Retinoblastoma protein as an intrinsic BRD4 inhibitor modulates small molecule BET inhibitor sensitivity in cancer

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Bromodomain and extraterminal (BET) proteins including BRD4 play important roles in oncogenesis and immune inflammation. Here we demonstrate that cancer cells with loss of the retinoblastoma (RB) tumor suppressor became resistant to small molecule bromodomain inhibitors of BET proteins. We find that RB binds to bromodomain-1 (BD1) of BRD4, but binding is impeded by CDK4/6-mediated RB phosphorylation at serine-249/threonine-252 (S249/T252). ChIP-seq analysis shows RB knockdown increases BRD4 occupancy at genomic loci of genes enriched in cancer-related pathways including the GPCR-GNBIL-CREB axis. S249/T252-phosphorylated RB positively correlates with GNBIL protein level in prostate cancer patient samples. BET inhibitor resistance in RB-deficient cells is abolished by co-administration of CREB inhibitor. Our study identifies RB protein as a bona fide intrinsic inhibitor of BRD4 and demonstrates that RB inactivation confers resistance to small molecule BET inhibitors, thereby revealing a regulatory hub that converges RB upstream signaling onto BRD4 functions in diseases such as cancer.

Bromodomain and extraterminal (BET) proteins, including BRD2, BRD3, BRD4, and BRDT (a testis-specific member), are important factors to recognize acetylated histones and play a critical role in transcriptional activation1. There are two bromodomains in BET proteins2 and it has been shown that the activity of bromodomain-1 (BD1) is primarily related to cancer whereas bromodomain-2 (BD2) appears to mainly function in immune response and inflammation3. A few small molecule inhibitors, especially those targeting BD1 domain have been developed and manifested some promising anti-cancer activities in a series of clinical trials4–6. However, resistance to BET inhibitors often occurs and the underlying mechanisms are not fully understood7. Thus, there is an urgent need to develop new strategies or compounds to overcome BET inhibitor resistance. Additionally, it has been reported that the function of BRD4 can be modulated by other factors through protein posttranslational modifications such as phosphorylation, methylation, and ubiquitylation8–10. However, it remains unclear whether there exists any endogenous protein(s) that functions as intrinsic inhibitors of BRD4.

Retinoblastoma (RB) protein, encoded by RB1 gene acts as a tumor suppressor by interacting with others proteins such as E2F...
family proteins to inhibit cancer-promoting activities such as accelerated cell cycle progression. Rb1 gene is frequently deleted in human cancers including prostate cancer (PCa). In addition to the deletion or mutation of the Rb1 gene, the cell cycle-inhibitory function of Rb protein is impeded by phosphorylation of Rb protein mediated by Cyclin-dependent kinases (CDKs) such as CDK4/6.21-23. Studies from us and others have showed that the Rb amino-terminal region (Rb-N) regulates cancer cell growth and immune response by interacting with an FXXV motif in client proteins including EID1 and p65, respectively. Notably, phosphorylation of serine 249/threonine 252 (S249/T252) in the linker region of Rb-N by CDK4/6 either enhances or inhibits the binding of its partners and the effect is dependent on the charge composition surrounding the FXXV motif.24-26. However, it remains largely unclear whether Rb-N could also regulate other cancer-relevant functions through undefined mechanisms.

In our current study, we find that Rb loss leads to BET inhibitor resistance in a manner independent of the Rb-E2F1 pathway in PCa cells. We show that Rb-N binds to the BD1 domain of Bd4 and restricts Bd4 occupancy in the genome; however, Rb-N-Bd4 binding is attenuated by Rb phosphorylation at S249/T252 residues. Our data also reveal that depletion of Rb enhances Bd4 occupancy at the loci of cancer-related genes including the GPCR-cAMP signaling gene Gnb1l. Finally, we demonstrate that loss of Rb protein confers resistance to small molecule BET inhibitor and this effect is abolished by co-inhibition of Gnb1l-CreB signaling.

Results

RB deficient cells are resistant to small molecule BET inhibitors

The Rb1 gene is frequently deleted in many human cancers including neuroendocrine PCa (NEPC), an advanced form of PCa which is resistant to most existing treatments in clinic.24-26. We, therefore, sought to investigate how Rb loss affects drug resistance in PCa cells with NEPC traits. It has been reported previously that PC-3 PCa cells exhibit cancer stem cell characteristics surrounding the FXXV motif.27,28. However, it remains unclear whether Rb-N could also regulate other cancer-relevant functions through undefined mechanisms.

In our current study, we find that Rb loss leads to BET inhibitor resistance in a manner independent of the Rb-E2F1 pathway in PCa cells. We show that Rb-N binds to the BD1 domain of BD4 and restricts BD4 occupancy in the genome; however, Rb-N-BD4 binding is attenuated by Rb phosphorylation at S249/T252 residues. Our data also reveal that depletion of Rb enhances BD4 occupancy at the loci of cancer-related genes including the GPCR-cAMP signaling gene Gnb1l. Finally, we demonstrate that loss of Rb protein confers resistance to small molecule BET inhibitor and this effect is abolished by co-inhibition of Gnb1l-CreB signaling.

