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Loss of TRIM33 causes resistance to BET bromodomain inhibitors through MYC- and TGFβ-dependent mechanisms

Xiarong Shi[1], Valia T. Mihaylova[1], Leena Kuruvilla[1], Fang Chen[1], Stephen Viviano[1], Massimiliano Baldassarre[1], David Sperandio[2], Ruben Martinez[2], Peng Yue[2], Jamie G. Bates[2], David G. Breckenridge[2], Joseph Schlessinger[1]*, Benjamin E. Turk[1]*, David A. Calderwood[1,3]*

* Correspondence

joseph.schlessinger@yale.edu, benjamin.turk@yale.edu, david.calderwood@yale.edu

Department of Pharmacology[1] and of Cell Biology[3], Yale University School of Medicine, 333 Cedar St, New Haven CT, USA 06520 and Gilead Sciences[2], Foster City, CA, USA.

Classification: BIOLOGICAL SCIENCES: Pharmacology

Keywords: Bromodomain inhibitor, TRIM33, JQ1, Drug resistance, TGFβ

ABSTRACT

Bromodomain and extraterminal domain protein inhibitors (BETi) hold great promise as a novel class of cancer therapeutics. As acquired resistance typically limits durable responses to targeted therapies, it is important to understand mechanisms by which tumor cells adapt to BETi. Here, through pooled shRNA screening of colorectal cancer cells, we identified tripartite motif-containing protein 33 (TRIM33) as a factor promoting sensitivity to BETi. We demonstrate that loss of TRIM33 reprograms cancer cells to a more resistant state through at least two mechanisms. TRIM33 silencing attenuates downregulation of MYC in response to BETi. Moreover, loss of TRIM33 enhances TGFβ receptor expression and signaling, and blocking TGFβ receptor activity potentiates the anti-proliferative effect of BETi. These results describe a mechanism for BETi resistance and suggest that combining inhibition of TGFβ signaling with BET bromodomain inhibition may offer new therapeutic benefits.

SIGNIFICANCE STATEMENT

Inhibitors of BET family bromodomain proteins (BETi) have generated considerable excitement and are in clinical trials for treatment of several cancers. Cancers treated with targeted therapies eventually become resistant, yet molecular mechanisms underlying resistance to BETi are poorly understood. To discover novel molecular mechanisms mediating resistance to BETi we performed an shRNA-based loss-of-function genetic screen. We found that loss of TRIM33, a chromatin-associated E3 ubiquitin ligase and established tumor suppressor, confers resistance to BETi. TRIM33 loss diminished BETi-mediated reduction in MYC expression and enhanced TGFβ signaling. Notably, inhibition of TGFβ signaling increased sensitivity of cells to the anti-proliferative effects of BETi. In particular, a TGFβ receptor inhibitor potentiated growth suppression by BETi, suggesting a clinically viable strategy for combination therapy.

INTRODUCTION

Epigenetic regulation of transcription is central to control of cell fate and proliferation. Addition or removal of a variety of specific post-translational modifications of histones affect the recruitment of epigenetic “readers”, proteins that selectively bind to modified sites and recruit transcriptional activators or repressors. Alterations in this complex epigenetic code contribute to a range of diseases, including cancer (1). Consequently pharmacological modulation of enzymes that generate or remove epigenetic modifications and their readers offer new therapeutic opportunities for cancer treatment (2).

The bromodomain and extraterminal domain (BET) proteins are one important class of epigenetic readers involved in transcriptional control (1, 3). The small family of BET proteins (BRD2, BRD3, BRD4 and BRDT) are characterized by tandem bromodomains, that bind acetylated lysine residues in histones and other proteins, and a C-terminal extraterminal domain responsible for interactions with chromatin regulators. BET proteins, in particular BRD4, have been implicated as general regulators of transcription through recruitment of the elongation factor P-TEFb to gene promoters and through interaction with the mediator complex. In addition, high-level recruitment of BRD4 to enhancer regions has been implicated in gene-specific transcriptional activation. Evidence from a variety of approaches has implicated BET proteins, in particular BRD2 and BRD4, in a range of cancers (1, 3-5) and inhibition of BET proteins offers a novel strategy for the treatment of cancer (3, 6). BET inhibitors (BETi) are small molecules that interact with the acetylated lysine binding pocket of the BET family bromodomains (6, 7), interfering with BET protein binding to chromatin and consequent modulation of transcription. BETi were initially shown to be effective in a mouse xenograft model of midline carcinoma, a rare cancer driven by a chromosomal translocation producing a BRD4-NUT fusion protein (6). BETi have subsequently proven to be effective in multiple models of hematologic malignancies (5, 8-13) and solid tumors (14-17) that are not characterized by genetic alterations in BET proteins. One key mechanism by which BETi suppress growth and survival of at least some types of cancer cells is by preferentially repressing transcription of the proto-oncogene MYC, which is often under the control of BRD4 (5, 10, 12, 18). Thus, BETi may provide a new mechanism to target MYC and other oncogenic transcription factors, which lack obvious binding pockets for small molecules and are thus typically considered to be “undruggable”.

The potential of targeting BET proteins in cancer has fueled the development of a variety of BETi, some of which are currently undergoing clinical trials (3). However, lessons from other targeted cancer therapies suggest that acquired resistance will limit long-term responsiveness to BETi treatment. Identification of specific molecular lesions leading to BETi resistance may suggest specific therapeutic strategies for re-sensitizing cells to BETi or for prolonging therapeutic response to BETi. Here, we have performed an shRNA-based genetic screen to identify factors whose loss promoted resistance of colon carcinoma cells to two structurally unrelated BET bromodomain inhibitors. Through this screen, we identified TRIM33 as a factor promoting sensitivity to BETi.

