SMARCA4 expression as a biomarker predicting sensitivity to epigenetic therapies

by

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Preface

Manuscript-based thesis content

This doctoral thesis was written according to the Guidelines for Preparation of a thesis for McGill University. The thesis represents a manuscript-based thesis format and comprises first-authored articles published or in preparation for submission. The thesis consists of five chapters. Chapter I represents a general introduction based on literature review. Chapter II is also an introductive part and based on first-authored review in preparation for submission. The review is written by Tatiana Shorstova and revised by supervisor Dr. Michael Witcher. It is expected to be published in a peer-reviewed journal. This chapter is presented as a separate chapter respecting McGill University guidelines. Chapter III and Chapter IV represent research content performed during my PhD studies and based on one already published article and an article under revision. These chapters include full articles. Chapter V represents general discussion and conclusions based on performed studies and literature background. References are listed after each section and a master reference list for all the chapters is presented at the end of the thesis.

Contribution to original knowledge

The contributions to original knowledge are listed below:

- Identification of bromodomain inhibitors (BET inhibitors) as novel potential therapies against highly aggressive SMARCA4-deficient Small Cell Carcinoma of the Ovary, Hypercalcemic Type (SCCOHT) and Non-Small Cell Lung Cancer (NSCLC) models.
- Establishing orthotopic ovarian cancer models including the first *in vivo* model for SCCOHT.
- Identification of active Mitogen Activated Protein-Kinase (MAPK) signaling as an intrinsic resistance mechanism to BET inhibitors based on SMARCA4 expression profile.
- Characterization of the down-regulation of Receptor Tyrosine Kinase (RTK) dependent pathways (PI3K-AKT and RAS-MAPK) in the response to BETi and the role of RTK, HER3 in partial intrinsic resistance to BETi.
- Identification of the sensitivity to combinatorial treatment of BETi and MEK inhibitors against SMARCA4-deficient SCCOHT and SMARCA4-expressing ovarian adenocarcinoma models *in vitro* and *in vivo*.
- Identification of nucleotide metabolism genes (TYMS, DUT and RRM1) as effectors to BETi/MEKi combination therapy.
- Proposition of SMARCA4 loss as a biomarker for therapeutic intervention with BETi and combination BETi/MEKi as new therapeutic approach to target both SMARCA4-expressing and SMARCA4-deficient tumor models.

Abstract

SMARCA4 is an ATP-dependent chromatin remodeler embedded within the SWI/SNF complex which plays an important role in gene transcription through shifting or evicting repressive nucleosomes, generally leading to transcriptional activation. *SMARCA4* has been found mutated in a spectrum of cancers. Loss-of-function mutations within SMARCA4 have been reported in almost 100% of Small Cell Carcinoma of the Ovary, Hypercalcemic Type (SCCOHT) and in ~ 10% of Non-Small Cell Lung cancer (NSCLC). In both cases, *SMARCA4* mutations are associated with a very poor clinical outcome and the current standard of care has proven ineffective against these cancers. SMARCA4 and the bromodomain protein, BRD4, independently co-regulate a transcriptional network of proliferation-related genes in a redundant manner. We proposed that inactivating BRD4 with bromodomain inhibitors (BETi) may represent a rational therapeutic approach to target SMARCA4-deficient cancers because this should critically repress BRD4-dependent oncogene expression in the absence of a SMARCA4 backup mechanism of gene activation.

We demonstrated that SMARCA4-deficient SCCOHT and NSCLC cells are acutely sensitive to BETi at low nanomolar concentrations *in vitro*. Next, we established for the first time, an orthotopic ovarian xenograft SCCOHT model. These tumors showed significant response to BETi at doses of only 20mg/kg/day. Mechanistically, our RNA-seq analysis revealed that BETi downregulates genes involved in oncogenic receptor tyrosine kinase (RTK) signaling primarily in sensitive cells. This includes repression of the RTK, HER3. Western blotting validated BETi-mediated repression of HER3 and downstream effectors preferentially through the MAPK pathway. Importantly, we found that re-expression of SMARCA4 or HER3 leads to partial resistance to BETi. Consistent with this, constitutive BRAF and KRAS activation dictate intrinsic resistance to BETi.

Overall, this work indicated that BETi may cooperate with RTK, PI3K, or MEK inhibitors to completely shut down these pathways, thereby overcoming intrinsic resistance to BETi. As such, we explored whether these combinations work *in vitro*. Among all the inhibitors tested, we discovered a clear synergy between the MEK inhibitor (cobimetinib) and BETi (OTX015) in

both SMARCA4-deficient SCCOHT and SMARCA4-expressing ovarian carcinoma cells. We validated this combination *in vivo* using orthotopic xenograft models of SCCOHT and ovarian adenocarcinoma models. To find effectors of the response to the combination of BETi/MEKi, we conducted SILAC mass spectrometry experiments on cells treated with DMSO, OTX015, Cobi or OTX015/Cobi for 24 hours. Interestingly, the analysis revealed that the OTX015/Cobi combination specifically repressed the expression of proteins involved in nucleotide metabolism such as TYMS, DUT and RRM1.

Overall, our data demonstrates a potent anti-tumorigenic effect of BETi against SMARCA4-deficient cancers. Thus, we suggest that the loss of SMARCA4 may act as a biomarker for therapeutic intervention with BETi. The BETi/MEKi combination studies potentially highlight new therapeutic approaches to treat multiple tumor types.

Résumé

SMARCA4 est une hélicase ATPase-dépendante du complexe de remodelage de la chromatine, SWI/SNF. SMARCA4 joue un rôle essentiel dans la transcription des gènes par le déplacement ou l'expulsion de nucléosomes obstruant les éléments régulateurs conduisant généralement à une activation de la transcription. Des mutations de *SMARCA4* ont été identifiées dans plusieurs cancers. Ainsi, les mutations de perte de fonction de *SMARCA4* ont été reportées dans presque 100% des carcinomes de l'ovaire à petites cellules de type hypercalcémiant (COPCH) et dans environ 10% des cancers du poumon non à petites cellules (CPNPC). Dans les deux cas, les mutations de *SMARCA4* sont associées à un pronostic non favorable et la thérapie standard n'est pas suffisamment efficace. SMARCA4 régule la transcription de certains gènes impliqués dans la prolifération cellulaire, gènes également ciblés par une protéine à bromodomaines, BRD4. Ces deux facteurs ont un rôle redondant, mais agissent indépendamment et ne sont pas simultanément présents à la chromatine de ces gènes. Nous proposons que dans les cancers arborants une perte de fonction de SMARCA4 (cancers SMARCA4 négatifs), l'inactivation de BRD4 par les inhibiteurs de bromodomaines (BETi) présenterait une nouvelle approche thérapeutique, les BETi ayant la capacité de réprimer les oncogènes régulés par BRD4 en absence de SMARCA4.

Premièrement, nous avons démontré que les cancers SMARCA4 négatifs, COPCH et CPNPC, sont très sensibles aux BETi *in vitro*, ce, à de faibles concentrations de l'ordre du nano molaires. Nous avons ensuite, pour la première fois, modélisé le cancer COPCH dans la souris par xénogreffes ovariennes orthotopiques. Ces tumeurs répondent significativement au traitement par les BETi à la très faible dose de 20mg/kg/jour. L'analyse du séquençage d'ARN a démontré que dans les cellules sensibles au traitement, les gènes réprimés par les BETi, sont impliqués dans la voie de signalisation oncogénique des récepteurs tyrosine kinase (RTK). Cela inclut la répression du RTK, HER3. L'analyse par immunobuvardage de type Western a validé la répression de HER3 et de ses effecteurs par les BETi, révélant une inhibition préférentielle de la voie des MAPK. De manière intéressante, nous avons démontré que la réexpression de SMARCA4 ou de HER3 conduit à une résistance partielle de ces cellules aux BETi. De même, une activation constitutive de BRAF et de KRAS confère une résistance intrinsèque aux BETi.

Dans l'ensemble, ces études indiquent que les BETi pourraient coopérer avec des inhibiteurs de RTK, PI3K ou MAPK pour complètement inactiver ces voies de signalisation et contrecarrer la résistance intrinsèque aux BETi. Ainsi, nous avons exploré ces combinaisons de traitement *in vitro*. Parmi tous les inhibiteurs testés, nous avons mis en évidence une synergie très claire entre un inhibiteur de MEK (cobimetinib) et un inhibiteur de bromodomaines (OTX015), ce, dans les deux types cancéreux, COPCH (SMARCA4 négatif), adenocarcinome ovarien (SMARCA4 positif). Nous avons validé cette combinaison *in vivo* sur les modèles de xénogreffe du COPCH et de l'adénocarcinome ovarien. Afin d'identifier les effecteurs de la réponse à la combinaison de BETi/MEKi, nous avons effectué des expériences de spectrométrie de masse SILAC sur les cellules traitées par du DMSO, OTX015, Cobi ou par la combinaison OTX015/Cobi pendant 24 heures. L'analyse a démontré que la combinaison OTX015/Cobi réprime spécifiquement l'expression des protéines impliquées dans le métabolisme telles que TYMS, DUT et RRM1.

En conclusion, nos données mettent en évidence un effet anti-tumorigénique des BETi dans les cancers SMARCA4 négatifs. Ainsi, nous proposons que la perte de SMARCA4 serait un biomarqueur thérapeutique pour un traitement aux BETi. Également, les études sur la combinaison de BETi/MEKi présentent de nouvelles approches thérapeutiques contre plusieurs types du cancer.

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List of Abbreviations

AKT: rac-alpha serine/threonine-protein kinase

BD: bromodomain

BET: bromodomain and extra-terminal

Bp: base pair

BRAF: v-raf murine sarcoma viral oncogene homolog B

BRD4: bromodomain-containing protein 4

cDNA: complementary deoxyribonucleic acid

ChIP: chromatin immunoprecipitation

DMSO: dimethylsulfoxide

DNA: deoxyribonucleic acid

dNTP: deoxynucleoside triphosphate (deoxynucleotide)

Dox: Doxycycline

dTMP: deoxythymidine monophosphate

dUMP: deoxyuridine monophosphate

DUT: deoxyuridine triphosphatase (dUTPase)

dUTP: deoxyuridine triphosphate

ERK: extra-cellular signal regulated kinase

FBS: fetal bovine serum

GSEA: gene set enrichment analysis

HAT: histone acetyl transferase

HER3: human epidermal growth factor receptor 3

HDC-aSCR: high-dose chemotherapy with autologous stem cell rescue

IgG: immunoglobulin G

IHC: immunohistochemistry

Kb: kilobase

Ki-67: Proliferation Marker Protein Kiel-67

KRAS: v-ki-ras2 kirsten rat sarcoma 2 viral oncogene homologue

MAPK: mitogen-activated protein kinase

MEK: mitogen-activated protein kinase

mTOR: mammalian target of rapamycin

MYC: V-Myc Avian Myelocytomatosis Viral Oncogene Homolog

NMC: NUT-midline carcinoma

NSCLC: non-small cell lung cancer

PI: propidium iodide

PI3K: phosphatidylinositol-4,5-bisphosphate 3-kinase

Pol II pS2: Polymerase II phosphorylated on serine 2

pTEFb: positive trabscription-elongation factor-b complex

RAS: guanosine-nucleotide-binding protein (small GTPase)

RNA: ribonucleic acid

RRM1: ribonucleotide reductase catalytic subunit M1

RTqPCR: reverse transcription quantitative polymerase chain reaction

S6: ribosomal protein S6

SCCOHT: small cell carcinoma of the ovary, hypercalcemic Type

shRNA: short hairpin RNA

SILAC: stable isotope labeling by/with amino acids in cell culture

SMARCA2: swi/snf-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 2

SMARCA4: swi/snf-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4

SWI/SNF: switch/sucrose nonfermentable

TSS: transcription start site

TYMS: thymidylate synthase

WB: western blotting

WT: wild type

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Contribution of authors and Included Articles

PhD student Tatiana Shorstova under the supervision of Dr. Michael Witcher and Dr. William Foulkes carried out most of the research work presented in the thesis which includes the articles listed below:

- **Chapter II: Betting on bromodomain inhibitors** (review in preparation)

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Contribution: T.S. wrote the manuscript and made Tables 1 and 2. M.W. designed Figure 9 and revised the manuscript.

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- **Chapter III: SWI/SNF-compromised cancers are susceptible to bromodomain inhibitors** (article published in Cancer Research)

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Contribution:

T. S. and M.W. conceptualized and designed the research study. T.S. performed the lab experiments and data analysis, prepared figures and tables and wrote the manuscript. M.M. performed RNAseq data analysis and wrote materials and methods on transcriptomics analysis. J.S. established ovarian xenograft models. J.J. took part in cell viability, clonogenic assay and western blot analysis. C.L.C., N. H., S. H., M.A-J., W.D.F. provided critical reading of the manuscript. M. W. and W.D.F. revised the manuscript. The study was supervised by M.W. and W.D.F.

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- **Chapter IV: Reprogramming of nucleotide metabolism mediates synergy between epigenetic therapy and MAP Kinase inhibition** (manuscript under revision).

  Authors: Tatiana Shorstova[1], Jie Su[1], Michael Dahabieh[1], Mariana De Sa Tavares Russo[3], Daina Avizonis[3], Shivshankari Rajkumar[4], Ian Watson[4], Sonia del Rincon[1], Wilson Miller[1], William D. Foulkes[1,2] and Michael Witcher[1]

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Contribution: T.S and M.W. conceptualized the research project and designed lab experiments. T.S. performed the lab experiments, analyzed the data, prepared figures and wrote the manuscript. J.S. performed *in vivo* experiments. M.D. participated in the SILAC mass spectrometry analysis. M.T.R and D.A. performed nucleotide metabolism analysis and wrote materials and methods on metabolomics analysis. S.R. and I.W. generated BRAF plasmid. S.D.R., W.M. and W.D.F. reviewed the manuscript. M.W. and W.D.F. supervised the study and revised the manuscript.
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Rationale and Objectives

SMARCA4 is an ATP-dependent chromatin remodeler playing an important role in gene transcription by moving repressive nucleosomes, generally leading to transcriptional activation. *SMARCA4* was found mutated in a spectrum of cancers. These mutations are variable in nature (nonsense, missense or frameshift), but generally lead to a loss of protein. Recently, loss of function mutations of *SMARCA4* have been reported in almost 100% of Small Cell Carcinoma of the Ovary, Hypercalcemic Type (SCCOHT) and in 10% of Non-Small Cell Lung cancer (NSCLC). Further, immunostaining shows the loss of SMARCA4 expression in nearly 100% of SCCOHT and in 10-30% of NSCLC. For SCCOHT and NSCLC, treatment generally involves surgery and adjuvant chemotherapy, most commonly platinum-based agents. However, this approach is clearly sub-optimal as SCCOHT is an extremely aggressive tumor, with long term survival rates of early stage diagnoses at ~33%, clearly more effective therapies are needed.

It has been demonstrated that SMARCA4 and another bromodomain protein, BRD4, independently co-regulate a transcriptional network of proliferation-related genes in a redundant manner. Thus, it is reasonable to hypothesize that in SMARCA4-deficient cells BRD4 is solely responsible for driving an oncogenic network that is otherwise controlled in a redundant fashion by SMARCA4 and BRD4. We further propose that inactivating BRD4 with bromodomain inhibitors (BETi) may represent a rational therapeutic approach to target *SMARCA4* mutant cancers because this should critically repress BRD4 dependent oncogene expression.

The objectives of my project were: 1) to determine the sensitivity of SMARCA4-deficient cancer models to bromodomain inhibitors and 2) to identify the most efficient combination treatment of BETi and inhibitors of RTK-dependent oncogenic signaling in SMARCA4-deficient and SMARCA4-expressing ovarian cancer models.

Chapter I: General introduction (part 1)

Introduction to SMARCA4 biology and Small Cell Carcinoma of the Ovary, Hypercalcemic Type (SCCOHT)

1.1 Preface

Part 1 of general introduction is based on literature review and describes the role of SWI/SNF chromatin remodeler, SMARCA4 in cancer particularly within the context of Small Cell carcinoma of the Ovary, Hypercalcemic Type (SCCOHT).

1.2 Ovarian cancer

1.2.1 Epidemiology, risk factors and prognosis

Ovarian cancer is considered as one of the most lethal cancers in women. In 2018, 240 000 new cases were reported worldwide that places this cancer type as the seventh most common cancer among women (1). In 2019, the Canadian Cancer Society Statistics estimated that 3000 women were diagnosed with ovarian cancer in Canada and 1900 deaths resulted from this pathology (2). More than 70% of ovarian cancer cases are diagnosed at advanced clinical stages III and IV due to variable symptoms appearing late when the disease is already at the advanced level (3). These symptoms can arise from fluid production (ascites) or from tumor infiltrating abdominal and pelvic organs. These symptoms may be mild and are often present in patients with non-malignant conditions that makes early diagnosis challenging. The average age of diagnosis for ovarian cancer resides within the postmenopausal period of 50-70 years (1,4). Among risk factors, clinical research revealed possible genetic predispositions in families with a history of ovarian cancer, Lynch syndrome, attributable to mismatch repair gene pathogenic variants (hereafter, mutations) and *BRCA1/2* mutations (1,5,6). Endometriosis has also been linked to the development of ovarian cancer in some patients (7). Further, a high number of ovulation cycles can increase the risk of developing of ovarian cancer (8). Prognosis depends on the stage when diagnosis was made. Patients at early stages have a 90% chance of 5-year survival while only 25% of patients with metastatic disease survive for this period (1,9). The average 5-year survival rate for all the stages is established at 47% (1,10).

1.2.2 Classification

Ovarian cancer is classified into two primary subtypes: epithelial and non-epithelial (Fig.1) (11,12). The epithelial subtype is the most common form of ovarian cancer which comprises 90% of all the ovarian cancers (1,11). Histologically, epithelial tumors can be classified into serous, endometroid, clear cell and mucinous (Fig.1). Serous tumors are subdivided into high-grade serous carcinomas (HGSC) and low-grade serous carcinomas (LGSC) (1). Epithelial ovarian cancer may develop from ovarian tissue, endometrium, fallopian tubes or abdominal epithelium (1,11). However, convincing clinical evidence and murine modeling indicates that a majority of serous ovarian tumors derive from fallopian epithelial cells (13,14). Two major categories highlighting clinicopathological features of epithelial cancer are simply referred to as type I and type II (1,15,16). Type I tumors are usually associated with a more favorable prognosis than type II (1). The origin of type I tumors usually resides in the ovaries and endometrium as a result of inflammation, endometriosis or intensive ovulation. Type II most commonly originate from fallopian tubal epithelium and represent around 70% of epithelial cancers. These tumors are characterized by aggressive behaviour (15). This type of ovarian cancer is characterized by a high degree of genomic instability as evidenced by a wide spectrum of genetic mutations. Most commonly, *TP53* and *BRCA1/2* are among the most commonly mutated genes in HGSC, described in detail below. HGSC tumors represent the majority of type II category while LGSC, endometrioid, clear cell and mucinous tumors belong to type I (1,15).

Non-epithelial ovarian tumors are rare representing, about 5-10% of all ovarian cancers (1,12). They generally arise from germ cells or sex cord-stromal cells. Germ cell tumors are often diagnosed in young women and the median age of diagnosis for sex cord-stromal tumors is 50 years. They are also characterized by non-specific symptoms, as described above for other types of ovarian cancer, but have distinct morphologic and genetic features. Among other ovarian tumors there is also a category of miscellaneous tumors with unknown cell origin which includes Small Cell Carcinoma of the Ovary, Hypercalcemic Type (SCCOHT) (Fig.1) (17).

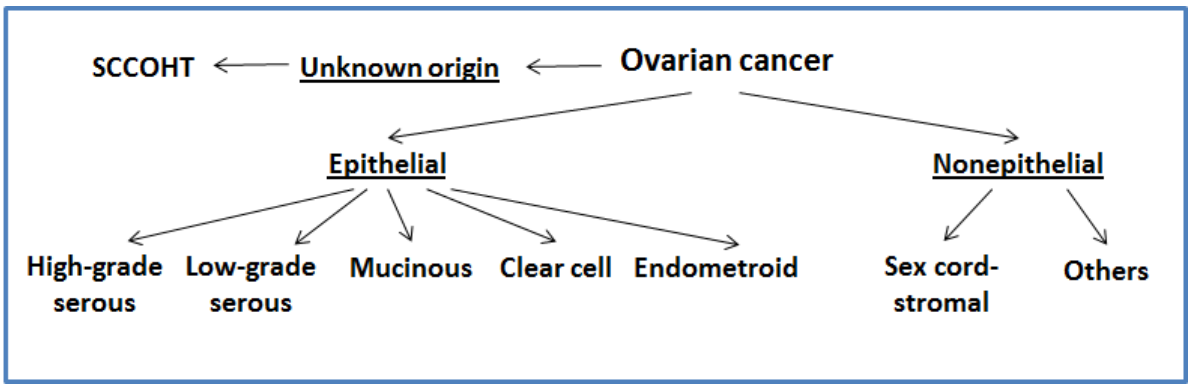

Figure 1. Ovarian cancer classification

1.2.3 Molecular defects and treatment options

Numerous genetic alterations have been reported in ovarian cancer including somatic and germline mutations in the tumor suppressor genes *BRCA1* and *BRCA2*. Women with germ-line heterozygous *BRCA1/2* mutations have a high risk of developing ovarian and breast cancer (1,15). *BRCA1* and *BRCA2* play role in DNA damage repair (18). In BRCA-mutant cancer cells, repair via homologous recombination (HR) is defective. Instead, such damage is repaired through non-homologous end joining (NHEJ) (19,20). Repair through NHEJ leads to the accumulation of mutations contributing to genomic instability and malignant transformation.

The gene encoding the tumor suppressor p53 is known as *TP53* and the most commonly mutated gene across ovarian cancers and found mutated in 90% of HGSCs (21). Mutations of *TP53* lead to escape from apoptosis and resistance to chemotherapeutic agents. *TP53* and *BRCA1/2* mutations are usually associated with poorly differentiated high-grade serous carcinomas (15). Among other molecular events occurring in ovarian cancer, recurrent activating mutation of Receptor Tyrosine Kinase Pathway (RTK) genes such as *HER4, PIK3CA, PTEN, KRAS* and *BRAF* are observed at frequencies of 5-13% each, variable across subtypes (15). HER4/EGFR4 is a member of the HER family of RTKs responsible for the activation of the PI3K/AKT and RAS oncogenic signaling pathways (22). These pathways, including downstream effectors, PI3K, BRAF and KRAS, are necessary for cell proliferation and survival leading to malignant transformation, invasion and metastases. Further, HER2 overexpression has been reported in 20-30% of ovarian cancers (22,23) and a significant percentage of epithelial ovarian cancers (19-77%) are characterized by EGFR overexpression (22,24). EGFR and HER2 overexpression are

correlated with poor survival and development of advanced tumors. 30% of ovarian tumors demonstrate the activation of PI3K/AKT (22,25). These mutations are common across most ovarian subtypes. For example, *KRAS* mutations have been found in 50% of mucinous ovarian tumors (22,26).

Treatment options for ovarian cancer are multimodal and basically consist of surgical cytoreduction (debulking surgery) followed by adjuvant chemotherapy (1,11). Neoadjuvant therapy is utilized for purposes of tumor burden reduction in patients with advanced disease (1). The purpose of debulking surgery is to completely remove bulky tumor masses and to stage the disease more accurately. Surgical staging is necessary for therapeutic management of patients and prognosis. Cytoreductive surgery also plays role in the improvement of patients' outcome with recurrent disease. Palliative surgery can be employed in order to improve quality of life of patients. Laparoscopic surgery may take place to explore whether debulking surgery is needed (1). The volume of surgical intervention depends on the area of lesions and in addition to tumor removal may include hysterectomy, bilateral salpingo-oophorectomy and removal of any affected tissue in pelvis and abdomen. In young women with early stage of ovarian cancer, less invasive surgery (for example unilateral salpingo-oophorectomy) should be considered in order to preserve fertility (8).

Therapeutic adjuvant and neoadjuvant regiments include taxanes (paclitaxel, docetaxel) and platinum-based (carboplatin, cisplatin) compounds (1,27). Thus, carboplatin with paclitaxel is the most preferred regimen for the treatment of primary ovarian cancer. Following debulking surgery, intraperitoneal chemotherapy can be offered to patients with low-volume residual disease (1,28). In the case of recurrent disease, patients most commonly obtain second-line chemotherapy (1). Recent advances in precision medicine studies have resulted in the development of a variety of possible treatments for ovarian cancer. Among medications recently obtaining FDA approval are PARP inhibitors (olaparib, rucaparib) that have achieved efficiency in patients with *BRCA1* and *BRCA2* mutations (29).

Survival for ovarian cancer patients depends on the stage of the disease. 18 months is recognized as the average period for median progression-free survival (1,30). Generally, prognosis for patients with ovarian cancer remains poor with high probability of relapse.

1.3 Small Cell Carcinoma of the Ovary, Hypercalcemic type (SCCOHT)

1.3.1 Epidemiology

Small Cell carcinoma of the Ovary, Hypercalcemic Type (SCCOHT) represents an ovarian cancer with an undefined cell of origin and currently, is classified as a miscellaneous tumor type. SCCOHT is a rare ovarian malignancy (31). However, it is one of the most common ovarian cancer of poor prognosis in women below 40 years of age (32). The mean age of diagnosis is 24 years. Prognosis depends on stage of the disease, but overall, is very poor. Development of this type of cancer is highly aggressive with an overall 5-year survival of around 16% (33). Long term survival at early stages is approximately 33% (33,34). The majority of patients die within 2 years of diagnosis because patients usually present at advanced stages.

1.3.2 Pathology and diagnosis

The aetiology of SCCOHT remains unknown. In 1979, Robert E Scully M.D. at the Massachusetts General Hospital was the first to document important pathological aspects of this carcinoma (35). According to his observations, SCCOHT is characterized by the presence of small hyperchromatic cells with scant cytoplasm and brisk mitotic activity, an early age of onset and hypercalcemia (35). Histologically, classic “small” SCCOHT tumors consist of small round cells with limited cytoplasm and follicle-like spaces with eosinophilic content (Fig.2) (36). The presence of necrosis is commonly observed. In addition to the “small” SCCOHT type, a “large” cell variant has been described in almost 50% of the SCCOHT cases and includes the appearance of large cells with abundant eosinophilic cytoplasm (Fig.2) (36). There is an opinion stating that the large cell variant of SCCOHT is morphologically and genetically similar to rhabdoid tumors (pediatric soft tissue tumors) (36-38).

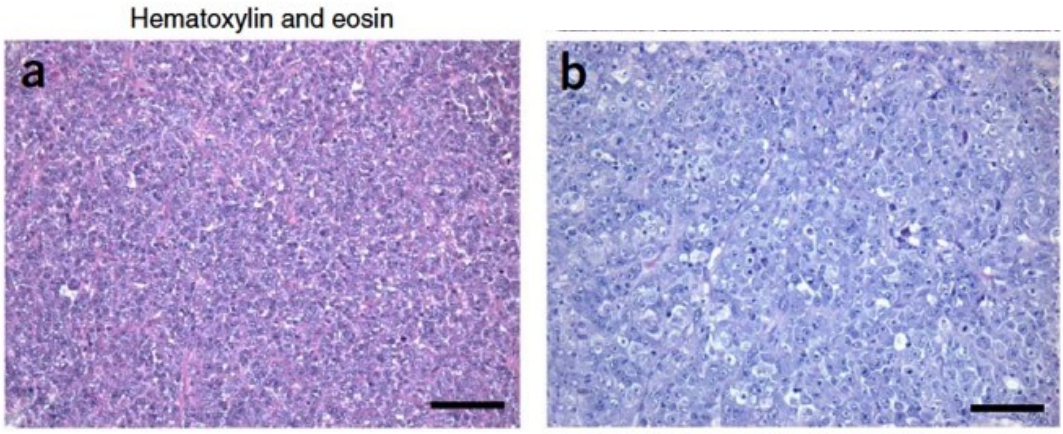

Figure 2. Morphology of SCCOHT tumors. A) SCCOHT, classic type; **B)** SCCOHT, large cell variant (from (38)).

The undifferentiated nature of the tumor makes diagnosis difficult. In most of the cases, the clinical symptoms of SCCOHT are associated with abdominal or pelvic mass and with hypercalcemia in two-thirds of cases (39,40). As mentioned previously, patients very often present at advanced stages with large solid tumors and metastatic disease is usually revealed by palpation and at laparotomy. The location of tumors is mostly unilateral (41). As SCCOHT has two variants "small" and "large", the differential diagnosis can be challenging. This tumor type can be confused with both primary neoplasms and metastatic tumors to the ovaries of epithelial and non-epithelial origin including germ cell tumors, neuroblastoma, granulosa cell tumors, lymphoma, metastases from small cell lung carcinoma and others (36,40,42).