RB-N interacts with BD4 and the interaction is diminished by Rb phosphorylation

It is known that Rb binds to and regulates the activity of its target proteins, such as E2F1 and p65. We hypothesized that Rb modulates BET inhibitor sensitivity by binding to and regulating BET protein activity. To test this hypothesis, we first performed co-IP experiments. We demonstrated that the ectopically expressed HA-Rb interacted with BD2, BD3, and BD4 in 293T cells (Supplementary Fig. 3a). RB interaction with these BET proteins were confirmed at the endogenous level in both C4-2 and PC-3 cells (Fig. 2a, b). Rb protein is highly phosphorylated in proliferating cells. We sought to determine whether Rb phosphorylation affects Rb-BET protein interaction. 293T cells were transfected with HA-Rb and cell lysate was treated with an antibody to anti-HA antibody. We demonstrated that phosphatase treatment largely enhanced Rb interaction with BD4 (Fig. 2c, d). Moreover, we found that treatment of CDK4/6 inhibitor palbociclib or co-knockdown of CDK4 and CDK6 enhanced Rb-N interaction with BD4 (Supplementary Fig. 3b). Similar results were obtained at the endogenous level in both C4-2 and PC-3 cell lines (Fig. 2c, d). Moreover, we found that RbΔCDK mutant, in which fifteen CDK serine/threonine phosphorylation residues were mutated to alanine,29 had greater interaction with BD4 than the WT counterpart (Supplementary Fig. 3c). These data indicate that Rb phosphorylation impairs Rb-BD4 interaction.

To define which region in Rb mediates its interaction with BET proteins, we constructed three glutathione-S-transferase (GST)-Rb recombinant protein vectors (Supplementary Fig. 3d) and purified the recombinant proteins from bacteria. Results from the GST pull-down assay showed that BD4 specifically bound to the N-terminal portion of Rb (Rb-N) in vitro (Fig. 2e). In contrast, BD2 and BD3 did not interact with both N- and C-terminal portions of Rb, but the interactions were much weaker compared to the BD4-Rb-N interaction (Fig. 2e). Therefore, we focused on Rb regulation of BD4 in further studies. Co-IP assay not only confirmed that BD4 interacted with Rb-N in cells, but also demonstrated that their interaction is regulated by phosphorylation (Fig. 2f).

RB serine-249/threonine 252 phosphorylation disrupts Rb-N-BD4 interaction

Since dephosphorylation of Rb-N enhances its interaction with BD4, we decided to determine which phosphorylation sites are involved in this process. GST pull-down assay revealed that BD4 bound to the N-terminal portion of Rb-N which contains CDK4/6 phosphorylation sites serine-249 and threonine-252 (S249/T252), but no interaction between BD4 and the C-terminal portion of Rb-N was detected (Fig. 2g, h). We also found that treatment of CDK4/6 inhibitor palbociclib or co-knockdown of CDK4 and CDK6 enhanced Rb-N interaction with BD4 (Supplementary Fig. 3e, f). Moreover, the BD4-Rb-N interaction was largely enhanced by the S249/T252 phosphorylation-resistant mutant S249A/T252A or deletion of the S249/T252-containing motif (SPRT) (RB-NASPTRT) (Fig. 2i) and Supplementary Fig. 3g).
**Fig. 1 | RB loss confers resistance to BET inhibitors independent of E2F1 signaling in PCa cells.** a Heatmap showing the effects of a set of small molecule inhibitors of cancer-related pathways on growth of control and RB knockdown PC-3 cells. b Cell viability analysis of control and RB knockdown C4-2 cells after the treatment with different concentrations of JQ1 or I-BET726 for 72 h. Western blot (WB) analysis of cleaved-PARP (c-PARP) and cleaved caspase-3 (c-Caspase-3) level in control and RB knockdown PC-3 (a) and C4-2 cells (d) treated with different doses of JQ1 for 72 h. WB analysis of indicated proteins (e) in DU145 cells after transfected with empty vector (EV), HA-RB or mutant HA-RB R661W for 72 h followed by cell viability analysis (f) after treatment with different doses of BET inhibitors for 72 h. WB analysis of indicated proteins (g) in PC-3 cells after infected with lentivirus for control shRNA or gene-specific shRNAs for RB1 or E2F1 for 48 h followed by cell viability analysis (h) after treatment with different doses of BET inhibitors for 72 h. Data in a, b, f, and h represented as mean ± s.d. from triplicates. Statistical significance in b, f, and h was assessed by two-way ANOVA.
These results suggest that S249/T252 phosphorylation inhibits BRD4-RB-N interaction. To mimic the RB-N phosphorylation at S249 and T252, we mutated S249 and T252 to aspartic acid (D). By performing GST pulldown assay, we demonstrated that the S249D/T252D mutant had less binding ability to BRD4 compared to the WT counterpart (Fig. 2j). In contrast, phosphorylation-mimicking mutation T356D/T373D had no effect on BRD4-RB-N binding (Fig. 2j), consistent with the observation that the C-terminal portion of RB-N does not interact with BRD4 (Fig. 2h). Next, we performed in vitro kinase assay prior to GST pulldown assay. We demonstrated that CDK4/6 diminished RB-N-BRD4 interaction; however, this effect was abolished by S249A/T252A or RB-NΔSPRT mutant (Fig. 2k and Supplementary Fig. 3h).
Collectively, these data suggest that S249/T252 phosphorylation by CDK4/6 impedes RB binding of BRD4.

The 157FLQK161 motif in BRD4 BD1 domain mediates BRD4-RB-N interaction. To determine the region in BRD4 that is required for RB binding, we generated four FLAG-tagged truncation mutants of BRD4 for co-IP assay. We found that only the BRD4 N-terminal region which contains two bromodomains (amino acids 1 to 470) bound to RB (Fig. 3a, b). Both GST pulldown and co-IP assays further showed that bromodomain 1 (BD1), but not BD2 binds RB (Fig. 3c, d and Supplementary Fig. 4a).

We and others have previously shown that an FXXV motif in the RB-binding partners is important for their binding with RB[20,21]. In this study, we sought to determine whether there is any functional FXXV motif in BRD4 that mediates binding to RB. We noticed that there is one typical FXXV motif (157FLQPV161) and an atypical FXXV motif (83FQQPV87) in BRD4, both within the RB-binding region in BD1 (Fig. 3e). To test the functionality of these two FXXV motifs, we generated a series of alanine mutants (E/A, R/L mutant) (Fig. 3f) and utilized them for in vitro GST pulldown assay. While the 83AQQPA87 mutant alone had no effect on BRD4 BD1 binding of RB, the 157ALQKA161 mutation alone largely diminished and 157ALQKA161 double mutant almost completely lost the binding of RB (Fig. 3g). Similar results were obtained in co-IP assay performed in cell lysate (Fig. 3h). Given that RB interacts with BRD4 through BD1, a domain critical for BRD4 recognition of acetylated histone and non-histone proteins[22,23], we examined whether inhibition of the bromodomain activity by JQ1 affects the RB-BRD4 interaction. We found that administration of JQ1 to the GST pulldown and co-IP assay buffers had no impact on BRD4 binding of RB (Supplementary Fig. 4b–e).

