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Predicting novel therapies and targets: regulation of Notch3 by the bromodomain protein BRD4

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Author contribution:

JR: initial conceptualization of the study, creation of the TPT, and intellectual contribution to and editing of the manuscript. AKS and SYW: initial conceptualization of the study and intellectual contribution to and editing of the manuscript. CL, KAC, MAC, SM, and MIE: completion and analysis of *in vitro* and *in vivo* experiments completed in this paper as well as editing of the manuscript. KB and YW: mining and analyzing TCGA data sets used in this manuscript. CI: analysis of RPPA data. PR: analysis of RPPA data and training in the use of the NetWalker analysis software. SMY, DJM, MY and JWS generated and provided the novel BETi, CN210. CRA and GLB: generated the DOPC nanoparticles loaded with the BRD4 siRNA sequences.

Disclosure and Potential Conflict of Interest:

No potential conflicts of interests are disclosed.

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Abstract

Background: Systematic approaches for accurate re-purposing of targeted therapies are needed. We developed and aimed to biologically validate our Therapy Predicting Tool (TPT) for the re-purposing of targeted therapies for specific tumor types by testing the role of Bromodomain and Extra-Terminal motif inhibitors (BETis) in inhibiting BRD4 function and downregulating Notch3 signaling in ovarian cancer.

Methods: Utilizing established ovarian cancer pre-clinical models, we carried out *in vitro* and *in vivo* studies using clinically relevant BETis to determine their therapeutic effect and impact on Notch3 signaling.

Results: Treatment with BETis or siRNA mediated BRD4 knockdown resulted in decreased cell viability, reduced cell proliferation and increased cell apoptosis *in vitro*. *In vivo* with orthotopic mouse models demonstrated that treatment with BETi decreased tumor growth. Additionally, knockdown of BRD4 with doxycycline inducible shRNA increased survival up to 50% ($p < 0.001$). Treatment with either BETis or BRD4 siRNA decreased Notch3 expression both *in vitro* and *in vivo*. BRD4 inhibition also decreased the expression of *NOTCH3* targets, including *HES1*. Chromatin immunoprecipitation revealed that BRD4 was present at the *NOTCH3* promoter.

Conclusion: Our findings provide biological validation for the TPT by demonstrating that BETis can be an effective therapeutic for ovarian cancer by downregulating Notch3 expression.

Impact: The TPT could rapidly identify candidate drugs for ovarian or other cancers along with novel companion biomarkers.

Introduction:

In the past decades, there has been a rapid surge in the development of targeted cancer therapies designed to inhibit specific molecular pathways in a particular tumor type (1). Although some of these therapies have been successful, most of them have failed to improve patient outcomes and/or survival (1). Instead of discarding these therapies, a shift in focus is needed whereby the ideal tumor types for specific drugs can be systematically identified. To this end, we developed a novel Therapy Predicting Tool (TPT) with the initial intent of determining which currently available targeted therapies in early-stage clinical development can be re-purposed for other tumor types to improve patient outcomes. Our algorithm incorporates gene and protein expression data from various databases, such as The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression project (GTEx) for normal tissue gene expression, to simultaneously detect genes with altered expression in various cancer types, the relationship of these alterations to patient survival, and specific drugs in phase I development that are available to target those genes (2). Furthermore, our algorithm

identifies potential functional relationships using mRNA sequencing and reverse phase protein array (RPPA) data by comparing expression of the target gene queried with that of other upregulated genes in the cancer types of interest. This expression data is further complemented by associating gene alterations of a specific cancer with those of normal tissues by using copy number and protein expression data. This algorithm thus allows rapid and rational search for potential drug candidates for specific tumor types.

In this study, we set out to biologically validate the TPT using ovarian cancer as a biologic disease model. Ovarian cancer remains the deadliest gynecologic disease in women (3, 4). Despite recent U.S. Food and Drug Administration approval of targeted therapies for ovarian cancer such as bevacizumab and poly(ADP-ribose) polymerase inhibitors, mortality in this disease remains high owing to drug resistance (3). New therapies that can work alone or in combination with established treatments to reduce disease burden and ultimately increase patient survival in this disease are urgently needed. Here, we report that the TPT predicted that the use of Bromodomain and Extra-Terminal motif inhibitors (BETis) will inhibit cell growth and increase animal survival in pre-clinical ovarian cancer models, providing a feasible therapeutic avenue. Through the TPT, we also uncovered a functional relationship between BRD4 and Notch3, a known player in ovarian cancer pathogenesis, where BRD4 inhibition directly promotes Notch3 transcription (5–7). This mechanism presents an attractive avenue by which targeting BRD4 to downregulate Notch3 signaling can induce anti-tumor effects in ovarian cancer. The biological validation of the TPT sets up the basis for rapid drug and target identification which can be expanded to various tumor types.

Materials and Methods:

Development of TPT

To predict novel indications for targeted therapeutics, we applied an integrated visualization interface using publicly available TCGA and GTEx data. The tool contains the following main components: (i) Survival analysis from Kaplan-Meier was performed using log-rank p-values and median overall and progression-free survival times to compare sample sets with low and high protein expression in the tumor. The low and high groups are defined using the median expression level, or the lowest and highest quartiles. (ii) Tumor mRNA expression levels are analyzed using a heat map showing median-centered median expression levels. (iii) Association of targets with copy number gain or loss in the tumor is analyzed in a manner similar to that of mRNA expression. (iv) Normal tissue expression levels, which can also help identify ideal cancer-specific targets, are examined using a heat map similar to that for tumor mRNA expression. The data for this normal tissue component is available in the Genotype Tissue Expression (GTEx) project. To create the interface, we used the established Tableau Desktop business intelligence software which we have successfully applied to analyze similar large data sets (8).

The TPT also includes large-scale correlation calculations (Spearman rank correlation coefficients) to provide information and insight on possible associations with protein expression and phosphorylation. There are about 200 total or phosphorylated protein expression data available in TCGA for each cancer type. Furthermore, using TCGA copy number and gene expression data, the TPT determines which copy number changes are

associated with significant alterations in gene expression. This component of TPT helps identify the impact of copy number variation on gene expression, providing justification for knock-down or over-expression studies. In this part of TPT, the user can quickly change the cancer type to compare associations of interests among cancer types. The method currently allows for quick visual inspection of large datasets and efficient identification of associations and alterations that may predict the relevance of a drug/target. Automatic prioritization will be provided in the next version of the tool.

TPT is available at https://public.tableau.com/profile/jason.roszik#!/vizhome/TPT_1/TPT.

Reagents and antibodies

For *in vitro* experiments, we used the BETi CPI203, purchased from Sigma-Aldrich (#SML1212), and the BETi CN210, kindly provided by ConverGene. The inhibitors were re-suspended in dimethyl sulfoxide (DMSO; #D2650; Sigma-Aldrich) to make a working stock of 10 mM. DMSO diluted at 1:1,000 was used as the vehicle control for all *in vitro* experiments. For Western blots, we used the following antibodies: Notch3 (#D11B8; Cell Signaling Technology; 1:1,000), Hes1 (#D6P2U; Cell Signaling Technology; 1:700), BRD4 (#A700-004; Bethyl Laboratories; 1:5,000), and vinculin (#V9131; Sigma-Aldrich; 1:10,000). For IHC analysis of paraffin sections, the following antibodies were used: BRD4 (#A700-004; Bethyl Laboratories; 1:100), Notch3 (#ab23426; Abcam; 1:100), Ki67 (#RB-9043-P1; NeoMarkers, 1:200), and cleaved caspase-3 (#9661S; Cell Signaling Technology; 1:100). For doxycycline induction of shRNA *in vitro*, we treated cells with 100 ng/mL of doxycycline (#D9891; Sigma-Aldrich). For *in vivo* induction of BRD4 shRNA, mice harboring OVCAR 5 tumors were placed on a daily 200 mg/kg doxycycline-chow diet (#14727450; Fisher Scientific).

