DHODH inhibition enhances the efficacy of immune checkpoint blockade by increasing cancer cell antigen presentation

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Abstract

Pyrimidine nucleotide biosynthesis is a druggable metabolic dependency of cancer cells, and chemotherapy agents targeting pyrimidine metabolism are the backbone of treatment for many cancers. Dihydroorotate dehydrogenase (DHODH) is an essential enzyme in the de novo pyrimidine biosynthesis pathway that can be targeted by clinically approved inhibitors. However, despite robust preclinical anticancer efficacy, DHODH inhibitors have shown limited single-agent activity in phase 1 and 2 clinical trials. Therefore, novel combination therapy strategies are necessary to realize the potential of these drugs. To search for therapeutic vulnerabilities induced by DHODH inhibition, we examined gene expression changes in cancer cells treated with the potent and selective DHODH inhibitor brequinar (BQ). This revealed that BQ treatment causes upregulation of antigen presentation pathway genes and cell surface MHC class I expression. Mechanistic studies showed that this effect is 1) strictly dependent on pyrimidine nucleotide depletion, 2) independent of canonical antigen presentation pathway transcriptional regulators, and 3) mediated by RNA polymerase II elongation control by positive transcription elongation factor B (P-TEFb). Furthermore, BQ showed impressive single-agent efficacy in the immunocompetent B16F10 melanoma model, and combination treatment with BQ and dual immune checkpoint blockade (anti-CTLA-4 plus anti-PD-1) significantly prolonged mouse survival compared to either therapy alone. Our results have important implications for the clinical development of DHODH inhibitors and provide a rationale for combination therapy with BQ and immune checkpoint blockade.

Keywords

Pyrimidine biosynthesis, DHODH, brequinar, antigen presentation, immunotherapy, MHC class I, P-TEFb

Introduction

Deranged cellular metabolism is a universal feature of cancer cells [1,2]. One particularly cancer-essential metabolic aberration is the hyperactive synthesis and utilization of nucleotide triphosphates; this phenotype is a critical driver of cancer cell malignant behaviors, including uncontrolled proliferation, evasion of the host immune response, metastasis to distant organs, and resistance to antineoplastic therapy [3]. The de novo pyrimidine biosynthesis pathway, which generates pyrimidine nucleotides from aspartate and glutamine, is consistently hyperactive in cancer cells and druggable by clinically approved inhibitors [4]. Dihydroorotate dehydrogenase (DHODH) catalyzes the fourth step in this pathway and is essential for de novo pyrimidine synthesis. DHODH inhibitors have shown robust preclinical anticancer activity across diverse cancer types [5-14] and have recently entered clinical trials for multiple hematologic cancers (NCT04609826 and NCT02509052). Although there is a vast literature on DHODH inhibitors dating back to the early 1990s, and despite the "rediscovery" of DHODH in recent years as a critical cancer cell metabolic dependency, important questions about the cellular response to DHODH inhibition remain unanswered.

While combination chemotherapy is highly effective and potentially curative against certain cancers (e.g. Hodgkin lymphoma, testicular cancer, childhood leukemia, and others), many common malignancies are refractory to chemotherapy (e.g. lung cancer, pancreatic cancer, colorectal cancer, etc.) [15]. In some chemotherapy-refractory cancers (most prominently melanoma, mismatch repair deficient colorectal cancer, bladder cancer, and non-small cell lung cancer), immunotherapeutic strategies have demonstrated strong efficacy and led to durable remissions in a subset of patients [16]. The efficacy of immunotherapy agents is dependent on multiple factors, including tumor antigen presentation, limited immune cells in the tumor milieu, and T-cell activation status [17,18]. Adoptive cell therapies and immune checkpoint blockade (ICB) can address the issues of limited immune cell recruitment into tumors and limited T-cell activation, respectively. However, adequate antigen presentation by tumor cells is still required for immunotherapy efficacy, which relies on T-cell-mediated adaptive immunity.

The antigen presentation pathway (APP) mediates the presentation of endogenous peptide antigens to CD8 T-cells via MHC class I (MHC-I). This pathway entails the degradation of cellular proteins into small peptides by the proteasome, the import of these peptides into the endoplasmic reticulum by transporter associated with antigen presentation proteins (*TAP1* and *TAP2*), and the loading of these peptides into the MHC-I complex, which consists of a heavy chain (encoded by *HLA-A*, *HLA-B*, or *HLA-C*) and a light chain (encoded by *B2M*) [19]. APP genes are often downregulated in cancer cells, and this impedes the recognition of immunogenic MHC-I restricted cancer cell antigens by infiltrating T-cells [20]. Antigen presentation and T-cell recognition are crucial for T-cell-mediated killing of cancer cells [21-23], and forced MHC-I expression enhances immunotherapy efficacy in preclinical models [24-27]. Furthermore, high tumoral expression of MHC-I, MHC-II, and other APP genes correlates with better overall survival in patients with melanoma treated with ICB therapies [28-31].

While previous reports have shown that pyrimidine nucleotide depletion triggers the expression of innate immunity-related genes and induces an interferon-like response [32-34], the role of pyrimidine starvation in antigen presentation has not been reported. Herein, we report that DHODH inhibition induces the robust upregulation of APP genes and increases tumor cell antigen presentation via MHC-I. We further explored the mechanism and functional consequences of DHODH inhibitor-mediated APP induction in cancer.

Results

Brequinar induces upregulation of MHC-I and antigen presentation pathway genes

We examined gene expression changes following transient or prolonged DHODH inhibition by culturing human pancreatic ductal adenocarcinoma cell lines S2-013 and CFPAC-1 in the presence of BQ at two different doses for 16 hours and for a two-week duration (Fig 1A). Gene set enrichment analysis (GSEA) using Hallmark and KEGG gene sets from MSigDB [35,36] revealed 17 gene sets that were significantly upregulated (FDR q < 0.25) across both cell lines following two-week BQ exposure

125 (Fig 1B). Twelve of these gene sets (highlighted in purple) are ontologically related to antigen
126 presentation and contain MHC class I, MHC class II, and/or APP genes such as *TAP1* in the leading
127 edge. Certain gene sets, such as allograft rejection (KEGG), graft versus host disease (KEGG), and
128 antigen processing and presentation (KEGG) are composed almost entirely of APP genes (Fig 1C).
129 Heatmap analysis showed that APP genes were robustly upregulated in a dose- and duration-
130 dependent manner in CFPAC-1 (Fig 1D) and S2-013 (Fig S1A) cells. The effect size was generally
131 smaller for S2-013 cells, likely because they are resistant to DHODH inhibition due to efficient
132 nucleoside salvage, as we previously reported [37]. Publicly available RNA-seq data from human A375
133 melanoma cells treated with the clinically approved DHODH inhibitor teriflunomide [38] corroborated our
134 findings, as teriflunomide caused a rapid (within 12 hours) and duration-dependent increase in MHC-I/II
135 and APP transcript levels (Fig 1E).
136   We validated these gene expression changes in CFPAC-1 cells by RT-qPCR (Fig S1B) and
137 then performed RT-qPCR to assess the mRNA levels of genes coding for MHC-I across a panel of
138 human cancer cell lines treated with BQ for 24 hours (Fig 1F). This confirmed that MHC-I heavy chain
139 transcripts (*HLA-A, HLA-B*, and *HLA-C*) are consistently upregulated in response to BQ across diverse
140 cancer types (Fig 1F). To optimize conditions for *in vivo* studies, we tested the long-term response and
141 observed that two-week BQ treatment of B16F10 murine melanoma cells also caused dramatic APP
142 gene upregulation (Fig S1C). Flow cytometry confirmed a marked increase in cell surface MHC-I levels
143 in nonpermeabilized live CFPAC-1 (Fig 1G) and B16F10 (Fig 1H) cells following a two-week BQ
144 treatment, confirming that transcriptional upregulation of APP genes results in greater cell surface
145 antigen presentation.
146   In parallel, we confirmed pyrimidine nucleotide depletion upon treatment with BQ at different
147 doses by performing metabolomics analysis of CFPAC-1 and B16F10 cells following BQ treatment. The
148 results demonstrated a rapid (8-hour treatment) and dose-dependent accumulation of dihydroorotate
149 and N-carbamoyl-aspartate (upstream of DHODH) as well as depletion of pyrimidine nucleotides UTP
150 and CTP (Fig 1I-J) and other pyrimidine species (Fig S1D-E). These results confirm that on-target
151 DHODH inhibition and resultant pyrimidine nucleotide depletion correlates with the transcriptional
152 induction of APP genes and enhanced antigen presentation via MHC-I.
153
154 **BQ-mediated APP induction depends on pyrimidine nucleotide depletion**
155   To confirm that BQ- or teriflunomide-mediated APP induction was specifically caused by
156 DHODH inhibition (i.e., on-target effect), we asked whether the effect could be reversed by restoring
157 pyrimidine nucleotides. As we previously observed [37], media supplementation with uridine rescued cell
158 viability (Fig 2A) and pyrimidine levels (Fig 2B) following BQ treatment and partially rescued viability
159 following teriflunomide treatment (Fig S2A). Uridine supplementation likewise blocked mRNA induction
160 of MHC-I transcripts (*H2-Db*, *H2-Kb*, and *B2m*), as well as *Nlrc5* (a major MHC-I transcriptional
161 coactivator) and *Tap1* (required for peptide import into the ER, a key step in MHC-I antigen
162 presentation) by BQ (Fig 2C) or teriflunomide (Fig S2B), while uridine alone had no effect (Fig S2C).
163 This same phenotype was observed in HCT116 human colorectal cancer cells (Fig S2D). Concordantly,
164 cell surface MHC-I upregulation by BQ or teriflunomide (24-hour treatment) was abrogated by uridine
165 supplementation (Fig 2D), while uridine alone again had no effect (Fig S2E). These results demonstrate
166 that DHODH inhibitor-mediated APP induction is caused by pyrimidine nucleotide depletion.
167   To further validate this finding, we assessed MHC-I heavy chain mRNA levels in S2-013 cells
168 with DHODH deletion (sgDHODH). We have previously demonstrated that these cells require
169 exogenous uridine for viability and experience profound pyrimidine depletion (>95% depletion of UTP
170 and CTP) after 8-hour incubation in nucleoside-free media [37]. After growing these cells with
171 supplemented uridine (1 mM), we withdrew exogenous nucleosides by changing to new media
172 containing 10% dialyzed FBS. After 72-hour exposure to nucleoside-free media, sgDHODH cells
173 upregulated *HLA-A*, *HLA-B*, and *HLA-C*, and this was reversed by adding back uridine (Fig 2E).
174 Importantly, treatment with BQ did not further increase MHC-I mRNA expression (Fig 2E, compare blue
175 and red bars). Together with our other data, these results indicate that BQ-mediated APP induction is
176 an on-target phenomenon with respect to DHODH inhibition.
177   Since uridine addback rescued BQ- and teriflunomide-mediated loss of viability (Fig 2A, S2A),
178 we queried whether BQ-mediated APP induction was caused by pyrimidine depletion *per se*, or if it was