Among the three members of the pocket protein family, we found that only RB, but not two other related proteins p107 and p130 can bind BRD4 (Fig. 3i), implicating that there is a unique motif in RB for its preferential binding of BRD4. In agreement with this observation, RB protein is the only member of the pocket protein family that harbors R-linker which contains BD4-interacting sites S249/T252. Notably, there are a number of positively charged arginine (R) residues within the R-linker in RB-N and several negatively charged glutamic acid (E) residues surrounding the 157FLQK161 motif in BRD4 (Fig. 3j, top). As described above, phosphorylation of RB-N S249/T252 impedes the BRD4-RB-N interaction. We hypothesized that the negative charges in the proximity of 157FLQK161 are critical for BRD4 interaction with RB R-linker (Supplementary Fig. 4f, left panel). To test this hypothesis, we made an E-to-A mutant by replacing E residues with alanine (E/A mutants) surrounding 157FLQK161 and an E-to-R mutant by replacing E residues with positively charged arginine (E/R mutant) (Fig. 3j, bottom) and performed GST pulldown assay. We demonstrated that the interaction between BRD4 BD1 and RB was attenuated by the E/R mutant and the interaction was further reduced by the E/R mutant (Fig. 3k).

Together, we provide evidence that the 157FLQK161 motif in BD1 and the negative charge in the adjacent residues are important for BRD4 binding of RB and that this interaction is diminished by the addition of negative charges in the RB R-linker due to S249/T252 phosphorylation by CDK4/6 (Supplementary Fig. 4f, right). To investigate the effect of RB on the binding ability of BRD4 to chromatin, we carried out the chromatin-binding assay and found that RB loss increased BRD4 binding of chromatin while the restoration of RB in RB-deficient cells reduced BRD4 binding on chromatin (Fig. 3l, m).

RB loss activates the GNB1L-CREB signaling axis through BRD4 BD1 is critical for BRD4 to recognize the acetylation moiety on histones and participates in transcription control of cancer-related genes[24,25]. Our data support the notion that RB binds to BD1 and limits BRD4 activity in chromatin binding and that RB loss renders resistance to BET inhibitors by increasing BRD4 engagement with chromatin and inducing transcriptional reprogramming. To interrogate this hypothesis, we performed BRD4 ChIP-seq in control and RB KD C4-2 cells. By running the Cistrome pipeline, we revealed that the quality of the BRD4 ChIP-seq data obtained from both control and RB KD C4-2 cells were very comparable to the BRD4 ChIP-seq results of approximately 300 different human cell samples in the Cistrome database (Supplementary Fig. 5a–1 and Supplementary Data 1). We demonstrated that RB knockdown increased BRD4 occupancy in the genome (n = 107, Log2 (fold change) > 1, p < 0.05), but only reduced BRD4 binding at 4 loci (Fig. 4a, b, Supplementary Data 2 and 3). The 107 peaks with enhanced occupancy of BRD4 were related to 152 genes, some of which were enriched in the cAMP pathway (Fig. 4c, d). cAMP production functions downstream of the GPCR signaling. We demonstrated that RB depletion increased BRD4 occupancy at certain region(s) in the loci of genes in the GPCR-cAMP pathways such as GNB1L (Fig. 4d and Supplementary Fig. 6a, b). Using ChIP-qPCR assay, we confirmed the increased BRD4 occupancy in the loci of GPCR-cAMP genes such as EDNRA, GNB1L, GRIN3A, GRM4, and SST1R in both PC-3 and C4-2 cells (Fig. 4e, f). Consistent with the ChIP-qPCR data, expression of these five genes was upregulated after RB KD in both PC-3 and C4-2 cells except GRIN3A and GRM4 expression in PC-3 cells (Fig. 4g, h). GNB1L protein was also substantially upregulated in RB KD PC-3 and C4-2 cells (Fig. 4i). We further showed that knockdown of BRD4 alone not only decreased GNB1L expression and phosphorylation of CREB, a downstream effector of the GPCR-cAMP pathway, but also completely abolished RB KD-induced upregulation of GNB1L protein and CREB phosphorylation (Fig. 4j, k), suggesting that RB deficiency-induced upregulation of GNB1L is mediated through BRD4. Notably, similar results were obtained in PCA patient samples from the TCGA database[26]. We found that GNB1L mRNA expression was much higher in RB deletion samples compared to WT samples (Fig. 4j, m), and their expression was inversely correlated (Fig. 4n). Among other four GPCR-cAMP pathway genes expression of two of them (e.g., GRIN3A and GRM4) was also upregulated in RB-deficient tumors compared to WT counterparts in the TCGA cohort and the difference was statistically significant (Supplementary Fig. 6c–f). These data suggest that RB can regulate expression of these GPCR-cAMP genes in cultured PCA cells and patient samples, but the regulation at certain gene loci could be influenced by the cellular contexts, especially in PCA samples from patients.

