HHS Public Access

Author manuscript

Clin Cancer Res. Author manuscript; available in PMC 2018 April 15.

Published in final edited form as:

Clin Cancer Res. 2017 April 15; 23(8): 2027–2037. doi:10.1158/1078-0432.CCR-16-0453.

The MAPK pathway regulates intrinsic resistance to BET inhibitors in colorectal cancer

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Abstract

Purpose—The bromodomain and extra-terminal domain (BET) family proteins are epigenetic readers for acetylated histone marks. Emerging BET bromodomain inhibitors have exhibited antineoplastic activities in a wide range of human cancers through suppression of oncogenic transcription factors, including MYC. However, the preclinical activities of BET inhibitors in advanced solid cancers are moderate at best. To improve BET-targeted therapy, we interrogated mechanisms mediating resistance to BET inhibitors in colorectal cancer (CRC).

Experimental Design—Using a panel of molecularly defined CRC cell lines, we examined the impact of BET inhibition on cellular proliferation and survival as well as MYC activity. We further tested the ability of inhibitors targeting the RAF/MEK/ERK (MAPK) pathway to enhance MYC suppression and circumvent intrinsic resistance to BET inhibitors. Key findings were validated using genetic approaches.

Results—BET inhibitors as monotherapy moderately reduced CRC cell proliferation and MYC expression. Blockade of the MAPK pathway synergistically sensitized CRC cells to BET inhibitors, leading to potent apoptosis and MYC downregulation *in vitro* and *in vivo*. A

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Conflict of interest: The authors have declared that no conflict of interest exists.

combination of JQ1 and trametinib, but neither agent alone, induced significant regression of subcutaneous CRC xenografts.

Conclusions—Our findings suggest that the MAPK pathway confers intrinsic resistance to BET inhibitors in CRC and propose an effective combination strategy for the treatment of CRC.

Keywords

BET bromodomain protein; colorectal cancer; RAF; MEK; ERK; MAPK

Introduction

Aberrant activation of WNT signaling through loss-of-function of *adenomatous polyposis coli* (*APC*) or mutation of β-catenin (*CTNNB1*) is the key oncogenic event in colorectal cancer (CRC) (1). The pluripotent transcription factor MYC is arguably the most important target gene of the WNT/β-catenin pathway in CRC (2). Data from the Cancer Genome Atlas suggest that MYC-dependent transcription program is activated in nearly all CRC (1). Depletion or reduction of MYC impairs tumorigenesis in *APC* loss-driven mouse models of colorectal cancer (3, 4). Therefore, targeting MYC has the potential to disrupt key oncogenic functions in CRC. While direct pharmacological intervention for transcription factors like MYC remains difficult, small molecule compounds targeting the bromodomain and extra-terminal domain (BET) family epigenetic readers recently emerged as alternative approaches to suppress oncogenic transcription factors, including MYC (5).

The BET family includes ubiquitously expressed BRD2, BRD3, BRD4, and a testis-specific BRDT. These proteins contain two evolutionarily conserved bromodomains that recognize acetylated lysine residues on histone tails (5). Through this interaction, BET proteins direct assembly of nuclear macromolecular complexes, such as the mediator complex and the transcription elongation complex, to acetylated chromatin (6). Hence, BET proteins have important roles in transcription initiation and elongation. Small molecule BET bromodomain inhibitors, such as JQ1, PFI-1, MS417 and i-BET762, exhibit promising antineoplastic activities in a range of preclinical models of human cancers (7–13). The antineoplastic activities of BET bromodomain inhibitors are associated with suppression of prominent oncogenic transcription programs frequently activated in human cancers, such as MYC (9–11), MYCN (12, 14), GLI1/2 (15), and NF-κB (16). As such, BET bromodomain inhibitors represent an appealing therapeutic option for cancers dependent on oncogenic transcription factors. Oncogenic transcription programs driven by β-catenin and MYC are key players in the molecular pathogenesis of CRC. Hu and colleagues recently reported that BET inhibition by MS417 attenuated CRC liver metastasis, although the impact on xenograft tumor growth was limited (17). In addition, McCleland et al. showed that knockdown of BRD4 induced MYC downregulation, differentiation and growth inhibition in CRC xenograft models (18). This study also suggested that CRC with the CpG island methylator phenotype (CIMP) were preferentially sensitive to BET inhibitors (18), although this link was not confirmed by a more recent study (19). Nevertheless, response to BET inhibitors in CRC is modest in general, suggesting that CRC tumors are intrinsically resistant to BET inhibition. In this study, we assessed the therapeutic potential of BET inhibitors in CRC and interrogated mechanisms conferring resistance to BET inhibitors. Our results demonstrated that blockade

of the RAS/RAF/MAPK pathway rendered CRC cells significantly more sensitive to BET inhibitors. Concurrent inhibition of BET proteins and the MAPK pathway was necessary and sufficient to effectively downregulate MYC and to induce significant tumor regression in xenograft models. Collectively, our findings suggest that the combination of BET inhibitors and MEK inhibitors is a promising therapeutic strategy for CRC.

MATERIALS AND METHODS

Cell culture

CRC cell lines were purchased from ATCC. MC38 mouse colon adenocarcinoma cells and DLD1 derivative lines, DKO1 and DKS8, were provided by Dr. Robert Coffey, Vanderbilt University. RKO derivative lines were provided by Dr. Daniel Liebeler, Vanderbilt University, which were originally purchased from Horizon Discovery (Cambridge, MA). All cells used were amplified from early passages and maintained in cultures for no more than 2 months. These lines were not further authenticated. Mycoplasma test were performed regularly using PCR detection kit from American Type Culture Collection (ATCC #30-1012K). All cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 U/mL penicillin-streptomycin (Life Technologies) at 37°C in 5% CO_2. The immortalized non-tumorigenic immortalized young adult mouse colon (YAMC) cells were obtained from Dr. Robert Whitehead, Vanderbilt University. YAMC cells were maintained and analyzed at 33°C to activate a temperature-sensitive mutant of SV40 large T antigen (20). Additional supplements were added in the growth medium for YAMC cells as previously described (20). The integrity of cell lines used in this study is described in our recent publications (21). The patient-derived xenograft line, CR-IGR-0034P, was purchased from Oncodesign, France. This line is serially passaged in immunocompromised mice without *in vitro* culture.

Plasmids, antibodies and lentivirus production

The pLKO.1 lentiviral vectors directing expression of shRNA sequences specific to BRD2 (2a: TRCN000006309, 2b: TRCN000006310), BRD3 (3a: TRCN0000021374; 3b: TRCN0000021376), and BRD4 (4a: TRCN0000196576; 4b: TRCN0000199427) have been described in our previous publications (13). KRAS knockdown constructs (sh-KRAS-1: TRCN000003260 and sh-KRAS-2L: TRCN000003262) were purchased from Sigma. Lentivirus was produced by co-transfection of the lentiviral vectors with the packaging vectors psPAX2 and pCI-VSVG (Addgene) into 293FT cells. Viral supernatants were collected 2 days after transfection and applied to cells at an approximate MOI of 5. Cells were selected with 1 μg/ml puromycin for at least 48 hours prior to experiments. Most antibodies used in this study are described in our recent publications, except that KRAS-specific antibody was purchased from Santa Cruz Biotechnology (#sc-30) (13). Immunohistochemical staining of xenograft tumor sections were performed with primary antibody against MYC (#sc-40, Santa Cruz Biotechnology) at a 1:500 dilution, Ki67 (#VP-K451, Vector Laboratories) at a 1:2000 dilution or cleaved Caspase-3 (# 9664, Cell Signaling) at a 1:300 dilution. Staining was visualized by the Bond Polymer Refine detection system.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using the Illustra RNAspin kit (GE Healthcare) and reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). Gene expression was measured by real-time PCR using universal SYBR-Green Mastermix (Bio-Rad). The reaction comprises 40 cycles of 95°C for 20 seconds and 60°C for 45 seconds. Primers are listed in Supplementary Table 1. Beta-actin was used as the loading control.

