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Inhibition of BRD4 attenuated IFNγ-induced apoptosis in colorectal cancer organoids

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Abstract

Background This study aimed to analyze the functional role of Brd4 in colorectal cancer (CRC) organoids. *Brd4* was identified as a CRC-related gene by our previous *Sleeping Beauty* mutagenesis transposon screening in mice. Brd4 is a transcriptional regulator that recognizes acetylated histones and is known to be involved in inflammatory responses. The role of Brd4 in CRC development remains largely unknown.

Methods We knocked out *Brd4* in tumor organoids carrying mutations in *Apc* and *Kras* to generate Brd4KO organoids, and performed RNA-seq. The response of Brd4KO organoids to IFNγ was analyzed via a cell viability assay, an apoptosis assay, and RNAseq. The results were validated by pharmacological inhibition experiments with JQ1 in human CRC organoids.

Results In Brd4KO organoids, the IFNγ signaling genes Il33 and Myc target genes were downregulated. The addition of IFNγ to the colon organoids induced apoptosis, but IFNγ-induced apoptosis was attenuated in the Brd4KO organoids compared with the control organoids (two-sided t-test, $P < 0.05$). Similar results were obtained from pharmacological inhibition with JQ1 in human CRC organoids; IL33 expression was decreased, and IFNγ-induced apoptosis was attenuated in the presence of JQ1.

Conclusions Our results showed that the inhibition of Brd4 suppressed IFNγ-induced cytotoxicity by modulating the Jak-Stat pathway. These data suggested that the inhibition of Brd4 could increase cell viability in the cancer microenvironment where IFNγ is abundant, revealing a new aspect of the molecular mechanism of CRC development. Our results may help in evaluating the application of Bet inhibitors in treating CRC. Additionally, our RNA-seq data sets will be helpful for clarifying the relationship between Brd4 and immunomodulators, such as Il33, or for studying the responses of colonic epithelial cells to IFNγ.

Keywords Organoid, Colorectal cancer, Brd4, Apoptosis, JQ1, IFNγ, IL33

Introduction

Colorectal cancer (CRC) is the second most common cancer worldwide. In CRC, loss-of-function mutations in *APC* are observed in nearly 80% of cases, and activating point mutations in *KRAS* are observed in 40% of cases [1]. CRC tissues exhibit genetic heterogeneity, with mutations identified in more than 70 genes [2]. Many of these genes are infrequently mutated, making distinguishing between passenger genes and driver genes simply from genomic information challenging.

To identify cancer-driver genes involved in CRC development and progression, we used *Sleeping Beauty* (SB) transposon mutagenesis screening in mice [3–5]. SB mutagenesis is a potent approach for identifying cancer driver genes that are also frequently mutated in human patients. This has led to the identification of many

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cancer-related genes [7–9], anticancer drug resistance genes [10], and inflammation-related cancer genes [4] in various organs.

In this study, we focused on bromodomain 4 (*Brd4*), which is frequently identified in SB screens in the colon [3, 4]. BRD4 is a member of the bromodomain and extraterminal domain (BET) family, with two bromodomains recognizing acetylated lysine residues in histones, and acts as an epigenetic reader with broad specificity for transcriptional control. BRD4 is involved in many physiological and pathological processes and has become an important therapeutic target for immune and inflammatory diseases [11]. In cancer, pharmacological inhibition of the BET bromodomain has been shown to be effective in a mouse multiple myeloma model via downregulation of Myc [12] or Myc-amplified medulloblastomas [13]. In CRC cell lines, promoter hypermethylation of *BRD4* was observed, which was correlated with reduced expression of *BRD4* mRNA. Furthermore, the restoration of *BRD4* expression was shown to reduce the efficiency of tumor development in vivo [14]. However, what gene expression is regulated by Brd4 in CRC cells remains unknown. In this study, we conducted genetic and pharmacological inhibition of Brd4 in mouse and human CRC organoids and showed that inhibition of *Brd4* could increase cell survival under IFNγ-enriched conditions.

Results

IFNγ signaling is deregulated in *Brd4* knockout organoids

To analyze the function of *Brd4*, we generated *Brd4* KO organoids since the transposon insertions in the SB screen disrupted the function of *Brd4*. We used mouse colon tumor organoids harboring loss-of-function mutations in *Apc* [15] and an activating point mutation in *Kras* [16] (AK organoids) [17] and knocked out *Brd4* via CRISPR-Cas9 using two different gRNAs [18, 19] (Fig. 1A). The knockout efficiency was confirmed by sequencing the gRNA target loci (Supplementary Figure A). First, we subcutaneously transplanted the organoids to validate the oncogenic potential of Brd4KO organoids in NSG mice. The frequency of tumor development was not different between control and Brd4KO organoids (Supplementary Figure B), suggesting that disruption of *Brd4* did not confer oncogenic ability in colonic organoids.

