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Title:

Highly expressed STING in liver metastasis of uveal melanoma can lead to synergistic killing

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Abstract:

BRCA1-associated protein 1 (BAP1) is frequently mutated in metastatic uveal melanoma (MUM). Since BAP1 loss of function was previously linked to changes of expression of Stimulator of Interferon Genes (STING), a DNA damage and viral DNA sensor, the expression of STING protein was examined by immunohistochemistry. It was discovered that the MUM cancer cells expressed high level of STING protein universally, while the hepatocytes stained negative. In addition, when tumor samples from primary UM patients were examined, the BAP1 wildtype cancer cells expressed STING weakly, while some cancer cells in a portion of the BAP1 deficient tumors expressed STING strongly. Analysis of single cell sequencing of UM tumors revealed that BAP1 loss is associated with high STING mRNA expression. MUM cell lines with either wildtype or mutant BAP1 all expressed STING that can be activated by a STING agonist at low concentrations. In addition, STING activation last longer in BAP1-deficient MUM cell lines. The STING agonist is cytostatic against UM cancer cell lines. Cotreatment of Bortezomib, a proteasome inhibitor and an FDA approved oncology drug, enhances phospho-STING, the active form of STING after the cells are treated with a STING agonist. This combination also leads to a large-scale cell death of MUM cancer cells at low concentrations, and strong synergy was detected. Interestingly, synergistic killing by this combination is not observed in normal human PBMC or multiple cancer cell lines with different tissue origins. Thus MUM cancer cells express high level of functional STING protein that can be therapeutically exploited.

Background: In adults, Uveal melanoma (UM) is the deadliest form of eye cancer. Mutations that inactivate the gene encoding BAP1 in UM tumors, and/or loss of expression of BAP1 protein, are strongly associated with an increased risk of metastasis (1, 2). Once metastasis occurs, the life expectancy of the patients is usually quite short. The locoregional therapies including percutaneous hepatic perfusion using recently approved Hepzato Kit marginally prolonged progression-free survival of MUM patients (3). Overall response rates to immunotherapies including tebentafusp remain low (3.6-18%) and long-lasting disease control has not been achieved(4). BAP1 loss is known to be associated with slower proliferation of UM cells *in vitro*, alteration of the S6 signaling (5), glycolytic phenotype (6), and upregulated cell adhesion molecules (7). It was also reported that BAP1 regulates epigenetic switch from pluripotency to differentiation (8) and its loss leads to DNA methylomic repatterning (9). However, it is not known mechanistically how BAP1 mutations contribute to tumor growth and metastasis, and there is no effective therapy against metastatic UM targeting BAP1 loss.

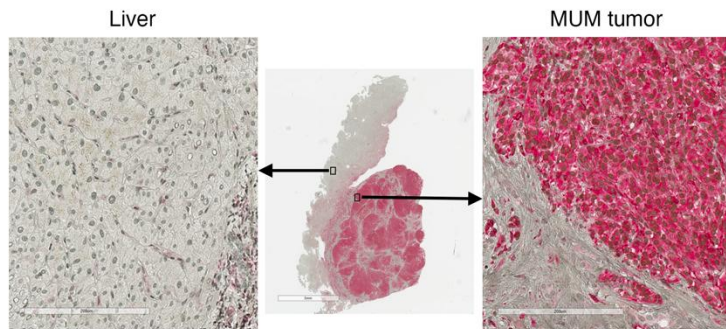
BAP1 mutations or loss of expression are also frequently present in other cancer types such as clear cell renal cell carcinoma (ccRCC) and mesothelioma (10, 11). To investigate how BAP1 loss might confer the risk of cancer patients, we studied the molecular consequence of BAP1 loss in ccRCC and mesothelioma. We found that loss of BAP1 in ccRCC cancer cells, primary mouse kidney cells, all led to the reduction of the expression of STING, suggesting that the connection between BAP1 and STING expression is well conserved from mouse to human, and from benign to cancerous tissues (12-14). The STING pathway is a key immune pathway that can play a role in cancer in both positive and negative ways (15), as it promotes distinct immune effector responses that impact many aspects of tumorigenesis, from malignant cell transformation to metastasis. Molecularly, STING activated by DNA damage or viral infection promotes the activation of Nuclear Factor Kappa B (NF- κ B) and Interferon Stimulated Gene Factor 3 (ISGF3), two transcription factors that induce the expression of inflammatory genes and interferons. In addition, STING was reported to be activated by chromosomal instability during UM progression

(16) and promotes invasion and migration of UM through p38-MAPK signaling (17). Given the strong connection between BAP1 deficiency and STING expression, and the fact that BAP1 is commonly lost in metastatic UM, either through somatic mutations, gene copy loss, and/or loss of protein expression (2, 18), we examined the expression of STING in the patient samples from metastatic UM.

Results:

STING is expressed at very high level in metastatic uveal melanoma cancer cells

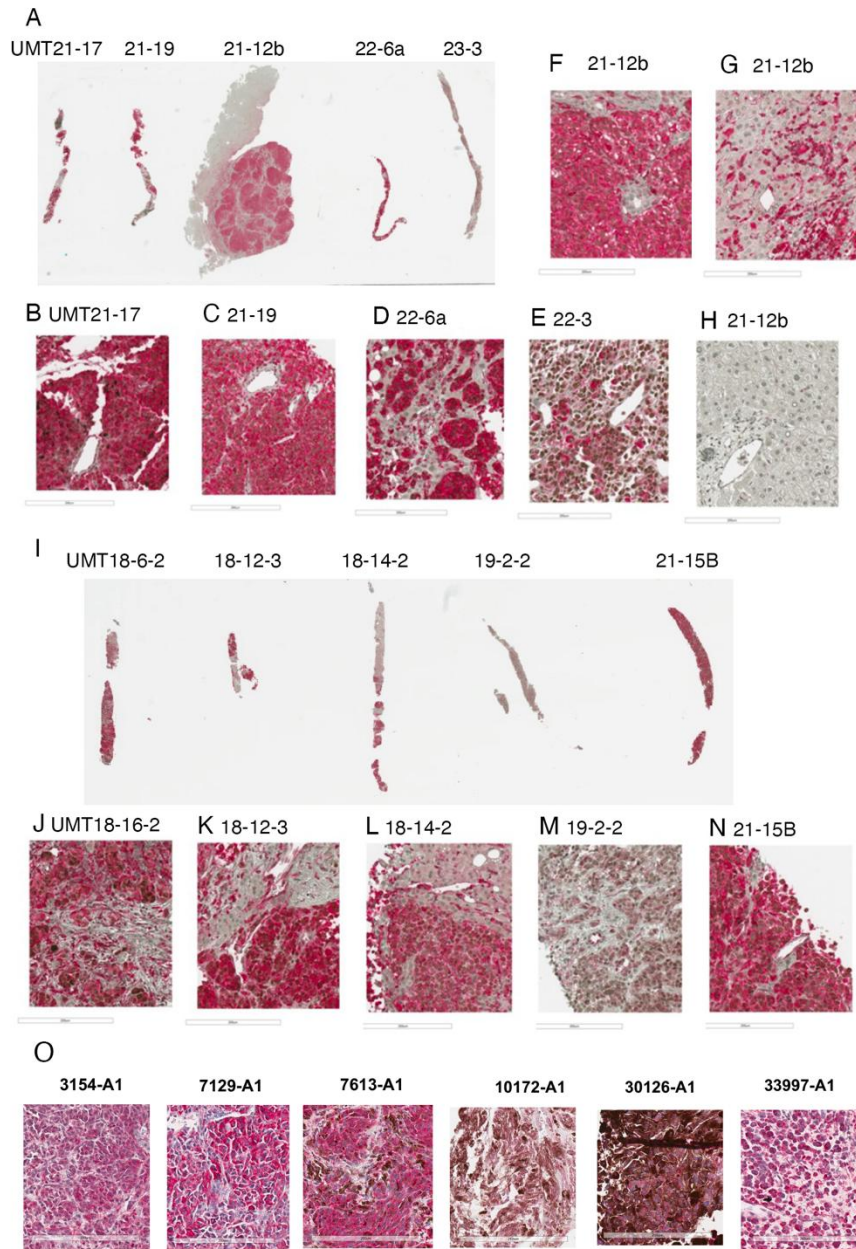
IHC: SOX10 (Brown), STING (Red)



Since BAP1 is frequently mutated in metastatic uveal melanoma (MUM) (2), and BAP1 regulates STING expression in other types of cancer, we examined if STING is expressed in MUM tumors. To this date, we have

Fig. 1. STING is highly expressed in MUM cancer cells. A laparoscopic evaluation of a liver met sample of MUM was examined with IHC with antibodies against SOX10 and STING. The slide was stained with anti-SOX10, and the cancer cells' nucleus are stained brown. Next the slides were stained with anti-STING and developed with pink chromophore. In the middle it is the image of the stained sample at low magnification. The left panel is the amplified image of the stained liver cells. The right panel is the amplified image of the stained MUM cancer cells.

examined its expression in human liver metastasis specimens. All the MUM cancer cells, as confirmed by positive staining of SOX10, expressed very high level of STING protein in their cytoplasm, while the normal hepatocytes expressed no detectable STING (**Fig. 1**). This observation is consistent among sixteen human liver metastasis specimens (**supplementary Fig. 1**). High STING protein expression in cancer cells but not in hepatocytes was also observed in four Patient-Derived Xenograft (PDX) models of MUM (**Fig. 2**), suggesting that this feature is a strong and consistent trait of the MUM cancers that is preserved in the PDX models.



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Supplementary Fig. 1. STING is highly expressed in MUM cancer cells. Needle biopsied liver metastatic samples or a laparoscopic evaluation (21-12b) of MUM were examined with IHC with antibodies against SOX10 and STING. The slides were stained with anti-SOX10, and the cancer cells' nucleus are stained brown. Next the slides were stained with anti-STING and developed with pink chromophore. The images of the stained biopsies at low power of magnification were shown (A and I). The enlarged images from each sample with most cancer cells were presented (B-G, and J-N), together with the stained liver cells from sample 21-12b (H). Five MUM liver biopsy samples were only stained with anti-STING and developed with pink chromophore (O).