Cell lines and tissue culture

The human ovarian cell lines used in this study were obtained from the American Type Culture Collection (ATCC) and The University of Texas MD Anderson Cancer Center Characterized Cell Line Core Facility, except for OVCAR 432, which was kindly provided by Dr. Ronny Drapkin (Dana-Farber/Harvard Cancer Center). Cell lines' identities were validated via short tandem repeat DNA profiling carried out by the Characterized Cell Line Core Facility in MD Anderson. Routine mycoplasma testing was done using the ATCC PCR Universal Mycoplasma Detection Kit (#30-1012K). The cell lines OVCAR 3, OVCAR 432, A2780, HeyA8, and OVCAR 8 were cultured in HyClone Roswell Park Memorial Institute 1640 medium (RPMI; #SH30027.01; GE Healthcare Life Sciences) media supplemented with 15% fetal bovine serum (FBS) (Sigma-Aldrich) and 0.2% gentamicin (#50146970, Fisher). OVCAR 4 cells were grown in RPMI supplemented with 10% FBS and 0.2% gentamicin (#50146970, Fisher). OVCAR 5 cells were cultured in Dulbecco Modified Eagle Medium (DMEM; #10-013-CV; Corning) supplemented with 10% FBS and 0.2% gentamicin. Ishikawa and Hec1-b cells were grown in Minimum Essential Medium Eagle (MEM; #10-010-CV; Corning) supplemented with 10% FBS, non-essential amino acids (#11140-050; Gibco), sodium pyruvate (#13-115E; Lonza) and 0.2% gentamicin. All cells were grown in incubators kept at 37°C with 5% CO₂. Cells expressing doxycycline-inducible shRNA constructs were grown in complete media supplemented with Tet System

Approved FBS (#631107; Takara) to avoid induction of BRD4 shRNA expression in culture. For all *in vitro* experiments, cells were used at a passage number below 20.

Cell siRNA transfections

All siRNA sequences were purchased from Sigma-Aldrich. Cells were seeded in six-well plates at a density that yielded 50% confluency after 24 hours of plating (150,000 to 200,000 cells/well). The next day, 2.6 µg (0.196 nmol) of siRNA sequences against *BRD4* or *NOTCH3* were mixed at a 1:3 ratio with Lipofectamine RNAiMAX (#13778500; Thermo Fisher Scientific) prepared in serum-free media for 30 minutes. The transfection complex was added to cells that had been washed twice with PBS and re-fed with serum-free media beforehand. Cells were incubated with the siRNA/RNAiMAX complex for 6 hours in a 37°C, 5% CO₂ tissue culture incubator and re-fed with complete media after the 6-hour incubation. Cells were then harvested for both qPCR and Western blot analysis to verify BRD4 knockdown.

For transfections in 96-well plates, cells were plated at a density of 7,000 to 10,000 cells per well in technical replicates of 10 wells per siRNA sequence. The next day, cells were transfected with 0.13 µg of siRNA in serum-free media and incubated for 6 hours in the tissue culture incubator, as described above. Cells were then re-fed with complete media and subjected to MTT viability assays. For specific siRNA sequences, please refer to Supplementary Materials and Supplementary Table S1.

MTT viability assays

Specified cell lines were seeded in 96-well plates at an initial density of 3,000 cells per well in quadruplicate technical replicates. Once cells were attached, after 24 hours, culture media were removed and replaced with 150 µL of culture media with escalating concentrations of CPI203 or CN210 (0.01, 0.1, 0.5, 1.0, 5, and 10 µM). We used DMSO (1:1000 dilution) as a vehicle control. After 72 hours of cell growth in the tissue culture incubators, 50 µL of 1.5 mg/mL MTT (#J19265; Affymetrix) solution was added to each well of the 96-well plates and incubated for 2 hours in the tissue culture incubator. Then, media with MTT reagent were discarded, and 100 µL of DMSO was added to each well, completely re-suspending the MTT byproduct metabolized formazan. Plates were then analyzed using a plate spectrophotometer reader at an absorbance of 540 nm. To calculate cell viability percentage, the mean absorbance values for each treatment were normalized to DMSO vehicle-treated wells.

Colony formation assays

Cells were plated in six-well plates in single-cell suspensions at a density of 1,000 cells per well in technical triplicates. Twenty-four hours after seeding, cells were re-fed with fresh media containing either DMSO (1:1000 dilution) or the specified BETi (CPI203 or CN210) at 1 µM. Cells were left growing in a tissue culture incubator for 7 to 10 days. Afterward, the cells were washed three times with ice-cold PBS, fixed with ice-cold 100% methanol for 10 minutes. After 10 minutes, the methanol was discarded, and cells were stained with crystal violet solution (Sigma-Aldrich; 0.5% crystal violet with 20% methanol in Milli-Q water [EMD Millipore]) for 30 minutes at room temperature with light agitation. Lastly, the crystal

violet was discarded, and the cells were washed with de-ionized Milli-Q water three times and left to dry at room temperature overnight.

EdU incorporation assay and Annexin V staining

Cells were plated in technical duplicates per experiment in six-well plates at a density of 50,000 cells per well. The next day, cells were treated with the specified BETi or were transfected with siRNA. BETi-treated cells were harvested 72 hours after treatment. siRNA-transfected cells were harvested 96 hours after transfection. Harvested cells were then pulsed with EdU for 1.5 hours and processed using the Click-iT Plus EdU Alexa Fluor 488 Flow Cytometry Assay Kit (#C10632; Thermo Fisher) following the manufacturer's protocol. For annexin V staining, we used the BD Biosciences FITC Annexin V Apoptosis Detection Kit I (#556547) and followed the manufacturer's protocol. For flow cytometry data collection and analysis, we used the Beckman Coulter Gallios Flow Cytometer.

RNA extraction, qRT-PCR and Western blot analysis

Cells that were either treated with a BETi or transfected with siRNA were washed with PBS and placed in TRIzol (Ambion TRIzol Reagent, #15596018; Thermo Fisher Scientific). For RNA extraction and purification, the Direct-zol column kit (#11–331; Genesee Scientific) was used following the manufacturer's protocol (including the DNase step). RNA was eluted with DNase- and RNase-free water, and the amount of RNA was quantified with the NanoDrop 2000 (Thermo Scientific). Any RNA that was not used immediately for reverse transcriptase reaction to create complementary DNA (cDNA) was stored at -80°C .

For cDNA synthesis, 1 μg of purified RNA was converted to cDNA using the Verso cDNA Synthesis Kit (#AB1453/B; Thermo Fisher Scientific) following the manufacturer's protocol. If not used immediately, newly synthesized cDNA was stored at -20°C . For qPCR analysis, we used Power SYBR Green PCR Master Mix (#4367659; Applied Biosystems) using the primers listed in Table S1 loaded in 96-well plates (#AB-1100; Thermo Scientific) and ran samples using the Applied Biosystems 7500 Real-Time PCR System. Data analysis was done using Applied Biosystems 7500 software version 2.3 to determine relative CT quantification (fold change, $2^{-\text{CT}}$) using *RPLP0* (also known as 36B4) as a housekeeping gene. For all primer sequences, see Supplementary Materials Table S1.