1.3.3 Molecular underpinnings

Several studies have shown that SCCOHT is invariably characterized by germline and/or somatic mutations in the *SMARCA4* gene (38,43). SMARCA4 (BRG1) is an ATP-dependent chromatin remodeler embedded within the SWI/SNF chromatin remodeling complex, playing a role in histone-DNA interactions by moving or restructuring nucleosomes generally required for transcriptional activation of genes involved in various cellular processes including cell proliferation and DNA repair (44-46). In familial cases of SCCOHT, the inheritance pattern is autosomal dominant. Almost all the identified mutations are deleterious leading to a truncated protein (38). In familial cases with germline *SMARCA4* mutations, somatic mutation or loss of heterozygosity (LOH) of the wild-type allele have been reported in tumor tissue (38,47). In fact,

where appropriate samples have been available, somatic LOH or a second, usually truncating, mutation in *SMARCA4* have been detected in almost all SCCOHT cases, whether the first hit *SMARCA4* is in the germline or tumor. Two commonly used tools to study SCCOHT are the cell lines BIN67 and SCCOHT1. BIN67 and SCCOHT1 carry biallelic mutations in *SMARCA4* (47). Although most of the mutations are deleterious, missense mutation within helicase domain of *SMARCA4* have also been reported (38).

In comparison to other types of ovarian cancer, SMARCA4 appears to be the single recurrent mutation, and in most cases, may represent the sole coding mutation, suggesting a driving role in the tumorigenesis (38,43). Immunohistochemical staining revealed the absence of SMARCA4 in almost 100% of tumors (38,48). In addition to SMARCA4, another ATPase component of the SWI/SNF complex, SMARCA2 has been shown to be lost, or underexpressed, in a significant number of SCCOHT cases (48). As *SMARCA2* has not been found mutated, the cause of SMARCA2 deficiency remains unknown but likely is a consequence of epigenetic silencing (37). Thus, concomitant loss of SMARCA4 and SMARCA2 represents a distinct molecular feature for SCCOHT and may facilitate a precise diagnosis of this pathology.

1.3.4 Therapy and survival

Treatment strategies for SCCOHT are variable and most often multimodal involving surgery and adjuvant chemotherapy (33,49). Based on retrospective analysis, it has been shown that surgery alone is not an appropriate mode of treatment (33). Thus, surgery is usually combined with platinum-based compounds achieving some improvement in survival rates. It is recommended to perform radical surgical resection of the primary tumor with bilateral salpingo-oophorectomy and debulking surgery of metastatic sites. In the absence of germline *SMARCA4* mutations and young age of patients, fertility preserving surgical approaches might be considered (50,51). Most of the treatment modalities remain ineffective and the percentage of relapse is quite high at around 85% (33) . However, it has been shown that surgery along with the high dose chemotherapy followed by autologous stem cell rescue can be beneficial (33). Of 14 patients who received this therapeutic avenue, 10 patients demonstrated complete response while of the 14 patients who did not receive high-dose chemotherapy with autologous stem cell rescue (HDC-

aSCR), 11 relapsed. While these data are encouraging, the reproducibility of these observations from other centres is still missing.

For SCCOHT, a more favorable prognosis depends on many factors including early stage of the disease, small tumors, normal concentration of calcium and exposure to chemotherapy (33,52). In the case of advanced disease, it has been suggested to administer neoadjuvant chemotherapy. The most common chemotherapeutic agents used for SCCOHT in the neoadjuvant setting include cisplatin, etoposide and cyclophosphamide (51). In general, the efficiency of SCCOHT therapy is limited, and recurrent disease is inevitable. Thus, new therapeutic options are strongly needed. One of the recent observations showed that anti-PD1 immunotherapy might be promising against SCCOHT tumors (53). A limited cohort of patients revealed that patients with recurrent disease treated with anti-PD1 inhibitor stayed in remission for 1.5 years. A possible explanation for the efficiency of this therapeutic approach resides in the elevated expression of PD-L1 and T-cell infiltration found in SCCOHT tumors (53).

1.4 SMARCA4 and SWI/SNF chromatin remodeling complex

1.4.1 Structure and functions of SWI/SNF complex

Epigenetics is defined as molecular factors and processes beyond DNA sequence that regulate genome activity, including chromosome organization and transcription. Epigenetic factors are independent of DNA sequence, and are generally mitotically stable. The basic subunit of chromatin, the nucleosome, is comprised of 147bp of DNA wrapped around an octamer of core histones, two each of H2A, H2B, H3 and H4 (54). Histone H1 bound to linker DNA between nucleosomes participates in forming a tightly compacted chromatin fiber (55). In eukaryotes, chromatin is highly organized, and epigenetic regulation of chromatin may represent a major barrier or facilitator of gene transcription. Dynamic changes in chromatin compaction and decompaction are regulated by two major mechanisms having considerable cross-talk; covalent modification of histones (e.g. methylation, acetylation, ubiquitination, ADP-ribosylation) and the enzymatic activity of chromatin remodeling complexes. One chromatin remodeling complex central for both homeostasis, development and tumor prevention is the multi-subunit chromatin remodeling complex SWI/SNF (Switch/Sucrose Non-Fermentable) (44,54,56). The mechanism of action of this complex is dependent on the energy of ATP hydrolysis which alters

DNA/histone interactions and allows sliding or evicting nucleosomes thereby controlling accessibility of DNA to transcriptional machinery (46). In yeast studies, SWI/SNF complex has shown selectivity towards H2A/H2B dimers eviction (57). In addition to the direct action on chromatin configuration, these proteins also possess interaction surfaces for other proteins including transcription factors.

This complex is evolutionary conserved and 29 different SWI/SNF members have so far been described (58). The SWI/SNF complex as a whole is 2MDa comprising 12-15 subunits. Depending on context and cell type, the subunit composition may vary, providing unique gene targeting capabilities (59). The chromatin remodeling complex contains core subunits that are universal across the SWI/SNF complexes and variable subunits that are present in subtypes of SWI/SNF (46). Among the variants of SWI/SNF, there are three major, distinct SWI/SNF subtypes: canonical BAF, pBAF and non-canonical ncBAF (Fig.3) (46,59,60). BAF and pBAF SWI/SNF members are consistent across subtypes and include the mutually exclusive catalytic ATPases, SMARCA4 (BRG1) or SMARCA2 (BRM) and core proteins SMARCB1 (SNF5), SMARCC1 (BAF155) and SMARCC2 (BAF170) (46,59). The BAF (BRG1-associated) subtype of SWI/SNF is specifically characterized by the presence of mutually exclusive ARID1A or ARID1B while pBAF (polybromo BRG1-associated factor) type is determined by PBRM1, BRD7 and ARID2 (46,59). In comparison to BAF and pBAF complexes, the ncBAF core complex is distinct and only contains SMARCA4/A2, SMARCC1/2 and SMARCD1/2/3. BRD9 and GLTSR1/L1 are specific subunits of the ncBAF complex (60).

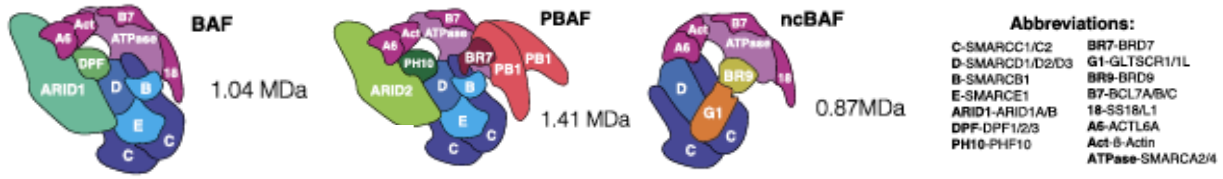

Figure 3. Mammalian SWI/SNF complex. Schematic representation of three main subtypes of SWI/SNF chromatin remodeling complex, BAF, pBAF and ncBAF (from (60)).

Fine tuning of SWI/SNF complex function is partially determined by mutually exclusive subunits represented by paralogue proteins sharing structural similarity. In such cases, only one

of the proteins can be incorporated into the SWI/SNF complex. The catalytic activity of SWI/SNF is dependent on a set of mutually exclusive subunits, SMARCA4 and SMARCA2. SMARCA4 and SMARCA2 contain ATPase domain which is essential for ATP energy conversion in order to move or restructure nucleosomes generally required for transcriptional activation (61). These ATPase paralogues share 75% identity and only one of them may be found within the same complex (62-64). It has been shown that they can have compensatory roles for each other meaning that some of their functions are overlapping (63,65-67). Two independent shRNA screens revealed that SMARCA2 is an essential gene driving proliferation in *SMARCA4*-mutant cancer models (65,67). Moreover, by co-IP it was shown that in the absence of SMARCA4, SMARCA2 was actively associated with SWI/SNF subunits and SMARCA2 knockdown was synthetically lethal resulting in the cancer cell death (67). Overlapping roles for SMARCA4 and SMARCA2 were also observed in RB-mediated cell cycle arrest (66). In the absence of two proteins, cancer cells were resistant to the RB re-expression while in the presence of either SMARCA4 or SMARCA2 or both, RB activity was conserved. Another study showed that SMARCA4 and SMARCA2 may participate in both distinct and overlapping functions (63). By employing knockdown experiments against SMARCA4 and SMARCA2, it has been shown that SMARCA4 and SMARCA2 depleted liver cells were morphologically different. SMARCA4 and SMARCA2 knockdown led to altered transcription of a distinct set of genes but genes co-regulated by SMARCA4/A2 were also detected, albeit these genes were less common. Notably, genes concurrently co-regulated by SMARCA4/A2 may be either upregulated or repressed by these catalytic proteins and the occupancy sites at chromatin for both proteins were similar. Interestingly, the depletion of one subunit led to the inhibition of recruitment of the second protein that possibly may explain why certain SMARCA4 negative cancers show concomitant deficiency for SMARCA2. The distinct functions of the two ATPase chromatin remodelers may be explained by unique amino acid sequences within the N-terminal domain of SMARCA4 and SMARCA2 providing a different landscape for protein interactions (68).

The SWI/SNF complex is involved in diverse cellular functions including cell cycle regulation, DNA repair and lineage specification (69-71). It has been shown that individual SWI/SNF subunits may both negatively and positively regulate the cell cycle. For example, SMARCA2 was found as a negative regulator of cell cycle. By co-immunoprecipitation analysis, SMARCA2

was found to directly interact with the growth suppressor RB and was present within the same complex with RB at its target gene, E2F1. This resulted in repression of E2F1, a protein necessary for the transition from G1 to S phase of cell cycle (72). In contrast, another SWI/SNF ATPase subunit, SMARCA4, demonstrated a positive regulation of cell cycle, but perhaps in a tissue specific fashion. SMARCA4 knockout in liver tissue impairs liver proliferation upon hepatectomy (70). That response was partially explained by the weak expression of cell cycle regulators, Cyclin B1 and Cdk1 and increased p53 expression in response to SMARCA4 depletion. However, negative cell cycle regulation by SMARCA4 has also been described, consistent with tissue-specific functions. SMARCA4 re-expression in SMARCA4-deficient breast cancer cell lines led to cell cycle arrest due to upregulation of p21, an inhibitor of CDK2 (73). Thus, SWI/SNF proteins coordinate cell cycle in a context dependent manner but precise molecular mechanisms to explain this phenomenon are still being uncovered.

The SWI/SNF complex is an important regulator of DNA damage repair that is necessary to prevent genomic instability and cancer. In particular, SWI/SNF proteins have been shown to play a role in double strand break (DSB) repair. One study revealed that SWI/SNF with the core subunit SMARCB1 was recruited to double strand breaks (74). This may indicate SMARCB1 is important for such recruitment. Another study showed that SMARCA4 and SMARCA2 are also recruited to chromatin at DSB and that the downregulation of these proteins leads to compromised γH2AX phosphorylation and DNA repair suggesting a role for genomic surveillance and maintenance of genomic integrity (75). The SWI/SNF complex was also found associated with a DNA repair protein, BRCA1. Here, it co-operated with BRCA1 to induce p53 upregulation, a function dependent on SMARCA4 (76).

On an organismal level, SWI/SNF complex directs the differentiation of many tissues. It may accomplish this feat through integration of specific subunits. During embryogenesis, SWI/SNF plays a critical role and the knockouts of several subunits result in embryonic lethality (77). The SWI/SNF functional variability has been demonstrated using mouse models where the inactivation of various SWI/SNF subunits results in distinct responses. SMARCB1 or SMARCA4 knockout are lethal at embryonic day 3 (E3) (78-80). In ARID1A knockout mice, mesoderm is absent and lethality occurs at day E6.5 (81). An important role of SWI/SNF within

the embryonic stem cell (ES) niche is demonstrated by an ES-specific complex, esBAF, which was purified from ES cells and was shown responsible for self-renewal and pluripotency (82). Interestingly, this complex is specifically characterized by the presence of SMARCA4 but not SMARCA2. In comparison to SMARCA4, SMARCA2 knockout mice are viable (83) and do not display early embryonic defects suggesting that SMARCA2 and SMARCA4 play distinct roles in cell development. This is consistent with a study showing that SMARCA4 was preferentially expressed in proliferating tissues with high self-renewal potential (e.g. lymphoid germinal centers, gastrointestinal crypts) while SMARCA2 expression is elevated in less proliferating tissues such as brain, muscle and liver (84). It may also suggest that SMARCA4 is involved in earlier differentiation processes in comparison to SMARCA2. Other evidence suggests that these two proteins may have antagonistic functions in osteoblast differentiation where SMARCA4 has been found to promote cell fate progression while SMARCA2 was performing repressive role in cell differentiation (85). Specific functions of the SWI/SNF complex within embryonic tissues are also achieved through differential integration of various subunits. For example, within the embryonic heart, SMARCD3 expression is greatly enriched (86). Tissue specificity amongst SWI/SNF subunits may impact recruitment to lineage-specific genes and facilitate specialized functions. Recent ChIP-seq studies performed on WT, SMARCA4 or SMARCB1-deficient mouse embryonic fibroblasts (MEF) showed the SWI/SNF complex represented by SMARCA4 and SMARCC1 was preferentially recruited to lineage-specific enhancers of genes important for cell differentiation and developmental processes (71).

Interestingly, specific subunits within the same SWI/SNF complex appear to regulate divergent lineages in the process of T-cell differentiation. *SMARCE1* was shown essential for CD4 (T helper) silencing while *SMARCA4* heterozygous mutant impaired CD8 (T-killer) activity in mouse models suggesting lineage specific transcriptional regulation during T-cell development (87).

SMARCB1 is considered a scaffold protein within SWI/SNF. It plays an important role in hepatocyte differentiation (88). Mice with SMARCB1 depletion specifically in hepatocytes were not viable due to impaired metabolism of glycogen and altered epithelial cell differentiation in liver tissue. A high number of genes responsible for normal hepatocytes differentiation and

functioning are affected in SMARCB1-depleted cells highlighting a wide role for the transcription of lineage specific factors by SWI/SNF subunits.

BAF and pBAF are two separate complexes but may be present within the same cell type (89-91) suggesting non-overlapping functions. However, the composition of these complexes may vary between cell types suggesting tissue specific roles. Several subtypes of BAF and pBAF complexes such as neural progenitor npBAF, neuron nBAF and embryonic stem cell esBAF were found assembled in a highly selective manner during cell differentiation (92,93) . These data support the idea that the SWI/SNF complex directs differentiation of various tissues through complex diversification. For example, changes to the composition of SWI/SNF from npBAF to nBAF occurs during cell differentiation from neuronal progenitors to neurons. In this case, ACTL6B, BAF45B, BAF45C are replaced by ACTL6A, BAF45A, and BAF53A (92). During this type of switch, distinct transcriptional programs are activated to determine cell fate. Beyond this, SMARCA4 can regulate specific interactions between chromosomes in myogenesis (94), but this role for SMARCA4 needs to be further explored. SMARCA4 also binds enhancer regions in B-cells suggesting a role in the lymphocyte differentiation process (95). Thus, the SWI/SNF complex displays multiple roles in differentiation and suggests that SWI/SNF deregulation may result in an imbalance between precursor and mature cells leading to tumorigenesis.

1.4.2 SMARCA4, a core ATPase SWI/SNF subunit

The SWI/SNF catalytic subunit, SMARCA4 (BRG1) was described first in S.cerevisae during studies of sucrose fermentation and yeast switch mating (96-98). In humans, SMARCA4 is located on chromosome 19p13.2 and is represented by several mRNA isoforms that are possibly tissue specific (99). The SMARCA4 (BRG1) protein contains several well-defined domains: ATPase domain, C-terminal bromodomain, AT motif and N-terminal region with QLQ (glutamine-leucine-glutamine) motif, HSA and BRK domains (Fig.4) (45,99,100). The ATPase domain consists of two motifs, DEXHc and HELICc. ATP molecules recognize the cleft formed by the ATPase domain which catalyzes ATP hydrolysis (101,102). This reaction converts ATP potential energy into mechanical movement by altering interactions between DNA and histones. The SWI/SNF complex is able to move nucleosomes along DNA, displace histone octamers

between nucleosomal arrays and even exchange H2A/H2B dimers between nucleosomes (103-106). This in turn results in conformational chromatin changes where nucleosomal DNA becomes hypersensitive to nuclease digestion and exposed for the binding of appropriate transcription factors to cognate response elements required for gene expression (107-109). The enrichment of SMARCA4 is found primarily at open chromatin regions solidifying its role in promoting nucleosome-free regions (110). Through chromatin remodeling activities, SMARCA4 can change nuclear structure and establish long-range chromatin interactions within chromatin topologically associated domains (TAD) thereby keeping high order chromatin structure and regulating specific gene expression (111).

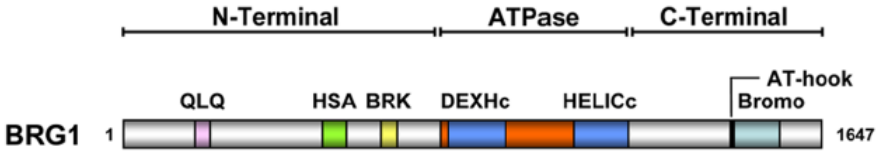

Figure 4. Structure of SMARCA4 (BRG1) protein (without modifications from (45), © This article is distributed under the terms of the Creative Commons Attribution License 4.0)

An important element of recruiting SMARCA4 to chromatin is represented by a bromodomain which is necessary for recognizing and binding to acetylated histones, most commonly to N-terminal acetylation marks of H3 and H4 (112,113). Surprisingly, a recent study showed that SMARCA4 might directly bind to DNA (114). It was demonstrated that AT hook motifs and bromodomains of SMARCA4 and SMARCA2 were both able to recognize DNA. Thus, the precise type of binding of SMARCA4 to chromatin still remains an active topic of investigation. The functions of the HAS, BRK (TCH) and QLQ domains are less studied and their functions remain unclear. However, the QLQ domain might participate in protein-protein interactions (115).

The chromatin remodeling activities of SMARCA4 regulate gene transcription and this role is contextual. The mechanism through which SMARCA4 is recruited to precise loci to remodel chromatin may be complex and involves multiple interactions between SWI/SNF subunits, histone modification and interactions with transcription factors (116-118). It has been shown that SMARCA4 purified from HeLa cells interacts with ARID1A/B proteins (subunits of BAF

complex) through integration within the same SWI/SNF complex, and that ARIDA/B recruits the complex to steroid hormone receptors leading to transcriptional activation (117). The recruitment of SMARCA4 to promoters was studied in MEF cells where SMARCA4 is found enriched at promoter regions and found essential for nucleosome occupancy around transcription start sites (TSS) (119). Upon SMARCA4 depletion, the nucleosome occupancy at promoter was reduced. Interestingly, gene expression in SMARCA4 deficient cells was only modestly changed in comparison to wild type cells despite significant changes in chromatin structure. This suggests that the loss of nucleosomes at promoter regions induced by SMARCA4 depletion might not be sufficient for gene activation. Instead, these nucleosome depleted regions may recruit lineage specific transcription factors. Importantly, SMARCA4 occupancy was found enriched at distal enhancer regions in embryonic stem cells and T cells which was important for transcriptional activation (82,120). Here, SMARCA4 binding is overlapping with H3K4me1 and H3K27ac, marks of enhancer elements (121). It is likely that SMARCA4 is recruited to this region through its bromodomain. It has been shown in leukemia cells that SMARCA4 established enhancer-promoter chromatin interactions at MYC gene and contributed to the recruitment of specific transcription factors (122). Thus, these data indicate to the significant contribution of SMARCA4 occupancy at enhancer regions to transcriptional activation.

As a transcriptional regulator within the SWI/SNF complex, SMARCA4 is implicated in numerous biological functions. On a cellular level, one of the important roles of SMARCA4 is the control of cell growth. SMARCA4 controls cell cycle checkpoint functions of RB. SMARCA4 can bind to RB through the RB-binding motif LxCxE (123). It has been shown that the ectopic expression of constitutively active RB did not stop the cell cycle at G1 phase in SMARCA4-negative cells and that the introduction of SMARCA4 decreased proliferation suggesting SMARCA4 and RB can cooperate in order to inhibit cell growth (124). In addition to the control of cell cycle checkpoints, SMARCA4 can exhibit growth inhibitory activity through the control of cell cycle regulators including cyclin E and CDK2. Cyclin E can bind to SMARCA4 which results in SMARCA4 phosphorylation by cyclin E-CDK2 complex leading to the inability of SMARCA4 to induce growth arrest (125). Growth inhibitory effects of SMARCA4 were proven in the studies where ectopic re-expression of SMARCA4 reduced cell proliferation in lung cancer cells having SMARCA4 deficiency (126). However, in contrast,

SMARCA4 promotes cell growth in acute leukemia models where it regulates transcription of MYC gene (122). Thus, SMARCA4 can exhibit opposing effects on cell proliferation that will be discussed in the following sections. SMARCA4 also plays a role in cell morphology and cellular adhesion. It has been demonstrated that the cells with ectopic expression of an ATPase-dead mutant *SMARCA4* changed morphology and display an increase in nuclear and cell size which was associated with the upregulation of integrins and the urokinase-type plasminogen activator receptor (127). Focal adhesion was also studied and revealed changes to paxillin distribution. Another study supported SWI/SNF playing a role in cell adhesion by showing the recruitment of SMARCA4 and SMARCA2 to the promoters of cell adhesion genes, CD44 and E-cadherin which results in hypomethylation and transcriptional activation (128). Among other functions of SMARCA4, a role in immune mobilization has been defined. Variable-diversity-joining (VDJ) recombination is partially controlled by SMARCA4 which binds to T-cell receptor and immunoglobulin genomic loci, and is required for DNA cleavage required for recombination in lymphocytes (129,130). Also important for immune surveillance, major histocompatibility complex class I and II genes are controlled by SMARCA4. SMARCA4 interacts with the CIITA gene from MHC class 2 and activates the "enhancer A" of the MHC class 1 promoter (131,132).

1.4.3 SWI/SNF aberrations in cancer

With the emergence of whole exome sequencing, SWI/SNF members have now been found mutated in nearly 20% of cancer (133,134) making members of this complex among the most highly mutated across cancers. The spectrum of SWI/SNF mutations is broad including both solid and hematological cancers. Among these mutations, a meta-analysis of whole exome sequencing studies revealed the highest prevalence (39%) of deleterious (frameshift, nonsense, rearrangement, splice-site) mutations, followed by missense mutations. These studies support a tumor suppressor role for the SWI/SNF complex. The SWI/SNF complex targets up to one third of all the genes and it is not surprising that mutations of at least 10 subunits have been reported across different tissue types (133,134). The most frequently mutated SWI/SNF subunits are *ARID1A, ARID2, PBRM1* and *SMARCA4* while *SMARCD1/D2/D3, SMARCE1* and *ACTL6A/B* are infrequently mutated (133).

Specific SWI/SNF mutations are often linked to different types of cancer. The gene encoding the most frequently mutated SWI/SNF subunit, *ARID1A*, was reported to be mutated in 45% of ovarian clear cell and endometroid carcinoma (133,135,136). *PBRM1* mutations occur in renal carcinoma, breast and pancreatic cancer (59,137-139). *ARID2* has been found mutated in melanoma and hepatocellular carcinoma (140,141). *SMARCB1* (SNF5) loss of function mutation characterizes malignant rhabdoid tumors (MRT) in children and familial schwannomatosis (142,143). Another SWI/SNF subunit, *SMARCE1* has been reported to be inactivated in spinal meningioma (144). Interestingly, *SMARCA2* mutations are rarely detected in cancer but missense mutations occur in neural disorders such a Nicolas-Baraitser syndrome (145). It is important to note that the SMARCA2 deficiency commonly observed in NSCLC and SCCOHT was not caused by genetic mutation (48,146). In contrast to *SMARCA2*, *SMARCA4* is frequently mutated in diverse cancer types including NSCLC, SCCOHT, pancreatic cancer and Burkitt's lymphoma (38,146-148).

1.4.4 SWI/SNF family members are context-dependent tumor suppressors

As expected from the mutational profile of SWI/SNF subunits in cancer, several mouse models support a tumor suppressor role for the SWI/SNF complexes in many contexts. These mouse models include homozygous and heterozygous SWI/SNF compromised mice.

The first reports suggesting a tumor suppressive activity for SWI/SNF appeared in the 1990s when *SMARCB1* mutation was detected in rhabdoid tumors (149). A genetically modified SMARCB1 knockout mouse model showed embryonic lethality at early stages of mouse development (78,79) complicating the study of this protein. However, heterozygous loss of *SMARCB1* led to the formation of sarcomas and nervous system tumors suggesting a possible tumor suppressor role of SMARCB1 (78,79). Furthermore, *SMARCB1* conditional deletion led to the appearance of rhabdoid-like tumors and lymphomas with a penetrance of 12-15% (150). Despite the fact that this is a core scaffold protein, the assembly of SWI/SNF complex can occur in its absence (151). As SMARCB1 provides an interaction surface for multiple proteins needed for gene transcription, the mechanism of malignant transformation in *SMARCB1*-mutant cancer is related to aberrant gene expression profiles. The loss of SMARCB1 reduces expression of RB target genes, p16 and E2F that results in cell cycle alterations (152,153). It has been shown that

SMARCB1 mutation also aberrantly upregulates MYC and WNT oncogenes (154,155). Importantly, SMARCB1 has an antagonistic activity towards EZH2, a member of the histone H3K27 methyltransferase Polycomb complex, PRC2. In the scenario of SMARCB1 loss, repressive activity of EZH2 is not suppressed by SMARCB1 which leads to altered gene expression programs, notably activation of a stem cell program, and tumor development (156).

The gene encoding the catalytic subunit of SWI/SNF, *SMARCA4*, is found recurrently mutated in a variety of cancers including 100% of SCCOHT and around 10% of lung adenocarcinomas (38,157,158). Notably, the majority of *SMARCA4* mutations are inactivating mutations leading to a truncated protein. However, missense mutations have also been reported (38). Loss of function mutations are most commonly homozygous and represented by frameshift or nonsense mutations of one allele and/or the absence of the second allele (LOH) which is a hallmark of tumor suppressor activity (38,159,160). Animal studies revealed that *SMARCA4* null mice die at the pre-implantation stage (80) and haploinsufficiency of *SMARCA4* leads to the developmental disorders and the formation of mammary gland tumors (77). Conditional knockout of *SMARCA4* in the lung potentiates carcinogen induced tumors (161). While the impact of complete SMARCA4 loss has been widely studied, the role of missense mutations remains obscure. Some studies show that *SMARCA4* heterozygous mutation allow increased binding of Polycomb Repressive Complexes 1 and 2 (PRC1,2) at transcription start sites (162). Here, opposing functions between activating SMARCA4 and transcriptionally repressive PRC1,2 have been observed, and evidence suggests that SWI/SNF evicts Polycomb complexes from chromatin. EZH2 of the PRC2 complex, places the repressive epigenetic modification H3K27me3, which in turn, is bound by PRC1 complex which acts to diminish transcription initiation (163-166). Through this mechanism, SMARCA4 prevents oncogene activation. The ATPase domain of SMARCA4 is most commonly the target for missense mutations (38,43). Recently, it has been demonstrated that heterozygous ATP-mutations may impair open chromatin configuration genome-wide (167). Notably, a significant number of altered sites are represented at enhancer regions resulting in less accessible chromatin for transcriptional activation.

SMARCA4 is primarily known for its tumor suppressor role. However, recent evidence suggests that it can act as a context dependent oncogene. Increased acetylation levels in cancer may

aberrantly recruit SMARCA4 protein to chromatin and activate transcription of oncogenes such as MYC (122). Importantly, it has been demonstrated that SMARCA4 exhibits tumor suppressor activity in mature pancreatic cells but at later stage of pancreatic cancer it promotes tumorigenicity by enhancing EMT transition (168). In addition, it has been also demonstrated that high levels of SMARCA4 expression dictate poor clinical prognosis in breast cancer (169).

In comparison to other *in vivo* SWI/SNF models described above, SMARCA2 null mice are viable but larger in size (83). SMARCA2 knockout mice are susceptible to the formation of lung tumors in response to ethylcarbamate and the development of melanoma upon UV irradiation suggesting a tumor protective role of SMARCA2 (170,171). *SMARCA2* is rarely mutated in cancer. However, the deficiency of SMARCA2 mRNA concomitant with SMARCA4 is observed across many cancers (172). The mechanism of SMARCA2 deficiency is usually not dictated by genetic mutation but perhaps can be explained by epigenetic silencing. Indeed, a study on lung cancer models identified the downregulation of SMARCA2 correlated with promoter hypermethylation (173). Similar to SMARCA4, in addition to its tumor suppressor activity, SMARCA2 may also exhibit oncogenic properties. In a background of *SMARCA4* mutation, SMARCA2 was shown as an essential gene driving proliferation (67). Again, this supports a partial compensatory, or overlapping roles between SMARCA2 and SMARCA4.