The high-level expression of GNB1L mRNA also significantly associated with poor survival of PCA patients (Fig. 4o). Accordingly, we showed that ectopic overexpression of GNB1L caused resistance to
Fig. 3 | BRD4 interacts with RB through an FXXXI motif in the BD1 domain. 

a) Diagram showing different mammalian expression vectors for full-length (FL) and truncated BRD4 proteins. b) WB analysis of RB protein in input and co-IP samples from C4-2 cells transfected with different fragments of BRD4. c) Diagram showing GST-BRD4 recombinant proteins containing two or each single BD domain. d) Top, WB analysis of RB proteins from C4-2 cell lysate pulled down by different GST-BD1 and mutants. Bottom, Coomassie blue staining of GST and GST-BD1 recombinant proteins. e) Diagram showing the location of FXXXV and FXXXI motifs in BD1 of BRD4. f) Diagram showing single or double mutants of FXXXV and FXXXI motifs. g) Top, WB analysis of RB proteins from C4-2 cell lysate pulled down by different GST-BD1 and mutant recombinant proteins. Bottom, Coomassie blue staining of GST and GST-BD1 mutant recombinant proteins. h) WB analysis of RB proteins from the co-IP samples from C4-2 cells expressing indicated FLAG-tagged BRD4 WT or mutants. i) WB analysis of indicated protein in input and co-IP samples from PC-3 cells. j) Diagram showing the charge composition in the RB-N R-linker region surrounding S249/T252 and residues surrounding the FXXXI motif in BRD4 BD1. k) Top, WB analysis of RB proteins from C4-2 cell lysate pulled down by different GST-BRD4 BD1 and mutants. Bottom, Coomassie blue staining of GST and GST-BRD4 BD1 recombinant proteins. l) WB analysis of indicated proteins in C4-2 cells transfected with indicated plasmids. m) WB analysis of soluble and chromatin fractions isolated from PC-3 cells expressing the indicated proteins. Western blot assays in b, d, g, h, i, k, l, and m were repeated two times independently with similar results.
BET inhibitors in both PC-3 and C4-2 cells (Supplementary Fig. 7a–d). Moreover, knockout of GNB1L re-sensitized RB-deficient PC-3 and C4-2 cells to BET inhibitors (Supplementary Fig. 7e–h). Similar to the findings in LNCaP/AR cell line30, RB KD not only decreased AR mRNA expression, but also induced resistance to the antiandrogen enzalutamide in C4-2 cells (Supplementary Fig. 7i, j). However, co-knockdown of GNB1L failed to abolish RB KD-induced AR downregulation and enzalutamide resistance (Supplementary Fig. 7i, j). Moreover, similar to the findings in LNCaP/AR cell line30, we found that RB KD in C4-2 cells induced upregulation of neuron-specific enolase (NSE), a neuroendocrine cell marker (Supplementary Fig. 7i); however, this effect was not affected by co-knockdown of GNB1L (Supplementary Fig. 7i).
Fig. 4 | cAMP pathway gene GNB1L is a BRD4 binding target gene whose expression is upregulated upon RB loss. a Volcano plot showing BRD4 Chip-seq peaks up- (red) or down-regulated (blue) in C-4-2 cells expressing control (shcon) or RB-specific shRNAs. b Heatmaps show the signaling intensity of 107 enhanced BRD4 binding peaks in C-4-2 cells due to RB KD. c KEGG pathway analysis of 152 genes related to 107 genome loci with increased BRD4 occupancy upon RB loss. d UCSC Genome Browser screenshots showing the enhanced BRD4 occupancy in the GNB1L gene locus in shRB C-4-2 cells compared to shcon cells. ChiP-qPCR analysis of BRD4 occupancy at GNB1L and other GPCR-CAMP gene loci after RB loss in PC-3 (e) and C-4-2 cells (f). RT-qPCR analysis of the mRNA level of the indicated GPCR-CAMP pathway genes in control or RB knockdown PC-3 (g) and C-4-2 (h) cells. WB analysis of expression of GNB1L and phosphorylation (activation) of CREB after RB knockdown in PC-3 and C-4-2 cells. RT-qPCR analysis (j) and WB analysis (k) of expression of indicated genes and proteins in PC-3 cells stably expressing the indicated shRNAs. Meta-analysis of RNA-seq data showing the association of increased expression of RB1 (l) or GNB1L (m) with the WT or deletion statuses of RB1 gene in PCa samples of the TCGA cohort. n The correlation between GNB1L and RB1 mRNA expression levels in PCa samples of the TCGA cohort. o Kaplan–Meier Survival curve showing the association of high GNB1L mRNA expression with poor overall survival of PCa samples of the TCGA cohort. Western blot assays in i, k were repeated two independent times with similar results. Data in e-h and j were shown as mean ± s.d. from three independent experiments with two-sided Student’s t test for the statistical analysis. Data was performed by two-sided Fisher’s exact test from Enrichr (https://maayanlab.cloud/Enrichr/) in (c). Statistical analysis for data in a, i, m, n, and o was indicated in the method section.

These data support the observation reported previously30,31 that loss of RB promotes antitumor resistance and neuroendocrine phenotype. However, none of these effects was mediated through RB loss-induced upregulation of GNB1L. Of course, our data cannot completely rule out the possibility that other GPCR-CAMP-related genes upregulated by RB loss-induced aberrant activation of BRD4 may contribute to anti-tumor resistance and neuroendocrine phenotype in RB-deficient cells. E2F1 knockdown did not alter GNB1L expression in both RB-proficient and -deficient PC-3 cells (Supplementary Fig. 8a, b), indicating that its expression is not regulated by the RB-E2F1 axis. Together, our data show that RB deficiency induces upregulation of GNB1L and activation of the GPCR-CAMP-CREB axis in PCa cells and that this effect is mediated through BRD4.