For Western blots, cells treated with the indicated BETi or transfected with siRNA were harvested and lysed with radioimmunoprecipitation assay buffer (RIPA) (25 mM Tris [pH 7.5], 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X) supplemented with single-use phosphatase and protease inhibitors (#78442; Thermo Scientific). Protein concentrations for lysed samples were determined using the Micro BCA Protein Assay Kit (#23235; Thermo Fisher Scientific) following the manufacturer's protocol. For all Western blots, 30 μg of total cell lysate diluted with 2 $\times$ Laemmli sample buffer (#1610737; Bio-Rad) was loaded in either 6% or 10% sodium dodecyl sulfate denaturing polyacrylamide gels. Protein was then transferred to a nitrocellulose membrane, blocked with 5% non-fat dry milk (#AB10109–01000; AmericanBio) in Tris-buffered saline (TBS)-T (0.1% Tween-20) for 1 hour at room temperature, and incubated with the indicated antibodies diluted in 5% milk in TBS-T overnight at 4°C with light agitation. The next day,

the membrane was washed three times with TBS-T for 10 minutes with light agitation. After the last wash, the membrane was incubated with species-specific secondary antibodies conjugated to horseradish peroxidase (#NA931V and #NA934V; GE Healthcare), diluted 1:2,500 in 5% milk in TBS-T, for 1 hour at room temperature with light agitation. The membrane was then washed three times in TBS-T and finally developed using Western Lightning Plus ECL (#NEL105001EA; Perkin Elmer) on x-ray film (#F-BX57; Phenix). For re-probing of Western blots, we stripped membranes with Restore PLUS Western Blot Stripping Buffer (#46430 Thermo Fisher Scientific), re-blocked them with 5% milk TBS-T, and incubated them with primary antibody.

Cloning of inducible shRNA constructs and lentiviral production

To generate and clone shBRD4 sequences into doxycycline-inducible lentiviral expression vectors, we used validated shRNA sequences from the Sigma-Aldrich MISSION shRNA library (Supplementary Materials, Table S1). Oligonucleotides (oligos) were cloned into a Tet-pLKO-puro lentiviral vector (plasmid #21915; Addgene) in accordance with Addgene's online protocol (<https://www.addgene.org/tools/protocols/plko/>) with slight modifications. For oligo duplex annealing, 1 µL of each oligo (100 µM) was combined with 1 µL of 10× T4 ligase buffer (#B0202S; New England Biolabs [NEB]), 1 µL of T4 polynucleotide kinase (#M0201S; NEB), and 6 µL of water, and the reaction was incubated at 37°C for 30 minutes in a Master cycler (Eppendorf). Next, the temperature was increased to 95°C and decreased by 5°C every 1 minute until reaching 25°C. The oligo duplex was then ligated to the Tet-pLKO-puro vector cut previously with BshTI (#FD1464; Thermo Fisher Scientific) using 2 µL of the newly formed oligo duplex, 3 µL of gel-purified cut Tet-pLKO-puro vector, 1 µL of T7 ligase (#M0318S; NEB), 1 µL of 2× T7 ligase buffer, and 3 µL of nuclease-free water. The ligation was incubated at 25°C for 1 hour. The ligation product was then transformed in NEB Stable Competent *Escherichia coli* (#C3040I) following the NEB protocol, plated on ampicillin plates, and grown at 30°C for 16 hours. Colonies were picked and grown in LB media at 30°C (#12795027; Thermo Fisher Scientific) supplemented with ampicillin (#BP1760-5; Fisher BioReagents). Plasmid DNA was purified using the QIAGEN Plasmid Plus Midi Kit (#12945) following the manufacturer's protocol and sequenced in the MD Anderson Cancer Center Sequencing and Microarray Facility to confirm the presence of the BRD4 shRNA sequence using the sequencing primer 5' GGCAGGGATATTCACCATTATCGTTTCAGA.

For lentiviral production, HEK 293T cells (grown in DMEM, 10% FBS, and 0.2% gentamicin) were transfected using Lipofectamine 2000 (#11668500; Thermo Fisher Scientific) with 10 µg of Tet-pLKO-puro plasmid containing either the shBRD4 sequence or the shControl non-targeting sequence (Table S1), along with 5 µg of psPAX2 (#12260; Addgene) and 2.5 µg of pMD2.G (#12259; Addgene) lentiviral helper plasmids. Supernatant containing newly generated virus was centrifuged to clear any cell debris and syringe-filtered using a 0.45-µm filter (#190-2545; Thermo Fisher Scientific). For infection of OVCAR 5 cells, cells were plated at 50% confluency in six-well plates, were incubated with 2 mL of newly produced virus along with polybrene at a 1:1,000 dilution (#sc-134220; Santa Cruz Biotechnology) for 24 hours, and were then re-fed using complete media. Lastly, cells

were positively selected using puromycin (#A11138–03; Gibco). Surviving cells were expanded and used for subsequent *in vitro* and *in vivo* experiments.

For labeling cells with luciferase for IVIS imaging, cells were infected with the lentiviral vector pGreenFire1-CMV (#TR011PA-1; System Biosciences), which co-expresses green fluorescent protein, using the protocol described above. Cells were then sorted at the MD Anderson Flow Cytometry and Cellular Imaging Core Facility for green fluorescent protein positivity and used for subsequent *in vivo* experiments.

IHC analysis

Harvested tumor nodules were embedded in paraffin blocks and sectioned by the MD Anderson Research Histology, Pathology, and Imaging Core. Slides were de-paraffinized as follows. First, slides were placed in 60°C for 30 minutes. Next, we did one incubation in 100% xylene (#C8H10, Fisher Chemical) for 4 minutes followed by another xylene incubation for 3 minutes. Next were two incubations with 100% ethanol for 2 minutes, two incubations with 95% ethanol for 2 minutes, one incubation in 80% ethanol for 1 minute, and two final incubations in Milli-Q water for 5 minutes each. For antigen retrieval to stain for Notch3 and cleaved caspase-3, slides were placed in a vegetable steamer (Hamilton Beach) in sodium citrate (pH 6) buffer for 25 minutes. For antigen retrieval for staining with Ki67, we used Diva Decloaker (#DV2004MX; Biocare Medical) instead of sodium citrate. Endogenous tissue peroxidase activity was quenched with 3% hydrogen peroxide in 100% methanol for 12 minutes. Slides were then washed and blocked with 5% goat serum in PBS for 1 hour at room temperature and we added primary antibody diluted in 5% goat serum in PBS overnight at 4°C in a humidified chamber. BRD4, Notch3, and cleaved caspase-3 were diluted at 1:100, while Ki67 was diluted at 1:200. For Notch3, BRD4, and cleaved caspase-3, slides were washed three times with PBS for 5-minute intervals and then incubated with biotinylated anti-rabbit antibody (#GR602H; Biocare Medical) for 20 minutes at room temperature. Then, slides were washed three times with PBS for 5 minutes at room temperature and then incubated with a streptavidin horseradish peroxidase label (#HP604H; Biocare Medical) for 20 minutes at room temperature and washed three times with PBS for 5 minutes at room temperature. For Ki67 IHC staining, we incubated slides with secondary anti-rabbit antibody conjugated to horseradish peroxidase (#111–036-047; Jackson ImmunoResearch) diluted in 5% goat serum in PBS at a 1:500 dilution for 1 hour at room temperature. After secondary antibody incubation, slides were again washed with PBS and then briefly washed with PBS containing Brij 35 (#858366; Sigma-Aldrich) and placed in DAB (#750118; Thermo Fisher Scientific). Upon color change, slides were rinsed in de-ionized Milli-Q water, counter-stained with hematoxylin (#GHS316; Sigma-Aldrich) for 15 seconds, rinsed in water again, and left to dry. When completely dry, slides were mounted with coverslips with permount (#sp15–100; Fisher Scientific). Slides were imaged using the Leica DM4000 B LED microscope. For quantification of cleaved caspase-3, Ki67, and BRD4 staining, five random high-power field photos were taken, and stained cells were counted manually using ImageJ software.

