, *BAP1* knockdown in human lung fibroblasts and human bronchial epithelial cells (HBECs) did not affect expression of DR4 and DR5 or sensitivity to rTRAIL suggesting this effect is specific to malignant cells (Fig. S7).

196

197 **BAP1 negatively regulates transcription of *DR4* and *DR5***

198

199 To test if BAP1 deubiquitinase activity is required for transcriptional regulation of
200 DR4 and DR5 expression, we next transduced a *BAP1*-null early passage MPM cell
201 line, Meso-8T, with a lentiviral construct expressing wild-type *BAP1* (wt-BAP1) or
202 *BAP1* with an inactivating mutation in the deubiquitinase site C91A-BAP1 or A95D-
203 BAP1. Various mutations at C91 have been reported and shown in COSMIC
204 (https://cancer.sanger.ac.uk/cosmic)(49). A95D is a naturally occurring mutation in
205 MPM tumours in patients (6). Transduction with wt-BAP1 but not C91A-BAP1
206 resulted in a decrease in DR4 and DR5 expression (Fig. 3A). Flow cytometry
207 confirmed a decrease in surface expression of DR4 and DR5 in cells transduced with
208 wt-BAP1 but not C91A-BAP1 or A95D-BAP1 (Fig. 3B). Cell survival assays
209 confirmed transduction with wt-BAP1, but not C91A-BAP1 or A95D-BAP1, resulted
210 in a significant reduction in rTRAIL sensitivity (Fig. 3C). Concordantly, we saw
211 decreased activation of caspase-8, caspase-3 and reduced PARP cleavage in wt-
212 BAP1 transduced relative to C91A-BAP1 transduced cells when treated with rTRAIL
213 (Fig. 3A) reflective of reduced activation of the extrinsic apoptotic pathway in the
214 presence of wt-BAP1. Quantitative PCR analysis demonstrated that DR4 and DR5
215 mRNA expression were both decreased in cells transduced with wt-BAP1 relative to
216 those transduced with C91A-BAP1 suggesting regulation of DR4 and DR5
217 expression by catalytically active BAP1 is at the transcriptional level (Fig. 3D).
218 These results were confirmed in a further MPM cell line, H28 which harbours a *BAP1*
219 splice site mutation commonly found in MPM tumours (Fig. S8) (6). We have also
220 previously confirmed reduced DR4 and DR5 expression in C91A BAP1 transduced
221 relative to *BAP1*-wild-type-transduced H226 cells using flow cytometry analysis (23).
222 Subsequently, we tested the effect of BAP1 on *DR4* and *DR5* transcription more
223 directly. Meso-8T cells were transduced with lentiviral vectors with luciferase
224 reporters under the control of *DR4* or *DR5* promoters (50). These reporter cells
225 were also transduced with either wt-BAP1 or A95D-BAP1. Cells transduced with wt-
226 BAP1 displayed a significantly lower luciferase activity than those transduced with
227 A95D-BAP1 or the parental cell line reflecting decreased *DR4* and *DR5*
228 transcriptional activity in the presence of functional BAP1 (Fig. 3E).

229

Together the above results support that the deubiquitinase activity of BAP1 mediates transcriptional repression of *DR4* and *DR5*. To determine whether this in turn determines rTRAIL sensitivity, we used two complementary approaches. First, we knocked down *DR4* or *DR5* in *BAP1* wild-type H2869 MPM cells transduced with BAP1 shRNA. BAP1 knockdown increased the sensitivity of H2869 cells to rTRAIL as expected (Fig. 3F). Interestingly, DR5, but not DR4 knockdown, in shBAP1-H2869 cells abolished the effect of BAP1 knockdown, resulting in rTRAIL resistance (Fig. 3F). Second, we knocked down *DR4* or *DR5* in the BAP1-null, rTRAIL-sensitive Meso-8T cell line. *DR5* knockdown only slightly decreased rTRAIL sensitivity but *DR4* knockdown reduced it to a similar level as transduction with wild-type *BAP1* (Fig. 3G). These data are in line with previous reports showing preferential use of one of the two receptors by distinct cell types (31). For example, haematological cancers seem to prefer DR4 for induction of apoptosis (51, 52), whereas solid tumours appear to exhibit heterogeneity in death receptor preference (31, 53, 54).

YY1 negatively regulates transcription of *DR4* and *DR5*

As BAP1 does not bind to DNA directly (5), we aimed to identify transcription factors that bind to the promoter regions of *DR4* and *DR5*. Bioinformatic analysis of 2000 nucleotides of the promoter region of *DR4* and *DR5* was conducted. From candidates identified (Fig. S9), YY1 was selected for further analysis as it has previously been shown to negatively regulate *DR5* expression in prostate cancer (55, 56). Furthermore, YY1 has been shown to bind directly to BAP1, with the C-terminal region of BAP1 essential for this interaction, forming a complex capable of regulating gene expression (57). *YY1* knock down with two different shRNA clones in *BAP1* wild-type MPM and CCRCC cells resulted in increased expression of both DR4 and DR5 without affecting steady-state levels of BAP1 (Fig. 4A and Fig.S10). shRNA knockdown of *BAP1* in MPM cells also did not affect steady-state levels of YY1 (Fig.S11A). In addition, we did not observe any difference in YY1 expression based on BAP1 mutational status and BAP1 expression level (Fig. S11B). qPCR analysis confirmed increased mRNA expression of *DR4* and *DR5* in cells transduced with YY1 shRNA (Fig. 4B). *YY1* knockdown also significantly increased sensitivity to rTRAIL and the DR5 agonist Medi3039 in MPM and CCRCC cells (Fig. 4C)(58). We also determined if YY1 is able to regulate DR4 and DR5 expression in the absence

of BAP1. We knocked down YY1 in *BAP1*-mutant MPM cell lines and *BAP1*-null early passage MPM cells and assessed the expression of DR4 and DR5, and rTRAIL sensitivity. Neither DR4/DR5 expression nor TRAIL sensitivity increased in the YY1 knock down cells (Fig. 4D and E) in these *BAP1* mutant cells, unlike in *BAP1* wild-type cells, suggesting that BAP1 is required for *DR4/5* regulation by YY1. These data demonstrate that YY1, in addition to BAP1, modulates expression of DR4 and DR5. As YY1 and BAP1 have been shown to form a complex capable of regulating gene expression, it is likely that this complex regulates *DR4* and *DR5* expression (57)