1.4.5 Compromised SWI/SNF as a biomarker

Synthetic lethality is defined as a genetic interaction where simultaneous inactivation of two genes leads to cell death while the presence of either of two allows survival. Synthetic lethality is of great interest to the field of cancer biology. Identifying a synthetic lethality reveals that the loss of one gene successfully predicts cell death upon the loss of a second, compensatory gene. Thus, the loss of either gene may act as a biomarker, predicting tumor sensitivity to inhibitors that disrupt the function of the second. Recently, synthetic lethalities for SWI/SNF subunits have been described. In *SMARCB1* mutant tumors, EZH2 activity is responsible for disease progression (156). It has been demonstrated that EZH2 inhibitors reduced cell proliferation in MRT cell lines carrying mutant *SMARCB1* whereas wild type cells were impervious to the treatment (174). Also, studies revealed that in *SMARCB1* mutant MRT cancer models, a residual complex with SMARCA4 determines cell growth and that knockdown of SMARCA4 leads to

deprivation of cell growth (175). This concept has been also demonstrated *in vivo* using lymphoma models. This is consistent with the fact that SMARCA4 directs lymphoid cell fate (175). ARID1A and ARID1B also demonstrate a synthetically lethal relationship (176). *ARID1A* mutations are more frequent in cancer than mutations of *ARID1*B (133). In *ARID1A* mutant tumor models, the absence of ARID1B results in a loss of SWI/SNF complex integrity which in turn, leads to impaired proliferation (176). However, this phenomenon does not explain the reason of co-occurrence of both *ARID1A* and *ARID1B* mutations in some types of cancer. Beyond other SWI/SNF members, *MAX* (MYC-associated factor X gene) in mutated small cell lung cancer models also showed a dependency on SMARCA4 (144). siRNA screening revealed that *SMARCA4*-mutant esophageal cancer cells are dependent on the bromodomain protein, BRD4 for survival (177). This widens the spectrum of synthetic lethality partners for SWI/SNF subunits and opens new avenues for therapeutic approaches to treat aggressive SWI/SNF mutated cancers.

A compensatory mechanism of action was described in *SMARCA4*-mutant cancer models where SMARCA2 was found essential for cancer cell viability (67). For example, in SMARCA4-negative lung cancer, growth inhibition was quite pronounced in response to SMARCA2 knockdown (67). Two independent shRNA screens performed on a large panel of human cancer cell lines, revealed SMARCA2 as a top hit determining cell proliferation in *SMARCA4* mutant cancers including lung, ovarian and endometrial cancer models (65,67). In line with this, SMARCA4 exogenous expression rescued this cancer phenotype and restored survival. Interestingly, the loss of SMARCA2 in SMARCA4-deficient cancers does not disrupt the integrity of the SWI/SNF complex suggesting that the ATPase activity plays a major role in cancer cell proliferation (65,67). While SMARCA4 and SMARCA2 represent a synthetic lethality in some contexts, but clearly this relationship is not universal, as some tumors are devoid of both SMARCA2 and SMARCA4.

Several hypotheses have been proposed that may explain the synthetic lethality relationship between SMARCA4 and SMARCA2 (46). The paralog insufficiency model states that SMARCA4 and SMARCA2 may possess redundant functions responsible for cell proliferation but not for tumor protection. In this scenario, the cells lose tumor protective functions of

SMARCA4 and at the same time residual SMARCA2 complexes compensate for SMARCA4 and drive cancer cell proliferation (46). Paralog antagonism model raises the possibility that SMARCA4 and SMARCA2 have antagonistic functions where SMARCA4 acts as a tumor suppressor and SMARCA2 determines oncogenesis. Thus, in the absence of SMARCA4, SMARCA2 induces oncogenic transcription program. Another model highlights that the mutation of *SMARCA4* may lead to the release of SMARCA4-associated complexes that participates in the formation of *de novo*, aberrant SMARCA2-containing complexes (46).

1.4.6 SMARCA4 as an oncogene

The mechanism whereby SMARCA4 acts as an oncogene is controversial and remains an active area of investigation. It is clear that SMARCA4 acts as a tumor suppressor in normal tissue but also promotes tumor growth in transformed cancer cells. This concept has been demonstrated in multiple types of cancer, but the mechanism underlying this switch remains ambiguous. Most definitively, this concept has been shown in pancreatic tissue. Here, SMARCA4 may switch between tumor protective and oncogenic roles during the course of pancreatic cancer development (168). It was shown that SMARCA4 loss promoted dedifferentiation of mature pancreatic ductal cells derived from mouse models. The re-expression of SMARCA4 in these cells rescued dedifferentiation by increasing mature duct markers including Sca1. In contrast, SMARCA4-deficient pancreatic ductal adenocarcinoma cells with ectopically expressed SMARCA4 demonstrated increased cancer cell growth that was validated using an *in vivo* model. Therefore, this study highlights that even within the same cancer cell origin, SMARCA4 activity is context dependent. This is consistent with observations demonstrating that SMARCA4 is usually expressed in highly proliferating cells (84). Moreover, SMARCA4 has been found upregulated at advanced stages of neuroblastoma and responsible for promoting cell proliferation. By using transcriptomic analysis, this study revealed SMARCA4 as a positive regulator of oncogenic PI3K/AKT signaling that was confirmed on protein level (178). In line with this, other study demonstrated high expression of SMARCA4 induced resistance to gemcitabine treatment in pancreatic cancer by upregulating phosphorylation of AKT (179). In acute myeloid leukemia (AML) mouse and cell models, oncogenic cell proliferation was dependent on SMARCA4 (122,180). These studies showed that SMARCA4 knockdown reduces cell growth and induces apoptosis.

Underlying these proliferative effects, SMARCA4 promotes the transcriptional upregulation of oncogenes such as MYC and HOXA9 (122). Enhancer elements of MYC have been found occupied by SMARCA4 which opens chromatin at enhancers and promoters to facilitate the loading of factors that allow a "loop' to form, joining promoter and enhancer to promote transcriptional elongation (122). It is unclear how SMARCA4 is recruited to these regions exclusively in cancer cells but it may be due to aberrant programming of histone acetylation surrounding these regions which, in turn, would recruit SMARCA4 through its bromodomain. Interestingly, by employing RNA sequencing, a study showed that SMARCA4 and another bromodomain protein BRD4, upregulate overlapping set of genes including oncogene MYC responsible for the proliferation of acute myeloid leukemia (122). ChIP-qPCR analysis further showed that SMARCA4 and BRD4 independently occupy a cluster of lineage-specific enhancers downstream of MYC suggesting that SMARCA4 and BRD4 co-regulate transcription of the same genes in a redundant manner. Additionally, it has been shown that SMARCA4 established chromatin looping interactions between enhancers and promoters which was associated with specific hematopoietic transcription factor occupancy (122). Overall, it is likely that SMARCA4 acts as a context-dependent oncogene through facilitating the aberrant activation of enhancers that transcriptionally upregulate oncogenes.

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Chapter II: General Introduction (part 2)

Betting on bromodomain inhibitors

Tatiana Shorstova and Michael Witcher (review in preparation)

2.1 Preface

The introductory part 2 is based on the review focusing on BET protein family proteins and recent advances in BET inhibitors development.

2.2 The players: BRD family members

Over the past decade, the influence of post-translational modification of core histones on transcription and how this process is deregulated in cancer has been widely studied. Chromatin modifications establish a connection between repressive or permissive chromatin structure and transcriptional outputs (181,182). It is clear that deregulation of such modifications including both transcriptional activating or repressing marks lead to aberrant transcriptional outputs such as heightened expression of oncogenes. One classical example of this is the accumulation of transcriptionally activating lysine acetylation at enhancer regions of oncogenes such as c-MYC (122).

Lysine acetylation is a chemical post-translational process, enzymatically carried out by histone acetyl-transferases (HATs), which utilize the cofactor acetyl-CoA to catalyze the covalent transfer of an acetyl group to multiple lysine residues of histone tails (181,183). Acetylation in turn is recognized (read) by proteins carrying bromodomains (BRDs) (184-186) and may be subsequently eliminated by histone deacetylases (HDACs) (183). The accumulation of histone acetylation at promoters or enhancers acts to recruit bromodomain proteins that generally enhance the rate of transcription of associated genes (187). Bromodomain proteins achieve this goal through multiple mechanisms including chromatin remodeling to displace nucleosomes as is observed upon recruitment of SWI/SNF complexes that are enriched for several bromodomain complex members (122,188,189). Additionally, histone acetylation is commonly read by

bromodomain proteins, such as the Bromo- and Extra Terminal (BET) subfamily proteins, which attract the mediator complex to enhance transcriptional elongation (190,191).

Bromodomains have now been identified in at least 46 unique human proteins (192,193). Based on their structure, they have been divided into 8 subfamilies (193). Bromo- and Extra terminal (BET) subfamily proteins are considered as "readers" of lysine acetylation and are represented by four members BRD2, BRD3, BRD4 and BRDT (Fig.5) (193). These proteins are comprised of two N-terminal bromodomains, BD1, BD2, extra terminal recruitment motif, ET and a C-terminal motif, is present in only a subset of BRD isoforms (194,195).

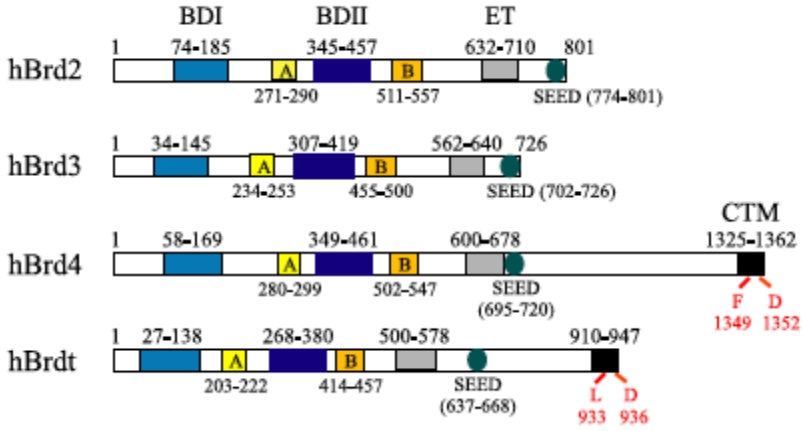

Figure 5. Structure of human BET proteins (without modifications from (196), © This article is distributed under the terms of the Creative Commons Attribution License 4.0).

BET proteins promote mRNA production through the recruitment of co-activating complexes, factors such as P300 that acetylates histones (197), and recruiting the mediator complex, that bridges enhancers and promoters facilitate phosphorylation of RNA Pol II thereby engaging transcriptional elongation complexes (190,198). At enhancers, BRD4 recruitment is often found flanking transcription factors that facilitate histone acetylation through histone acetyltransferases recruitment (199). BRD proteins may facilitate a link, via a DNA loop, bridging enhancers, and their associated chromatin complexes, with the core transcription machinery found at promoters (198,200). At both proximal promoters and enhancers, the recruitment of pTEFb by BRD family proteins plays an important role in enhancing transcriptional outputs. pTEFb mediates

phosphorylation of RNA polymerase II on serine 2 of the C-terminal repeat region, and promotes transcriptional elongation (Fig.6).

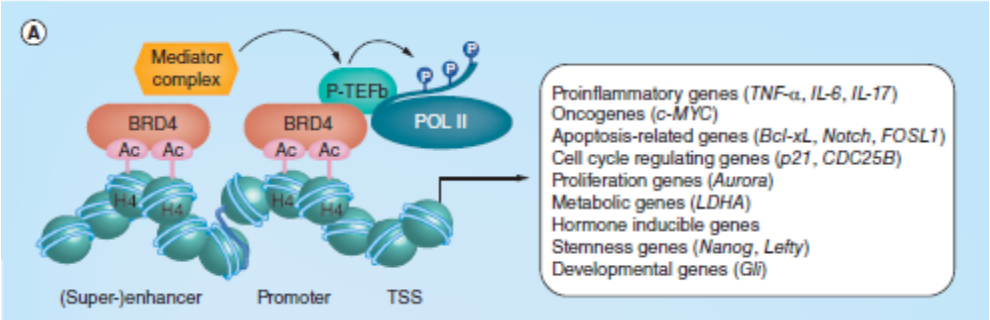

Figure 6. BET protein BRD4 promotes transcription (without modifications from (201), © This article is distributed under the terms of the Creative Commons Attribution License 4.0).

Interestingly, it has been proposed that BRD4 acts not only as a transcription regulator but also as a HAT. Recent evidence suggests that BRD4 acetylates H4 and H3 including H3122, a mark which promotes chromatin decompaction through nucleosome destabilization (202). Previous evidence suggests BRD4 further enhances histone acetylation through P300 recruitment (193). Thus, by acetylating specific histones, BRD4 may trigger nucleosome eviction thereby activating the transcription of target genes, especially those involved in survival and proliferation. The necessity of this domain in mediating pro-oncogenic phenotypes remains to be explored.

BET proteins are commonly recruited to specific loci through the binding of acetylated lysines via their bromodomains (184,186). The bromodomain contains four left-handed α-helices (αZ, αA, αB, αC) linked by loops (BC, ZA) that together structure a hydrophobic binding pocket for the acetyl-lysine groups where they link to asparagine residues, stabilizing the association (Fig.7) (193,194,203). Each bromodomain of BET proteins preferentially allows the recognition of multiple histone acetylated lysines. This was clearly shown for a single bromodomain of BRDT which simultaneously recognized H4K5ac and H4K8ac37 (204). The affinity of BD1 and BD2 for acetylated lysines is different. For example, the BD1 of BRD4 preferentially binds H4 acetylated lysines, while BD2 recognizes di- and tri- acetylation marks in histone 3 (186,193). This might be determined by the read-out of specific histone marks necessary for appropriate cell division (205).

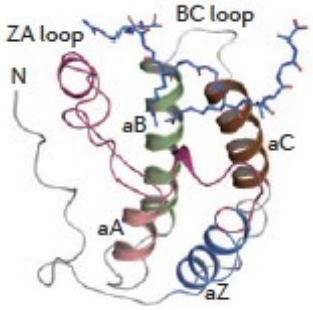

Figure 7. Structure of bromodomain BD1 of BRD4 protein in complex with di-acetylated histone H4 (from (206)).

BRD2, BRD3 and BRD4 are ubiquitously expressed across tissue types while BRDT is restricted to testis tissue (207,208). Underscoring the important physiological role of BET proteins, BRD2 and BRD4 homozygous knockout is embryonic lethal (209,210). Haploinsufficiency of BRD4 leads to alterations of cell cycle and severe organ defects including head malformations, liver necrosis and skin lesions. In contrast, mice with BRDT loss are viable but display defects in spermatogenesis (211).

Cancer is a heterogeneous disease which is characterized by abnormal cell proliferation and a range of molecular defects acquired during tumorigenesis. These "Hallmarks of Cancer" include sustained proliferative signaling, insensitivity to growth-suppressive signals, resistance to apoptosis, replicative immortality, angiogenesis and the capacity to invade and metastasis (212). More recently, these characteristics were complemented by emerging hallmarks such as dysregulation of energy metabolism and avoiding immune response. Tumor promoting inflammation, genome instability and mutation, are also described as tumor-enabling characteristics. To translate these seminal findings to the clinic, it is important to identify transcriptional networks that drive these diverse oncogenic mechanisms in order to pinpoint nodes for therapeutic intervention.

Appropriately controlled transcriptional regulation of gene expression is critical for homeostasis and genome stability. Dysregulated transcriptional programs trigger two major events promoting cancer; the activation of oncogenes and conversely, the silencing tumor suppressor genes. Both

of these processes may subsequently impact cancer hallmarks, influencing both cancer initiation and progression. These oncogenic transcriptional events may be driven by either genetic rearrangements or epigenetic mechanisms. Epigenetic reprogramming of DNA methylation and histone modifications, in particular, are well-characterized and contribute to aberrant oncogenic transcriptional networks.

As readers of histone acetylation marks, BET proteins are important regulators of transcriptional outputs. It is therefore not surprising that this family of proteins play important roles in homeostasis and cell survival, and that their deregulation may promote cancer. BET proteins are known facilitators of the cell cycle (213). For example, progression through G1 is dependent on the recruitment of the elongation complex pTEFb by BRD4. This interaction is mostly pronounced in the end of mitosis and entering to G1 phase. Consistent with this, subsequent knockdown of BRD4 leads to G1 arrest and apoptosis. Interestingly, the depletion of BRD4 resulted in the downregulated expression of G1 activated genes such as JunB, c-MYC and cyclin D1 (213). Another bromodomain protein BRD2 has been shown to activate Cyclin A in S phase which was correlated with increased acetylation levels at promoter (214). Further, the ectopic expression of BRD2 displayed accelerated progression through the cell cycle in fibroblasts. BRD2 was also found associated with E2F which is important for S phase progression suggesting that BRD2 may acts as a E2F coactivator to potentiate cell cycle progression (214). Thus, by tightly controlling the cell cycle, BET proteins help establish a balance between proliferative and growth arrest, an important component of cell homeostasis.

As a part of its homeostatic function, BET proteins regulate tissue specific gene expression. While it is not entirely clear how BET proteins activate genes in a tissue-specific manner, interaction with lineage-specific factors that recruit bromodomain proteins to precise loci, appears important. For example, it has been shown that BRD3 controls the differentiation of erythroid cells through binding to transcription factor GATA-1 (215). BRD2 specifically regulates neuronal differentiation through E2F1 pathway (216,217) and BRDT coordinates spermatogenesis (218,219). BRD4 was shown co-localized with lineage-specific transcription factors at active enhancer regions essential for adipogenesis and myogenesis (220). BRD4 binding to enhancer elements activates transcription factors necessary for differentiation of

adipocytes (221). Metabolism and BET activity are also tightly related. Increased respiration through oxidative phosphorylation is observed upon BET inhibition and BRD4 is a key transcriptional regulator of OXPHOS genes (222). Beyond the requirement for its transcriptional regulation as a homeostatic protein, BET proteins also exhibit insulator activity in multiple settings. Insulator activity is necessary for protecting individual chromatin domains from the surrounding environment through the establishment of specific DNA boundary elements called insulators. BRD4 facilitates insulator activity surrounding sites of DNA double strand break repair by recruiting the Condensin chromatin complex (223). BRD2 also cooperates with CTCF to establish transcriptional boundaries that prevent aberrant promoter-enhancer contacts (200).

There is evidence showing that aberrant expression of BET proteins leads to cancer initiation. The first example of this was the discovery that BRD4 or BRD3 translocate to form a fusion protein with NUT (Nuclear Protein in Testis, also known as NUTM1), t(15;19). This BRD/NUT fusion is highly oncogenic and initiates the development of NUT-midline carcinoma (NMC) having an aggressive phenotype with a very poor prognosis (224). NUT-midline carcinoma is categorized as a squamous cell carcinoma subtype and as its name suggests, arises in organs along the midline of the body. This pathology predominantly affects children and young adults. Under normal conditions the NUT protein is exclusively expressed in post-meiotic spermatids. The product of BRD4-NUT fusion is driven by the BRD promoter. The driving oncogenic nature of this translocation was confirmed by whole exome sequencing where BRD/NUT appears as a unique event. Further, treatments with the bromodomain inhibitor, JQ1 (discussed below) reduced tumor cell proliferation and contributed to squamous cell differentiation and apoptosis. BRD4/NUT is implicated in the recruitment of histone acetyltransferases such as p300 and is found co-localised with the transcriptionally active H3K27Ac mark (225), leading to the activation of pro-survival genes such as MYC (226).

In cancer cells, BRD4 plays a critical role in maintaining cell proliferation and repressing apoptosis. This shift from maintaining homeostasis to promoting proliferation is likely dependent on the mis-targeting of BRD4 to the regulatory regions of oncogenes due to reprogramming of histone acetylation. It appears that BRD4 acts as an important driver of tumor growth in some settings. An shRNA screen revealed that BRD4 is an essential gene for the proliferation of

epithelial ovarian cancer and BRD4 depletion significantly reduced cancer cell viability (227). In renal cell carcinoma, BRD4 has been found upregulated (228). Subsequent inhibition of BRD4 with shRNAs and BET inhibitors caused cell cycle arrest at the G1 phase and a subsequent increase in apoptosis that was associated with elevated BAX, cleaved caspase 3 and a down-regulation of the MYC oncogene (228). BRD3 and BRD4 also appear to play a role in promoting cell cycle progression and resistance to apoptosis in cancer. Chromatin Immunoprecipitation showed the displacement of both BRD3 and BRD4 from BCL-2 and CDK6 loci upon treatment with BET inhibitor IBET151 resulting in the transcriptional downregulation of these potent tumorigenic factors (233). In NSCLC models, BET proteins promote survival and BET inhibition results in the induction of apoptosis and the repression of anti-apoptotic factors c-FLIP and XIAP (229). BRD4 has also been implicated in cell invasion and migration. This was demonstrated in a breast cancer model where BRD4 inhibition abrogated the invasion of breast cancer cells and downregulated expression of the EMT transcription factor Snail (230). However, studies aimed at understanding in what context BRD4 acts as an oncogene are lacking. Minimally, ectopic expression of BRD4 in non-transformed cells would add insights into its role as a tumor promoter.

The proto-oncogene MYC drives proliferation and survival and is found over-expressed in many types of cancer through both focal amplification and transcriptional upregulation. c-MYC, L-MYC and N-MYC members of the MYC family of proteins share high sequence homology and differentially overexpressed in cancers (231). c-MYC is primarily found overexpressed in hematologic malignancies and breast cancer (232,233), N-MYC in brain tumors (234) and L-MYC in small cell lung tumors (235). BRD4 is known as a critical regulator of c-MYC expression within tumors where c-MYC is transcriptionally upregulated. It has been observed in MLL-rearranged acute myelogenous leukemia (AML) that BRD4 protein binding is highly enriched at distal enhancer elements called super enhancers that activate MYC transcription (236,237). In multiple myeloma, super-enhancers located within 50kb of the c-MYC were found occupied by BRD4, H3K27Ac and Mediator complex which generated aberrant c-MYC transcription (187). A direct role of BRD4 in controlling c-MYC expression was demonstrated through treatment with bromodomain inhibitors (BETi) that displaced BRD4 from chromatin and led to the inhibition of c-MYC expression (187).

2.3 Ante up (intro to small molecule inhibitors of bromodomain proteins, BETi)

The intensive development of BETi first gained traction in 2010 upon the successful synthesis of 2 classes of highly selective BETi; thienodiazepines and benzodiazepines (Table 1) (192,238). The Bradner lab in collaboration with the SGC identified a novel thienotriazolodiazepine-based, selective BETi, termed JQ1, that was derived from less potent compounds patented by the Mitsubishi-Tanabe company in 2006 and 2009. Differential scanning fluorimetry revealed that of the 46 human bromodomains, JQ1 binds most tightly to the Bromodomain and Extra-Terminal domain (BET) protein subfamily, with the highest affinity to BRD4 (192). Crystallographic studies demonstrate that this small molecule mimics acetylated lysine and competitively fits into the binding pocket of the BET bromodomain (192). This small molecule inserts entirely within the acetyl-lysine-binding pocket of the bromodomain and forms a hydrogen bond with a conserved asparagine. The complex of BET bromodomain and JQ1 is stabilized by hydrophobic interactions in BC and ZA loop regions (Fig.8). Thus, perfect complementarity results in a closed structure of the pocket (192). As a result of this tight-fit, JQ1 can displace BRD4 from chromatin, confirmed by fluorescence recovery after photobleaching cell assay (FRAP) and by chromatin immunoprecipitation (ChIP) (192). The association of JQ1 with chromatin has also been confirmed with a Chem-seq approach (239).

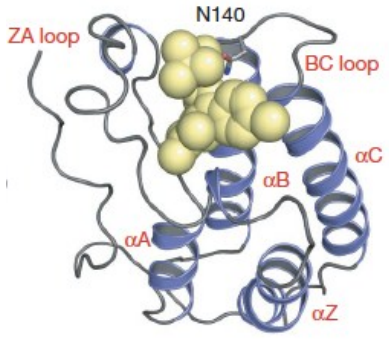

Figure 8. Complex of BET protein, BRD4 with JQ1. The complex is highlighted in yellow**.** N140: conserved asparagine residue (from (192)).

The development of JQ1 offered a great opportunity to validate the oncogenicity of BRD4 and to test its mettle as an anti-cancer target. JQ1 showed anti-cancer properties against NUT midline

carcinoma models described in previous section. JQ1 induced growth arrest and cell differentiation in NMC-derived cell lines consistent with genetic knockdown studies (192). Furthermore, JQ1 has also shown significant anti-tumorigenic activity in mouse xenograft models where the compound inhibited tumor growth and improved survival rates. RNA interference screening detected dependency of AML leukemia models on BRD4 expression and JQ1 treatment lead to anti-cancer effects *in vitro* and *in vivo* settings by inhibiting cell proliferation and inducing myeloid differentiation (240). Many other cancer models including medulloblastoma, breast and lung cancer also showed anti-tumorigenic response to JQ1 (229,241,242). Despite anti-tumor activity, JQ1 is known to have a poor pharmacokinetic profile and low oral bioavailability (243). It has a short half-life of only 1 hour and to obtain a therapeutic effect, the drug would need to be administered twice per day. Thus, optimization of the molecule was necessary and has resulted in the synthesis of a JQ1 analogue named TEN-010 (JQ2) (244). Currently, TEN-010 is undergoing clinical trials for patients with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) and solid tumors (NCT02308761, NCT01987362).

In parallel, a benzodiazepine I-BET762 (GSK525762A), has been developed by a GlaxoSmithKline group (245). This compound demonstrated pan-affinity profile to BET proteins, targeting BRD2, BRD3 and BRD4. The first study claimed that I-BET762 downregulated a pro-inflammatory set of genes in activated macrophages *in vitro* and reduced inflammation in murine sepsis models (238). Afterwards, this compound was shown efficient in tumor models including neuroblastoma and pancreatic cancer (246,247). Since the discovery of these initial BETi, the development of this class of compounds has broadened leading to the synthesis of a diverse array of novel BET targeting molecules structurally similar to JQ1, but with improved pharmacological properties including OTX015 and I-BET151, both inhibitors of BRD2, BRD3 and BRD4 (248,249). Importantly, OTX015 can be administered orally. In preclinical studies, these inhibitors showed efficacy against hematological malignancies including myeloma, lymphoma, myeloproliferative neoplasms and some solid types of cancer (250-253).

A novel and BD2 selective quinazolone, RVX-208, recognizes the BD2 domain of BRD2 and BRD3, and inhibits these proteins at IC50 of approximately 500nM (254). RVX-208 increases ApoA1 and reduces vascular inflammation in manner dependent on BRD inhibition (255). RVX-208 is currently under clinical evaluation for the treatment of atherosclerosis and other high-density lipoprotein-linked diseases (256-259). More potent BETi, MS417 binds to BD1 and BD2 of BRD4 at *Kd* value of 25-36nM (260,261). MS417 has been shown to completely inactivate transcription of NF-kb at 1uM in human embryonic HEK293T cells. In breast cancer *in vivo* model, MS417 showed more pronounced anti-tumor effect at only 20mg/kg than JQ1 used at 50mg/kg. (262). The same dose of MS417 significantly decreased liver metastasis in colorectal cancer preclinical models (263). Another BETi, ABBV-075 demonstrates binding to BRD2, BRD4 and BRDT at Ki of only 1-2.2nM/L (264). Interestingly, this small-molecule compound is quite specific for BRD2, BRD4 and BRDT but showed a weak affinity to BRD3. A large screen of 147 hematological and solid cancer cell lines demonstrated ABBV-075 inhibiting cell proliferation at IC50 of around 100nM with more pronounced efficiency in hematological cancer models (264).

Beyond selectively targeting a single bromodomain, another interesting strategy to target BRD family members is via PROTACs (protein-targeting chimeric molecules) where a BET inhibitor is coupled to a molecule trapping bromodomain proteins to E3 ubiquitin ligase complex leading to protein degradation (265). These compounds are still being optimized on the preclinical level in lymphomas and breast cancer models (266,267). In order to potentiate optimal BETi activity, the latest generation of bivalent compounds such as AZD5153 recognize simultaneously both bromodomains (268). AZD5153 enhanced the displacement of BRD4 from chromatin at lower concentrations in comparison to IBET762. AZD5153 showed significant efficiency in inhibiting cell growth in hematological cancer models at GI50 <25nM for most of the tested cell lines. Importantly, preliminary data with AZD5153 shows encouraging results *in vitro* and *in vivo* against hematologic and thyroid malignancies (268,269).

The progress in BETi development has led to decreasing concentrations being required to achieve efficacy in preclinical studies. The average doses for the first BETi such as JQ1, I-BET762, I-BET151, OTX015 range between 300nM-1uM when employed *in vitro* and 30-50mg/kg in murine models of cancer (192,247,250,251,253). PROTACs molecules ARV-825

and ARV-771 are more potent and inhibit cell proliferation at 100-250nM and exhibit anti-tumorigenic effect at 10-30mg/kg *in vivo* (267). Currently, in terms of dose optimization, compounds MS417 and AZD5153 appear quite promising. AZD5153 demonstrates efficacy *in vitro* at only 10-100nM. *In vivo*, MS417 was effective against breast cancer models at 20mg/kg and AZD5153 at 10mg/kg in hematological and solid cancers (262,268,269).