In vivo experiments

For all *in vivo* mouse experiments, female athymic (NCR-nude) mice were used, purchased from Taconic Biosciences. Mice were taken care of in accordance with the American Association for Assessment and Accreditation of Laboratory Animal Care and the U.S. Public Health Service policy on Humane Care and Use of Laboratory Animals. All studies and experiments carried out in this work were also supervised and approved by the MD Anderson Institutional Animal Care and Use Committee. All mice used were 10 to 15 weeks old at the time of tumor intraperitoneal injection cell injections. For OVCAR 5 cells, we injected 1×10^6 cells intraperitoneally per mouse. For OVCAR 4ip1 cells, we injected 2×10^6 cells per mouse. Tumor cells to be injected were first grown in the indicated media in tissue culture incubators until reaching 70% confluency. The cells were then trypsinized, washed twice with PBS (to remove any residual trypsin) and finally re-suspended in ice-cold Hank's Balanced Salt Solution (#21-021-CV; Cellgrow). For determining the presence of tumors, mice were injected with 200 μ L of 14.7 mg/mL luciferin (#LUCK-1G; Gold Bio) and imaged using the IVIS. Any non-tumor bearing mice were removed from the experiment. After injection and confirmation of tumor presence, mice were randomly assigned to the treatment groups described in main text. During the course of all *in vivo* experiments, the primary investigator was not blinded to the allocation of each treatment group, but at the time of tumor dissections, all investigators involved were blinded to group allocations to determine the outcome of the experiment.

For CPI203 experiments, 10 mice were initially calculated for each treatment group since this sample size gave an 80% power to detect a 50% decrease in tumor weights and nodules with a 95% confidence. Tumor cell take rates were not 100%, thus the final mouse number per group are specified in the figure legends of each experiment. Mice were treated daily with either 10 mg/kg CPI203 diluted in PBS or with PBS alone, injected intraperitoneally. After 21 days of treatment, mice were euthanized, and tumor nodule numbers and weights were recorded accordingly. Harvested nodules were then placed in 10% buffered formalin phosphate (#SF100-4; Fisher Scientific) for subsequent embedding in paraffin blocks for IHC staining. For tumor weights and nodule analysis, we tested for and excluded outliers using the ROUT analysis provided by Graph Pad Prism 7 software package to identify for multiple outliers present in each treatment group ($Q = 1\%$). One outlier was removed from CPI203 treated OVCAR 5 tumor bearing mice (Fig. 3C and Fig. S3A).

For CN210 *in vivo* experiments, we re-suspended CN210 in 30% solution of Kolliphor (#42966-1KG; Sigma-Aldrich) diluted in water and adjusted with 1.05 equivalent of 0.1 N HCl for dosing. Sonication was done to completely dissolve all CN210. CN210 was delivered orally at 50, 100 or 200 mg/kg daily for 2 days to OVCAR 5 tumor-bearing mice, with $n = 3$ mice per group. Twenty-four hours after the final dose, mice were euthanized, and tumors were harvested for Notch3 IHC staining.

For survival experiments, luciferase-labeled OVCAR 5 cells expressing either doxycycline-inducible shBRD4 or non-targeting shControl (described in supplementary material) were grown in DMEM, 10% Tet System approved FBS, and 0.2% gentamicin to 70% confluency and injected into the peritoneal cavities of mice. The mice were then imaged 7 days later using IVIS imaging and, upon confirmation of tumor establishment, were started on a 200

mg/kg doxycycline-chow diet. Mice were monitored daily, and once they became moribund owing to disease burden, they were euthanized and tumors were harvested for IHC staining.

Liposome nanoparticle preparation and delivery in vivo

In vivo delivery of siBRD4 2 was achieved by incorporating the siRNA in a previously described DOPC liposomal particle delivery system (9). Briefly, DOPC and siRNA were combined at a ratio of 1:10 (siRNA to DOPC) in the presence of excess tertiary butanol. Tween-20 was then added to the mixture at a ratio of 1:10 (Tween-20 to DOPC/siRNA mixture). This combination was mixed in a vortexer, frozen in a bath of acetone and dry ice, lyophilized, and kept at -20°C until needed for *in vivo* injections. For *in vivo* administration, the DOPC/siRNA mixture was hydrated in sterile PBS without calcium or magnesium at room temperature to a concentration of $25\text{ }\mu\text{g/mL}$ and injected in doses of $200\text{ }\mu\text{L}$, for a total of $5\text{ }\mu\text{g}$ per OVCAR 5 tumor-bearing mouse, in the peritoneal cavities of mice twice a week. After three treatments, mice were euthanized, and tumor nodules were harvested for IHC staining.

Generation of OVCAR 4ip1 cells

OVCAR 4 parental cells were labeled with luciferase, as described above, and injected (2×10^6) into the peritoneal cavities of nude athymic mice. Three weeks after injection, tumor nodules were harvested, placed in sterile serum-free RPMI, and processed in the laboratory under sterile conditions. Briefly, tumor cells were dissociated using the gentle MACS Dissociator (Miltenyi Biotec). Recovered tumor cells were then cultured in RPMI with 10% FBS and 0.2% gentamicin. Cells were then expanded and underwent short tandem repeat DNA fingerprinting analysis to confirm the identity of OVCAR 4 cells and underwent mycoplasma testing before their injection into mice.

ChIP and ChIP PCR

For ChIP experiments, cells were plated in 15-cm tissue culture dishes and grown until reaching 70% to 80% confluency. Then, the cells were harvested, and chromatin DNA was according to the protocol of the Active Motif ChIP-IT Express kit (#53008). For each ChIP reaction, $25\text{ }\mu\text{g}$ of isolated chromatin was used with $5\text{ }\mu\text{g}$ of BRD4 antibody (#A700-004; Bethyl Laboratories) or normal rabbit immunoglobulin G (IgG) (#2729S; Cell Signaling Technology). For regular PCR reactions, we used the AccuPrime GC-Rich DNA Polymerase kit (#12337016; Thermo Fisher Scientific). The following thermocycler (Eppendorf vapo.protect Mastercycler) conditions were used: 94°C for 2 minutes of initial denaturing, then 36 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. PCR products were then loaded in a 2% agarose gel made with $1\times$ TBE buffer (1.0 M Tris, 0.9 M boric acid, and 0.01 M ethylenediaminetetraacetic acid; #15581-028; Invitrogen UltraPure) and run for 120 volts alongside a 100- to 3,000-bp DNA ladder (#DNAL-100BP; Phenix). The gel was stained with ethidium bromide and imaged under ultraviolet light. For qPCR reactions, isolated DNA was further purified using the Active Motif ChIP DNA Purification Kit (#58002), and qPCRs were run using SYBR Green reagent using the Applied Biosystems 7500 Real-Time PCR System. To calculate fold enrichment, the DNA amount of normal rabbit IgG was used as a normalizing value. For complete primer sequences, see Supplementary Materials Table S1.