BAP1 and YY1 act at DR4 and DR5 promoters to facilitate transcriptional repression

BAP1 has been shown to form a ternary complex with YY1 and HCF-1 (Host Cell Factor 1) in HeLa cells (57). Through its coiled-coil motif, BAP1 directly interacts with the zinc fingers of YY1 while HCF-1 interacts with the middle region of YY1 and is essential for the formation of the ternary complex in vivo (57). Therefore, we aimed to determine if BAP1 and YY1 also interact directly in MPM and CCRCC cells. Protein extracts from H2818, MPP89 and Caki-1 cells were co-immunoprecipitated (co-IP) using anti-YY1 antibody or IgG as a control. Immunoblot confirmed the interaction of endogenous YY1 with BAP1 (Fig. 5A). To verify the specificity of these results, we compared results of co-IP assay in BAP1-null MPM cells (Meso-8T) that were transduced with wt-BAP1 or a control vector alone. A strong interaction of YY1 and BAP1 was detected only in cells transduced with wt-BAP1 but not the control vector, confirming the specificity of the YY1/BAP1 interaction (Fig. 5B). Here, co-IP assay demonstrates that YY1 also interacts with HCF-1 in *BAP1* wild-type CCRCC cells (Fig S12). However, we have previously shown that MPM cells expressing BAP1 that lacks the binding domain for HCF-1 are not significantly different in their TRAIL sensitivity compared to cells expressing wtBAP1 (23). This suggests that the HCF-1/BAP1 interaction does not determine TRAIL sensitivity and is unlikely to be involved in death receptor regulation.

In addition to physical interactions, we sought to examine the functional interaction between YY1 and BAP1. As BAP1 does not have a DNA binding domain, but directly interacts with the transcriptional repressor YY1, we hypothesized that BAP1 and YY1 are recruited to the promoter regions of DR4 and DR5. Chromatin immunoprecipitation (ChIP) assays were performed with antibodies for BAP1, YY1 or IgG as a control. The immunoprecipitated DNA was analysed with probes for DR4 or DR5 by qPCR in Meso-8T cells transduced with wt-BAP1, C91A-BAP1 or a control vector. Both BAP1 and YY1 were enriched in the promoter regions of DR4 and DR5 in cells transduced with wt-BAP1 but not the control vector (Fig. 5C). Interestingly, BAP1 and YY1 were also enriched in these promoter regions in cells transduced with C91A-BAP1 indicating BAP1 and YY1 are recruited to these promoter regions regardless of deubiquitinase activity. This finding is consistent with previous reports that catalytically inactive BAP1 is also recruited to FoxK2-binding regions (59). Catalytically inactive BAP1 has also previously been shown to form a complex with YY1 (57). Taken together we show that BAP1 and YY1 are recruited at the promoters of TRAIL receptors and are necessary to initiate transcriptional regulation of TRAIL receptors.

Discussion

We have recently reported that loss of BAP1 function is a predictive biomarker for rTRAIL sensitivity in cancer (23). In this study, we delineate the underlying molecular mechanism. We demonstrate BAP1 and the transcriptional regulator YY1 act at the promoter regions of *DR4* and *DR5* where they facilitate transcriptional repression of *DR4* and *DR5*, which requires BAP1 deubiquitinase activity. Decreased cell surface expression of DR4 and DR5 and reduced activation of the apoptotic pathway in turn mediates rTRAIL resistance in *BAP1* wild-type cells. Conversely, increased cell surface expression of DR4 and DR5 in *BAP1* mutant cells mediates the observed increased sensitivity to rTRAIL. Various mechanisms of resistance to rTRAIL and other death receptor agonists have been suggested (60). Evidence supports that low expression of DR4 and DR5 due to mutations, promoter methylation, constitutive endocytosis or deficient transport to the cell surface is important (60–63). Indeed, strategies to enhance the efficacy of rTRAIL treatment, such as a combination with

chemotherapeutic drugs, have been demonstrated to mediate these effects through increased death receptor expression (25). Our results are consistent with these data and support the centrality of death receptor expression in TRAIL therapeutics.

YY1 inhibition has previously been shown to upregulate DR5 expression and enhance rTRAIL sensitivity in prostate cancer and B-non-Hodgkin lymphoma cells (55, 56, 64). Here however we show that YY1 is involved in the transcriptional regulation of both DR4 and DR5 and is enriched at the promoters of both DR4 and DR5 when BAP1 is present. BAP1 is known to form multiprotein complexes including as many as ten partners which in turn determine the precise targets of its deubiquitinase activity (5, 65). It has previously shown that BAP1 forms a multi-protein complex with YY1 and the transcriptional cofactor HCF-1 (57). Although not investigated here, further work might identify additional cofactor(s) that direct BAP1 and YY1 to the DR4 and DR5 promoters. We have previously shown that mutation of the ASXL binding site on BAP1 and ASXL1 knockdown also increases rTRAIL sensitivity (23). The BAP1/ASXL1 complex is a polycomb repressor deubiquitinase complex capable of deubiquitination of histone 2A at lysine 119 (H2A119Ub), a process which modulates expression of the polycomb genes (5). Interestingly, YY1 has also been shown to interact with polycomb proteins (57, 66). It may therefore be that YY1 interacts with both BAP1 and ASXL1 to modulate death receptor expression through the deubiquitination of H2A119Ub or that BAP1 and YY1 form an alternate complex with a different target that modulates histone and chromatin structure death receptor expression.