4

179 the result of some nonspecific downstream consequence of pyrimidine starvation, such as DNA
180 damage or loss of cell viability. To address this, we screened a panel of genotoxic chemotherapy
181 agents and small molecule inhibitors for their ability to induce APP genes following 72-hour exposure at
182 previously determined cytotoxic doses in CFPAC-1 cells (Fig 2F). Besides interferon gamma (a positive
183 control), BQ, teriflunomide, and GSK983 (another DHODH inhibitor), the only agent that induced APP
184 gene transcription in this assay was mycophenolate, a clinically approved inhibitor of the *de novo* GTP
185 synthesis enzymes inosine monophosphate dehydrogenase 1 and 2 (IMPDH1/2). The effect of
186 mycophenolate on APP gene expression was subsequently validated in B16F10 cells (Fig S2F),
187 demonstrating that either purine or pyrimidine nucleotide depletion can induce cancer cell APP mRNA
188 expression *in vitro*.
189   The other drugs screened included nucleotide synthesis inhibitors (5-fluorouracil, methotrexate,
190 gemcitabine, and hydroxyurea), DNA damage inducers (oxaliplatin, irinotecan, and cytarabine), a
191 microtubule targeting drug (paclitaxel), a DNA methylation inhibitor (azacytidine), and other small
192 molecule inhibitors (Fig 2F). While we cannot rule out the possibility that these agents induce APP
193 transcription in other cell lines or under other dose/duration conditions, the inertness of these
194 compounds (with respect to APP gene expression) in our screen suggests that BQ-mediated APP
195 induction in CFPAC-1 cells is not a general phenomenon that occurs downstream of DNA damage or
196 some other response to therapy-induced stress.
197
198 **BQ-mediated APP induction does not depend on canonical APP transcriptional regulators**
199   To elucidate the molecular pathway leading to APP induction downstream of pyrimidine
200 depletion, we extended our findings to HEK-293T cells, which also display rapid (within 4 hours)
201 transcriptional induction of MHC-I upon BQ treatment (Fig S3A). Reasoning that the mechanism of this
202 phenomenon in HEK-293T cells is less likely to involve idiosyncratic genetic aberrations than in cancer
203 cell lines, we chose to conduct our initial mechanistic studies in this system and then extend our
204 findings to cancer cell lines if possible.
205   We used a candidate-based chemical biology screening approach to ask if drugs targeting
206 suspected pathways might block BQ-mediated APP induction in HEK-293T cells. We first interrogated
207 pathways that are known to control MHC/APP expression, including IFN-JAK-STAT [39], NF-κB [27,40], and
208 cGAS-STING-TBK1 [41]. Neither ruxolitinib (a JAK1/2 inhibitor with activity against STAT3) nor GSK8612
209 (a TBK1 inhibitor) [42], nor TPCA-1 (an IKK2 inhibitor) [43] abrogated BQ-mediated APP induction (Fig 3A),
210 despite blocking APP induction downstream of poly(dA:dT) and interferon gamma (Fig S3B) as
211 expected. This indicates that these canonical regulators of MHC/APP expression are dispensable for
212 APP induction downstream of DHODH inhibition.
213   Interestingly, the IKK2 inhibitor BMS-345541 [44] mostly abrogated BQ-mediated APP induction
214 (Fig 3A). BMS-345541 effectively blocked BQ- and Ter-mediated APP induction at concentrations of 10
215 µM and 40 µM, but not 2.5 µM (Fig 3B). The effect of BMS-345541 was confirmed in B16F10 (Fig 3C),
216 CFPAC-1 (Fig 3D), and HCT116 (Fig 3E) cells. Furthermore, BQ treatment (24 hours) of HCT116 cells
217 caused increased cell surface expression of MHC-I, which could be reversed by either uridine
218 supplementation or by treatment with BMS-345541; neither uridine nor BMS-345541 alone affected cell
219 surface MHC-I expression (Fig 3F).
220   Given that TPCA-1 (an established IKK2 inhibitor [43]) did not block BQ-mediated APP induction
221 (Fig 3A, 3C), we suspected that this effect of BMS-345541 was independent of IKK2. To test this, we
222 used previously reported MiaPaCa2 cells with CRISPR-Cas9 deletion of *IKK2* (MiaPaCa2-*IKK2*-KO) [45].
223 Increased APP mRNA expression was observed upon BQ, teriflunomide, or GSK983 treatment (all
224 DHODH inhibitors) of either wild-type or *IKK2*-KO MiaPaCa2 cells (Fig S3C). However, while TNF-
225 alpha stimulation induced APP and *CCL5* (a canonical NF-κB target gene downstream of TNF-alpha [46])
226 expression in wild-type cells, this was not observed in *IKK2*-KO cells, as expected (Fig S3C, far right).
227 Finally, BQ-mediated APP induction in *IKK2*-KO cells was significantly reversed with concurrent BMS-
228 345541 treatment (Fig 3G). Together, these results demonstrate that IKK2 is dispensable for BQ-
229 mediated APP induction and that the observed reversal effect of BMS-345541 is independent of IKK2.
230
231 **Nucleotide starvation induces APP transcription in a P-TEFb-dependent manner**

232 To further investigate the mechanism by which BMS-345541 blocks APP induction downstream
233 of pyrimidine starvation, we leveraged publicly available data on the target profile of BMS-345541 and
234 other agents tested in the cell-free KINOMEscan assay [47]. BMS-345541 reproducibly bound more than
235 20 kinases, with dissociation constants (k_d) ranging from 130-8100 nM (Fig 4A). We prioritized potential
236 targets with a k_d in the low micromolar range, given that 2.5 µM BMS-345541 did not block BQ-
237 mediated APP induction in our previous experiments, and the effect seemed to be maximal at 10 µM,
238 with no significant increase in the magnitude of the effect between 10 µM and 40 µM (Fig 3B).
239 Additionally, we prioritized targets that were >50% inhibited with 10 µM BMS-345541 treatment. These
240 two conditions correspond to the upper left quadrant of Fig 4A.
241 One potential target that met the selection criteria was CDK9, which together with cyclin T1 or
242 T2 forms positive transcription elongation factor B (P-TEFb). P-TEFb is required for the release of
243 promoter-proximal paused RNA polymerase II (Pol II) into productive elongation and therefore is
244 essential for Pol II transcription from paused promoters [48,49]. The potent P-TEFb inhibitor flavopiridol [50]
245 phenocopied BMS-345541 in our assays, as it blocked APP induction downstream of DHODH,
246 IMPDH1/2 (by mycophenolate), or CTP synthase (by 3-deazauridine [51]) inhibition (Fig S4A). This
247 suggests that APP induction downstream of nucleotide starvation requires P-TEFb-mediated paused
248 Pol II release. It also suggests that the BMS-345541 effect of reversing BQ-induced APP upregulation
249 is due to P-TEFb inhibition.
250 Within the list of kinases bound by BMS-345541 (Fig 4A) we eliminated those that were a) not
251 expressed by CFPAC-1 cells in our RNA-seq data, b) not bound by flavopiridol in KINOMEscan data, or
252 c) bound by ruxolitinib in KINOMEscan data with Kd < 500 nM (as 2.5 µM ruxolitinib failed to reverse
253 BQ-mediated APP induction (Fig 3A)). Five candidates (besides CDK9) remained that were bound by
254 both BMS-345541 and flavopiridol in KINOMEscan assays. Of these, three are CDKs known to play a
255 role in transcription (CDK7, CDK13, and CDK16). Inhibition of any of these CDKs could theoretically
256 account for the observed effects of flavopiridol and BMS-345541. However, previous studies suggest
257 that flavopiridol inhibition of these CDKs in vivo is much less efficient than in cell-free assays because it
258 is competitive with ATP (and thus less efficient in living cells where the ATP concentration is in the 1-10
259 mM range, which is much higher than in cell-free assay conditions), while its inhibition of P-TEFb is not
260 affected by ATP concentration [50]. Furthermore, flavopiridol and the CDK7 inhibitor THZ1 have very
261 different (and mutually exclusive) effects on transcriptional processes [52], arguing against CDK7
262 inhibition as the mechanism of flavopiridol's effect.
263 To further probe whether the observed effect of flavopiridol was due to CDK9 inhibition, we
264 tested two other CDK9 inhibitors (AT7519 and dinaciclib). Both CDK9 inhibitors phenocopied
265 flavopiridol in our assays (Fig 4B). Furthermore, a previously characterized CDK9-targeted proteolysis
266 targeting chimera (PROTAC), termed PROTAC2 [53], had the same effect (Fig 4B). PROTAC2 consists
267 of a CDK9-binding aminopyrazole warhead conjugated to pomalidomide, which recruits the E3 ubiquitin
268 ligase Cereblon (*CRBN*). Cereblon in turn ubiquitinates CDK9, resulting in its proteasomal degradation.
269 Co-treatment of HEK-293 cells with PROTAC2 and pomalidomide prevents PROTAC2-mediated CDK9
270 degradation, as free pomalidomide competes with PROTAC2 for Cereblon binding [53]. We observed that
271 PROTAC2 (1 µM) blocked BQ-mediated APP induction, and this effect was reversed by co-treatment
272 with 10-fold excess pomalidomide (10 µM); however, when we increased the concentration of
273 PROTAC2 to 10 µM (so that PROTAC2 and pomalidomide concentrations were equal), pomalidomide
274 no longer had this effect (Fig 4C). Consistently, immunoblot analysis showed that 10 µM pomalidomide
275 prevents CDK9 degradation upon 1 µM but not 10 µM PROTAC2 treatment (Fig 4D). When we
276 repeated the experiment shown in Fig 4B with HCT116 cells, we found that all CKD9 inhibitors reversed
277 BQ-mediated APP induction, but PROTAC2 did not (Fig 4E). Concordantly, immunoblot analysis
278 showed that PROTAC2 did not cause CDK9 depletion in HCT116 cells treated in parallel (Fig 4F).
279 Taken together, these results demonstrate that CDK9 degradation is necessary for the reversal effect
280 of PROTAC2 and that CDK9 is required for BQ-mediated APP induction.
281 The dependence of BQ-mediated APP induction on CDK9 strongly suggests that nucleotide
282 starvation enforces nascent transcription of APP genes, as opposed to increased mRNA stability. This
283 is further supported by the rapid buildup of APP transcripts following DHODH inhibitor treatment (within
284 4 hours, Fig S3A). Additionally, ChIP-seq analysis of global Pol II occupancy following 48-hour
285 teriflunomide treatment in A375 cells [38] shows increased Pol II occupancy across many APP genes,

286 and fold change in Pol II occupancy significantly correlated with fold change in mRNA expression under
287 the same conditions (Fig 4G). Overall, these results show that nucleotide starvation induces an antigen
288 presentation gene expression program that is independent of canonical APP regulators but depends on
289 CDK9/P-TEFb.
290
291 **BQ suppresses tumor growth, induces MHC-I expression, and increases immunotherapy**
292 **efficacy in a syngeneic melanoma model**
293 Enforced MHC-I upregulation by various interventions can facilitate anticancer immunity and
294 enhance the efficacy of immune checkpoint blockade (ICB) by antibodies directed at PD-(L)1 and/or
295 CTLA-4 [24-27]. Moreover, high MHC-I expression has been proposed as a predictor of ICB response [28-31],
296 and high expression of MHC-I and other APP genes, including *NLRC5* and *TAP1*, correlates with better
297 survival in patients with melanoma (Fig S5A), for whom ICB is a first-line therapy. Therefore, we asked
298 if BQ could improve anticancer immunity in the B16F10 melanoma immunocompetent mouse model,
299 which is typically refractory to dual ICB (i.e., anti-PD-1 plus anti-CTLA-4) [54].
300 BQ (10 mg/kg daily IP injection) markedly suppressed tumor growth and led to reduced tumor
301 burden (Fig 5A-B). Historically, the lead tool compound that was ultimately modified to BQ (called NSC
302 339768) was prioritized in part based on its activity against B16 melanoma [55]; however, to our
303 knowledge, this is the first direct demonstration of BQ activity in this model system. Consistent with our
304 *in vitro* metabolomics data (Fig 1I-J, S1B), BQ treatment caused marked buildup of metabolites
305 upstream of DHODH and depletion of downstream pyrimidine nucleotide species in B16F10 tumors
306 (Fig 5C), confirming target engagement *in vivo*. Metabolomics analysis of BQ- and vehicle-treated
307 tumors separated in principal component analysis (Fig S5B) and unsupervised hierarchical clustering
308 (Fig S5C), confirming the perturbation of tumor metabolism following DHODH inhibition.
309 BQ-treated B16F10 tumors showed increased mRNA expression of MHC-I (*H2-Db* and *H2-Kb*)
310 and *Nlrc5* (Fig 5D). We thus addressed whether BQ could augment the efficacy of dual ICB (anti-CTLA-
311 4 plus anti-PD-1) with the knowledge that enforced MHC-I antigen presentation has also been shown to
312 boost the effect of ICB [24,26,27]. While BQ is not an approved medication, two FDA-approved low potency
313 DHODH inhibitors (leflunomide, teriflunomide) are effective in treating autoimmune conditions such as
314 rheumatoid arthritis and multiple sclerosis and act to decrease the activity of auto-reactive
315 T-lymphocytes [56-58]. It was possible that BQ treatment may actually impair the effectiveness of ICB by
316 inhibiting T-lymphocytes despite augmented cancer cell antigen presentation. We, therefore, tested
317 both concurrent, upfront administration of BQ plus dual ICB and sequential administration of BQ
318 followed by dual ICB (Fig S5D).
319 Similar to its impressive activity in our first experiment (Fig 5A-B), BQ monotherapy conferred
320 marked survival benefit. This was significantly enhanced by subsequent dual ICB, while dual ICB alone
321 conferred only marginally prolonged survival, and concurrent BQ plus dual ICB did not significantly
322 improve survival versus BQ monotherapy (Fig 5E). This suggests that sequential (rather than
323 concurrent) administration of DHODH inhibitor and ICB may be superior. Hypotheses that may explain
324 these findings include: a) Concurrent BQ dampens the initial anticancer immune response generated
325 by dual ICB, or b) cancer cell MHC-I and related genes are not maximally upregulated at the time of
326 ICB administration with concurrent treatment. Taken together, these results show that BQ causes
327 pyrimidine nucleotide depletion, MHC-I and APP gene transcriptional upregulation, and additive survival
328 benefit with dual ICB in a highly aggressive and ICB-refractory mouse melanoma model.
329
330 **Discussion**
331
332 Our results demonstrate that pyrimidine nucleotide depletion by DHODH inhibition causes
333 increased expression of APP genes and increased antigen presentation via MHC-I across a diverse
334 panel of cancer cell lines (Fig 1). This effect of BQ and teriflunomide is strictly dependent on pyrimidine
335 nucleotide depletion, as it was abrogated by restoration of pyrimidine levels with exogenous uridine (Fig
336 2B-D and Fig S2B-E). Furthermore, genetic deletion of *DHODH* recapitulated this effect, and treatment
337 of DHODH knockout cells with BQ did not further increase MHC-I mRNA expression (Fig 2E). Our
338 inhibitor reversal studies determined that BQ-mediated APP induction is independent of several
339 canonical APP regulatory pathways, including IFN-JAK-STAT, cGAS-STING-TBK1, and NF-κB (Fig 3