Reverse phase protein array (RPPA) and NetWalker analysis

OVCAR 4 and OVCAR 5 cells were cultured in either DMSO or 1 μ M CPI203 for 48 hours. Then, cells were harvested and lysed with RIPA supplemented with phosphatase and protease inhibitors and diluted to a concentration of 1.5 μ g/ μ L, which was mixed with 4 $\times$ sample buffer (40% glycerol, 8% sodium dodecyl sulfate, and 0.25 M Tris, pH 6.8) with no bromophenol blue, to which β -mercaptoethanol was added in a 1:10 ratio before use. Samples were prepared in biologic duplicates, and a total of 40 μ L of samples was submitted to the MD Anderson RPPA Core Facility and run using a validated set of antibodies (10). Initial protein expression analysis provided by the core was done using the R statistical software package (<http://cran.r-project.org>). To create a *NOTCH3* gene signature in our ovarian cancer samples and study changes in expression of those genes after CPI203 treatment, we used NetWalker analysis software (11). Changes in expression were determined by means of normalized log2 of DMSO-treated cells compared with CPI203-treated cells. The generation of the final network is described in the Results.

Statistical analysis

Statistical analyses of *in vitro* and *in vivo* experiments were done using GraphPad Prism 7. To determine whether differences between two groups were significant, we used a two-tailed Student *t*-test (equal variance) or one-way analysis of variance (ANOVA) for multiple group comparisons. For these analyses, a *p* value < 0.05 was considered significant. Results are presented as the mean $\pm$ standard deviation of the mean. To test for outliers in our *in vivo* experiments, we used ROUT analysis provided by Graph Pad Prism 7 software package to identify for multiple outliers present in each treatment group (*Q* = 1%). For survival curve comparison analysis, we used the Log-rank (Mantel-Cox) test. For TCGA analysis, Spearman's rank correlation test was performed using 309 to evaluate the correlation between expression levels of *BRD4* and other genes of specified in Fig. S1. The same test was applied to investigate the correlation between *BRD4* gene expression and copy number within 303 ovarian cancer patients. For these analysis, we chose a *p* value of 3×10^{-4} as the cutoff to evaluate significant correlations.

Results:

TPT identifies BRD4 as a therapeutic avenue to target Notch3 in ovarian cancer

We first employed the TPT to identify promising, clinically relevant targeted therapies at the phase I clinical trial stage that can be re-purposed for ovarian cancer (Fig. 1A). Our analysis revealed BETis which inhibit the bromodomain containing the protein BRD4 and have shown promise in other tumor models, including ovarian cancer. (Fig. 1A) (12–14). On the basis of these findings, we proceeded with an *in silico* validation of the TPT in which we explored the role of BRD4 in ovarian cancer. Independently probing gene expression data of TCGA and GTEx databases revealed that mRNA expression of BRD4 is highly upregulated in ovarian cancer compared with that in normal ovarian tissues (Fig. 1B). In validation of this result, using patient data sets from the TCGA independent of the TPT, we found that in ovarian cancer, an increase in BRD4 copy number strongly correlated with increased BRD4 mRNA expression (Fig. S1A). TCGA analysis also revealed that increased BRD4 expression in ovarian cancer correlated with a significant decrease in survival (*p* < 0.02 Fig. 1C). Taken

together, these computational analysis results provided the initial justification to investigate the biologic impact of BRD4 inhibition in pre-clinical ovarian cancer models.

A critical feature of the TPT is that it correlates expression of a target gene with the expression of oncogenic proteins associated with the cancer of interest to reveal potential novel functional relationships. The anti-tumor effect of BETis has been attributed to downregulation of the expression of oncogenes such *MYC*, which cannot be directly targeted (12). Thus, using our tool, we sought to identify novel downstream molecular targets that could be impacted by BRD4 inhibition in ovarian cancer. Comparing BRD4 mRNA levels with RPPA data on proteins in TPT, we determined that BRD4 levels strongly correlate with expression of Notch3 (Fig. 1D). Notch3 has been shown to be highly upregulated in ovarian cancer and correlates with increased proliferation, increased chemoresistance, and decreased survival but has proven difficult to target directly (5–7, 15–17). To investigate whether this correlation between BRD4 and Notch3 upregulation is exclusive to the Notch3 receptor, we probed the ovarian cancer TCGA data set. Our analysis revealed that there is indeed a high correlation between Notch3 and BRD4 expression in ovarian cancer (Spearman coefficient, 0.5052; $p < 2.2 \times 10^{-16}$) (Fig. S1E), but increased *BRD4* expression did not significantly correlate with expression of *MYC* (Spearman coefficient, 0.00067; $p = 0.91$), *NOTCH1* (Spearman coefficient, 0.1505; $p = 0.005$) and *NOTCH4* (Spearman coefficient, 0.1575; $p = 0.003$) receptors (Fig. S1B, S1C and S1F). We did observe a significant correlation between *BRD4* expression and the *NOTCH2* receptor (Spearman coefficient, 0.2237, $p = 7.58 \times 10^{-5}$), but this was not as strong when compared to *BRD4* and *NOTCH3* (Fig. S1E and S1D).

BRD4 inhibition has a therapeutic effect in pre-clinical ovarian cancer models

To biologically validate our *in silico* findings, we evaluated the therapeutic effect of BRD4 inhibition in ovarian cancer *in vitro*. BETis have proven effective in other tumor models, including multiple myeloma and NUT midline carcinoma, which propelled the use of BETis in clinical trials (12, 18, 19). We first tested BRD4 expression in a panel of well-established ovarian cancer cell lines. All ovarian cancer cell lines tested had substantially higher BRD4 mRNA and protein levels than did freshly isolated patient-derived primary fallopian tube epithelial (FTE4) cells (Fig. 2A and 2B). FTE4 cells were used as a control given the growing evidence of fallopian tube epithelial cells being one etiological source for ovarian epithelial cancer (20, 21). This overexpression of BRD4 in ovarian cancer is also in accordance with our *in silico* results described in the previous sections (Fig. 1B and Fig. S1A). Next, we tested cell viability using the clinically relevant BRD4 inhibitors CPI203 and CN210, a novel, un-characterized pan-BETi (refer to Supplementary Materials for the chemical structure and synthesis of CN210 and Tables S2 and Table S3 for K_d and IC50 values) (22, 23). Like other BETis, such as JQ1, these inhibitors bind directly to the BRD4 bromodomains and block BRD4's ability to interact with acetylated chromatin, stopping the expression of target genes (24). Both 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and colony formation assays using either compound showed a significant decrease in cell viability and growth, in association with treatment (Fig. 2C–2F). To determine whether these effects were due to BRD4 inhibition and not due to off-target effects of the compounds, we used siRNA to reduce BRD4 levels and then carried out MTT

assays (Fig. S2A and S2B). There was again a significant decrease in cell viability in MTT assays upon BRD4 knockdown (Fig. S2B).