YY1 and BAP1 may be involved more widely in the transcriptional regulation of the TNF receptor superfamily. Nitric oxide has been shown to inhibit YY1 binding to the *Fas* promoter resulting in Fas upregulation and cell sensitisation to Fas-ligand induced apoptosis in prostate cancer (67). YY1 has also been shown to supress the Fas promoter activity in B-non-Hodgkin lymphoma and colon cancer (68, 69). We have also previously demonstrated that BAP1 knockdown sensitises MPM cells to Fas ligand and TNF-alpha (23).

Although *BAP1* was originally identified as a tumour suppressor gene, accumulating evidence has revealed roles in multiple clinically targetable pathways (70–73).

Indeed we have proposed BAP1 expression to be a stratifying biomarker for sensitivity to death receptor agonists (23) and our work here provides a biological rationale for this. The current study also demonstrates that YY1 knockdown enhances the sensitivity to TRAIL and DR5 agonist. YY1 is overexpressed in many types of cancer and high expression correlates with poor clinical outcomes and resistance to chemotherapy and immunotherapy making it an attractive therapeutic target (74, 75). Thus, targeting the BAP1/YY1 axis may be an additional novel therapeutic strategy in TRAIL therapeutics.

Experimental procedures

Cell Culture

All cancer cell lines were obtained from the Wellcome Trust Sanger Institute (Cambridgeshire, UK) except the H226 line that was kindly gifted from Dr. P. Szlosarek (Barts Cancer Institute, London, UK). Cancer cell lines were cultured in RPMI-1640, Dulbecco's modified Eagle's medium (DMEM) or DMEM and nutrient mix 12 medium (DMEM:F12) supplemented with 10% fetal bovine serum (FBS), penicillin/ streptavidin and sodium pyruvate. Early passage human mesothelioma cells were purchased from MesobanK (39) and cultured in RPMI-1640 medium supplemented with 5% FBS, 25 mM HEPES, penicillin/ streptavidin and sodium pyruvate. Primary human lung fibroblasts (kind gift from Dr. R. Chambers at UCL) were cultured in DMEM media supplemented with 10% FBS and penicillin/streptavidin in an incubator with 10% CO_2 (76). Experiments were conducted on cells between passage 6 and 8. Primary human bronchial epithelial cells (HBECs) were obtained from endobronchial biopsies with patient consent as previously described (77). Ethical approval was obtained through the National Research Ethic Committee (REC reference 06/Q0505/12). All studies involving human subjects abide by the Declaration of Helsinki Principles. HBECs were cultured in bronchial epithelial growth medium (BEGM; Lonza) on top of 3T3-J2

mouse embryonic fibroblast feeder cells inactivated by mitomycin-C treatment (0.4 μg/ml, Sigma-Aldrich, Merck, Darmstadt, Germany).

XTT cell viability assay

Cells were seeded in 96-well plates in 100 µl media per well at a density of 40,000 cells/ml one day prior to treatment with soluble recombinant TRAIL (PeproTech, Rocky Hill, NJ, USA) or MEDI3039 (Medimmune, AstraZeneca, Cambridge, UK). XTT reagent and the activation solution (Applichem,Akron Biotech, Boca Raton, FL, USA 88) were mixed and added to the cells at the end of treatment. The plate was returned to a CO_2 incubator to incubate for 2 hours, the absorbance at a wavelength of 490nm was measured using a microplate reader. Relative cell viability was calculated as a fraction of viable cells relative to untreated cells.

Immunoblotting

Cells were lysed in RIPA lysis buffer (Sigma-Aldrich) with protease inhibitors (Complete-mini; Roche, Basel, Switzerland) on ice to extract protein. 30 µg of protein samples were separated by SDS–PAGE and transferred onto nitrocellulose membranes using iBlot2 Dry Blotting System (Thermo Fisher Scientific, Waltham, MA, USA). Membranes were incubated with specific primary antibodies, washed, incubated with secondary antibodies and visualised using an ImageQuant LAS 4000 imaging system (GE Healthcare, Chicago, IL, USA). A list of antibodies used for immunoblotting is provided in Table S1. Quantification of bands was performed using ImageJ (Image Processing and Analysis in Java).

Immunoprecipitation (IP)

Cells were lysed in IP buffer containing 0.2% NP-40, 20mM Tris-HCl (pH 7.4), 150mM NaCl, 10% glycerol and protease inhibitors. The lysates were incubated overnight with gentle rocking with anti-YY1 antibody (ab38422, Abcam, Canmbridge, UK) or IgG (2729, Cell Signaling Technology, Danvers, MA, USA). Protein-A magnetic beads (Pierce Biotechnology, Thermo Fisher Scientific, Waltham, MA, USA) were added and incubation was continued for 1 hour. The beads were washed

with IP buffer and proteins eluted from the beads by heating with SDS sample buffer. Proteins were separated by SDS-PAGE and immunoblotting was performed as described above with anti-BAP1 antibody (sc-28383, Santa Cruz).

Plasmids

Full-length *BAP1* cDNA was amplified by PCR from pCMV6-AC *BAP1* plasmid (SC117256, Origene, Rockville, MD, USA) and cloned into the lentiviral plasmid pCCL-CMV-flT vector. Vectors expressing mutant *BAP1* constructs were generated by site-directed mutagenesis (E0554, New England Biolabs, Ipswich, MA, USA) of the pCCL-CMV-BAP1 vector as previously described (23).

RNA interference

Short hairpin RNAs (shRNAs) were expressed as part of a mir30-based GIPZ lentiviral vector (Dharmacon, Lafayette, CO, USA). The clones used in this study include BAP1 (clone#1; V2LHS_41473, clone#2; V2LHS_41478), DR4 (V3LHS_383718), DR5 (V3LHS_328891), YY1 (clone#1; V3LHS_412955, clone#2; V3LHS_412955) and the empty GIPZ control vector.