Table 1. BET inhibitors

BET inhibitor	Company
JQ1	Bradner lab
TEN010 (JQ2)	Hoffmann-La Roche
I-BET762 (GSK525762A)	GlaxoSmithKline
OTX015	Merck Sharp and Dohme Corp.
IBET-151(GSK1210151A)	GlaxoSmithKline
RVX-208	Resverologix Corp
MS417	Zhou lab
ABBV-075	AbbVie
AZD5153	AstraZeneca
ARV-825	Arvinas
ARV-771	Arvinas

2.4 The big blind – Are there robust predictive biomarkers?

BETi treatment has been suggested to be an efficient therapeutic strategy in multiple preclinical cancer models including NUT midline carcinoma, acute myeloid leukemia, myeloma, lung, breast and pancreatic cancer (192,240,242,250,270,271). However, not all cells show sensitivity and it has been challenging to identify the tumor subtypes, or predictive biomarkers, to reveal tumors that will show the highest degree of growth inhibition after BETi exposure. This is partially due to variability in defining precise concentrations of BETi that represent "sensitive" cells. Most of the studies published to date employed high concentrations ranging between

500nM-1uM for *in vitro* studies and ≈50mg/kg in animal studies (192,242,250,270,271). Such concentrations of drug could make the discovery of robust biomarkers challenging, because biomarkers predicting sensitivity to these concentrations are unlikely to hold water clinically, where such doses are unattainable. Thus, there is currently an emerging need to carry out BETi research utilizing lower doses of BETi in preclinical experiments in order to properly stratify tumors into responders and non-responders. Such approaches will also help future clinical trials avoid undesirable toxicities. Perhaps, the development of new more potent bivalent compounds targeting two bromodomains of BET proteins such as AZD5153 could also address these concerns.

The primary target of BETi, BRD4, is oncogenic in nature and perhaps identifying tumor types that are dependent on BRD4 for survival is one key to unlocking the identity of tumor subtypes that will be most sensitive to BRD4 inhibition. For example, it is clear that BRD4 plays a major role in driving the oncogenic progression of NUT midline carcinoma (224,272). BRD4 has been also shown as an essential gene for cancer cell proliferation in AML and ovarian cancer which could potentially be employed as a strategy for BETi treatments (227,240). A topic that is underexplored is whether overexpression of BRD family members themselves influence the sensitivity to BETi. While there are many scenarios, it is possible that tumors overexpressing BRD4 depend on its expression for survival. A caveat here involves the selectivity profile of BETi. Currently, most of BETi target all the members of BET subfamily including BRD2,3,4 and BRDT (192,238,248,249). These proteins may have both distinct and overlapping functions which requires deeper understanding of the mechanism of action, and oncogenic driver activity of these proteins in different cancers. Exogeneous expression of each BET protein in distinct cell types, either alone or in combination, would help to elucidate tissue-specific oncogenic properties of these chromatin binding proteins and allow to examine their impact on sensitivity to BETi.

A key discovery regarding the anti-tumor activity of BETi is that this class of drugs silence the expression of the c-MYC (273). It has been documented that treatment with BETi leads to dissociation of BET proteins from the MYC locus. Through repression of MYC-mediated transcription, BETi are able to achieve cell cycle arrest and apoptosis. This has been validated in

several cancers where MYC overexpression is thought to be a primary driver including multiple myeloma, MLL-fusion leukemia, ALL, and glioblastoma (236,273-275). Therefore, MYC amplification might represent a potential biomarker for BET-inhibitor sensitivity. Considering MYC amplification is often associated with a poor prognosis, targeting MYC amplified cancers would theoretically have a profound effect on clinical outcomes for these cancers.

While MYC is commonly downregulated in BETi exposed cells, it is not always clear that this event mediates the anti-proliferative effects of BETi and a number of recent studies show that c-MYC may not necessarily act as a mediator of BETi sensitivity. For instance, ectopic expression of MYC would be expected to confer BETi resistance if its down-regulation was the primary mediator of BETi-induced growth inhibition. However, such experiments give highly mixed results suggesting that other pathways beyond MYC may be determinants of the drug response. In glioblastoma models, MYC reconstitution did not significantly protect the cells from JQ1 induced growth arrest (274). In this model, factors including exogenous BCL-XL expression or p21 knockdown were able to rescue the sensitivity to BETi. Another study reported FOSL1 as a key determinant of the response to BETi in lung adenocarcinoma cells and here, response to JQ1 was independent of MYC-expression (276). In small-cell lung cancer cells, JQ1 treatment resulted in the inhibition of the lineage-specific transcription factor ASCL1 while MYC expression was not affected (277). Similarly, MYC was not found to be a determinant of BETi sensitivity in patient derived xenografts of pancreatic tumors (278). It should be noted that most of the studies reporting MYC as a principal factor in the sensitivity to BETi, were conducted on hematological malignancies models. However, even clinical studies using BETi directed against MM, AML and DLBCL have so far not found MYC amplification to predict sensitivity to this class of compounds (279,280). Again, these studies question the validity of MYC as a predictive biomarker.

As cancer represents a very heterogeneous disease on the genomic and epigenomic levels, the response to BETi may not be necessarily dictated by the same targets across tumor types. Furthermore, even in a few studies where MYC has been shown as a marker of sensitivity to BETi in solid cancers including lung cancer and neuroblastoma (229,281), the drug dosage employed was much higher in comparison to the doses used in preclinical blood cancer models

(282,283). Thus, it is important to identify critical, specific targets of BETi for each type of cancer rather than applying the same approach to all the tumor subtypes.

After the initial discovery of MYC as a predictive biomarker and potent target for BETi, numerous studies focused on understanding the anti-oncogenic properties of the BETi observed inactivation of transcriptional programs involved in RTK dependent pathways (284-288). RTKs mediate proliferation and survival through the activation of downstream PI3K-AKT and RAS-MAPK pathways. Many types of cancer are characterized by the upregulated transduction through these signaling cascades. One mechanism for this is through upregulation of cell surface growth factor receptors such as EGFR. Alternatively, downstream effectors are often mutated across a spectrum of cancers. This includes breast and colorectal cancers that commonly carry activating *PIK3CA* mutations, melanoma, which is often characterized by *BRAF* and *NRAS* mutations and pancreatic cancers, among others, that are characterized by frequent *KRAS* mutations.

BETi have been shown to down-regulate RTK dependent PI3K and RAS signaling pathways in sensitive cells (172,285). Related to this, evidence suggests that the constitutive activation of PI3K and MAPK signaling may dictate intrinsic resistance to BETi (172,287). A genome wide shRNA screen revealed that PI3K pathways along with BRD4 are essential drivers of tumor cell proliferation in luminal breast cancer (285). However, the sensitivity of luminal cells to BRD4 knockdown did not consistently correlate with the JQ1 sensitivity. This suggests that BRD4-independent mechanisms of drug response or BRD4-independent resistance mechanisms, such as PI3K activation, dictate responsiveness to BETi (285). Some insight into this phenomenon may be provided by findings linking PI3K and MAPK signaling to BETi resistance. Using CRISPR screens in neuroblastoma models, it was revealed that PI3K activation imparts acquired and intrinsic resistance to BETi. It has been also revealed that in resistant cells, genes upregulated by PI3K pathway showed a gain in enhancer elements potentially explaining resistance mechanisms (286). Consistent with this, ovarian cancer cells demonstrate acquired resistance to BETi thorough kinome reprogramming, where elevated signaling through PI3K and RAS were correlated with resistance (288) and combination treatment of BETi with PI3K inhibitors partially overcame resistance. Similarly, an intrinsically BETi-resistant *NRAS*-mutant melanoma

model showed sensitivity to the combination therapy of BETi and MAPKi (287). Mutation of *LKB1*, which modulates downstream effectors in the PI3K pathway, was also suggested to impart resistance to BETi. However, this was not formally proven through the introduction of dominant-negative *LKB1* mutations into WT cells (271). Together, these data suggest that gain of function mutations and constitutive activity through the PI3K and RAS axes might act as biomarkers of resistance to BETi, that may be overcome with combination therapies.

Understanding mechanisms through which BRD4 driven-transcription programs are regulated will likely reveal vulnerabilities that may be exploited using BETi. BRD4 and a second bromodomain protein, SMARCA4, independently, but concurrently, activate gene expression through simultaneous binding at regulatory elements (122). It is likely that SMARCA2, a SMARCA4 paralogue, may compensate for SMARCA4 loss. Thus, in SMARCA4/A2-deficient cancers, BRD4 may act as the primary driver of SMARCA4/BRD4-dependent oncogenes, inferring that the exposure to BETi might eliminate this network to promote cell cycle arrest or apoptosis. Consistent with this hypothesis, our data demonstrated that SMARCA4/A2-deficient SCCOHT and NSCLC models are acutely sensitive to BETi at low nanomolar concentrations *in vitro* as well as *in vivo* (20mg/kg/day) (172). Further support for this model was supported through ectopic expression of SMARCA4 that was found to confer partial resistance to BETi. These findings demonstrate that the loss of SMARCA4 may act as a potential predictive biomarker for BETi. Notably, in OTX-015 sensitive cells harboring dual SMARCA4/A2 loss, repression of the MAPK pathway was tightly correlated with drug efficacy. Again, this suggests that the RAS/MAPK pathway plays an important role in dictating the response to BETi.

SPOP is a protein responsible for the ubiquitination and degradation of BET proteins that is found mutated in 6-15% of prostate tumors across cohorts (289). Recent findings demonstrate that *SPOP* mutations in prostate cancer are associated with resistance to BETi (290,291). The underlying mechanism appears to be that *SPOP* mutation impedes the rate of BET protein degradation resulting in an accumulation of BRD4. *SPOP* mutations confer intrinsic resistance to BETi both *in vitro* and *in vivo*, but depletion of BET proteins re-sensitizes cells to this treatment (290,291). This reinforces the notion that BRD4 levels within tumors modulate responsiveness to BETi. This data also suggests that the activity of BET proteins might be regulated by

proteasomal degradation across tissue types which opens new therapeutic perspectives. *SPOP* mutations may act as biomarkers for BETi resistance, and when treating prostate cancers with BETi, either this (289)cohort might be avoided. Perhaps, a PROTACs strategy leading to BET proteins degradation might be a potential choice (267). Recent evidence also suggests that a new combination BRD4/CBP-p300 inhibitor overcomes SPOP resistance (292).

2.5 Raising the stakes: Identifying pharmacodynamic biomarkers

Pharmacodynamic (PD) biomarkers allow clinical monitoring of drug activity *in vivo*, ideally using simple PCR or ELISA-based assays that act in lieu of more complex approaches to measure the accumulation of an administered drug within plasma or the tumor itself. Identifying robust pharmacodynamic biomarkers for BETi in preclinical settings will undoubtedly facilitate the optimization of their clinical utility. Establishing such biomarkers may be enhanced through the preclinical use of orthotopic xenografts that mimic a relevant tumor microenvironment thereby more accurately predicting drug response in a time and dose-dependent manner.

Based on initial studies in hematopoietic malignancies, it was thought that MYC repression may act as a pharmacodynamic biomarker. A phase 1 study using OTX015 against AML was unable to validate MYC as a PD biomarker in the bone marrow of AML patients after 1 week of treatment (280). Considering that at least one study shows that downregulation of MYC in response to BETi treatment is restricted primarily to hematological cancer cell lines (293), it seems essential to identify alternative PD assessment criteria. This study identified upregulation of HEXIM1 as a potential PD biomarker in numerous tumor xenografts, whole blood samples and skin in response to ABBV-075 treatment. HEXIM1 is an inhibitory protein of pTEFb and its upregulation leads to the inhibition of transcriptional elongation by RNA Pol II (294). This represents an indirect mechanism through which BETi may repress transcription. An increase of HEXIM1 was reported in blood and skin samples at 4 and 6 hours respectively upon exposure to BETi which was consistent with the plasma concentration of the drug (293). A small clinical trial using BAY1238097 against mixed, refractory malignancies, showed a trend of both MYC repression and HEXIM1 increase in the plasma of exposed patients (295). This trend was only observed when plasma concentrations reached approximately 20ug/L or greater. Another preclinical study reported that MYC and HEXIM1 act mostly as tumor-based biomarkers for

BETi and these mRNAs did not show optimal correlation with drug accumulation within blood samples (296). In this study, CCR2 and CD180 were found to be significantly down-regulated in whole blood samples exposed to AZD5153 from patients with multiple types of cancer (296). Originally identified as BETi targets using transcriptomic analysis, the down regulation of these immune components may link BETi activity with modulation of immune infiltrates. Further clinical trials are required to validate these findings.

Notably among other potential whole blood PD markers, an increase of high-density lipoprotein cholesterol and apolipoprotein A1 have been found in the blood of atherosclerosis patients exposed to RVX-208 (256,297). Despite the preferential using of these markers for the patients with cardiovascular disease, they might also be tested as supplementary markers for cancer patients.

2.6 All in on clinical trials. We Fold: aborted clinical trials

After extensive preclinical evaluation, but without robust predictive biomarkers, multiple clinical trials against solid and hematological types of cancer have been initiated. To date, ~ 25 clinical trials are either ongoing or completed (Table 2). Beyond NUT-midline carcinoma, these trials are not targeting specific pathologies, and this lack of biological rationale, plus unexpected toxicities, may limit clinical efficacy in the short term.

In particular, strong results from preclinical evaluation of BETi against multiple myeloma led to elevated expectations for achieving clinical responses in this malignancy. The first published clinical trials for BETi all employed the widely studied drug, OTX015 (MK-8268) (279,280,298,299). One of these Phase I trials included a cohort of multiple myeloma patients. Here, OTX015 was tested in 45 lymphoma and multiple myeloma patients where 2 patients with DLBCL showed complete response and 1 patient responded partially to the treatment (279). Unfortunately, there were no reports of patients with multiple myeloma who displayed responsiveness. Among 5 patients with MYC-positive DLBCL, only 1 patient showed a favorable outcome to BETi exposure. These studies established a drug dose of 80mg/day for a schedule of 14 days on, 7 days off (21-day regimen). This dose may not be universally achievable because almost all of the patients on this trial displayed non-DLT toxic effects

including thrombocytopenia, anemia, neutropenia, gastrointestinal events and fatigue. Some patients treated with higher doses of 120-160mg/day of OTX015 had severe DLTs such as grade 4 thrombocytopenia, grade 3 gastrointestinal events, grade 3 fatigue and high bilirubin. Again, these studies highlight the importance of identifying PD biomarkers for BETi, which could help guide dose escalation studies. Once maximum drug activity is identified in the plasma or intratumorally, higher doses are likely to elicit strong off-target effects.

Similar results have been reported for a clinical trial using CPI-0610 in a phase I trial against relapsed/refractory lymphoma (NCT01949883). Patients were treated with a range of doses from 6 to 230mg. Among 44 patients, 2 patients showed complete response and 1 patient was partially responsive. TEN010 has also shown partial response in 2 DLBCL patients expressing MYC (NCT01967362).

As described above, NMC are characterized by BRD4 fusions leading to aberrant BRD4 activity. Again, based on preclinical data, there were high expectations that BETi would show efficacy against these tumors and as a result, the first published clinical trial utilizing BETi was focused on NMC (298). Here, a small series of 4 patients carrying diverse, advanced stage tumors harboring BRD4-NUT fusions received OTX015. Clinical responses were observed in 2 patients, but all 4 patients succumbed to their disease between 5 and 19 months post-diagnosis. Complementing this study, Lewin et al (299) observed partial responses in 3 of 10 NMC patients receiving OTX015 at 80 mg once daily, for a duration ranging from 1.8 to 8.4 months. It might be noted here that 83% of patients enrolled in this study displayed treatment-related side effects. Two other BETi, TEN010 and GSK525762, have been tested against NMC and both showed partial response in a small number of patients (NCT01987362, NCT01587703). While somewhat encouraging, these trials have not met expectations and causality for the gap between preclinical observations and clinical results remains obscure.

Beyond MYC and BRD4/NUT driven malignancies BETi have been tested against a number of acute myeloid leukemias and diverse solid tumors. An initial study using OTX015 tested the drug against a cohort of 41 AML patients (280). However, only 3 patients showed partial responses and 2 patients showed complete response lasting 2-5 months. Importantly, an attempt

to study potential biomarkers including mutations in 42 genes failed to identify clear molecular pathologies among responders.

Published reports of BETi against solid tumors have been less than encouraging thus far. Perhaps the most promising preclinical data against solid tumors beyond NMC has come from prostate cancer models (299). However, 26 patients harboring castrate-resistant prostate cancer showed little response to OTX015 on either continuous or discontinuous regimens (299). A published clinical trial using a new BETi, BAY1238097, against solid cancers has been terminated because of DLT side effects including headache and back pain (295). Likewise, other clinical trials involving OTX015 against glioblastoma and other advanced solid tumors have been terminated because of the lack of clinical activity (NCT02698176) and severe adverse events have been reported in glioblastoma patients (NCT02296476).

Based on these data, it is difficult to formulate a clear picture of clinical response to BETi due to the limited cohort of patients. However, it is clear that predictive biomarkers will be essential moving forward, and perhaps more importantly, it will be critical to overcome the in-class DLTs that have led to many trials being terminated. As described above, BRD proteins are important for multiple cellular processes required for homeostasis. This may explain why complete bromodomain inhibition leads to unwanted effects such as gastrointestinal lesions, neurological disorders including memory loss, problems with glucose uptake, decreased hematopoiesis and others. The most common DLT observed across BETi clinical trials to date are thrombocytopenia, hyperbilirubinemia and gastrointestinal events. Advances in the medicinal chemistry of BETi may be required to dissociate anti-cancer effects from the inhibition of physiological pathways required for homeostasis.

Table 2. Clinical trials with BET inhibitors.

BET inhibitor	Reference	Sponsor	Combination	Phase/ Status	Indications	Results
FT-1101	NCT02543879	Forma Therapeutics, Inc.	Azacitidine	Phase I, Recruiting	Hematologic malignancies	NA
RO6870810	NCT03068351	Hoffman-La Roche	Daratumumab	Phase I, Recruiting	Advanced Multiple Myeloma	NA

CPI-0610	NCT02157636	Constellation Pharmaceuticals	Alone	Phase I, Completed	Multiple myeloma	NA
CPI-0610	NCT01949883	Constellation Pharmaceuticals	Alone	Phase I, Active, not recruiting	Lymphoma	Abstract
CPI-0610	NCT02158858	Constellation Pharmaceuticals	Rixolitinib	Phase I/II, Recruiting	Hematologic malignancies, myelofibrosis	NA
CPI-0610	NCT02986919	Texas Southwestern Medical Center	Alone	Phase II, Withdrawn	Peripheral nerve tumors	NA
GSK525762	NCT01943851	GlaxoSmithKline	Alone	Phase I/II, Recruiting	Hematologic malignancies	NA
GSK525762	NCT03266159	GlaxoSmithKline	Trametinib	Phase I/II, Withdrawn	Solid tumors	NA
GSK525762	NCT01587703	GlaxoSmithKline	Alone	Phase I/II, Active, not recruiting	NUT midline carcinoma	Abstract
ZEN003694	NCT02711956	Zenith Epigenetics	Enzalutamide	Phase I/II, Recruiting	Prostate cancer	NA
ZEN003694	NCT02705469	Zenith Epigenetics	Alone	Phase I, Completed	Prostate cancer	NA
INCB054329	NCT02431260	Incyte Corporation	Alone	Phase I/II, Completed	Solid and hematologic malignancies	NA
BMS-986158	NCT02419417	Bristol-Myers Squibb	Nivolumab	Phase I/II, Recruiting	Advanced solid tumors and hematologic malignancies	NA
MK-8628 (OTX015)	NCT02303782	Oncoethix GmbH	Azacitidine	Phase I/II, Withdrawn	Acute myeloid leukemia	NA
MK-8628 (OTX015)	NCT02698189	Merck Sharp & Dohme Corp.	Alone	Phase I, Active, not recruiting	Hematologic malignancies	NA
MK-8628 (OTX015)	NCT02698176	Merck Sharp & Dohme Corp.	Alone	Phase I, Terminated (limited efficacy)	Advanced solid tumors	NA
MK-8628 (OTX015)	NCT02296476	Oncoethix GmbH	Alone	Phase II, Terminated (lack of clinical activity)	Glioblastoma Multiforme	Abstract
MK-8628 (OTX015)	NCT02259114	Oncoethix GmbH	Alone	Phase I, Completed	Advanced solid tumors	Abstract
MK-8628 (OTX015)	NCT01713582	Oncoethix GmbH	Alone	Phase I, Completed	Hematologic malignancies	2 reports posted
RO6870810/ TEN010	NCT02308761	Hoffmann-La Roche	Alone	Phase I, Completed	Hematologic malignancies	NA
RO6870810/ TEN010	NCT01987362	Hoffmann-La Roche	Alone	Phase I, Completed	Solid tumors	2 abstracts

BAY1238097	NCT02369029	Bayer	Alone	Phase I, Terminated	Solid tumors, non-Hodgkin lymphomas	Abstract

2.7 A winning hand: combination therapies

Preclinical research demonstrating efficiency of BETi as a single agent often utilize drug concentrations of 500nM to micromolar concentrations and *in vivo* concentrations that are unattainable in a clinical setting. The necessity of using such high doses to combat cancerous proliferation strongly suggests a high level of intrinsic resistance to these compounds across tumor types that has not been widely studied or appreciated. The precise mechanisms of resistance to BETi remain unclear, but multiple studies suggest activation of oncogenic signaling pathways including PI3K and RAS pathways may be involved and that repression of this pathway may act as a key mediator of BETi activity. Upregulation of MAPK signaling through *KRAS* or *NRAS* mutations or PI3K/AKT signaling through *PIK3CA* mutation have been correlated with drug resistance in lung, melanoma and breast cancer respectively (271,284,285,287). We suggest that these pathways also play a role in presenting intrinsic BETi resistance that may be overcome with combination therapy.

Several studies reported the PI3K pathway as a determining factor influencing BETi resistance, and that this resistance could be successfully overcome through the combination treatment with BETi with PI3K inhibitors (285,286). *PIK3CA* is one of the most mutated genes in cancer, with *PIK3CA* mutations occur in approximately 30% of breast, endometrial and colorectal cancer (300-302). *PIK3CA* mutations lead to the activation of PI3K/AKT pathway resulting in enhanced tumor cell proliferation and survival. *PIK3CA*-mutated breast cancer models are resistant to either PI3K or BETi alone due to feed-back activation of multiple RTKs and re-activation of the PI3K pathway (284,285). A recent study suggests that PI3K signalling, in particular, AKT activation, confers considerable resistance to BETi in neuroblastoma. Here, upregulation of RTK-related genes is associated with chromatin remodeling of enhancer regions leading to aberrant transcriptional upregulation (286). In 45% of these aberrantly activated enhancers, enrichment for BRD4 binding was observed. This molecular pathology rendered cells sensitive to PI3K inhibition and even more to combination therapy with BETi.

Consistent with BRD4 playing a role in regulating RTK signaling, BETi have been shown to displace BRD4 from certain RTKs such as IGFR and INSR in murine mammary tumors (284). Here, BETi and PI3K inhibitors also showed synergy. ChIP-seq studies indicate BRD4 binds primarily at regulatory regions of upstream regulators of RTK signaling, while interactions at promoter/enhancer regions of downstream effectors such as PIK3CA and AKT appear not to play a major role in their activation.

Acquired resistance to BETi may also be imparted through activation of PI3K in ovarian adenocarcinoma cell lines (288). In this study, acquired resistance to JQ1 was conferred by RTK reprogramming where upregulated PI3K and ERK signaling stabilized MYC and FOSL1 transcription factors preventing them from protein degradation promoting resistance to BETi (288).

It is interesting to note that a previous study indicated that triple negative breast cancer (TNBC) models are more sensitive to BETi than the luminal subtype (242). However, the drug concentrations used in that study were not optimal (2-20uM) (242) and off-target effects are likely. In addition, another study proposed an opposite hypothesis underlying the sensitivity of luminal subtype to BETi treatment with a number of *PIK3CA* mutant cells showing resistance (285). Thus, further studies allowing us to interpret mechanisms of intrinsic resistance are needed.

There are also strong indications that the oncogenic MAPK pathway plays a role in BETi resistance. Melanomas carrying *NRAS* mutations frequently present high BRD4 mRNA and protein expression levels associated with poor outcome (287). These cells display a weak response to relatively high concentration of BETi of 500nM. In contrast, the combination of BETi with only 100nM of MEK inhibitor, PD901 showed efficacy in inhibiting cell proliferation which was validated in mouse models (287). It remains to be seen if the ectopic expression of constitutively active KRAS or BRAF to sensitive cells will impart resistance. In colorectal models, it does not appear that *KRAS* mutations render cells more resistant to BETi (303). However, it appears that these cells harbor a high degree of intrinsic resistance to BETi

regardless of their mutational profile. In *KRAS*-mutant carrying NSCLC models, sensitivity to BETi was reported, but 2.5uM of JQ1 was required. Thus, in reality, these cells are quite resistant to BETi. In these cells, MYC downregulation was observed at 500nM of JQ1 at concentration insufficient to elicit an anti-proliferative effect, indicating MYC down-regulation on its own could not prompt cells to stop dividing (271). Notably, this study also revealed that the loss of LKB1, an upstream modulator of mTOR activity led to BETi resistance.

Considering what we know thus far regarding BETi resistance, and evidence from preliminary studies, it is logical to predict that BETi may act in synergy with inhibitors of MEK or PI3K to overcome intrinsic resistance. Considerable evidence indicates that BETi, similar to targeted inhibitors, repress these pathways, thus the combination might be expected to more effectively repress these classical oncogenic processes. Further, the inclusion of BETi within this combination would be expected to target additional transcriptional targets, including MYC. Multiple studies demonstrate that combining BET inhibitors with tyrosine kinase dependent inhibitors effectively overcomes drug resistance in several types of cancer (284,287,288). A chemical combinatorial screening of JQ1 with around 1900 compounds aimed at finding effective small molecule combination therapies with BETi revealed PI3K inhibitors to be the most potent partner against neuroblastoma both *in vitro* and in animal models (286). The combination of PI3K and BETi was also efficient in ovarian cancer cells with acquired resistance to BETi (288). The efficiency of this strategy was confirmed by the down-regulation of pAKT protein expression. Additionally, the expression of oncogenic factors activated by MAPK signaling, MYC and FOSL1 was also decreased indicating to a potential crosstalk between PI3K and MAPK pathways.

Combined inhibition of PI3K and BETi is able to potently block PI3K/AKT signaling as evidenced by the inhibition of a key node of this pathway, phosphorylated AKT (284). But it remains unclear how BETi represses PI3K signaling or enhances the effects of PI3K inhibitors, but this may stem from transcriptional repression of RTKs themselves, or downstream effectors.

MEK inhibitors also showed synergy with BET inhibitors in melanoma models (287) and cooperatively in colorectal models (303). In *NRAS*-mutated melanoma models, BRD4 levels

correlated with sensitivity to BETi and synergy between JQ1 and MEK inhibitor PD0325901 was observed across *NRAS*-mutated cell lines at concentrations of approximately 500nM JQ1 or higher (287). In vivo, effective synergy was observed in PDX models at only 25 mg/kg using OTX-015 as a combination therapy with PD0325901. The combined treatment down-regulated proteins implicated in cell cycle regulation and apoptosis, however, the precise mechanism whereby MEKi acts in synergy with BETi remains to be delineated.

Beyond these approaches, additional rational combinations with BETi show promise. JQ1 and HDAC inhibitors have been shown to act in synergy against pancreatic cancer models (270). Pancreatic ductal adenocarcinoma (PDAC) with *KRAS* mutation represent tumors with a very poor prognosis. The study showed that *KRAS* mutation was correlated with the overexpression of BET proteins including BRD2,3 and 4. The efficiency of JQ1 alone was mainly due to the inhibition of MYC and inflammation genes including IL6. The same study showed that the HDAC inhibitor SAHA, in combination with JQ1, induced a synergistic effect in animal models and an indicator of the efficacy was upregulation of the tumor suppressor p57. Again, the doses of drugs employed in this study were quite high (50mg/kg/d), adding uncertainty to the translation of this combination to a clinical setting. An interesting combination of BETi with vitamin C was demonstrated effective using both *in vitro* and *in vivo* triple negative breast cancer models. Here, simultaneous treatment resulted in the decrease of histone acetylation (304), perhaps consistent with the findings of efficacy between BETi and HDACi. This strategy of combining BETi with vitamin C in TNBC and melanoma significantly improved EC50 of BETi by reducing it to nanomolar range (304,305).