TRIM33 (also called TIF1γ) belongs to a subfamily of tripartite motif-containing (TRIM) E3 ubiquitin ligases that also includes TRIM24 (TIF1α) and TRIM28 (TIF1β). TRIM33 and its relatives are chromatin-associated transcriptional repressors characterized by an N-terminal RING domain and a C-terminal PHD (plant homeodomain)-bromodomain cassette that interacts with post-translationally modified histone tails. TRIM33 has been

characterized as a key factor controlling cell fate decisions during embryonic development (19, 20) and is an established tumor suppressor in pancreatic cancer, hepatocellular carcinoma and chronic myelomonocytic leukemia (21-23). Roles of TRIM33 in development and as a tumor suppressor have been attributed to its ability to strongly modulate transforming growth factor β (TGFβ) signaling through interactions with SMAD family transcription factors (24, 25). TRIM33 can also positively regulate cell cycle progression and survival independently of TGFβ through interactions with the anaphase-promoting complex (26) and lineage-specific transcription factors in leukocytes (19, 27, 28). Here, we find that TRIM33 silencing can inhibit BETi function by attenuating down-regulation of MYC and by potentiating TGFβ signaling. These results identify potential mechanism of clinical resistance to BETi, and suggest avenues for enhancing the efficacy of BETi through combination therapy.

RESULTS

Pooled shRNA library screening identifies TRIM33 as a negative regulator of BETi resistance

To identify genes whose loss confers resistance to the anti-proliferative effects of BET bromodomain inhibitors, we performed a pooled shRNA screen in a BETi-sensitive colorectal cancer cell line (RKO). Screening was carried out in the presence of one of two structurally unrelated inhibitors: the widely used compound JQ1 and a novel BETi GS-626510 (Figure 1A). GS-626510 binds with high affinity and specificity to BET family bromodomains (Figure 1B, Table S1), and a detailed description of its development will be published elsewhere. Both JQ1 and GS-626510 potently inhibited growth of RKO cells with IC_{50} values of 81 nM and 33 nM respectively (Figure 1C). As anticipated for BRD4 inhibition, both compounds strongly decreased MYC levels in RKO cells (Figure 1D). RNAseq analysis showed a strong correlation between genes up- and down-regulated following 3 h treatment of RKO cells with 1 µM of JQ1 or 0.3 µM of GS-626510 (Figure S1A), suggesting that growth suppression by these compounds is attributable to a common mechanism of action.

We generated a custom lentiviral shRNA library containing 5634 shRNA constructs targeting 517 genes annotated as protein kinases and 85 non-targeting control shRNAs. RKO cells were infected with the pooled shRNA virus, and following puromycin selection for infected cells, 6×10^6 cells were removed for genomic DNA extraction to serve as a reference (T0) population. The remaining cells were placed into each of 5 different inhibitor conditions: DMSO vehicle control and low and high doses of either JQ1 or GS-626510 (Figure 1E). Cells were allowed to proliferate and were passaged when they approached confluence. This treatment was maintained until cells reached passage 4 (T4). Genomic DNA was extracted and the relative abundance of each shRNA in each treatment condition at T4, and in the reference T0 condition, was assessed by PCR amplifying the integrated shRNA followed by next generation sequencing (Figure 1E). This allowed calculation of the relative enrichment or depletion of each individual shRNA at T4 compared with T0. As the library contains multiple shRNAs targeting each gene, we used RIGER analysis (29) to identify and rank genes preferentially targeted by hairpins enriched upon drug treatment but not in the DMSO-treated control cells. These genes presumably encode proteins that promote susceptibility to BETi. Silencing expression of these genes thus causes drug resistance, resulting in cells harboring their

respective hairpins being enriched at the end of the screen. Strikingly, TRIM33 was the top ranked enriched target gene in all four BETi-treated conditions, but was not enriched in the absence of inhibitor (Figure 1F). Tracking individual shRNAs revealed clear enrichment of most shRNAs targeting TRIM33 at T4 in the presence of JQ1 or GS-626510 (Figure 1G). By contrast, TRIM33 hairpins appear to be preferentially depleted in the DMSO vehicle control sample. An independent replicate of this screen, carried out to passage 5 (T5) produced very similar results with TRIM33 ranked in the top 3 of all four drug conditions (Figure S1B). Thus, data from two independent screens, each performed with two doses of two chemically unrelated BET bromodomain inhibitors, indicate that TRIM33 knockdown confers a selective growth advantage in BETi-treated RKO cells. Notably, TRIM24, the most closely related TRIM33 family member, was also highly enriched in all four inhibitor treated conditions but not in the DMSO control (Figure 1F, S1B), supporting the potential functional relevance of TRIM33 to modulation of BETi sensitivity. TRIM33 and TRIM24 were included in our shRNA library on the basis of early reports identifying TRIM24 and TRIM28 as protein kinases (30, 31), but the absence of a recognizable kinase catalytic domain and lack of subsequent verification suggests that these proteins are unlikely to have such activity.