Lentivirus production and cell transfection

Lentiviral particles were produced by co-transfection of 293T cells with construct plasmids and the packaging plasmids pCMV-dR8.74 and pMD2.G (kind gifts from Dr Adrian Thrasher, University College London) using a DNA transfection reagent jetPEI (Source Bioscience, Nottingham, UK). The viral particles were concentrated by ultracentrifugation at 17,000 rpm (SW28 rotor, Optima LE80K Ultracentrifuge, Beckman, Brea, CA, USA) for 2 hours at 4°C. To determine the titres of prepared lentivirus, 293T cells were transduced with serial dilutions of viruses in the presence of 8 µg/mL polybrene and protein expression was assessed by flow cytometry and immunoblotting.

Flow cytometry

All flow cytometry analysis was performed on an LSR Fortessa analyser (Becton Dickinson, Franklin Lakes, NJ, USA). For analysis of BAP1 expression, cells were fixed, permiabilized and stained with primary antibody to BAP1 (1:50, SC28383, Santa Cruz) and then with an AlexaFluor 488-conjugated anti-mouse antibody (1:200, A-21202, Invitrogen, Carlsbad, CA, US). For analysis of surface expression of DR4 and DR5, cells were stained with 1:100 dilution of PE-conjugated antibody (#307205 for DR4, #307405 for DR5, #400112 for isotype, Biolegend, San Diego, CA, USA). FlowJo software was used to analyse all data.

Quantitative RT-PCR

Total RNA was extracted from the cells using SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacture's instructions. cDNA was synthesized using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative PCR was performed using TaqMan probes (DR4: Hs00269492_m1, DR5: Hs00366278_m1, beta-2-microglobulin: Hs00187842_m1) and TaqMan Gene Expression Master Mix (Life Technologies, Carlsbad, CA, USA) as per the manufacture's protocol. Relative expression of DR4 and DR5 was calculated using comparative CT method with a reference gene, beta-2-microglobulin.

ChIP assay

The ChIP assay was carried out using EZ ChIP^{TM} Chromatin Immunoprecipitation kit (Merck-Millipore, Burlington, MA, USA) according to the manufacture's instruction. Briefly, the cells were cross-linked, quenched and lysed then the chromatin was fragmented by sonication shearing. Protein/DNA complexes were diluted, pre-cleared with Protein G agarose beads, then immunoprecipitated (IP) by incubation with antibodies against BAP1 (#78105, Cell Signaling), YY1 (#ab38422, Abcam 422) or IgG (#2729, Cell Signaling) overnight with rotation, followed by incubation with protein G agarose beads for 1 hr. After washing beads, protein/DNA complexes were eluted, reverse crosslinked to free DNA, which was then purified using spin columns

and analysed by quantitative PCR (qPCR). Primer pairs for ChIP assays were as follows: DR5; forward 5'-GGGAAGGGGAGAAGATCAAG-3', reverse 5'-GAAGGGACCGGAACTAACCT-3'. DR4; forward 5'-CCGAATGCGAAGTTCTGTCT-3', reverse 5'-AAGAGCCCCACACTTTGCT-3'.

Luciferase Reporter assay

Meso-8T cells were transduced with lentiviral vectors expressing a firefly luciferase reporter plasmid containing either DR4 promoter (upstream -1773/+63) or DR5 promoter (upstream -1400), plus control Renilla luciferase reporter under a control of CMV promoter (pDR4-FireflyLuc-CMV-RenillaLucDsRed2 or pDR5-FireflyLuc-CMV-RenillaLucDsRed2) vectors (50). Cells were seeded in 96 wells plate and luciferase activities were measured using Dual-Luciferase Reporter Assay System kit as described by the manufacture (Promega). Fluc/Rluc ratios were determined as relative luciferase activities.

Immunohistochemistry (IHC)

Tumor biopsies taken from patients with MPM in the MS01 trial (NCT00075699) were stored as formalin fixed paraffin embedded (FFPE) blocks or as unstained mounted sections as previously described (38). The TMA slides containing tumour samples from patients with MPM were purchased from MesobanK UK. All studies involving human subjects abide by the Declaration of Helsinki Principles. To assess expression of DR4, DR5, CK5 and calretinin, samples were first incubated in the oven at 60 °C for 30min, then deparaffinised and rehydrated using an automated tissue processor (Tissue-Tek, Alpena an den Rijn, The Netherlands). Antigen retrieval was achieved by immersion in 10mM Citric acid buffer (pH.6.0) at 95 °C for 15 min. After washing with PBS and blocking with 2.5% normal goat serum, samples were incubated with primary antibody: anti-DR4 (1:500, ab8414, abcam), anti-DR5, (1:500, ab8416, abcam), anti-calretinin (1:200, NCL-L-CALRET-566, Leica Biosystems, Wetzlar, Germany), anti-keratin 5 (Biolegend: 905501, 1:500) in 1% BSA / 4% serum overnight at 4 °C. Samples were incubated with ImmPRESS polymer reagent (VECTOR Laboratories, Burlingame, CA, USA) for 30min and stained with ImmPACT Nova RED (VECTOR Laboratories). Hematoxylin and eosin

(H&E) staining was carried out using an automated tissue processor (Tissue-Tek). Staining for BAP1 was performed as described before using anti-BAP1 antibody (1:150, sc-28282, Santa Cruz Biotechnology) (38). Images were acquired using a NanoZoomer 2.0HT whole slide imaging system (Hamamatsu Photonics, Hamamatsu, Japan). Histology and nuclear BAP1 assessment was performed by two consultant pathologists. Intensity of DR4 and DR5 expression was assessed blindly by three independent observers and scored as follows (no staining=0; low staining=1; medium staining=2; strong staining=3).