The combination of PARP inhibitors with BETi has been proven efficient across many cell lines including ovarian, breast and pancreatic cancer models *in vitro* and *in vivo* (306-309). It has been shown that BRD4 inhibition led to defects in the repair of DNA lesions through the homologous recombination repair pathway rendering cells sensitive to PARP inhibition (306). BRD4 transcriptionally regulates the resection protein CtIP, which is a requisite step in the repair of DNA double strand breaks through homologous recombination. BRD4 inhibition led to decreased CtIP levels thereby impairing homologous recombination. The synergy between BETi and PARPi appears independent of defects in BRCA1/2, TP53 or KRAS, suggesting this

approach may have widespread clinical potential. Another study revealed that BETi decreased the binding of BET proteins to BRCA1 and RAD51 genes and thereby repressed their transcription. This may further explain BETi/PARPi synergy and explain why synergy appears independent of BRCA status (308). These studies are consistent with data obtained using ovarian adenocarcinoma models having BRCA1/2 deficient and proficient profile (307). The study showed that both *BRCA1/2*-mutated and wild type models were highly sensitive to BETi/PARPi combination. Importantly, the resistance of *BRCA1/2* mutant cells to PARP inhibitor, olaparib was significantly reduced by BETi exposure.

Other noteworthy drug partners for BETi are immune checkpoint inhibitors. To avoid immune detection and destruction, cancer cells need to avoid immune surveillance. Especially important is the suppression of the T cell response. Antibody-based immune checkpoint therapies, such as anti-PD-L1 or anti-PD1, work by "turning off" T-cell checkpoint receptors utilized by tumors to repress immune activity. PD-L1 appears to be a direct target of BRD4 (310) and BETi suppress the expression of this cell surface protein. Thus, in this setting, tumors could not engage with the PD1 receptor on T-cells, thereby facilitating immune detection of neoantigens. Synergy has been observed between BETi and the PD-1 antibody in *KRAS* mutant non-small lung cancer models (311). *KRAS* mutant tumors are characterized by compromised immunosuppressive mechanisms. This is represented by the elevation of suppressive regulatory T cells (Treg) and induced expression of PD-1 on tumor infiltrating T cells which impairs anti-tumor functions of T cells. The combination of JQ1 with anti-PD1 antibody was able to reduce the proportion of Treg cells by 60% and inhibited PD-1 expression in tumor infiltration T cells. Furthermore, the analysis of tumor niche fluid revealed the decrease of Th1 cytokines profile, IL12, TNFα, and IFNγ. All this resulted in significant tumor growth inhibition.

Despite some encouraging results from this combination treatment, a clear mechanism explaining the drug interaction is missing as are biomarkers predicting sensitivity to this combinatorial approach. Further studies across cancer types are required to provide additional insights (Fig.9).

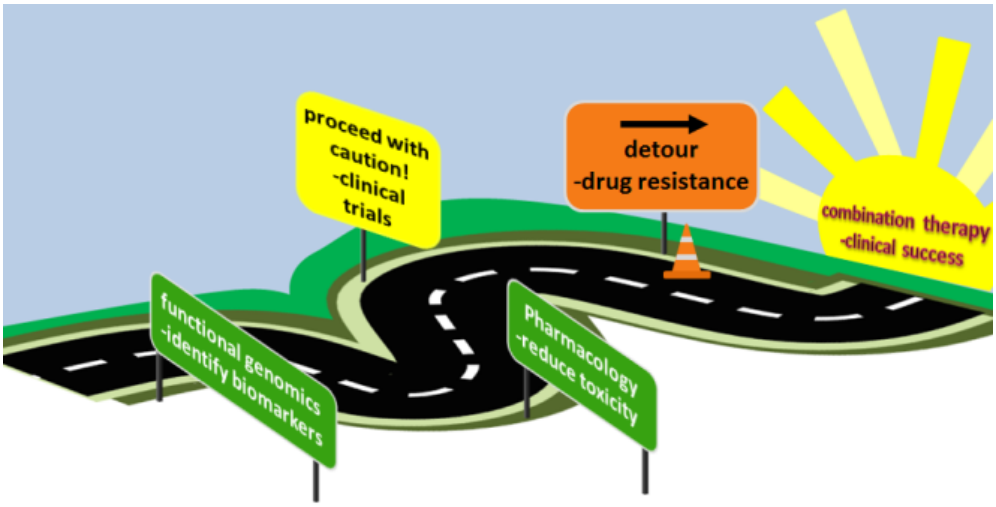

Figure 9. Schematic representation of BETi treatment strategy.

2.8 Conclusion

The development of the BET inhibitors has provided important insights into the key role of BET proteins in transcriptional control of proto-oncogenes, and highlighted their potential as therapeutic targets. Anti-tumorigenic effects of BETi has been shown in many types of cancer, including several incurable subtypes. Preclinical studies have demonstrated a remarkable anti-proliferative activity in tumors. However, a lack of biomarkers predicting sensitivity to BET inhibitors is limiting their application in clinical practice. Thus, further mechanistic studies are necessary. Undoubtedly, the development of novel, highly selective bromodomain inhibitors will help uncover novel bromodomain networks and new clinical applications for targeting BET proteins. Further, in the long term, BETi hold the most clinical potential as a part of combination regimens.

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Chapter III: SWI/SNF-compromised cancers are susceptible to bromodomain inhibitors

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3.1 Preface

Small Cell Carcinoma of the Ovary, Hypercalcemic Type (SCCOHT) and Non-Small Cell Lung Cancer (NSCLC) display inactivating mutations in *SMARCA4* gene, generally leading to a complete loss of the protein. Both of the diseases have poor outcome and have been proven challenging to treat. Here, we propose a novel therapeutic strategy employing BET inhibitors where SMARCA4-deficiency is considered as a predictive biomarker of the sensitivity to BETi.

3.2 Abstract

The antitumor activity of bromodomain and extra-terminal motif protein inhibitors (BETi) has been demonstrated across numerous types of cancer. As such, these inhibitors are currently undergoing widespread clinical evaluation. However, predictive biomarkers allowing the stratification of tumors into responders and non-responders to BETi are lacking. Here we showed significant anti-proliferative effects of low dosage BETi in vitro and in vivo against aggressive ovarian and lung cancer models lacking SMARCA4 and SMARCA2, key components of SWI/SNF chromatin remodeling complexes. Restoration of SMARCA4 or SMARCA2 promoted resistance to BETi in these models, and conversely, knockdown of SMARCA4 sensitized resistant cells to BETi. Transcriptomic analysis revealed that exposure to BETi potently downregulated a network of genes involved in receptor tyrosine kinase (RTK) signaling in SMARCA4/A2-deficient cells, including the oncogenic RTK HER3. Repression of signaling downstream of HER3 was found to be an important determinant of response to BETi in SMARCA4/A2-deficient cells. Overall, we propose that BETi represent a rational therapeutic strategy in poor prognosis, SMARCA4/A2-deficient cancers.

3.3 Introduction

A growing number of potent, selective small molecule compounds targeting epigenetic enzymes and transcriptional regulators have been developed, including inhibitors targeting bromodomain and extra-terminal motif containing proteins (BETi). This class of bromodomain proteins include BRD2, BRD3, BRD4, and BRDT (312). Of these, BRD4 has been the most extensively studied and a wealth of evidence has been acquired, revealing it to be an important therapeutic target (236,240). BRD4 is considered as a "reader" of lysine acetylation that specifically binds to acetylated histone tails thereby stimulating the recruitment of transcriptional machinery to promoter and/or enhancer regions of target genes most commonly implicated in tumorigenesis (206). Preclinical studies in diverse cancer types point to BRD4 as the primary target of most BETi (192,242). However, these inhibitors generally bind other BET family members as well. Importantly, a number of BETi have been proven highly selective, showing minimal off-target binding to bromodomain-containing proteins outside the BET family (192,249). BETi act as acetyl-histone mimetic compounds that disrupt BRD4 function by competitively inhibiting its binding to chromatin, which in turn, inactivates transcription of proliferation-related gene networks. In particular, the oncogenic c-MYC network is known to be repressed after exposure to BETi (273). However, not all responses to BETi are dependent on c-MYC repression and down-regulation of additional pathways, such as the PI3K signal transduction cascade, undoubtedly mediate the anti-proliferative effects of BETi in some tissues (313,314).

Selective inhibitors of BET proteins, such as JQ1, OTX015 and I-BET762 have shown anti-cancer activity against a plethora of tumor types, both *in vitro* and in murine tumor models of NUT midline carcinoma, multiple myeloma, myeloid leukemia, pancreatic, lung and breast cancer among others (192,242,250,270,271). However, the doses of BETi employed in these studies were generally quite high ranging from 500nM to 1uM for *in vitro* experiments and employing a typical dose of 50mg/kg or more per day *in vivo* against solid tumors. To identify patient cohorts likely to show durable clinical response to BETi, it is likely that biomarkers predicting substantially lower doses will be required. This is especially important considering that numerous trials are currently underway against both solid and hematopoietic tumors and results thus far have not met expectations (279,280,315).

Preclinical studies have focused primarily on c-MYC downregulation as the principal mediator of the anti-proliferative effects of BETi. In some cases, intrinsic resistance to BETi may be conferred through ectopic expression of c-MYC (282) . While encompassing a limited cohort of patients, preliminary clinical trials have not yet substantiated elevated c-MYC as a strong predictive biomarker of BETi activity (279,280). Preclinical data clearly show that inhibition of c-MYC-independent targets of BETi may also drive growth arrest. Interestingly, these include signaling pathways such as phosphatidylinositol-3 kinase (PI3K) or Hedgehog signaling, which may mediate the anti-proliferative effects of BETi in a tissue-specific manner (284,313,314,316). To date, biomarkers predicting response to BETi are still lacking, but their identity would obviously enhance the therapeutic potential of this class of drugs. Beyond acting as single agent, the full potential of BETi as anti-cancer agents may not be realized until their utility as combination therapies is fully explored. In particular, recent data indicates BETi in combination with PARP or MAPK inhibitors hold great promise (287,306).

SMARCA4 (SWI/SNF-related, matrix-associated, actin dependent regulator of chromatin, subfamily a, member 4; also known as BRG1) is a core catalytic component of the multisubunit SWI/SNF (SWItch/Sucrose Non-Fermentable) chromatin remodeling complex (44). Using energy generated through ATP hydrolysis, SMARCA4 shifts or evicts nucleosomes, generally leading to transcriptional activation across a spectrum of genes. SWI/SNF complexes may utilize either SMARCA4 or another, highly homologous ATPase, SMARCA2 (also known as BRM), in a mutually exclusive manner. Physiologically, these two ATPases are often expressed in a mutually exclusive manner across tissues, but in cancer their relationship is more complex. SMARCA4/A2 may coregulate target genes and each subunit has been demonstrated to compensate for the loss of the other (63). In cancer, SMARCA4 plays a Janus-like role, acting most commonly as a tumor suppressor, but also as a context-specific oncogene (168,317) . The role of SMARCA4 as a tumor suppressor has been widely studied, and mice heterozygous for the gene are predisposed to tumors of the mammary gland and lung (77,161). *SMARCA4* deletion or loss of function mutations are thought to promote tumorigenesis through complementary mechanisms including metabolic reprogramming and an incapacity to evict Polycomb repressors from target genes (162,318).

Clinical data also supports this tumor suppressor role of SMARCA4. SMARCA4 protein is lost in almost 100% of Small Cell Carcinoma of the Ovary, Hypercalcemic Type (SCCOHT), generally because of loss of function mutations (38). SCCOHT is a deadly cancer with an overall 5-year survival of only 16% (41 patients out of 257), with slightly better outcomes expected for disease diagnosed at early stages (319). Immunohistochemical staining shows a complete loss of SMARCA4 protein in nearly 100% of SCCOHT. Notably, SMARCA2 was observed concurrently lost in 100% of SCCOHT, albeit, in a limit cohort of 46 patients (48). A similar pathology was observed for a subset of NSCLC patients where ~15% show a complete loss of SMARCA4 and a concurrent loss of SMARCA2 in 10% of NSCLC (146), again based on a small cohort of 41 tumors. This same study indicated that the concurrent loss of these two chromatin remodelers is associated with a poor clinical outcome. SMARCA4/A2-deficient tumors, especially SCCOHT and NSCLC, have proved challenging to treat, and these cancers respond weakly to the current standard of care. For SCCOHT, treatment generally involves surgery and adjuvant chemotherapy, most commonly platinum-based agents (49,319). Emerging evidence suggests that high dose chemotherapy followed by autologous stem cell rescue prolongs the survival of SCCOHT patients (319). Similarly, for NSCLC beyond early stages, radical surgery, followed by platinum-based chemotherapy and/or radiotherapy is provided. But for both diseases there is a dire need for new therapeutic approaches.

In contrast to its role as a tumor suppressor, SMARCA4 is also a known oncogene and is overexpressed in several cancers. It has been suggested that a switch in SMARCA4 function takes place during the course of tumor progression allowing it to promote cell proliferation, and possibly confer drug resistance (168,179). It is currently unclear what molecular events trigger this switch. Mechanistically, there is evidence that SMARCA4 acts as a coactivator for oncogenic transcription factors such as MITF or ZEB1 (320,321). The oncogenicity of SMARCA4 may further rely on the activation of survival and proliferation related genes such as c-MYC, or activation of the PI3K pathway (122,178). Importantly, in the case of c-MYC, it has been shown that SMARCA4 and BRD4 independently occupy distal enhancer elements and co-activate transcription in a redundant manner (122). Thus, it is reasonable to hypothesize that in SMARCA4-deficient cells BRD4 is solely responsible for driving an oncogenic network that is

otherwise controlled in a redundant fashion by SMARCA4 and BRD4. Consistent with this, a siRNA screen revealed that *SMARCA4*-mutant esophageal cancer models depend on BRD4 for survival (177). These data led us to test the hypothesis that SWI/SNF-compromised cancers may show sensitivity to BETi. We expected that this approach may be especially relevant to SCCOHT tumors which carry a light mutational burden and are invariably characterized by the loss of SMARCA4 (322).

Here, we demonstrate that SMARCA4/A2-deficient SCCOHT and NSCLC models are highly sensitive to BETi *in vitro* at doses of BETi in the low nanomolar range, significantly lower than those previously reported for cells derived from solid tumors (192,242,270,271). In contrast, mutation of SMARCB1 does not predict such sensitivity. Add-back of SMARCA4/A2 leads to acquired BETi resistance. Using an orthotopic xenograft model of SCCOHT, we also show hypersensitivity to BETi *in vivo*, again at doses well below those utilized to target solid tumors in previous reports. RNA-sequencing revealed that BETi represses the transcription of the receptor tyrosine kinase (RTK) *ERBB3* (HER3) and downstream signaling events in BETi-sensitive cells lacking SMARCA4/A2. HER3 down-regulation was found to be at least partially responsible for the anti-proliferative effects of BETi in SCCOHT models. Overall, our findings suggest that BETi may represent a potential therapeutic strategy against aggressive SMARCA4/A2-deficient cancers.

3.4 Results

3.4.1 SWI/SNF compromised cancer cells demonstrate enhanced sensitivity to BETi

SMARCA4/A2 deficiency has been previously characterized in both SCCOHT and NSCLC (38,48,146). Based on median RNA-seq values from TCGA datasets, the molecular phenotype of concurrently low SMARCA4/A2 expression appears common across many cancers and may be associated with poor clinical outcomes (Fig.10A,B (38,319,323) and Supplementary Fig.S1A). We predicted that the absence of SMARCA4 may dictate cell sensitivity to BET inhibitors. This hypothesis was based on data from a siRNA screen indicating *SMARCA4*-mutant esophageal cancer models depend on BRD4 for survival (177) and other work suggesting the proliferation and survival of cancer cells may be controlled in some tumors by co-regulation of an oncogenic network by BRD4 and SMARCA4 (122). Thus, in SMARCA4-deficient cells, inhibition of

BRD4 with BETi might lead to a shutdown of these co-regulated genes, culminating in a loss of proliferation. To test this hypothesis, we first employed a panel of control cell lines expressing SMARCA4/A2 including the serous ovarian cancer lines OVCAR8, SKOV3, OVCAR4 and IGROV1, as well as the NSCLC lines H358 and H2228. As a comparison to these, we tested the SMARCA4/A2-deficient SCCOHT cell lines SCCOHT1, OVK18, BIN67 and the lung cancer cell line H522 (Fig.10B; Supplementary Fig.S1B, Supplementary Table S3), each carrying mutations with the coding region of *SMARCA4*. Two widely used BETi, JQ1 (192) and the clinically relevant OTX015 (279,280) were then tested for anti-proliferative effects against these cells. We tested the relative sensitivity and resistance to BETi using short term (5 day) cell viability assays (Fig.10C; Supplementary Fig.S1C). Here, we see that SMARCA4/A2-deficient cells show IC50 values between 50-100 nM for both BETi. Complementing these data, long term clonogenic assays using a range of drug concentrations revealed that SMARCA4/A2-deficient cells show hypersensitivity to both BETi at IC50 concentrations ≤50nM (Fig.10D; Supplementary Fig. S1D). These concentrations were substantially lower than thresholds previously established for BETi sensitivity in solid tumors (<750 nM) including ovarian cancer (285,288). BETi sensitivity was accompanied by an accumulation of Sub-G1 cells and positive staining for Annexin V, indicating BETi is inducing a pro-apoptotic, rather than a cytostatic, response (Supplementary Fig.S2A, S2B). Consistent with a cytotoxic response, cells continued to undergo apoptosis after cell washout, and cell numbers did not recover (Supplementary Fig.S2C).

In parallel with the testing of BETi efficacy, we undertook experiments to ensure the results observed with BETi could be closely recapitulated upon BRD4 knockdown. Using lentiviral-mediated delivery of two independent shRNAs targeting BRD4, we demonstrated that SCCOHT1 cells respond in a dose-dependent manner to BRD4 knockdown (Fig.10E). Consistent with our BETi data, OVCAR8 cells were quite resistant to growth inhibition upon BRD4 knockdown. We further found that BETi exposure in BRD4 knockdown cells continued to decrease viability, suggesting BET proteins beyond BRD4 are being targeted by the compounds (Supplementary Fig.S2D).

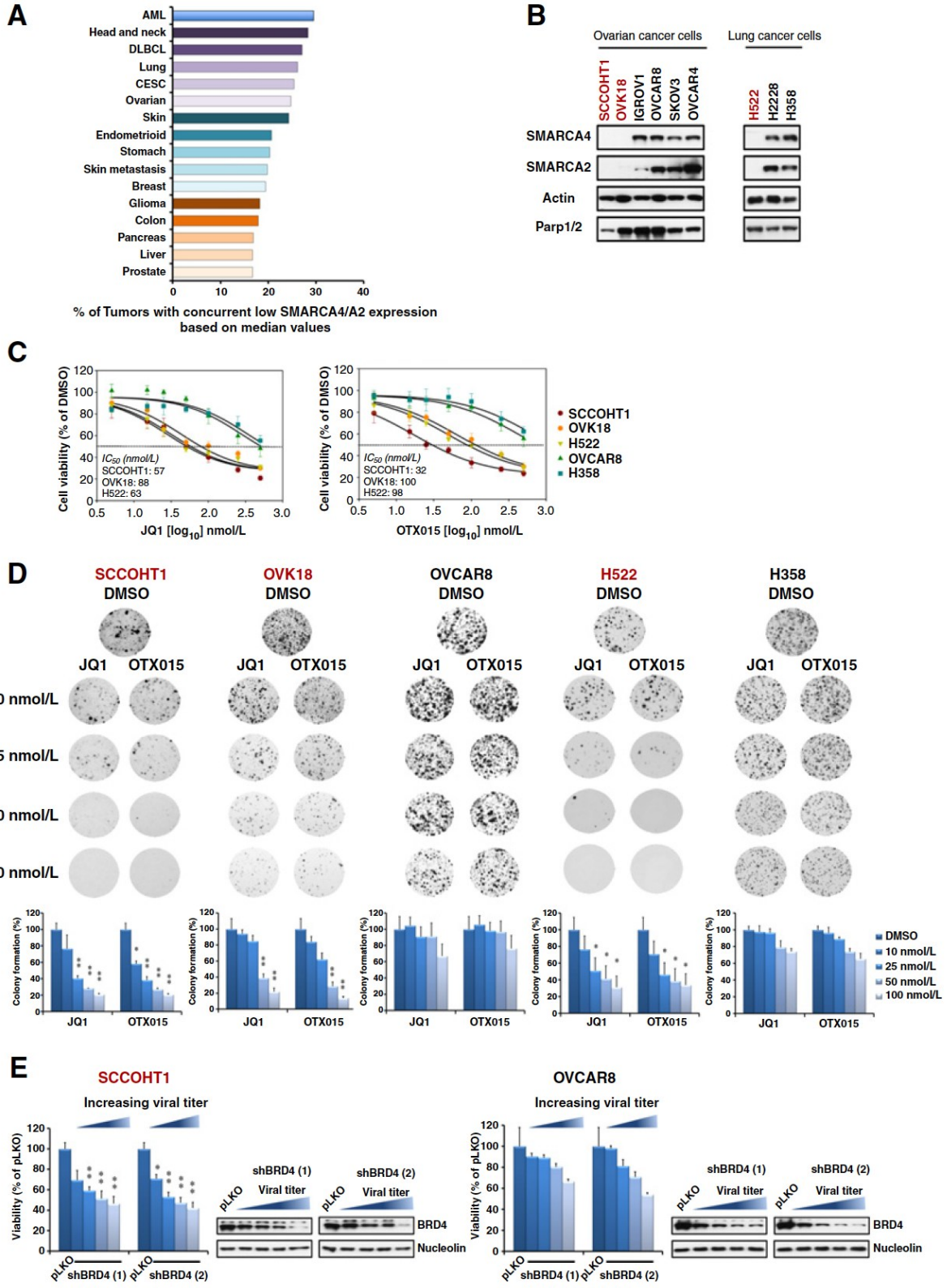

A
AML
Head and neck
DLBCL
Lung
CESC
Ovarian
Skin
Endometrioid
Stomach
Skin metastasis
Breast
Glioma
Colon
Pancreas
Liver
Prostate
0 10 20 30 40
% of Tumors with concurrent low SMARCA4/A2 expression based on median values
B
Ovarian cancer cells
Lung cancer cells
SCCOHT1
OVK18
IGROV1
OVCAR8
SKOV3
OVCAR4
H522
H2228
H358
SMARCA4
SMARCA2
Actin
Parp1/2
C
Cell viability (% of DMSO)
IC50 (nmol/L)
SCCOHT1: 57
OVK18: 88
H522: 63
JQ1 [log10] nmol/L
IC50 (nmol/L)
SCCOHT1: 32
OVK18: 100
H522: 98
OTX015 [log10] nmol/L
SCCOHT1
OVK18
H522
OVCAR8
H358
D
SCCOHT1
OVK18
OVCAR8
H522
H358
DMSO
JQ1
OTX015
10 nmol/L
25 nmol/L
50 nmol/L
100 nmol/L
Colony formation (%)
DMSO
10 nmol/L
25 nmol/L
50 nmol/L
100 nmol/L
E
SCCOHT1
OVCAR8
Increasing viral titer
Viability (% of pLKO)
pLKO
shBRD4 (1)
shBRD4 (2)
Viral titer
BRD4
Nucleolin

Figure 10. SWI/SNF compromised cell lines are highly sensitive to BET inhibitors. A, TCGA data analysis representing the percentage of cancers with concurrent low expression of SMARCA4 and SMARCA2 mRNA based on median values. **B,** Western Blotting of SMARCA4 and SMARCA2 in SCCOHT1 and OVK18, SCCOHT cell lines; IGROV1, OVCAR8, SKOV3, and OVCAR4, serous ovarian carcinoma cells; H522, H2228 and H358, NSCLC cells. **C,** Cell viability assays using ovarian and lung cancer cells exposed to 5-500nM concentrations of BETi for 5 days (n=3, error bars denote SEM). **D,** Clonogenic assays of ovarian and lung cancer cells in response to BETi. DMSO (control); JQ1, OTX015: BET inhibitors. The data is normalized to % of DMSO (n=3, error bars represent SEM, two-tailed Student t test, *P≤0.05, **P≤0.01). **E,** Cell viability assays showing survival of SCCOHT1 and OVCAR8 cells after 5 days of BRD4 knockdown (n=3, error bars: SEM, two-tailed Student t test, *P≤0.05, **P≤0.01). pLKO: control vector; shBRD4: shRNA targeting BRD4. Western blots show decreased BRD4 expression upon the knockdown using increasing viral titers in SCCOHT1 and OVCAR8.

SMARCA4-mutant SCCOHT tumors clinically and morphologically mirror atypical teratoid rhabdoid tumors (AT/RT) (38). AT/RT cancers are characterized by inactivating mutations to the SWI/SNF subunit SMARCB1. We tested two such cell lines (BT12 and CHLA266) for sensitivity to BETi. In contrast to SCCOHT cells, AT/RT lines displayed a strong intrinsic resistance to BETi exposure (Supplementary Fig.S3A), suggesting that SMARCB1 deficiency confers oncogenic properties distinct from SMARCA4 loss.

Beyond BETi, we tested our ovarian and lung cell lines panel with other epigenetic therapies, either FDA approved, or undergoing clinical evaluation. These included the DNA methylation inhibitor Decitabine (5-Aza-2-deoxycytidine), the histone deacetylase inhibitor Vorinostat, and the EZH2 inhibitor, GSK343. Over the course of our relatively short term, five-day assays, these inhibitors did not exert the robust anti-proliferative effects against SMARCA4/A2-deficient SCCOHT1 and H522 cells at concentrations of 50–100nM as is seen with BETi (Supplementary Fig.S3B). These results underscore the selectivity of SMARCA4/A2-deficient tumor cells for BETi. Amongst the three additional epigenetic therapies tested, the most potent was Vorinostat, consistent with a previous report indicating SCCOHT cells show responsiveness to HDACi *in vitro* (48).

3.4.2 OTX015 shows anti-tumor activity against an orthotopic xenograft model of SCCOHT

To extend our *in vitro* data, we next aimed to test the efficacy of BETi against an orthotopic model of SCCOHT. Considering that OTX015 has been evaluated in numerous clinical trials and demonstrates favorable bioavailability characteristics in pre-clinical studies, we employed this compound for our *in vivo* studies. Previous murine studies of SCCOHT exclusively employed subcutaneous models. Here, to more closely mimic tumor microenvironment and perhaps to better predict future, clinical drug responses, we developed orthotopic ovarian xenograft models of SCCOHT (SCCOHT1) and serous ovarian carcinoma (OVCAR8). Previous reports showing BETi efficacy against solid tumors *in vivo* generally administered 50 mg/kg/day or even higher doses (192,271). However, due to the hypersensitivity of SMARCA4/A2-deficient tumors to BETi, we reasoned that this dose could be considerably reduced.

Following tumor development at the ovary, mice were treated with vehicle control or OTX015 at doses of 20 mg/kg/day, by oral gavage, for a period of 3 weeks (Fig.11A). OTX015 was well tolerated by mice at this dose without body weight loss (Supplementary Fig.S3C). Quantification of tumor growth showed BETi to act as an effective anti-neoplastic agent against SCCOHT *in vivo*, consistent with our *in vitro* results described above. While SMARCA4/A2-deficient SCCOHT tumors were highly susceptible to OTX015-mediated growth inhibition, OVCAR8 tumors were unresponsive (Fig.11A). BETi reduced tumor weight and volume by almost 80% across all of the SCCOHT tumors. No anti-tumor response was observed at this dose in any of the tumors arising from OVCAR8 cells. Consistent with our macroscopic tumor measures, OTX015 nearly ablated the expression of the classical marker of cell proliferation Ki-67 in SCCOHT tumors (Fig.11B). In contrast, no significant changes were observed in OVCAR8 tumors, again indicating a complete lack of response at the doses suitable for treating SCCOHT tumors.