RNA seq and Gene Set Enrichment Analysis (GSEA)

CRC Cells were treated with DMSO or 1 μmol/L JQ1 for 24 hours. Total RNA was extracted using the Illustra RNAspin kit with on-column DNA digestion according to the manufacturer's instructions. RNAseq were performed by Vanderbilt Technologies for Advanced Genomics. All RNA samples were quantified using the QuBit RNA assay kit. RNA quality was checked using Agilent Bioanalyzer. The Illumina TruSeq RNA Sample Preparation kit (Illumina) was used for library preparation. Samples were sequenced on HiSeq 2500 to obtain at least 30 million paired end (2 × 50 bp) reads per sample. The RNAseq data went through multiple stages of thorough quality control as recommended (22). Raw data and alignment quality control were performed using QC3, gene quantification quality control was conducted using MultiRankSeq. Raw data were aligned with TopHat2 against human transcript genome HG19. Gene expression were quantified using Cufflinks(23). Differentially expression analysis is performed using Cuffdiff command from Cufflinks package. False discovery rate < 0.05 was used as the significant threshold.

We employed the GSEA method (http://www.broadinstitute.org/gsea) for statistical analysis of changes in gene expression patterns (24). GSEA determines whether a defined set of genes shows statistically significant differences between two phenotypes. The complete list of genes and their scores were used in GSEA with a focus on the C6 MSigDB collection (oncogenic signatures). According to the developer's instructions, the false discovery rate (FDR) q value represents 'the estimated probability that a gene set with a given normalized enrichment score represents a false positive finding' and the nominal p value estimates 'the statistical significance of the enrichment score for a single gene set.

Cell cycle distribution

Cells were treated for 24 hours, fixed in 75% ethanol, treated with RNase A, and stained with 10 μg/ml propidium iodide. Cell cycle distribution was measured by flow cytometry on a BD LSRFortessa cell Analyzer. The percentage of cells in each cell cycle phase was assessed by the ModFit LT software.

Caspase activation assays

To measure caspase activity, 2500 cells were plated in 96-well plates and treated for 72 hours prior to analysis. Activities of caspase-3/7 were measured using a Caspase-Glo 3/7 assay kit (Promega) following the manufacturer's instructions. Relative caspase 3/7 activities were calculated by normalizing the values of caspase-3/7 activities to the values of cell titers measured by a CellTiter-Glo assay kit (Promega) in replicated wells.

Colony formation assay

CRC cells were plated at 200 cells per well in 6-well plates in triplicates and treated the next day with 500 nmol/L JQ1 ± 25 nmol/L trametinib. However, RKO was treated with 100 nmol/L JQ1 and 25 nmol/L trametinib, because this line appeared to be highly sensitive to BET inhibitors alone in vitro. Drugs were withdrawn after 5-day incubation. Cells were allowed to grow for 7 additional days prior to be fixed and stained with 0.5% crystal violet. Colonies were counted using the Image J software.

Cell viability assay

CRC cells were plated 2500/well in 96-well plates and treated with BET inhibitors with or without the MAPK pathway inhibitors following a 2-fold serial dilution. Five days later, cell viability was measured using a Sensolyte Cell Viability and Proliferation Assay kit (Anaspec). The dose-response curves were fitted and IC_{50} values were calculated using GraphPad Prism following a nonlinear regression (least squares fit) method.

Subcutaneous xenograft tumor assays

All animal experiments were performed under protocols approved by the Vanderbilt University Institutional Animal Care and Use Committee. The xenograft assays used female athymic nude mice 6–8 weeks old. CRC cells were trypsinized, suspended in PBS and mixed with equal volume of growth factor reduced Matrigel (BD Biosciences). Both flank sites of mice received injection of two million tumor cells. Tumor size were measured by a digital caliper and calculated following a formula of Size = Length × Width × Width/2. Treatment began when the median tumor size reached approximately 100 mm^3. Prior to treatment, tumor-bearing mice were randomized into 4 arms that the median tumor sizes of each arm were roughly the same. JQ1 was first dissolved in DMSO and diluted to 9 volumes of 10% β-cyclodextrin in sterile saline and administrated through intraperitoneal injection. Trametinib was prepared in 1% β-cyclodextrin and administrated through oral gavage. Gefitinib was prepared in 1% Tween-80 and administrated through oral gavage. Tumor-bearing mice were treated daily with vehicle, 50 mg/kg/day JQ1, trametinib (0.5 mg/kg for SW480 and Difi, or 1 mg/kg for RKO), or both. Mice bearing the patient-derived xenograft tumors were treated with 100 mg/kg/day JQ1 and 1 mg/kg trametinib, of which JQ1 was given twice a day and trametinib was given once a day. In addition, a group of mice bearing Difi xenograft tumor were treated with 50 mg/kg gefitinib daily. Tumors were measured two to three times a week. Animals were monitored for significant adverse effects. Mice accidentally died of injury caused by oral gavage or intraperitoneal injection was excluded from analyses.

Other statistical analyses

GraphPad Prism 5.0 was used to determine statistical significance. P-values of less than 0.05 were considered significant. The combination index values were calculated using the Chou-Talalay method with the Compusyn software following the developer's instructions.

Results

BET inhibition leads to moderate MYC downregulation in CRC

It has been increasingly documented that inhibition of BET proteins results in potent downregulation of MYC in hematologic cancers, such as multiple myeloma, Burkitt's lymphoma, and acute myeloid leukemia (9–11). However, regulation of MYC by BET proteins in solid cancers appears to be heterogeneous and context-dependent (13, 14, 25). MYC is commonly activated in CRC and plays a critical role in disease initiation and progression. Therefore, we asked whether MYC expression in CRC was sensitive to BET inhibition. Our results showed that BET inhibition by JQ1 commonly downregulated MYC at both protein and mRNA levels in CRC cell lines (Fig. 1A and 1B). However, the reductions of MYC at protein levels were moderate and less significant compared with changes at mRNA levels, suggesting implications of post-transcriptional regulations. Following BET inhibition, expression of MYC targets, such as Bcl-2, Bcl-xL and hTERT, was decreased; while the MYC-repressed tumor suppressor, p21 (a.k.a. *CDKN1A*), was increased, albeit to various extents in different lines (Fig. 1C and Supplementary Fig. S1A–S1C). Selective depletion of individual BET family members by RNA interference suggested that BRD4 was the primary MYC regulator within the family, but BRD2 and BRD3 might also contribute to MYC regulation (Supplementary Fig. S1D–1G). We further assessed the impact of JQ1 on gene expression profiles in CRC cells using RNA seq and employed GSEA to analyze the changes in MYC-dependent gene signatures. In DLD1, KM12C and RKO cells, JQ1 significantly suppressed MYC-dependent transcription modules (Fig. 1D and Supplementary Fig. S1H). However, JQ1-induced repression of MYC signature was not statistically significant in SW480 cells (FDR q = 0.127, NES = −1.27). These results collectively suggest that BET proteins are commonly implicated in regulation of MYC expression and activity in CRC. However, in heterogeneous solid caners like CRC, MYC regulation by BET proteins can be context-dependent.