Bioinformatical Analysis

To identify the common transcription factors which potentially regulate these genes, the 2000 nucleotide sequence of the promoter regions of DR4 and DR5 are entered into Human Core-Promoter Finder (http://rulai.cshl.org/tools/genefinder/CPROMOTER/human.htm).

Statistical Analysis

Data were evaluated using the statistical analysis and indicated with *P* values. $P<0.05$ was considered statistically significant. Using Prism 8 (GraphPad, CA, USA), student's *t*-test was performed to analyse differences between two groups whilst one-way ANOVA was used to determine the differences between three or more independent groups.

For the statistical analysis of TMAs, linear mixed modelling was used to account for multiple samples per patient, including the patient ID as a random effect. Linear mixed models were implemented using the Bioconductor *lme4* and *lmerTest* packages. Pairwise *t*-test confirmed that there was no systematic bias between the score of different observers.

Data availability

All data are contained within the article.

Supporting information

This article contains supporting information.

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Conflict of interest:

The authors declare no potential conflict of interest.

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Figure Legends

Figure1: Expression levels of DR4 and DR5 are inversely correlated with BAP1 expression in malignant pleural mesothelioma

A, Representative images of IHC of DR4 and DR5 in a core from an MPM tissue microarray (TMA) with or without nuclear BAP1 expression (from 88 cores of 32 patients). *B*, Semi-quantitative analysis of DR4 and DR5 expression in MPM TMA cores with (n=42) and without (n=46) nuclear BAP1 expression. Each dot represents an average score per patient (n=32). t-test; p=0.024 (DR4) and p=0.0092 (DR5). See method section for details. *C*, Immunoblots of DR4, DR5 and BAP1 protein expression in BAP1 mutant (n=7) vs BAP1 wild-type (n=7) MPM cell lines. Duplet bands of DR5 represent two isoforms, DR5-short (DR5-S) and DR5-long (DR5-L). Sensitivity to rTRAIL treatment is indicated as font color: green sensitive (S); orange partially sensitive (PS); red resistant (R). *D*, Quantitative analysis of immunoblot intensity of DR4 and DR5 in wild type BAP1 and mutant BAP1 MPM cell lines (DR4 t-test; p=0.046, DR5 t-test; p=0.009). Dots color indicates the sensitivity to rTRAIL treatment as shown in (C). *E*, Quantitative analysis of immunoblot intensity of DR4 and DR5 in early-passage MPM cells with (+) and without (-) nuclear BAP1 expression. (DR4 t-test; p=0.033, DR5 t-test; p=0.049). *F*, Flow cytometry analysis of DR4 and DR5 cell surface expression in early passage MPM cells with (BAP1+) and without (BAP1-) nuclear BAP1 expression alongside an isotype control.

Figure2: BAP1 knockdown increases death receptors expression and TRAIL sensitivity in cancer cells

A, Immunoblots of pro-apoptotic proteins in parental, BAP1 shRNA (shBAP1-clone#1) or empty vector shRNA (EV) transduced BAP1-wild-type MPM cells (H2818) across multiple time points (0, 6, 12, 24 and 48 hours) post rTRAIL treatment (100ng/mL). Duplet bands of DR5 represent two isoforms, DR5-short (DR5-S) and DR5-long (DR5-L). The bands were quantified and normalized to an average of parental cells data. *B*, Cell viability assay of parental, shBAP1- or EV-transduced H2818 cells following treatment with a dose range of rTRAIL (0-1000ng/ml) for 72 hours. *C*, Immunoblot analysis in BAP1-wild-type- clear cell renal cell carcinoma (CCRCC) cells (BB65 and Caki-1) and MPM cells (H2818)

transduced with BAP1 (shBAP1#1 or shBAP1#2) or empty vector (EV) shRNA. The bands were quantified and normalized to EV. *D*, Cell viability assay of EV- or shBAP1- transduced BAP1 wild-type CCRCC cells following treatment with a dose range of rTRAIL (0-1000ng/ml) for 72 hours. *E*, Relative expression of DR4 and DR5 mRNA in CCRCC cells transduced with EV or shBAP1 assessed by qPCR. Relative mRNA expression was normalized to beta-2-microgloblin (B2M) expression. Data shown are the mean ± s.d. of two experiments performed in triplicates. t-test; *p<0.05, **p<0.01.

Figure 3: The deubiquitinase function of BAP1 regulates the transcription of DR4 and DR5

A, Immunoblots of pro-apoptotic proteins in BAP1 null early passage mesothelioma cells (Meso-8T) transduced with constructs expressing wild type-BAP1 (wt-BAP1), deubiquitinase mutant BAP1 (C91A-BAP1) or a control vector (cont-vec) untreated and after 5 hours of rTRAIL treatment (50 ng/mL). *B*, Flow cytometry analysis of cell surface expression of DR4 and DR5 in Meso-8T cells transduced with constructs expressing wild type-BAP1 (wt-BAP1) or one of two deubiquitinase mutant BAP1 vectors (C91A or A95D). One-way ANOVA; ***p<0.001. *C*, Cell viability assay of Meso-8T cells transduced with wt-BAP1 or one of two deubiquitinase mutant BAP1 vectors (C91A or A95D) following treatment with a dose range of rTRAIL (0-1000ng/ml) for 72 hours. *D*, Relative DR4 and DR5 mRNA expression in parental Meso-8T cells and cells transduced with wt-BAP1 or C91A-BAP1. Relative mRNA expression was normalized to beta-2-microgloblin (B2M) expression. Data are shown as the mean ± s.d. of two experiments performed in triplicates. *, P<0.05; **, P<0.01. *E*, Reporter assay for promoter activities of DR4 and DR5 in parental Meso-8T cells transduced with a luciferase reporter under the control of DR4 or DR5 promoter and cells further transduced with wt-BAP1 or A95D-BAP1. Firefly luciferase/Renilla luciferase ratios were determined as relative luciferase activities. Data are shown as the mean ± s.d. of two experiments (n=6 in each experiment). *, P<0.05; **, P<0.01. *F*, Cell viability assay of BAP1 wild-type H2869 cells transduced with EV or shBAP1 following treatment with a dose range of rTRAIL (0-1000ng/ml) for 72 hours and for shBAP1 cells further transduced with DR4 (shDR4) or DR5 shRNA (shDR5) following the same treatment. *G*, Cell viability assay of parental

BAP1 null Meso-8T early passage MPM cells transduced with wild-type BAP1 (wtBAP1) or DR4 (shDR4) or DR5 shRNA (shDR5) following treatment with a dose range of rTRAIL (0-1000ng/ml) for 72 hours. +,++; lentiviral titer. Error bars represent the standard deviation.