7

340 and Fig S3). We showed that this effect relies on P-TEFb-mediated release of Pol II from promoter-
341 proximal paused state to productive elongation (Fig 4). These findings were extended to inhibition of
342 IMPDH (which depletes cellular GTP) and CTPS (which depletes cellular CTP), as these effects were
343 also reversible with P-TEFb inhibition (Fig S4A). This suggests that pharmacologic depletion of these
344 nucleotide species also triggers APP upregulation in a P-TEFb-dependent manner.
345   Since T-cell recognition of antigens via MHC-I is required for T-cell-mediated elimination of
346 cancer cells or virus-infected cells, these results have important implications for the development of
347 nucleotide synthesis inhibitors as anticancer/antiviral therapies. We provide proof of concept evidence
348 that pretreatment with DHODH inhibitors can improve the efficacy of immune checkpoint blockade in a
349 highly aggressive and ICB-refractory mouse melanoma model (Fig 5 and Fig S5). Because BQ-
350 mediated APP induction does not require interferon signaling, this strategy may have particular
351 relevance for clinical scenarios in which tumor antigen presentation is dampened by the loss or
352 silencing of cancer cell interferon signaling, which has been demonstrated to confer both intrinsic [59] and
353 acquired [23] ICB resistance in human melanoma patients.
354   Emerging evidence suggests that cancer cell MHC-I expression predicts favorable response to
355 ICB, and several recent studies have shown that enforced cancer cell MHC-I expression enhances
356 anticancer immunity and ICB efficacy in various mouse models. Accordingly, functional genomic
357 screens for regulators of cancer cell MHC-I expression have recently been undertaken, and these
358 efforts have revealed novel molecular targets to induce cancer cell APP activity [27,60]. Agents shown to
359 increase cancer cell antigen presentation include hydroxychloroquine (by autophagy inhibition) [24],
360 poly(I:C) (by NF-κB activation downstream of dsRNA sensing) [26], SMAC mimetics (by NF-κB activation)
361 [27], CDK4/6 inhibitors (by activation of endogenous genomic retroviral elements) [25], and others. It is very
362 likely that many other anticancer drugs perturb cancer cell antigen presentation and/or have other
363 immunomodulatory properties in addition to their cell-intrinsic antiproliferative activity [61], and this area
364 requires further scrutiny. In this study, we identified DHODH inhibition as a powerful inducer of antigen
365 presentation and MHC-I expression in diverse cancer cell lines and in HEK-293T cells.
366   Previous studies have linked pyrimidine depletion with upregulation of innate immunity and
367 interferon-stimulated genes [33,34], and this was confirmed by our transcriptomic profiling experiments
368 (Fig 1B-C). Multiple mechanistic explanations for these observations have been suggested. Lucas-
369 Hourani et al. proposed that interferon-stimulated gene expression requires the DNA damage
370 checkpoint kinase ATM [33], while Sprenger et al. conclude that pyrimidine depletion causes
371 accumulation of mitochondrial DNA in the cytosol, which is sensed by the cGAS-STING-TBK1 pathway
372 to promote innate immunity [34]. In our models, neither ATM/ATR nor TBK1 inhibition blocked BQ-
373 mediated APP induction (Fig 4A). It is possible that pyrimidine nucleotide shortage leads to APP
374 induction by multiple redundant mechanisms, any of which may predominate based on the cellular
375 context. We speculate that cells may have evolved multiple means of sensing acute pyrimidine
376 shortage as a way to detect viral infection or malignant transformation, as both viral replication and
377 uncontrolled cell proliferation avidly consume nucleotides.
378   Our focused chemical screen for MHC-I inducers (Fig 2F) identified the approved IMPDH1/2
379 inhibitor mycophenolate, which was subsequently validated in multiple other cell types (Fig S2D, S4A).
380 This is consistent with a recent study in which IMPDH inhibition was shown to enhance ICB efficacy by
381 favorably altering the MHC-I peptide repertoire and increasing immunoproteasome expression [62].
382 However, in this study, the cancer cells were pretreated with IMPDH inhibitor before implantation into
383 syngeneic hosts, and so possible countervailing immunosuppression by systemic IMPDH inhibitor
384 treatment was not addressed [62]. Our *in vivo* results (Fig 5F) highlight the importance of timing/sequence
385 when administering immunotherapy in combination with nucleotide synthesis inhibitors and suggest that
386 upfront BQ followed by ICB may be superior to concurrent administration.
387   Thymidylate synthase inhibition was recently shown to induce MHC-I in a model of diffuse large
388 B cell lymphoma [60]. The failure of thymidylate synthase inhibitors 5-fluorouracil and methotrexate to
389 induce MHC-I in our screen (Fig 2F) may be attributable to cell line differences (PDAC vs DLBCL),
390 dose/duration considerations, or the use of different thymidylate synthase inhibitors than in their study
391 (which used pemetrexed and raltitrexed). Thus, it appears that the abundance of multiple nucleotide
392 species can exert context-dependent influence on MHC and APP gene expression, and key details of
393 this relationship remain to be elucidated.

394   Overall, our study establishes P-TEFb and Pol II elongation control as a mechanistic link
395 between nucleotide depletion and APP induction. We provide proof of concept evidence for
396 combinatorial benefit of DHODH inhibition and immune checkpoint blockade in an aggressive and
397 poorly immunogenic mouse model of melanoma. A deeper understanding of metabolic control of
398 antigen presentation will enable rational therapy development for cancer and viral infection.
399
400 **Materials and Methods**
401

402 **Cell culture and cell lines**
403 The S2-013 cell line is a clonal derivative of the Suit2 cell line and was a kind gift from the Tony
404 Hollingsworth laboratory at the University of Nebraska Medical Center. The MiaPaCa2 *IKK2*-KO and
405 parental wild-type MiaPaCa2 cell lines were a kind gift from the Amar Natarajan laboratory at the
406 University of Nebraska Medical Center. All other cell lines in this study were obtained from American
407 Type Culture Collection (Manassas, VA, USA). All human cell lines were authenticated by STR profiling
408 by the Genetics Core at the University of Arizona. Cells were routinely (at the time of initial revival from
409 liquid nitrogen storage and at least every 6 months) determined to be free of mycoplasma
410 contamination by PCR-based methods. Cells were cultured in Dulbecco's modified Eagle medium
411 (Sigma-Aldrich, St Louis, MO, USA) supplemented with 50 IU/mL penicillin, 50 μg/mL streptomycin,
412 and incubated at 37 °C in a humidified incubator with 5% CO_2. Cells were maintained at 10% fetal
413 bovine serum (FBS). Upon reaching 70–80% confluency, cells were passaged by washing with
414 phosphate-buffered saline (PBS) before adding 0.25% trypsin (Caisson Labs, Smithfield, UT, USA) and
415 plating at 25% confluency.
416

417 **Drug treatment of cultured cells for RT-qPCR and flow cytometry experiments**
418 Drug treatment dose and duration are indicated for each experiment. A table of manufacturer and
419 catalog number for each agent described can be found in Supplementary Table 1. For stimulation with
420 poly(dA:dT), 2μg of poly(dA:dT) and 2μl of Lipofectamine2000 (Invitrogen #11668027) were incubated
421 in 400μl Opti-MEM (Gibco #11058021) for 30 minutes at room temperature and then added to cells in
422 2ml final volume of complete media.
423

424 **Cell viability assays**
425 Cells were seeded in 96 well plates (1000 cells per well in 90μl media) and allowed to equilibrate
426 overnight. Cells were then treated with indicated compounds (final volume 100μl) for 72 hours, and
427 viability was assessed by CellTiter-Glo assay (Promega, Madison, WI). Luminescence values for each
428 condition were normalized to the average luminescence of the vehicle-treated control replicates.
429 Experiments were performed at least three times using biological triplicates for each condition. Dose-
430 response curves were fit to nonlinear regression models using Prism9 software.
431

432 **Liquid chromatography – tandem mass spectrometry-based metabolomics analysis**
433 For in vitro metabolomics experiments, 5×10^5 cells were seeded in 6-well plates and allowed to
434 equilibrate overnight. At the start of each assay, the cell culture media was changed, and fresh media
435 with desired conditions was added (to eliminate metabolite depletion from overnight equilibration as a
436 confounding variable). Following 8-hour treatment of cancer cell lines with BQ (or in the case of Fig 2B,
437 24-hour treatment with BQ +/- 100μM uridine), polar metabolites were extracted and quantified as
438 previously described [63]. For B16F10 tumor metabolomics, subcutaneous tumors were harvested at
439 necropsy and immediately snap frozen in liquid nitrogen and stored at -80 °C. Tumors were
440 subsequently ground into fine powder in liquid nitrogen using a mortar and pestle, and metabolites were
441 extracted using the same method as for cultured cells. Peak areas were normalized to the mass of
442 tumor tissue that was input.
443   Datasets were processed using Skyline (MacCoss Lab Software), and Metaboanalyst5.0 web
444 tool was used to generate principal component analysis and heatmap visualizations of resulting
445 datasets. Raw LC-MS/MS data and R-command history for Metaboanalyst analysis are available upon
446 request. Relative metabolite abundances were normalized to the average peak area of the