BET inhibition impair proliferation and survival of CRC cells

We next assessed activities of BET inhibitors in a subset of CRC cell lines harboring genetic alterations commonly found in CRC (26, 27). JQ1 dose-dependently decreased cell growth in all of the tested CRC cells (Fig. 2A and Supplementary Table 2). Similar response was observed using distinct BET bromodomain inhibitors, such as PFI-1 (Supplementary Fig. S2A). The anti-growth effects of BET inhibitors were partially phenocopied by knockdown of BRD4, while knockdown of BRD2 and BRD3 had less significant impact (Fig. 2B, Supplementary Fig. S2B, S2C). Inhibition of BET proteins was associated with cell cycle arrest in G1/G2 phases and moderate caspase activation (Fig. 2C, 2D, and Supplementary Fig. S2D–S2F). While these findings are consistent with recently published studies and underscore the potential of BET proteins as drug targets in CRC (17–19), BET inhibitors alone were largely cytostatic in CRC cultures, raising the concern whether they may be clinically effective as monotherapy.

Blockade of the RAS/RAF/MAPK pathway sensitizes CRC cells to BET inhibitors

The modest response to BET inhibitors and the lack of significant cell death suggest that intrinsic resistance to BET inhibitors is common in CRC, which can be a critical obstacle for

successful translation. The RAS/RAF/MAPK pathway is activated in most CRC tumors via activating mutations of KRAS, NRAS, BRAF, amplification of EGFR, in addition to other mechanisms (1). Among the CRC cell lines used in our study, phosphorylation levels of ERK were the highest in $BRAF^{V600E}$–expressing RKO and HT29, whereas KRAS mutations did not always result in hyperactivation of the MAPK pathway (Supplementary Fig. S3). Activation of RAS/RAF and the downstream MAPK pathway through mutations or other mechanisms is a common key oncogenic event in CRC. Introduction of an oncogenic KRAS mutant drastically promotes tumor formation and progression in *APC* deficient mouse model of intestinal tumors (28). RAS activation augments WNT signaling (29) and also promotes MYC protein stability, through mechanisms such as ERK-mediated MYC phosphorylation (30). Hence, we asked whether blockade of the MAPK pathway might affect CRC response to BET inhibitors. We treated CRC cells using a FDA-approved MEK inhibitor, trametinib, with or without JQ1 at a fixed molar ratio of 1:100 following a 2-fold serial dilution. This combination treatment induced significantly more potent reduction of cell growth than either agent alone in all tested lines across a wide range of concentrations (Fig. 3A–3C, Supplementary Fig. S4A and Table 2). Among the 14 CRC cell lines that we have tested, the median IC_{50} value of JQ1 was approximately 330 nmol/L. The median IC_{50} value of JQ1 was reduced to 130 nmol/L when combined with trametinib (Fig. 3C and Supplementary Table 2). The median IC_{50} value of trametinib was approximately 11 nmol/L as monotherapy, while it was reduced to merely 1 nmol/L in the presence of JQ1 (Fig. 3C and Supplementary Table 2). The interaction between JQ1 and trametinib was further assessed by the Chou-Talalay statistical method (31). In KM12C, RKO and SW480, the combination index (CI) values of JQ1 and trametinib were substantially less than 1 at concentrations across multiple orders of magnitude, indicating strong drug synergism (Fig. 3A, 3B, and Supplementary Fig. S4A, green cross, right Y axis). Similar synergistic interactions between JQ1 and the BRAF inhibitor vemurafenib were shown in $BRAF^{V600E}$ HT29 cells (Supplementary Fig. S4B). In addition, combinations of JQ1 with an ERK inhibitor SCH772984, or i-BET762 with an MEK inhibitor selumetinib also produced synergistic response (Supplementary Fig. S4C and S4D). In contrast, non-tumorigenic YAMC cells were refractory to JQ1 and trametinib, either alone or in combination (Supplementary Fig. S4E), whereas MC38, a cell line derived from genetic mouse models of colon cancer, was highly sensitive to the combination (Supplementary Fig. S4F). Clonogenic assays showed that transient exposure to JQ1 and trametinib almost completely abolished the ability of CRC cells to form colonies at concentrations that single-agent had limited impact (Fig. 3D and Supplementary Fig. S5), suggesting that this combination therapy induced prolonged damage to the tumorigenic potential of CRC cells. To further validate the specificity of actions of chemical probes, we showed that selective depletion of BRD4 sensitized KM12C and RKO cells to trametinib (Supplementary Fig. S6). Conversely, knockdown of BRD2 or BRD3 did not significantly altered response to MEK inhibition, suggesting that BRD4 plays a major role in regulation the crosstalk with the MAPK pathway. In addition, knockdown of KRAS decreased phosphorylation levels of ERK in both KRAS-mutant SW480 cells and KRAS-wild type KM12C cells and rendered cells more sensitive to JQ1 (Supplementary Fig. S7). These results collectively suggest that the synergism between of BET inhibitors and MAPK pathway inhibitors are primarily mediated via their respective on-target effects.

Our results showed that the combination of BET inhibitors and MEK inhibitors induced synergistic response in all tested CRC cell lines, irrespective of KRAS and BRAF mutations. These findings suggest an intrinsic role of MAPK signaling in mediating resistance to BET inhibitors that is not dependent on the mechanisms by which the MAPK pathway is activated. To further test this hypothesis, we investigated two sets of isogenic CRC lines that were genetically modified to deplete either the mutant allele of KRAS/BRAF or the wild type one. DLD1 cells has one copy of wild type KRAS and one copy of $KRAS^{G13D}$. This line had low MAPK activity compared with other KRAS-mutant CRC lines (Supplementary Fig. S3). The derivative line DKS8 lost the mutant $KRAS^{G13D}$ allele. Interestingly, phosphorylation of ERK in DKS8 was moderately higher than the parental line, despite loss of the mutant KRAS (Supplementary Fig. S8A). The response to JQ1, trametinib or the combination was similar between DKS8 and the parental DLD1 (Supplementary Fig. S8B and S8C). In contrast, the other derivative line depleted of the wild type KRAS allele, DKO1, showed much more potent activation of ERK (Supplementary Fig. S8A). DKO1 appeared to be more resistant to JQ1 than two other isogenic lines, but the resistance was essentially abolished by trametinib (Supplementary Fig. S8D and Table 2). In BRAF-mutant RKO cells, loss of the V600E allele significantly reduced phosphorylation of ERK (Supplementary Fig. S9A), but did not affect the synergistic interaction between JQ1 and trametinib (Supplementary Fig. S9B–9D). However, RKO-$BRAF^{wt}$ cells had lower cell viability in the presence of JQ1 at micromolar concentrations (Supplementary Fig. S9E). Taken together, these results suggest that modulations of MAPK activity may affect CRC cell sensitivity to BET inhibitors. However, at least in CRC, the synergistic interaction between BET inhibitors and MEK inhibitors is not genotype-dependent.

Concurrent inhibition of MEK and BET results in potent MYC downregulation

We next assessed changes in MYC expression when BET proteins, MAPK, or both were targeted. Our results showed that MYC protein was more effectively decreased following exposure to the combination of JQ1 and trametinib compared with either agent alone (Fig. 4A). However, the combination did not result in a more effective reduction of MYC mRNA (Fig. 4B and Supplementary Fig. S10A). Hence, the ability of trametinib to augment JQ1-induced downregulation of MYC protein appeared to be primarily mediated through post-transcriptional mechanisms. Expression of a constitutively active MEK1 mutant, $MEK1^{C121S}$, resulted in accumulation of MYC protein and counteracted JQ1-induced MYC repression (Fig. 4C). $MEK1^{C121S}$ also attenuated the effects of JQ1 and trametinib in Difi and SW480 cells, either alone or in combination (Fig. 4D and Supplementary Fig. S10B).