Figure 4: YY1 knockdown increases the expression of death receptors and rTRAIL-induced cell death

A, Immunoblot analysis in BAP1-wild-type MPM cells (H2818, MPP89 and H2591) or CCRCC cells (BB65 and Caki-1) transduced with YY1 shRNA-clone#1 (+) or an empty vector shRNA (-). Quantitative analysis of DR4 and DR5 bands from three independent experiments was performed. Average data after normalization to tubulin were shown as bar graphs. *B*, Relative DR4 and DR5 mRNA expression in MPM cells (H2818) and CCRCC cells (Caki-1 and BB65) transduced with YY1-shRNA or EV-shRNA. Relative mRNA expression was normalized to beta-2-microgloblin (B2M) expression. Data are shown as the mean ± s.d. of two experiments performed in triplicates. *, P<0.05; **, P<0.01. *C*, Cell viability assays of BAP1-wild-type MPM and CCRCC cells transduced with EV shRNA or shYY1 (clone#1) following treatment with a dose range of rTRAIL (0-1000ng/ml) or MEDI3039 (0.1-100pM) for 72 hours. Error bars represent the standard deviation. *D*, Immunoblot analysis in BAP1-null early passage MPM Meso-8T cells and BAP1-mutant MPM cell lines (H28, H226) transduced with YY1 (clone#1) or empty vector (EV) shRNA. *E*, Cell viability assay of EV- or shYY1 (clone#1) - transduced cells described in D, following treatment with a dose range of rTRAIL (0-1000ng/ml) for 72 hours.

Figure 5: YY1 recruits BAP1 to the promoter regions of DR4 and DR5 and represses their transcriptional activities

A, Co-Immunoprecipitation (Co-IP) of endogenous YY1 and BAP1 in MPM (H2818, MPP89) and CCRCC (Caki-1) cells. *B*, Co-IP of YY1 and BAP1 in BAP1 null early passage MPM Meso-8T cells transduced with wild-type BAP1 (wt-BAP1) or a control vector. *C*, Enrichment of BAP1 and YY1 in the promoter regions of DR4 and DR5. Meso-8T cells were overexpressed with wt-BAP1, catalytically inactive mutant-BAP1 (C91A-BAP1) or a control vector (cont). Chromatin Immunoprecipitation (ChIP) was

performed against BAP1, YY1 or IgG control followed by qPCR using primers specific for promoter regions of DR4 or DR5. Error bars represent the standard deviation. P-values are calculated to compare against IgG control using student t-test (n=3); *p<0.05, **p<0.01. *D*, Schematic model of the transcriptional regulation of TRAIL death receptors by BAP1 and YY1.