Next, we performed RNA-seq on control and Brd4KO organoids. GSEA revealed that several gene sets, including 'Myc targets' and 'IFNγ response', were deregulated in Brd4KO organoids (Fig. 1B and C). A heatmap of genes involved in the IFNγ response revealed that the expression of *Socs3*, *Stat3*, and *Jak1/2/3* was decreased in Brd4KO organoids, but the expression of *Cd274*, encoding PD-L1, was not downregulated in Brd4KO organoids (Fig. 1D). Since Brd4 is known to directly regulate the transcription of these genes by binding to their promoters [11, 20], we examined the binding of BRD4 in CRC cells via a previously published data set for ChIP-seq with a BRD4 antibody [21]. The IGV image revealed that BRD4 binds to the transcriptional start site of JAK2 (Fig. 1E). JAK2 is a nonreceptor tyrosine kinase that transduces signals from the IFNγ receptor. These data suggest that IFNγ signaling is downregulated in Brd4KO organoids by partially attenuating Jak2-mediated signal transduction.

We generated a volcano plot to enrich genes whose expression levels were significantly changed. Several genes encoding MHC genes were significantly upregulated in Brd4KO organoids (Fig. 1E), indicating that Brd4 regulates MHC gene expression and that immunogenicity in colon tumor cells may be increased by Brd4 deficiency, as previously described in prostate cancer cells [22]. We also found that the expression of IL33 and IL18 was significantly suppressed among the interleukins in the Brd4KO organoids (Fig. 1F), suggesting that Brd4 positively regulates the expression of these cytokines. Moreover, quantitative PCR confirmed that *Il33* was significantly downregulated in the Brd4KO-1 and Brd4KO-2 organoids (Fig. 1G). IL33, a member of the IL-1 family, is involved in innate and adaptive immune responses, including autoimmune disease and allergic reactions. IL33 is highly expressed in gut epithelial cells and localizes to the nucleus because of its chromatin-binding domain. During immune responses, damaged epithelial cells release IL33, which binds to ST2, an IL33 receptor [23]. Our data suggest that Brd4 tightly regulates IL33 expression.

Notably, Hoxa cluster genes were downregulated, which is consistent with a previous report showing the recruitment of BRD4 to *HOXA* genes to activate gene expression [24].

Brd4 knockout organoids are resistant to IFNγ-induced cell death

Next, we analyzed the functional relationship between Brd4 and IFNγ signaling. IFNγ secreted by immune cells such as T cells plays essential roles in host defense and antitumor immune responses. Colonic epithelial cells stimulated with IFNγ exhibit increased expression of MHC [25] and induce apoptosis [26]. When 1 ng/ml IFNγ was added to control AK organoids, the morphology of the organoids collapsed, and the organoids eventually died by day 4 (Fig. 2A and B). Compared with that in the absence of IFNγ, cell viability in the presence of IFNγ was decreased to 0.4 (Fig. 2C). Interestingly, Brd4KO organoids could proliferate in the presence of IFNγ and were resistant to IFNγ-induced cell death (Fig. 2B and C).