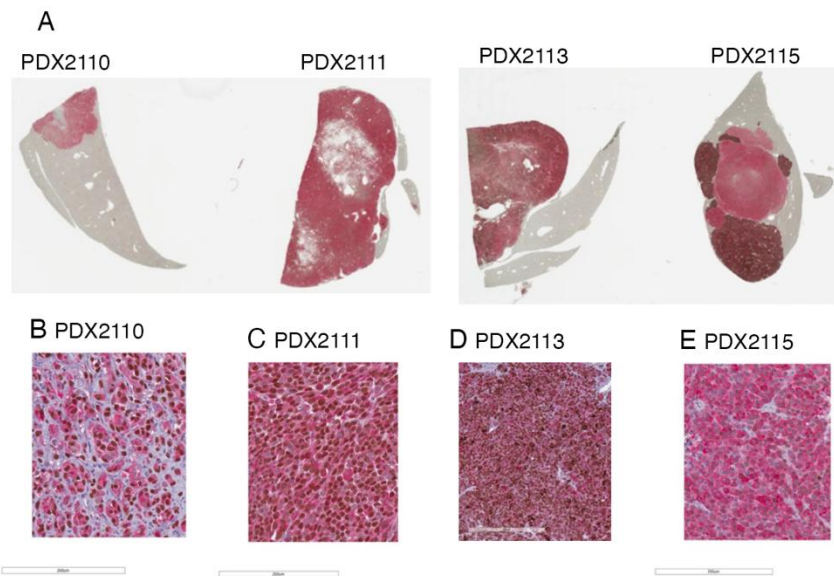


Fig. 2. MUM cancer cells in PDX models also express high levels of STING protein. FFPE PDX samples of MUM were sectioned and subjected to anti-SOX10 (brown) and anti-STING (pink) IHC as described in Fig. 1. The images of the stained PDX samples at low power of magnification were shown in A). The representative and enlarged images from each PDX samples with the most cancer cells were presented for PDX2110 B), PDX2111 C), PDX2113 D), and PDX2115 E).

STING expression in primary uveal melanoma is weaker than that in MUM, with strong expression in some cases with BAP1 loss

Next, we examined if STING is expressed in primary UM tumors in eyes. The BAP1 status is determined by a combination of anti-BAP1 IHC (**Fig. 3A**) and BAP1 mutational analysis in some cases. To examine STING expression with IHC, we included PDX MUM samples as positive controls (**Fig. 3B** and supplementary **Fig. 3A**). We also stained seven primary UM samples from

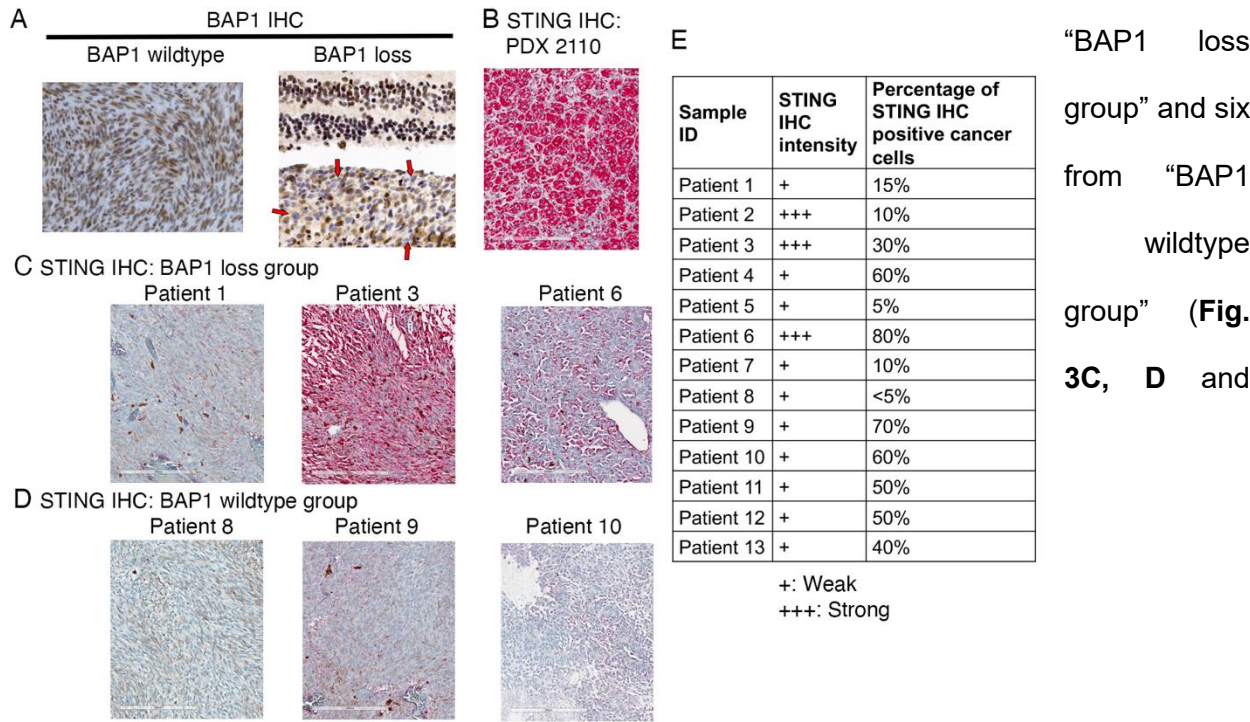
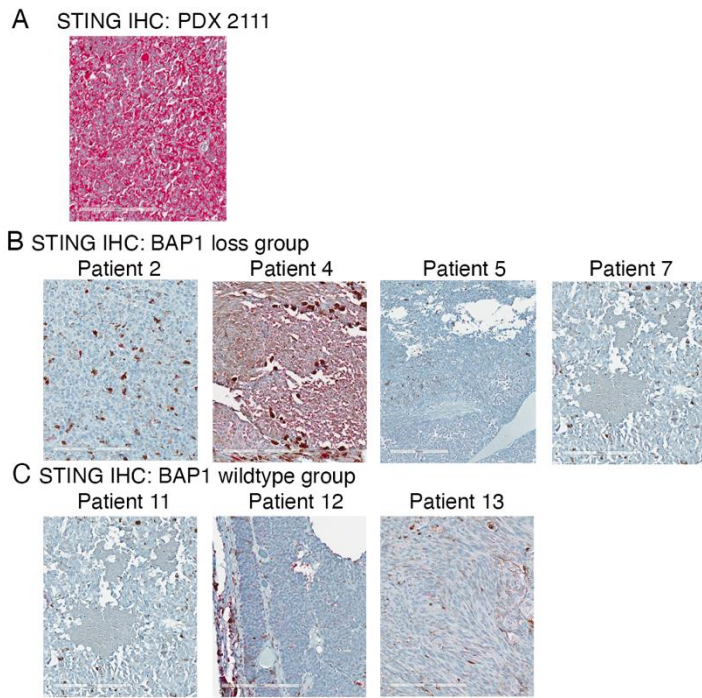


Fig. 3. STING expression in primary uveal melanoma is weaker than in MUM cancer cells in PDX models.

Samples of primary UM were sectioned and IHC stained with anti-BAP1 (brown), and anti-STING (pink) antibodies as described in Fig. 1. The BAP1 status in the samples was determined by anti-BAP1 IHC **A**) and genomic sequencing to identify BAP1 mutations in some cases. anti-STING (pink) IHC was performed on the samples of positive control **B**) and several samples with BAP1 loss **C**) and several with wildtype BAP1 **D**). The results of STING expression in primary UM samples were examined by a pathologist and summarized in **E**).

supplementary **Fig. 3B, C**). Examination by a pathologist determined that patients 2, 3, and 6, all belonging to the “BAP1 loss group”, had strong STING expression in a portion of their cancer cells. In other samples, portions of the cancer cells had weak STING expression (**Fig. 3E**).



Supplementary Fig. 2. STING expression in primary uveal melanoma is weaker than in MUM cancer cells in PDX models. Samples of primary UM were sectioned and IHC stained with anti-STING (pink) antibody. Anti-STING (pink) IHC on the samples of positive control A) and several samples with BAP1 loss B) and wildtype BAP1 C).

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BAP1 loss is associated with increased STING mRNA in human uveal melanoma

As metastatic UM in liver tends to have occurred after BAP1 mutations, and the strong expression of STING was observed in the primary UM cases with BAP1 loss, we sought to examine if there is an association between BAP1 status and STING expression in human UM samples. The examination of the TCGA database revealed that UVM in general express high level of STING mRNA (**Fig. 4A**). To further investigate if BAP1-STING association exists in UM cancer cells, analysis of single cell sequencing database was performed on UM scRNA-seq data derived from GEO (GSE139829) (19). The cells of different types, including BAP1 wildtype and mutant cancer cells, endothelial cells, fibroblasts, immune cells, were successfully separated (**Fig. 4B**). The expression of STING (TMEM173) was determined for each cell population (**Fig. 4C**), and the quantification revealed that BAP1 wildtype cancer cells, be that they are primary or metastatic, had significantly less STING mRNA expression than that in BAP1 mutant cancer cells (**Fig. 4D-E**). This correlation was further confirmed by the tumor data generated from Jefferson patients. In MUM samples with SF3B1 mutations, BAP1 expression was high and STING expression was low compared to the tumors in which BAP1 was mutant (**Fig. 4F-G**). This is consistent with what was reported by Bakhoun et al. (16), suggesting that there is a strong association between BAP1 loss and higher STING mRNA expression in UM from databases from different sources.