RB S249/T252 phosphorylation promotes GNB1L expression and CREB activation

Since RB S249/T252 phosphorylation inhibits RB-BRD4 interaction, we sought to determine whether S249/T252 phosphorylation regulates GNB1L expression via BRD4. Consistent with the regulation of GNB1L by BRD4 knockdown, we found that in a dose-dependent manner treatment of PC-3 cells with the BRD4 inhibitor JQ1 inhibited GNB1L expression at both mRNA and protein levels (Supplementary Fig. 8c, d). As RB-BRD4 interaction can be enhanced by RB S249/T252 dephosphorylation, we examined whether the expression of GNB1L and activation of CREB were influenced by RB dephosphorylation. We demonstrated that activation (dephosphorylation) of RB by treatment with CDK4/6 inhibitor palbociclib decreased GNB1L expression at protein and mRNA levels and inhibited CREB phosphorylation in a dose-dependent fashion in both PC-3 and C-4-2 cell lines (Fig. 5a, b and Supplementary Fig. 5e, f). In agreement with this observation, ChiP-qPCR assay showed that BRD4 binding on chromatin at the GNB1L gene locus was attenuated after palbociclib treatment in both cell lines (Fig. 5c and Supplementary Fig. 8g). Furthermore, we showed that ectopic expression of WT RB suppressed GNB1L mRNA and protein expression in DU145 cells in which endogenous RB is dysfunctional (Fig. 5d, e). Compared to the WT RB, the inhibitory effect of RB S249/T252 phosphorylation-resistant mutant RBΔCDK was much greater as evident by the reduced expression of GNB1L protein and CREB phosphorylation of CREB (Fig. 5d, e).

To verify these findings, we knocked down endogenous RB and performed rescue experiments. We found that RB knockdown-induced upregulation of GNB1L expression and CREB phosphorylation was reversed by restored expression of shRNA-resistant WT RB and this effect was largely enhanced by expression of phosphorylation-resistant mutant RBΔCDK (Fig. 5f, g). In line with these observations, enhanced expression of CDK4 and Cyclin D1 increased GNB1L expression and induced BET inhibitor resistance in C-4-2 and LNCaP cells (Fig. 5h–k). In various PCa cell lines with different levels of total and phosphorylated RB, we further demonstrated that PC-3 and DU145 cell lines (in which little/no RB protein or high level of phosphorylated RB was expressed) were much insensitive to BET inhibitors compared to C-4-2 and LNCaP (in which low level of phosphorylated RB was expressed) (Supplementary Fig. 8j–k). Notably, these results are consistent with the previous report that DU145 and PC-3 cells were relatively insensitive to JQ1 compared to LNCaP cells32. We also performed immunohistochemistry (IHC) analysis using a tissue microarray (TMA) containing 115 cores resulting from 51 patients with advanced PCa to investigate the correlation between RB phosphorylation and GNB1L expression. We found that high expression of GNB1L positively correlated with elevated RB S249/T252 phosphorylation and their correlation was statistically significant (Fig. 5l, m). Collectively, our data suggest that RB S249/T252 phosphorylation can promote GNB1L expression in PCa cells in culture and patient samples.

CREB inhibitor 666-15 overcomes JQ1 resistance in RB-deficient PCa cells in vitro and in vivo

Next, we sought to determine whether targeting GPCR-CAMP signaling enables to overcome BET inhibitor resistance in RB-deficient PCa cells. To this end, we treated control or RB KD PC-3 cells with JQ1 or CREB inhibitor 666-15 individually or both prior to MTS assays. We demonstrated that while RB KD cells were highly resistant to JQ1, both control and RB KD cells were equivalently sensitive to 666-15 (Fig. 6a). Importantly, 666-15 co-treatment completely abolished JQ1 resistance in RB-deficient cells (Fig. 6a). Similar results were obtained in C-4-2 cells (Supplementary Fig. 8k). Using colony formation assay we further validated that co-treatment of 666-15 completely abolished JQ1 resistance in these two cell lines (Fig. 6b–d and Supplementary Fig. 8l, m).

We also examined the anti-cancer effect of JQ1 and the CREB inhibitor in RB-deficient tumors in vivo. As expected, RB depletion largely enhanced PC-3 tumor growth in mice (Fig. 6e–g). Importantly, RB-deficient PC-3 tumors were resistant to JQ1 in comparison to control tumors (Fig. 6e–g), consistent with the results obtained in PC-3 cells cultured in vitro (Fig. 6a). However, co-administration of 666-15 abolished JQ1 resistance of RB-deficient PC-3 tumors (Fig. 6e–g), suggesting that there was seemingly no obvious drug-drug interaction between these two compounds under these conditions. Analysis of Ki67 and cleaved Caspase-3 IHC in tumor tissues showed that JQ1 treatment only resulted in a minimal effect on proliferation of RB-deficient tumors, but Ki67 expression was largely inhibited by the combined treatment of JQ1 and 666-15 (Fig. 6h, i). Although JQ1 treatment alone had no obvious effect on the expression of cleaved Caspase-3, an indication of apoptosis, the combination of JQ1 and 666-15 drastically induced expression of cleaved Caspase-3 (Fig. 6j, k). In agreement with the result of cell death, there was almost no effect of JQ1 treatment on expression of BCL-2, a known transcription target of CREB and a well-studied anti-apoptotic protein33 (Fig. 6d). In contrast, treatment with 666-15 in combination with JQ1 completely abolished RB depletion-induced upregulation of BCL-2 (Fig. 6d). Together, these data indicate that activation of CREB plays an essential role in mediating BET inhibitor resistance in RB-deficient PCa cells and that inhibition of CREB overcomes BET inhibitor resistance.
**Discussion**