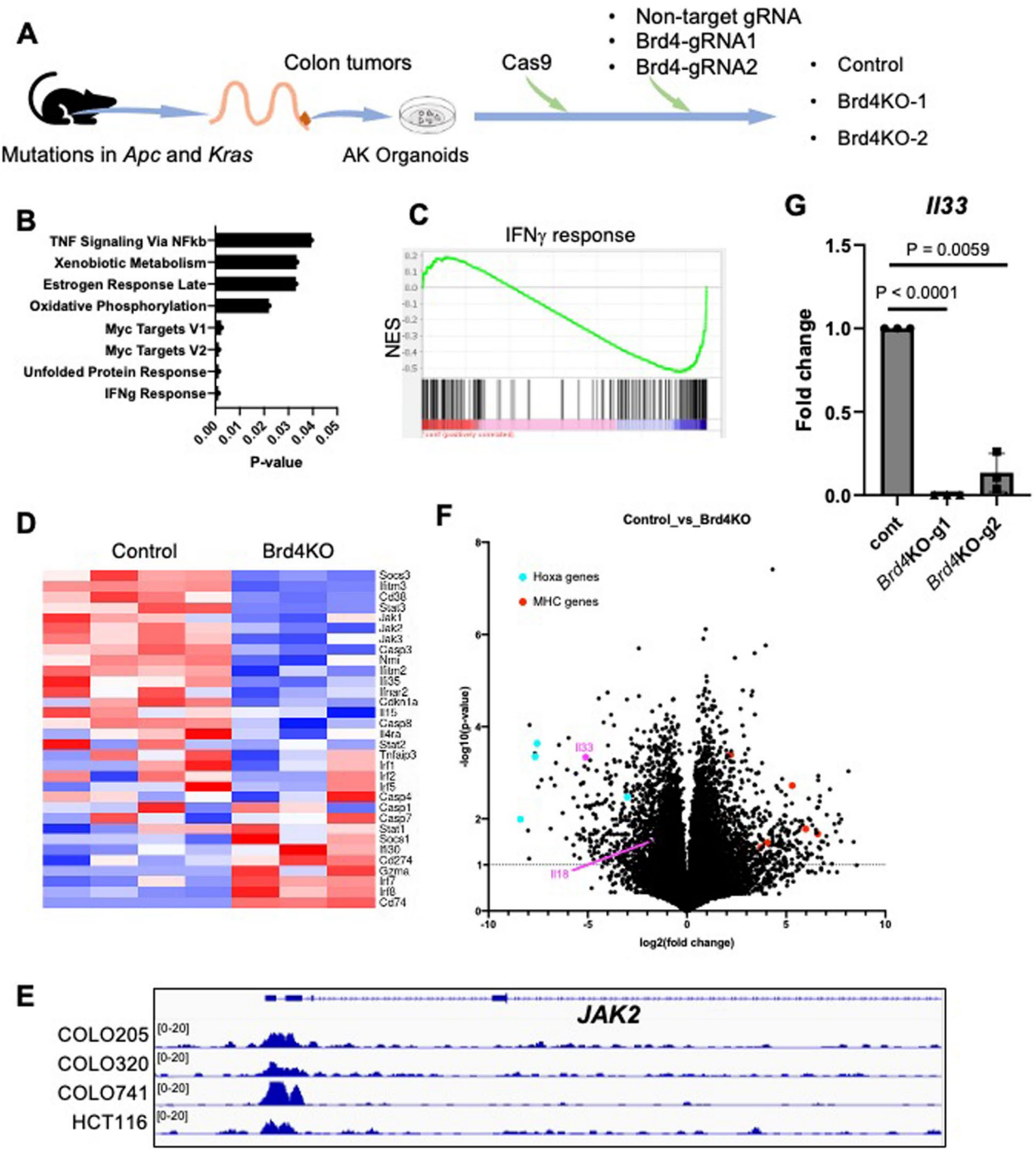

Fig. 1 Generation and analyses of *Brd4* knockout organoids. **A** An experimental design to generate *Brd4* knockout mouse organoids. Colonic tumor organoids were established from mice [17]. Cas9 was introduced into AK organoids to establish Cas9-expressing AK organoids. Nontargeting gRNA and gRNAs targeting *Brd4* were subsequently introduced to generate control organoids and Brd4KO organoids, respectively. **B** Bar graph showing the GSEA results for RNA-seq. Two-sided Fisher's exact test was used. **C** GSEA showing the downregulation of IFNγ signaling in Brd4KO organoids. **D** Heatmap of genes involved in IFNγ signaling between control and Brd4KO organoids. **E** An IGV image for ChIP-seq with an anti-BRD4 Ab at the *JAK2* locus. **F** Volcano plot showing the genes differentially expressed between control and Brd4KO organoids. Data plots showing MHC genes, Hoxa genes, and Il33 are highlighted in red, light blue, and magenta, respectively. **G** qPCR analysis of mouse *Il33* in control and Brd4KO organoids