The combination of BET inhibitors and MEK inhibitors shift the balance among apoptosis regulators

While either JQ1 or trametinib alone was largely cytostatic in CRC cells, we noticed that targeting both BET and the MAPK pathway induced massive cell death and potent caspase activation (Fig. 5A and 5B). Several anti-apoptosis genes, Bcl-2 in particular, have been found to be targets of BET inhibitors in various cancers (9–11). Bcl-2 may play a primary role in neutralizing apoptotic stresses in some hematologic cancers, as the selective Bcl-2 inhibitors navitoclax and venetoclax generate significant response in patients with chronic lymphocytic leukemia (32, 33). However, the apoptosis regulatory network in solid cancers

is more complex and heterogeneous. Our results showed that BET inhibition induced downregulation of multiple anti-apoptotic Bcl-2 family members, Bcl-2 and Bcl-xL, as well as the inhibitor of apoptosis (IAP) family member, survivin (Fig. 5C and Supplementary Fig. S11A). In SW480 cells, BET inhibition also increased expression of BIM, a pro-apoptotic member of the Bcl-2 family (Fig. 5D). However, BET inhibition resulted in downregulation of certain pro-apoptotic genes, such as BIK in SW480 cells and BIK and PUMA in KM12C cells, which are expected to mitigate the impact of loss of anti-apoptotic genes (Fig. 5D and Supplementary Fig. S11B). Trametinib exhibited context-dependent effects on expression of these apoptosis regulators. Combining trametinib with JQ1 resulted in more effective suppression of anti-apoptotic genes, as exemplified by survivin in SW480 cells (Fig. 5C), and further increased BIM expression (Fig. 5D and Supplementary Fig. S11B). Interestingly, JQ1-induced suppression of BIK and PUMA was neutralized by trametinib (Fig. 5D and Supplementary Fig. S11B). Although changes of individual apoptosis regulators following treatments of JQ1 and/or trametinib were not identical across CRC lines with different genetic backgrounds, our results showed a pattern that BET inhibitors and MEK inhibitors in combination induced a more effective downregulation of anti-apoptosis genes and upregulation of pro-apoptosis genes compared with monotherapies, leading to strong apoptotic cell death.

A combination of JQ1 and trametinib induces CRC xenograft tumor regression

We next sought to assess the *in vivo* efficacy of the combination of JQ1 and trametinib using subcutaneous xenograft models. A few recent studies showed that BET inhibitors, JQ1 or MS417, had limited impact on CRC xenograft tumor growth (17, 19). Our data demonstrated that either JQ1 or trametinib alone moderately reduced the rate of tumor growth. In contrast, the combination of these two agents decreased median tumor size by 45% in $KRAS^{G12V}$ SW480 xenografts and 60% in $BRAF^{V600E}$ RKO xenografts following 2 weeks of treatment (Fig. 6A, 6B). Tumor regression was associated with decreased proliferation and increased apoptosis as shown by Ki67 and cleaved caspase 3 staining (Supplementary Fig. S12A). Animals treated with the combination experienced approximately 15% loss of body weight in the RKO experiment, while changes in the SW480 experiment were minor (Supplementary Fig. S12B and S12C). Using a patient-derived $BRAF^{V600E}$-expressing CRC xenograft model (34), we further showed that JQ1 or trametinib alone decreased tumor growth, whereas the combination induced a 75% reduction in median tumor size (Fig. 6C). Anti-EGFR therapy has been approved for the management of KRAS- and BRAF-wild type CRC. We asked whether the efficacy of the combination of BET inhibitors and MEK inhibitors was comparable to anti-EGFR agents in EGFR-dependent CRCs, such as Difi. Daily administration of 50 mg/kg gefitinib over 15 days decreased growth of Difi xenografts from 176% to 20%. In comparison, the combination of JQ1 and trametinib reduced median tumor volume by approximately 40% (Fig. 6D). Immunohistochemical staining of MYC in tumors treated for 5 days showed that JQ1 or trametinib alone only moderately decreased MYC-expressing cells in RKO tumors and marginally affected MYC expression in SW480 cells. However, MYC-expressing cells were drastically decreased in tumors treated with both compounds (Figure 6E and Supplementary Fig. S12D and S12E). Taken together, our results strongly suggest that a combination of BET bromodomain inhibitors and MEK inhibitors have broad therapeutic potential for

treating CRC, including those therapeutically refractory tumors that harbor BRAF and KRAS mutations.

Discussion

BET bromodomain inhibitors are a class of emerging drug candidates with attractive anti-cancer and anti-inflammatory activities through modulation of aberrantly activated transcription factors. Favorable clinical response was recently reported in hematologic cancers (35). However, the efficacy of BET inhibitors in preclinical models of advanced solid cancers is generally moderate. Mechanisms mediating acquired resistance to BET inhibitors just began to emerge. Acute myeloid leukemia may reactivate MYC expression through WNT signaling that confers resistance to BET inhibitors (36, 37). Alternatively, triple negative breast cancer may gain resistance through increasing phosphorylation of BRD4 that renders BRD4 maintain its transcription regulatory activities independent of bromodomains (38). Mechanisms mediating intrinsic resistance to BET inhibitors remain elusive. Identification of these mechanisms may direct development of more effective combination therapy and expedite translation of BET inhibitors as viable therapeutic options for advanced solid cancers. In the present study, we showed that activation of the MAPK pathway conferred intrinsic resistance to BET inhibitors. Thus, a combination of BET inhibitors and the MAPK pathway inhibitors generated synergistic response in CRC cell cultures and xenograft models. This approach is particularly appealing for CRC tumors carrying mutations in the RAS/RAF/MAPK pathway, which is highly resistant to currently available therapeutics.

A hallmark of CRC is prevalent co-activation of β-catenin/MYC-driven transcription and the RAS/RAF/MAPK pathway that act synergistically to promote CRC pathogenesis (1, 28, 39). The RAS/RAF/MAPK pathway may augment β-catenin and MYC activity through a multitude of mechanisms (30, 40, 41). Alternatively, activation of the WNT/β-catenin/MYC pathway is implicated in resistance to BRAF and MEK inhibitors (42–45). As such, accumulating evidence support the rationale to co-target both pathways for the treatment of CRC and a range of other cancers. However, the master regulators of these two pathways, KRAS and β-catenin, are both difficult drug targets. Our findings suggest that BET bromodomain proteins represent a druggable signaling hub in the oncogenic network driven by the WNT pathway and the RAS/RAF/MAPK pathway. As a result, the combination comprising a BET inhibitor and an inhibitor blocking the MAPK pathway holds great promise to treat cancers affected by aberrations of these two pathways, such as CRC.