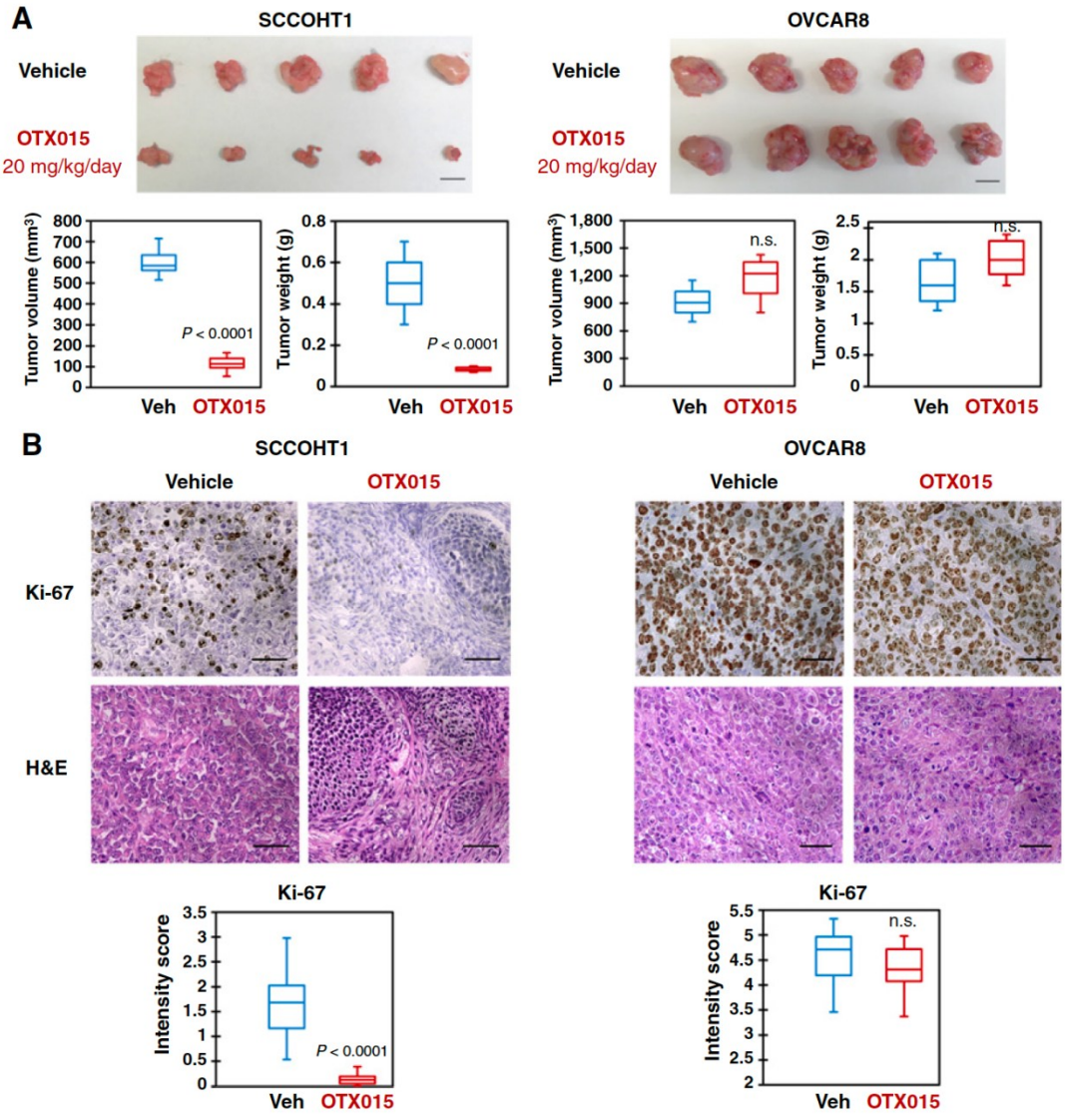


Figure 11. OTX015 ablates growth of SWI/SNF compromised, SCCOHT orthotopic tumor xenografts. A, Top, Photos documenting tumors after 3 weeks of treatment with 20 mg/kg/day of OTX015 (scale bars, 1cm). Veh: vehicle (control); OTX015: BET inhibitor. Bottom, box plots showing tumor volume and tumor weight quantification in response to the OTX015 treatment (vehicle group, n=5; OTX015 group, n=5, two-tailed Student t test, n.s.: not significant). **B,** Top immunohistochemistry analysis of SCCOHT1 and OVCAR8 tumors for proliferation marker, Ki-67 upon 3 weeks of treatment with 20 mg/kg/day of OTX015 (scale bars, 50µM). HE: hematoxylin eosin staining. Bottom, box plots representing intensity score of Ki-67 expression in SCCOHT1 and OVCAR8 tumors after the application of OTX015. Ranking system: 0=no staining, 1=very weak staining, 2=weak staining, 3=intermediate staining, 4=strong staining, 5=very strong staining (vehicle group, n=3; OTX015 group, n=3, two-tailed Student t test, n.s.: not significant).

3.4.3 SMARCA4 and SMARCA2 expression determines responses to BET inhibitors

At this point, our data supported the hypothesis that SMARCA4/A2-deficient cells rely on BET family members for survival, and that the loss of SMARCA4 sensitizes cells to BRD4 inhibition. Therefore, we next further interrogated this hypothesis using complimentary approaches of SMARCA4 re-expression and SMARCA4 knockdown. If our model is correct, then ectopic expression of SMARCA4 in deficient cells should impart BETi resistance. Conversely, knockdown of SMARCA4 in resistant cells, might lead to inhibitor sensitization. First, we restored wild-type SMARCA4 via a doxycycline-inducible lentiviral vector system in ovarian SCCOHT1 cells and the lung cancer cell line H522 (Fig.12A). SMARCA4 restoration rendered both cell lines significantly more resistant to both JQ1 and OTX015 across a range of concentrations.

Complimentary experiments using SMARCA4 knockdown also supported the concept that this protein is partially responsible for intrinsic cell resistance to BETi (Fig.12B). Here, SMARCA4 knockdown was achieved using a lentiviral system to deliver two independent shRNAs targeting SMARCA4 to the BETi resistant cell line, OVCAR8. Subsequent viability analysis found that SMARCA4 depletion altered the response of OVCAR8 to JQ1 and OTX015, allowing a dose-dependent reduction of cell survival. These data have several implications. First, there is clear indication that SMARCA4 expression confers resistance to BETi. Second, our work shows that re-expression of SMARCA4 partially prohibits the anti-proliferative effects of BRD4 inhibition. This data supports previous work characterizing coordinated gene expression by BRD4 and SMARCA4 to promote proliferation and survival (122,177). The data further suggests that small molecule inhibitors of SMARCA4, if developed, might act in synergy with BETi.

The SWI/SNF chromatin remodeling complex may integrate SMARCA4 or SMARCA2 in a mutually exclusive manner. However, as mentioned above, evidence demonstrates that these remodelers are capable of compensating for the loss of the other ATPase in a context-dependent manner, indicating a degree of functional redundancy (63). Thus, it is not surprising that a dual repression of SMARCA4/A2 is commonly observed in a number of cancers (Fig.10A and

(48,323,324)). We noted that the cells showing acute sensitivity to BETi have concurrent SMARCA4/A2 loss (Fig. 10B, 12A).

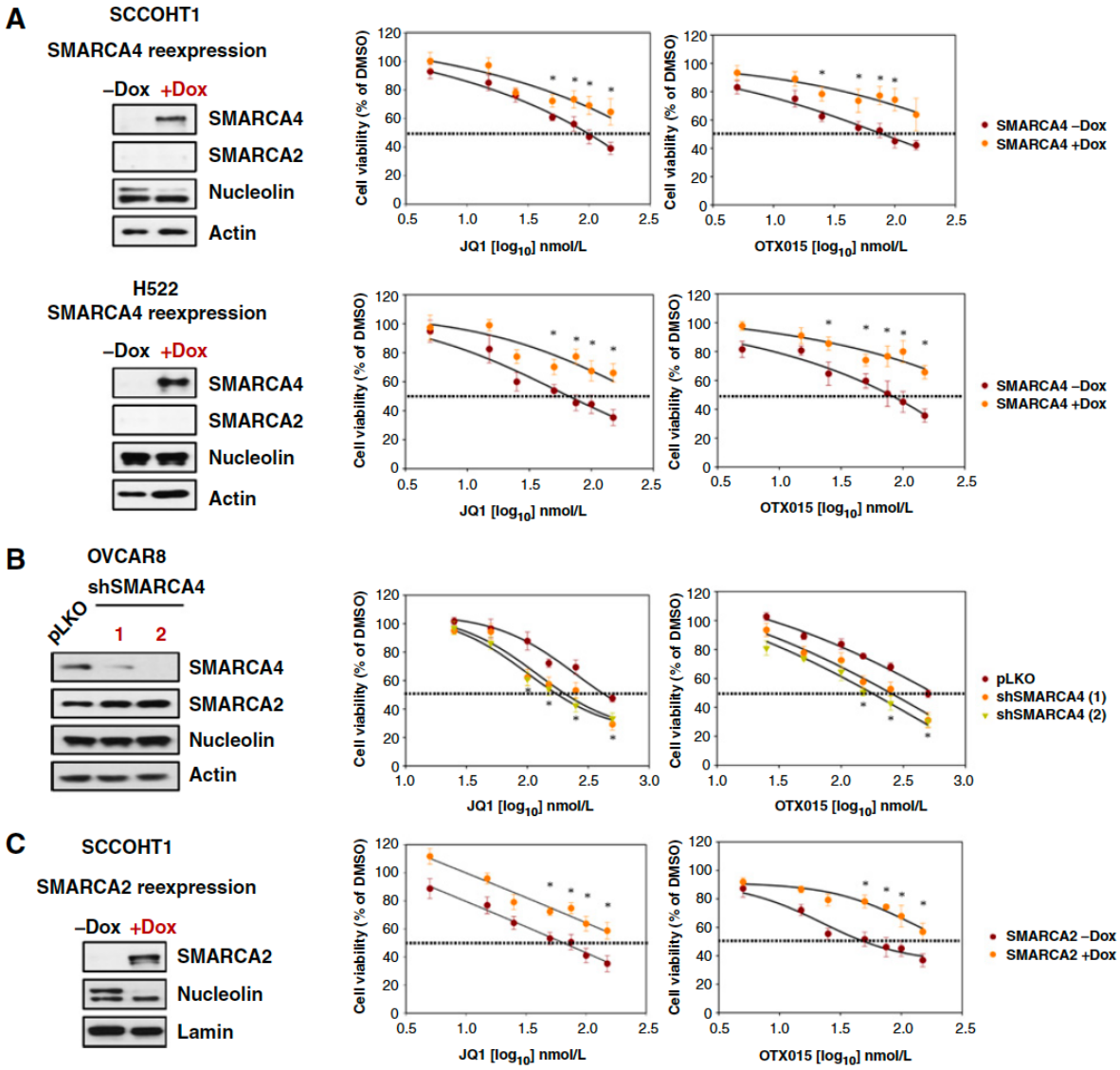


Figure 12. SMARCA4 and SMARCA2 restoration mediates resistance to BETi and SMARCA4 depletion sensitizes to BETi. A, Left, Western blotting analysis of SMARCA4 and SMARCA2 protein levels after inducible SMARCA4 re-expression in ovarian and lung cancer cells. Right, Cell viability assay in response to 5 days of treatment with BETi and inducible SMARCA4 ectopic expression (n=3, error bars: SEM, *P≤0.05). SCCOHT1: SCCOHT cell line; H522: lung carcinoma (NSCLC) cells; DMSO: control; JQ1, OTX015: BETi; Dox: doxycycline for SMARCA4 induction. **B**, Left, SMARCA4 and SMARCA2 protein expression after the SMARCA4 knockdown in ovarian carcinoma cells, OVCAR8. Right, Cell viability assay upon 5 days of SMARCA4 knockdown with two shRNAs and exposure to BETi (n=3, error bars: SEM, *P≤0.05). pLKO: control vector; shSMARCA4: shRNA against SMARCA4. **C,** Left, Western blot for SMARCA2 in SCCOHT1 upon inducible ectopic expression of SMARCA2. Dox:

doxycycline for SMARCA2 induction. Right, Cell viability graphs showing the response of SMARCA2 restored SCCOHT1 cells to 5 days of treatment with BETi (n=3, error bars: SEM, *P≤0.05).

Therefore, we wanted to examine whether SMARCA2 re-expression might mediate resistance to BETi in a similar fashion as SMARCA4. Using the same inducible approach as described above for SMARCA4 restoration, we transduced SCCOHT1 with SMARCA2 and tested for the sensitivity to BETi (Fig.12C). SMARCA2 restoration rendered cells considerably less responsive to the two bromodomain inhibitors in comparison to the parental controls. Our findings indicate that SWI/SNF activity modulates the response to BETi and that concurrent low expression of both SMARCA4 and SMARCA2 may act as a predictive biomarker for tumor sensitivity to BETi.

3.4.4 OTX015 suppresses HER3 expression and oncogenic signaling in SMARCA4/A2 deficient cells

BET family members are key activators of genes controlling proliferation and survival. Therefore, using RNA-seq we aimed to identify potential BRD4 target genes whose repression mediates the anti-proliferative effect of BETi in SMARCA4/A2-deficient cells. Until now, transcriptomic analysis of BETi targets in cells derived from solid tumors have primarily utilized drug doses ≥ 500nM or even micromolar ranges (277,282). Due to the exquisite sensitivity of SMARCA4/A2-compromised cells to BETi, we used treatments of 100nM to distinguish gene expression profiles between SCCOHT1 and BETi-insensitive OVCAR8 cells. Such exposures for 4 and 24 hours revealed a distinct transcriptional profile imposed by BETi in SCCOHT1 cells that was absent in OVCAR8, highlighting the differential response of these cell lines to 100nM OTX015 (Fig. 13A,B, Supplementary Fig.S4, GEO accession #GSE102908). Analysis of the most strongly 150 downregulated genes from our RNAseq data (>2-fold decrease) using Kegg pathway analysis revealed a network of genes potentially regulating signal transduction through the PI3K and RAS pathways (Kegg pathway, PI3K-AKT, $p<0.005$, MAPK, $p<0.006$, GSEA Receptor Tyrosine Kinase gene set (14 of 18 genes within this custom set are found within the Gene Ontology_Phosphorylation set (biological processes), Supplementary Fig.S4A, S4B). Notably, the oncogenic receptor tyrosine kinase (RTK), *ERBB3* (HER3 protein) was strongly suppressed by OTX015 in SCCOHT1 cells (Fig.13A, 13B, $p \leq 0.05$). HER3 is a key initiator of signal transduction through the PI3K and RAS signaling cascades (325). Thus, suppression of

ERBB3 levels represented a rational candidate for a BRD4 target gene whose repression might be responsible for mediating some of the anti-proliferative effects of BETi.

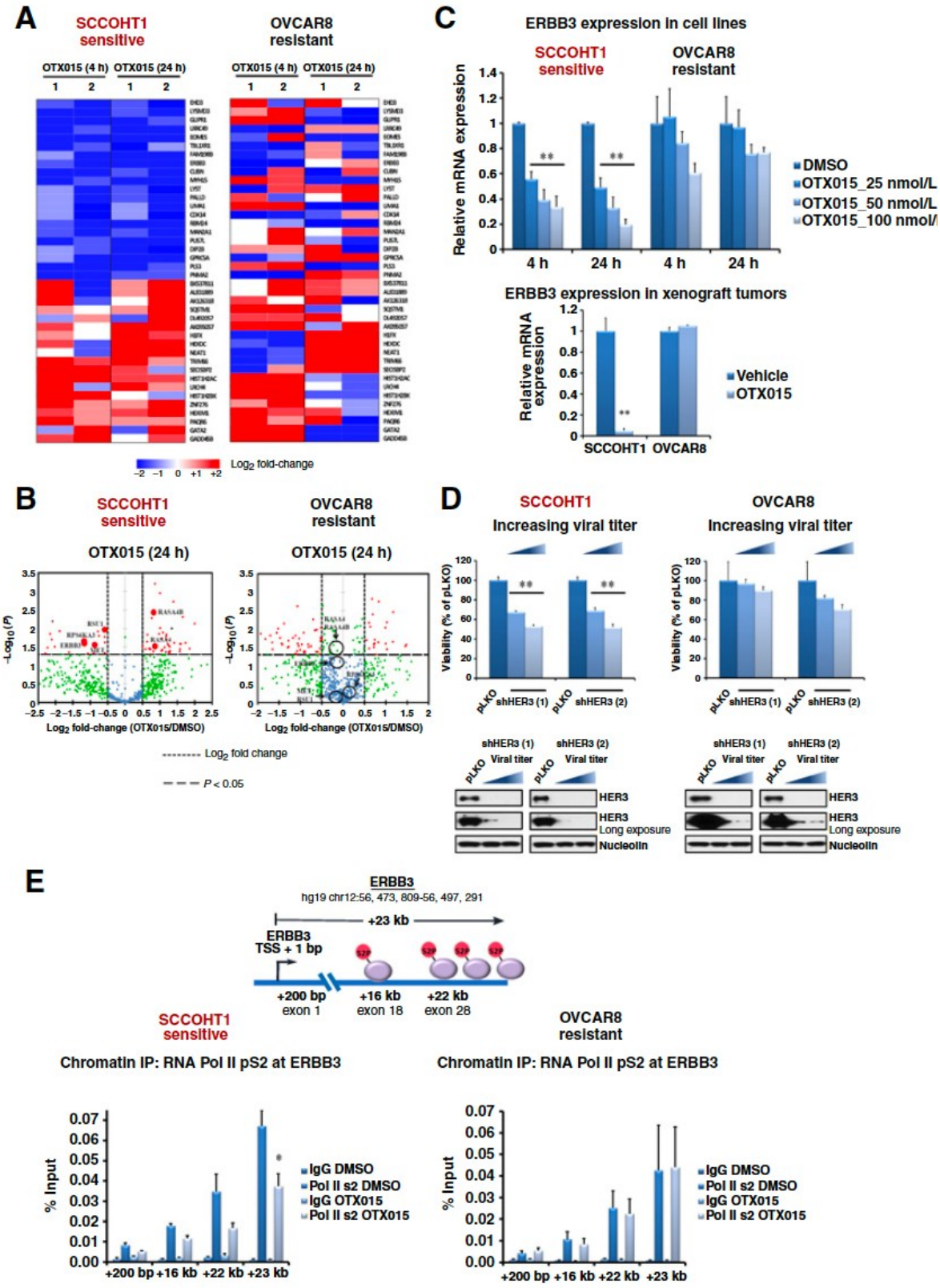

A
SCCOHT1 sensitive
OVCAR8 resistant
OTX015 (4 h)
OTX015 (24 h)
Log2 fold-change
B
SCCOHT1 sensitive
OVCAR8 resistant
OTX015 (24 h)
Log2 fold-change (OTX015/DMSO)
Log2 fold change
P < 0.05
C
ERBB3 expression in cell lines
SCCOHT1 sensitive
OVCAR8 resistant
Relative mRNA expression
DMSO
OTX015_25 nmol/L
OTX015_50 nmol/L
OTX015_100 nmol/
4 h
24 h
ERBB3 expression in xenograft tumors
Vehicle
OTX015
SCCOHT1
OVCAR8
D
SCCOHT1
OVCAR8
Increasing viral titer
Viability (% of pLKO)
pLKO
shHER3 (1)
shHER3 (2)
Viral titer
HER3
HER3 Long exposure
Nucleolin
E
ERBB3
hg19 chr12:56, 473, 809-56, 497, 291
+23 kb
ERBB3 TSS + 1 bp
+200 bp exon 1
+16 kb exon 18
+22 kb exon 28
SCCOHT1 sensitive
OVCAR8 resistant
Chromatin IP: RNA Pol II pS2 at ERBB3
% Input
IgG DMSO
Pol II s2 DMSO
IgG OTX015
Pol II s2 OTX015
+200 bp
+16 kb
+22 kb
+23 kb

Figure 13. OTX015 transcriptionally represses the *ERBB3* gene. A, RNA-seq analysis of mRNA samples from SCCOHT1 and OVCAR8 cells exposed to 100nM of OTX015 at 4 and 24 hours. Heat maps demonstrate distinct gene expression pattern of sensitive and resistant cells in response to OTX015 compared to DMSO controls (log_2 fold change >1, P ≤0.05). **B,** Volcano plots demonstrating transcriptional response of genes involved in the RTK pathway in SCCOHT1 and OVCAR8 upon 24h of treatment with OTX015 (log_2 fold change >0.5, P≤0.05). **C,** Top, Quantitative PCR analysis of *ERBB3* in SCCOHT1 and OVCAR8 cells treated with a range of OTX015 concentrations at 4 and 24h. The relative mRNA expression was normalized to 36B4 and DMSO control (n=3, error bars: SEM, two tailed Student t test, **P≤0.01). Bottom, mRNA expression of *ERBB3* in SCCOHT1 and OVCAR8 tumor tissues in response to 3 weeks of treatment with vehicle (control) or 20mg/kg/day of OTX015 (n=2, error bars: SEM, two tailed Student t test, **P≤0.01). **D,** Top, 4 days cell viability assays in response to HER3 knockdown using shRNAs, infected into SCCOHT1 and OVCAR8 cells (n=3, error bars: SEM, two tailed Student t test, **P≤0.01). Bottom, Western blotting with an anti-HER3 antibody confirmed protein depletion. **E,** OTX015 represses active transcription of the *ERBB3* gene in sensitive cells. Top, Map of the *ERBB3* gene from +1 to +23 kb, with positions of qPCR amplicons used to examine the association of RNA Pol II phosphorylated on serine-2 (RNA Pol II pS2) at each position by ChIP-qPCR. Bottom, ChIP-qPCR data of RNA Pol II pS2 at *ERBB3* in SCCOHT1 and OVCAR8 treated with 0.01% DMSO or 100nM of OTX015 for 24 hours. The qPCR data is represented as % of input (n=3, Student two-tailed test, *P≤0.05).

Therefore, we further explored the regulation of *ERBB3* by BETi. We validated our RNA-seq data by qPCR (Fig.13C, upper panel) and observed a significant down-regulation of *ERBB3* in SCCOHT1 cells exposed to BETi in a both time and dose-dependent manner. In contrast, *ERBB3* mRNA levels in OVCAR8 were largely invariant across the range of conditions tested, and the changes that could be observed at 4 hours were quite transient. Similar results were observed in the H522, BETi sensitive lung cancer cells, compared to the resistant line H358 (Supplementary Fig.S4C). *ERBB3* was considerably down-regulated in SCCOHT1 tumors exposed to OTX015, but this regulation was not observed in OVCAR8 tumors (Fig.13C, lower panel). These data suggest that *ERBB3* repression may mediate a portion of the anti-proliferative effects of BETi in acutely sensitive cells. Consistent with this, knockdown of HER3 led to a significant decrease in the proliferation of SCCOHT1 cells, but a far more muted response was seen in SMARCA4/A2-expressing OVCAR8 cells (Fig.13D).

The strong repression of *ERBB3* by BETi indicates the gene is a direct target of BET family members. BRD4 activates gene expression through facilitating the recruitment of an elongation complex that phosphorylates RNA POL II on serine 2 of its C-terminal domain. Consistent with *ERBB3* being a direct transcriptional target of BRD4, we detect multiple BRD4 binding sites are

observed flanking the *ERBB3* gene (Supplementary Fig.S4D). Further, using chromatin immunoprecipitation (ChIP) with an anti-phospho-serine2-POL II antibody, we see that OTX015 mitigates the serine 2 phosphorylation of RNA POL II at *ERBB3* in SCCOHT1 cells (Fig.13E). Exposure to 100nM OTX015 effectively reduced the enrichment of phospho-serine2-POL II throughout the gene body of *ERBB3* from +200bp relative to the transcription start site, through +23kb, near the 3' terminus. Again, this data supports the conclusion that the reduction of *ERBB3* mRNA seen after BETi exposure results from diminished transcriptional activity. Our ChIP data further revealed that BETi did not reduce the enrichment of RNA POL II serine 2 phosphorylation at *ERBB3* in BETi-resistant OVCAR8 cells. Overall, the data suggests that a compensatory mechanism, possibly dependent on SMARCA4, maintains *ERBB3* expression in OVCAR8 cells after inactivation of BRD4. It also suggests that the sensitivity of SCCOHT1 cells to BETi is reflective of the capacity of low doses of BETi to repress the transcription of proliferation-related genes, including *ERBB3*.

Further validating our transcriptional data, the reduction of HER3 (encoded by the *ERBB3* gene) on the protein level was also confirmed by Western blotting in BETi sensitive SCCOHT1, OVK18 and H522 cells (Fig.14, Supplementary Fig.S4C). HER3 protein was diminished at concentrations of only 25-50nM in sensitive cells, but such a decrease was not observed in any of the resistant cell lines. The HER3 protein is a critical mediator of survival and proliferation in a range of cancers including ovarian. These pro-tumorigenic activities are carried out by signal transduction through both the PI3K/AKT and RAS/MAPK signaling axis (325). To explore whether these classical effector pathways are repressed by BETi we exposed a panel of sensitive and resistant ovarian cell lines to OTX015 and evaluated phospho-S6 and phospho-AKT from the PI3K pathway, and phospho-ERK1/2 from the RAS/MAPK pathway. In SCCOHT1 cells we detected a significant decrease of phospho-AKT and phospho-S6 in response to OTX015 that was not observed in the three resistant ovarian cell lines tested (OVCAR8, SKOV3, IGROV1) (Fig.14). Likewise, we also observed a dose dependent reduction in phospho-ERK1/2 in sensitive cells that was not apparent in resistant cells. In BETi-sensitive OVK18 cells we also observed a reduction in these phosphorylation events. However, the reduction in phospho-ERK1/2 was considerably more pronounced, indicating repression of MAPK signaling may be critical for the anti-proliferative effects of BETi in these cells.

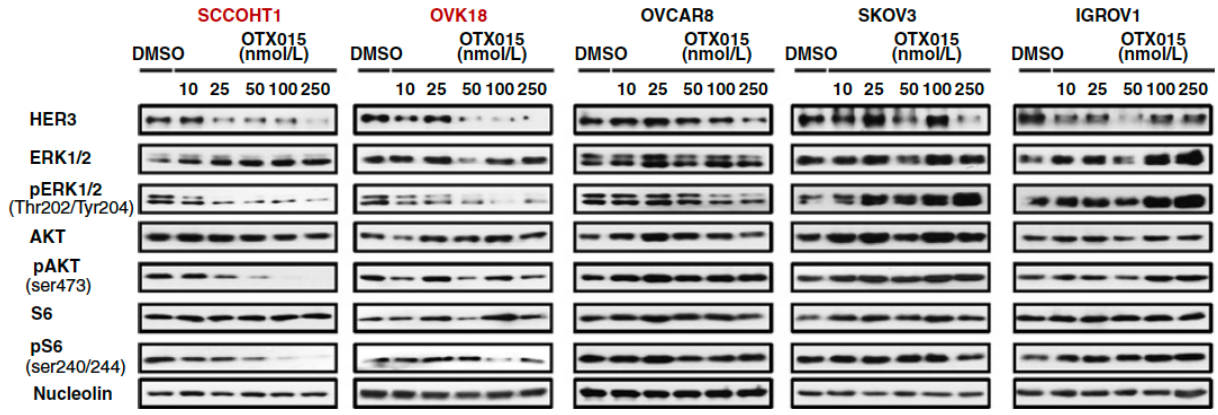


Figure 14. BETi dampens signaling downstream of receptor tyrosine kinases. Western blotting of HER3 and downstream effectors in response to increasing doses of OTX015 at 24h. SCCOHT1, OVK18: SCCOHT cells; OVCAR8, SKOV3, and IGROV1, serous ovarian carcinoma cells.

A majority of the reports investigating the role of BRD4 in cancer progression have focused on c-MYC as a principal target mediating these effects. However, we saw little effect on c-MYC mRNA levels over a 24 hours period after BETi exposure in SCCOHT1 cells (Supplementary Fig.S5A, GEO accession #GSE102908). Our data suggests that BET family members may also regulate PI3K and MAPK signaling, in part, through upregulation of the HER3 oncogene. Conversely, the loss of BET proteins, such as BRD4, would be expected to dampen signaling through the HER3 axis. To validate that the repression of HER3 and downstream signaling cascades by BETi is directly dependent on BRD4, we next knocked down BRD4 via shRNA and again tested for HER3 expression and RTK-dependent signaling events. Consistent with our BETi data, depletion of BRD4 lowered HER3 protein levels and repressed signaling through both the PI3K and MAPK pathways in ovarian SCCOHT cells (Supplementary Fig.S5B).

Collectively, our data support a model where cells lacking SMARCA4/A2 are highly sensitive to BETi, in part, through inactivation of HER3 RTK signaling. If this model has merit, we expect that forced expression of HER3 would confer resistance to BETi. To test this concept, we generated stable expression of the *ERBB3* gene in SCCOHT1 and OVK18 SMARCA4/A2 compromised cells, leading to elevated HER3 protein levels (Fig.15). The cells were subsequently exposed to either JQ1 or OTX015 at increasing doses and changes to cell viability quantified.