BETi resistance in shTRIM33 cells is due to the specific loss of TRIM33 protein

To verify our screening data suggesting that TRIM33 promotes sensitivity to BETi in cancer cells, we established stable TRIM33 knockdown RKO cells by lentiviral transduction and evaluated their sensitivity to JQ1 or GS-626510. Among four individual shRNAs tested, we chose shTRIM33-B5 (hereafter referred to as shTRIM33 unless otherwise noted) to silence expression of TRIM33 as it produced the most efficient TRIM33 knockdown at the protein level (Figure 2A). Comparison of cell proliferation of shCTRL and shTRIM33 cells in 15-day cultures confirmed that knocking down TRIM33 conferred a growth advantage in the presence of BETi (Figure 2B). Notably, consistent with the screening data, shTRIM33 cells cultured in the absence of inhibitor exhibit a growth disadvantage (Figure 2B), suggesting that the effect of TRIM33 on growth in the presence of BETi is not due to a basal increase in cell proliferation. We extended these studies to compare the potency of JQ1 and GS-626510 in shCTRL and shTRIM33 cells. Cells were incubated with varying concentrations of JQ1 or GS-626510 for 5 days and the relative cell number was determined. TRIM33 knockdown produced a rightward shift in the growth inhibition curves for both JQ1 and GS-626510 (Figure 2C). Multiple replicates revealed that the IC_{50} value of JQ1 and GS-626510 was increased by approximately 3-fold in shTRIM33 cells, suggesting the shTRIM33 cells are more resistant to BETi (Figure 2D). This effect is not limited to RKO cells as similar experiments performed in a panel of colorectal, breast and prostate cancer cell lines revealed that TRIM33 knockdown also decreased sensitivity to JQ1 and GS-626510 in a subset of the cell lines tested (Figure S2A,B). Finally, in prolonged culture TRIM33 knockdown facilitates outgrowth of BETi-treated RKO cells (Figure 2E). Similar effects were observed with a different shRNA targeting TRIM33 (A12) (Figure 2A, S2C), suggesting that results are not due to off target effects.

To further confirm that BETi resistance caused by TRIM33-directed shRNA is due to the loss of TRIM33 protein and not due to off target silencing of other genes, we generated rescue RKO cell lines re-expressing a knockdown-resistant TRIM33 cDNA (Figure 2F,G). shTRIM33 cells re-expressing TRIM33 (pLenti-TRIM33), but not those infected with an empty vector (pLenti-EV) became more sensitive to both JQ1 and GS-626510 in long-term culture assays (Figure 2F, bottom panel, S2D). Furthermore, in these

experiments, overexpression of TRIM33 in shCTRL cells increased sensitivity to both compounds (Figure 2F, top panel, S2D). Together, our data support the idea that TRIM33 promotes sensitivity to BET bromodomain inhibition.

TRIM33 knockdown maintains MYC expression following BETi

Given the established role of both TRIM33 and BET proteins as transcriptional regulators, we hypothesized that shTRIM33-mediated BETi resistance could be due to deregulated gene transcription. We therefore used RNAseq to investigate changes in gene expression resulting from treatment with BETi and with loss of TRIM33 (Table S2). RNAseq was performed in shCTRL and shTRIM33 cells after 3h treatment with JQ1 (1 μM), GS-626510 (0.3 μM) or vehicle control (DMSO). Results from two independent replicate experiments were analyzed by DESeq. Results consistent with RNAseq data were obtained by measuring mRNA levels for 15 genes by qRT-PCR (Figure S3A-B).

In keeping with previous reports (5, 10), 3-hour BETi treatment had a broad impact on gene expression: among the 11,277 genes reliably detected by RNAseq, approximately 1200 genes changed by greater than 2-fold (Figure 3A-B). Consistent with prior studies in other cell types (5, 10), BETi treatment of RKO cells strongly reduced levels of *MYC* (5 to 6-fold). Furthermore, gene set enrichment analysis (GSEA) of transcripts down-regulated by both inhibitors revealed significant enrichment for genes having target motifs for MYC or the MYC co-activator MAZ in their promoter regions (20% of downregulated genes, Figure 3C). In contrast to BET bromodomain inhibition, TRIM33 KD influenced the expression of a relatively small fraction of genes (Figure 3D). Following TRIM33 knockdown, 272 transcripts were up regulated by at least 2-fold, while only 84 were down-regulated by at least 2-fold, arguing that TRIM33 works preferentially as a transcriptional repressor rather than an activator (32). Notably, loss of TRIM33 had no effect on expression of BET genes (BRD2, BRD3 and BRD4) themselves and did not affect BRD4 protein levels (Table S2, Figure S3C).

Repression of MYC is believed to be a major mechanism by which BETi suppress growth of some cell types (10, 12). We therefore examined a potential role for MYC in mediating the effect of TRIM33 knockdown. Consistent with our RNAseq data (Figure 4A), 3 h of treatment with either JQ1 or GS-626510 strongly suppressed *MYC* mRNA levels as measured by qRT-PCR (Figure 4B). Furthermore, presumably due to the short (20-30 min) half-life of MYC protein (33), MYC protein levels were also strongly suppressed (Figure 4C). While basal levels of *MYC* mRNA and protein were modestly increased in shTRIM33 cells, we found that their downregulation by BETi was substantially attenuated (Figure 4B-4C). Furthermore, rescue of TRIM33 protein expression in shTRIM33 cells partially restored MYC sensitivity to JQ1 and GS-626510 (Figure 4B-4C). These results suggest that TRIM33 is required for the ability of BET inhibitors to maximally down regulate MYC. To determine whether stabilization of MYC may play a role in conferring resistance to BETi, we stably over-expressed MYC in RKO cells. Ectopically expressed MYC was resistant to BETi-mediated down regulation (Figure 4D). We found that while RKO cells overexpressing MYC proliferated at the same rate as control cells, possibly reflecting the high basal levels of MYC expression in this cell line, MYC over-expressing cells had a growth advantage in long-term culture in the presence of JQ1 or GS-626510 (Figure 4E, F). Thus, protection of MYC levels from downregulation is likely to contribute to BETi resistance in shTRIM33 RKO cells.