In our study, we observed synergistic interaction between BET inhibitors and MAPK inhibitors in all tested CRC cell lines, irrespective of mutational status of KRAS and BRAF. In addition, our data did not suggest a linear correlation between sensitivity to JQ1 and activation of the MAPK pathway. Although the broad response in CRC may raise concerns over off-target effects, it is in concordance with prevalent activation of the MAPK pathway in this disease, which can be induced through not only mutations of KRAS/BRAF but also many other mechanisms. Using two sets of isogenic CRC cell lines, we showed that depletion of the mutant KRAS or BRAF alleles might alter JQ1 sensitivity according to the changes in MAPK activation. However, the synergistic interaction between JQ1 and

trametinib was not compromised. Thus, the crosstalk between MAPK signaling and BET proteins appears to be an intrinsic phenotype of CRC that is independent of the mechanisms through which the MAPK pathway is activated or the degree of MAPK activation. On the contrary, non-tumorigenic YAMC cells was spared by this combination therapy. Several genetic approaches, such as selective depletion of individual BET genes or KRAS and expression of a constitutively active MEK1 mutant, also underscored the on-target actions of BET inhibitors and MEK inhibitors. It is an interesting question if this paradigm can be applied to additional cancers transformed by MYC and oncogenic KRAS.

The mechanisms mediating the synergistic effects of the combination of BET inhibitors and MEK inhibitors remain to be fully elucidated in different CRC subtypes. While many downstream signaling molecules are affected by the combination therapy, MYC is a particularly important target. Overexpression of MYC is extensively documented in CRC and associated with poor survival (46). The Cancer Genome Atlas (TCGA) data indicate that MYC-dependent transcription is activated in nearly all CRC samples (1). We found that trametinib did not augment JQ1-induced downregulation of MYC mRNA, but further decreased MYC protein levels in the presence of JQ1, suggesting that trametinib modulated MYC levels through posttranslational mechanisms. The detailed mechanisms through which the MAPK pathway regulates MYC protein levels remain an open question and can be context-dependent. Nonetheless, our findings suggest that targeting both BET proteins and the MAPK pathway is necessary to effectively downregulate MYC proteins in CRC, in contrast to observations in MYC-amplified hematopoietic cancers. The resistance of MYC to BET inhibition is even more obvious *in vivo*, as most cells in CRC xenografts remained positively stained for MYC following administration of JQ1. In contrast, the combination of JQ1 and trametinib drastically reduced the percentage of MYC-positive cells. Hence, this combination strategy has important indications to pharmacologically target oncogenic MYC in solid cancers.

Another phenotype consistently shown in CRC cells treated with both BET inhibitors and MEK inhibitors was the shift of the balance between anti-apoptotic genes and pro-apoptotic genes. BET inhibition unexpectedly reduced expression of certain pro-apoptotic genes, such as BIK and PUMA. As such, despite reduced expression of anti-apoptotic genes, the overall impact of BET inhibitors on apoptosis regulatory network might not be sufficient to induce significant cell death. Addition of trametinib neutralized JQ1-induced downregulation of BIK and PUMA, augmented induction of BIM, and further decreased survivin. Hence, blockade of both pathways appears to be necessary and sufficient to effectively shift the balance between pro-apoptotic genes and anti-apoptotic genes to activate apoptosis. A recent study shows that concurrent upregulation of NOXA/BIK and inactivation of Bcl-2/Bcl-xL is necessary to induce apoptosis in KRAS-mutant colon cancer cells, underscoring the importance of coordinated changes in the apoptotic regulatory network (47). In addition, Conery and colleagues recently reported that activity of BET inhibitors in preclinical cancer models largely correlated with apoptotic response (48). Collectively, the coordinated actions of BET inhibitors and MEK inhibitors on expression of apoptosis regulators may have an important role in mediating the synergistic response.

To date, BET inhibitors alone exhibit generally moderate preclinical activities in solid tumor models. To realize the therapeutic potential of BET inhibitors, it is crucial to identify the mechanisms of resistance and to develop rational combinations that can produce meaningful response in a preclinical setting. In the current study, we identified a role of the MAPK pathway in mediating intrinsic resistance to BET inhibitors in CRC. As a result, combinations of BET inhibitors with inhibitors targeting the MAPK pathway induced synergistic response *in vitro* and *in vivo*. These findings propose a combination strategy that may substantially promote the uses of BET inhibitors and FDA-approved BRAF and MEK inhibitors. Crosstalks between signal transduction pathways and epigenetic regulation have recently emerged as a key factor to determine therapeutic response to BET inhibitors (38, 49–51). As such, combining with additional targeted agents is likely essential to optimize BET-targeted therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We want to thank Drs. Robert Coffey, Daniel Liebler, and Robert Whitehead for providing important cell lines.

Grant Supports

J.W. is supported by the National Institutes of Health grant (R01CA166492) and the Voice Against Brain Cancer Foundation. E. L. is supported by NIH grants R01GM103926 and R01GM081635, and R. D. B. is supported by R01CA069457. S.K. is grateful for support by the Structural Genomics Consortium, a registered charity (No. 1097737) that receives funds from AbbVie, Bayer, Boehringer Ingelheim, Genome Canada through Ontario Genomics Institute Grant OGI-055, GlaxoSmithKline, Janssen, Lilly Canada, the Novartis Research Foundation, the Ontario Ministry of Economic Development and Innovation, Pfizer, Takeda, and Welcome Trust Grant 092809/Z/10/Z. L.N. is a recipient of NIH training grant 5T32HD007502.

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Translational Relevance

BET bromodomain inhibitors are promising drug candidates, particularly for cancers driven by aberrantly activated oncogenic transcription programs. However, the moderate preclinical efficacy of BET inhibitors in advance solid cancer models raises concerns over their clinical potential as monotherapy. Hence, identifying mechanisms of resistance and developing rational combination approaches are critical for successful clinical translation. In CRC, BET inhibitors alone was inadequate to induce significant cell death and depletion of MYC protein. Our findings showed that the MAPK pathway mediated intrinsic resistance to BET inhibitors. Consequently, a combination of BET inhibitors and MEK inhibitors effectively reduced MYC protein levels and induced significant tumor regression in xenograft models, including those bearing KRAS or BRAF mutations. These findings suggest that rationally designed drug combinations hold promise to optimize BET bromodomain-targeted therapy.