We next aimed to identify the biologic mechanisms underlying this decrease in cell viability. Ovarian cancer cells treated with either BRD4 inhibitor, compared with those treated with vehicle, demonstrated a significant decrease in 5-ethynyl-2'-deoxyuridine (EdU) incorporation in most of the cell lines tested, suggesting a decrease in proliferation (Fig. 2G and 2H). Furthermore, we observed a significant increase in apoptosis upon treatment with either inhibitor (Fig. 2I and 2J). We observed similar results upon siRNA knockdown of BRD4 (Fig. S2C and S2D). These results show that BRD4 inhibition has a positive therapeutic effect in pre-clinical ovarian cancer models and provide a biologic basis for our TPT.

BRD4 inhibition decreases tumor growth and prolongs survival

Based on our *in vitro* results, we next examined the anti-tumor and survival effects of BRD4 inhibition *in vivo*. We first asked whether targeting BRD4 using CPI203, which showed a more potent inhibitory effect than CN210 did *in vitro*, inhibits tumor growth in an OVCAR 5 orthotopic (intra-peritoneal) tumor model (25). We chose to use OVCAR 5 as an initial tumor model because it is a high grade serous model, which is the most common subtype of epithelial ovarian cancers (3, 25). Furthermore, the TCGA data set available for ovarian cancer, which is one of the data sets that our TPT utilizes, is mainly based on high grade serous ovarian cancer. Tumor-bearing mice were treated daily with 10 mg/kg CPI203 (injected intraperitoneally) for 21 days (Fig. 3A). Compared with vehicle-treated mice, CPI203-treated mice had significantly decreased tumor weights ($p < 0.05$; Fig. 3B and 3C) and non-significantly fewer tumor nodules ($p = 0.0596$; Fig. S3A). To identify the biologic mechanism by which BRD4 inhibition decreased tumor growth in the OVCAR 5 tumor model, we performed immunohistochemical (IHC) staining on tumor sections with either the proliferative marker Ki67 or the apoptosis marker cleaved caspase-3. Tumors treated with CPI203 had a significant decrease in Ki67-positive cells compared with vehicle-treated controls (Fig. 3D and 3E). No significant changes in cleaved Caspase-3 staining were observed between groups (Fig. S3B and S3C). Next, we conducted the same experiment using a second high grade serous ovarian cancer tumor model, OVCAR 4ip1. We observed a significant decrease in both tumor weights (Fig. S4A and S4B, $p < 0.05$) as well as the number of tumor nodules (Fig. S4C, $p < 0.05$) in mice treated with CPI203 when compared to PBS treated controls. Furthermore, CPI203 treated mice demonstrated a significant increase in cleaved Caspase-3 IHC staining (Fig. S4D and S4E, $p < 0.001$) and a significant decrease in IHC Ki67 staining (Fig. S4F and S4G, $p < 0.05$). These results indicate that decreased proliferation was the cause of decreased tumor growth in both OVCAR 5 and OVCAR4ip1 models as well as an increase in apoptosis in OVCAR 4ip1 model alone.

We then tested whether long-term BRD4 downregulation resulted in increased survival, as predicted by the TPT. To do so, we generated a doxycycline-inducible shRNA system for luciferase-labeled OVCAR 5 cells, validated its function *in vitro*, and injected the cells into nude mice (Fig. S5A and S5B). Upon tumor establishment, the mice were given a daily diet containing 200 mg/kg doxycycline to induce BRD4 shRNA expression in tumor cells and

were killed when moribund (Fig. 3F). Luciferase (IVIS) imaging revealed that induction of shBRD4 decreased growth of tumors compared with induction of control shRNA (Fig. 3G). We confirmed BRD4 knockdown *in vivo* through IHC staining of the harvested tumors (Fig. 3H and 3I). Strikingly, mice with BRD4 knockdown had a 50% increase in survival rate compared with controls ($p < 0.001$; Fig. 3J). These results are consistent with our *in vitro* assays described above and provide further biologic support of the prediction given by our algorithm.

BRD4 inhibition decreases Notch3 expression in ovarian cancer

Next, based on the predictions of the TPT, we tested whether there is a functional relationship between BRD4 and Notch3 in ovarian cancer. Given the TPT's prediction as well as BRD4's known role as a transcriptional regulator, we hypothesized that BRD4 can directly regulate Notch3 expression in ovarian cancer (26–28). Treatment of various ovarian cancer cells with the BETis CPI203 or CN210 revealed a decrease in Notch3 protein levels (Fig. 4A and 4C). Notch3 protein levels were also decreased in these cells upon BRD4 knockdown using two different siRNA sequences (Fig. 4E). Interestingly, the decrease in Notch3 protein expression after BRD4 siRNA knockdown is comparable to directly knocking down Notch3 using siRNA sequences against Notch3 (Fig. S6A). Additionally, we observed a decrease in cell viability by MTT assay upon Notch3 knockdown in the cell lines tested (Fig. S6B), although this decrease is not as substantial as the decrease in viability observed upon BRD4 knockdown (Fig. S2B).

To investigate whether the decrease in Notch3 protein levels was due to downregulation of *NOTCH3* transcription, we performed quantitative real-time polymerase chain reaction (PCR) (qRT-PCR). *NOTCH3* mRNA transcription was significantly decreased upon treatment with either CPI203 or CN210 (Fig. 4B and 4D) and upon BRD4 knockdown using siRNA (Fig. 4F). Next, we tested whether BRD4 knockdown downregulates Notch3 protein expression *in vivo* using our OVCAR 5 tumor model. Treating tumor-bearing mice with CN210 at 100 mg/kg resulted in decreased Notch3 staining on IHC analysis (Fig. 4G and Fig. S7). To further validate these results, we used a previously described dioleoylphosphatidylcholine (DOPC) liposomal particle delivery system to deliver BRD4 siRNA in OVCAR 5 tumors (9, 29). Tumor-bearing mice were injected intraperitoneally with DOPC particles containing siBRD4 on days 1, 4 and 7 for a total of 3 treatments every 3 days. On day 8 mice were euthanized and tumors were harvested for IHC. IHC analysis of harvested tumors showed that upon BRD4 knockdown, there was a marked decrease in Notch3 staining (Fig. 4H). These results are consistent with our *in vitro* findings and suggest that BRD4 has a direct role in Notch3 transcription and promotes Notch3 mRNA expression in ovarian cancer. Furthermore, these results suggest that targeting BRD4 serves as a strategy to indirectly downregulate Notch3 protein expression and that a portion of BRD4's pro-growth effects are mediated by promoting Notch3 expression.

BRD4 directly regulates NOTCH3 expression and impacts NOTCH3 downstream effectors and TPT prediction of additional BRD4 targets in endometrial cancer

Given our results so far, we wanted to test whether BRD4 directly promotes Notch3 transcription at the gene promoter level in ovarian cancer. Previous studies suggest that

BRD4 regulates expression of genes, including *MYC*, by recruitment to the gene promoter (30–32). We performed chromatin immunoprecipitation (ChIP) of BRD4 and conducted PCR using previously described primers that flank the transcription start site of Notch3 (Fig. 5A) (33). ChIP PCR demonstrated that BRD4 was enriched at the Notch3 promoter in both cell lines tested (Fig. 5B). These results were quantified using qRT-PCR (Fig. 5C). BRD4 was also present at the *MYC* transcription start site, as described in other tumor models (Fig. 5B) (30, 32). Interestingly, BRD4 was not present at the *NOTCH2* promoter, suggesting that BRD4 directly and preferentially regulates *NOTCH3* gene expression in ovarian cancer (Fig. 5B). These results indicate that BRD4 directly promotes Notch3 expression by enhancing its transcription at the gene promoter.