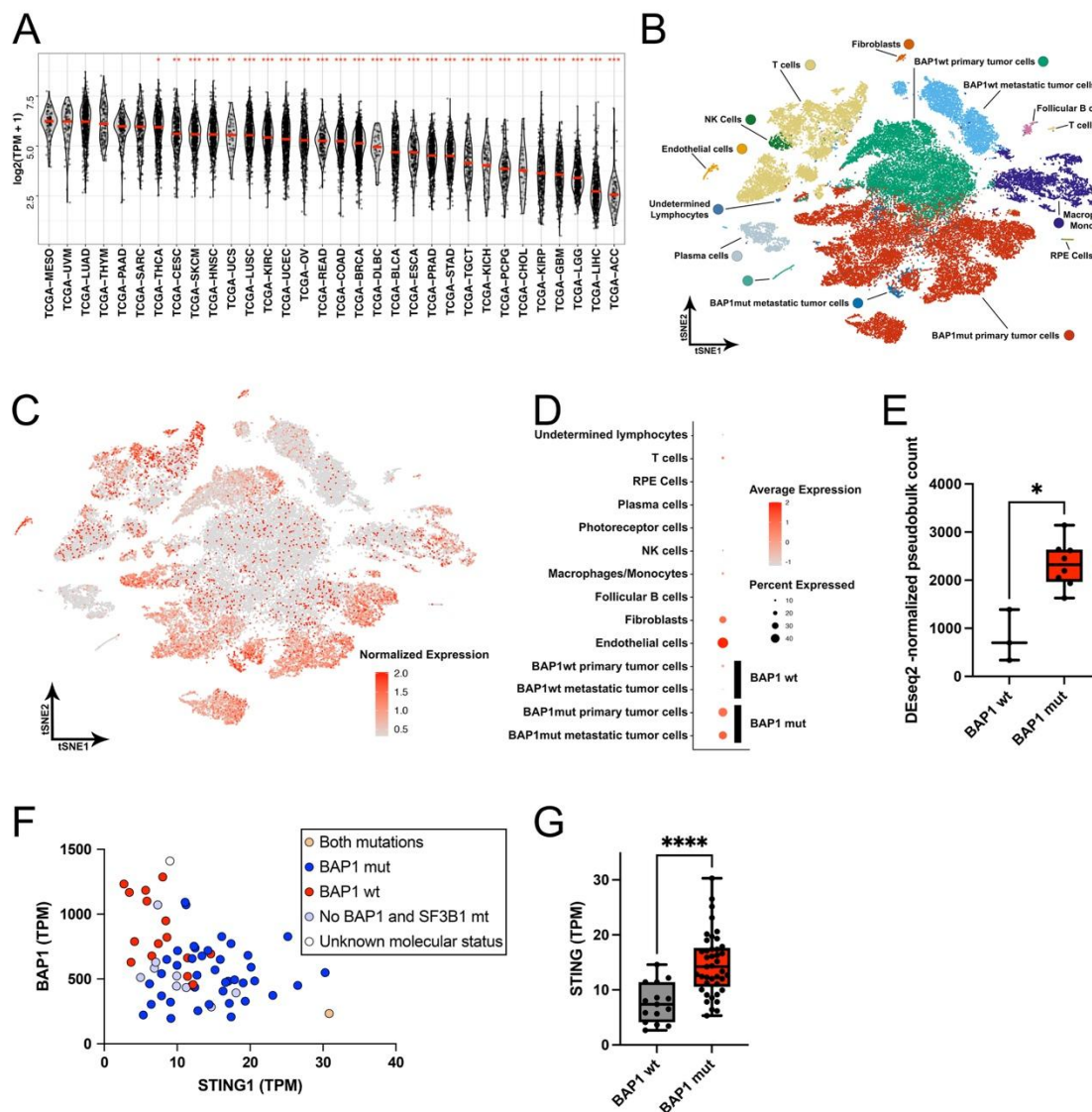


Fig. 4. BAP1 mutations are associated with higher STING1 (TMEM173) mRNA expression.

A) Bulk RNA-seq STING1 expression across TCGA tumor types. Violins show per-cohort distributions (log-scaled expression). Uveal melanoma exhibits relatively high STING1 compared with many other cancer types. B) t-SNE of 59,915 cells from 11 UM samples colored by cell-type annotations. C) STING1 expression (FeaturePlot) mapped onto the same t-SNE layout. Color indicates log-normalized expression. D) Dot plot of STING1 across annotated populations. Dot size reflects the fraction of cells with non-zero expression and color represents average expression (z-scaled per gene across groups). BAP1-mutant tumor cells show higher STING1 levels. E) Pseudobulk comparison of STING1 in BAP1-mutant versus BAP1-wild-type tumor cells. Each point is one patient sample. Boxplots summarize DESeq2-normalized counts as median (center line) and IQR (box). Significance from the DESeq2 Wald test ($*** P < 0.001$). F) Correlation between BAP1 and STING1 expression in metastatic uveal melanoma samples from Jefferson. The Y-axis shows BAP1 expression (TPM) and the X-axis shows STING1 expression (TPM) ($n=67$). G. Boxplots summary of STING1 mRNA expression in BAP1-mutated, SF3B1 mutated, and both wildtype specimens.

Human UM cancer cell lines all express STING proteins, and a STING agonist activates STING in human UM cancer cell lines and has cytostatic effects

Next, we examined the expression of STING and ISGF3 subunits (STAT1, STAT2, IRF9) in BAP1 wildtype and BAP1 mutant MUM cancer cell lines. All MUM cancer cells expressed STING protein and ISGF3 subunits (STAT1, STAT2, and IRF9) (**Fig. 5A**). We also found that the STING protein in BAP1-WT MUM cells (UM001, UM004 and 92.1) was degraded after one day of treatment with a STING agonist, diABZI (**Fig. 5B**). Interestingly, both the phosphorylated and unphosphorylated STING protein in BAP1-mutant MUM cells (UM005 and MM28) persisted after one day treatment. Consequently, higher levels of RIG-1, an ISGF3 target, were induced in BAP1-mutant cells (**Fig. 5B**). Titration of diABZI revealed that the MUM cancer cell lines responded to nanomolar levels of diABZI (**Fig. 5C**). In addition, overactivation of the STING pathway with diABZI had cytostatic effects on the MUM cells, as continuous treatment of 50nM of diABZI inhibited their growth (**Fig. 5D-G**). As controls, dosage titration and time course experiments of diABZI were performed on kidney cancer cells. 786-O cells were much less responsive to diABZI than RCC4 cells (**Supplementary Fig. 3A-B**). Consistent with this, the growth of RCC4 cells but not 786-O cells were slowed down by diABZI treatment (**Supplementary Fig. 3C-D**).