Consistent with a role for TRIM33 in regulation of MYC expression, chromatin immunoprecipitation (ChIP) revealed that TRIM33 associates with the MYC promoter in

BETi-treated RKO cells (Fig S4A). Notably, BRD4 ChIP showed that BRD4 associated with similar sites in the MYC promoter and that BRD4 was displaced following BETi treatment (Fig S4B). These data suggest that BETi may suppress MYC expression by displacing BRD4 from the MYC promoter to allow recruitment of the transcriptional repressor TRIM33 at that site. In the absence of TRIM33 this negative regulation would be lost, rendering cells less sensitive to BETi

TRIM33 knockdown potentiates TGFβ signaling and inhibition of TGFβ pathway increases BETi sensitivity

While the efficacy of BETi has been linked to down-regulation of MYC expression in hematopoietic cancers and a subset of solid tumors, in other tumor cells BETi-mediated growth suppression is independent of MYC (15, 34). Notably, in contrast to what we observed in RKO cells, MYC levels in another colorectal cancer cell line, SK-CO-1, were much less sensitive to either BETi treatment or TRIM33 knockdown (Fig S4C). Nonetheless, in this cell line TRIM33 knockdown conferred resistance to BETi (Fig S2A S2B).This observation suggests that other pathways in addition to MYC signaling can contribute to shTRIM33 cell resistance to BETi. Gene set enrichment analysis (GSEA) of the RNAseq data revealed that the two signatures most differentially regulated by BETi-treatment in shCTRL vs. shTRIM33 RKO cells corresponded to genes targeted by TGFβ signaling (Figure S5A). Modulation of TGFβ target genes in the context of BET inhibition was of interest because TRIM33 has been implicated as a regulator of TGFβ signaling (25, 35). Furthermore, as TGFβ signaling can promote resistance to other targeted therapies (36), we investigated how the pathway was altered in shTRIM33 RKO cells. Canonical TGFβ signaling involves TGFβ ligand-induced formation of heterotetramers containing dimers of the TGFβ receptor I (TβRI) and TGFβ receptor II (TβRII) serine-threonine kinases. Receptor clustering promotes TβRII phosphorylation of TβRI, leading to recruitment and phosphorylation of regulatory SMADs (SMAD2/3) by TβRI. Phosphorylated SMAD2/3 then binds to SMAD4 to form a complex that enters the nucleus to drive transcription of target genes. Stimulation of control and shTRIM33 cells with recombinant TGFβ1 ligand revealed that phosphorylation of SMAD2 was dramatically potentiated in the absence of TRIM33 (Figure 5A). Thus, under conditions where control cells exhibited barely detectable responses to TGFβ1, SMAD2 was robustly phosphorylated in shTRIM33 cells. These changes were not due to differences in the expression level of SMAD2, which appeared uniform in control and shTRIM33 cells (Figure 5A). TGFβ1-induced phosphorylated SMAD2 (pSMAD2) seen in shTRIM33 cells co-immunoprecipitated with SMAD4, suggesting that the pSMAD2 enters functional complexes with SMAD4 (Figure 5B). Previous reports have suggested that TRIM33 antagonizes TGFβ signaling by negatively regulating SMAD4 through either mono-ubiquitinating SMAD4 or competing with SMAD4 for phosphorylated SMAD2/3 (24, 25). However, knockdown of SMAD4 in shTRIM33 cells had no impact on the TGFβ1-mediated induction of pSMAD2 (Figure 5C). These results suggest that loss of TRIM33 in RKO cells potentiates TGFβ signaling upstream of SMAD4, at the level of SMAD2 phosphorylation.

Our RNAseq data showed that the TβRII mRNA is upregulated ~2 fold in shTRIM33 cells (Figure 5D). Furthermore, ChIP experiments revealed that TRIM33 association with the TβRII promoter is increased by BETi while BRD4 association is decreased (Fig S4D-E), similar to the manner that MYC is regulated by TRIM33 and BRD4. To investigate whether TβRII up-regulation could underlie the potentiation of TGFβ signaling that accompanies loss of TRIM33, we employed two different shRNAs to knockdown TβRII

and assessed SMAD2 phosphorylation. Both shRNAs efficiently reduced TβRII mRNA levels (Figure 5E) and in shTRIM33 cells they dramatically reduced TGFβ1-induced pSMAD2 levels (Figure 5F). Notably, when we assessed the sensitivity of these cells to JQ1 or GS-626510 growth inhibition we found that loss of TβRII re-sensitized the shTRIM33 cells to the BET bromodomain inhibitors (Figure 5G, right panel, S5B). TβRII knockdown also increased sensitivity of control cells to BETi (Figure 5G, left panel, S5B). These data suggest that a combination of TGFβ pathway inhibitors and BET bromodomain inhibitors may provide a more potent inhibition of cell growth and may provide a means to overcome resistance to BET bromodomain inhibitors. To test this possibility directly we used the small molecule TβRI inhibitor LY2157299 (galunisertib) (37, 38). Treatment with LY2157299 at a dose that can substantially block TGFβ1-stimulated pSMAD2 (Figure 5H) greatly increased the anti-proliferative effect of JQ1 or GS-626510 in shTRIM33 cells, yet alone had no effect on cell growth (Figure 5I, S5C). As with silencing of TβRII expression, chemical inhibition of TβRI also sensitized shCTRL cells to BETi. Interestingly, sensitization of shTRIM33 cells to BETi by treatment with LY2157299 was not accompanied by down regulation of MYC (Figure 5J). Thus, results with both TβRII knockdown and small molecule inhibitors of TβRI strongly suggest that TRIM33 promotes sensitivity to BETi at least in part through attenuation of TGFβ signaling.

