Neuronal differentiation and cell-cycle programs mediate response to BET-bromodomain inhibition in MYC-driven medulloblastoma

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BET-bromodomain inhibition (BETi) has shown pre-clinical promise for MYC-amplified medulloblastoma. However, the mechanisms for its action, and ultimately for resistance, have not been fully defined. Here, using a combination of expression profiling, genome-scale CRISPR/Cas9-mediated loss of function and ORF/cDNA driven rescue screens, and cell-based models of spontaneous resistance, we identify bHLH/homeobox transcription factors and cell-cycle regulators as key genes mediating BETi’s response and resistance. Cells that acquire drug tolerance exhibit a more neuronally differentiated cell-state and expression of lineage-specific bHLH/homeobox transcription factors. However, they do not terminally differentiate, maintain expression of CCND2, and continue to cycle through S-phase. Moreover, CDK4/CDK6 inhibition delays acquisition of resistance. Therefore, our data provide insights about the mechanisms underlying BETi effects and the appearance of resistance and support the therapeutic use of combined cell-cycle inhibitors with BETi in MYC-amplified medulloblastoma.
Myc-driven group 3 medulloblastoma is an aggressive pediatric brain tumor that is refractory to intensive multimodal therapy\(^1-3\). We and others have shown BET-bromodomain inhibition (BETi) to be a potential therapeutic strategy to target MYC-driven medulloblastomas and other cancers\(^4-9\). BET-bromodomain proteins bind to H3K27ac enhancers across the genome to recruit transcriptional complexes, thereby facilitating the expression of thousands of genes\(^10\). These include genes that regulate progression through the cell cycle and genes that mediate commitment of cell fate and differentiation in a context specific manner\(^4,10-14\). The transcriptional and phenotypic effects of BETi have been ascribed to reduced expression of MYC, cell-cycle regulators and stem-like transcriptional programs, in addition to global suppression of super-enhancer regulated genes\(^10\). However, it is currently unclear which of these BETi-modulated genes directly contribute to the growth-suppressing effects of BETi.

We hypothesized that genes that were required for medulloblastoma cell line growth and were sufficient to rescue the effects of BETi would represent key downstream effectors of this class of therapeutics. Here, we report a systematic approach to identify such genes using a combination of CRISPR/Cas9-based dependency screening, ORF/cDNA-mediated drug-rescue screens, and spontaneous models of drug resistance.

We found that BETi response is mediated by suppression of a combination of genes that regulate neuronal differentiation programs and of genes that regulate progression through the cell cycle. Furthermore, re-expression of these genes attenuates response to BETi by inducing a neuronally differentiated phenotype, while also maintaining proliferative capacity.

**Results**

**A genomics approach to identify mediators of BETi response.** Noting that BET-bromodomain inhibitors mediate their effects through suppression of genes across the genome (Fig. 1, top panel), we sought to identify specific mediators of response to BET-bromodomain inhibition (BETi). We applied an integrative genomics approach using data from three sources: first, expression profiling of genes that are suppressed following BETi; second, genome-scale CRISPR/Cas9 screens to determine which of the suppressed genes are also essential for cellular viability; and third, a near-genome scale open reading frame (ORF) rescue screen to determine which of the suppressed genes are also sufficient to drive resistance to BETi (Fig. 1, bottom panel). In this way, we systematically evaluated which BETi target genes are both required for cellular proliferation, and able to rescue BETi phenotypes. We considered genes that were nominated by all three assays to be responsible for BETi-induced reductions in cell viability. We also validated the role of these genes and pathways in cells that acquired spontaneous tolerance to BETi.

**BETi generates widespread changes in expression.** We characterized the extent and uniformity of transcriptional effects of BETi in medulloblastoma models through expression profiling of four MYC-driven medulloblastoma cell lines treated with the BET-bromodomain inhibitor JQ1, relative to vehicle controls (Supplementary Data File 1 and Supplementary Figs. 1 and 2). The transcriptomic effects of BETi were widespread. For example, within the D458 and D283 cell lines, we respectively observed 5241 and 4762 genes to be downregulated following treatment with JQ1 (FDR ≤ 0.1) (Supplementary Data File 1). However, many of these expression changes are unlikely to affect cell survival, and therefore do not mediate BETi proliferation and viability effects.

**Genes suppressed by BETi tend to be cell-essential.** We therefore determined which of the suppressed genes are cell-essential. We applied a pooled CRISPR/Cas9 screen targeting 18,454 genes (Supplementary Fig. 3A) to each of the D458 and D283 cell lines. Cas9-expressing cells were infected with a genome-scaled pooled guide RNA (sgRNA) lentiviral library and passed for 21 days. We considered genes that were depleted at the end of the assay relative to the early time point as cell-essential.

We identified 2455 and 2321 essential genes (Dependency score > 0.35 and FDR < 0.2 with no filtering of pan-essential genes; Supplementary Data File 2, Supplementary Figs. 4A, B, 5A, B), respectively. Of these, 876 (D458) and 760 (D283) genes were suppressed following BETi (Fig. 2a). The overlap between the two cell lines was significant (449 genes; p < 0.0001).

Across both lines, the essential genes exploited by BETi were enriched for members of 34 pathways, and most frequently included members of the cell-cycle (8 pathways), DNA replication/synthesis/elongation (5 pathways), and RNA processing/transcription pathways (5 pathways) (Supplementary Data File 3).

One gene-set associated with MYC-activation was also enriched. We validated these using two additional MYC-driven cell lines, D425, and D341. We applied the same genome-scale CRISPR screens to these lines, respectively and identified 2560 and 1980 essential genes (Supplementary Data File 2, Supplementary Figs. 4C, D and 5C, D). Among these, 1005 and 504 genes were among the 5000 genes that were most suppressed by JQ1 treatment (Supplementary Data File 1). This represented significant enrichment (p = 0.003 and p < 0.0001 respectively, Supplementary Fig. 3B). The 15 pathways that were most significantly suppressed by BETi (p < 0.05) included cell-cycle regulation (2 pathways), DNA replication (1 pathway) and MYC-activation (1 pathway), while another 3 pathways included members associated with chromatin regulation (Supplementary Data File 3).

We conclude that BETi consistently suppresses essential genes in sensitive MYC-amplified medulloblastoma cells, and that these genes consistently belong to cell cycle, DNA replication, and RNA processing pathways. However, the finding that these pathways are suppressed by BETi and are sufficient to generate cell death does not indicate that they are responsible for BETi’s phenotypic effects. Some of these may represent essential pathways that are indirectly affected by BETi, after cell fate has already been determined.

**Essential genes that are suppressed by BETi.** We therefore attempted to narrow the set of essential genes and pathways that BETi exploits to those that are required for BETi phenotypic effects by determining, which genes were sufficient to rescue cells from BETi. We applied a lentivirally delivered ORF library encompassing 12,579 genes to both the D458 and D283 cell lines, each treated with either of two structurally distinct BET-bromodomain inhibitors (JQ1 and IBET151) or vehicle control (See Methods and Supplementary Fig. 3C). We measured the abundance of each ORF at initiation and completion of each assay to determine log-fold changes following BETi treatment.

For both cell lines, log-fold-changes for each gene were highly correlated between the IBET151 and JQ1 experiments (D458 R\(^2\) = 0.76, p value < 0.0001; D283 R\(^2\) = 0.39, p value < 0.0001) (Supplementary Fig. 3D, E), supporting similar target specificity for the two compounds.