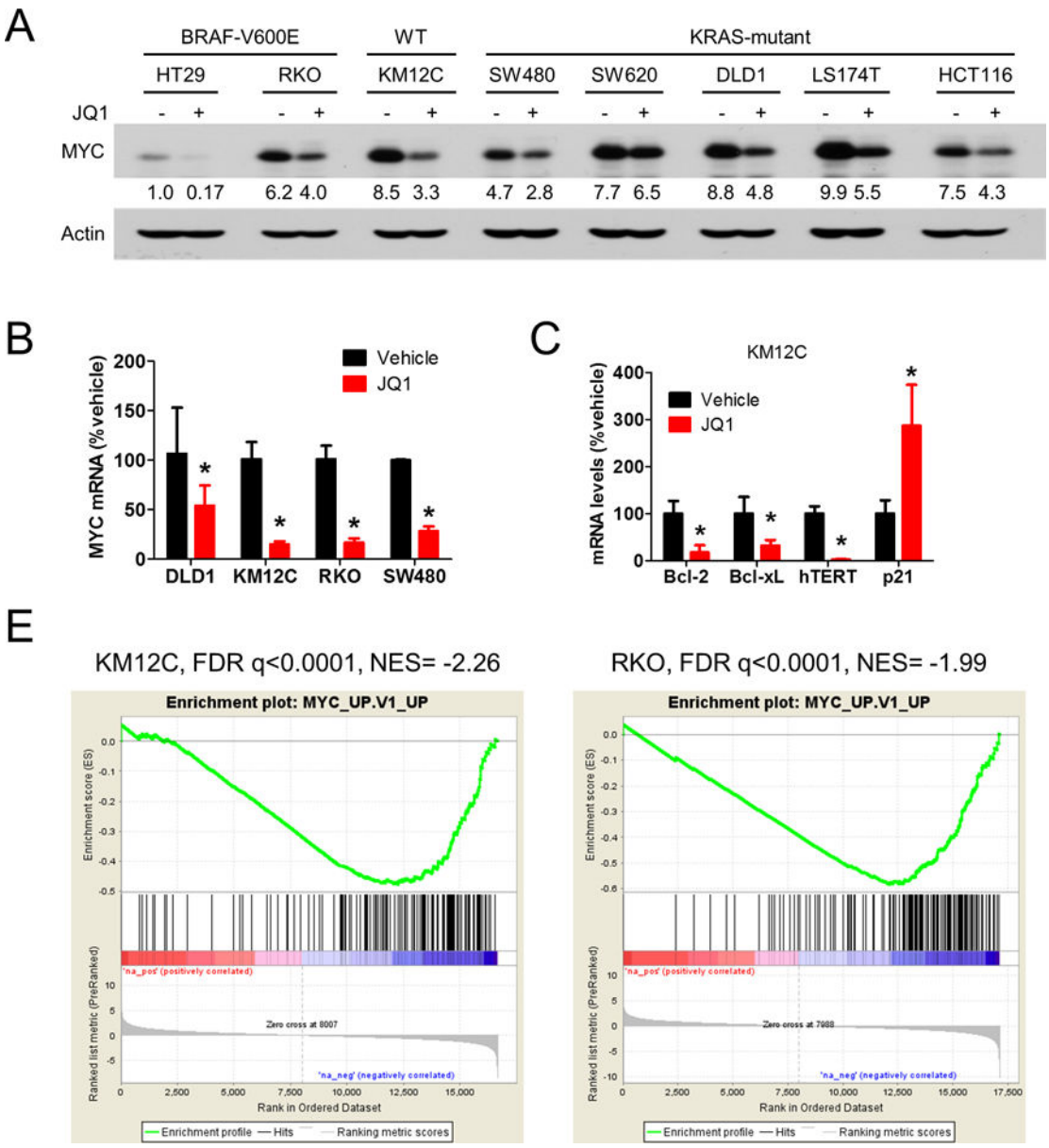

Figure 1. BET inhibition suppresses MYC in CRC

(A) CRC cells were treated with 2.5 μmol/L JQ1 for 24 hours and subject to immunoblotting. Similar experiments have been performed for at least three times unless otherwise indicated. Density of MYC bands were determined by Image J and normalized to the corresponding actin bands. The relative density of MYC in vehicle-treated RKO cells was assigned as 1. (B) CRC cells were treated with 1 μmol/L JQ1 for 24 hours. RNA was extracted and subject to qRT- PCR using beta-actin as the loading control. Error bars represent standard deviations in all figures unless otherwise indicated. Standard deviations were calculated from at least three technical replicates. *: $p < 0.05$ by Student's *t*-test. (C) Expression of MYC target genes in KM12C cells treated as described above. (E) KM12C and RKO cells were treated with 1 μmol/L JQ1 for 24 hours and subject to RNA seq. GSEA

enrichment plots show suppression of a MYC-dependent gene signature (MYC_UP.V1_UP, 200 genes). FDR: false discovery rate. NES: Normalized enrichment score.

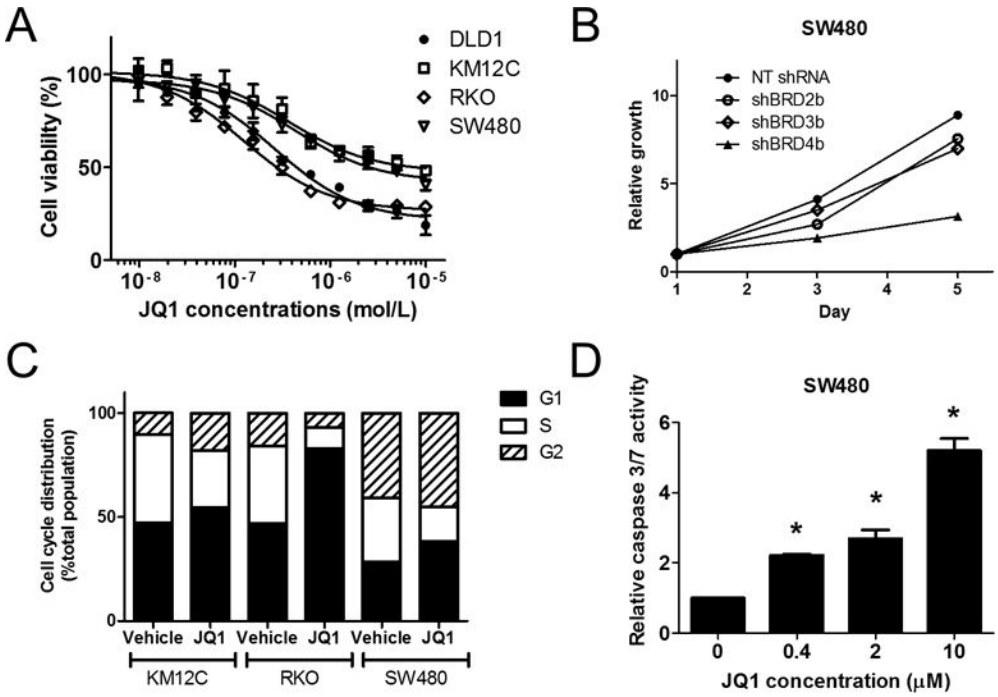

Figure 2. Inhibition of BET proteins impairs proliferation and survival of CRC cells

(A) CRC cells in 96-well plates were treated with JQ1 following a 12-point 2-fold serial dilution. Dose-response curves were determined following a three-parameter non-linear regression method. (B) Following lentiviral infection and puromycin selection, SW480 cell growth was measured every two days and normalized to the corresponding values of day 1. (C) CRC cells were treated with 1 μmol/L JQ1 for 24 hours. Cell cycle distribution was measured by flow cytometry and analyzed using the ModFit software. Representative plots are shown. (D) SW480 cells were treated with JQ1 for 72 hours at indicated concentrations. Caspase activities were determined using Caspase-Glo 3/7 assay kit (Promega) and normalized to cell titers determined using the CellTiter-Glo assay kit. *: $p < 0.05$ by Student's *t*-test compared with the corresponding control groups.