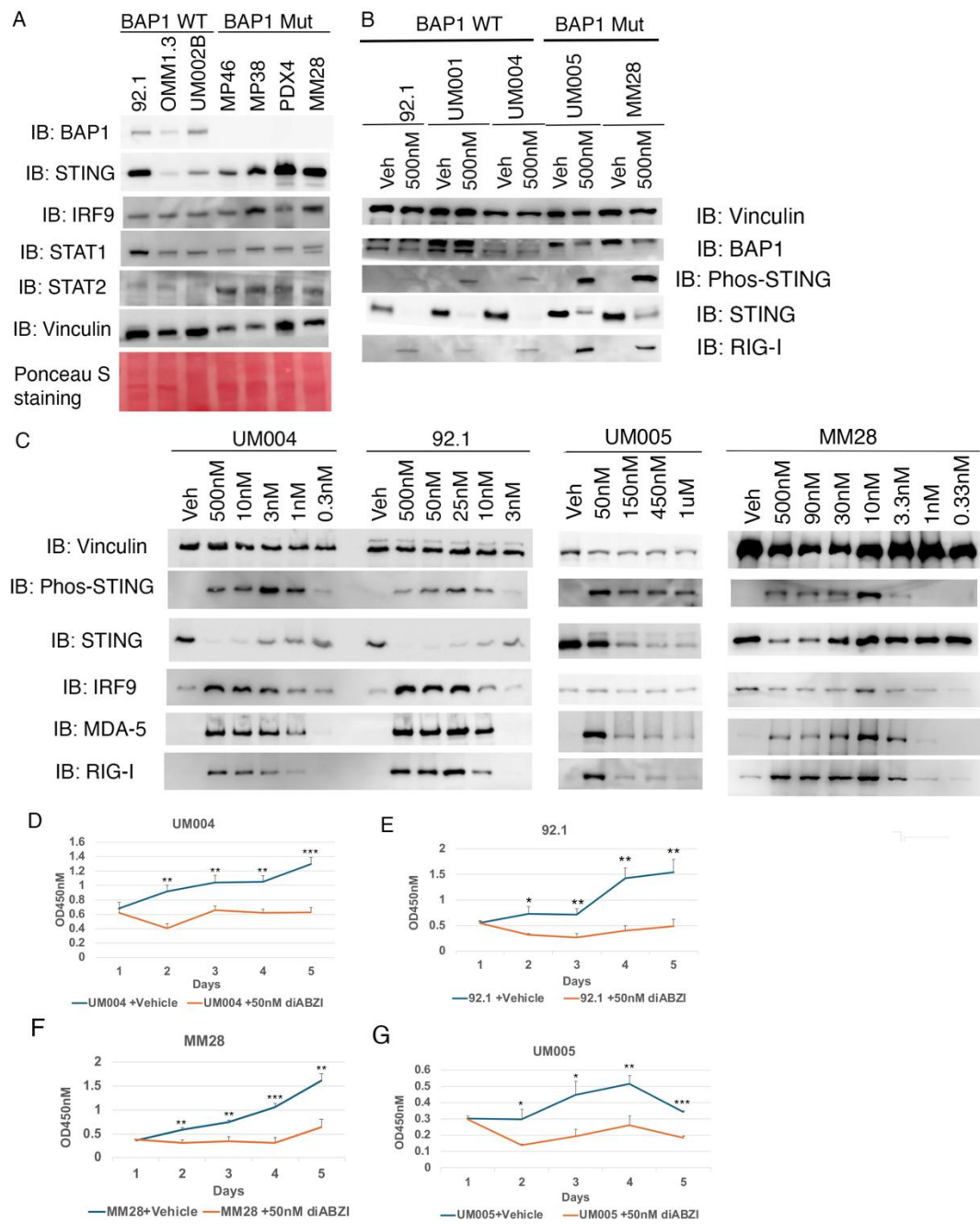
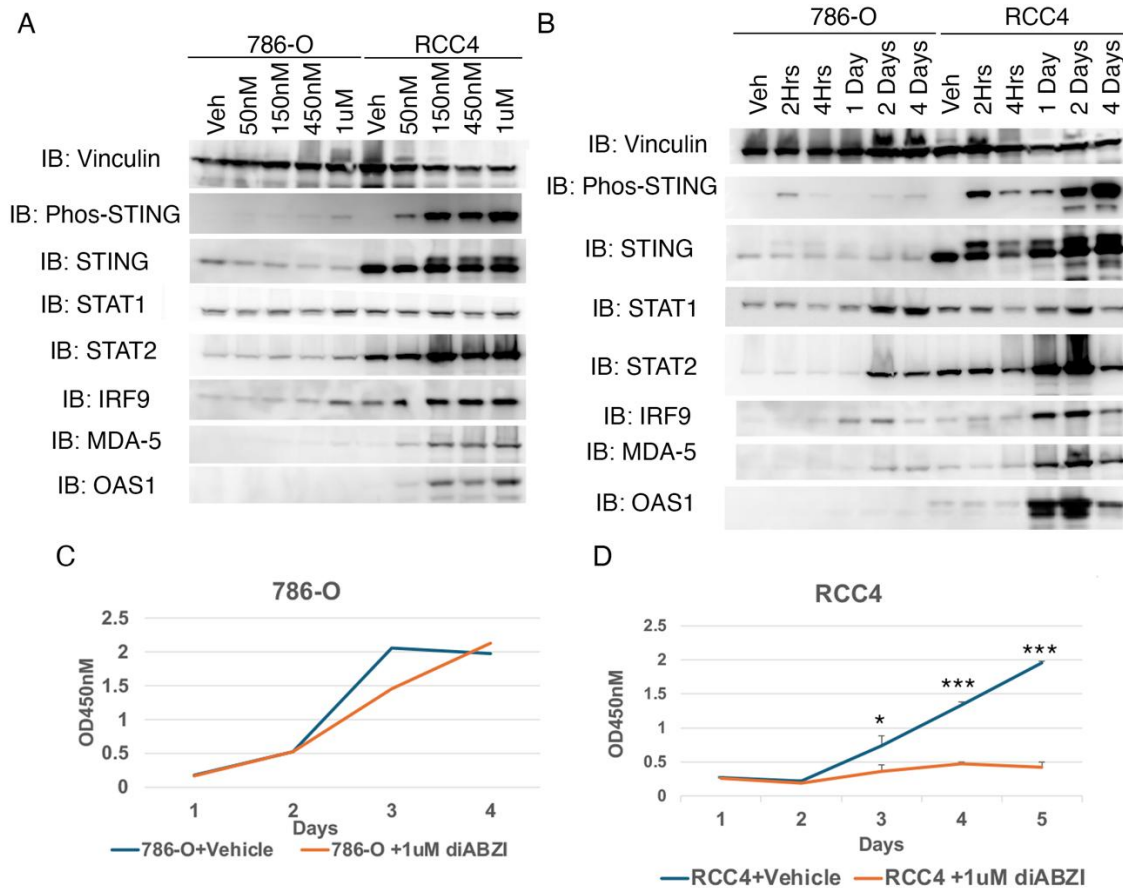


Fig. 5. Human UM cancer cell lines all express STING proteins, and a STING agonist activates STING in human UM cancer cell lines and has cytostatic effects. A) Cell lysates of BAP1-wildtype (WT) and BAP1 mutant (mut) MUM cells were immunoblotted with indicated antibodies. B) BAP1-wildtype UM001, UM004 and 92.1 cells, and BAP1 mutant UM005 and MM28 cells were treated with 500nM of diABZI for one day and subjected to western blots. C) UM004, 92.1, UM005, and MM28 cells were treated with decreasing concentrations of diABZI for one day and subjected to western blots. UM004 D), 92.1 E), MM28 F), and UM005 G) cells were treated with vehicle or 50nM diABZI in 96 well for indicated times and subjected to cck8 cell proliferation assay. The media was changed once after two days of treatment. *p* Values were calculated with two-tailed student *t* test. *: *p*<0.05. **: *p*<0.005. ***: *p*<0.001.



Supplementary Fig. 3. The response of Human ccRCC cancer cell lines to a STING agonist. A) 786-O or RCC4 ccRCC cancer cells were treated with diABZI of different concentrations for a day and subjected to western blots with indicated antibodies. B) 786-O or RCC4 ccRCC cancer cells were treated with 1uM diABZI for indicated times and subjected to western blots with indicated antibodies. 786-O C) and RCC4 D) cells were treated with 1uM diABZI for indicated times and subjected to cck8 cell proliferation assay. *p* Values were calculated with two-tailed student *t* test. *: *p*<0.05. **: *p*<0.005. ***: *p*<0.001.

1 The STING agonist alone did not kill most MUM cancer cells. A time course experiment
2 revealed that the total and phospho-STING proteins were quickly degraded in 92.1 and
3 MUM004 cancer cells after four hours of diABZI treatment (**Fig. 6A**), suggesting that this is a
4 negative feedback mechanism to limit the killing/inhibitory capability of the STING agonist. To
5 overcome this, we examined if a proteasome inhibitor Bortezomib (Bort), which is an FDA-
6 approved drug to treat multiple myeloma, was able to stabilize phospho-STING, as
7 ubiquitylation was reported to be required for degradation of activated STING (20). Two hours of
8 pretreatment of Bortezomib stabilized phospho-STING in multiple MUM cell lines (**Fig. 6B**).
9 Bortezomib blocked proteasome function which led to the accumulation of poly-ubiquitylated
10 protein species except at 3nM (**Fig. 6B**). The combination of clinically achievable low
11 concentrations of drugs, at low nMs, killed most MUM cells within a day (**Fig. 6C-D**). Further
12 titration revealed that 15nM Bort plus 10nM diABZI, but not 3nM Bort, killed 92.1 cells
13 (**Supplementary Fig. 4**). Similarly, 15nM Bort plus 50nM diABZI, but not 3nM Bort, killed
14 UM004 cells (**Supplementary Fig. 5**). Interestingly, 3nM Bort failed to boost active form of
15 STING (**Fig. 6B**), suggesting that hyperactive STING contributed to the effective killing of MUM
16 cancer cells.

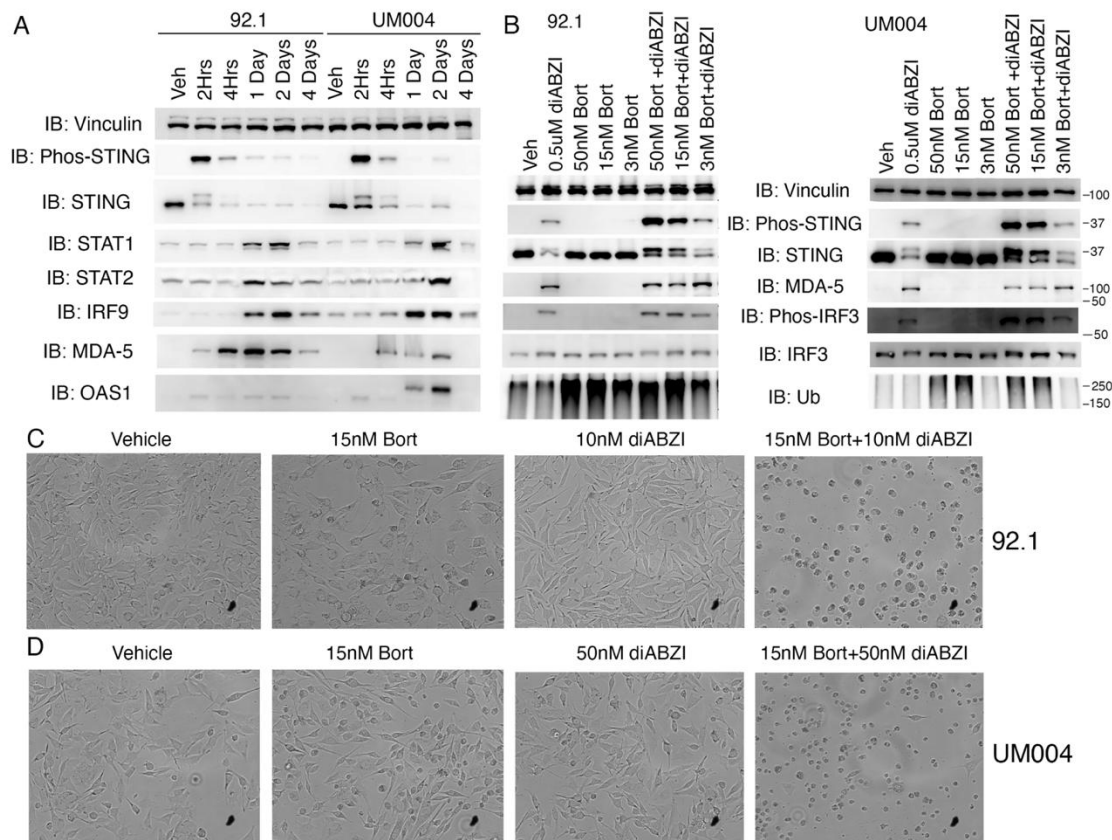
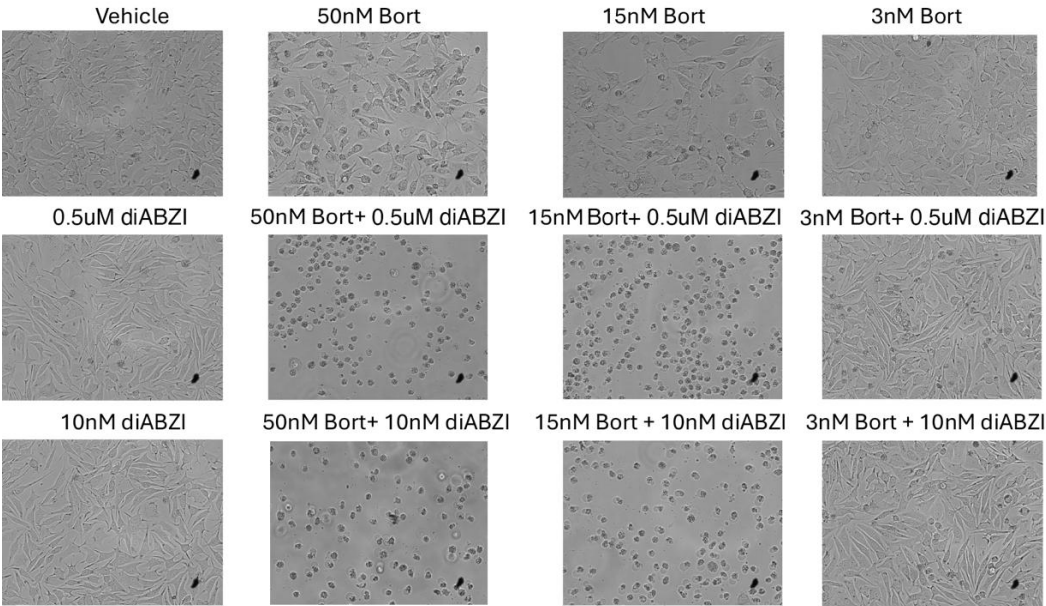