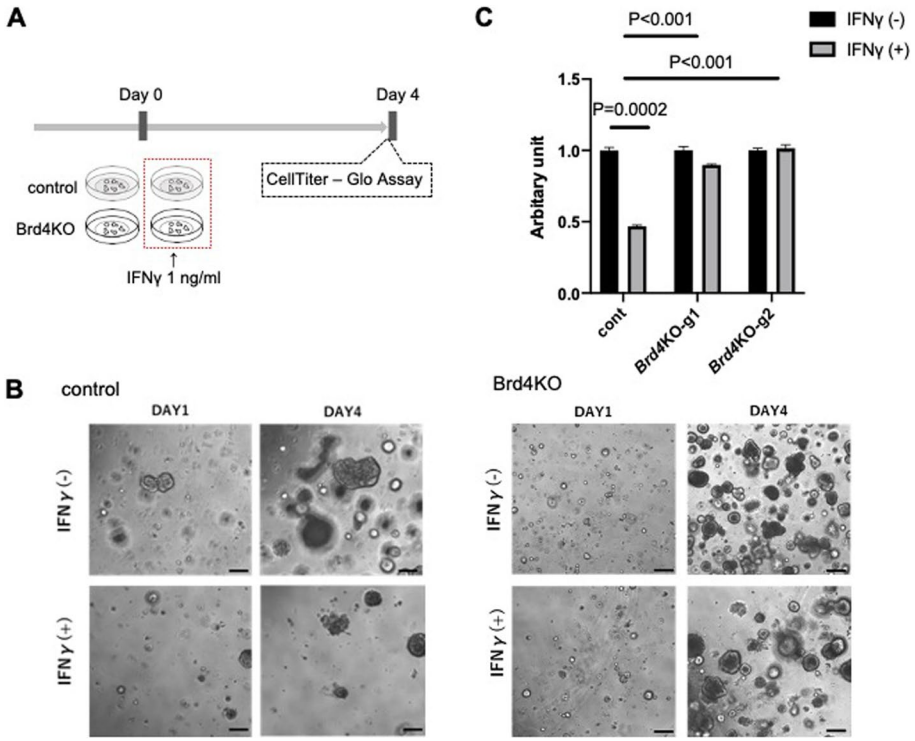

Fig. 2 Brd4 knockout is resistant to IFNγ-induced cell death. **A** Experimental schedule for the cell growth assay. **B** Photos of control and Brd4KO organoids in the presence or absence of IFNγ. **C** Cell viability was increased in the Brd4KO-1 and Brd4KO-2 organoids. Bars: 100 μm. Representative photos from 3 independent experiments are shown

IFNγ induced apoptosis in mouse colonic tumor organoids

To understand the mechanism underlying IFNγ-induced cell death, we collected the RNA samples 48 h after IFNγ addition, and performed RNA-seq using control and IFNγ-treated control organoids (Fig. 3A). A volcano plot revealed that the expression of known IFNγ-responsive genes, such as *Gbp2*, *Gbp6*, *Gbp10*, *Irf1*, *Irf7*, and *Irf8*, and genes encoding MHC genes was increased in IFNγ-treated organoids (Fig. 3B). GSEA revealed that genes involved in apoptosis were enriched in IFNγ-treated organoids (Fig. 3C, D). This finding is consistent with the fact that IFNγ can induce apoptosis in cancer cells via Jak-Stat-Caspase signaling [27]. In addition, the expression of genes involved in necroptosis and pyroptosis increased, suggesting that several cell death pathways might be involved in IFNγ-induced cell death (Fig. 3C).

To understand how Brd4KO organoids gain resistance to IFNγ, we also collected RNA from Brd4KO organoids which were cultured in the presence of IFNγ for 30 days (Fig. 3E). The volcano plot revealed that the expression of MHC genes increased, probably because of the synergistic effects of the loss of Brd4 and IFNγ stimulation (Fig. 3F). Interestingly, we observed that the expression of keratin genes, such as *Krt14*, *Krt19*, *Krt20*, *Krt33a*, and *Krt34*, significantly decreased in IFNγ-resistant Brd4KO organoids. IFNγ can induce stemness in intestinal epithelial cells in the context of regeneration [28]. Although the expression of several colonic stem cell markers did not significantly increase in Brd4KO + IFNγ organoids compared with Brd4KO organoids (Fig. 3G), decreased expression of keratin may be associated with dedifferentiation since keratin is one of the essential markers for epithelial cells.

GSEA revealed that apoptotic genes were not enriched in the IFNγ-resistant Brd4KO organoids (Fig. 3H and I), indicating that apoptotic signaling was not activated.

JQ1 partially rescues IFNγ-induced cell death in human CRC organoids

Using human CRC-derived organoids, we confirmed whether inhibition of BRD4 could rescue IFNγ-induced cell death. We used CRC20 organoids carrying mutations in *APC*, *CTNNB1*, and *TSC1* analyzed by the NCC oncopanel v4. JQ1, a BET bromodomain inhibitor, binds to the bromodomains of BRD4 and blocks the interaction between BRD4 and acetylated lysines in histones. BET inhibitors are being tested in clinical trials as anticancer drugs [29] because they suppress triple-negative breast cancer cell proliferation in vitro [30] and xenograft models of medulloblastomas in vivo [13]. The addition