Figure 1

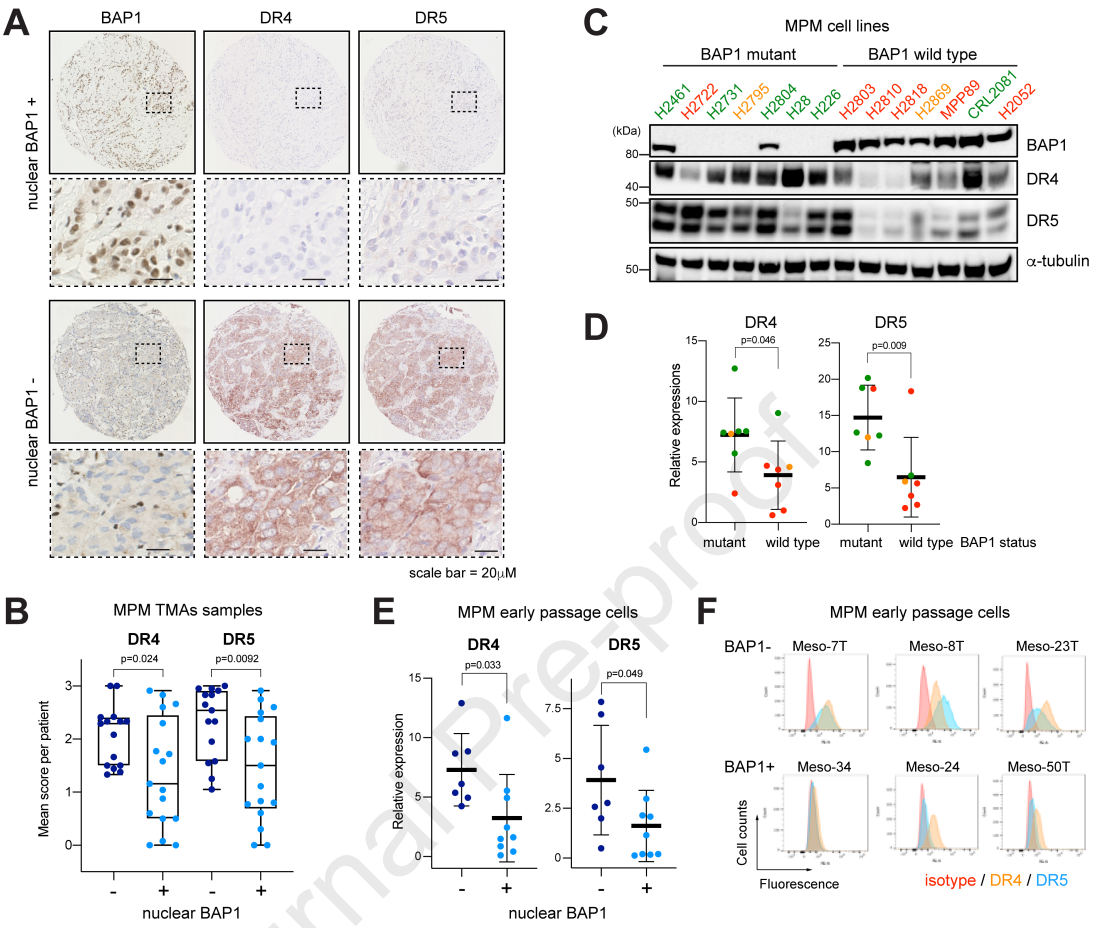

Figure 2

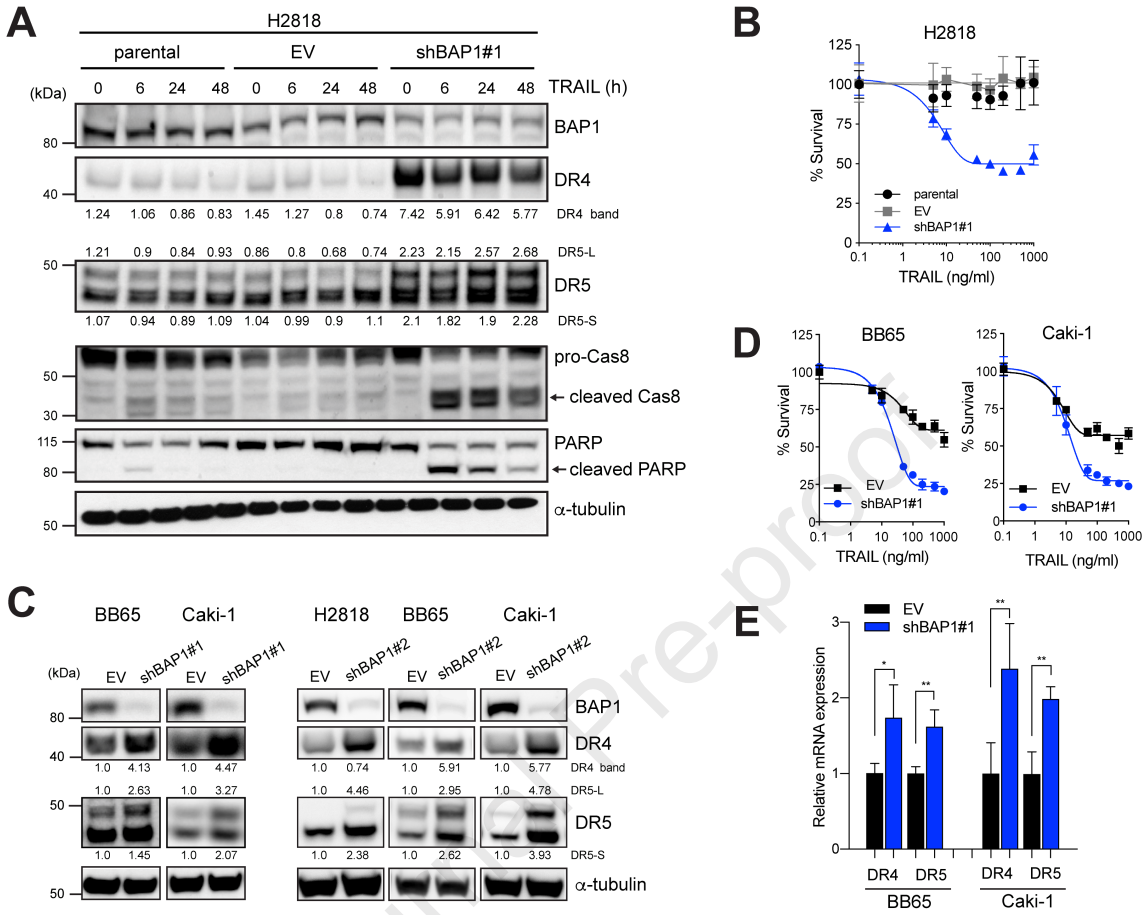