In each cell line, we identified ORF constructs encoding 18 different genes that significantly rescued cells from either JQ1 or IBET151 and these lists were partially overlapping (31 genes total in both cell lines; Fig. 2b). We defined “rescue ORFs” as those conferring >1.5 log-fold enrichment with q < 0.25. The results for
the two cell lines overlapped extensively, as evidenced in three analyses. First, five of these rescue ORFs (ATOH1, BCL2L1, BCL2L2, CCND3, and NEUROG1) were common to both cell lines, a statistically significant overlap ($p < 0.0001$). Second, another four ORFs that met our significance threshold in only one cell line (SPN, CCND2, NEUROG3, and MSX2) all scored in the second line with a $q < 0.25$, but had fold-changes ranging from 1.15 to 1.4. Third, the five genes that scored among both cell lines included cell-cycle regulators and bHLH transcription factors that regulate neuronal differentiation: gene families that also include six of the 26 rescue ORFs that scored in only one of the lines (CCND1, CCND2, NEUROG3, NEUROD6, NEUROD1, and NEUROD4). Additional genes in these same families were identified using more relaxed cutoffs to define rescue ORFs (Supplementary Data File 4).

Proteins related to cell fate commitment, transcription, and developmental processes were significantly enriched in the rescue gene network ($q < 0.0001$), as were MYC-type basic helix-loop-helix (bHLH) ($q < 0.001$), cyclin ($q < 0.01$) and myogenic basic muscle-specific protein domains ($q < 0.001$, Supplementary Fig. 3F). Protein network analysis (performed using String, see Methods section) revealed that the pathway enrichment was also reflected by a high connectivity for the ORF network as a whole, with 42 edges (referring to protein–protein interactions; expected number of edges is 8) between the 31 nodes (individual ORFs) and a clustering coefficient of 0.749 ($p < 0.0001$, Fig. 2c).

Integrating all three datasets—gene expression, CRISPR/Cas9 screen, and ORF rescue—cell-cycle genes (CCND2 and CCND3) scored in D458 and the anti-apoptosis gene BCL2L1 and bHLH transcription factor-encoding gene NEUROG1 scored in D283 (Fig. 2d). The cell-cycle gene CCND2 also scored as an essential gene that is suppressed by JQ1 in D283 but only met the $q$-value (not log fold-change) threshold for a rescue gene.

We validated these genes in low-throughput assays (Fig. 3a, b, Supplementary Figs. 6A–C, 7). We overexpressed eGFP, CCND2, CCND3, BCL2L1, MYOD1, MYOG, NEUROD1, NEUROG1, and NEUROG3, in medulloblastoma cells and assessed proliferation in 1 μM of JQ1 or DMSO control. Overexpression of CCND2, CCND3, and BCL2L1 rescued D458 cells from the effects of JQ1 ($p$ values 0.002, 0.002, and 0.01) and CCND3 and NEUROG1 rescued D283 cells ($p$ value = 0.002 and 0.01). There was a trend for overexpression of CCND2 and BCL2L1 in D283 to confer selective advantage in JQ1, but these did not reach statistical significance ($p = 0.08$ and 0.06, respectively). We also validated additional bHLH transcription factors as rescue genes: MYOD1 in D458 ($p = 0.04$) and NEUROD1 ($p = 0.027$), NEUROG1 (0.02) and NEUROG3 ($p = 0.02$) in D283. Overexpression of these ORFs did not confer growth advantages in any of the cell lines when passaged in DMSO (Supplementary Fig. 7). Expression of BCL2L1 and NEUROG3 attenuated JQ1-induced apoptosis relative to eGFP controls in both D458 and D283 ($p$ values D458 BCL2L1 0.085 and NEUROG3 0.012; D283 BCL2L1 <
Protein-protein interactions (edges) between ORF rescue genes (nodes) that scored in either D458 or D283 following treatment with JQ1 or IBET151 are suppressed by BETi in both D458 and D283 (Supplementary Data File 1).

Fig. 2 Rescue genes are enriched for cell-cycle regulators and bHLH/homeobox transcription factors. a Intersection of genes suppressed by 1µM JQ1 with those identified to be cell essential (green) in D458 (top) and D283 (bottom). p-values indicate significance of overlap as determined with a Chi Square test. Source data: Supplementary Data Files 1 and 2. b Rescue genes identified in D458 (left) and D283 (right) cell lines following treatment with either JQ1 or IBET151. Asterisks indicate genes that scored as statistically significant rescue genes in both cell lines, but only met fold-change thresholds in the cell line shown. p-value indicates significance of overlap as determined by Fisher’s Exact Test. Source data: Supplementary Data File 4. c STRING protein network analysis to identify direct and functional protein networks that exist between the entire set of candidate rescue ORFs identified across both cell lines. Protein-protein interactions (edges) between ORF rescue genes (nodes) that scored in either D458 or D283 following treatment with JQ1 or IBET151 are shown. p-value indicates significance of enrichment of protein-protein interactions. Source data: Supplementary Data File 4. d Venn diagram depicting overlap of genes that are suppressed by JQ1 (blue), score as dependancies in CRISPR-Cas9 screens (green) and are identified to be rescue genes (red) in D458 (top) or D283 (bottom). *CCND2 met both the q value threshold and the log-fold change threshold in D458, but only the q-value threshold in D283. Source data: Supplementary Data Files 1, 2, and 4.

We also validated these ORFs as rescue genes in other patient-derived MYC-driven medulloblastoma cell lines: D341 and two that are passaged in serum-free conditions: CHLA01 and the recently generated cell line MB002. In each line, we found responses to JQ1 were attenuated by overexpression of a cell cycle regulator, BCL2 family member, and at least one bHLH/homeobox transcription factor (Supplementary Figs. 6C, 7). Thus, while we observed cell specific differences in the magnitude of resistance for individual ORFs, we observed consistency at the pathway level (i.e., cell cycle, apoptosis avoidance, and bHLH/homeobox transcription factors).

BETi has been reported as a means to target MYC.\textsuperscript{4,6} MYC was not included in the ORF screens. However, we previously demonstrated that ectopic MYC expression rescues D283 cells from BETi,\textsuperscript{6} and our analysis here confirmed MYC to be an essential gene (Supplementary Data File 2) that is transcriptionally suppressed by BETi in both D458 and D283 (Supplementary Data File 1)—indicating that MYC also fulfills all three criteria of a key essential gene that is suppressed by BETi. However, our analysis indicates that MYC is not the sole mediator of BETi’s phenotypic effects.

Drug-tolerant D458 cells exhibit reversal of BETi effects. We next sought to determine if the rescue genes identified in our ORF screens were differentially expressed in medulloblastoma cells that acquire BETi tolerance. We therefore passaged D458 cells and the related D425 line\textsuperscript{15} in JQ1 until they exhibited growth in the presence of JQ1 and IBET151 (Supplementary Fig. 8A).

Drug-tolerant D425 and D458 cells maintained viability following treatment with JQ1, with reduced BETi-induced apoptosis and necrosis compared to drug naïve (or sensitive) control cells (Fig. 4a and Supplementary Fig. 8B, C), even when re-challenged with BETi after 30 days of drug withdrawal (Fig. 4b). We were unable to isolate drug-tolerant cells from the other medulloblastoma cell lines.