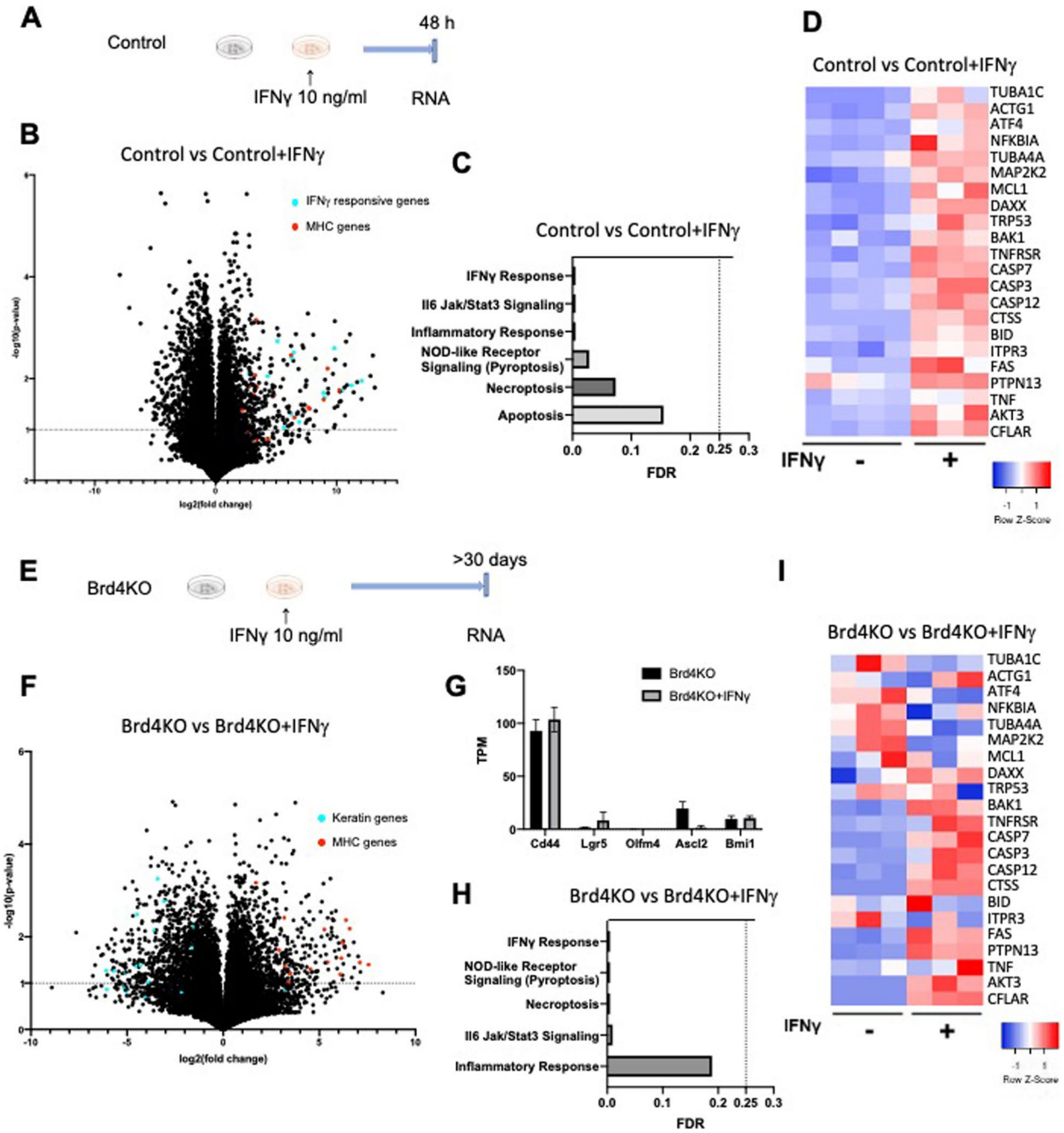

Fig. 3 Apoptosis- and necroptosis-related genes were enriched in IFNγ-treated organoids. **A** An experimental design to analyze the response of control organoids to IFNγ. **B** Volcano plot showing the genes differentially expressed between control and IFNγ-treated control organoids. Data plots showing MHC genes and IFNγ-responsive genes (Gbps and Irfs) are highlighted in red and light blue, respectively. **C** GSEA between control organoids and IFNγ-treated control organoids. **D** A Heatmap showing the expression of genes involved in apoptosis. **E** Experimental design for analyzing the response of Brd4KO organoids to IFNγ. **F** Volcano plot showing the genes differentially expressed between Brd4KO and IFNγ-treated Brd4KO organoids. Data plots showing the MHC and keratin genes are highlighted in red and light blue, respectively. **G** The expression of colonic stem cell markers in Brd4KO organoids and Brd4KO + IFNγ organoids. **H** GSEA between Brd4KO and IFNγ-treated Brd4KO organoids. **I** A Heatmap of RNA expression associated with the apoptosis of Brd4KO organoids in the presence or absence of IFNγ

of IFNγ (40 ng/ml) induced cell death in CRC organoids (Fig. 4A). We observed that IFNγ induced cell death in human CRC organoids at higher concentrations than in mouse tumor organoids. This may reflect the difference in the expression of genes in the IFNγ signaling pathway and the difference in the stage of the tumor from which the organoids are derived. The growth of CRC20 organoids was suppressed in the presence of 250 nM or 500 nM JQ1 (Fig. 4A), which is consistent with previous reports.