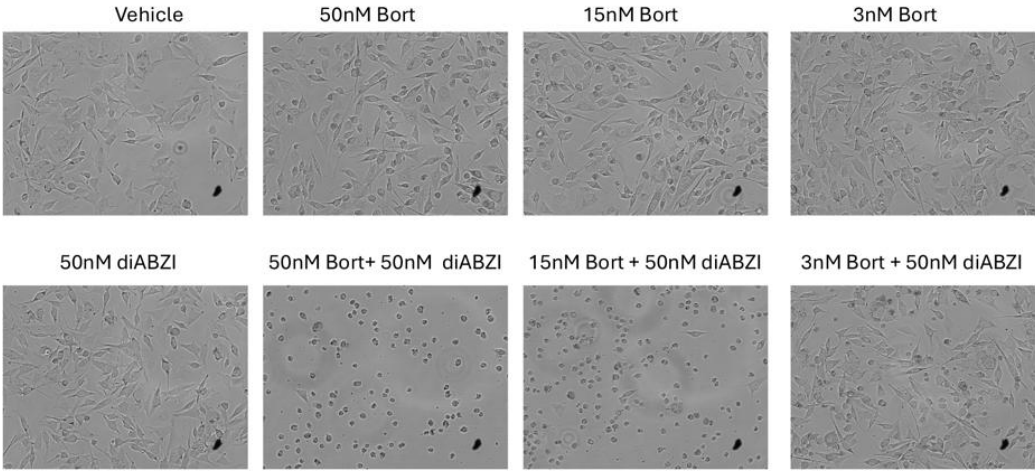
Fig. 6. Bortezomib stabilizes the active form of STING, Phospho-STING, and effectively kills UM cancer cells when combined with diABZI. A. 92.1 and UM004 cells were treated with 1 μ M of diABZI for indicated time points. The cellular lysates were blotted with indicated antibodies. B. 92.1 and UM004 cells were treated with indicated reagents. Bortezomib was added two hours before diABZI. 4 hours after diABZI addition, the cells were harvested and subjected to western blot analysis. 92.1 cells C), and UM004 cells D) were treated with indicated reagents. Bortezomib was added two hours before diABZI. The pictures were taken after one day of treatment.

92.1 cells under various treatment conditions



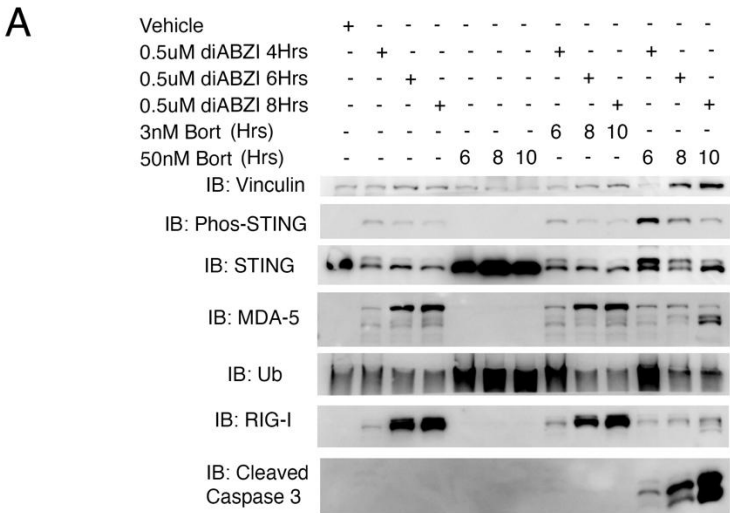
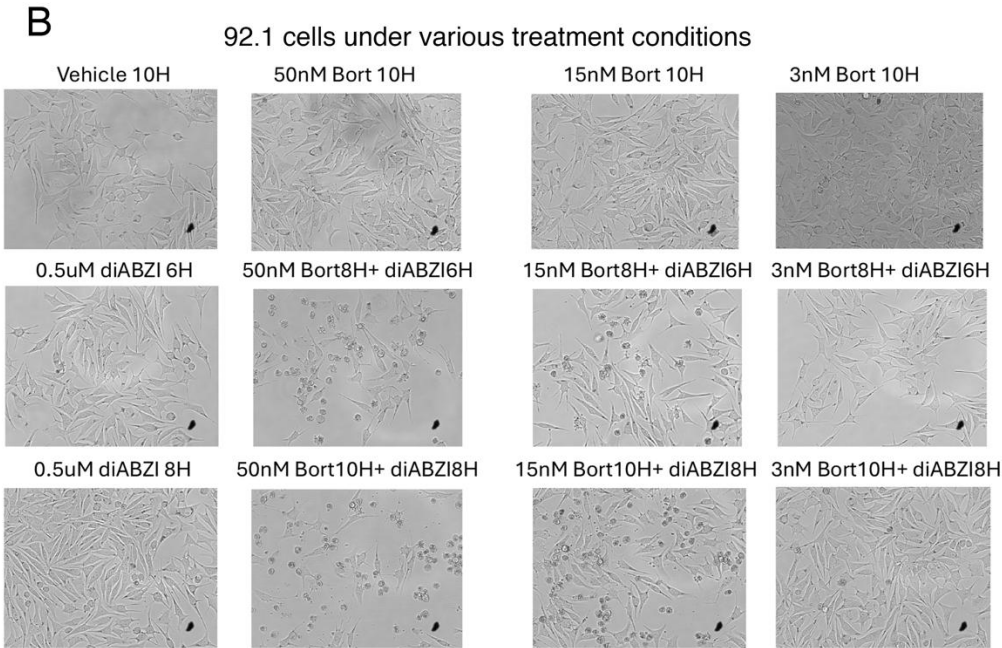
Supplementary Fig. 4. Bortezomib effectively kills 92.1 cancer cells when combined with diABZI. Cells were treated by indicated reagents for a day. Bortezomib was added two hours before diABZI. The pictures were taken after one day of treatment.

UM004 cells under various treatment conditions



Supplementary Fig. 5. Bortezomib effectively kills UM004 cancer cells when combined with diABZI. Cells were treated by indicated reagents for a day. Bortezomib was added two hours before diABZI. The pictures were taken after one day of treatment.

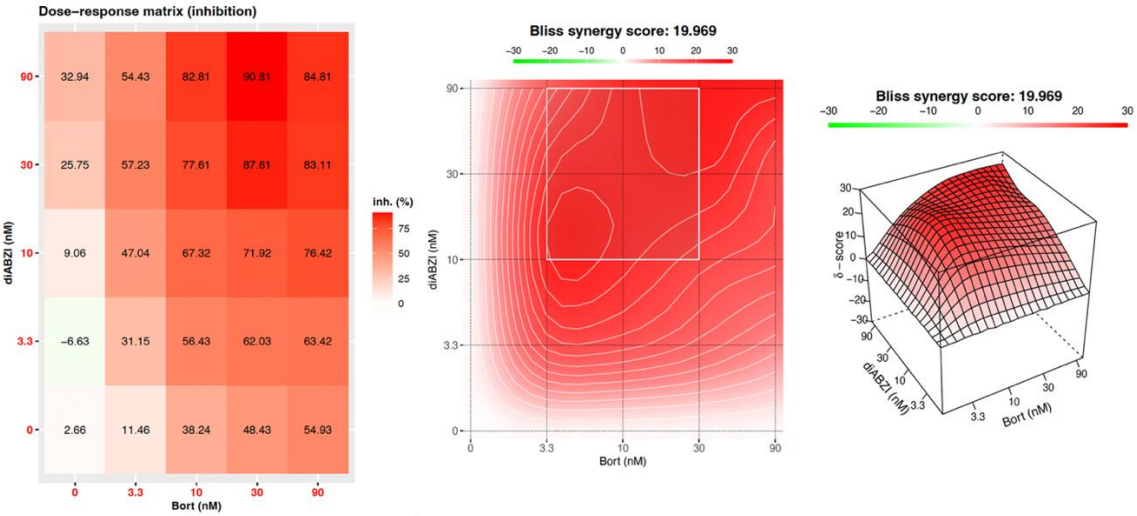
Next, we examined how fast the combination of Bort and diABZI could kill MUM cancer cells. Western blot revealed that after 92.1 cells were treated by both reagents, significant levels of cleaved caspase 3, an indicator of apoptosis, started to show up at 4 hours after diABZI addition, increased after six hours, and peaked at eight hours (**Supplementary Fig. 6A**). Interestingly, the cells treated with single reagents did not generate cleaved caspase 3. Furthermore, the morphology of the treated cells showed massive cell death after combined treatment for six and eight hours (**Supplementary Fig. 6B**). Thus this killing of 92.1 cells by the combination treatment occurred very quickly.