We hypothesized that drug-tolerant cells evade BETi effects by reversing its transcriptional consequences. In genome-scale expression profiles of sensitive and drug-tolerant cells following treatment with DMSO or JQ1, we found 3279 genes to be significantly upregulated in drug-tolerant cells cultured in JQ1...
**Fig. 3** Expression of cell-cycle regulators, anti-apoptosis genes and bHLH/homeobox transcription factors rescue BETi effects. a Low throughput rescue assays in D458 and D283 cells expressing eGFP, CCND2, CCND3, BCL2L1, NEUROD1, NEUROG3, MYOD1, or MYOG that were treated with JQ1 1 μM or DMSO control. Asterisks denote significant differences from eGFP controls (*p < 0.05, **p < 0.01, ***p < 0.001) as determined by Two-tailed unpaired t-tests. Error bars depict mean ± SEM. Source data: Source Data File. b Percentage (%) of apoptotic or dead cells in D458 and D283 cells expressing eGFP, CCND2, CCND3, BCL2L1, NEUROD1, or NEUROG3 treated with JQ1 2 μM for 72 h. Asterisks denote significant differences from eGFP controls (**p < 0.01, ***p < 0.001) as determined by two-tailed unpaired t-tests. Error bars depict mean ± SEM. Source data: Source Data File

**Fig. 4** Drug-tolerant cells exhibit attenuated responses to BETi. a Percentage of viable cells among sensitive and drug-tolerant D425 and D458 populations after 72 h of treatment with JQ1. Error bars depict mean across six independent experiments ± SEM. ***p < 0.001 as determined by two-tailed unpaired t-tests. Source data: Source Data File. b Fold change in cell proliferation relative to pre-treatment baseline following 48 h of treatment with JQ1 among sensitive and drug-tolerant D425 and D458 cells that had been maintained in continuous JQ1 treatment (“drug-tolerant continuously treated”) or in which JQ1 had been withdrawn for 30 days (“drug-tolerant treatment withdrawn”). Data depict 20 replicate measurements across 4 independent experiments; error bars depict mean ± SEM. Asterisks (***) depicts p < 0.0001 as determined by two-tailed unpaired t-test. Source data: Source Data File. c Expression of genes in the JQ1 consensus signature in sensitive and drug-tolerant D458 cells following 24 h of treatment with JQ1 (1 μM) or vehicle control. Data from five independent replicates are shown, error bars depict mean ± SEM. Asterisks denote significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) as determined by two-tailed unpaired t-tests. Source data: Source Data File. d Expression of JQ1 medulloblastoma target genes in sensitive and drug-tolerant cells treated with JQ1 or vehicle control. Values represent mean of five replicate experiments ± SEM. Asterisks denote significant differences (*p < 0.05, **p < 0.01, ***p < 0.001) as determined by two-tailed unpaired t-tests. Source data: Source Data File.
Fig. 5 Drug tolerant cells express BETi mediator and rescue genes a Immunoblots probing for BETi mediator proteins BCL2L1, CCND2, HLX in D458 sensitive and drug-tolerant cells following 24 h of treatment with JQ1 (1 μM) or vehicle control. Vinculin is included as a loading control. Source data: Source Data File. b Immunoblots probing for BETi rescue proteins NEUROD1 and NEUROG1 in D458 sensitive and drug-tolerant cells following 24 h of treatment with JQ1 (1 μM) or vehicle control. Vinculin is included as a loading control. Source data: Source Data File. c Fold change in proliferation (as measured by luminescence) of D458 sensitive cells (top panel) and D458 drug tolerant medulloblastoma cells (bottom panel) following lentiviral infection with short hairpins targeting eGFP (negative control), SF3B1 (positive control) and BCL2L1. Values represent mean of 15 independent measurements across three replicate experiments ± SEM. Asterisks (***) denotes p values < 0.0001 as determined by two-tailed unpaired t-tests. Source data: Source Data File. d Fold change in proliferation (as measured by luminescence) of D458 sensitive cells (top panel) and D458 drug tolerant medulloblastoma cells (bottom panel) following lentiviral infection with short hairpins targeting eGFP (negative control), SF3B1 (positive control), CCND2 and NEUROD1. Values represent mean of 15 independent measurements across three replicate experiments ± SEM. Asterisks (****) denotes p values of < 0.0001 as determined by two-tailed unpaired t-tests. Source data: Source Data File.

We next validated that rescue genes identified in our ORF screens, including cell-cycle regulators and bHLH transcription factors, were relevant in these models. Among the 18 rescue ORFs, five ORFs (BCL2L1, CCND2, HLX, NEUROD1, and NEUROG1) were either re-expressed in drug tolerant cells following suppression with BETi or exhibited increased expression with drug-tolerance. The cell-cycle regulator CCND2 was suppressed by BETi (p value 0.04, Fig. 5a and Supplementary Fig. 9C), while the bHLH transcription factor HLX and the anti-apoptotic protein BCL2L1 trended towards suppression with BETi (p value 0.05 and 0.059, respectively, Fig. 5a and Supplementary Fig. 9C–E). All three of these were re-expressed at both the mRNA and protein levels in drug-tolerant cells (Differential mRNA expression q < 0.1, Fig. 5a and Supplementary Figs 9C–E, 11B (mRNA), p values for proteins 0.0002, 0.02, and 0.01, respectively, suggesting that these were “mediator” genes whose expression had been reinstalled. The other 15 rescue ORFs had not been suppressed by BETi, but expression of two of these also increased in drug-tolerant cells: the bHLH transcription factors NEUROD1 and NEUROG1 (Fig. 5b, Supplementary Fig. 9E, G, p values 0.0005 and 0.002, respectively for sensitive and drug-tolerant cells in DMSO).

To further evaluate the functional significance of the anti-apoptosis, cell cycle and bHLH transcription factors in drug-tolerance, we suppressed BCL2L1, CCND2, and NEUROD1 using short hairpin RNAs, confirming each of these genes to be essential in both D458 drug-naive and drug-tolerant cells (p values < 0.0001 in all cases, Fig. 5c, d and Supplementary Fig. 11C, D). We concluded that rescue genes from cell-cycle, bHLH transcription factor, and anti-apoptotic pathways are cell-essential in D458 cells and are re-expressed in cells that acquire drug-tolerance, remaining genetic dependencies in those cells.
Drug-tolerant cells exhibit altered chromatin landscape. We next explored possible mechanisms by which drug-tolerant cells express rescue genes including the cell-cycle regulator CCND2 and bHLH/homeobox transcription factors. We first explored genetic mechanisms by performing whole-exome sequencing of drug-tolerant D458 and D425 cells relative to matched drug-sensitive controls. We found no mutations that recurred across two or more drug-tolerant replicates that were not present in drug-sensitive controls. We found no mutations that recurred across two or more drug-tolerant replicates that were not present in drug-sensitive controls. We found no mutations that recurred across two or more drug-tolerant replicates that were not present in drug-sensitive controls. We found no mutations that recurred across two or more drug-tolerant replicates that were not present in drug-sensitive controls. We found no mutations that recurred across two or more drug-tolerant replicates that were not present in drug-sensitive controls.