Finally, to determine whether enhanced TGFβ signaling is sufficient to induce resistance to BETi we examined the consequences of over-expressing TβRII. Robust TGFβ1-induced SMAD2 phosphorylation was detected in TβRII-overexpressing cells but not in the empty vector control cells (Figure S5D). However, this was insufficient to confer resistance to either JQ1 or GS-626510 (Figure S5E). TβRII over-expression also failed to protect MYC levels from downregulation by BETi treatment, even in the presence of exogenously added TGFβ1 (Figure S5F). Taken together, these results suggest that TRIM33 knockdown confers resistance to BETi through combined independent effects on MYC transcription and TGFβ signaling.

DISCUSSION

The recent discovery of small molecule BET bromodomain inhibitors and the demonstration of their potent anti-proliferative activity in hematological and solid tumors highlights the potential of BETi as anti-cancer agents. As a recurring limitation to targeted anti-cancer therapies is the acquisition of drug resistance, in this study we used pooled shRNA screening to identify genes whose silencing protects RKO colon cancer cells from two chemically distinct BETi: the originally characterized BET inhibitor, JQ1 (6), and a newly developed inhibitor GS-626510. The top hit from the screen was TRIM33, with its close family member TRIM24 also being identified. These data suggest that loss of TRIM33 confers resistance to BETi, and we confirmed this in both short and long-term growth assays. Mechanistically, loss of TRIM33 reduces BETi-mediated down-regulation of MYC and sensitizes cells to TGFβ signaling. Notably, inhibition of TGFβ signaling re-sensitizes TRIM33 knockdown cells to BETi, suggesting that combining TGFβ inhibitors with BETi may have therapeutic benefit.

Multiple studies have pointed to the oncogenic transcription factor MYC as a target of BETi in both hematopoietic and solid tumor cell lines (10, 12, 13). As shown previously for JQ1 treatment, we found that both BETi employed in our study strongly decreased MYC mRNA and protein levels in RKO colorectal cancer cells, and potently inhibited cell growth. Previously it was shown that ectopic expression of MYC partly protected a multiple myeloma cell line from the growth inhibitory effects of JQ1, affirming MYC suppression to be a major mechanism underlying growth suppression by BETi. By contrast, it was reported that in lung adenocarcinoma cell lines, JQ1 suppressed growth by downregulating the transcription factor FOSL1 rather than MYC (15), suggesting that alternative mechanisms may underlie the activity of BETi in solid tumors. We observed that MYC overexpression in RKO cells attenuated the efficacy of BETi. In addition, RNAseq analysis showed no reduction in FOSL1 transcript level upon BETi treatment of RKO cells. These observations support a central role for MYC as a key transcriptional target for BET bromodomains in colorectal cancer.

To identify genes whose loss conferred resistance to BETi, we performed a pooled shRNA screen with a library targeting genes annotated as protein kinases. We found that loss of TRIM33 conferred resistance to either JQ1 or GS-626510 treatment, indicating that TRIM33 is required, in at least some cell types, for cells to be fully sensitive to BETi. In such cells, TRIM33 appears to promote downregulation of MYC by BETi. Classically TRIM33, TRIM24 and TRIM28 act as potent transcriptional co-repressors when recruited to the promoters of target genes, and consistent with this mechanism, we found TRIM33 to associate with the MYC promoter. Notably this association is enhanced by BETi, possibly due to direct competition between BRD4 and TRIM33 for binding at these sites. Transcriptional modulation of MYC by TRIM33 could involve its E3 ligase activity, for example by triggering ubiquitin-mediated degradation of factors co-associated with promoter or enhancer regions. We attempted to test this model using TRIM33 mutants with impaired E3 ligase activity. Mutant TRIM33, while unable to restore JQ1 sensitivity in shTRIM33 cells, was also very poorly expressed, making it unclear whether its ligase activity was essential (data not shown).

While our study was underway, several other groups reported alternative mechanisms of BETi resistance in other cancer lines (39-43). While the details of the specific adaptive pathways vary across cell types, a common feature of BETi resistance appears to be reactivation of BRD4-dependent target genes. Most of these reported models of resistance involve the emergence of mechanisms to drive MYC expression in the presence of BETi. For example, up-regulation of the transcription factor GLI2 contributes to acquired BETi-resistance in pancreatic cancer cells (43) by driving MYC expression, and in models of acute myeloid leukemia (AML) (39, 41), increased WNT signaling apparently bypasses BET bromodomain-mediated transcription to maintain MYC expression through utilization of a cryptic enhancer region. Our data show that loss of TRIM33 partially protects MYC levels after BETi treatment, but we did not find that loss of TRIM33 affected β-catenin levels or localization in RKO cells (data not shown). Furthermore, as judged by RNAseq analysis, we found that TRIM33 knockdown did not induce GLI2 in RKO cells. Thus, while TRIM33 knockdown apparently confers BETi-resistance at least in part by preventing MYC downregulation, the pathways involved are distinct from those previously characterized. In cell lines where BETi function independently of MYC, reported mechanisms of resistance likewise appear to involve maintaining expression of BRD4-target genes. For example, triple negative breast cancer cells can acquire BETi-resistance through BRD4 hyperphosphorylation, which drives expression of target genes through interactions with the mediator complex in a

manner independent of the acetylated lysine binding pocket of its bromodomains(42). As with each of these described mechanisms of resistance, sparing of critical target genes appears to be an important component of BETi resistance caused by loss of TRIM33.