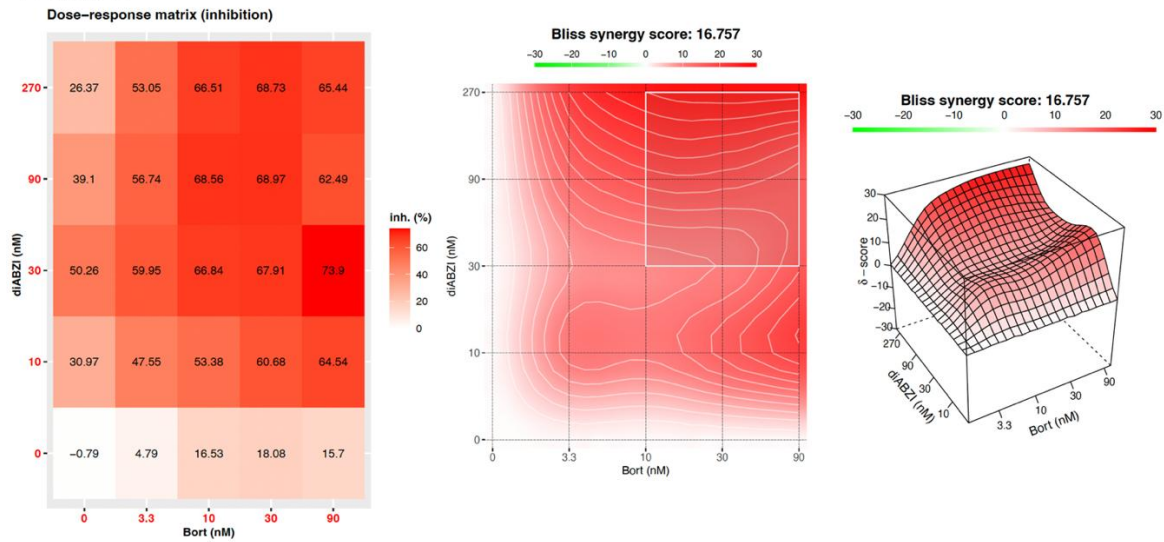
Supplementary Fig. 6. Bortezomib and diABZI combination leads to large scale apoptosis in 92.1 cells quickly. Cells were treated by indicated reagents for different length of time. Bortezomib was added two hours before diABZI. The pictures were taken at the end of treatment.

Next, we examined if the combination treatment synergistically kills UM cells. Using the platform of SynergyFinder (21), we found that Bortezomib and diABZI had very high synergy in killing of all five tested UM cancer cell lines within a day. With increasing concentrations, Bortezomib and diABZI each individually inhibited STING-expressing MUM cells to certain extent (**Fig. 7A-B and supplementary Fig. 7A-C**). When the two drugs are combined, the inhibition reached much higher level of inhibitor at various combination doses (**Fig. 7A-B and supplementary Fig. 7A-C**), and the synergy scores very high at 20, 17, 31, 28, 16 for UM004, MM28, 92.1, UM001, UM005 respectively (Synergy is strong if the score is greater than 10) (**Fig. 7A-B and supplementary Fig. 7A-C**). To learn if this is a generally toxic combination to STING-expressing normal human cells, peripheral blood mononuclear cells (PBMC) from two healthy donors were tested. The synergistic killing was absent (**Fig. 7C and supplementary Fig. 7D**). Furthermore, other STING-expressing human cancer cell lines originating from cervix, lung and kidney were not synergistically killed by this combination (**supplementary Fig. 8B**), suggesting that MUM cancer cells are uniquely sensitive to this combined treatment. Our data also indicate that the cytotoxic effects of diABZI + Bortezomib are independent of BAP1 mutation status and could be related to other MUM-specific mutations.

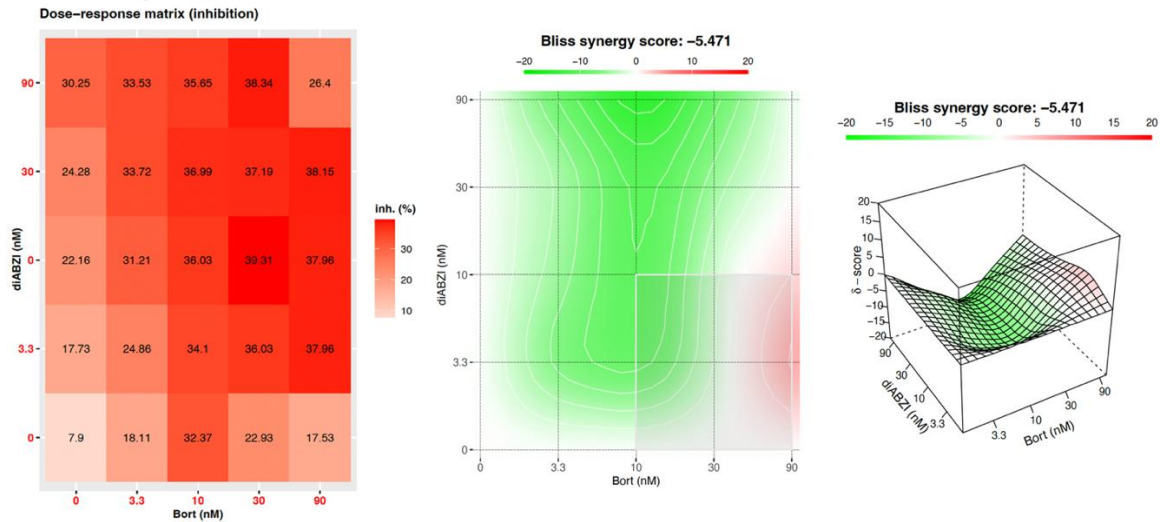
A UM004



B MM28



C PBMC-pt263



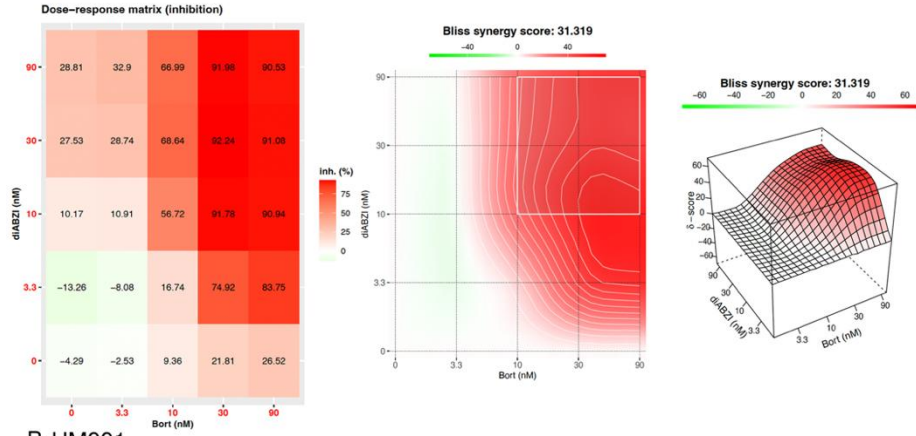
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Fig. 7. Bortezomib and diABZI display strong and selective synergistic killing of UM cancer cells.

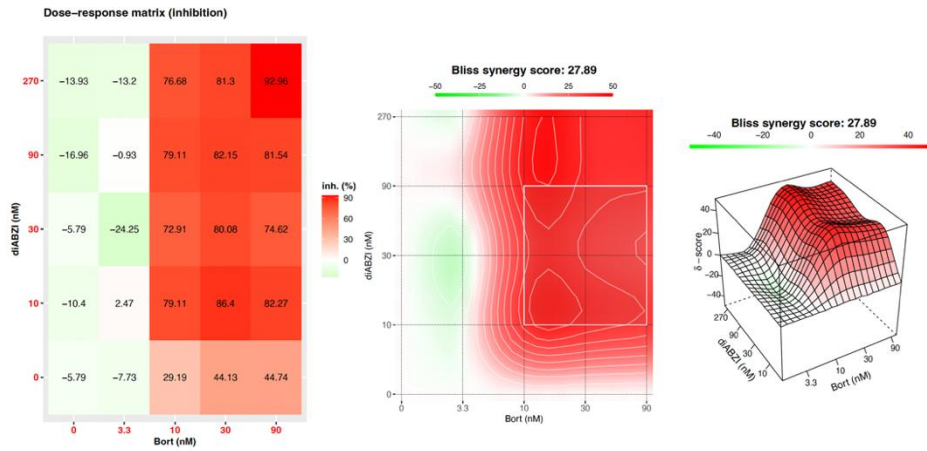
Bortezomib and diABZI display strong and selective synergistic killing of UM cancer cells. Synergistic killing

of UM cancer cell lines UM004 A), MM28 B) and human peripheral blood monocytes from a patient C). Left: Inhibition of cell growth by the combination of different concentrations of Bortezomib and diABZI. 2D map (Middle) and 3D map (right) of synergy scores of human cells treated with different combinations of reagents.