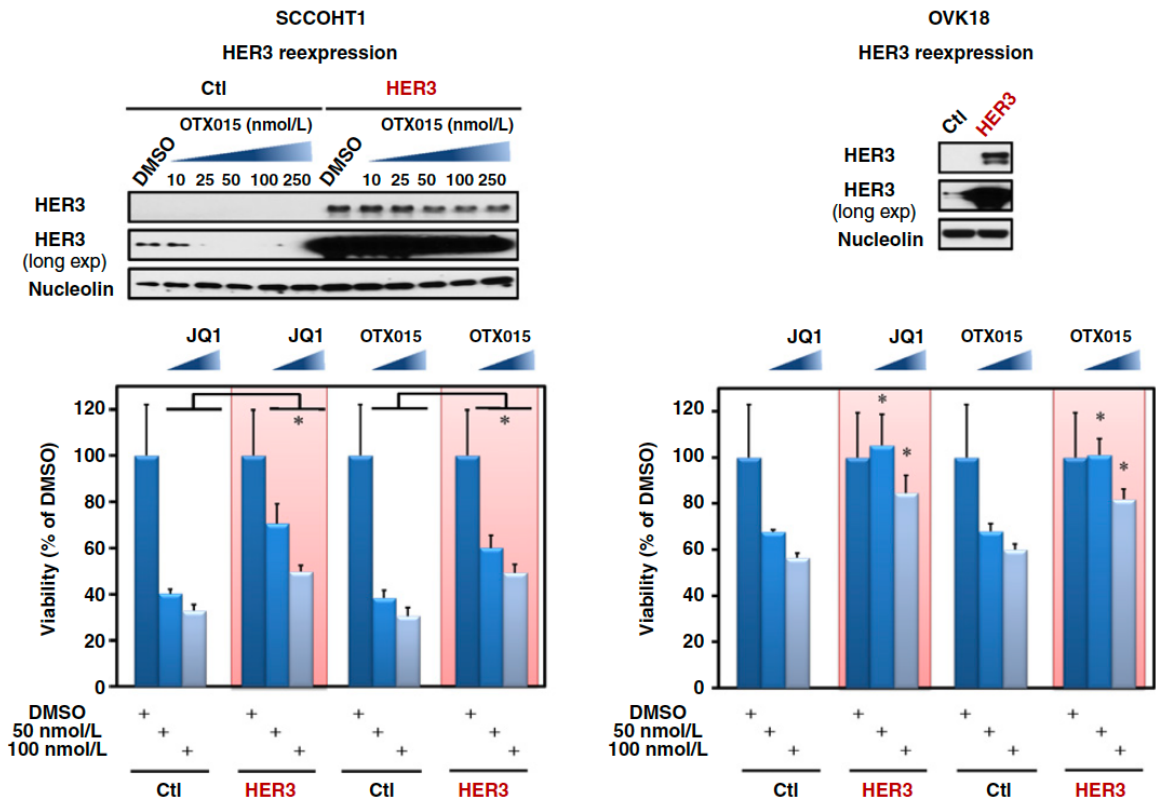


Figure 15. HER3 re-expression confers partial resistance to BET inhibitors. Top left, Western blot analysis of HER3 in SCCOHT1 cells after re-expression and dose-dependent treatment with OTX015 at 24h. Top right, Protein expression of HER3 upon ectopic expression in OVK18. Bottom, Cell viability assay of SCCOHT1 and OVK18 cells with ectopic expression of HER3 and exposed to BETi (JQ1, OTX015) for 5 days (n=3, error bars: SEM, Student two-tailed test, *P≤0.05). Ctl: control vector. HER3: HER3 (ERBB3) vector.

We found that maintaining the pool of HER3 through ectopic expression, imparted the cells with partial, but significant, BETi resistance in both cell lines. Thus, our experiments reveal that HER3 is a key target of BETi whose downregulation significantly contributes to the hypersensitivity to BETi in SMARCA4/A2 deficient cancer cells.

3.5 Discussion

All cancers, even those with comparatively low mutational burdens, such as SCCOHT, show deregulated transcription. Thus, targeting transcriptional processes, particularly through 'epigenetic therapy' offers an attractive approach to treat many cancers. Indeed, two such drugs, Decitabine and Vorinostat are approved by the FDA. While the panel of drugs targeting

transcriptional and epigenetic processes has grown substantially in the past decade, and many clinical trials are ongoing, biomarkers predicting the efficacy of such drugs are sorely lacking. Included among the list of small molecules aimed at "drugging the genome" being tested in clinical trials, but without clear predictive biomarkers are the BET inhibitors.

Exciting preclinical studies demonstrating potent anticancer activity of BET inhibitors have prompted the rapid clinical development of this class of compounds. So far, Phase I trials using BETi against myeloma, acute leukemia and NMC have not met expectations, but complete responses have been reported (279,280). These clinical trials have been unable to identify biomarkers predicting patient responses. Further studies of a longer duration may be required to determine whether c-MYC holds the same strength as a biomarker of BETi efficacy in a clinical setting as it does in some preclinical investigations. Other preclinical studies demonstrate that not all cells responding to BETi are reliant on repression of c-MYC (326,327) suggesting that BETi may regulate other key targets depended on the cancer type. Our findings reveal for the first time that SCCOHT and NSCLC cells with compromised SMARCA4/A2 show hypersensitivity to BETi. Importantly, add-back of SMARCA4 or SMARCA2 leads to acquired resistance and SMARCA4 knockdown sensitized cells to BETi demonstrating the specificity of SMARCA4 as a predictive biomarker. Our *in vivo* work validated the exquisite sensitivity of SCCOHT cells that we observed *in vitro*. Again, using SCCOHT as a relevant *in vivo* model, we found that only 20 mg/kg of OTX015 delivered orally 5 days per week was sufficient to ablate tumor growth. To our knowledge, this is the lowest dose of any BETi that has shown efficacy against solid tumors *in vivo*. These data potentially have important clinical implications, but with several caveats.

While our study indicates SMARCA4/A2-deficient cancers may be highly sensitive to BETi and warrants further clinical investigation, our data also reveal potential resistance mechanisms. First, we show that SMARCA2 may compensate for the loss of SMARCA4, as previously described. Based on this, we predict that BETi will show the highest degree of antitumor activity in a setting where both SMARCA4 and SMARCA2 are concurrently compromised by either mutation or loss of expression. In fact, SCCOHT patients are invariably characterized by concurrent loss of SMARCA4/A2 (48). Further, in NSCLC and prostate cancer, concurrent low expression of these proteins has also been reported (323,324), as well as in numerous other

cancers based on TCGA (Fig.10A). Considering that SMARCA4/A2 compromised cancers are associated with poor clinical outcomes (Supplementary Fig.S1A), BETi may represent a new answer for an unmet clinical need.

The ERBB3 RTK acts as a potent oncogene mediating tumor growth and drug resistance whose expression is elevated in several types of cancer (325). We see that BETi represses *ERBB3* mRNA production in a dose and time dependent fashion in SMARCA4/A2-deficient cells. This repression is concomitant with reduced signal transduction through the PI3K and MAPK pathways. These data are consistent with previous reports indicating BETi dampens signal transduction through these pathways (284,285,288). Mechanistically, our add-back studies confirm that HER3 repression is a key effector of BETi, and whose repression mediates at least a part, of their anti-proliferative response. Therefore, our data suggests that the repression of oncogenic signaling observed in BETi-exposed cells is at least partially dependent on HER3 downregulation. Further, our data predicts that aberrant activation of PI3K or MAPK signaling may promote resistance to the anti-tumor effects of BETi. This concept is indeed supported by previous findings. Knockdown of the tumor suppressor LKB1, leading to elevated PI3K signaling, confers resistance to JQ1 (271). Likewise, kinome analysis of ovarian cells selected for acquired resistance to chronic BETi exposure, revealed increased signaling through the PI3K/RAS pathways (288). However, further work where constitutively active AKT or MAPK mutants are introduced into SMARCA4/A2-deficient cells will be required to solidify the hypothesis.

While our data, and that of others, suggests that aberrant activation of PI3K/RAS signaling may impose resistance to BETi, this model also indicates that BETi in combination with HER3/PI3K/MAPK inhibitors represent a rational approach to overcome this resistance. In fact, multiple studies have shown a synergy between inhibitors of PI3K, and more recently MAPK, and BETi against a variety of cancers, but key questions remain (284,287,313). Currently, defined molecular pathologies predicting sensitivity to these combination therapies are lacking. Our data suggests SMARCA4/A2 deficient tumors with additional oncogenic hits activating the MAPK pathway may be especially sensitive to such combinatorial therapeutic intervention. Further, it is possible that combination therapy with BETi and drugs targeting the MAPK

pathway might represent a more effective therapeutic avenue than BETi with PI3K inhibitors. Our work also indicates that therapeutic antibodies targeting HER3 may hold promise as combination therapy with BETi. Systematic comparison of such combinations will be of great interest, and necessary to optimize the clinical efficacy of BETi.

Based on the work described herein and current literature, it is clear that repression of the PI3K and RAS signaling pathways contribute to the anti-proliferative effects of BETi. Undoubtedly, *ERBB3* is not the only gene responsible for facilitating the anti-proliferative effects of BETi, and we predict further targets of BET family members are involved in modulating the activity of the PI3K and RAS signaling pathways. Our RNA-seq data indicates other potential targets include *KRAS, BRAF,* and upregulation of MAPK repressors such as RASA4 (Supplementary Fig.S4A). Future work will be required to assess whether modulation of these targets also influences the anti-proliferative effects of BETi and whether BRD4 and SMARCA4 cooperate to control their transcription.

3.6 Materials and methods

3.6.1 Cell culture and chemicals

SCCOHT1 cell line was provided by Dr. Ralf Hass. OVCAR8, SKOV3, NCI-H522, NCI-H2228, HEK293T were purchased from the American Type Culture Collection (ATCC). OVCAR4 and IGROV-1 cells were obtained from Dr. Edwin Wang and NCI-H358 was a gift from Dr. Moulay A. Alaoui-Jamali, originally purchased from ATCC. The OVK18 cell line was obtained from the RIKEN Cell Bank. BIN67 cell line was provided by Dr. Barbara Vanderhyden. Ovarian and lung cancer cells were cultured in RPMI-1640 1x medium supplemented with 10% FBS. HEK293T cells were cultured in DMEM/10% FBS. BT12 and CHLA266 cells were provided by the Children's Oncology Group Cell Culture and Xenograft Repository (Texas Tech University Health Sciences Center) and cultured in Iscove's medium supplemented with 10% FBS, 4mM L-Glutamine, 1X ITS (5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenous acid). The cell The lines were maintained in culture for no more than 10 passages from initial stock (prepared from early passages). All cells were tested for Mycoplasma contamination by DAPI staining monthly and not otherwise authenticated.

Compounds used in the studies included OTX015 (MedChem Express, #HY-15743), JQ1 (MedChem Express, #HY-13030), Decitabine (Selleckchem, #S1200), GSK343 (Selleckchem #S7164), Vorinostat (Cayman Chemical, #10009929) and Cisplatin (Accord, #DIN 02355183).

3.6.2 Plasmids

For cDNA expression of ERBB3 (HER3), ERBB3-pReciever-LV120 (GeneCopeia, #EX-M0854-Lv120) was employed, along with the control vector pReciever-LV120 (GeneCopeia, #EX-EGFP-Lv120). Inducible SMARCA4 expression was achieved using the vector pInducer-20 carry full length SMARCA4 cDNA, kindly provided by Dr. Jannik N. Andersen (The University of Texas MD Anderson Cancer Center). Similarly, a SMARCA2 inducible plasmid was obtained by subcloning full length SMARCA2 cDNA into pInducer-20 lentiviral vector (Addgene, #44012). shRNAs directed against BRD4, HER3 and SMARCA4 are held in vector TRC1.5/PLKO.1 and were obtained from Sigma (Supplementary Table S1).

3.6.3 Lentiviral production and cells transduction

Lentiviral particles were packaged in HEK293T cells as described previously (328). For viral transduction, $0.5x10^6$/mL of SCCOHT1, OVK18 or H522 cells were seeded in 10cm petri dishes. To achieve stable ERBB3 (HER3), or inducible, ectopic expression of SMARCA4, or SMARCA2, cells were incubated with the viral suspension at ratios of 1 ml of virus for each 4 ml of cell media, with a final concentration of hexadimethrine bromide (Polybrene) at 5ug/mL. After 24 hours, the media was changed and stable selection carried out using appropriate selective antibiotic. ERBB3 (HER3) stable cell lines were obtained after selection with 0.5ug/mL of puromycin, while 200ug/mL of neomycin was used to generate SMARCA4 or SMARCA2 inducible cells. For BRD4 or ERBB3 (HER3) knockdown experiments, SCCOHT1 and OVCAR8 cells were plated in 6-well plates and transduced with lentiviral particles at increasing dilution ratios (1:50, 1:20, 1:10, 1:6). Expressing cells were elected for using puromycin. For SMARCA4 knockdown, OVCAR8 cells were incubated with shSMARCA4-containing lentivirus followed by selection with puromycin.

3.6.4 Cell viability and clonogenic assays

Ovarian or lung cancer cells were seeded in 24-well plates at 10^4-10^5 cells/mL and treated for a period of 5 days with the indicated concentrations of 5 compounds (JQ1, OTX015, GSK343, Decitabine and Vorinostat). 0.01% DMSO was used as a control. Culture media was changed each day. For washout experiments, SCCOHT1 and OVCAR8 cells were plated and treated with DMSO, Cisplatin or OTX015 for 5 days. At day 5, the drugs were washed out and the cells were grown for additional 3 days (day 8 time point). For knockdown of HER3 or BRD4, cells were infected at increasing viral titer and analyzed at day 4 or 5 respectively, after transduction. For SMARCA4 or BRD4 knockdown, cells were exposed to viral particles at a ratio of 1ml virus per 7 ml culture media, followed by selection using 0.5ug/mL of puromycin. Next, treatments with BETi were conducted for 5 days. For ectopic expression of HER3, cells stably expressing ERBB3 were plated and treated with the indicated concentrations of BETi. To assess the BETi resistance of SCCOHT1 or H522 cells expressing SMARCA4 or SMARCA2, cells were first seeded in 24-well plates followed by treatment for 1 day with 1ug/mL of doxycycline prior to the BETi exposure. For quantification of cell viability curves, crystal violet staining was measured as previously described (329).

For clonogenic assays, either 400-500 cells/mL (for fast-proliferating) or 2500 cells/mL (for slow-proliferating) cells were seeded in each well of 6-well plates. The next day, treatments with BETi were initiated at 10nM-100nM every 3-4 days until the cells reached 70-80% confluency. The plates were fixed with 4% formaldehyde/1xPBS and stained with 0.1% crystal violet/10% ethanol. Quantification step was performed by application of 10% acetic acid followed by absorbance measurements. Colony formation value was normalized to DMSO.

3.6.5 Annexin V staining

$0.5x10^6$ /mL cells were seeded in 6-well plates. Apoptosis analysis was performed with FITC Annexin-V Apoptosis Detection kit I (BD Biosciences, cat #556547) and cell cycle analysis was assessed via propidium iodide staining as described previously (328). The samples were analyzed by flow cytometry at day 3 post-incubation with DMSO (0.01%), Cisplatin (5uM) or OTX015 (50nM) and day 5 (washout of compounds at day 3).

3.6.6 Quantitative PCR

Total RNA was extracted using Gene Elute Mammalian Total RNA kit (Sigma, #RTN350) according manufactured procedure. 1ug of RNA was subjected to reverse transcription carried out with 5X All-In-One RT MasterMix (ABM, #G490) following commercial instructions. The resultant cDNA was subsequently analyzed by qPCR. GoTaq® qPCR Master Mix (Promega, #A6002) was used for qPCR reactions according to manufacturer protocol. PCR product was amplified with specific primers at 250nM per reaction (Supplementary Table S2). mRNA levels were normalized to human 36B4 expression.

3.6.7 RNA sequencing and Bioinformatics

Cells were exposed to 0.01% DMSO or 100nM OTX015 for 4 and 24 hours. After treatments, RNA extraction was performed as described above and 1ug of RNA by replicate was sent to the "Genome Quebec Innovation Center" for RNA sequencing, with each condition being sequenced in duplicate. mRNA stranded library preparation was carried out using Illumina TruSeq rRNA-depleted stranded library preparation followed by paired-end 100bp sequencing with a HiSeq 2500. Using Trimmomatic v0.32, low quality bases (phred33 < 30) and adapters were removed, the first three bases were clipped and only reads with a minimum length of 35 bases, and in pairs, were kept for the next step. Clean paired sequences were aligned using STAR v2.3.0e then the number of reads per gene using UCSC hg19 annotation and featureCount was obtained and used for differential expression analysis with DESeq2. The selection of differentially expressed genes was based on fold change >0.5 and FDR <0.1. The data is available on the GEO database (GEO accession #GSE102908). The heatmaps and volcano plots were generated using XLSTAT 2014. Validation of ERBB3 expression was carried out by RT-qPCR as described above. Gene Set Enrichment Analysis tool (GSEA, http://www.broad.mit.edu/gsea/) was used to determine the enrichment of the custom 18 gene signature "Receptor Tyrosine Kinase gene signatures". 14 of these genes are held within the Gene Ontology_Phosphorylation set (biological processes). The percentage of tumors with concurrently low SMARCA4/A2 was analyzed from TCGA data website by using the median expression to cut the data in low and high. For each cancer type, SMARCA2 and SMARCA4 expression levels under the median values were considered as low, and the percent of patients with "low" expression for these two genes was computed across

multiple cancer type. Total number of analyzed samples was 9144. Kaplan Meier plot was generated from TCGA data. Total number of patients studied was 239 from LUAD gene expression (IlluminaHiSeq) data set.

3.6.8 Chromatin Immunoprecipitation

Cells were seeded in 4, 15cm culture plates and treated with 0.01% DMSO or 100nM of OTX015 for 24 hours. Chromatin IP was carried out as previously described (328). DNA was extracted by QIAquick PCR purification Kit (Qiagen) according to manufacturer instructions. The samples were analyzed by qPCR with the results being represented as % of input. The list of primer sequences can be found in Supplementary Table S2. Further information is available to readers upon request.

3.6.9 Western blotting

Whole cell lysates were collected and Western blotting analysis was performed precisely as previously described (330).

3.6.10 Xenograft experiments

Animal studies were conducted in accordance with guidelines of the Canadian Council of Animal Care and approved by the Animal Resources Centre (ARC) at McGill University. $2x10^7$ of SCCOHT1 cells or $3x10^6$ of OVCAR8 cells in PBS were injected into the left ovary of 4-week-old female NOD/SCID mice. After 3 weeks for SCCOHT1 and 2 weeks for OVCAR8, mice were randomized into groups of 5. Next, treatments with 20 mg/kg/day of OTX015 dissolved in 2% DMSO, 30% PEG300, 5% Tween 20 or vehicle (same solvent) were carried out by oral gavage, 5 days per week. After 3 weeks of treatment mice were sacrificed and tumors were collected and weighted. Tumors were measured with caliper and tumor volume was calculated using following formula: $\frac{1}{2} \times ((\text{length in mm}) \times (\text{width in mm})^2)$. Immunohistochemical procedures were carried out at the Segal Cancer Centre Research Pathology Facility (Jewish General Hospital) as previously described (331). Slides with tissue samples were incubated in 1:100 dilution of primary Ki-67 rabbit antibody. Sections were analyzed by conventional light microscopy. Each part of the tissue section was analyzed

individually and the average value was used as a final score. The intensity of staining was measured by ImageJ and was scored by assessing five-tiered ranking (0=no staining, 1=very weak staining, 2=weak staining, 3=intermediate staining, 4=strong staining, 5=very strong staining).

3.6.11 Antibodies

Antibodies for Western Blotting were as follows: anti-SMARCA4 (Santa Cruz, #sc17796), anti-SMARCA2 (Abcam, #ab12165), anti-SMARCB1 (Bethyl, #A301-087A), anti-Actin (Sigma, #A5316), anti-Lamin A (Santa Cruz, #sc-20680), anti-Parp1/2 (Santa Cruz, #sc-7150), anti-c23-MS3-nucleolin (Santa Cruz, #sc8031), anti-BRD4 (Cell Signaling, #13440s), anti-HER3 (Cell Signaling, #12708s), anti-AKT (Cell Signaling, #2920s), anti-pAKT (Cell Signaling, #4060s), anti-S6 (Cell Signaling, #2217s), anti-pS6 (Cell Signaling, #2215s), anti-ERK1/2 (Cell Signaling, #4695s), anti-pERK1/2 (Cell Signaling, #9101s). For ChIP-qPCR, anti-RNA polymerase II subunit B1 phospho-CTD Ser-2 (Millipore, #04-1571) was employed. Anti-Ki-67 (SP6) (Abcam, #ab16667) was used for IHC analysis.

3.6.12 Statistical analysis

XLSTAT 2014 and SigmaPlot 12.0 were used for statistical analysis. All the error bars represent SEM. Significance of results was calculated with Student *t* test. Data was compiled from at least three replicates and was considered significant by obtaining a P value ≤ 0.05.

3.7 Supplementary Figures and Tables

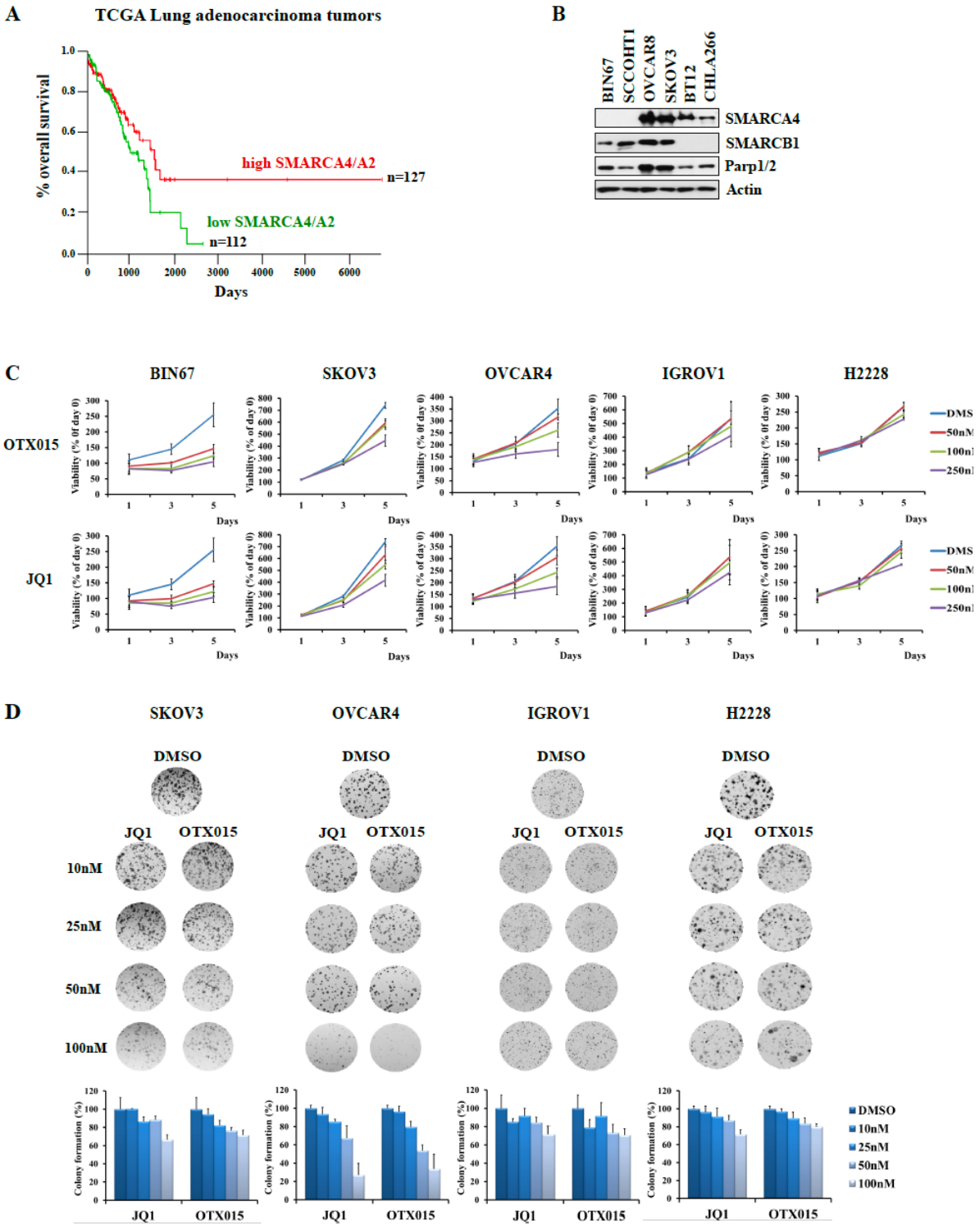
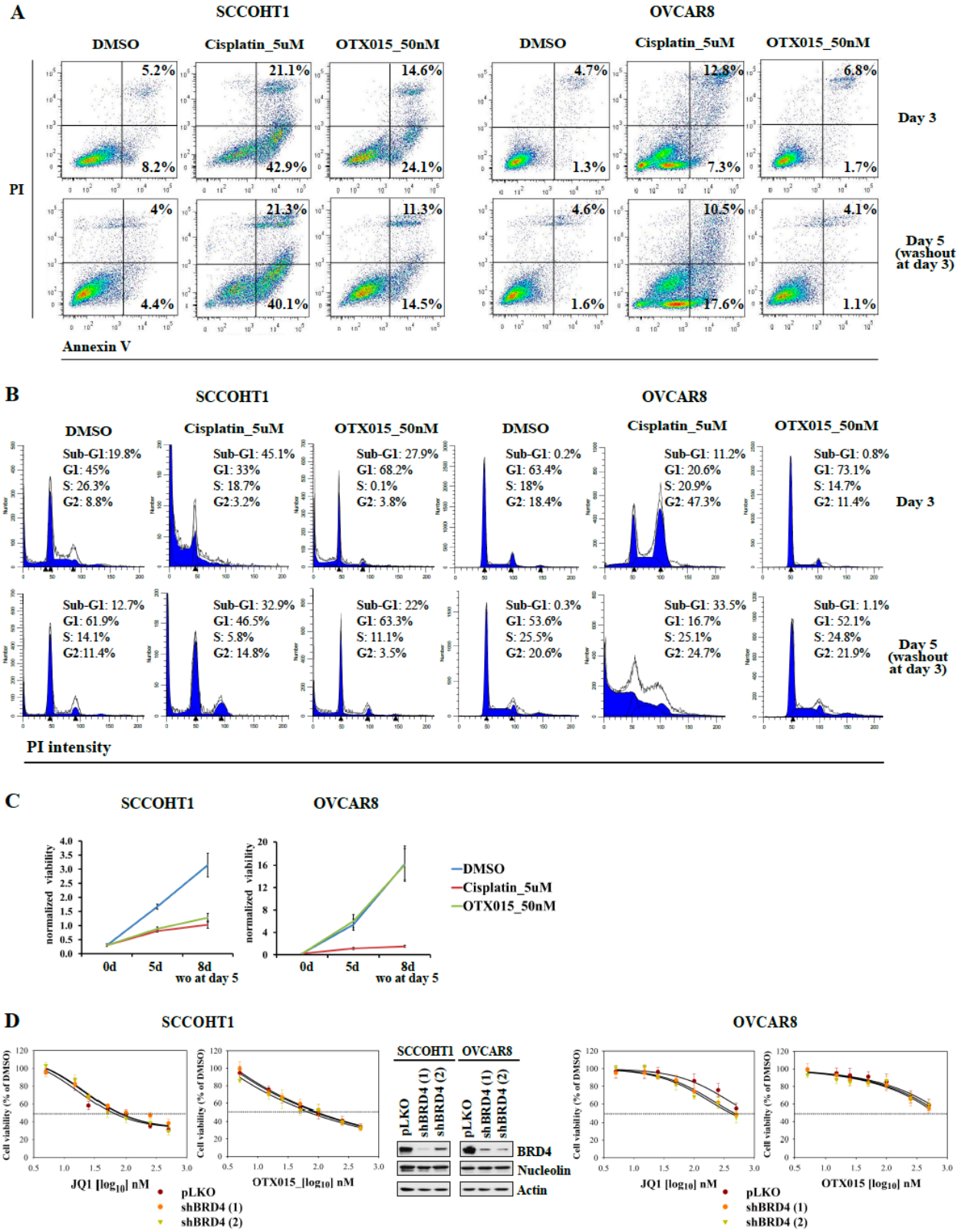


Figure S1. Response of cancer cells to epigenetic targeting molecules. A, Kaplan-Meier survival plot (TCGA database, RNA-seq) for lung adenocarcinoma patients, split by median values for SMARCA4/SMARCA2 mRNA levels. Total number of patients in cohort = 239. **B,** Western Blot showing SMARCA4 and SMARCB1 expression in ovarian and AT/RT cells. **C,** Cell viability of SCCOHT cells (BIN67), ovarian carcinoma cells (SKOV3, OVCAR4, IGROV1) and lung carcinoma cells (H2228) treated with JQ1 or OTX015 for 1, 3 and 5 days. The data is normalized to % of cells at day 0 (n=3, error bars: SEM). **D,** Clonogenic assay of ovarian carcinoma cells (SKOV3, OVCAR4, IGROV1) and lung carcinoma cells (H2228) in response to BETi treatments. The data is normalized to % of DMSO (n=3, error bars: SEM).