Through a small-scale screening of signaling pathway inhibitors such as BET inhibitor, CBP/p300 inhibitor, PARP inhibitor, and AKT inhibitor, we uncovered that RB-deficient PCa cells are resistant to BET inhibitors. It has been shown previously that RB-null NUT midline carcinoma cells are resistant to BET inhibitors and the resistance is believed to be mediated through the RB-E2F1 pathway. Different from the finding in NUT carcinoma cells, however, we provide evidence that E2F1 knockdown had little or no obvious effect on the BET inhibitor sensitivity in both RB WT and deficient PCa cells. This difference can be explained, at least in part by the differential effects of JQ1 on the expression of E2F1 in these two cancer types.
treatment decreased E2F1 expression in NUT carcinoma cells, no such effect was observed in PCA cells, reinforcing the notion that BET inhibitor resistance in PCA cells is mediated through the mechanism independent of E2F1. It has been shown previously that the CDK4/6 inhibitor palbociclib can largely sensitize cells to JQ1 treatment in RB-proficient cells in a dosage-dependent manner. However, such combination is not applicable in RB-deficient PCA cells, highlighting that a new strategy is needed to overcome BET inhibitor resistance. By identifying RB protein as a binding partner and an intrinsic inhibitor of BRD4, we further show that loss of RB results in aberrant occupancy of BRD4 in the loci of GPCR-cAMP pathway genes. Importantly, we convincingly show that co-targeting this signaling pathway overcomes BET inhibitor resistance in RB-deficient cells. Hence, our work not only provides molecular insights into the mechanism of BET inhibitor resistance in RB-deficient PCA cells, but also define a viable therapeutic strategy to overcome the resistance.

We and others have previously reported a FXXXV-containing motif in client proteins mediating the binding by RB-N19,20. While there is a quite a few glutamic acid residues present surrounding the BRD3, and BRD4, to our surprise, such motif is not required for BET protein binding of the R-linker region in RB-N (Fig.7). Our findings in cultured PCa cells and xenograft tumors in mice invariably suggest that CREB is also a potential therapeutic target of PCA, especially those in which the RBP gene is deleted or protein becomes hyperphosphorylation due to aberrant activation of CDK4/6 (Fig. 7).

In summary, our work identifies RB protein as a bona fide endogenous inhibitor of BRD4 and demonstrates that RB loss or phosphorylation at S249/T252 by CDK4/6 confers PCA cell resistance to small molecule BET inhibitors by upregulating GNB1L expression and activating GNB1L-CREB signaling cascade. Our results imply that loss of RB, aberrant activation of CDK4/6 as well as CREB high expression could serve as biomarkers to predict the BET inhibitors in PCA. Furthermore, our studies suggest that RB-deficient cells become susceptible to BET inhibitors after co-administration of the CREB inhibitor. Therefore, our findings not only illustrate the molecular mechanism of BET inhibitor resistance caused by RB loss, but also reveal a potential therapeutic strategy for prostate cancers with abnormalities in RB, CDK4/6, and/or CREB signaling.

**Methods**

**Antibodies, plasmids, and chemicals** The following antibodies are used in the experiments: Anti-RB (1:2000 in dilution, 554136, BD bioscience), p107/RBL1 (1:1000 in dilution, SC-318, Santa Cruz Biotechnology), p130/RBL2 (1:1000 in dilution, SC-317, Santa Cruz Biotechnology), ERK2 (1:2000 in dilution, SC-1647, Santa Cruz Biotechnology), Histone H3 (1:1000 in dilution, SC-8035, Santa Cruz Biotechnology), CDK6 (1:1000 in dilution, SC-601, Santa Cruz Biotechnology), CDK4 (1:1000 in dilution, SC-601, Santa Cruz Biotechnology), p130/RBL2 (1:1000 in dilution, SC-601, Santa Cruz Biotechnology), BRD2 (1:1000 in dilution, SC-1647, Santa Cruz Biotechnology), Histone H3 (1:1000 in dilution, 9715, Cell Signaling Technology), BRD2 (1:1000 in dilution, ab139690, Abcam), BRD3 (1:1000 in dilution, A302-368A, Beyhl), BRD4 (1:1000 in dilution, A301-9851A00, Beyhl), BRD4 (1:1000 in dilution, ab128874, Abcam), GNB1L (1:1000 in dilution, HPA034627, Sigma), Cleaved PARP (1:1000 in dilution, 5625, Cell Signaling Technology), Cleaved Caspase-3 (Asp175) (1:1000 in dilution, 9661, Cell Signaling Technology), p50 (1:1000 in dilution, 13856, Cell Signaling Technology). Secondary antibodies including anti-mouse (light chain) secondary antibody were purchased from Jackson Immunoresearch at 1:5000 of dilution when used. Plasmids for HA-RB and related mutants, HA-Cyclin D1 and V5-CDK4 are described previously19,40. FLAG-GNB1L plasmid was purchased from GenScript (#OHU05623D). Detailed information of the shRNAs and sgRNAs used in this study is found in Supplementary Data 4. LentiCRISPR v2-dCas9 plasmid was obtained from Addgene (#112233). JQ1 was purchased from TargetMol (#T2110). I-BET726 was obtained from Cayman (#16872). 66-15 was purchased from Millipore (#538341). Palbociclib was purchased from APEXBio (#A8316).

**Cell lines and cell culture** PC-3, DU-145, LNCaP, and 293T cells were purchased from American Type Culture Collection (ATCC), C4-2 cells were purchased from Uro Corporation. PC-3, DU145, LNCaP, and C4-2 cells were cultivated in different doses of BET inhibitors for 72 h. Representative images of GNB1L and phosphorylated RB at S249/T252 (pRB) IHC staining on a TMA from a cohort of metastatic PCA patient samples. Scale bar in ‘50 x’ represented 150 µm while that in ‘100 x’ represented 50 µm. Positive correlation between GNB1L and pRB protein expression in the cohort of PCA patient samples. Data in b, c, e, and g were as mean ± s.d. from three independent experiments. The statistic test was performed by two-sided Student’s t test. Data in i and k were analyzed by two-way ANOVA. The Spearman’s correlation was performed in (m).
RPMI 1640 media (Corning) with 10% fetal bovine serum (FBS) (Invitrogen/Thermo Fisher Scientific). 293T cells were grown in DMEM media (Corning) supplemented with 10% FBS (Millipore). All the cells were incubated at 37 °C supplied with 5% CO₂. Cells were treated with plasmocin (Invivogen) to anti-mycoplasma before the subsequent experiments.