Interestingly, when both JQ1 and IFNγ were added to CRC20 organoids, cell viability was significantly greater than that in IFNγ-treated organoids (Fig. 4A and B). To determine whether apoptosis was induced by IFNγ, the activity of Caspase3/7 was measured. The ratio of Caspase3/7 activity to cell viability was greater in the

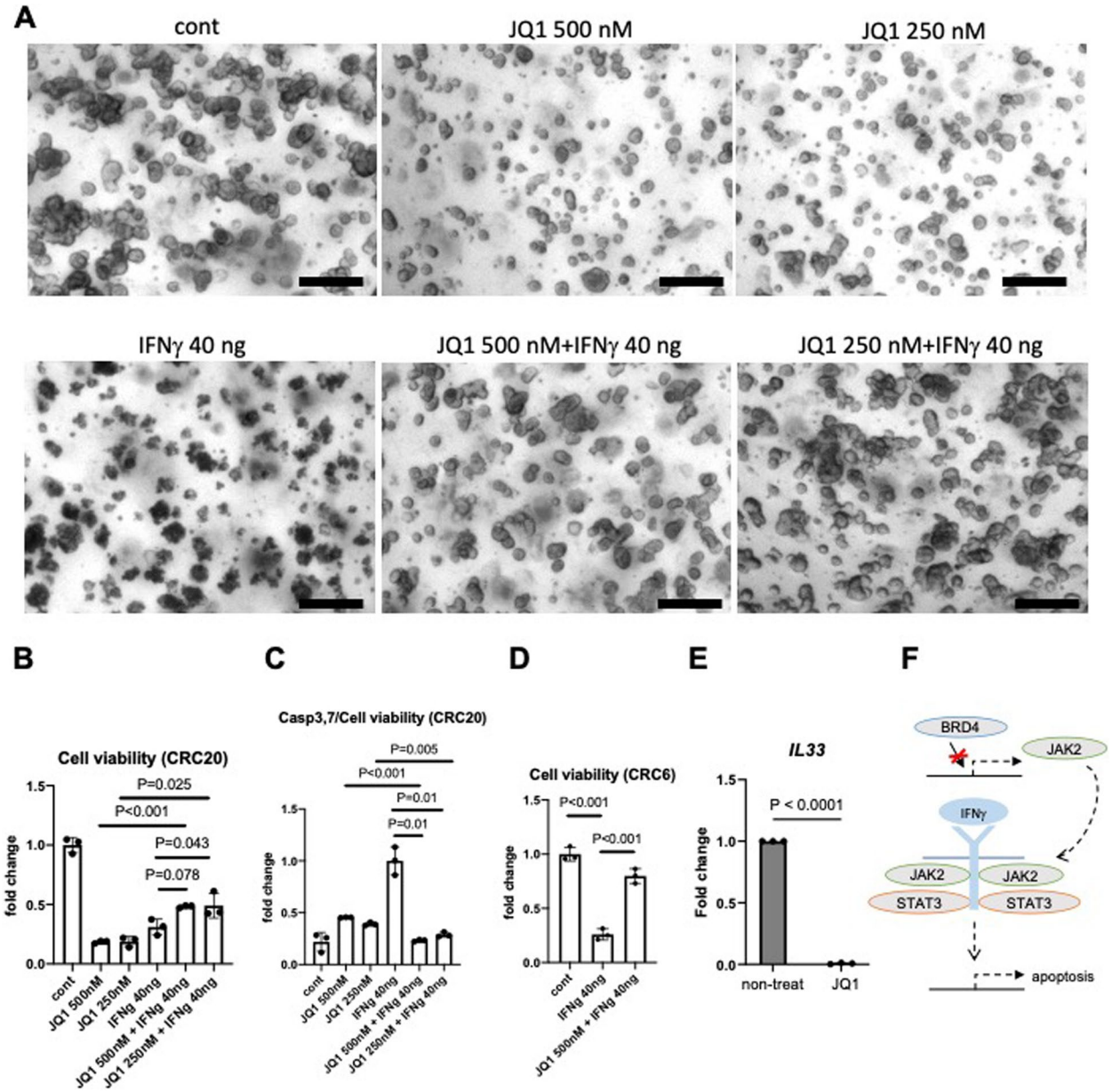

Fig. 4 JQ1 partially rescued IFNγ-induced cell death in human CRC organoids. **A** Photos of CRC20 organoids treated with JQ1 and IFNγ. Scale bars: 250 μm. **B** Cell viability assay for CRC20 organoids treated with JQ1 and IFNγ. A representative result from three independent experiments is shown. **C** Caspase 3/7 assay in CRC20 organoids treated with JQ1 and IFNγ. **D** Cell viability assay for CRC6 organoids treated with JQ1 and IFNγ. A representative result from three independent experiments is shown. **E** Expression of IL33 in CRC20 organoids in the absence and presence of JQ1. **F** Picture showing the relationship between Brd4 and the IFNγ pathway