447 experimental control group and were compared using two-way ANOVA with Bonferonni's post-test
448 correction for multiple comparisons. P < 0.05 was considered significant.
449
450 **Mice studies**
451 All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the
452 University of Nebraska Medical Center. For tumor xenograft studies, 10^4 B16F10 cells in a 1:1 vol/vol
453 ratio (100µl final volume) with Matrigel were injected subcutaneously into the right flank of 10-week-old
454 female C57BL/6J mice (Jackson labs). Tumors of live mice were serially measured in two dimensions
455 using digital calipers, and tumor volume for Fig 5A was calculated as ($0.5L \times W^2$), where L is the long
456 dimension and W is the short dimension. For Fig 5B-C, tumors were harvested at necropsy, weighed on
457 an analytical balance (for Fig 5B), and measured in three perpendicular dimensions by calipers to
458 generate volume measurements for Fig 5C, which were calculated as (dimension 1 x dimension 2 x
459 dimension 3).
460   For survival experiments (Fig 5F), mice were monitored daily for signs of euthanasia criteria or
461 actual demise. When tumor volume reached $2000cm^3$ as determined by the above formula for live mice
462 ($0.5L \times W^2$), mice were sacrificed according to protocol euthanasia criteria.
463   Brequinar was obtained from Clear Creek Bio and dissolved in 0.9% NaCl. For both endpoint
464 and survival studies, BQ (10mg/kg) or vehicle solvent (0.9% NaCl) was injected intraperitoneally daily.
465 Anti-CTLA-4 and anti-PD-1 antibodies, as well as their respective isotype controls, were obtained from
466 BioXCell. Antibodies were dosed at 100µg/mouse IP twice per week.
467
468 **RNA sequencing and gene set enrichment analysis**
469 For RNA sequencing experiments, S2-013 or CFPAC-1 cells were treated with brequinar for the
470 indicated dose and duration (Fig 1 and S1). For two-week drug treatment experiments, cells were
471 passaged every 3 days and 5×10^5 cells were reseeded in a new 10cm tissue culture dish. RNA was
472 isolated using RNEasy Mini kit (Qiagen, catalog number 74104).
473   Samples were processed by BGI Genomics (San Jose, California, USA) according to their
474 proprietary method. Briefly, RNA quality check was performed using Agilent 2100 Bioanalyzer. Poly-A-
475 containing mRNA was isolated using magnetic beads and then fragmented using divalent cations under
476 elevated temperature. cDNA synthesis was performed using reverse transcriptase and RNase H.
477 Adapter sequences were then ligated onto cDNA fragments, purified, enriched by PCR, quantified by
478 Qubit, and pooled to generate the final library. Libraries were then sequenced using the BGI DNBseq
479 platform. Reads mapped to rRNA, low quality reads, and reads with adaptors were removed. The
480 resulting clean reads were mapped to the reference genome (hg19_UCSC_20180115) using HISAT2
481 program (http://www.ccb.jhu.edu/software/hisat/index.shtml) and converted to fragments for kilobase
482 per million mapped reads (FPKM).
483   Fold change FPKM (brequinar/vehicle control) values for all expressed genes (FPKM > 1) were
484 subjected to gene set enrichment analysis [36] with GSEA prerank using HALLMARK and KEGG genes
485 sets from the Molecular Signatures Database (MSigDB) as previously described [64]. Gene sets positive
486 enriched with FDR q < 0.25 are shown in Fig 1B.
487
488 **Real time quantitative PCR analysis for mRNA expression**
489 For in vitro RT-qPCR experiments, RNA was harvested using Trizol reagent (Thermo Fisher Scientific,
490 Waltham, MA, USA) according to manufacturer's instructions. For tumor RT-qPCR, tumors were
491 crushed with mortar and pestle in liquid nitrogen, and Trizol was used to extract RNA from the resulting
492 powder, just as for cultured cells. cDNA synthesis was performed (1µg RNA input) using BioRad
493 (Hercules, CA, USA) iScript cDNA synthesis kit (catalog number 1708891) according to manufacturer's
494 instructions. For RT-qPCR reactions, 3µl of diluted cDNA, 2µl of primer mix (diluted to a final
495 concentration of 200 nM for forward and reverse primers), and 5µl SYBR green master mix (cat #)
496 were mixed (10µl final volume), and reactions were analyzed using Applied Biosystems QuantStudio5
497 instrument with previously reported thermocycling parameters [65].
498   18S rRNA was used as a loading control to generate delta Ct values, and each sample was
499 normalized to the experimental control delta Ct values to generate delta delta Ct values which were
500 converted to fold change by (2^-ddCt). For all experiments, *ACTB* (beta-actin) mRNA expression was

501 quantified and used as an additional loading control, and results were concordant regardless of whether
502 18S or *ACTB* was used for normalization. For Fig 5E, each data point represents the average
503 expression value of 4 technical replicates for an individual tumor.
504   For pairwise comparisons, an unpaired student's t-test was used. For comparisons of 3 or more
505 conditions, a two-way ANOVA with Bonferonni's post-test correction for multiple comparisons was
506 used. P < 0.05 was considered significant. Primer sequences for RT-qPCR reactions are provided in
507 Supplementary Table 2.
508
509 **Flow cytometry measurement of cell surface MHC-I**
510 Cells were treated as described and then detached with Accutase (Sigma Aldrich #A6964), washed
511 twice with PBS, stained with fluorescent dye-conjugated antibodies against H2-Db (BioLegend
512 #111508) or HLA-A/B/C (BioLegend #311418, BioLegend #311406) for 30 minutes at 4 °C in PBS,
513 washed once more with PBS, and then resuspended in FACS buffer and subjected to flow cytometry
514 analysis for fluorescence intensity. Aqua live/dead dye (Invitrogen #L34957) or propidium iodide was
515 used to exclude dead cells from the analysis.
516
517 **Western blot**
518 Protein isolation from cultured cells and western blotting procedure were described previously [63]. CDK9
519 antibody was obtained from Cell Signaling Technology (catalog number 2316, clone C12F7), HSP70
520 antibody was obtained from Cell Signaling Technology (catalog number 4872), and beta-actin antibody
521 was obtained from Santa Cruz Biotechnology (catalog number sc-4778, clone C4). Blots were
522 incubated with primary antibody overnight at 4°C, washed, incubated with secondary antibody
523 conjugated with horseradish peroxidase for 45 min at room temperature, washed, developed with ECL
524 reagent and visualized by autoradiography using plain film.
525
526 **Procurement and analysis of previously published datasets**
527 All datasets reported by Tan and colleagues [38] were obtained from Gene Expression Omnibus,
528 accession numbers GSE68053 and GSE68039. Processed RNA sequencing data for human A375
529 melanoma cells treated with DMSO vehicle control (GSM1661518, GSM1661518), or teriflunomide
530 (25μM) for 12 hours (GSM1661510, GSM1661511), 24 hours (GSM1661512, GSM1661513), 48 hours
531 (GSM1661514, GSM1661515), or 72 hours (GSM1661516, GSM1661517) was downloaded as an
532 Excel file from GSE68039 (GSE6809_A375.FPKM.xls) and directly analyzed by manual inspection. The
533 two FPKM values for each experimental condition were averaged, and these average values were used
534 to calculate the fold change (teriflunomide/DMSO) values presented in Figure 1E and Figure 4G.
535   For chromatin immunoprecitation sequencing (ChIP-seq) datasets (used to generate Fig 4G),
536 Fastq files for human A375 melanoma cells treated for 48 hours with DMSO (GSM1661790) or
537 teriflunomide (GSM1661791) for were downloaded from GSE68053, trimmed of adapter sequences at
538 the 3'ends with trim_galore v0.6 (https://github.com/FelixKrueger/TrimGalore), and aligned to hg38
539 using Bowtie (v1.2.3) (ref = https://genomebiology.biomedcentral.com/articles/10.1186/gb-2009-10-3-
540 r25) with parameters --minins 18 --maxins 1000 --fr --best --allow-contain. Reads overlapping with the
541 longest transcript of each gene (Genecode v32 and
542 https://github.com/GeoffSCollins/PolTools/blob/master/PolTools/static/longest_transcript_with_downstr
543 eam_start_codon.txt) were counted with BEDtools intersect (v2.27.1). Library size correction factors
544 were calculated separately for the ChIP-seq datasets. The correction factor for a given ChIP-seq
545 sample was computed by dividing the number of mapped reads in that sample by the average number
546 of mapped reads across all ChIP samples (DMSO, A771726). After normalization, the total number of
547 read counts (now corrected for total number of mapped reads per sample) aligned to each gene of
548 interest were used to calculate fold change (teriflunomide/DMSO) in Pol II occupancy values presented
549 in Figure 4G.
550
551
552 **ACKNOWLEDGMENTS**

556
557
558   **Conflict of Interest Statement:** DBS is a co-founder and holds equity in Clear Creek Bio. The other
559   authors declare no competing interests.

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560

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728
729

Figure legends

Figure 1: Brequinar induces mRNA expression of antigen presentation pathway genes and upregulates cell surface MHC-I in diverse cancer cell lines. A) Schematic of RNA sequencing experiment for panels C-D, with de novo pyrimidine pathway shown to highlight the role of DHODH **B)** Normalized enrichment scores for gene sets commonly enriched (FDR q < 0.25) in S2-013 (500nM) and CFPAC-1 (250nM) cells following two-week BQ treatment, as assessed by gene set enrichment analysis. **C)** GSEA plots for indicated gene sets following two-week BQ treatment of CFPAC-1 (left) or S2-013 (right) cells at the indicated doses **D)** Heatmap showing log2 fold change mRNA expression of APP genes in CFPAC-1 cells treated with BQ for indicated dose and duration. **E)** Heatmap showing log2 fold change mRNA expression for APP genes in A375 melanoma cells treated with the DHODH inhibitor teriflunomide (25µM) for indicated durations, data extracted from [38]. **F)** RT-qPCR quantification of *HLA-A*, *HLA-B*, and *HLA-C* mRNA levels in cancer cell lines after 24-hour BQ treatment. Numbers represent fold change relative to vehicle control for each cell line. Data are representative of at least 3 independent experiments. *HLA-B* was not detectable in MiaPaCa2 cells. **G-H)** Flow cytometry analysis of cell surface MHC-I in live CFPAC-1 (G) or B16F10 (H) cells following 10-day treatment with BQ (250nM for CFPAC-1 and 10µM for B16F10). **I-J)** LC-MS/MS metabolomics quantification of de novo pyrimidine pathway metabolites in CFPAC-1 (I) or B16F10 (J) cells following 8-hour BQ treatment at indicated doses. Data represent mean +/- SEM of four (CFPAC-1) or six (B16F10) biological replicates. # indicates p < 0.0001 by two-way ANOVA with Bonferonni's post-comparison test.

Figure S1: BQ treatment upregulates APP genes and depletes pyrimidine nucleotides. A) Heatmap showing log2 fold change mRNA expression of APP genes in S2-013 cells treated with BQ for indicated dose and duration. **B-C)** RT-qPCR quantification of APP genes after two-week BQ treatment of CFPAC-1 (250nM) (B) or B16F10 (10µM) (C) cells. **D-E)** Quantification of pyrimidine metabolites in CFPAC-1 (C) or B16F10 (D) cells treated with BQ for 8 hours at indicated doses. Data represent mean +/- SEM of four (CFPAC-1) or six (B16F10) biological replicates. * indicates p < 0.05, ** p < 0.01, *** p < 0.001, and # p < 0.0001 by unpaired t-test (B, C) or two-way ANOVA with Bonferonni's post-comparison test (D, E).

Figure 2: BQ-mediated APP induction requires pyrimidine nucleotide depletion. A) Dose-response cell viability experiment in B16F10 cells treated with BQ +/- uridine (100µM) for 72 hours. Data represent mean +/- SEM of three biological replicates. One representative result of three independent experiments is shown. **B)** Quantification of pyrimidine metabolites following 24-hour treatment of B16F10 cells with vehicle, BQ (10µM), or BQ + uridine (1mM). Data represent mean +/- SEM of six biological replicates. # indicates p < 0.0001 by two-way ANOVA with Bonferonni post-comparison test. **C)** RT-qPCR of indicated genes in B16F10 cells following 24-hour treatment with BQ (10µM) +/- uridine (1mM). Data represent mean +/- SD of three technical replicates. One representative result of three independent experiments is shown. **D)** Left: flow cytometry analysis of cell surface MHC-I (H2-Db) on live B16F10 cells following 24-hour treatment with indicated agents (BQ 10µM, teriflunomide 100µM, uridine 1mM). Right: quantification of H2-Db mean fluorescence intensity normalized to vehicle control. Data represent mean +/- SEM of three independent experiments. # indicates p < 0.0001 with two-way ANOVA with Bonferroni post-comparison test. **E)** RT-qPCR analysis of indicated genes in S2-013 cells with DHODH knockout (sgDHODH) or non-targeting control vector (sgNT) treated with indicated agents for 72 hours. Data represent mean +/- SD of four determinations. One representative result of three independent experiments is shown. **F)** RT-qPCR analysis of indicated genes in CFPAC-1 cells following 72-hour treatment with indicated agents. Numbers in the heatmap represent mean fold change versus vehicle control with four determinations.

Figure S2: Uridine rescues B16F10 cells from teriflunomide toxicity but does not alter APP expression by itself. A) Dose-response cell viability experiment as in Fig 2A but with teriflunomide (Ter) instead of BQ. **B)** RT-qPCR analysis of indicated genes following treatment with teriflunomide +/- uridine (1mM) for 24 hours. Data represent mean +/- SEM of three determinations. One representative result of three independent experiments is shown. **C)** RT-qPCR analysis of indicated genes after

17

784   treatment with vehicle or uridine (1mM) for 24 hours. Data represent mean +/- SEM of four
785   determinations. One representative result of three independent experiments is shown. **D)** RT-qPCR
786   analysis of indicated genes following treatment with BQ (10µM) +/- uridine (1mM) for 24 hours. Data
787   represent mean +/- SD of three independent experiments. **E)** Flow cytometry analysis of cell surface
788   MHC-I (H2-Db) following 24-hour uridine (1mM) treatment. Data represent mean +/- SEM of three
789   independent experiments. **F)** RT-qPCR analysis of indicated genes in B16F10 cells following 24-hour
790   treatment with MPA (5µM). Data represent mean +/- SEM of four determinations. One representative
791   result of three independent experiments is shown.