Figure 3

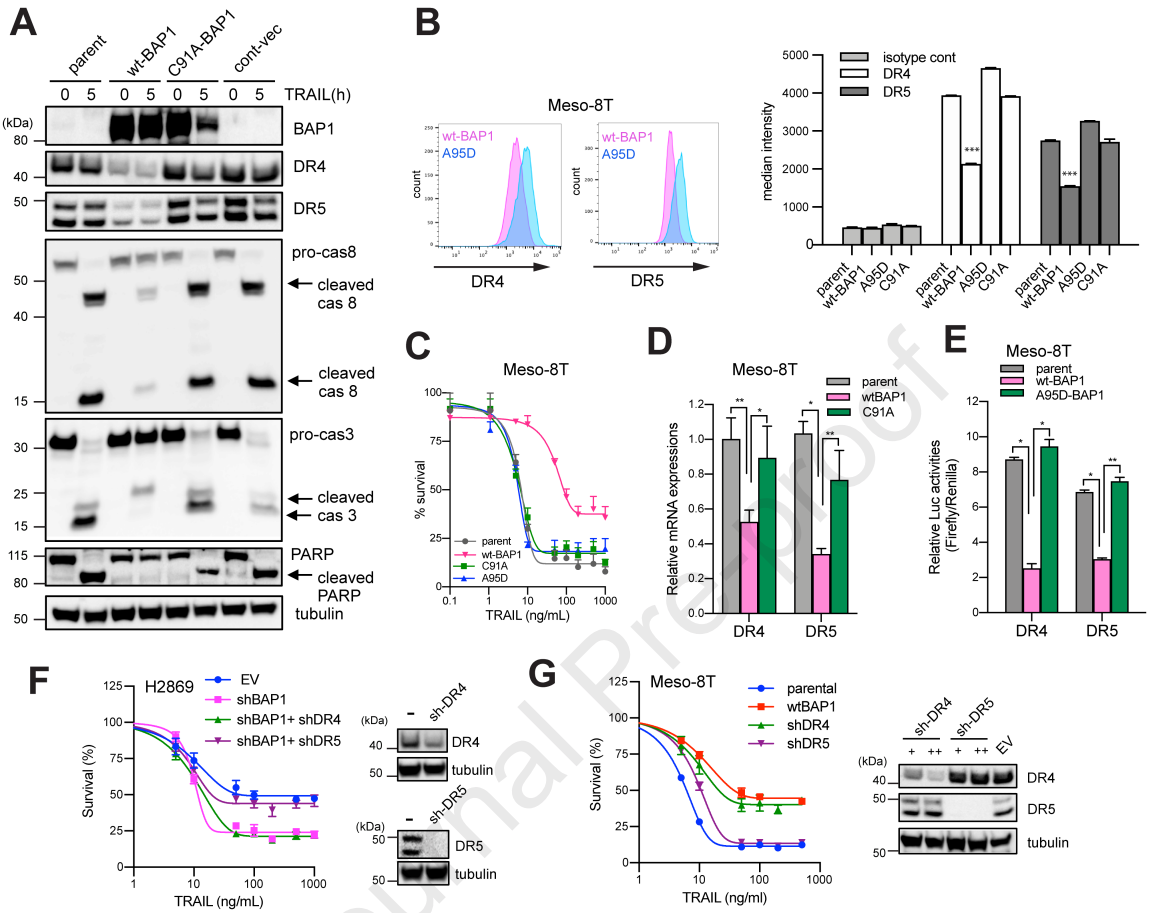

Figure 4

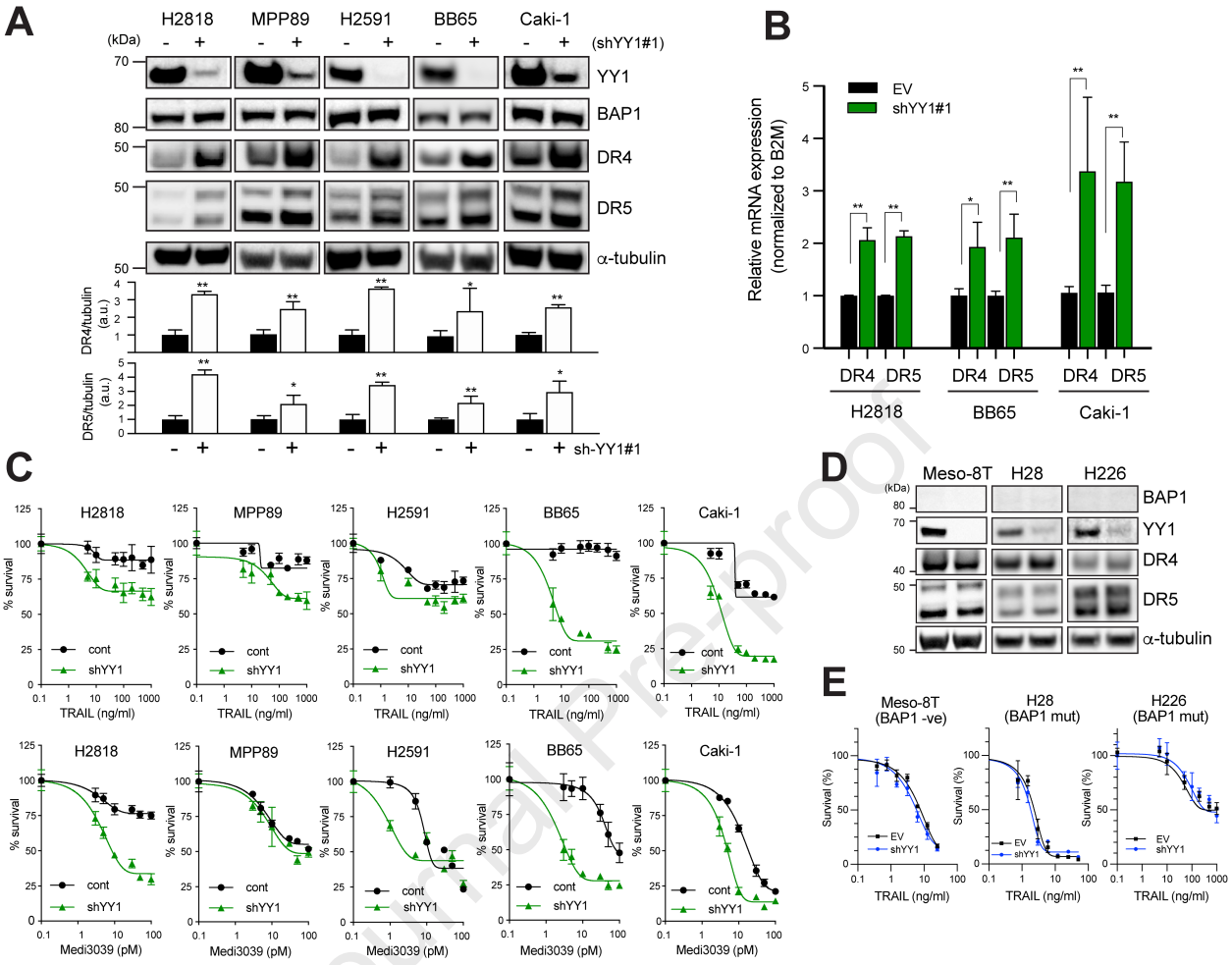

Figure 5