Next, to understand the broader downstream effects of BRD4 inhibition, we conducted reverse phase protein array (RPPA) analysis in OVCAR 4 and OVCAR 5 cells that were treated with CPI203 for 48 hours (Table S4 and S5). RPPA allows for the rapid assessment of protein level changes in various cancer relevant signaling pathways, including Notch signaling. Based on our RPPA results, we generated a *NOTCH3* gene signature by combining previously identified Notch3 targets in ovarian cancer as well as the Broad Institute *NOTCH* gene signature to determine how protein levels of those targets would change in our RPPA data set after treatment with CPI203 compared with vehicle (Fig. 5D and 5E) (7, 15). The results showed that OVCAR 4 cells had an overall decrease in levels of Notch3 targets, including the previously described target Hes1 (Fig. 5D) (7). In OVCAR 5 cells, these changes were less prominent, but we also observed a downregulation of the Hes1 (Fig. 5E). We validated these results using both Western blot as well as qPCR analysis and observed that after BETi treatment, Hes1 protein (Fig. 5F and 5G) and mRNA (Fig. 5H) levels decrease in both OVCAR 4 and OVCAR 5 cells after BETi treatment. These results suggest that in ovarian cancer, BRD4 can directly regulate *NOTCH3* transcription and regulate *NOTCH3* downstream gene targets in ovarian cancer.

Subsequently, to further test the accuracy of the TPT, we set out to uncover a second BRD4 downstream target in a second cancer type. We chose a second gynecological cancer, endometrial adenocarcinoma. BRD4 upregulation was associated with worse patient survival, with a trend towards significance ($p = 0.06$, Fig. S8A). Treatment of endometrial cancer cells, Hec1-b and Ishikawa, with BETis decreased cell viability by both colony formation and MTT assays (Fig. S8B–E). When searching for novel downstream targets, we observed that BRD4 overexpression positively correlated with expression of Cyclin B1 (Fig. S8F). Cyclin B1 upregulation has been previously observed to be associated with worse patient survival (34). Treatment with BETis reduced mRNA expression of Cyclin B1 in both Hec1-b and Ishikawa cells, with CPI203 producing a more significant decrease than CN210 (Fig. S8G and H). These results suggest that the TPT may have broader applications for accurately predicting novel drug/targets in other tumor types.

Discussion:

In this study, we took a multidisciplinary approach to identify and re-purpose clinically relevant targeted therapies. Using the TPT, we identified that BRD4 overexpression in ovarian tumors is associated with poor survival and correlates with Notch3 expression,

therefore BETis may provide therapeutic and survival benefits in pre-clinical ovarian cancer models (Fig. 6A and 6B). Furthermore, using *in vitro* and *in vivo* experiments, we uncovered BRD4's ability to regulate Notch3 expression both *in vitro* and *in vivo* in ovarian cancer models (Fig. 6B).

The development of cancer targeted therapies for cancer rests upon the idea of inhibiting a specific molecule or pathway and impacting a multitude of downstream factors (1). To re-purpose and extend the use of these compounds, researchers are mining readily available databases such as TCGA, Cancer Cell Line Encyclopedia (CCLE), and Genomics of Drug Sensitivity in Cancer to generate accurate drug and target predictions (35–38). A recent study reported a promising *in silico* tool that uses TCGA and CCLE data sets to match cancer gene signatures with cell lines with similar alterations and test whether specific targeted therapies are effective in pre-clinical models (37). Our TPT builds on a similar premise but differs by compiling patient survival data, comparing gene expression alterations of a specific cancer with those of normal tissues of gene candidates by using copy number and protein expression data. The tool can then identify cancer-specific targets and couples them with readily available phase I clinical trial drugs. Additionally, previously published tools only use *in vitro* models for biological validation (37, 38). For our study, we complemented this by also using *in vivo* preclinical ovarian cancer models (25).

A novel function of the TPT is that it predicts potentially new downstream targets of the re-purposed targeted therapy in the tumor type of interest. In our initial analysis, we observed that BRD4 overexpression strongly correlated with Notch3 upregulation in ovarian cancer. Notch signaling regulates many processes in both normal development and disease (39). The biologic influence of Notch activation at a cellular level is highly tissue- and context-dependent (39, 40). In the context of ovarian cancer, Notch3 overexpression correlates with cancer progression, proliferation, and chemo-resistance but is challenging to directly target (5, 7, 15, 41). The use of γ -secretase inhibitors, which inhibit the ability of Notch receptors to be cleaved and translocate to the nucleus, can sensitize Notch3-overexpressing cells to cisplatin (42). Unfortunately, the γ -secretase inhibitors have not been effective in the clinic owing to their toxic adverse effects (43). Our study suggests that BRD4 can directly regulate Notch3 expression and impact expression of known Notch3 downstream factors such as Hes1 in ovarian cancer cells (7). Our pre-clinical findings support the idea that ovarian cancer patients with high tumor Notch3 expression would benefit from BETis such as CN210. We also confirmed the utility of this feature by observing the regulation of Cyclin B1 by BRD4 in endometrial cancer. These findings further suggest that the TPT can uncover novel molecular targets that are biologically relevant for the tumor of interest for the re-purposed therapy. This feature readily allows quick identification of candidate biomarkers that could guide clinicians to determine which patients could benefit the most from a specific re-purposed targeted therapy during the design of a clinical trial.

While our findings provide biologic validation of our TPT, the accuracy of our tool must be tested further using other tumor models and therapy combinations. The lead candidate molecule identified by the TPT in ovarian cancer, BRD4, is a well-studied transcriptional regulator of multiple genes (28, 44). In the context of ovarian cancer, other groups have observed that BRD4 regulates other key players such as FoxM1, MYCN, c-Myc, WEE1,

TOPBP1 and ALDH (45–49). The advantage of utilizing the TPT approach is that we were able to identify a novel molecular relationship between BRD4 and Notch3. By using the TPT, we also identified Cyclin B1 to be another novel target for BRD4 in endometrial cancer, despite previous groups describing the use of BETis in this tumor type (50, 51). For ovarian cancer, it is likely that the pro-tumorigenic activity of BRD4 is not exclusive to promoting Notch3 expression in ovarian cancer. Nevertheless, our *in vitro* findings did suggest that downregulating Notch3 directly decreases cell viability to similar levels of BRD4 siRNA knockdown. These results are consistent with previously published work from our group and suggests that part of BRD4's pro-tumorigenic effect is by promoting Notch3 expression in ovarian cancer (7). For future studies, generating specific gene networks and signatures encompassing all these identified BRD4 downstream targets may hold promise in further personalizing the use of BETis and combining them with other targeted therapies in ovarian cancer. Lastly, in this study, we determined by ChIP PCR that BRD4 is enriched at the Notch3 promoter. BRD4 may be able to regulate Notch3 expression by binding to other DNA regulatory sequences, such as enhancers, which may also be identified by using ChIP sequencing (52).