In contrast, drug-tolerant cells exhibited changes in their chromatin landscape. We profiled global histone marks using a targeted, quantitative mass spectrometry approach, in sensitive and drug-tolerant D425 and D458 cells, in both the presence and absence of BET-bromodomain inhibitors (Fig. 6a and Supplementary Data File 8). Of the 76 histone marks included in the assay, 18 were differentially altered in drug-tolerant lines, of which were up-regulated (Supplementary Data File 8). Drug-tolerant cells were enriched for methyl modifications and for those that facilitate transcription (p < 0.05 in both cases). These include the promoter-associated mark H3K4me3 (and the related mark, H3K4me2), H3K9me1 (which has been associated with active enhancers), and upregulation of H3K79me1, H3K9me2, and H3.3, all of which have been described to be associated with transcription. In contrast, drug sensitive cells had increased levels of the polycomb repressive marks H3K27me3 and the H3K9me3. We did not observe any statistically significant differences in the H3K27ac profiles of drug-sensitive and drug-tolerant cells. Taken together, these data suggest that, in the setting of BETi, drug-tolerant cells maintain gene transcription through upregulation of activating methyl marks and downregulation of repressive marks.

These changes in chromatin landscapes appear to facilitate the expression of rescue genes that we had observed in our drug-tolerant cell lines (and had scored in the D458 rescue screen, including cell-cycle regulators and bHLH/homeobox transcription factors). We performed ChIP-seq for the promoter-associated and enhancer-associated marks H3K4me3 and H3K27ac, respectively, in sensitive cells treated with DMSO or JQ1 and in drug-tolerant cells treated with JQ1. Rescue genes exhibited increased levels of total H3K4me3 in drug tolerant D458 medulloblastoma cells compared to sensitive cells in either DMSO (p value 0.03) or JQ1 (0.02, Fig. 6b). Treatment of D458 cells with JQ1 was associated with increased levels of total H3K27ac at rescue genes (p value 0.0002, Fig. 6c), persisting in drug tolerant cells, which maintained elevated levels of total H3K27ac at rescue genes relative to untreated D458 cells (p value 0.01, Fig. 6c). There was a trend for D458 drug-tolerant cells to exhibit increased levels of H3K27ac binding at rescue genes compared to sensitive cells treated with JQ1, but this did not reach statistical significance (p value 0.05, Fig. 6c). We did not observe similar changes in relation to genes that did not score as rescue genes in our ORF screens (Supplementary Fig. 11E). In aggregate, these data suggest that drug tolerance involves activation of promoter-associated and enhancer-associated marks preferentially for rescue genes.
Changes in cell-state and differentiation attenuate response to BETi. The findings that expression of bHLH transcription factors and altered chromatin landscapes were both associated with drug tolerance led us to hypothesize that the drug-tolerant cell lines exhibit altered differentiation states. bHLH transcription factors have been reported to play an essential role in neural development and regulation of cell-fate commitment. We confirmed overexpression of NEUROD1 and NEUROG1 to be associated with an increased drive towards neuronal differentiation, as measured by levels of the neuronal marker TUJ1 (TUBB3) (Supplementary Figs. 9H, 12A, p values 0.03 and 0.02, respectively).

Drug-tolerant D458 cells exhibited changes in differentiation status relative to sensitive cells (Figs. 7a, 5b and Supplementary Figs. 9, 10), with increased expression of neuronal markers NEUROD1 (p value < 0.001 sensitive and drug tolerant cells in DMSO), NEUROG1 (p value 0.002 sensitive and drug tolerant cells in DMSO), TUJ1 (0.049 sensitive and drug tolerant cells in DMSO and <0.001 between sensitive cells treated with DMSO or JQ1) and NF68 (p value < 0.001 between sensitive cells treated with DMSO or JQ1). Drug-tolerant cells also exhibited suppression of the stem marker MSI1 (p value 0.002 sensitive and drug tolerant cells in DMSO).

The finding that expression of bHLH genes leads to both differentiation and resistance raises a conundrum. While stem cells exhibit self-renewal capacity and progression through S-phase, cells on the path towards neuronal differentiation exit S-phase, with cell-cycle arrest corresponding with terminal differentiation. However, we found that BETi drug-tolerant cells continue to cycle through S-phase, even in the presence of BETi, albeit at lower rates than untreated drug-naïve controls. Treatment of drug-sensitive D425 and D458 with JQ1 results in significant reductions in the percentage of BrdU positive cells relative to those treated with DMSO vehicle controls (19.5% vs. 53%, p value < 0.0001, Fig. 7c).

Drug-tolerant D425 and D458 cells do not exhibit further changes in the proportion of BrdU positive cells when challenged with JQ1 or DMSO, with approximately 30% of cells staining positive for BrdU in both conditions. However, the percentage of BrdU positive drug tolerant cells in DMSO is significantly lower than sensitive cells (31% vs. 53%, p value 0.0017, Fig. 7c).

This conundrum may explain why cell-cycle regulators were also prominent “hits” in our integrative analysis (Figs. 1c, 2c, d) and why CCND2, a transcriptional target of BETi, was re-expressed in drug-tolerant cells (Fig. 5a). Thus, we conclude that drug tolerant cells have acquired a cellular state that is primed for differentiation (e.g., via upregulation of bHLH) and exhibits global alterations in chromatin structure, but paradoxically maintains its ability to progress through the cell cycle (e.g., by upregulation of cell cycle genes).

Inhibition of cell-cycling delays acquisition of resistance to BETi. Our finding that drug-tolerant cells exhibited upregulation of the cell-cycle regulator (and BETi rescue gene) CCND2 and continue to cycle through S-phase led us to hypothesize that cell-cycle inhibition would prevent the acquisition of drug tolerance.

We first evaluated the acute efficacy of the combination of JQ1 with the CDK4/CDK6 inhibitor LEE011. We found this combination to meet BLISS synergy criteria in D458, MB002, and D341 (Supplementary Fig. 12B–F). We further validated synergy (using the LOEWES model for synergy) between LEE011 and JQ1 across a wider range of concentrations in D458 (p value < 0.0001), MB002 (p value < 0.0001) and D283 (p value < 0.0001), along with an additional MYC-driven line HD_MB003 (p value < 0.0001, Supplementary Figs. 13, 14). With prolonged treatment of D458 cells, we also found the addition of LEE011 to JQ1 delayed the acquisition of drug tolerance relative to treatment with JQ1 alone (Fig. 8a).

Combined treatment with LEE011 and JQ1 also attenuated the acquisition of resistance in vivo. In mice harboring flank injections of D458 and MB002 (Fig. 8b, c), GSEA revealed seven pathways to be significantly downregulated in RNA from D458 tumors treated with combination therapy compared to those treated with JQ1 alone, including two associated with the cell cycle (E2F_Targets and G2M_Checkpoint), and two enriched with MYC targets (Fig. 5d), suggesting addition of LEE011 effectively suppressed the cell cycle. In mice bearing intracranial xenografts of D458 cells, treatment with LEE011 alone had no effect on tumor growth or survival, while single agent JQ1 reduced tumor growth (p = 0.0004 on day 14) and prolonged survival (p = 0.0002) relative to vehicle controls. However, the combination of LEE011 and JQ1 further attenuated tumor growth compared to those treated with JQ1 alone, with significantly less bioluminescence after one month (Supplementary Fig. 14C – D = 150, Supplementary Fig. 14C – D = 150, p < 0.05). We observed similar synergy in two additional patient-derived MYC-driven intracranial medulloblastoma models: Med-114FH (Fig. 9b) and Med-411FH (Fig. 9c), which exhibited significantly prolonged survival when treated with both LEE011 and JQ1 compared to vehicle controls (p values 0.01 and 0.02) or
Drug-tolerant cells occupy a mixed differentiation state. Our findings suggest a model whereby BETi suppresses essential bHLH lineage-specific transcription factors only in relatively undifferentiated cells, while more neuronally differentiated medulloblastoma cells maintain expression and viability in the presence of drug (Fig. 10a). However, we find that the cells do not terminally differentiate, maintaining expression of SOX2 (Fig. 7a and Supplementary Fig. 10) and re-expressing cell-cycle regulators such as CCND2 to promote cell-cycle progression (Fig. 5a). We therefore hypothesized that resistance accrues in populations of medulloblastoma cells that are somewhat, but incompletely, neuronally differentiated (Fig. 10a).