While multiple studies have addressed adaptive responses to BETi and mechanisms of acquired resistance, much less is understood about factors controlling intrinsic susceptibility of tumors to BETi. Mutations in PIK3CA appear to confer intrinsic resistance to BETi in breast cancer cell lines, yet the molecular basis for this phenomenon is currently unknown(40). Across a panel of cell lines tested, we found no correlation between the level of TRIM33 protein expression and sensitivity to BETi (data not shown), suggesting that TRIM33 status is not predictive of intrinsic resistance. It remains to be seen whether loss of TRIM33 will be a clinically important mechanism for acquired resistance to BETi.

A short isoform of BRD4 was recently shown to be an inhibitor of DNA damage response signaling by influencing chromatin structure independently of its role as a transcriptional activator(44). Resistance to BETi could thus theoretically arise by reduction of DNA damage signaling, bypassing growth arrest, We found, however, that TRIM33 knockdown did not alter DNA damage signaling as assessed by γH2AX staining (data not shown) suggesting that an alternative resistance pathway must be involved.

Consistent with prior reports implicating TRIM33 in TGFβ signaling we find that loss of TRIM33 sensitizes cells to TGFβ. However, in contrast to previous studies suggesting that TRIM33 acts as an E3 ubiquitin ligase for SMAD4, we find loss of TRIM33 strongly enhances SMAD2 phosphorylation independently of SMAD4 and is associated with increased expression of TβRII. TRIM33 may therefore act as a direct modulator of TβRII gene transcription. Importantly, downregulation of TGFβ signaling, either by silencing TβRII expression or with a small molecule inhibitor of TβRI, sensitizes TRIM33 knockdown cells to BETi. Notably, while overexpressing TβRII is sufficient to sensitize cells to TGFβ1, it does not prevent BETi-mediated suppression of MYC levels or cell growth. Thus while promoting TGFβ signaling cannot explain all of the effects of TRIM33 knockdown on BETi sensitivity, inhibition of TGFβ signaling is nonetheless sufficient to sensitize cells to BETi.

How increased TGFβ signaling contributes to BETi resistance is unclear, but it is noteworthy that in non-small cell lung cancer cell lines, knockdown of mediator complex component MED12 confers resistance to multiple kinase inhibitors through a transcription-independent mechanism that results in stabilization of TβRII (36). Likewise, knockdown of the transcription factor SOX10 in melanoma cell lines induces BRAF inhibitor resistance by induction of TβRII and TGFβ signaling, ultimately resulting in increased receptor tyrosine kinase expression (45). In both of these contexts, TGFβ-induced resistance to targeted therapies is associated with enhanced signaling through the ERK MAP kinase pathway. Notably, in addition to up-regulated Wnt signaling, BETi-resistance in AML was also associated with up-regulated TGFβ-dependent gene expression (39, 41). These observations are consistent with our finding that potentiated TGFβ signaling contributes to shTRIM33-mediated BETi resistance and suggests that TGFβ inhibitors may be valuable in combination with BETi in a range of malignancies. The ability of TGFβ inhibitors to potentiate the effect of BETi and to function in the

setting of TRIM33 loss provides a potential clinical strategy to overcome or delay acquired resistance.

EXPERIMENTAL PROCEDURES

Cell lines, antibodies and drugs. Cell lines 293T, RKO, HCT15, HCT116, LoVo, SW620, SW837, SK-CO-1, SW480, SW1463, MDA-MB-231, MDA-MB-415, MDA-MB-468, ZR-75-1, LNCap and PC-3 were obtained from ATCC and maintained as suggested. Antibodies were purchased from Cell Signaling Technology and Abcam: TRIM33 (#13387), SMAD2 (#5339), pSMAD2 (#3108), SMAD4 (#9515), BRD4 (#13440), actin (#3700) and MYC (ab32072). Recombinant human TGFβ1 was from Cell Signaling Technology (#8915LC). (+)-JQ1 (11187) was purchased from Cayman Chemical and LY2157299 (S2230) was purchased from Selleck Chemical. GS-626510 was synthesized at Gilead Sciences and details will be published elsewhere.

Stable knockdown and expression cell lines. Lentiviral expression vectors for shRNAs in the pLKO.1 puro vector (Sigma) were used to stably knockdown TRIM33, TβRII or SMAD4. For stable knockdown of two genes, the shTRIM33-B5 sequence was cloned into pLKO.1 blast (Addgene #26655) to silence TRIM33 expression. The shRNA target sequences used are in Table S3. For expression of TGFβRII and TRIM33, cDNAs from Addgene #19147 and Addgene #15734 respectively, were cloned into pLentiCMV-hygro(DEST) (Addgene #17454)) through Gateway cloning (Invitrogen). Seven silent mutations were made to TRIM33 cDNA to render resistance to shTRIM33-B5. MYC lentiviral expression vector is from Addgene (#46970).

Pooled shRNA screening. Pooled shRNA screens were performed similarly as described (29). Details are provided in Supplemental Experimental Procedures.