793   **Figure 3: IKK2 inhibitor BMS-345541 abrogates BQ-mediated APP induction in an IKK2-**
794   **independent manner. A-B)** HEK-293T cells were treated with indicated agents for 24 hours and then
795   subjected to RT-qPCR analysis for indicated genes. Numbers in the heatmap represent mean of four
796   determinations. **C-E, G)** B16F10 (C), CFPAC-1 (D), HCT116 (E) or MiaPaCa2-IKK2-KO (G) cells were
797   treated with indicated agents for 24 hours and subjected to RT-qPCR analysis of indicated genes. Data
798   in D,E, and G represent mean +/- SD of three independent experiments. * indicates $p < 0.05$, ** $p <$
799   0.01, *** $p < 0.001$, and # $p < 0.0001$ with two-way ANOVA with Bonferroni post-comparison test. For
800   C, numbers in the heatmap represent mean fold change versus vehicle with three determinations.
801   Representative result of three independent experiments is shown**. F)** Flow cytometry analysis of cell
802   surface MHC-I in HCT116 cells treated with indicated agents for 24 hours.

804   **Figure S3: A)** RT-qPCR time course analysis for MHC-I genes in HEK-293T cells treated for indicated
805   times with BQ (10µM). Data represent mean +/- SD of four determinations. **B)** RT-qPCR analysis for
806   *Nlrc5* (left) or *Tap1* (right) in B16F10 cells treated for 24 hours with indicated agents. Data represent
807   mean +/- SD of four determinations. # indicates $p < 0.0001$ with two-way ANOVA with Bonferroni post-
808   comparison test. Representative results for one of three independent experiments are shown. **C)**
809   Heatmap indicating RT-qPCR analysis for indicated genes in wild-type or *IKK2*-KO MiaPaCa2 cells
810   treated with indicated agents for 24 hours. Numbers in the heatmap represent mean of four
811   determinations.
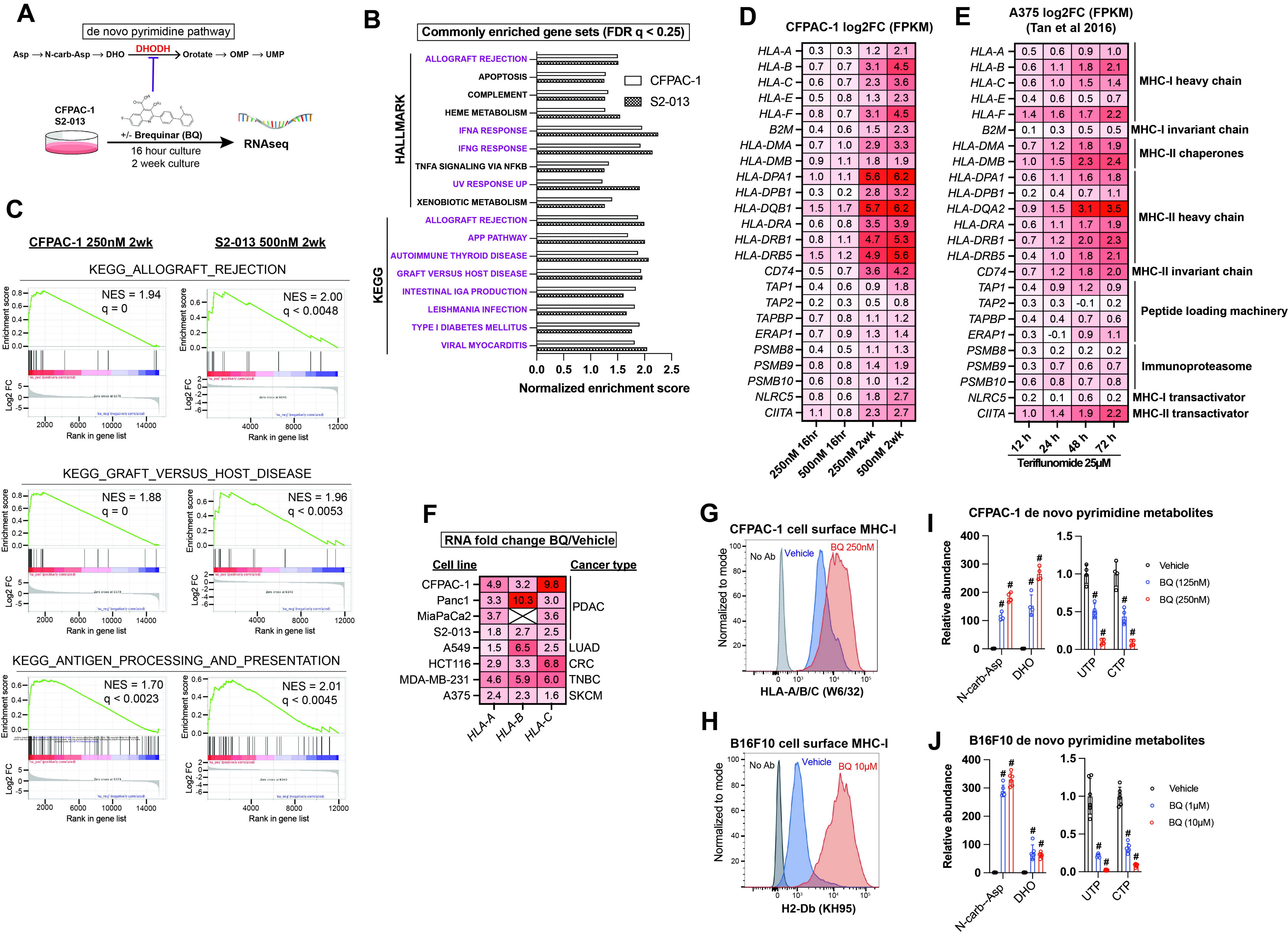
813   **Figure 4: P-TEFb inhibitor flavopiridol abrogates APP induction downstream of nucleotide**
814   **depletion. A)** Plot of percent inhibition (10µM treatment) vs -log(dissociation constant) for kinases
815   bound by BMS-345541 in KINOMEscan assays [47]. Each data point represents an individual kinase. **B-**
816   **C)** RT-qPCR analysis for indicated genes in HEK-293T cells treated with indicated agents for 24 hours.
817   Data represent mean +/- SD of three independent experiments. * indicates $p < 0.05$, ** indicates $p <$
818   0.01, *** indicates $p < 0.001$, and # indicates $p < 0.0001$ by two-way ANOVA with Bonferonni's post-
819   comparison test **D)** Western blot analysis for CDK9 performed on HEK-293T cells treated with CDK9-
820   targeted PROTAC (PROTAC2) and/or pomalidomide (POM) for 24 hours. Beta actin was used as a
821   loading control. **E)** RT-qPCR analysis of indicated genes after 24-hour treatment with indicated agents.
822   Data represent mean +/- SD of three independent experiments. **F)** Western blot analysis for CDK9