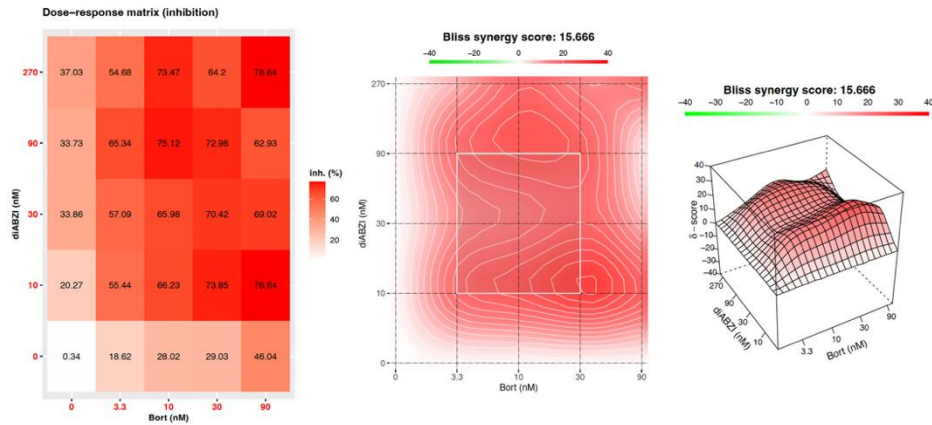
A 92.1



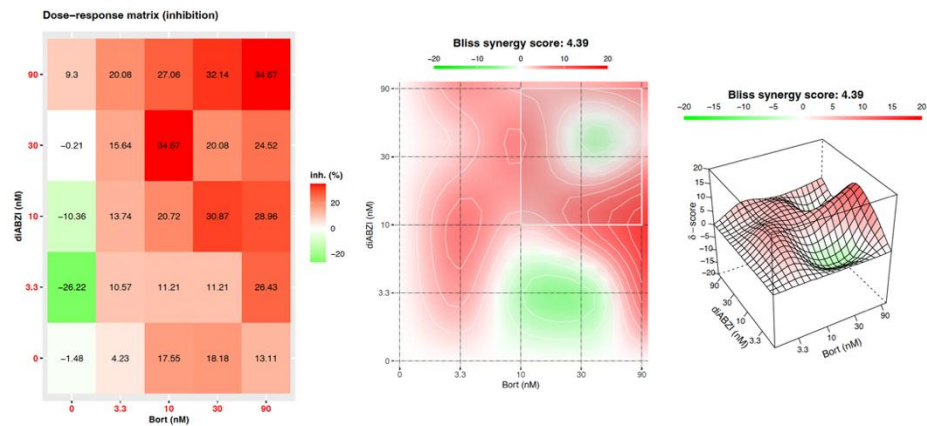
B UM001



C UM005



D PBMC-pt248

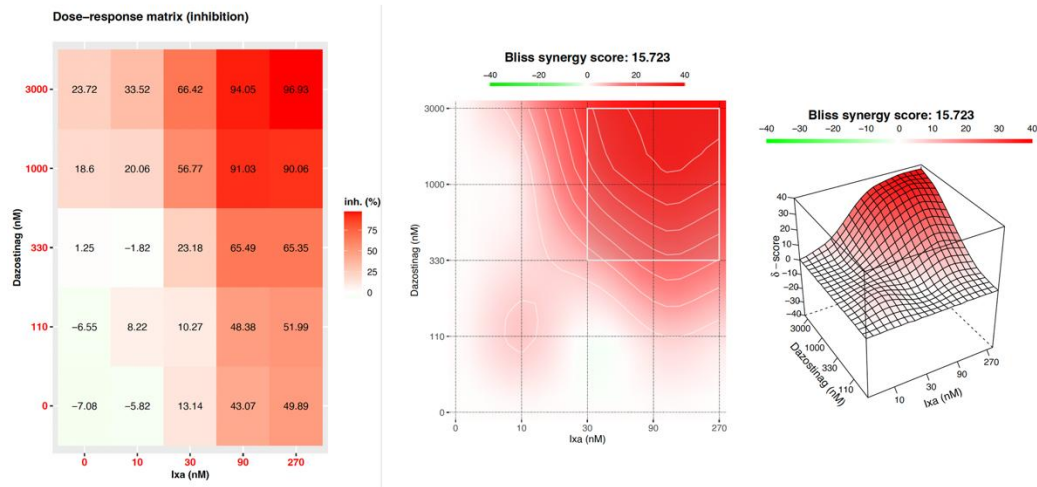


Supplementary Fig. 7. Bortezomib and diABZI display strong and selective synergistic killing of UM cancer cells. Synergistic killing of UM cancer cell lines 92.1 A), UM001 B), UM005 C) and human peripheral blood monocytes from a healthy donor D). Left: Inhibition of cell growth by the combination of different concentrations of Bortezomib and diABZI. 2D map (Middle) and 3D map (right) of synergy scores of human cells treated with different combinations of drugs.

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3 It is not known if the synergistic killing by bortezomib and diABZI was drug-dependent. To
4 address this, we tested the combination of Ixazomib (a proteasome inhibitor like Bortezomib)
5 and Dazostinag (a STING agonist like diABZI). It displayed a strong synergistic killing of 92.1
6 when these two reagents were combined (**supplementary Fig. 8A**), suggesting that the killing
7 was pathway-dependent, not specific drug-dependent.



B. Bliss synergy scores of various cells to Bortezomib and diABZI combination.

Human Cells	Bliss Synergy Score (Bort-diABZI)
92.1 (MUM)	26.41
UM001 (MUM)	27.89
UM004 (MUM)	19.97
MM28 (MUM)	16.76
UM005 (MUM)	15.67
PBMC (pt263, normal human)	-5.47
PBMC (pt248, normal human)	4.39
HeLa (cervical cancer)	-0.35
H1299 (lung cancer)	-2.65
786-O (kidney cancer)	-1.2
A498 (kidney cancer)	-2.57
Caki-1 (kidney cancer)	9.27
RCC4 (kidney cancer)	-1.2
Ren-02 (kidney cancer)	4.15

Supplementary Fig. 8. Proteasome inhibitors combined with STING agonists display strong and selective synergistic killing of UM cancer cells. A) 92.1 cells were treated with different combination of Ixazomib (a proteasome inhibitor) and Dazostinnag (a STING agonist) for a day before the cell viability was measured. Left: Inhibition of 92.1 cell growth by the combination of different concentrations of Ixazomib and Dazostinnag. 2D map (Middle) and 3D map (right) of synergy scores of 92.1 cells treated with different combinations of drugs. B) Bliss Synergy scores of various human cells treated with the combination of Bortezomib and diABZI.

Discussion:

cGAS/STING senses cytoplasmic DNA and responds to DNA damage, viral infection and many other stimuli (15, 22). Its activation produces type I interferons and inflammatory cytokines (22, 23). Biologically, it could lead to cytostasis (24), ER-mediated apoptosis (25), autophagy (26), senescence (23) based on cell types, strength and duration of STING activation. Because of the link of BAP1 and STING in other types of cancers, we probed the expression of STING in the cancer cells of metastatic uveal melanoma. Given that STING tends to negatively impact cellular growth and survival when activated, it was somewhat surprising to find that MUM cancer cells almost uniformly express high level of STING. The STING in MUM cancer cells seem to be highly functional as they can be activated by very low levels of a STING agonist, which had cytostatic effect on them. The basal state of unphosphorylated STING does not elicit much activity of its downstream targets, but it can quickly respond when the stimuli appear. The biological significance of high level of STING to MUM is currently unknown. It is unclear if it contributes to the growth and/or metastasis of the cancer cells. The primary UM cancer cells seem to express much lower level of it, and there was one report suggesting that STING might promote invasion and migration of UM cancer cells, which could help metastasis (17).

We discovered that BAP1-deficient MUM cancer cells expressed higher level of STING mRNA in MUM tumors. In addition, we also found that BAP1-deficient MUM cancer cells cleared activated and unphosphorylated STING protein more slowly after treatment with agonist. Thus it is likely that BAP1 might regulate STING expression and function at multiple levels. BAP1 could regulate the expression of STING via its H2AUB deubiquitinase activity, as it has been reported to modulate the expression of SLC7A11 through this activity (27). In addition, BAP1 can inhibit autophagy in melanoma cells (28), and STING is degraded via ESCRT-dependent microautophagy (20, 29, 30). The mechanisms of how STING is regulated by BAP1 will be investigated. In addition, BAP1-deficient MUM cancer cells grow slowly and are more resistant to nutrient stresses (5). It remains to be determined if the high level of STING contributes to such phenotypes.

1
2 It was a serendipitous finding that the combination of Bortezomib and a STING agonist was
3 deadly to MUM cancer cells, and such toxicity seemed to be highly unique to MUM cells as it
4 was not overly toxic to PBMC or cancer cells from other tissue origins. Since Bortezomib is an
5 oncology drug with acceptable side effects (31), and many STING agonists are being tested
6 with clinical trials, such a combination, if proven to be highly effective and relatively safe in
7 animal models of MUM, has the potential of being quickly tested in a clinical trial. STING
8 agonists alone tend to be relatively safe but lacked obvious efficacy (32-34). Such a potent cell
9 autonomous killing when it is combined with an anti-cancer drug could prove to be a highly
10 effective treatment. Interestingly, Bortezomib alone already activated cGAS/STING in multiple
11 myeloma cancer cells and a STING agonist boosted it (31) but did not do so in MUM cells,
12 suggesting that cellular context could lead to different response to the same drug.

13
14 The synergistic killing of MUM cells by this combination was not BAP1-dependent. It will be
15 interesting to find out which pathway(s) are quickly activated in MUM cells that led to quick cell
16 death, which are not induced in cells from other tissue origins. Given that G protein subunit
17 alpha q/11 (GNAQ/11) activating mutations occur in 90% or more of cases of Uveal melanoma,
18 and they are highly unique to UM cancer cells, it will be very informative to investigate if
19 GNAQ/11 mutations creates/enhances the unique sensitivity to the combination treatment.
20 Identifying the key pathways(s) that led to synergistic killing could assist in designing different
21 methods to activate the same pathway(s) to kill MUM cancer cells, and possibly identify useful
22 biomarkers when the combination is tested in a clinical trial.