Given that sensitivity to BETi is related to neuronal differentiation within cell lines, we reasoned that across cell lines, those with increased expression of neuronal differentiation markers would be inherently less sensitive to BETi. To test this, we interrogated the CTRP dataset\textsuperscript{27,28} and found Tuj1 expression to be correlated with resistance to JQ1 across 783 cancer cell lines (Supplementary Fig. 14C). Within lineages, expression of neuronal differentiation markers would be inherently less sensitive to BETi. To test this, we interrogated the CTRP dataset\textsuperscript{27,28} and found Tuj1 expression to be correlated with resistance to JQ1 across 783 cancer cell lines (Supplementary Fig. 14C). Within lineages, expression of neuronal differentiation markers to BETi sensitivity are not restricted to D283 and D458 results suggest that the relevance of neuronal differentiation led Tuj1 expression, in addition to the stem markers Nestin, and lineage markers within human medulloblastomas. We found that the cells do not terminally differentiate, maintaining expression of SOX2 (Fig. 7a and Supplementary Fig. 10) and re-expressing cell-cycle regulators such as CCND2 to promote cell-cycle progression (Fig. 5a). We therefore hypothesized that resistance accrues in populations of medulloblastoma cells that are somewhat, but incompletely, neuronally differentiated (Fig. 10a).

Given that sensitivity to BETi is related to neuronal differentiation within cell lines, we reasoned that across cell lines, those with increased expression of neuronal differentiation markers would be inherently less sensitive to BETi. To test this, we interrogated the CTRP dataset\textsuperscript{27,28} and found Tuj1 expression to be correlated with resistance to JQ1 across 783 cancer cell lines (Supplementary Fig. 14C). Within lineages, expression of neuronal differentiation markers to BETi sensitivity are not restricted to D283 and D458 results suggest that the relevance of neuronal differentiation led Tuj1 expression, in addition to the stem markers Nestin, and lineage markers within human medulloblastomas. We found that the cells do not terminally differentiate, maintaining expression of SOX2 (Fig. 7a and Supplementary Fig. 10) and re-expressing cell-cycle regulators such as CCND2 to promote cell-cycle progression (Fig. 5a). We therefore hypothesized that resistance accrues in populations of medulloblastoma cells that are somewhat, but incompletely, neuronally differentiated (Fig. 10a).

Drug-tolerant cells occupy a mixed differentiation state. Our findings suggest a model whereby BETi suppresses essential bHLH lineage-specific transcription factors only in relatively undifferentiated cells, while more neuronally differentiated medulloblastoma cells maintain expression and viability in the presence of drug (Fig. 10a). However, we find that the cells do not terminally differentiate, maintaining expression of SOX2 (Fig. 7a and Supplementary Fig. 10) and re-expressing cell-cycle regulators such as CCND2 to promote cell-cycle progression (Fig. 5a). We therefore hypothesized that resistance accrues in populations of medulloblastoma cells that are somewhat, but incompletely, neuronally differentiated (Fig. 10a).

**treatment with JQ1 alone (p values 0.017 and 0.007). No differences were observed with either single agent JQ1 or LEE011 compared to vehicle controls.**

Together, these in vitro and in vivo experiments across a number of cell lines indicate synergy between JQ1 and LEE011 in BETi naive cells, with the addition of LEE011 delaying the acquisition of resistance.
Tolerance to BETi is predetermined. The heterogeneity within medulloblastomas led us to hypothesize that pre-existing subpopulations of differentiated neuronal cells may be selected for by BETi due to their inherent resistance. We explored this using two approaches. First, using flow cytometry, we observed an enrichment of Tuj1 positive and MSI1 negative drug-tolerant D458 cells with BETi. Flow cytometry analysis revealed 0.7% of cells to Tuj1 positive and MSI1 negative for the stem cell markers, thus representing a more differentiated phenotype (Fig. 10c). In addition, we also observed subpopulations of cells that exhibited a mixed phenotype with co-expression of the neuronal marker Tuj1 with the stem markers.

Within and across tumor samples (Supplementary Data File 9). Across all tumors, medulloblastomas harbored a subpopulation of cells (median 9%, range 0–86%) that expressed Tuj1 but were negative for the stem cell markers, thus representing a more differentiated phenotype (Fig. 10c). In addition, we also observed subpopulations of cells that exhibited a mixed phenotype with co-expression of the neuronal marker Tuj1 with the stem markers.

Discussion
Small molecule inhibitors of transcriptional modulators are increasingly showing preclinical promise across a range of cancers. However, these compounds have pleiotropic effects, making it difficult to identify the genes and pathways that mediate their efficacy. Identifying genes that are suppressed, and whose suppression is both necessary and sufficient to generate specific phenotypes, has been used as an approach to detect mediators of mechanistic effects for decades. With the advent of high-throughput functional screening including genome-scale CRISPR and ORF rescue screens, it is now possible to test sufficiency and necessity on a genome-scale basis, which is the approach we have taken here. Similar integrative genomic approaches may also be effective in clarifying the primary targets of other modulators of transcription such as inhibitors of chromatin/transcriptional complexes.

Our analysis support heterogeneity within (and between) medulloblastomas, a finding that has also been reported in single-cell RNA-sequencing studies of medulloblastomas. We identified cells that express both genes associated with neuronal differentiation and stemness as being associated with drug-tolerance. Similar populations of cells have been identified in other normal developmental hierarchies and in cancer, and have been termed transit amplifying cells or in the neural context, “activated quiescent neural progenitor cells”. These cells have been shown to harbor a phenotype that is more differentiated than stem cells, and to harbor increased proliferative potential.
MYC to be re-expressed in drug-tolerant cells, and for rescue ORFs to be enriched with bHLH/homeobox transcription factors that contain MYC binding motifs, raising the possibility that MYC may regulate expression of rescue genes.

Changes in cell state have been shown to be associated with resistance to anticancer therapies across multiple settings\(^{45-48}\). In the setting of BETi, epithelial-mesenchymal transition has been reported to be associated with resistance in pancreatic cells\(^{49}\). Among leukemias, stem cells have been found to be most resistant\(^{50}\)—an opposite result to ours in medulloblastoma, where cells with a more (but not terminally) differentiated phenotype are less likely to respond to BETi.

Finally, our finding that BETi alters overall cell state towards a more differentiated neuronal phenotype is one that is highly relevant for medulloblastoma. The effect of BETi on the developing brain remains to be characterized; such studies are essential to determine potential developmental sequelae of BETi when used to treat pediatric patients with medulloblastomas and other cancers.