Cell lysis for immunoblotting and immunoprecipitation. For immunoblotting, cells in 6-well plates were quickly rinsed twice with PBS and directly lysed in 150 µL SDS lysis buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol). The lysate was then transferred to 1.5 mL Eppendorf tubes and heated for 10 min at 95-100 ºC with intermittent vortexing. After spinning to remove any undissolved material and measuring the protein concentration using BCA assay, 20-40 µg total lysate was fractionated by SDS-PAGE and transferred to nitrocellulose membrane for immunoblotting. For immunoprecipitation, cells were rinsed quickly with ice-cold PBS and lysed in buffer (50 mM HEPES pH7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 25 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF and Roche Complete Protease Inhibitor Cocktail) on ice for 15 min. Scraped cell lysate was centrifuged at 13,200 rpm for 10 min at 4 ºC and 1 mg of supernatant was incubated with 1-5 µg primary antibody overnight at 4 ºC. 25 µL of protein A sepharose 4B (Invitrogen) was added to the tube for another 2 h, and the precipitate was washed 3 times and then eluted in 60 µL of Laemmli sample buffer. 20 µL of the elution was used for immunoblotting.

Quantitative RT-PCR analysis. Total RNA was extracted using an RNeasy® mini kit (Source) with on-column DNA digestion. 1 µg of total RNA was used for cDNA synthesis with the iScript™ cDNA synthesis kit (Bio-Rad) as per manufacturer's suggestion. Real-time PCR was performed on a Bio-Rad CFX Connect™ Real-Time System and relative mRNA level was calculated in CFX Manager software using the 2^ (-ΔΔCt) method. GAPDH mRNA was used as internal control. PCR primer sequences are listed in Table S4

Cumulative cell growth assay. RKO cells (3 x 10^5) transduced with the indicated virus were plated in a single well of a 6-well plate at day 0 in the presence or absence of inhibitors. Three days later cells were detached, counted, and 3 x 10^5 cells were transferred to a new well. The process was repeated until day 15. The cumulative cell number was then calculated from fold changes and the individual cell counts at each passage.

Crystal violet cell proliferation assay. Cells (5-20 x 10^3) were plated in each well of a 6-well plate with 3 mL of media with or without inhibitors and cultured for 14 days undisturbed. Medium was aspirated, and cells were stained with crystal violet staining solution (0.05% w/v crystal violet, 1% formaldehyde, 1% methanol in PBS) for 30 minutes and washed with water several times. Stained plates were then air-dried and imaged with ChemiDoc® using Image Lab software (Bio-Rad). To quantify the crystal violet staining, 1 mL of 10% acetic acid was added to each well to solubilize the stain for 20 min and the stain was diluted 1:4 in water and absorbance was measured at 590 nm.

Growth inhibition assay and IC_{50} value determination. Cells (1000 per well) were plated in 96-well plates in duplicate with 1:3 serial dilutions of BETi ranging from 0.169 nM to 10 µM or 0.1% DMSO vehicle and cultured for 120 h. The end point relative viable cell number was determined using CellTiter Glo by quickly decanting the media, adding 100 µL of 1:2 CellTiter Glo reagent diluted in PBS to the well and incubating for 10 min. The luminescence of each well was read with a TECAN Infinite M1000Pro plate reader. IC_{50} values were calculated with Prism 6 (GraphPad) by fitting the data to the “3-parameter log (inhibitor) vs response” equation. At least three independent growth inhibition assays were performed for each pair of cell lines to derive mean IC_{50} values.

RNAseq data analysis and gene set enrichment analysis. See Supplemental Experimental Procedures.

AUTHOR CONTRIBUTIONS

X.S. carried out the majority of experimental work and data analysis. V.T.M. prepared pooled virus and performed the screen. L.K. helped prepare the shRNA library and performed RIGER analysis. F.C. performed cumulative cell growth assays, BETi dose-response assay on MYC level and validation of RNAseq by quantitative RT-PCR as well as CHIP-PCR experiments. S.V. provided technical support. M.B. helped establish the screening platform. D.S and R.M. synthesized GS-626510. P.Y. performed GSEA analysis. J.G.B. and D.G.B were involved in the development of GS-626510 and in project design. X.S., J.S., B.E.T. and D.A.C. conceived the project, designed experiments, analyzed data, and wrote the manuscript.

ACKNOWLEDGEMENTS

We thank Phillip B. Murray for help with the shRNA mapping pipeline and Francesc Lopez-Giraldez for help with RNAseq mapping software.

CONFLICT OF INTEREST

David Sperandio, Peng Yue, Jamie Bates, and David Breckenridge are employees of Gilead Sciences, Inc.

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FIGURE LEGENDS

Figure 1. shRNA screening reveals TRIM33 as a regulator of BETi resistance in cancer cells. (A) Structures of the two different BETi used in this study, JQ1 and GS-626510. (B) K_D values of GS-626510 for 40 bromodomains (Table S1) were determined using BROMOscan™ (DiscoveRx). The dendrogram Image was generated using TREEspot™ Software Tool and reprinted with permission from KINOMEscan DiscoveRx Corporation, © DISCOVERX CORPORATION 2010. (C) Dose-dependent inhibition of RKO cell proliferation by JQ1 and GS-626510 in a 5-day assay. Relative viable cell number was determined by CellTiter Glo assay. (D) GS-626510 and JQ1 both down-regulate MYC protein levels. RKO cells were treated with increasing concentrations of BETi for 3 h and MYC levels in whole cell lysates were assessed by immunoblotting. Actin was used as a loading control. (E) Scheme of shRNA screening procedure. Cells infected by the pooled shRNA library were propagated through 8 doublings in presence of either DMSO vehicle control or different concentrations of JQ1 or GS-626510. Genomic DNA was extracted from the T0 (reference) and T4 conditions for determination of proviral shRNA abundance. (F) Top 10 enriched target genes revealed by RIGER analysis in each condition. (G) Multiple individual TRIM33 shRNAs are enriched in BETi-treated, but not in DMSO control treated, conditions. Log_2 fold change (T4/T0) of each shRNA is plotted from the most depleted to the most enriched. Each red line represents a single shRNA targeting TRIM33.