IFNγ-treated organoids than in the control organoids (Fig. 4C), indicating the induction of apoptosis by IFNγ. In contrast, JQ1 does not cause apoptosis, but is thought to induce inhibition of cell proliferation. Furthermore, the concomitant addition of JQ1 and IFNγ did not induce apoptosis, indicating that pharmacological BRD4 inhibition can suppress IFNγ-induced cell death in human CRC cells (Fig. 4C). We performed similar experiments using another human CRC organoids, CRC6, which carry mutations in *APC*, *KRAS*, and *TP53*. IFNγ induced cell death in CRC6 organoids, which was partially rescued by JQ1. The result was consistent with that of CRC20, indicating the inhibitory effects of JQ1 in IFNγ-induced apoptosis.

We have identified Il33 as one of the significantly downregulated genes in Brd4KO organoids by RNA-seq (Fig. 1G). In the last few years, the important function of IL33 in pathological conditions has been reported in several papers [31]. We confirmed that the addition of JQ1 to human CRC organoids also suppressed the expression of IL33 (Fig. 4E), showing that BRD4 can regulate IL33 in human CRC cells.

Discussion

In this study, we showed that genetic and pharmacological inhibition of Brd4 attenuated IFNγ-induced cell death in mouse colon tumor organoids and human CRC organoids. One of the molecular mechanisms that may explain this phenomenon is that Brd4 regulates the expression of Jak2, which functions downstream of the IFNγ receptor, by binding to its transcription start site. Indeed, knockout of *Brd4* in organoids alleviated IFNγ-induced cell death in Brd4KO organoids, indicating that Brd4 positively regulates the IFNγ pathway (Fig. 4E).

Although mutations in *BRD4* are not frequent in CRC, there are cases in which termination mutations are induced, suggesting that the loss of *BRD4* may be selected in some cases of CRC. Our data indicate that Brd4 intact CRC cells may show more vulnerability in the IFNγ-rich environment compared with Brd4-inactivated CRC cells, although inactivation of Brd4 does not promote tumor development and progression. For example, in antitumor immune responses, activated T cells release IFNγ when killing tumor cells, but cancer cells with *BRD4* mutations may be resistant. We believe that in vivo validation using Brd4 knockout mouse cancer models will reveal new functions of Brd4 in CRC development. The role of IFNγ in cancer is controversial since IFNγ secreted by immune cells induces cell death in cancer cells, exerting tumor-suppressive effects, whereas IFNγ induces PD-L1 expression, leading to immune evasion [27, 32]. Our data show that CRC cells may selectively acquire *Brd4* mutations to increase their survival potential in the context of IFNγ.

In our studies, RNA-seq was performed to identify signaling pathways affected by the inhibition of Brd4 and genes whose expression is affected by IFNγ. With these data sets, we found that Brd4 regulates the expression of genes involved in immune responses and the regulation of Il33 expression, which has attracted attention in recent years because of its role in allergic reactions. These data sets will be helpful to many researchers in the future to clarify the relationship between Brd4 and Il33.

Materials and methods

Mice

NOD. Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}*/SzJ* (NSG) female mice were purchased from Charles River. The mice were anesthetized by isoflurane (Pfizer) inhalation. A total of 1×10^5 cells dissolved in 100 µl of 50% Matrigel (Corning) in DMEM/F12 were injected subcutaneously using a 26G needle. Four weeks later, the mice were euthanized with CO_2 to evaluate the efficiency of tumor development. All animal experimental protocols were approved by the Committee on Animal Experimentation of the National Cancer Center Research Institute (Study number: T19-006-M07).

Organoid culture

Colonic tumor organoids were established from Villin-CreERT2 [33], *ApcΔ716/+* [15] and *KrasG12D/+* [16] mice as previously described [18]. Organoids were cultured in AK media, which contained 10 mM HEPES, GlutaMax, penicillin/streptomycin, N2 (Invitrogen), B27 (Invitrogen), 50 ng/ml EGF (Peprotech), 5 nM A83-01 (Tocris Bioscience) and 1 µM N-acetylcysteine in advanced DMEM-F12 (Invitrogen). IFNγ (Wako) and JQ1 (Selleck) (1–40 ng/ml) were added.