823   performed on HCT116 cells treated with the indicated concentrations of PROTAC2 for 24 hours. Heat
824   shock protein 70 (HSP70) was used as a loading control **G)** Linear regression analysis of fold change
825   (teriflunomide/DMSO) in Pol II occupancy (assessed by ChIP-seq) versus fold change
826   (teriflunomide/DMSO) in mRNA abundance (assessed by RNAseq) following 48-hour treatment of A375
827   cells with teriflunomide or DMSO vehicle control; data derived from [38].
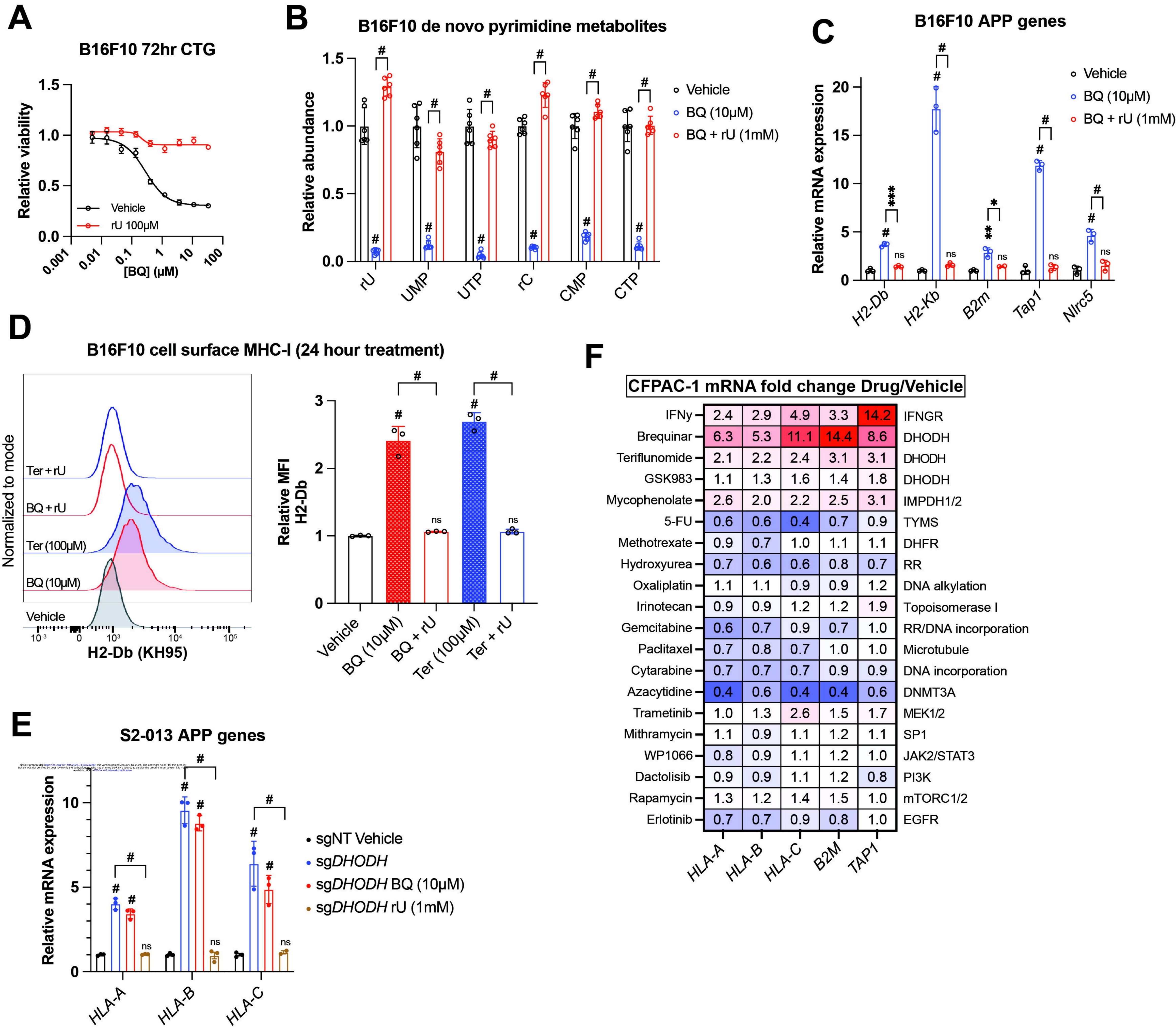
829   **Figure S4: A)** RT-qPCR analysis for indicated genes in HCT116 cells treated with indicated agents in
830   the presence or absence of flavopiridol (1µM). Numbers in the heatmap represent mean of three
831   determinations.
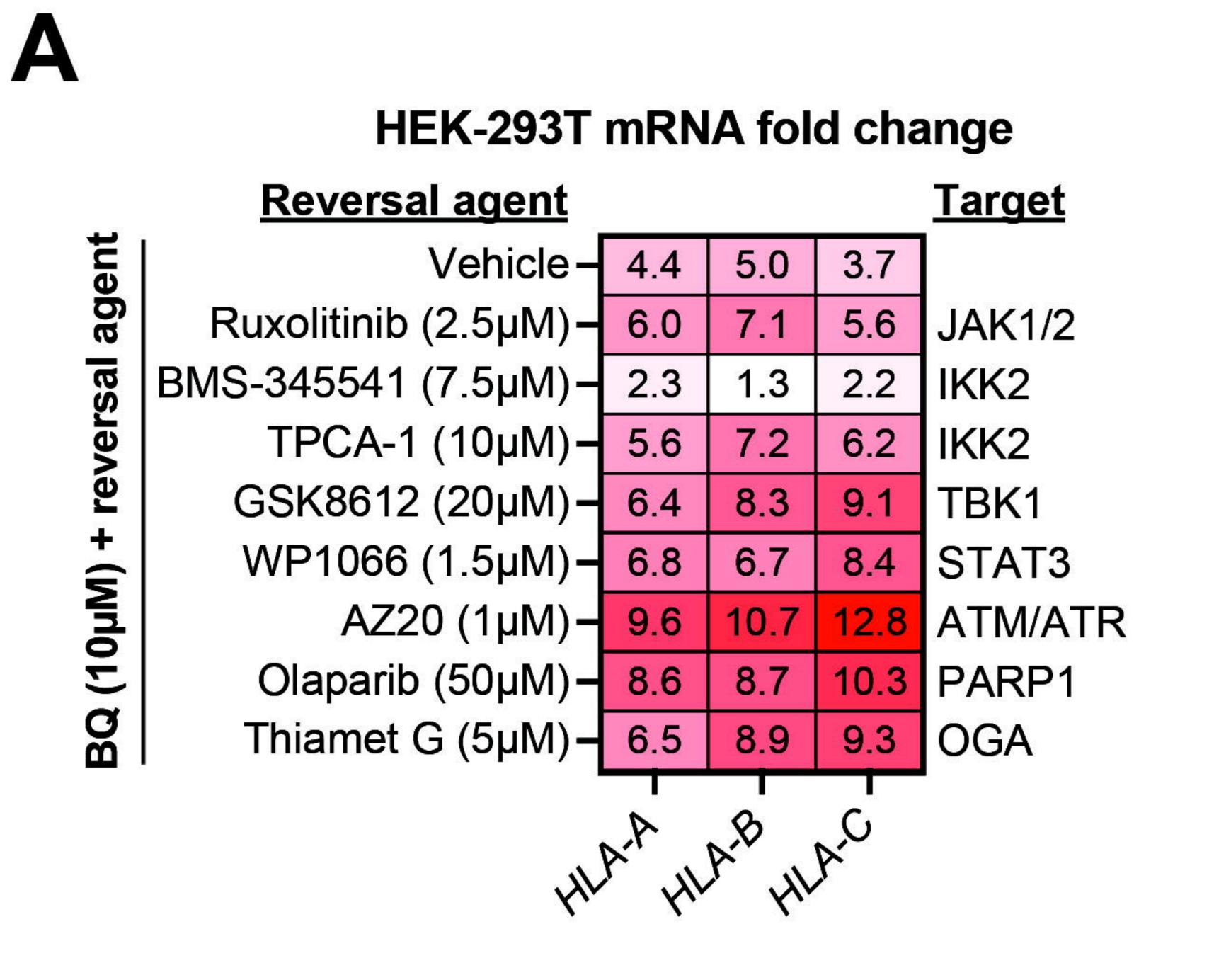
833   **Figure 5: BQ inhibits tumor growth, increases tumor MHC-I, and enhances immune checkpoint**
834   **blockade efficacy in B16F10 murine melanoma model. A-D)** B16F10 cells were injected
835   subcutaneously into syngeneic hosts and mice were treated with brequinar (BQ; 10mg/kg IP daily) or
836   vehicle control starting at day 7 post implantation. **A)** Longitudinal measurement of B16F10
837   subcutaneous tumors with BQ (10mg/kg IP daily) or vehicle treatment. **B)** Weight (left) and volume
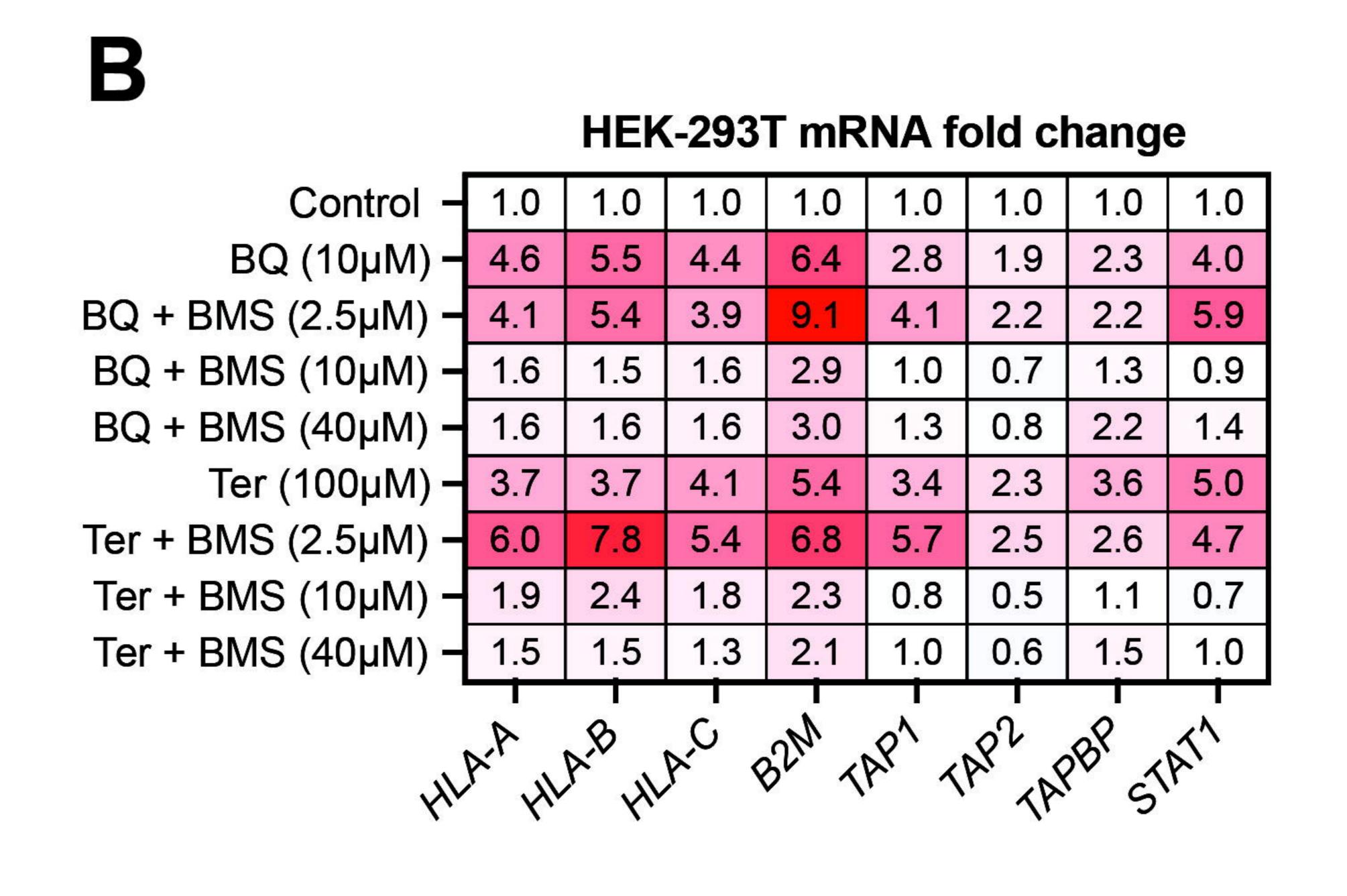
838   (right) of tumors at necropsy. **C)** Quantification of metabolites from B16F10 tumors harvested at
839   necropsy. **D)** RT-qPCR analysis of APP genes from tumors harvested at necropsy. For (A-D), data
840   represent mean +/- SD of n = 5 mice per group (n = 4 for BQ group in C). * indicates p < 0.05, **
841   indicates p < 0.01, *** indicates p < 0.001, and # indicates p < 0.0001 by unpaired t-test. **E)** Kaplan-
842   Meier survival analysis for mice implanted with B16F10 tumors as in (A-D) and treated with indicated
843   regimens; see Fig S5D for treatment timeline. * indicates p < 0.05, # p < 0.0001 by Mantel-Cox logrank
844   test. For vehicle, immune checkpoint blockade (ICB; Anti-CTLA-4 and anti-PD-1; 100µg/mouse each,
845   IP twice per week), and BQ (10mg/kg IP daily) + concurrent ICB, n = 15. For BQ, n = 7. For BQ +
846   delayed ICB, n = 8.
847
848   **Figure S5: A)** Overall survival in melanoma patients (SKCM) from the Cancer Genome Atlas (TCGA)
849   with above (indicated in red) and below median (indicated in blue) mRNA expression of indicated
850   genes. **B-C)** Principal component analysis (PCA) plot (B) and unsupervised hierarchical clustering (C)
851   from metabolomics analysis of B16F10 tumors at necropsy (Fig 5C). **D)** Treatment regimen of mice
852   from Fig 5E.
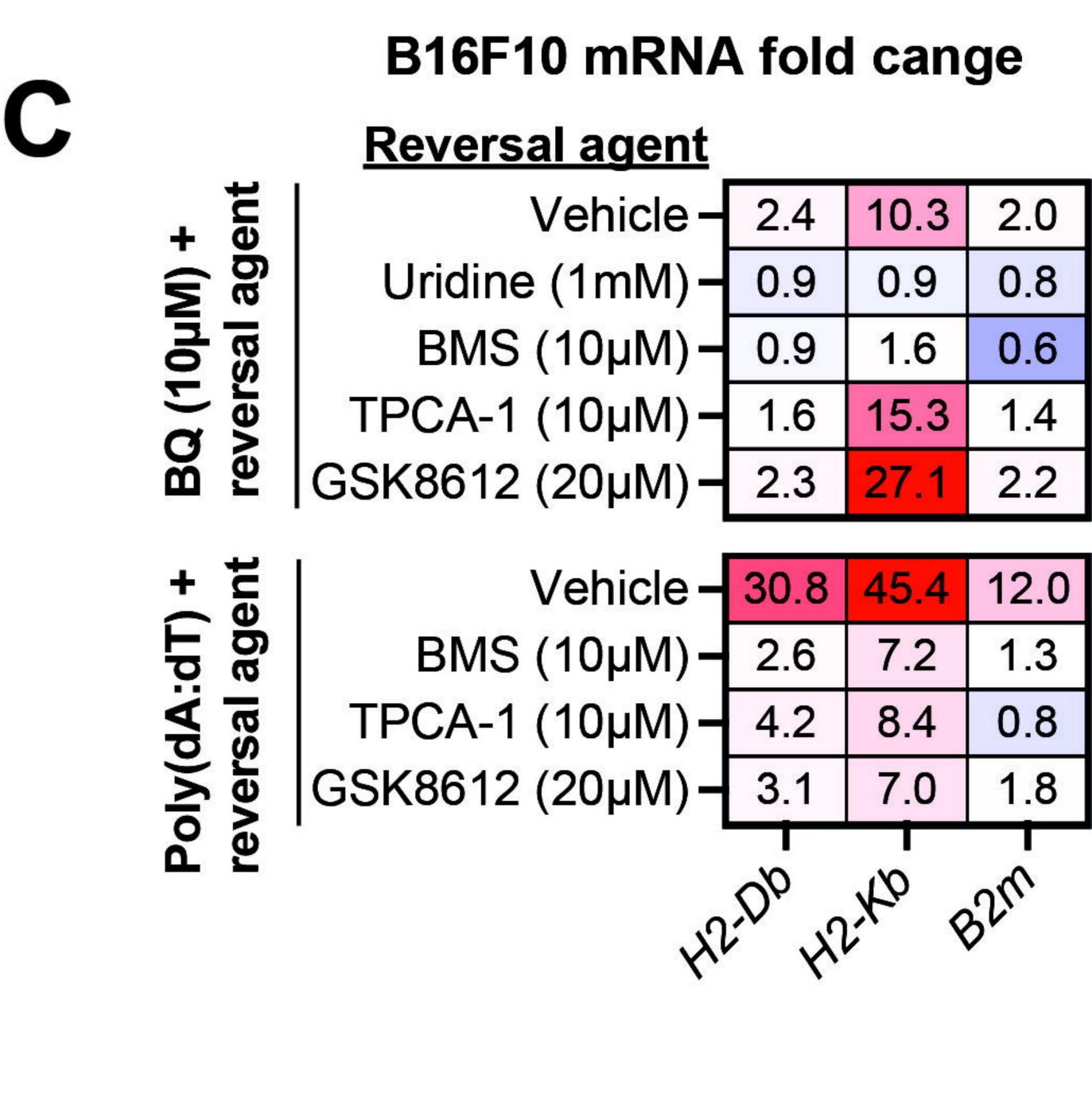
A

de novo pyrimidine pathway

Asp → N-carb-Asp → DHO →[DHODH]→ Orotate → OMP → UMP

CFPAC-1
S2-013

+/- Brequinar (BQ)
16 hour culture
2 week culture
→ RNAseq

B

Commonly enriched gene sets (FDR q < 0.25)