Despite the limitations described above, our study provides a strong basis for further exploration and eventual application of the TPT. Due to its potential biological accuracy, the TPT could serve as an integral part for clinical trial design. By systematically identifying clinically and biologically relevant targeted therapies that can be easily re-purposed, clinicians can create novel combinations with current standard of care therapy modalities for the cancer of interest. The goal of this to ultimately diminish the time and cost necessary for expanding personalized treatment options for cancer patients and prolong survival. The TPT could also prove useful for adaptive clinical trial approach, by identifying new therapies that can be easily incorporated in ongoing clinical trials (53). Lastly, the use of the TPT may also help uncover novel molecular mechanisms of the re-purposed drugs in the tumor of interest, providing further insight as to how effective the re-purposed therapy will be for patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Determining BRD4 as a therapeutic avenue for targeting Notch3 in ovarian cancer with the Therapy Predicting Tool.

(A) Screenshot of the TPT demonstrating where the user chooses tumor type, gene of interest (BRD4) and correlate expression to potential downstream target (Notch3) based on reverse phase protein array (RPPA) data available for ovarian cancer. TPT can also demonstrate the importance of the downstream target, Notch3, in ovarian cancer by comparing mRNA expression levels with protein levels provided by RPPA as well as correlate protein level (RPPA) with copy number alterations. Blue box pop up summarizes the query results. The scale bar shown in this figure panel only pertains to the highlighted row in the heat map and not to the entire heat map displayed in the screen shot. **(B)** GTEx analysis comparing expression of BRD4 in normal ovarian tissue (blue box) with that in ovarian cancer (red box). **(C)** Survival curves for patients with ovarian cancer from The Cancer Genome Atlas data sets comparing low BRD4 expression (green curve) with high

BRD4 expression (red curve) in ovarian cancer tumors ($p < 0.02$). Statistical analysis was done by applying the log-rank test in the “survival” R package. (D) RPPA data heatmap expanded from Figure 1A correlating BRD4 mRNA levels with protein expression levels of indicated proteins.

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Figure 2. BRD4 inhibition using BETis has a beneficial therapeutic effect in ovarian cancer cells. (A) Standard curve qRT-PCR analysis of relative BRD4 mRNA relative fold change levels in ovarian cancer cell lines compared with human isolated primary fallopian tube epithelial cells (FTE 4). Experiment done in 2 independent biological duplicates, **** $p < 0.001$. One-way ANOVA was used to test for significance. (B) BRD4 protein levels in ovarian cancer cells relative to those of FTE 4. Experiment done in 2 independent biological duplicates. (C and D) MTT viability assays of ovarian cancer cells treated for 72 hours with BETi CPI203 or CN210, respectively. Experiment done in 3 independent biological duplicates. (E and F) Colony formation assay of ovarian cancer cells treated for 10 days with BETi CPI203 or CN210, respectively. Experiment done in 3 independent biological duplicates. (G and H) EdU flow cytometry analysis of ovarian cancer cells treated with BETi CPI203 or CN210, respectively, for 72 hours. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns (non-significant).

Experiment done in 3 independent biological duplicates. Statistical significance was determined by conducting unpaired Student *t*-test comparing the mean of EdU incorporation of vehicle control versus BETi treated cells. Experiment done in 3 independent biological duplicates. **(I and J)** Annexin V flow cytometry analysis of ovarian cancer cells treated with BETi CPI203 or CN210, respectively, for 72 hours. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns (non-significant). Experiment done in 3 independent biological duplicates. Statistical significance was determined by conducting unpaired Student *t*-test comparing the mean Annexin V staining of vehicle control versus BETi treatments.

Figure 3. BRD4 inhibition reduces tumor growth and prolongs survival.

(A) Schematic for CPI203 treatment in OVCAR 5 tumor-bearing mice. (B) Tumor nodules (white arrows) in CPI203- or PBS-treated mice. (C) Mean tumor weights in PBS (n = 8) and CPI203-treated mice (n = 6), * p < 0.05. Statistical significance was determined using Student *t*-test for mean difference in tumor weights. (D) IHC staining of OVCAR 5 tumors for Ki67. Scale bar, 100 μ m. N = 4 per group. (E) Quantification of Ki67-positive cells from PBS- or CPI203-treated mice, n = 4 per group. Quantification was done using pictures of five random fields from OVCAR 5 tumors. Statistical significance was determined using Student *t*-test for mean difference in Ki67 positive cells. (F) Timeline for doxycycline-inducible shBRD4 survival experiment. (G) IVIS imaging of OVCAR 5 tumor-bearing mice 21 days after shRNA induction. (H) IHC staining for BRD4 in OVCAR 5 tumor-bearing mice to confirm BRD4 knockdown *in vivo*. Scale bar, 50 μ m. (I) Quantification of BRD4-

positive cells from tumors in (**H**), ** $p < 0.01$, *** $p < 0.001$. Quantification was done using pictures of five random fields from OVCAR 5 tumors and statistical significance was determined using Student *t*-test for mean difference in BRD4 positive cells ($n = 3$ per indicated group). (**J**) Survival curve of each indicated group after shBRD4 induction, *** $p < 0.001$. Comparison was made to test for any significant difference between curves using Log-rank (Mantel-Cox) test.

Figure 4. BRD4 inhibition decreases Notch3 expression in ovarian cancer.

(A, C, and E) Protein expression analysis at 72 hours of full-length (FL) and cleaved (C) Notch3 after BRD4 inhibition using a BETi (A and C) or siRNA-mediated knockdown (E). (B, D, and F) qRT-PCR analysis of Notch3 mRNA levels after BRD4 inhibition using a BETi (B and D) or siRNA-mediated knockdown (F). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ns (non-significant). Statistical analysis was done by applying unpaired Student t -test of mean fold change for vehicle control cells compared to BETi treated cells. Experiments (A) through (F) have been repeated at least in 3 independent biological replicates. (G) Notch3 IHC staining of OVCAR 5 tumor-bearing mice treated with either

vehicle control (n = 3) or CN210 (n = 3). **(H)** Notch3 IHC staining of OVCAR 5 tumors after Control (n = 5) or BRD4 (n = 5) siRNA delivery by DOPC nanoliposomes.

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Figure 5. BRD4 is present on the *NOTCH3* promoter and impacts Notch3 downstream signaling. (A) Schematic of the *NOTCH3* promoter and transcription start site. (B) Chromatin immunoprecipitation (ChIP) PCR reactions after BRD4 pulldown at the indicated gene promoter regions. Experiments repeated in 3 independent biological duplicates. (C) ChIP qPCR of *NOTCH3* transcription start site after BRD4 pulldown, * p < 0.05. Experiments repeated in 3 independent biological duplicates. Statistical analysis was done by applying unpaired Student *t*-test for mean fold enrichment of BRD4 over IgG control. (D and E) *NOTCH3* gene signature generated from RPPA data using NetWalker software. Fold change calculated based on NormLog2 expression difference of DMSO treated cells versus CPI203 treated cells. Samples were submitted as 2 independent biological duplicates. (F and G)

Western blot of cells treated with BETis. Experiments repeated in 2 independent biological duplicates (**H**) qRT-PCR analysis of *HES1* after BETi treatment. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Experiments repeated in 3 independent biological duplicates. Statistical analysis was done by applying unpaired Student *t*-test of mean fold change for vehicle control cells compared to BETi treated cells.

Figure 6. Summary model figure for use of BETis to for targeting Notch3 in ovarian cancer. (A) BRD4 promoting *NOTCH3* transcription and driving ovarian cancer tumor growth. (B) BETi inhibiting BRD4 mediated *NOTCH3* transcription and inhibiting ovarian cancer tumor growth.