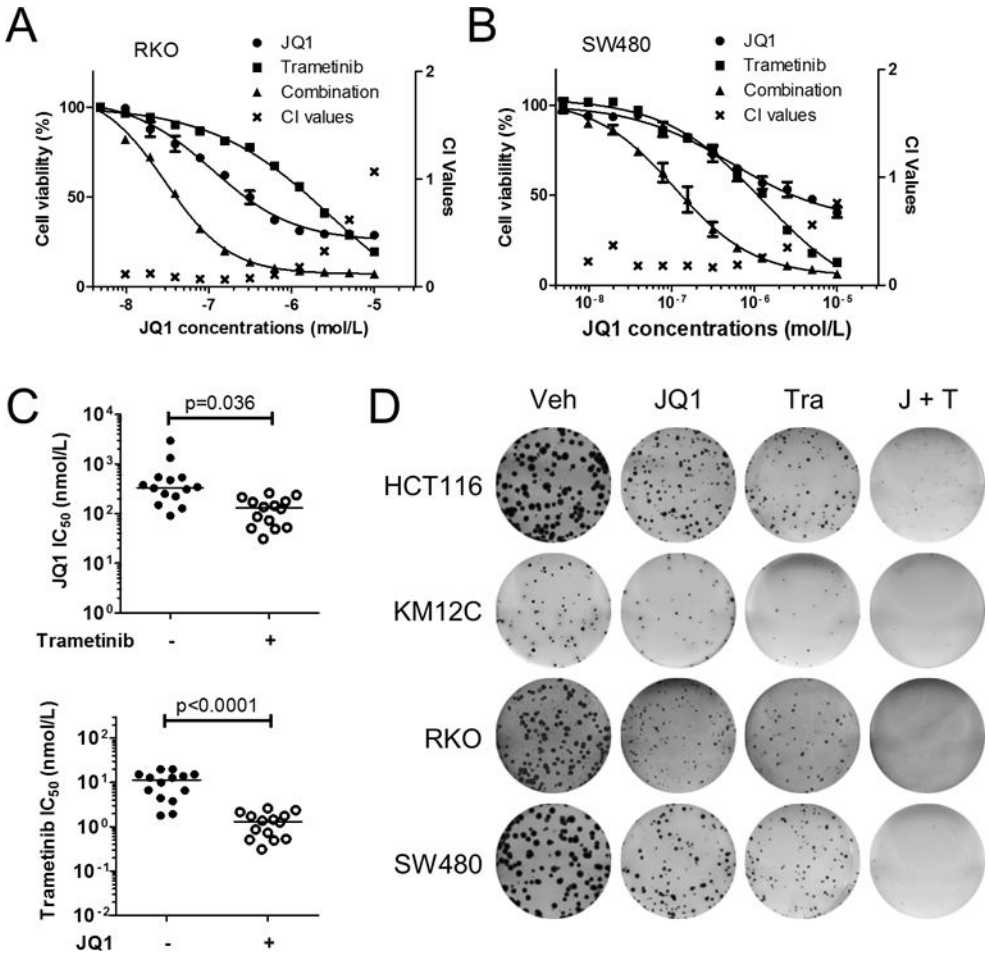

Figure 3. MEK inhibition synergistically improves the efficacy of BET inhibitors

(A) RKO and (B) SW480 cells were treated with JQ1 ± trametinib at a fixed ratio (100:1). Dose-response curves were determined as described in Fig. 2A. Combination index (CI) values were determined following the Chou-Talalay method. (C) IC_{50} values of JQ1 or trametinib either alone or in combination. Each dot represents mean of IC_{50} values of one CRC line generated from at least three independent experiments. (D) Colony formation by CRC cells treated for 5 days and cultured for additional 7 days after drug withdrawal. Veh: vehicle, Tra: trametinib, J + T: the combination of JQ1 and trametinib.

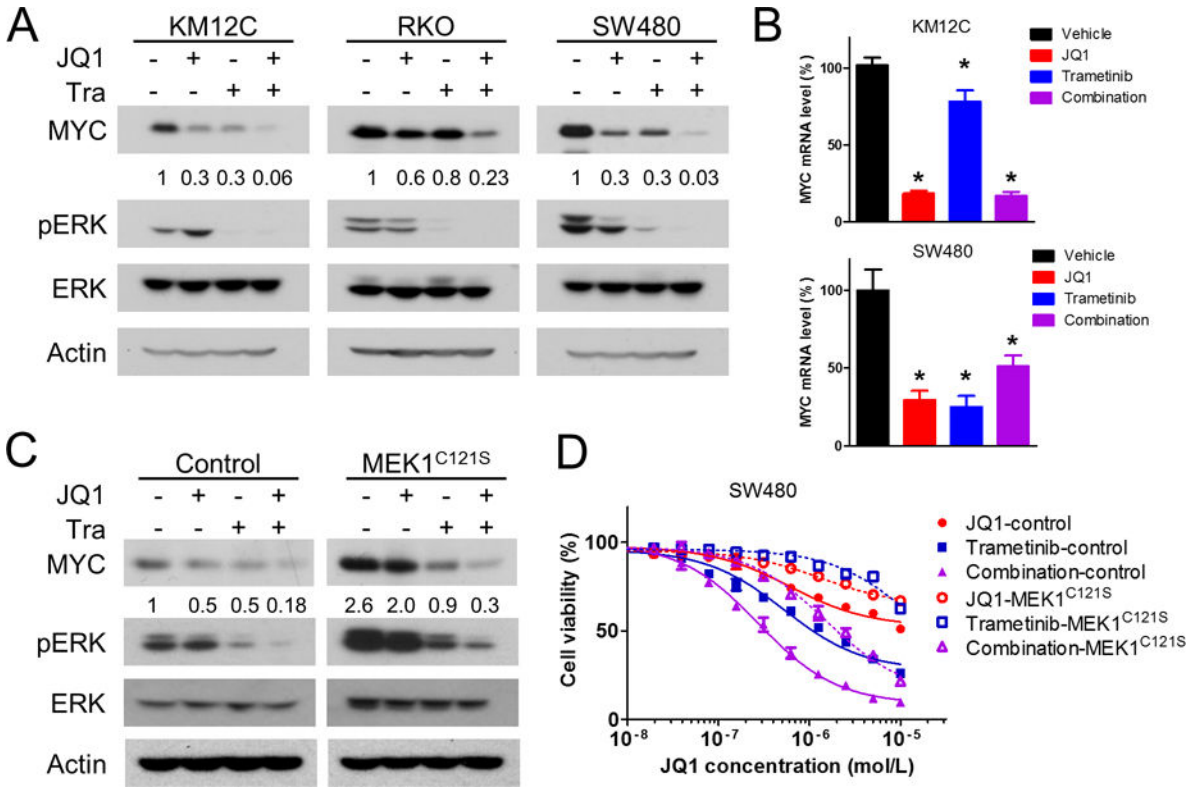

Figure 4. Co-targeting BET proteins and MEK induces potent downregulation of MYC

(A) CRC cells were treated with 500 nmol/L JQ1 ± 50 nmol/L trametinib for 2 days prior to lysis for immunoblotting. The relative density of MYC bands are calculated as described in Figure 1A. (B) KM12C and SW480 cells were treated as described above. MYC mRNA levels were determined by qRT-PCR using beta-actin as loading control. *: treated vs. control, $p < 0.05$ by Student's *t*-test. (C) SW480 cells were infected with control lentivirus or lentivirus directing expression of $MEK1^{C121S}$. After puromycin selection, cells were treated as described above and MYC protein levels were shown. (D) Dose response to JQ1 ± trametinib in SW480 cells infected with control lentivirus or lentivirus directing expression of $MEK1^{C121S}$.