□ CFPAC-1
▨ S2-013

HALLMARK:
- ALLOGRAFT REJECTION
- APOPTOSIS
- COMPLEMENT
- HEME METABOLISM
- IFNA RESPONSE
- IFNG RESPONSE
- TNFA SIGNALING VIA NFKB
- UV RESPONSE UP
- XENOBIOTIC METABOLISM

KEGG:
- ALLOGRAFT REJECTION
- APP PATHWAY
- AUTOIMMUNE THYROID DISEASE
- GRAFT VERSUS HOST DISEASE
- INTESTINAL IGA PRODUCTION
- LEISHMANIA INFECTION
- TYPE I DIABETES MELLITUS
- VIRAL MYOCARDITIS

Normalized enrichment score

C

CFPAC-1 250nM 2wk | S2-013 500nM 2wk

KEGG_ALLOGRAFT_REJECTION
- NES = 1.94, q = 0
- NES = 2.00, q < 0.0048

KEGG_GRAFT_VERSUS_HOST_DISEASE
- NES = 1.88, q = 0
- NES = 1.96, q < 0.0053

KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION
- NES = 1.70, q < 0.0023
- NES = 2.01, q < 0.0045

D

CFPAC-1 log2FC (FPKM)

Gene	250nM 16hr	500nM 16hr	250nM 2wk	500nM 2wk
HLA-A	0.3	0.3	1.2	2.1
HLA-B	0.7	0.7	3.1	4.5
HLA-C	0.6	0.7	2.3	3.6
HLA-E	0.5	0.7	1.3	2.1
HLA-F	0.8	0.7	3.1	4.5
B2M	0.4	0.6	1.5	2.3
HLA-DMA	0.7	1.0	2.9	3.3
HLA-DMB	0.9	1.1	1.8	1.9
HLA-DPA1	1.0	1.1	5.6	6.2
HLA-DPB1	0.3	0.2	2.8	3.2
HLA-DQB1	1.5	1.7	5.7	6.2
HLA-DRA	0.6	0.8	3.5	3.9
HLA-DRB1	0.8	1.1	4.7	5.3
HLA-DRB5	1.5	1.2	4.9	5.6
CD74	0.5	0.7	3.6	4.2
TAP1	0.4	0.6	0.9	1.8
TAP2	0.2	0.3	0.5	0.8
TAPBP	0.7	0.8	1.1	1.2
ERAP1	0.7	0.9	1.3	1.4
PSMB8	0.4	0.5	1.1	1.3
PSMB9	0.8	0.8	1.4	1.9
PSMB10	0.6	0.8	1.0	1.2
NLRC5	0.8	0.6	1.8	2.7
CIITA	1.1	0.8	2.3	2.7

E

A375 log2FC (FPKM) (Tan et al 2016)

Gene	12 h	24 h	48 h	72 h	
HLA-A	0.5	0.6	0.9	1.0	MHC-I heavy chain
HLA-B	0.6	1.1	1.8	2.1	
HLA-C	0.6	1.0	1.5	1.4	
HLA-E	0.4	0.6	0.5	0.7	
HLA-F	1.4	1.6	1.7	2.2	
B2M	0.1	0.3	0.5	0.5	MHC-I invariant chain
HLA-DMA	0.7	1.2	1.8	1.9	MHC-II chaperones
HLA-DMB	1.0	1.5	2.3	2.4	
HLA-DPA1	0.6	1.1	1.6	1.8	
HLA-DPB1	0.4	0.4	0.7	1.1	
HLA-DQA2	0.9	1.5	3.1	3.5	MHC-II heavy chain
HLA-DRA	0.6	1.1	1.7	1.9	
HLA-DRB1	0.7	1.2	2.0	2.3	
HLA-DRB5	0.6	1.0	1.8	2.1	
CD74	1.2	1.8	2.0		MHC-II invariant chain
TAP1	0.4	0.9	1.2	0.9	Peptide loading machinery
TAP2	0.3	0.3	-0.1	0.2	
TAPBP	0.4	0.4	0.7	0.6	
ERAP1	0.3	-0.1	0.9	1.1	
PSMB8	0.3	0.2	0.2	0.2	Immunoproteasome
PSMB9	0.3	0.7	0.6	0.7	
PSMB10	0.6	0.8	0.7	0.8	
NLRC5	0.2	0.1	0.6	0.2	MHC-I transactivator
CIITA	1.0	1.4	1.9	2.2	MHC-II transactivator

Teriflunomide 25μM

F

RNA fold change BQ/Vehicle

Cell line	HLA-A	HLA-B	HLA-C	Cancer type
CFPAC-1	4.9	3.2	9.8	
Panc1	3.3	10.3	3.0	PDAC
MiaPaCa2	3.7	✕	3.6	
S2-013	1.8	2.7	2.5	
A549	1.5	6.5	2.5	LUAD
HCT116	2.9	3.3	6.8	CRC
MDA-MB-231	4.6	5.9	6.0	TNBC
A375	2.4	2.3	1.6	SKCM

G

CFPAC-1 cell surface MHC-I

No Ab | Vehicle | BQ 250nM

HLA-A/B/C (W6/32)

H

B16F10 cell surface MHC-I

No Ab | Vehicle | BQ 10μM

H2-Db (KH95)

I

CFPAC-1 de novo pyrimidine metabolites

○ Vehicle
○ BQ (125nM)
○ BQ (250nM)

N-carb-Asp, DHO, UTP, CTP
(significance marks)

J

B16F10 de novo pyrimidine metabolites

○ Vehicle
○ BQ (1μM)
○ BQ (10μM)

N-carb-Asp, DHO, UTP, CTP
(significance marks)

A B16F10 72hr CTG

B B16F10 de novo pyrimidine metabolites

C B16F10 APP genes

D B16F10 cell surface MHC-I (24 hour treatment)

E S2-013 APP genes

F CFPAC-1 mRNA fold change Drug/Vehicle

	HLA-A	HLA-B	HLA-C	B2M	TAP1	
IFNγ	2.4	2.9	4.9	3.3	14.2	IFNGR
Brequinar	6.3	5.3	11.1	14.4	8.6	DHODH
Teriflunomide	2.1	2.2	2.4	3.1	3.1	DHODH
GSK983	1.1	1.3	1.6	1.4	1.8	DHODH
Mycophenolate	2.6	2.0	2.2	2.5	3.1	IMPDH1/2
5-FU	0.6	0.6	0.4	0.7	0.9	TYMS
Methotrexate	0.9	0.7	1.0	1.1	1.1	DHFR
Hydroxyurea	0.7	0.6	0.6	0.8	0.7	RR
Oxaliplatin	1.1	1.1	0.9	0.9	1.2	DNA alkylation
Irinotecan	0.9	0.9	1.2	1.2	1.9	Topoisomerase I
Gemcitabine	0.6	0.7	0.9	1.0	1.0	RR/DNA incorporation
Paclitaxel	0.7	0.8	0.7	1.0	1.0	Microtubule
Cytarabine	0.7	0.7	0.7	0.9	0.9	DNA incorporation
Azacytidine	0.4	0.6	0.4	0.4	0.6	DNMT3A
Trametinib	1.0	1.3	2.6	1.5	1.7	MEK1/2
Mithramycin	1.1	0.9	1.1	1.2	1.1	SP1
WP1066	0.8	0.9	1.1	1.2	1.0	JAK2/STAT3
Dactolisib	0.9	0.9	1.1	1.2	0.8	PI3K
Rapamycin	1.3	1.2	1.4	1.5	1.0	mTORC1/2
Erlotinib	0.7	0.7	0.9	0.8	1.0	EGFR

A — HEK-293T mRNA fold change

BQ (10µM) + reversal agent

Reversal agent	HLA-A	HLA-B	HLA-C	Target
Vehicle	4.4	5.0	3.7	
Ruxolitinib (2.5µM)	6.0	7.1	5.6	JAK1/2
BMS-345541 (7.5µM)	2.3	1.3	2.2	IKK2
TPCA-1 (10µM)	5.6	7.2	6.2	IKK2
GSK8612 (20µM)	6.4	8.3	9.1	TBK1
WP1066 (1.5µM)	6.8	6.7	8.4	STAT3
AZ20 (1µM)	9.6	10.7	12.8	ATM/ATR
Olaparib (50µM)	8.6	8.7	10.3	PARP1
Thiamet G (5µM)	6.5	8.9	9.3	OGA

B — HEK-293T mRNA fold change

	HLA-A	HLA-B	HLA-C	B2M	TAP1	TAP2	TAPBP	STAT1
Control	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
BQ (10µM)	4.6	5.5	4.4	6.4	2.8	1.9	2.3	4.0
BQ + BMS (2.5µM)	4.1	5.4	3.9	9.1	4.1	2.2	2.2	5.9
BQ + BMS (10µM)	1.6	1.5	1.6	2.9	1.0	0.7	1.3	0.9
BQ + BMS (40µM)	1.6	1.6	1.6	3.0	1.3	0.7	2.2	1.4
Ter (100µM)	3.7	3.7	4.1	5.4	3.4	2.3	3.6	5.0
Ter + BMS (2.5µM)	6.0	7.8	5.4	6.8	5.7	2.5	2.6	4.7
Ter + BMS (10µM)	1.9	2.4	1.8	2.3	0.8	0.5	1.1	0.7
Ter + BMS (40µM)	1.5	1.5	1.3	2.1	1.0	0.6	1.5	1.0

C — B16F10 mRNA fold cange

Reversal agent

BQ (10µM) + reversal agent

	H2-Db	H2-Kb	B2m
Vehicle	2.4	10.3	2.0
Uridine (1mM)	0.9	0.9	0.8
BMS (10µM)	0.9	1.6	0.6
TPCA-1 (10µM)	1.6	15.3	1.4
GSK8612 (20µM)	2.3	27.1	2.2

Poly(dA:dT) + reversal agent

	H2-Db	H2-Kb	B2m
Vehicle	30.8	45.4	12.0
BMS (10µM)	2.6	7.2	1.3
TPCA-1 (10µM)	4.2	8.4	0.8
GSK8612 (20µM)	3.1	7.0	1.8

D — CFPAC-1 APP genes

Relative mRNA expression

Legend: Vehicle; BQ (10µM); BQ + BMS (10µM)

Genes: HLA-A, HLA-B, HLA-C, B2M, TAP1, TAP2, TAPBP, CCL5

E — HCT116 APP genes

Relative mRNA expression

Legend: Vehicle; BQ (10µM); BQ + BMS (10µM)