23
24 The finding that the synergistic killing can be triggered by a different proteasome inhibitor and a
25 different STING agonist suggested that the killing was pathway-dependent, not drug-dependent.
26 This also suggests that the effect is on-target, and the functions of proteasome and STING
27 activation is critical for the killing. Since STING activation could lead to cytokine storm and
28 serious side effects in some patients, it will be worth checking if the activity of ISGF3, which is

1 responsible for inducing interferon's targets and is critical for the cytokine storm, is necessary
2 for the synergistic killing. If not, JAK inhibitors, which are usually very safe, could be added to
3 the treatment to reduce toxicity to the patients.

1 Declarations

3 Abbreviations

4 BRCA1-associated protein 1: BAP1

5 metastatic uveal melanoma: MUM

6 Stimulator of Interferon Genes: STING

7 Uveal melanoma: UM

8 clear cell renal cell carcinoma: ccRCC

9 Nuclear Factor Kappa B: NF- κ B

10 Interferon Stimulated Gene Factor 3: ISGF3

11 Patient-Derived Xenograft: PDX

12 Bortezomib: Bort

13 G protein subunit alpha q/11: GNAQ/11

15 Ethics approval and consent to participate

16 This study was approved by the University Institutional Review Board of each institution, and all
17 the samples were obtained with informed consent from patients.

19 Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

20 No AI tool was used in the manuscript preparation process.

22 Consent for publication

23 All the authors consent to the submission of this manuscript for publication

25 Availability of data and materials

26 Data generated in this study are available from the corresponding author upon reasonable
27 request.

Competing Interests

The authors declare no competing interests.

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Authors' contributions

ZZ: Investigation, Formal analysis, Validation, Visualization, Writing - review and editing. QL: Investigation, Visualization. MT: Investigation, Formal analysis, Validation, Visualization, Writing - review and editing. SK: Investigation, Formal analysis, Validation, Visualization, Writing - review and editing. RO: Investigation. WJ: Investigation. MEM: Investigation, Formal analysis, Resources. TM: Investigation, Formal analysis, Resources. FW: Resources. AA: Resources, Writing - review and editing. UA: Resources. TS: Conceptualization, Writing - review and editing, Resources, Supervision, Funding acquisition. HY: Conceptualization, Resources, Writing - original draft, Writing - review and editing, Supervision, Funding acquisition.

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Materials and methods:

Immunohistochemistry (IHC)

FFPE blocks were sectioned into 4 µm slices, deparaffinized, and rehydrated on a Varistain Gemini ES Autostainer (Thermo Shandon). Heat-induced epitope retrieval (HIER) or HIER-based antibody stripping was performed with the PT-Link (DAKO, 97 °C for 20 min). Double IHC stain for SOX10 and STING was performed on the IntelliPATH FLX® Autostainer (Biocare Medical) at room temperature with 1X TBST washes between steps. Following HIER in citrate buffer (pH 6.0), sections were blocked with H₂O₂ and normal serum, then incubated with an anti-SOX10 antibody (Abcam, ab227680, 1:200) for 30 min and detected with anti-rabbit peroxidase polymer (Vector, MP-6401-15), and visualized using DAB (Vector, SK-4103). Antibody stripping was then performed by HIER in Tris-EDTA buffer (pH 9.0), followed by normal serum blocking and incubation with an anti-STING antibody (Proteintech, 19851-1-AP, 1:1000) for 30 min. Detection was carried out with anti-rabbit alkaline phosphatase polymer (Vector, MP-5401-50), and visualized using ImmPACT Vector Red substrate (Vector, SK-5105). Slides were counterstained with hematoxylin, dehydrated and cleared on Varistain Gemini ES Autostainer, and coverslipped on a ClearVUE Coversliper.

Cell culture:

Human PBMC and cancer cell lines HeLa, H1299, 786-O, A498, Caki-1, RCC4, Ren-02 were maintained in Gibco glutamine-containing DMEM (Thermo Fisher, 11995-065, Waltham, MA, USA), which is supplemented with 10% fetal bovine serum (Bio-technie's R&D Systems, S11150, Minneapolis, MN, USA) and 1% penicillin and streptomycin (Thermo Fisher, 15140-122). Cells were cultured at 37 °C in incubators with 5% CO₂. 786-O cells were purchased from ATCC (CRL-1932, Manassas, VA, USA). Ren-02 cells were gifts from Dr. Daniel Lindner (Cleveland Clinic). HeLa, H1299, 786-O, A498, Caki-1 and RCC4 cells were gifts from Dr. William Kaelin, Jr. (Dana-Farber Cancer Institute). Uveal melanoma cell lines 92.1 UM001,

UM004, UM005, and MM28 were gifts from Dr. Takami Sato (Thomas Jefferson University). 92.1 UM001, UM005, and MM28 were maintained in RPMI1640 supplemented with 20% FBS plus 1% penicillin and streptomycin. UM004 cells were maintained in EMEM supplemented with 10% FBS plus 1% penicillin and streptomycin. Medium is changed once every three days or the cells are passaged. The pictures of the cells were taken with a Floid® Cell Imaging Station (Life technologies).

Western blotting

Cells were washed with PBS once, and resuspended in EBC buffer (120 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.5% NP-40) protease inhibitors (Thermo Fisher, A32963). Samples were boiled for 5 min in 5X loading buffer. Proteins were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Blots were developed using Immobilon Western Chemiluminescent HRP substrates (Millipore Sigma, WBKLS0500) and images were captured on an ImageQuant LAS 4000 (GE Healthcare Life Sciences, Marlborough, MA, USA). If the membranes were re-probed, they were stripped with Restore Western Blot Stripping Buffer (Thermo Fisher, 21063). The antibodies and dilutions used are listed in Table I.

Table I. Antibodies used for western blots in this study

Antibody	Catalog number	Company	Dilution
BAP1	sc-28383	Santa Cruz Biotechnology	1:200
Vinculin	sc-73614		
Ubiquitin	sc-8017		
VHL	2738	Cell Signaling Technology	
STAT1	9172S		
STAT2	72604		

p-STAT2 (Y690)	4441		1:1000
IRF9	76684S		
RIG-I	3743S		
MDA5	5321S		
OAS1	14498S		
STING	13647S		
p-STING (S366)	19781		
p-IRF3 (S396)	4947S		
IRF3	11904S		
anti-mouse IgG	62-6520	Invitrogen	1:5000
anti-rabbit IgG	31460		

1

2 Single-cell RNA sequencing (scRNA-seq)

3 UM scRNA-seq data were obtained from GEO (GSE139829), comprising 59,915 cells from 11

4 uveal melanoma (UM) samples, with library preparation, primary processing, and malignant-cell

5 identification as described previously (Durante et al., *Nat Commun* 2020). Cells were filtered by

6 library complexity and mitochondrial content ($nFeature_RNA \geq 100$, $nFeature_RNA \leq 8,000$,

7 $nCount_RNA \geq 400$, $percent.mito \leq 10$). Counts were normalized with Seurat's LogNormalize

8 (scale factor 10,000). Highly variable genes were selected with vst, and data were scaled while

9 regressing $nCount_RNA$ and $percent.mito$. Neighborhood graphs were built from principal

10 components 1–20 for Louvain clustering, and two-dimensional embeddings were generated with

11 t-SNE (Rtsne; perplexity ≈ 30) using fixed random seeds with random initialization. Cell-type

12 annotations followed the original publication. Differential expression at the cell level used Seurat's

two-sided Wilcoxon rank-sum test on log-normalized counts. Dimensional-reduction and dot plots were generated in Seurat.

To account for patient-level replication, we generated pseudobulk profiles by summing raw counts per group-sample using Seurat's AggregateExpression. Differential expression between conditions (BAP1mut vs BAP1wt) was then tested at the sample level with DESeq2. DESeq2-normalized pseudobulk counts were used to generate the box-plot figures.

Expression of a gene of interest across TCGA tumors

DESeq2-normalized, log2-transformed expression data from the UCSC Xena TCGA-GTEX-TARGET resource were analyzed in R. For each cancer type, distributions of the gene of interest were visualized with violin plots overlaid with individual samples and median crossbars. Pairwise differences between TCGA-UVM and other cancer types were assessed by Mann-Whitney U test with Bonferroni adjustment. Statistical significance was annotated as *, **, and *** for adjusted $P \leq 0.05$, ≤ 0.01 , and ≤ 0.001 , respectively.

Statistical analysis.

Single-cell differential expression was performed in Seurat using the two-sided Wilcoxon rank-sum test (FindMarkers on log-normalized data); P values were adjusted for multiple testing by Bonferroni correction across all tested genes, and adjusted $P < 0.05$ was considered significant. Pseudobulk differential expression was carried out with DESeq2 using the Wald test, with multiple-testing correction by the Benjamini-Hochberg procedure (reported as FDR/padj). Genes with $FDR < 0.05$ were considered significant.

Cell growth and drug synergism analyses

1 Cell proliferation/killing was measured using the Cell Counting Kit-8 (CCK-8) (Apexbio, K1018),
2 with slight modifications to the manufacturer's protocol. Briefly, cells were seeded into 96-well
3 plates, treated with indicated drug concentrations for the indicated length. For measurement, the
4 CCK-8 reagent was added to the wells in triplicate. After incubation for 2-4 hours, the
5 absorbance was measured at 450 nm wavelength on a CLARIOstar plus microplate reader
6 (BMG Labtech) with background subtraction.
7 To calculate the synergism scores, each cell line or thawed PBMC were split into 96 wells in
8 triplicates.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [BMCCancersupplementaryfile2.pdf](#)