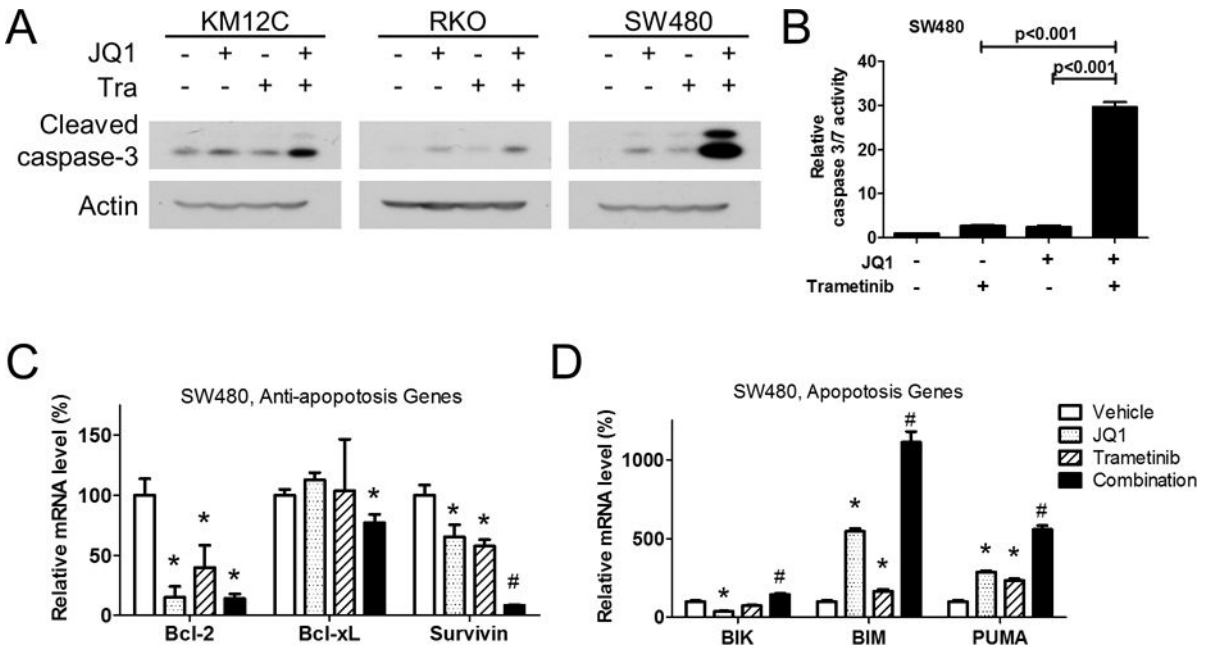

Figure 5. Combining BET inhibitors and MEK inhibitors induces potent apoptosis

(A) CRC cells were treated with 500 nmol/L JQ1 ± 50 nmol/L trametinib for 2 days. Immunoblotting of cleaved caspase 3 was presented. (B) Activation of caspase was assessed using the Promega Caspase-Glo 3/7 kit in SW480 cells. P values were determined by student's *t*-test. (C) SW480 cells were treated as described above. Expression of indicated target genes was determined by qRT-PCR using beta-actin as loading control. *: treated vs. vehicle, $p < 0.05$ by Student's *t*-test. #: combination vs. JQ1, $p < 0.05$ by Student's *t*-test.

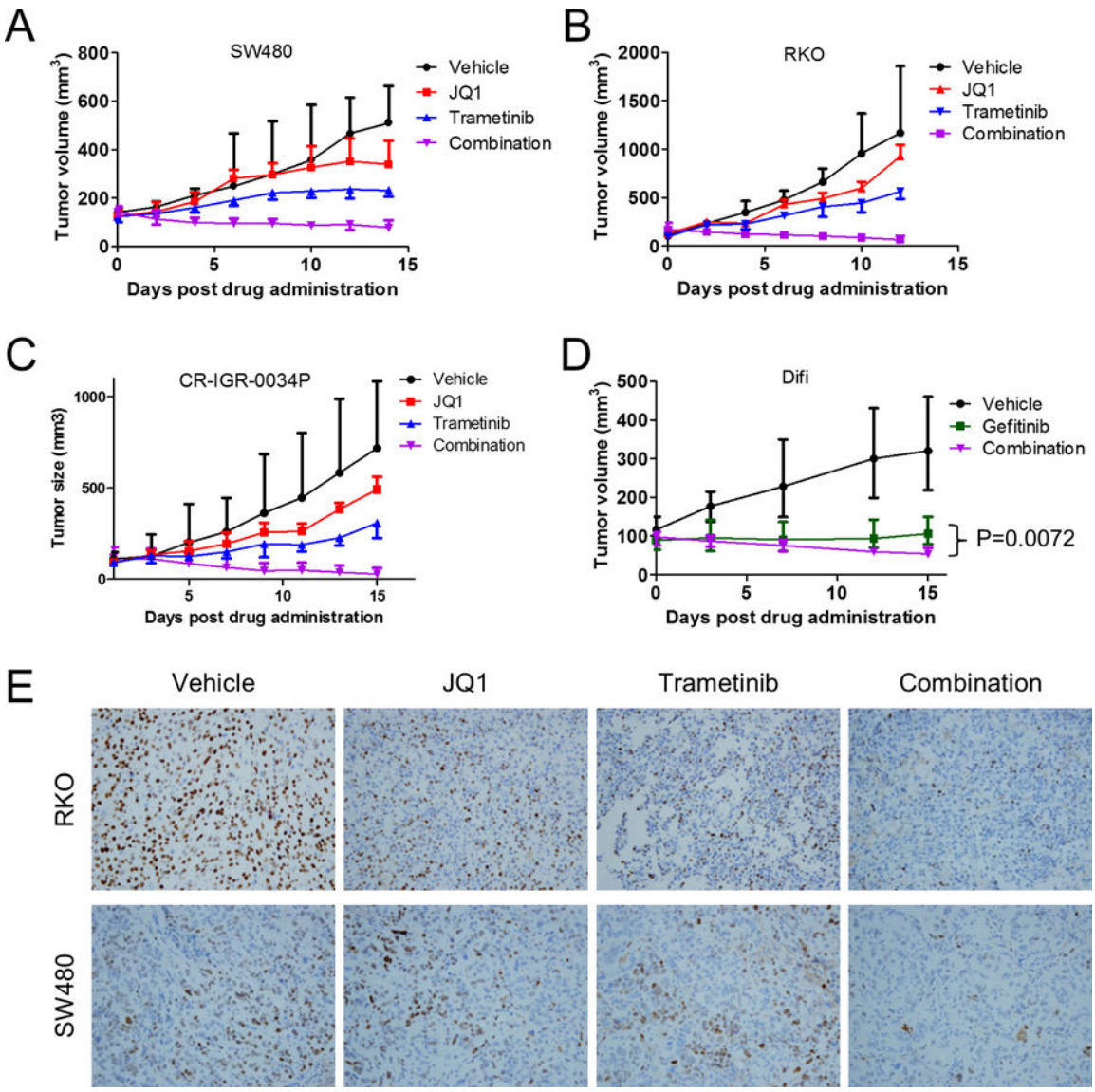

Figure 6. The combination of JQ1 and trametinib induces CRC xenograft tumor regression

(A) SW480 subcutaneous xenograft tumors (n=7, 14 tumors) were treated with 50 mg/kg/day JQ1 (i.p., once per day) ± 0.5 mg/kg/day trametinib (p.o., once per day) for 14 days. (B) RKO subcutaneous xenograft tumors (n=6, 12 tumors) were treated with 50 mg/kg/day JQ1 ± 1 mg/kg/day trametinib for 12 days. (C) CR-IGR-0034P subcutaneous tumors (n=7, 14 tumors) were treated with 100 mg/kg/day JQ1 (twice per day) ± 1 mg/kg/day trametinib (once per day) for 15 days. (D) Difi subcutaneous xenograft tumors (n=5, 10 tumors) were treated with 50 mg/kg/day gefitinib (p.o.) or 50 mg/kg/day JQ1 + 0.5 mg/kg/day trametinib as described above for 15 days. All tumor size data presented are median tumor size ± interquartile range. (E) After 5 days of treatment, selected tumors were resected for immunohistochemical staining of MYC. Representative images are presented (200×).