Transfection and lentivirus infection
Lipofectamine 2000 (Thermo Fisher Scientific) was used for transient transfection with indicated plasmids according to the manufacturer’s instruction. The supernatant containing lentivirus was collected from 293T cells co-transfected with plasmids of psPAX2, pMDG2 and control or gene-specific shRNAs and filtered using 0.22 μm filter
mice at 23 days after treatment with vehicle or indicated inhibitors. b–k IHC images and quantitative data of Ki67 (h, i) and cleaved Caspase-3 (j, k) in xenografts in mice as treated in (e). Scale bar in the IHC images in h, j represented 100 μm. Data in a shown as means ± S.D. from five replicates. Data in c shown as means ± S.D. from three independent experiments. Data in f and g shown as means ± S.D. from six tumors (n = 6) in each group. Data in l and k shown as mean ± s.d. from three independent experiments. For each experiment, five independent fields were enrolled for the calculation. Two-sided Student’s t test was used for a, c, g, i, and k. Two-way ANOVA was performed in (f).

Cell proliferation assay
For IC50 assay, DU145 (1000/well), PC-3 (1000/well), C4-2 (2000/well) or LNCaP cells (2000/well) were seeded in 96-well plates overnight. Cells were collected after transfected with indicated plasmids. After 72 h of incubation, MTS reagent (Promega) was added to each well and the plates were incubated at 37 °C for 2 h. Optical density (OD) of cells was measured at 490 nm using microtiter reader (Biotek).

Chromatin immunoprecipitation (ChIP), high throughput sequencing and ChIP-qPCR
Control and RB-knockdown PC-3 and C4-2 cells were treated with disuccinimidyl glutarate (DSG) (A33932, Thermo Fisher Scientific) for 30 min at room temperature before being fixed in 1% formaldehyde. Cells were quenched with 2.5 μM glycine and collected for sonication. The supernatant was obtained after centrifuge and mixed with protein A/G beads (Thermo Fisher). The elution was further crosslink was performed at 65 °C for 2 h. The elution was further analyzed by Western blot.

Relative gene expression was normalized to the expression of house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The sequence information of the primers used for RT-qPCR are provided in Supplementary Data 4.
treated with RNase A and proteinase K. Enriched DNA was collected for high throughput sequencing (ChIP-seq) or quantitative PCR (ChIP-qPCR). For ChIP-seq, the high-throughput sequencing was performed using the Illumina HiSeq 4000 platform by Genome Analysis Core at Mayo Clinic. Primers for different BRD4 target genes were designed based on the BRD4 binding peaks identified by ChIP-seq. BRD4 binding of specific target loci was validated by ChIP-qPCR using the SYBR Green Mix (Bio-Rad) and CFX96 Real-Time System on C1000 Touch Thermal Cycler (Bio-Rad) according to manufacturer’s instructions. The sequence information of the primers used for ChIP-qPCR are provided in Supplementary Data 4.

ChIP-seq data analysis

The raw reads of ChIP-seq were mapped to the human reference genome (GRCh37/hg38) using bowtie2 (version 2.2.9). MACS2 (version 2.1.1) was run to perform the peak calling with a p value threshold of 1 × 10⁻⁸. BigWig files were generated for visualization using the UCSC Genome Browser. The assignment of peaks to potential target genes was performed by the Genomic Regions Enrichment of Annotations Tool (GREAT). DiffBind software from an open source (https://bioconductor.org/packages/release/bioc/html/DiffBind.html) was utilized to determine the differential binding of BRD4 in the genome of the control and RB knockdown C4-2 cells. Data was collected for differential analysis by DEseq2, calculated for p value by two-sided Wald test after modeling the count data by logistic regression, and p value was adjusted by the Benjamini and Hochberg method for multiple comparison. The ChIP-seq data have been deposited into the NCBI GEO data repository with the accession code GSE191263.

Cistrome database BRD4 ChIP-seq samples, the peak files and QC metrics were downloaded by batch download function at http://cistrome.org/db/#/bdown. In total, 294 human BRD4 ChIP-seq samples available in the batch download regardless of cell type or disease type. ChIPs pipeline was used to run BRD4 ChIP-seq samples, and QC report was generated by ChIPs pipeline (https://julab-dfci.github.io/resources/publications/F1000ResCh10_517.pdf).

Preparation of chromatin fraction

PC-3 cells transfected with the indicated plasmids were collected for the fractionation as described. After washing with cold 1 × PBS, cells were incubated with lysis buffer (50 mM Tris-HCl, pH 7.5, 5% glycerol, 150 mM NaCl, 1.5 mM MgCl₂) supplied with 0.5% NP-40 at 4°C for 30 min. The lysate was centrifuged at 1500 × g for 5 min. The supernatant was then spun at 20,000 g for 15 min and the soluble fraction was collected. The pellet from the first-time centrifugation was resuspended in 2 volumes of lysis buffer supplemented with 0.2% SDS and 1:1000 dilution of Benzonase Nuclease (E1014, Sigma-Aldrich) after washing one time with lysis buffer, and incubated in an incubator shaker at 37°C at 850 rpm for 30 min. The chromatin fraction was spun down by 20,000 g for 15 min. The soluble and chromatin fractions were further analyzed by Western blot.

Crystal violet staining

PC-3 (1,000) or C4-2 cells (2000) were seeded in 6-well plates overnight. After 14 days of treatment, cells were washed with 1 × PBS and fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature. After washing with 1 × PBS for twice, cells were stained with crystal violet (0.5 g crystal violet in 80 ml H₂O with 20 ml methanol) for 30 min at room temperature. Cells were washed with distilled water gently to remove the remaining crystal violet.