**Methods**

**Ethics statement.** Ethics approval was granted by relevant human IRB and/or animal research committees (IACUC) of Dana-Farber Cancer Institute (DFCI),
Boston Children’s Hospital, The Broad Institute, Fred Hutchinson Cancer Research Center and Massachusetts Institute of Technology (MIT).

**Cell culture.** All cell lines included in this study have been shown to harbor features that recapitulate Group 3 human medulloblastomas. D425 and D458 cells were a kind gift from Dr. Bigner, D283, CHLA01 and D341 cells were obtained directly from ATCC. MB002 cells were a kind gift from Dr. Cho. HDBM003 cells were a kind gift from Drs. Pfister and Milde. D425, D458, D283, and D341 cells were cultured in DMEM/F12 with 10% serum and 1% glutamate/pen-strep in ultra-low attachment flasks and plates. CHLA01 and MB002 cells were cultured in serum-free and growth factor supplemented media as previously described. SNP-based fingerprinting assays were used prior to screens and sequencing assays to ensure authenticity. Mouse neural stem cells were generated and cultured as previously described. All cells were routinely monitored for mycoplasma infection.

**Generation of drug-tolerant cells.** One million cells (D458 and D283) were passaged in 6-well plates in 1 μM JQ1 (or DMSO) control for greater than 30 days until they started to exhibit growth. DMSO treated cells were passaged every 3–4 days. Media was changed for the cells treated with the BET-bromodomain inhibitors every 3–4 days. Cells were counted weekly until BETi treated cells exhibited proliferation at which point cells were challenged with increasing doses of BETI (JQ1 and IBET151) to confirm the acquisition of drug tolerance.

**Generation of Cas9 expressing cells.** 1.5 × 10^6 cells with 4 μg/ml polybrene were seeded in one well of a 12-well plate, then spin-infected with pLX311-Cas9 virus. Cells were selected for 7 days in blastidin (commencing 24 h infection). Cas9 activity was confirmed using eGFP reporter assays as previously described.

**Infection of pooled libraries:** CRISPR-Cas9 screen and ORF screen. Pooled lentiviral libraries were infected with a 30–50% infection efficiency, corresponding to a multiplicity of infection (MOI) of ~0.3–1. Spin-infections (2000 rpm for 2 h at 37 °C) were performed in 12-well plate format with 1.5 × 10^6 cells each well. Approximately 24 h after infection, cells were trypsinized and 3 × 10^4 cells from each infection were seeded in two wells of a six-well plate, each with complete medium, one supplemented with the appropriate concentration of puromycin. Cells were counted four days post-selection to determine the infection efficiency. Volumes of virus that yielded ~30–50% infection efficiency were used for screening.

Screening-scale infections of the ORFeome pLX317 barcoded library (contains ~17,255 barcoded ORFs overexpressing 12,579 genes) and the Avana barcoded library (contains 73,087 confirmed ORFs and 18,454 genes and 1000 non-targeting guides). Cells were infected to achieve a representation of at least 1000 cells per ORF and 500 Cas9 expressing cells per CRISPR following puromycin selection. Cells within a replicate were harvested, pooled and split into T225 flasks 24 h after infection. Following selection, cells were seeded in T225 flasks in media. For the CRISPR-cas9 screens, cells were passaged for ~21 days. For the ORF screens, 3 μM JQ1 and 5 μM of iBET-151 were added to the cells on Day 0. Cells were passaged in drug or fresh media containing drugs every 3–4 days. Cells were harvested ~21 days after initiation of treatment. Each sgRNA vector and ORF vector harbor unique DNA barcodes that allow the tracking of abundance of each vector through the assays. Genomic gDNA extraction, PCR and sequencing were performed as previously described. Samples were sequenced on a HiSeq2000 (Illumina). CRISPR-Cas9: The processing of sgRNA read count data, quality control filters and modeling of guide activity, gene-knockout, and copy-number effect with the CERES algorithm were performed as previously described.

**Determination of significance:** 73,372 guides that passed quality control (including approximately 1000 guides that do not target any location in the reference genome as negative controls) were included in the analysis. To calculate the probability that a gene dependency score represents a true dependency in a given cell line, we fit a two-component mixture model in each cell line. The two components were (1) the empirically determined distribution of true dependent scores, identified using the pan-essential gene scores in that cell line, and (2) the empirically determined distribution of true non-dependent scores, identified as genes that were not expressed in that line. We defined as pan-essential genes 1607 genes whose dependency scores fall in the bottom 25% of gene scores in at least 90% of cell lines analyzed in the Achilles Avana dataset 18Q1, which includes 391 cell lines. The probability of dependency for each gene score is the probability that it was generated from the distribution of true dependent gene scores. To correct for noise in the tails of the distributions, all gene scores below ~1.5 were assigned probability of dependency. Score distributions were taken at a score threshold of 0.5 as assigned probability 0. A Gaussian smoothing kernel with width 0.15 was applied to the final probability scores to further reduce noise. Genes with a dependency probability >0.35 with a FDR <0.2 were deemed to represent a dependency within each line.

**ORF rescue screen.** Read counts were normalized to reads per million and then log2-transformed and fold-change of each ORF was determined relative to the initial time point for each biological replicate. Abundance of each ORF was measured at the initiation and completion of each assay to determine log-fold changes following treatment with BET-bromodomain inhibitors. We defined “ rescue ORFs” as those conferring >1.5 log-fold enrichment with a q value of <0.25. Whole-genome CRISPRi screening using the CRISPRiBase tool was performed to identify rescue ORFs that exist between the entire set of candidate rescue ORFs identified across both cell lines.

**ORF rescue assays.** Medulloblastoma cells were transduced with lentivirus with pLEX-307 lentiviral vectors to overexpress eGFP, CCND2, CCND3, BCL2L1, MYOD1, MYOG, NEUROD1, NEUROG1, and NEUROG3 in individual infections. Genes were delivered using a spin protocol (3 million cells per infection, 200 μg of DNA). Cells were harvested the following day and subjected to puromycin selection (1 μg/ml) at 48 h for three days. Cells were treated with 1 μM JQ1 or DMSO control. Cells were counted with trypan blue and cumulative doubling of JQ1 treated cells (relative to DMSO) between seven to 14 days. Genes that significantly attenuated BETi response (relative to eGFP controls) were deemed to “ rescue” the BETi proliferative phenotype.

**Immunoblotting.** Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors on ice for 30 min. Lysates for centrifuged at 13,000 g for 10 min and the supernatant was harvested. Supernatant was mixed with 4 × SDS loading buffer and heated at 70 °C for 10 min and subject to SDS-PAGE on 4–12% gradient gels. See Supplementary Data File 10 for antibodies used.

**Flow cytometry for apoptosis and cell-cycle.** Sensitive and drug-tolerant cells were treated with JQ1 (1 μM) or ORF1 (1 μM) for 72 h. Medulloblastoma cells overexpressing ORFs were selected in puromycin for 48 h before being treated with JQ1 (500 nM) or vehicle control for 72 h. Annexin V/Propidium iodide apoptosis assays were performed as previously described. Proportion of cells in S phase was determined by flow cytometry assay of BrdU/Propidium Iodide (BD biosciences) as per manufacturer’s instructions.