Figure 2. Loss of TRIM33 confers resistance to BETi. (A) Top, schematic of TRIM33 domain organization and positions of two pairs of RT-PCR primers. Middle, *TRIM33* mRNA levels determined by RT-PCR in shCTRL cell line and cell lines expressing four different TRIM33-targeting shRNAs. Bottom, TRIM33 protein levels in these cell lines. (B) shCTRL or shTRIM33 cells were seeded in a 6-well plate (3×10^5 cells per well) in the presence of DMSO, 100 nM JQ1 or 50 nM GS-626510 and cumulative cell numbers

were assessed every 3 days for up to 15 days. (C) Growth inhibition assay. shCTRL and shTRIM33 cells were cultured with different concentrations of JQ1 or GS-626510 for 120 h and relative cell numbers were determined using CellTiter Glo. (D) IC_{50} values (mean ± SEM) were calculated from 5 independently performed growth inhibition assays using shCTRL and shTRIM33 cells. *P* values are based on paired *t*-test. (E) 2×10^4 shCTRL or shTRIM33 cells were plated in 6-well plates, treated with DMSO, 100 nM JQ1, or 50 nM GS-626510 for two weeks and then stained with crystal violet. The crystal violet staining was quantified at 590 nm absorbance. (F) shCTRL or shTRIM33 cells were transduced with either an empty vector control or TRIM33-expressing lentivirus and cell growth was assessed as in (E). (G) TRIM33 expression levels in cells from (F) were assessed by immunoblotting .

Figure 3. RNAseq analysis of vehicle or BETi-treated shCTRL or shTRIM33 cells. Waterfall plots show gene expression changes induced by 3 h treatment of shCTRL RKO cells with 1 μM JQ1 (A) or 0.3 μM GS-626510 (B). *MYC* (red) is down-regulated by both JQ1 and GS-626510. (C) Top 10 sequence motifs enriched in promoter regions of genes down-regulated >2-fold by JQ1 and GS-626510 in shCTRL cells were determined by Gene Set Enrichment Analysis (Broad Institute). (D) Gene expression changes induced by shTRIM33 in RKO cells.

Figure 4. TRIM33 modulates MYC sensitivity to BETi. (A) Normalized RNAseq reads of *MYC* mRNA from two replicate experiments before and after JQ1 or GS626510 treatment. (B) RT-PCR quantification of *MYC* mRNA in shCTRL, shTRIM33 and shTRIM33 rescued ($shTRIM33^{RES}$) cells, either untreated or treated with BETi for 3 h. (C) cells treated similarly as in panel (B) were analyzed for MYC protein. (D) MYC protein level in control or MYC over-expressing cells before and after BETi treatment for 3 h. (E) Crystal violet staining of control or MYC over-expressing cells growing with DMSO, JQ1 or GS-626510 for two weeks. (F) Cumulative cell growth of control or MYC-overexpressing cells over 15 days.

Figure 5. Inhibition of TGFβ signaling potentiates the anti-proliferative effects of BETi. (A) TGFβ1 ligand stimulated phosphorylation of SMAD2 is potentiated in shTRIM33 cells. shCTRL or shTRIM33 RKO cells were treated with increasing doses of TGFβ1 for 25 min (left panel) or with 2 ng/ml TGFβ1 for various times (right panel), cells were lysed and immunoblotted for phospho-SMAD2 (pSMAD2), total SMAD2 and TRIM33. (B) shCTRL or shTRIM33 cells were untreated or treated with 100 pM of TGFβ1 for 25 min and SMAD4 was immunoprecipitated. Co-precipitating pSMAD2 was assessed by immunoblotting. (C) shCTRL or shTRIM33 cells were infected with lentivirus encoding shCTRL or one of two hairpins targeting SMAD4 (shSMAD4-3 or shSMAD4-4). Cells were untreated or treated with 100 pM of TGFβ1 for 25 min SMAD4, pSMAD2 and total SMAD2 levels were assessed by immunoblotting. (D) TGFβ receptor II (*TβRII*) mRNA from RNAseq in shCTRL and shTRIM33 cells. (E-G) Inhibition of TGFβ pathway by silencing TβRII increases the magnitude of cell growth inhibition by BETi. (E) RT-PCR quantification of *TβRII* mRNA levels in shCTRL and shTRIM33 cells expressing control (shCTRL) or two different TβRII-targeting shRNAs (shTβRII-3 and shTβRII-4). (F) Cells from (E) were stimulated with 100 pM of TGFβ1 for 25 min and pSMAD2 levels assessed by immunoblotting. (G) shCTRL cells (left) or shTRIM33 cells (right) expressing control and TβRII-targeting shRNAs were cultured for 2 weeks with DMSO or different concentrations of BETi (as indicated) and then stained with crystal violet. (H-J) The TβRI inhibitor LY2157299 potentiates BETi-mediated inhibition of cell proliferation. (H) shTRIM33 cells were pre-treated with increasing doses of LY2157299 and then

exposed to 100 pM TGFβ1 for 25 min. Immunoblotting shows dose-dependent inhibition of pSMAD2 by LY2157299. (I) shCTRL and two shTRIM33 KD cell lines were cultured in the presence of JQ1 or GS-626510, with or without LY2157299 for 2 weeks and stained with crystal violet. (J) shCTRL or shTRIM33 cells were treated with 1 μM JQ1 or 0.3 μM GS-626510 with or without 5 μM LY2157299 overnight and MYC protein levels were assessed by immunoblotting.