GST-tagged recombinant protein purification

Plasmids for GST-tagged recombinant proteins were transformed into E. coli BL21. The successfully transformed BL21 cells were cultured in flasks in an incubator shaker and treated with 100 μM IPTG (Sigma-Aldrich) at 18°C overnight. After IPTG induction, bacteria were collected and resuspended in lysis buffer (50 mM Tris-HCl, pH 8.0) supplied with protease inhibitor (Sigma-Aldrich) and sonicated. Glutathione Agarose (Thermo Fisher Scientific) was added to enrich the GST-tagged protein. The 10 nM reduced glutathione (Sigma-Aldrich) in 50 mM Tris-HCl, pH 8.0 was added and incubated with agarose for 1 h at room temperature. The eluted protein was collected by centrifuge and saved at -80°C for further use.

In vitro kinase assay

Cyclin D1 and CDK4 proteins were synthesized using the in vitro transcription and translation system (LI70, Promega) according to the manufacturer’s instructions. These proteins were then incubated with the mixture of GST-RBN or GST-RBN (S249A/T252A) proteins and GST beads in the kinase reaction buffer (25 mM HEPES pH 7.5, 25 mM β-glycerophosphate, 25 mM MgCl₂, 2 mM dithiothreitol, 0.2 mM ATP and 0.1 mM NaVO₃) at 30°C for 2 h. After washing with wash buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂) twice, the beads were incubated with lyses of 293T cells at 30°C for 2 h. The beads were washed with wash buffer for five times, and beads were boiled in sample loading buffer and subjected to the Western blot analysis.

Prostate cancer patient samples

With the approval of the institutional review board (IRB), metastatic PCA specimens were obtained from patients undergoing standard-of-care biopsies at Mayo Clinic (Rochester, MN). Informed consent was obtained for use of the samples for research by the time the specimens were collected by the Mayo Clinic. A tissue microarray was constructed from the formalin-fixed paraffin-embedded (FFPE) samples of metastatic PCA identified after a search of pathologic and clinical databases of archival tissues. The Mayo Clinic institutional review board approved the experimental protocols for retrieving pathology blocks/slides and accessing electronic medical records. Cores in which greater than 50% of the tissue was lost during IHC were excluded from analysis. A total of 115 tissue microarray cores resulting from 53 samples (20 bone metastases and 33 nonbone metastases) of 51 patients were used for analysis. The study design and conduct complied with all relevant regulations regarding the use the specimens of human study participants. The study was conducted in accordance to the criteria set by the Declaration of Helsinki.

Generation and treatment of prostate cancer xenografts in mice

Mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Mayo Clinic. Six-week-old male SCID mice were generated in house. Control and RB knockdown PC-3 cells (5 × 10⁶) mixed with Matrigel mixture (1 × PBS: Matrigel (BD Biosciences) = 1:1) were injected subcutaneously into SCID mice. After the average size of the xenografts reached approximately 100 mm³, mice were treated intraperitoneally with vehicle (10% β-cyclodextrin (Sigma-Aldrich), 1% N-methylpyrrolidone (NMP) (Thermo Fisher Scientific), 5% Tween-80 (Sigma-Aldrich) in 1 × PBS) in 1 × PBS, Q1 (Sigma-Aldrich, dissolved in 10% β-cyclodextrin) at 50 mg/kg or 666-15 (TargetMotol, dissolved in 1% N-methylpyrrolidone (NMP), 5% Tween-80 in 1 × PBS) at 10 mg/kg 5 days a week. Tumor length (L) and width (W) were measured every 4 days, and tumor volumes were calculated by the formula (L × W²)/2. Mice were sacrificed by euthanasia and tumors were collected and photographed. One portion of the tumor tissues was used for FFPE and the rest was frozen for RNA and protein extraction.

Immunohistochemistry (IHC)

The FFPE xenograft tissues and patient specimens were consecutively cut at 4 μm. The tissues on slides were rehydrated, the activity of endogenous peroxidase was inhibited and antigen retrieval was performed as previously described. The tissues were incubated with

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primary antibody overnight at 4 °C. The following primary antibodies were used: anti-Ki67 (ab15580, Abcam) and anti-cleaved Caspase-3 (9661, Cell Signaling Technology). After washing, the tissues were incubated with secondary antibody (BA1000, Vector Lab) for 1h at room temperature. After counterstaining, the tissues were dehydrated and covered with a coverslip. For quantification, cells with positive staining in the nucleus were identified and included to calculate the percentage of Ki67 positive-staining cells.

Meta-analysis of patient data
The patient data from TCGA database was obtained from cBioPortal (https://www.cbioportal.org/). The Z-score (FPKM) for mRNA level of each gene of interest was downloaded from the database for further analysis. N = 267 for the RB ‘WT’ group and n = 218 for the RB ‘Deletion’ group. Data were shown as mean ± s.d., and two-tailed Mann–Whitney U test was carried out to calculate p value for the comparison. Relative level of GNB1L and RB expression in 485 patients was included for correlation analysis, which was determined by two-tailed Spearman’s rank correlation coefficient. Log-rank (Mantel–Cox) test was performed to determine the statistical differences between stratified groups used for Kaplan–Meier Survival curve analyses.

Statistical analysis
P values were determined by a two-tailed Student’s t test, two-way ANOVA test, log-rank test, Mann–Whitney U test. All data are shown as mean values ± S.D. for experiments representing three independent experiments except particular indication. P values < 0.05 were considered statistically significant.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data that support this study are available from the corresponding authors upon reasonable request. The ChIP-seq data generated in this study have been deposited into at the NCBI’s GEO data repository with the accession code GSE191263. The Human reference genome (GRCh38/hg38) (GenBank/RefSeq assembly accession numbers GCA_000001405.15/GCA_000001405.26) was used for mapping of ChIP-seq reads. Source data are provided with this paper.

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Author contributions
H.H. conceived the study. D.D. and L.S. performed experiments, data collection, and analysis. R.J. supervised histological and IHC analysis in patient samples. X.H. and S.J.W. provided reagents and participated in experimental design. L.W., R.Z., and Y.T. performed bioinformatics analysis. H.H., L.S., and D.D. wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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