**Whole-exome sequencing.** DNA was extracted from sensitive and drug-tolerant D425 and D458 cells (Qiagen DNeasy Blood and Tissue kit). DNA was subjected to whole-exome Illumina sequencing. Libraries with a 250 bp average insert size were prepared by Covaris sonication, followed by double-size selection (Agencourt AMPure XP beads) and ligation to specific barcoded adapters (Illunina TruSeq) for multiplexed analysis. Exome hybrid capture was performed with the Agilent Human All Exon v2 (44 MB) bait set.

Sequence data were aligned to the hg19 (b37) reference genome with the Burrows-Wheeler Aligner (28) with parameters [-q 5 - 32 -k 2 - t 4 - o 1]. Aligned data were sorted, duplicate-marked, and indexed with Picard tools. Base-quality score recalibration and local realignment around insertions and deletions was achieved with the Genome Analysis Toolkit.

Mutations were called from chronically passaged cells with MuTect, filtered against DNA from pretreatment samples, and annotated to genes with Oncotator.

**Gene-expression profiling.** Sensitive and drug-tolerant cells were treated with DMSO or JQ1 (1 μM) for 24 h in independent experiments (D283 = 3 replicates per condition, D458 = 5 replicates per condition) and RNA extracted (Qiagen RNeasy kit, with DNase treatment as per protocol). Gene expression profiles were assayed using Affymetrix Human Gene 2.0 ST microarrays. CEL files were RMA normalized. Comparative marker selection analysis was performed in GenePattern using default settings. Genes with p <0.05 and q <0.1 were considered to have significant changes in expression unless otherwise specified. GSEA was performed using the C2 (CP) gene sets (MsigDB). Gene sets with nominal q < 0.25 were considered to be significantly altered. Principle component analysis was performed in R studio using the pracma function.

**CyCIF staining of human tumors.** Formalin-fixed and paraffin-embedded (FFPE) tissue micro arrays (TMAs) containing medulloblastoma tissues from 50 patients and 10 normal brain controls were obtained from the Department of Pathology at Boston Children’s Hospital according to IRB approval. TMAs were stained and analyzed on a single-cell basis using the open source t-CyCIF methodology (http://www.cycif.org). The BOND RX Automated IHC/ISH Stainer (Leica Biosystems, Buffalo Grove, IL) was used to bake slides at 60 °C for 30 min, dewax with Bond Dewax Solution at 72 °C, and perform antigen retrieval with Epothioid Retrieval 1 (ERI) solution at 100 °C for 20 minutes. TMAs underwent multiple cycles of antibody incubation, imaging, and fluorophore inactivation. Tissues were incubated overnight at 4 °C using commercially available, fluorophore-conjugated antibodies listed in Supplementary Data File 10. Nuclei were stained in each cycle using Hoechst 33342 (Catalog No. 4082S, Cell Signaling Technologies, Danvers, MA) and Cell Signaling Cell Imaging System (GE Healthcare Life Sciences, Pittsburgh, PA) at 20× magnification using the DAPI, FITC, dRed, and Cy5 channels. Exposure times ranged from 0.0750 to 1.000 ms. Fluorophores were inactivated by submerging slides in a 4.5% H2O2, 20 mM NaOH in PBS solution and exposed to LED light source for 2h at room temperature. Images were processed and single-cell data was quantified using customized versions of ImageJ and MATLAB scripts from the Sorger Lab GitHub repository.
Global chromatin profiling. Quantitative targeted mass spectrometry was performed on sensitive and drug-tolerant D425 and D458 cells passaged in DMSO or BETI (Q1 or IBET151) for 24 h. Cell lysate, histone extraction and mass spectrometry (Chromatin Profiling) was performed as previously published[11]. Data was log normalized, and differentially altered markers were determined using comparative marker selection in GenePattern, correcting for cell-line, with a threshold of a Bonferroni FDR of <0.1.

ChIP-seqencing. Sensitive and drug-tolerant D425 and D458 were passaged in DMSO or Q1 (1 µM) for 24 h. Sheared chromatin from each cell line was subjected to ChIP-seq as previously described[12], enriching for H3K27ac (Cell Signaling Technologies, 817355), H3K4me3 (Cell Signaling Technologies, 975155). Histone H3 (Cell Signaling 4499S) and BRD4 (Bethyl, A301–985A100). ChIP libraries were indexed, pooled and sequenced on Illumina Hi-seq-2000 sequencers. Raw data was aligned to the human reference genome Hg19 using Picard tools (https://broadinstitute.github.io/picard/). Read count data was mapped to the reference genome using bowtie2 version 2.2.1 with parameters -p 4 -k 1. Peaks were called using MACS version 1.4.2 over an input control. Reads were extended 200-bp and normalized to read-density in units of reads per million mapped reads per bp (rpm/bp). To calculate ChIP-binding score for each gene, read-density in units for reads per million were aggregated for each gene (extending to 500 kb in each direction). Z-scores of ChIP-seq scores for each gene within each sample was calculated. Peaks and alignments were converted to TDFs by IGV tools and visualized by IGV.

Drug assays. JQ1, IBET151, and LEE011 assays were performed by seeding 1 million cells per well in 6-well ultra-low attachment flasks with DMSO controls. Doses (final concentration of the specified) were JQ1 1 µM, IBET151 1 µM, LEE011 500 nM or 1 µM. Total number of viable cells at designated time points were determined by trypsin blue assays. DBET experiments were performed by seeding 1000 cells per well in 96-well plates (DMSO control or Q1 2 µM for drug-tolerant cells) at the doses specified. Luminescence measurements of ATP content (Cell-Titer-Glo) were performed as a marker of cell viability.

Loewe’s synergy testing. Experimental details: Cell lines were seeded into 384-well, white-walled, clear bottom plates at a density of 500 cells/well. Twenty-four hours after seeding, combination drugs were administered using an HP D300 Digital Dispenser in matrix format. Drug was administered such that the final volume of DMSO did not exceed 0.5%. The cells were then incubated for seven days and cell luminescence was measured using the Cell Titer-Glo assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions.

For calculation of drug combination effects: Curves fit were deviation from the null model (Loewe additivity) assessed using the BIGL package in R (reference: PMID 29263342). General-purpose optimization (Nelder-Mean algorithm) was used for single-agent fits, and the “model” option was used to predict variance. Overall significance was assessed using the bootstrapped mean±R test (n = 1000 iterations), and per-concentration significance using maxR, as described by the package authors.

Animal studies. In vivo studies were performed in compliance with IACUC approved protocols at Dana-Farber Cancer Institute (FLank), Fred Hutchinson Cancer Research Center (Patient Derived Xenografts) or Massachusetts Institute of Technology (D458 intracranial experiments). Cells were tested for mycoplasma and subjected to IMPACT testing for pathogens prior to use in experiments.

Flank. Flank xenografts were established by injecting five million D458 cells or ten million MB002 cells (matrigel:PBS at a 1:1 ratio) in NSG mice (Jackson Labs). Mice were treated with Q1 (50 mg/kg/daily intraperitoneal injection), LEE011 (75 mg/ kg/daily oral gavage), combination therapy (LEE011 75 mg/kg/daily with Q1 50 mg/kg/daily intraperitoneal injection) or vehicle control. Tumor growth was monitored by caliper measurements.