Photographs of the organoids were taken with a BZ-X810 (Keyence) or IX73 inverted microscope equipped with a DP80 digital camera via CellSens software (Olympus).

Brd4 knockout organoids

The generation of knockout organoids via CRISPR-Cas9 was described in previous papers [4, 18]. Briefly, to generate gRNA vectors, gRNAs targeting *Brd4* (gRNA-1: ATTAAAACACCCATGGATA; gRNA-2: ATTCAT GAGCCACCCTCAC) were cloned and inserted into the lentiviral vector pKLV2.0 [34]. The nontarget gRNA vector was described previously [18]. HEK293FT cells (ATCC Cat# PTA-5077, RRID: CVCL_6911) were plated on polylysine-coated dishes in high-glucose DMEM (Invitrogen) to generate lentiviruses. The pKLV2.0 vectors carrying gRNA sequences were cotransfected with packaging vectors (ViraPower Lentiviral Expression Systems, Invitrogen) via polyethylenimine. The next day, the

culture medium was replaced with 5 ml of high-glucose DMEM. Ninety-six hours after transfection, the viral supernatants were collected and concentrated with Lenti-X (Takara). These viral particles were transduced into colonic AK organoids carrying Cas9 [18], which was generated previously. As previously described, the knockout efficiency was confirmed by PCR amplification of target loci and next-generation sequencing (NGS) [18]. The primers used for the experiment are described in Supplementary Table 1.

Organoid growth assay

The media were removed from the wells of 48-well plates, the plates were washed with PBS, 100 µl of cell recovery solution (Corning) was added, and the plates were shaken for 30 min on ice. Then, 100 µl of CellTiter-Gro (Promega) was added, the mixture was vortexed for 5 min, incubated for 15 min, the mixture was transferred to a 96-well plate, and the luminescence was measured with a microplate reader (Agilent BioTek, Synergy H1).

RNA sequencing

RNA was collected via ISOGEN (Nippon Gene) and treated with DNaseI. A total of 10 ng of RNA was used for RNA-seq. Following the manufacturer's protocol, the library was prepared via QIAseq UPX 3' Transcriptome Kits (Cat No. 333088).

Moreover, the MGI DNBSEQ-G400 was sequenced via PE150. Reference sequences for mapping were prepared via rsem-prepare-reference of RSEM version 1.3.1 and STAR version 2.7.9a. The sequence reads were trimmed via Trim Galore version 0.6.7, Trimmomatic version 0.39, and Cutadapt version 3.7. The processed reads were mapped to the reference sequences, and TPM (transcripts per million) was calculated via rsem-calculate-expression for RSEM version 1.3.1 and STAR version 2.7.9a. Gene and isoform TPM values were extracted from the TPM columns of the RSEM result files. The GRCm39 genome construct was used for RNA-seq analyses. The accession number for the data is GSE217170.

For GSEA, the software GSEA_4.3.2 was used. A Chip platform (Mouse_Gene_Symbol_Remapping_MSigDB.v2023.1.Mm.chip) was downloaded from the GSEA website.

qPCR

The RNA was purified via ISOGEN (Nippon Gene) and treated with DNaseI. cDNA was synthesized from 200 to 600 ng of RNA via the PrimeScript RT Reagent Kit (Takara) following the manufacturer's protocol. The primers used were from PrimerBank (https://pga.mgh.harvard.edu/primerbank/). The sequences of the qPCR primers are provided in Supplementary Table 1. qPCR was performed via SYBR Premix Ex Taq (Thermo Fisher) on a QuantStudio 3 (Thermo Fisher). The fold change was calculated via the ΔΔCt method. At least three independent experiments were performed using three or more samples to determine reproducibility.

Abbreviations

IFNγ	interferon gamma
Il	interleukin
BET	bromodomain and extraterminal domain
CRC	colorectal cancer

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-025-13544-y.

Supplementary Material 1.

Supplementary Material 2.

Acknowledgements

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Authors' contributions

A.K. and K.S. performed experiment. A.K. prepared all figures. K.O. provided human CRC organoids. H.T. wrote the main manuscript text.

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Data availability

The RNA-seq data have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE217170.

Declarations

Ethics approval and consent to participate

All experiments were performed in accordance with our institutional guidelines and regulations. All protocols for animal experiments were reviewed and approved by the institutional animal care and use committee of National Cancer Center (Study number: T19-006-M07). The study protocol for experiments on human-derived samples was approved by the Ethics Committees of the National Cancer Center (Study number: 2020 – 393, 2008-097). Written informed consent was obtained in advance for participants, and no compensation was paid.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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