Genes: HLA-A, HLA-B, HLA-C, B2M, TAP1, TAP2

F — HCT116 cell surface MHC-I

Normalized to mode

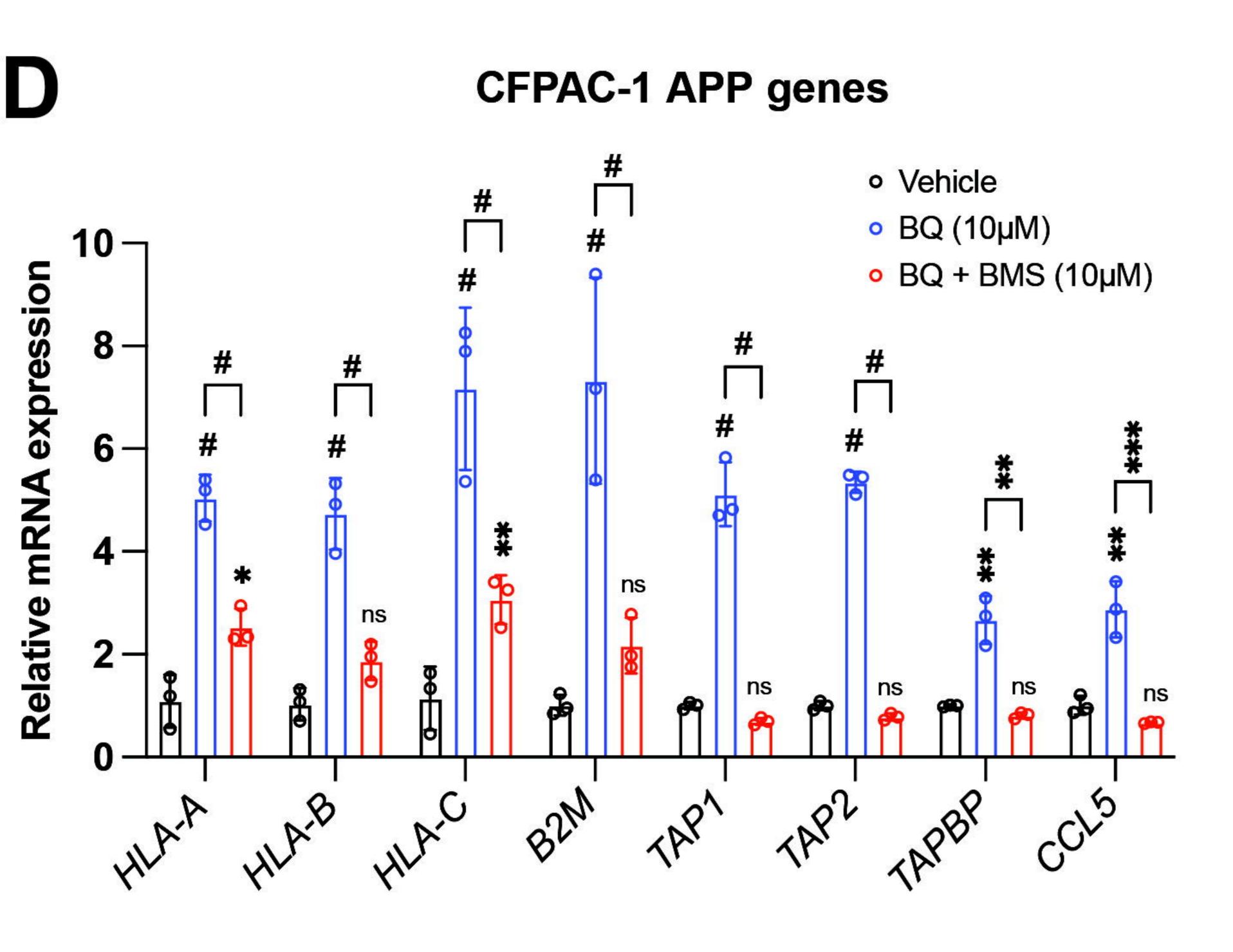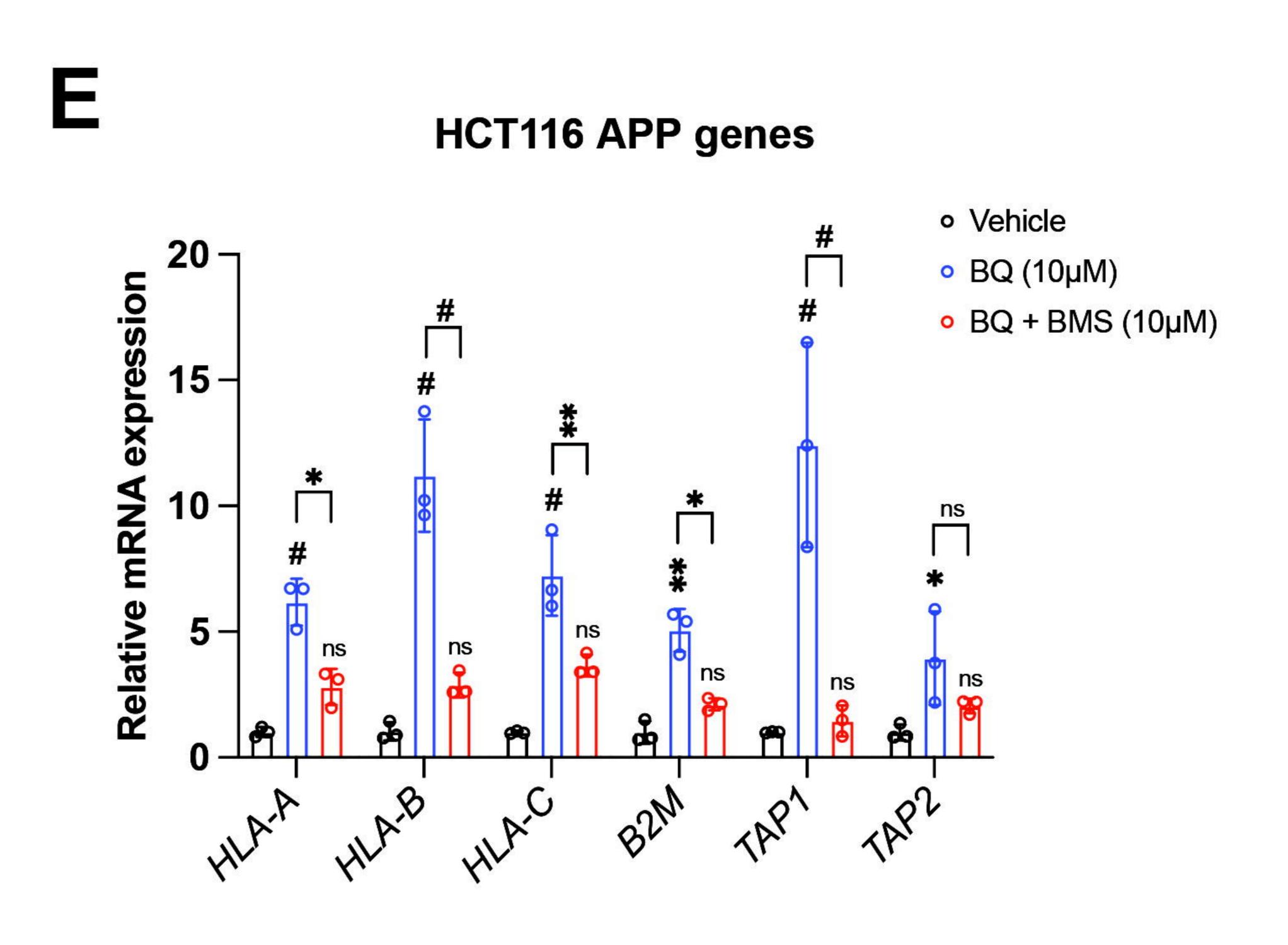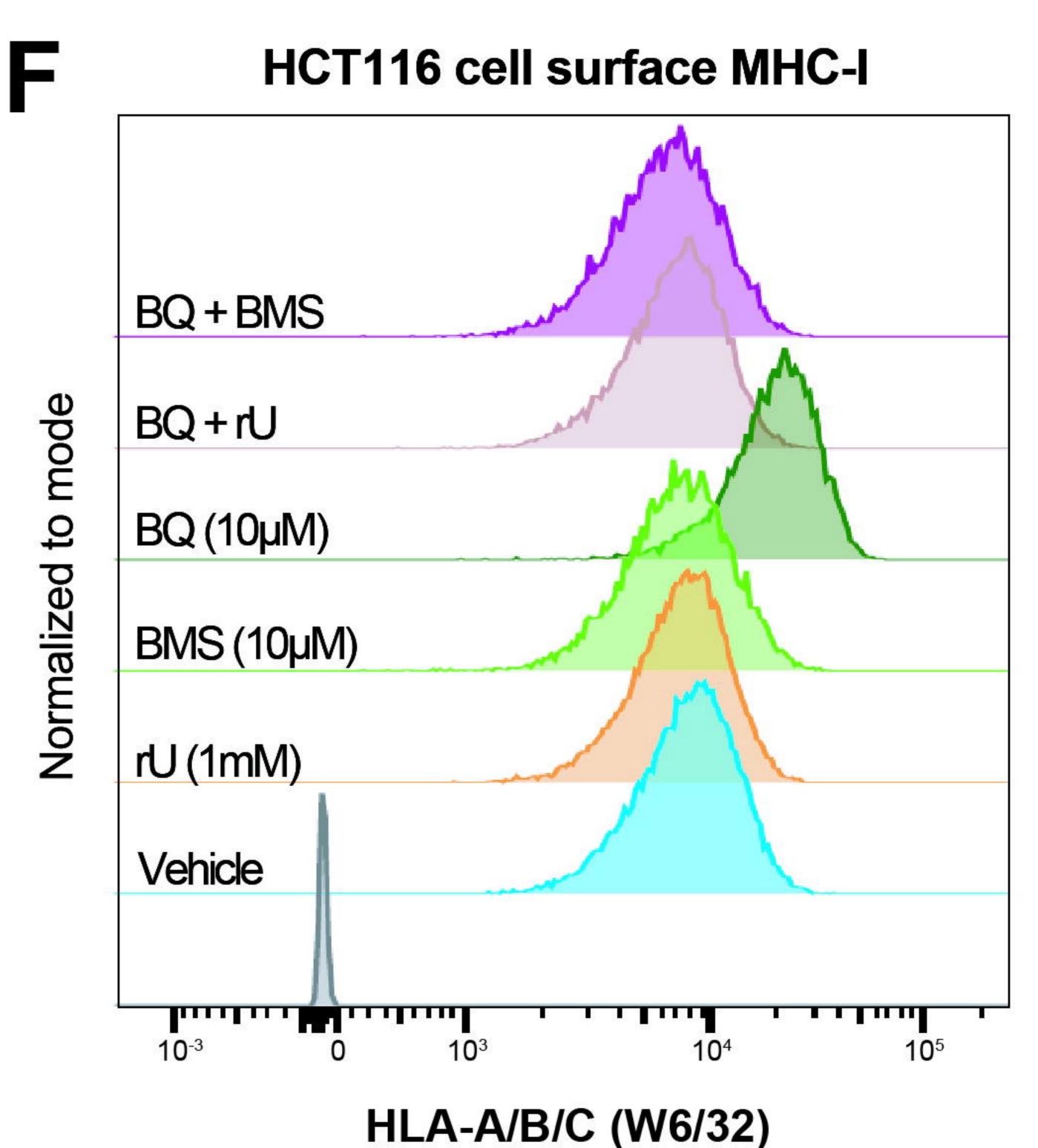Conditions (top to bottom): BQ + BMS; BQ + rU; BQ (10µM); BMS (10µM); rU (1mM); Vehicle

HLA-A/B/C (W6/32)

G — MiaPaCa2-IKK2-KO APP genes

Relative mRNA expression

Legend: Vehicle; BQ (10µM); BQ + BMS (10µM)

Genes: HLA-A, HLA-C, B2M, TAP1, NLRC5

A BMS-345541 KINOMEscan

○ Not inhibited by flavopiridol
○ Ruxolitinib Kd < 500 nM
○ Not expressed in CFPAC-1 cells
○ Ruled out experimentally

B HEK-293T

- Vehicle
- BQ (10μM)
- BQ + Flavopiridol (1μM)
- BQ + AT7519 (1μM)
- BQ + Dinaciclib (1μM)
- BQ + PROTAC2 (1μM)
- BQ + PROTAC2 (10μM)

C HEK-293T

- Vehicle
- BQ (10μM)
- BQ + PROTAC2 (1μM)
- BQ + PROTAC2 (1μM) + POM (10μM)
- BQ + PROTAC2 (10uM) + POM (10μM)

D HEK-293T

IB: CDK9
IB: ß-Actin

E HCT116

- Vehicle
- BQ (10uM)
- BQ + Flavo (1uM)
- BQ + AT7519 (1uM)
- BQ + Dinaciclib (1uM)
- BQ + PROTAC2 (1uM)
- BQ + PROTAC2 (10uM)

F HCT116

IB: CDK9
IB: HSP70

G A375 48hr (Tan et al, 2016)

$R^2 = 0.3891$
$P = 0.00015$

A B16F10 subcutaneous

B B16F10 tumor measurements at endpoint

C B16F10 tumor de novo pyrmidine metobolites

D B16F10 tumor APP genes

E B16F10 subcutaneous implantation