D458 Intracranial: D458 medulloblastoma cells were maintained in 1:1 DMEM/ F12 media (Biocoro) supplemented with 10% FBS (HyClone), 1x Glutamax (Biocoro), and 1x Pen/Strep (Sigma). Cells were transduced with a lentiviral pLMP-GFP-Luc vector to allow for staining expression of GFP and luciferase prior to implantation. Six-week-old NCR nude mice (Tacoi) were used to generate intracranial orthotopic right cerebellar D458 medulloblastoma tumors.

In brief, mice were anesthetized using 2% isoflurane and their heads immobilized in a stereotactic headframe using ataractic ear bars. A Burr hole was made using a steel drill bit (Plastics One, Roanoke, VA, USA) 2 mm right of the sagittal and 2 mm posterior to the lambda suture. 105 D458 cells were injected stereotactically into the right cerebellar hemisphere. Tumors were allowed to grow for 14 days prior to commencement of treatment (same doses as those used for flank injections). Intracranial tumor growth was monitored in vivo using bioluminescence IVIS® imaging (Xenogen, Alameda, CA) equipped with LivingImage® software (Xenogen). Tumor response to treatment was tracked every 3–5 days using IVIS imaging. Mice were given 150 µL I.P. of 30 mg/mL n-luciferin (PerkinElmer) dissolved in PBS 10 min prior to IVIS imaging. Signal intensity was quantified using a Living Image software.

Intracranial patient derived xenograft: Med-114FH and Med-411FH models were implanted directly from the human patient into mouse cerebellum and propagated serially in mice for 5 (Med-114FH) or 6 (Med-411FH) passages as previously described[13]. To transduce with lentiviral mCherry-Luciferase, cells were briefly maintained in Neurocult media supplemented with their proprietary additive plus EGF and FGF before re-implantation in mice and continued serial passage in vivo. The cells used for this study were on mouse passage 4 (Med-114FH) or 7 (Med-411FH) after transduction. 100,000 cells per mouse were implanted orthotopically in the cerebellum of 5 (Med-114FH) or 7 (Med-411FH) week-old ICD-Athymic Nude Foxn1nu #069 Envigo mice. Twenty-five days after implant mice underwent bioluminescent imaging for luciferase expression and were assigned treatment groups so as to normalize the luminescence across all groups, mice with luminescent signal <16 were excluded from the study, n = 10 per group. Mice were weighed and dosed daily with vehicle, LEE011 (75 mg/kg PO), Q1 (50 mg/kg IP), or the combination. Hydrogel was provided as needed. Mice were euthanized post sacrifice or when mice being cold, hunched, or lethargic. The genomic characterization of these models as well as implant procedures have been previously reported[14].

Short hairpin RNA (shRNA) suppression experiments. D458 sensitive and BETI drug tolerant cells were transduced with lentivirus encoding shRNAs targeting BCL2L1, CCND2, or NEUROD1, in addition to SF3B1 (positive control) or eGFP (negative control). Cells were plated in puromycin selection 24 h after infection. On day 3 (48 h post-selection), 1000 cells were plated in each well of 96-well plates (five replicate wells per condition). Cell viability was measured on subsequent days by assessing ATP content with Cell Titre-Glo (Promega). Results were normalized to baseline.

Barcoding. 600K barcode library production: Five sets of primers were designed (Supplementary Data File 10) to incorporate a six nucleotide sub-pool barcode followed by 24 basepair degenerate sequence flank by overhanging 5’Agel site and 3’ EcoRI sites. Pairs of complementary pairs of oligos were annealed ligated into a modified pLKO.1 backbone. The modified pLKO.1 backbone had the human U6 promoter deleted (PmuPl-Agel) and replaced with a short sequence (GGAGGCTTAAGATCTATGCTGACACCTAACTGAGAGATTTC CGAATTCTGCTAGTTATATCGGCAGAGGACCGGT). This substitution will not transcribe the barcode sequence (https://portals.broadinstitute.org/gpp/public/resources/protocols). The ligations were amplified and plasmid preps generated and sequence verified for barcode diversity as previously described. Sub-pool 1 had a diversity of ~54,000 barcodes, while sub-pools 2–5 had a diversity of ~130,000–153,000 barcodes. (https://portals.broadinstitute.org/gpp/public/resources/protocols) Virus was generated and titered from each subpool, and mixed based on barcode diversity within the plasmids pools and titer levels to maximize a homogeneous distribution of representation of each barcode. Cells were barcoded with the pooled lentiviral barcoding library. D283 and D458 cells were transduced with a low MOI (of 30%) with the aim of labeling single cells with individual barcodes. Each transduced cell line was expanded as single pools before being divided into replicate drug treatment or vehicle control experiments. We extracted DNA from each pool of cells to determine individual DNA barcode abundance prior to drug treatments. Cells were passaged in the presence of Q1 (or DMSO control) for 40 days. Genomic DNA extraction, PCR and sequencing were performed as previously described[15] to determine the presence of each barcode (absence of expression relative to the early time point control) in each replicate experiment. Barcodes with a minimum read count of 3 at the early time point were included for analysis.

Statistical analysis. Log-rank (Mantel-Cox) tests were performed to analyze survival analysis of animal experiments. Unless otherwise described in the relevant results and methods, remaining p values were determined using two-tailed t-tests. p values of <0.05 were considered significant.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.
Data availability
Gene expression profiling data has been deposited in GEO under accession number GSE123248. ChiP-seq data has been deposited in GEO under accession number GSE129521. Data from genome scale modifier screens and barcoding assays have been included in Supplementary Data Files and Data Source Files. Data from which figures were generated are included in Supplementary Data or Source Data Files as indicated in individual figure legends. Uncorrected western blots are included in the Data Source File.

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Author contributions
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Author contributions
P.B., C.J., and R.B. conceived the study. P.B., F.P., R.O., P.H., E.G.Z., G.B., K.Q., G.G., L.B., M.C., E.G., T.M., S.P., J.O., F.D., B.R.P., G.A., G.R., A.B., A.C., B.T., P.K., A.T., H.T., J.R.L., A.H., S.C., R.R., G.C., A.G., Y.L., T.S.K., C.S., F.C.L., M.P., M.Y., S.C., M.S., A.M., W., C.H., M.W.K., S.S., K.L.L., A.T., J.B., J.Q., P.C.G., J.J., D.R., F.V., K.S., C.J., and R.B. designed and/or executed experiments, or generated cell lines used in experiments. P.B., F.P., O.S., M.C., E.G., J.O., F.D., N.G., N.D., M.R., A.C., B.T., S.C., R.R., M.S., A.T., and R. B. contributed to data analysis. All authors contributed to the preparation of the manuscript. C.J. and R.B. supervised the overall study.

Additional information
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Competing interests: JEB is now an executive and shareholder of Novartis AG, and has been a founder and shareholder of SHape (acquired by Medivir), Acetylex (acquired by Celgene), Tenisha (acquired by Roche), Syros, Regency and C4 Therapeutics. A.G., R.B., and K.S. also consulted for Novartis. P.B., K.S., and R.B. receive grant funding from Novartis. M.W.K is now an employee of Bristol Myer Squibb and C.M.J is now an employee of Novartis. W.C.H is a consultant for Thermo Fisher, Alujub, Parexel, MPM and is a founder and advisor to K.S.Q. Therapeutics. SS consulted for Rarebyte, Inc. All other authors declare no competing interests.

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