A
SCCOHT1
OVCAR8
DMSO
Cisplatin_5uM
OTX015_50nM
PI
Annexin V
Day 3
Day 5 (washout at day 3)
5.2%
8.2%
21.1%
42.9%
14.6%
24.1%
4.7%
1.3%
12.8%
7.3%
6.8%
1.7%
4%
4.4%
21.3%
40.1%
11.3%
14.5%
4.6%
1.6%
10.5%
17.6%
4.1%
1.1%
B
SCCOHT1
OVCAR8
DMSO
Cisplatin_5uM
OTX015_50nM
Sub-G1:19.8%
G1: 45%
S: 26.3%
G2: 8.8%
Sub-G1: 45.1%
G1: 33%
S: 18.7%
G2:3.2%
Sub-G1: 27.9%
G1: 68.2%
S: 0.1%
G2: 3.8%
Sub-G1: 0.2%
G1: 63.4%
S: 18%
G2: 18.4%
Sub-G1: 11.2%
G1: 20.6%
S: 20.9%
G2: 47.3%
Sub-G1: 0.8%
G1: 73.1%
S: 14.7%
G2: 11.4%
Sub-G1: 12.7%
G1: 61.9%
S: 14.1%
G2:11.4%
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G1: 46.5%
S: 5.8%
G2: 14.8%
Sub-G1: 22%
G1: 63.3%
S: 11.1%
G2: 3.5%
Sub-G1: 0.3%
G1: 53.6%
S: 25.5%
G2: 20.6%
Sub-G1: 33.5%
G1: 16.7%
S: 25.1%
G2: 24.7%
Sub-G1: 1.1%
G1: 52.1%
S: 24.8%
G2: 21.9%
Day 3
Day 5 (washout at day 3)
PI intensity
C
SCCOHT1
OVCAR8
normalized viability
0d
5d
8d
wo at day 5
DMSO
Cisplatin_5uM
OTX015_50nM
D
SCCOHT1
OVCAR8
Cell viability (% of DMSO)
JQ1 [log10] nM
OTX015_[log10] nM
pLKO
shBRD4 (1)
shBRD4 (2)
BRD4
Nucleolin
Actin

Figure S2. Response of sensitive and resistant cells to BETi. A, Apoptosis analysis by Annexin-V-FITC and PI staining in SCCOHT1 and OVCAR8 cells treated with DMSO, Cisplatin and OTX015 at indicated time points. **B,** Cell cycle analysis of SCCOHT1 and OVCAR8 cells treated with DMSO, Cisplatin and OTX015 at indicated time points. **C,** Cell viability assays at day 5 and day 8 (washout at day 5) in SCCOHT1 and OVCAR8 cells treated with DMSO, Cisplatin and OTX015. The values are normalized to % of cells at day 0 (n=3, error bars: SEM). **D,** Cell viability assays for SCCOHT1 and OVCAR8 cells upon BRD4 knockdown and treatment with BETi. pLKO: pLKO control, shBRD4: shRNA against BRD4. JQ1, OTX015: BETi (n=3, error bars: SEM). Western blotting showing reduced BRD4 protein expression in knock-down cells.

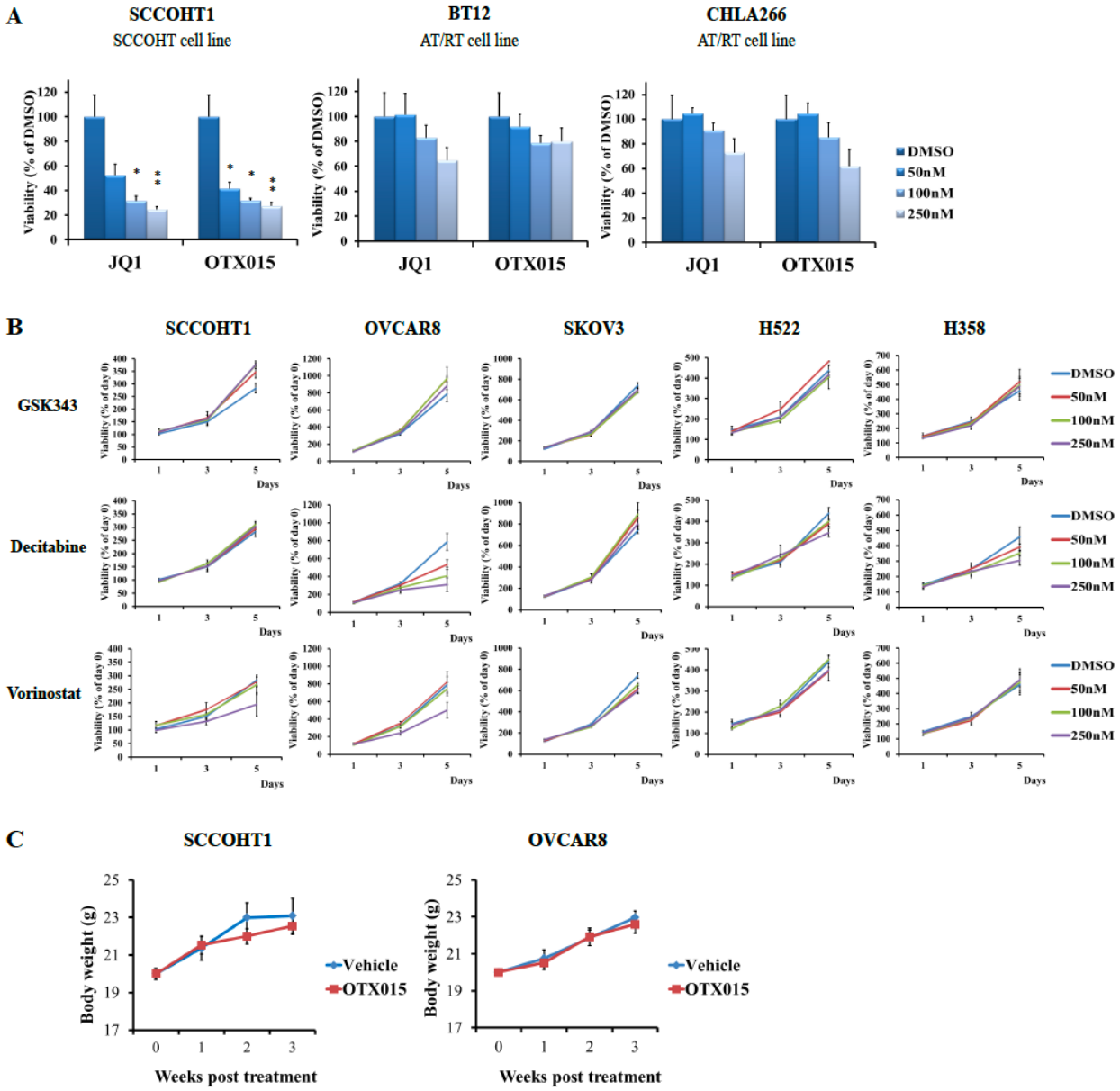
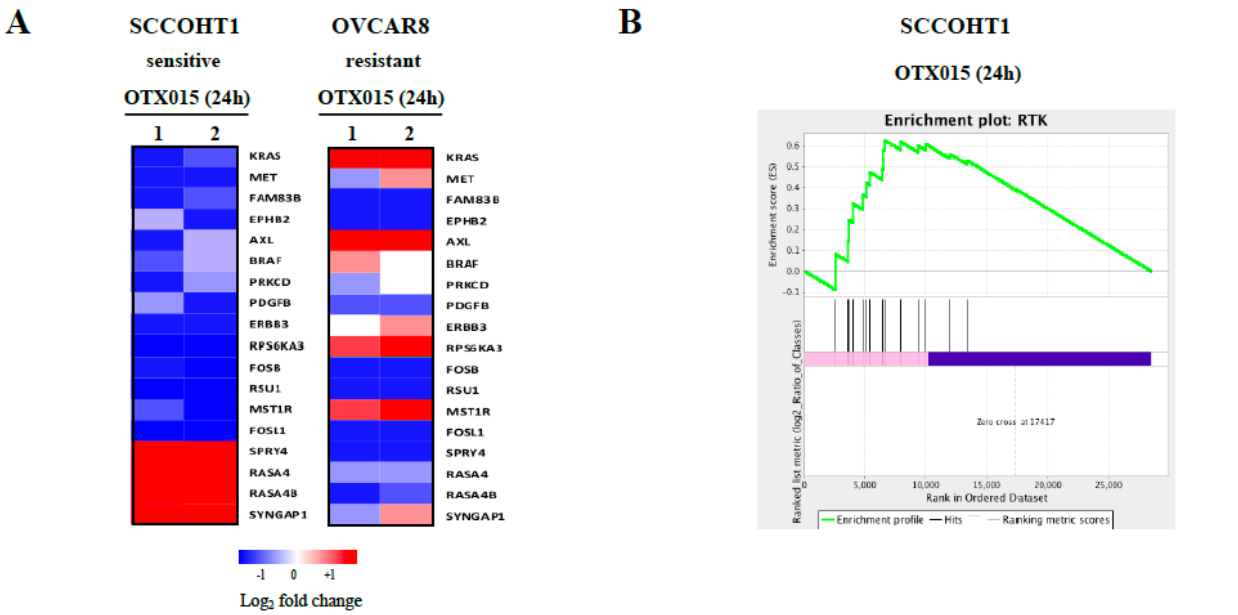


Figure S3. Variable responses to epigenetic drugs. **A,** Cell viability analysis of cells treated with JQ1 and OTX015 for 5 days. SCCOHT1: SCCOHT cells; BT12, CHLA266: AT/RT cells (n=3, error bars: SEM, Student two-tailed test, *P≤0.05, **P≤0.01). **B,** Cell viability data of SCCOHT1, OVCAR8, SKOV3, H522 and H358 cells exposed for 5 days to GSK343, Decitabine or Vorinostat (n=3, error bars: SEM). **C,** Body weight measurements at 0, 1, 2 and 3 weeks post treatment with vehicle or 20 mg/kg/day of OTX015 (vehicle group, n=5; OTX015 group, n=5, error bars: SEM). SCCOHT1: SCCOHT; OVCAR8: ovarian carcinoma.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SCCOHT1
sensitive
OVCAR8
resistant
OTX015 (24h)
OTX015 (24h)
1 2
1 2
KRAS
MET
FAM83B
EPHB2
AXL
BRAF
PRKCD
PDGFB
ERBB3
RPS6KA3
FOSB
RSU1
MST1R
FOSL1
SPRY4
RASA4
RASA4B
SYNGAP1
-1 0 +1
Log2 fold change
B
SCCOHT1
OTX015 (24h)
Enrichment plot: RTK
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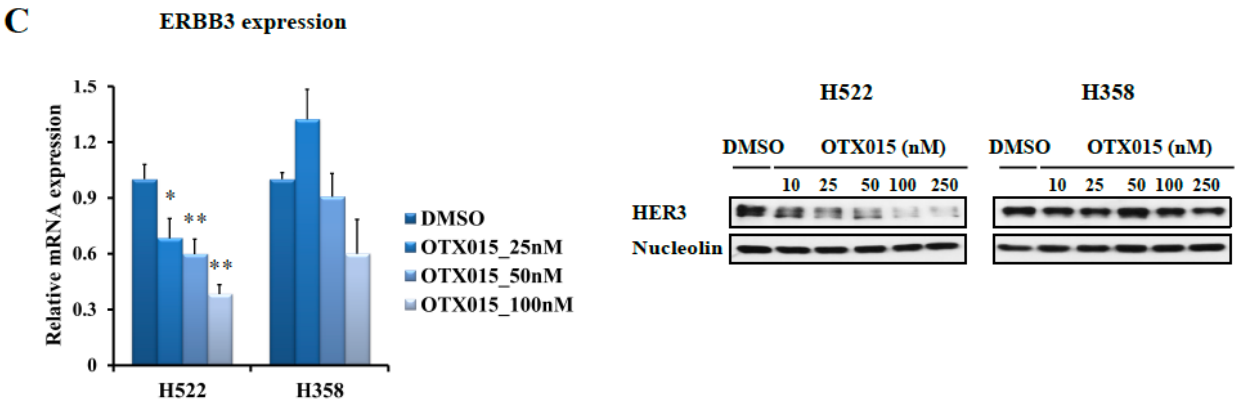
Ranked list metric (log2_Ratio_of_Classes)
Zero cross at 17417
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores


C
ERBB3 expression
Relative mRNA expression
1.5
1.2
0.9
0.6
0.3
0
*
**
**
H522
H358
DMSO
OTX015_25nM
OTX015_50nM
OTX015_100nM
H522
H358
DMSO
OTX015 (nM)
DMSO
OTX015 (nM)
10 25 50 100 250
10 25 50 100 250
HER3
Nucleolin

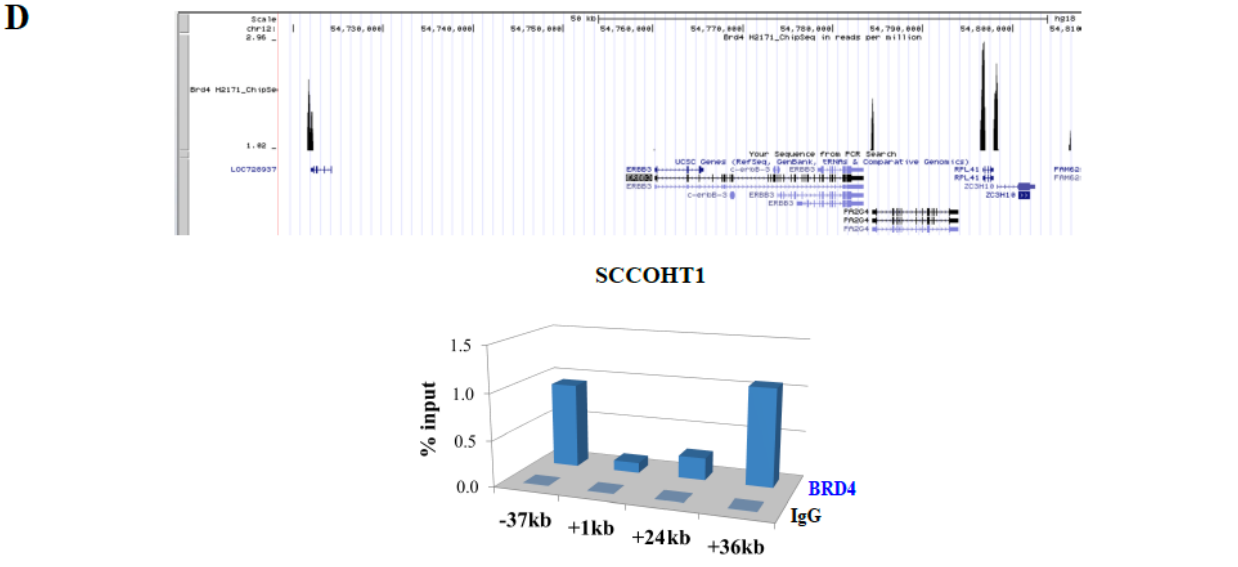

D
SCCOHT1
% input
1.5
1.0
0.5
0.0
BRD4
IgG
-37kb
+1kb
+24kb
+36kb

Figure S4. OTX015 transcriptionally regulates a network of genes involved in cell signaling. A, Genes known to either initiate, modulate, or act as downstream effectors of PI3K and RAS signaling pathways are shown, whose regulation by OTX015 is more significant in BETi-sensitive SCCOHT1 cells (log_2 fold change >0.5, P≤0.05). **B**, GSEA analysis showing RTK-dependent genes enrichment in SCCOHT1 treated with OTX015. **C,** OTX015 down-regulates HER3 in H522 (NSCLC) cells. Left, qPCR data of *ERBB3* mRNA expression in lung carcinoma cells, H522 and H358, in response to treatments with a range of concentrations of OTX015 at 24h. The relative mRNA expression was normalized to human 36B4 and DMSO control (n=3, error bars: SEM, Student two-tailed test, *P≤0.05, **P≤0.01). Right, Western blot analysis showing expression of HER3 upon dose-dependent treatment with OTX015 at 24h in H522 and H358 cells. **D,** Top, BRD4 binds to multiple sites at the *ERBB3* gene. ChIP-seq track adopted from the UCSC genome browser (GEO accession #GSM1038270). Bottom, Chromatin IP analysis showing BRD4 binding at *ERBB3* gene in SCCOHT1 cells (n=3).

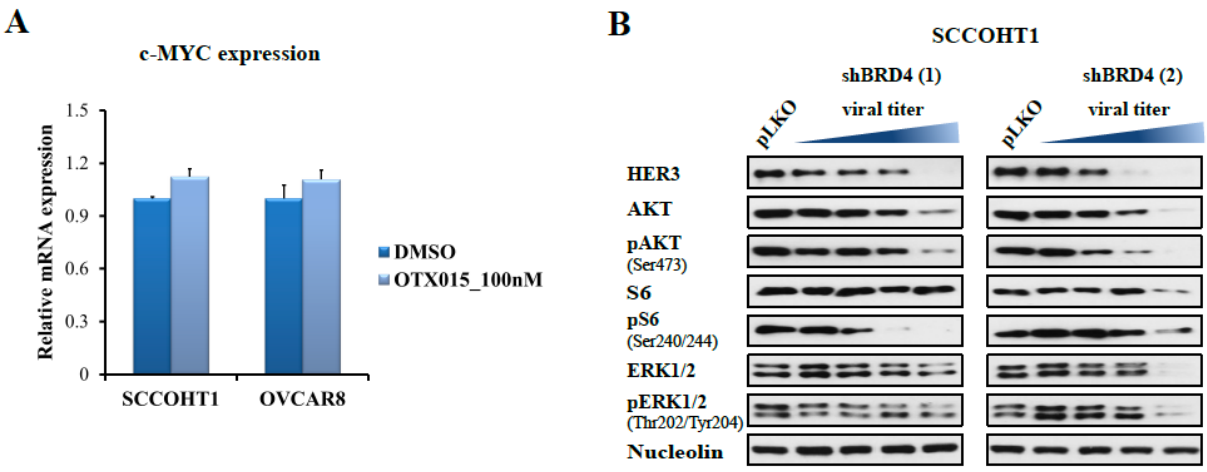


Figure S5. c-MYC expression in response to BETi treatment in ovarian cancer cells. BRD4 knockdown represses HER3 expression and downstream signaling. A, qPCR analysis of *c-MYC* mRNA expression in SCCOHT1 and OVCAR8 treated with DMSO and OTX015 at 24h (n=2, error bars: SEM). **B,** Western blot analysis of HER3 and downstream targets in SCCOHT1 upon 5 days of BRD4 knockdown using increasing viral titer. pLKO: control vector, shBRD4: shRNA targeting BRD4.

Table S1. List of shRNA plasmids

Plasmid name	TRC ID	Clone Name
pLKO control vector	TRC1.5/pLKO.1	Empty vector
BRD4 (1)	TRCN0000199427	NM_058243.1_1707s1c1
BRD4 (2)	TRCN0000196576	NM_058243.1_4626s1c1
HER3 (1)	TRCN0000040110	NM_001982.1_2068s1c1
HER3 (2)	TRCN0000009835	NM_001982.1_4705s1c1
SMARCA4 (1)	TRCN0000380723	NM_003072.3_3096s21c1
SMARCA4 (2)	TRCN0000231102	NM_003072.3_5279s21c1

Table S2. List of primers

Molecular test	Primer name	Sequence (5'-3')
RTqPCR	ERBB3 Forward	GTGGACTCGAGCAACATTGA
RTqPCR	ERBB3 Reverse	CCGTACTGTCCGGAAGACAT
RTqPCR	36B4 Forward	CGACCTGGAAGTCCAACTAC
RTqPCR	36B4 Reverse	ATCTGCTGCATCTGCTTG
ChIPqPCR	ERBB3 +200bp Forward	GCTCTTGCCTCGATGTCCTA
ChIPqPCR	ERBB3 +200bp Reverse	GCCAGGCTGAAAAGCAAG
ChIPqPCR	ERBB3 +16kb Forward	ACTCCTTCCCATTTGCTCCT
ChIPqPCR	ERBB3 +16kb Reverse	ATGGGTCACTCACTTTGTGC
ChIPqPCR	ERBB3 +22kb Forward	GCAAAATCCTCCCAATTCCT
ChIPqPCR	ERBB3 +22kb Reverse	CCACCACCACTTCCTGAGAT
ChIPqPCR	ERBB3 +23kb Forward	CTCCCTGTTCTCCCAGCTTC
ChIPqPCR	ERBB3 +23kb Reverse	GGCAGGGGTGGAGTAGAGT
ChIPqPCR	ERBB3 -37kb Forward	GGTGCAGACTCTGGGATTGT
ChIPqPCR	ERBB3 -37kb Reverse	ATCTCCTGTTTCTCCGCAAA
ChIPqPCR	ERBB3 +1kb Forward	TTCAGGCTTGAAGTTCTGGAG
ChIPqPCR	ERBB3 +1kb Reverse	CCTTGCATTTCAAATCCCTTA
ChIPqPCR	ERBB3 +24kb Forward	GGAGCAAACTATCGCTGAGG
ChIPqPCR	ERBB3 +24kb Reverse	CCTTTCCCTATCAGCCTTGA
ChIPqPCR	ERBB3 +36kb Forward	AGACATCTGACCTCGGCACT
ChIPqPCR	ERBB3 +36kb Reverse	CCTCGCTACTACTCGCCAAC

Table S3. Mutations in ovarian and lung cancer cell lines.

Cell line	Clinical subtype	SMARCA4	SMARCA2	TP53	PIK3CA	KRAS	Ref
SCCOHT1	SCCOHT	p.Pro1180fs p.Arg1077*	NA	NA	NA	NA	(47)
OVK18	SCCOHT	p.G1160fs p.P110fs p.P109fs*194	NA	p.P153fs	NA	p.A59G	(332)
BIN67	SCCOHT	c.2438+1G>A c.2439-2A>T	NA	NA	NA	NA	(38)
OVCAR8	Ovarian carcinoma	NA	NA	Splice site	NA	p.P121H	(333)
OVCAR4	Ovarian carcinoma	p.P324P (silent mutation)	NA	p.L130V	NA	NA	(333)
SKOV3	Ovarian carcinoma	NA	NA	p.P89fs	p.H1047R	NA	(333)
IGROV1	Ovarian carcinoma	p.D1416N p.E1212del	p.P101Q	p.Y126C p.S90fs	p.R38C p.*1069W	NA	(333)
H522	NSCLC	p.P270fs	NA	p.P191fs	NA	NA	(334)
H2228	NSCLC	NA	p.T59S	p.Q331*	NA	NA	(333)
H358	NSCLC	NA	NA	NA	NA	p.G12C	(333)
BT12	AT/RT	NA	NA	NA	NA	NA	(333)
CHLA266	AT/RT	NA	NA	NA	NA	NA	(335)

3.8 References Chapter (III)

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Chapter IV: Reprogramming of nucleotide metabolism mediates synergy between epigenetic therapy and MAP Kinase inhibition

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4.1 Preface

We and others have shown that cancer cells commonly display intrinsic resistance to BET inhibitors (BETi). Responsiveness to BETi is often accompanied by the down-regulation of RTK-dependent signaling. In order to potentiate the anti-proliferative effects of BETi we aimed to concurrently repress oncogenic signaling through the classical downstream effectors of RTK, MAPK and PI3K pathways. To this end, we carried out combination treatments with a panel of inhibitors targeting various nodes within the PI3K and MAPK pathways in both BETi-sensitive, and more resistant, ovarian cancer models. We found that inhibitors of MAPK represent the most potent combination with BETi. Unexpectedly, the anti-proliferative effects of this combination therapy appear to be mediated through inhibition of nucleotide synthesis.

4.2 Abstract

Small Cell Carcinoma of the Ovary, Hypercalcemic Type is a rare but often lethal cancer which is diagnosed at a median age of 24 years. Optimal management of patients is not well defined and current treatment remains challenging, necessitating the discovery of novel therapeutic approaches. The identification of *SMARCA4*-inactivating mutations invariably characterizing this type of cancer provided insights facilitating diagnostic and therapeutic measures against this disease. We show here that the BET inhibitor OTX015 acts in synergy with the MEK inhibitor cobimetinib to repress the proliferation of SCCOHT *in vivo*. Notably, this synergy is also observed in some SMARCA4-expressing ovarian adenocarcinoma models intrinsically resistant to BETi. Mass Spectrometry, coupled with knockdown of newly-found targets including thymidylate synthetase, revealed that the repression of a panel of proteins involved in nucleotide synthesis, underlies this synergy both *in vitro* and *in vivo*, resulting in reduced pools of

nucleotide metabolites and subsequent cell cycle arrest. Overall, our data indicate that dual treatment with BETi and MEKi represents a rational combination therapy against SCCOHT and potentially additional ovarian cancer subtypes.

4.3 Introduction

Small Cell Carcinoma of the ovary, Hypercalcaemic Type (SCCOHT) is an aggressive malignant tumor with a dismal prognosis (32). The mean age at diagnosis is ~24 years and most patients die within two years of diagnosis. For SCCOHT, treatment generally involves surgery and adjuvant chemotherapy, most commonly platinum based compounds. Despite combination chemotherapy approaches, however, the prognosis still remains poor with overall 5-year survival rates of only 16% (33). Moving forward, personalized therapies for SCCOHT will require proper diagnosis and the identification of oncogenic drivers of these carcinomas. Differentiating SCCOHT from morphologically similar tumors is challenging. The "small" and "large" variants are fairly analogous (36) and SCCOHT also needs to be distinguished from other primary and metastatic tumors that may be found within the same tissue including non-epithelial ovarian neoplasms and metastases from small cell lung carcinoma among others (36,40,42). The identification of a central role for *SMARCA4* mutations in the pathogenesis of this tumor (38,47,336,337), and subsequent use of SMARCA4 (BRG1) immunohistochemistry, with or without using antibodies raised against SMARCA2 (BRM) has greatly facilitated the diagnosis (48,338).

Most *SMARCA4* mutations in SCCOHT are deleterious resulting in a complete loss of protein expression, being confirmed by immunohistochemistry in almost 100% of cases (38,48). SMARCA4 and its paralogue SMARCA2 are essential ATPase components of the multisubunit SWI/SNF (SWItch/Sucrose Non-Fermentable) chromatin remodeling complex and modifies histone-DNA interactions by shifting or evicting nucleosomes to change the landscape of accessible regions on chromatin, thereby impacting transcriptional activation (44). SMARCA2 is concomitantly lost with SMARCA4 in almost all SCCOHT cases, and this profile now constitutes a molecular signature of the disease (48).

Previously, it was shown that SMARCA4 and another bromodomain protein BRD4 independently associate with distal enhancer elements of c-MYC in order to activate oncogene

transcription, suggesting some redundancy between these two proteins in gene regulation and tumorigenesis (122). This led us to hypothesize that in the absence of SMARCA4 and SMARCA2, BRD family members might represent essential proteins for driving transcriptional networks involved in proliferation and survival (172). Thus, targeting SMARCA4-deficient cancers with bromodomain inhibitors (BETi), that target multiple BRD proteins, might effectively shut down this BRD-driven oncogenic network. We demonstrated that SMARCA4/A2 deficient SCCOHT and Non-Small Cell Lung cancer (NSCLC) models were acutely sensitive to BET inhibitors *in vitro* and in mouse models at very low doses (172). Notably, this work revealed that BETi efficacy correlated with repression of PI3K and MAPK pathways. This is consistent with other studies suggesting that intrinsic resistance to BETi is conferred by constitutive signaling through receptor tyrosine kinase pathways including PI3K and MAPK (285-288,339). Recently, it was shown that *NRAS* mutant melanoma models displayed resistance to BETi. In turn, combining BETi with MEKi led to decreased cell proliferation *in vitro* and was also effective against melanomas carrying *NRAS* mutations *in vivo* (287). The precise mechanism through which this combination works, and whether this approach may be applicable to additional tumor types, remains unclear.

Here, we screened a range of inhibitors targeting PI3K and MAPK pathways for potential synergy with BETi against a panel of SMARCA4-deficient and SMARCA4-expressing ovarian cancer cell lines. Among all the tested compounds, we found a strong synergy between BETi (OTX015) and MEK inhibitors (cobimetinib and trametinib) at suboptimal doses in both ovarian cancer models. This combination also proved highly effective against orthotopic xenograft models of ovarian cancer. Using mass spectrometry to assess changes in protein content after exposure to combination therapy revealed that concurrent treatment with BETi/MEKi represses key enzymes involved in nucleotide metabolism. A concomitant decrease in nucleotide pools was validated using metabolomics profiling. This culminates in a reduced pool of nucleotide precursors and cell cycle arrest. Overall, the combination of BETi/MEKi highlights a potential new therapeutic approach to treat multiple subtypes of ovarian tumors.

4.4 Results

4.4.1 Ovarian cancer cell lines are sensitive to BETi/MEKi exposure

We previously published that the downregulation of PI3K and MAPK pathways correlates with the anti-proliferative effects of BETi in SMARCA4-deficient cells (172). However, SMARCA4-expressing cells were largely unresponsive to BETi exposure. In addition, BETi failed to reduce PI3K and MAPK activity in SMARCA4-proficient cells. We also previously found that re-expression of the RTK, HER3 conferred partial resistance to BETi indicating RTK signaling is indeed important for BETi mediating resistance (172). However, it remains unproven that downstream effectors of RTK directly confer resistance. As such, we introduced BRAF (V600E) and KRAS (G12D) into initially BETi sensitive, SMARCA4-deficient ovarian cancer cells and subsequently compared cell viability after BETi exposure (Supplementary Fig. S6A). As expected, expression of the constitutively active BRAF and KRAS induced ERK phosphorylation (Supplementary Fig. S6B) which was paralleled by decreased sensitivity to BETi (Supplementary Fig. S6A). These data confirmed that both upstream and downstream activators of RAS-MAPK oncogenic pathways may impart resistance to BETi. Thus, we hypothesized that BETi may cooperate with RTK or MAPK inhibitors to repress oncogenic growth in SMARCA4-deficient cells and that such combinations may potentially overcome resistance in SMARCA4-expressing models.

To explore this, we first carried out short-term (5-day) *in vitro* cell viability assays, with the degree of synergy being calculated using "Excess Over Bliss" (EOB) as previously described (340,341). These experiments were carried out on a panel of SMARCA4-deficient SCCOHT (SCCOHT1, OVK18) and SMARCA4-expressing ovarian adenocarcinoma cell lines (OVCAR4, OVCAR3, SKOV3, IGROV1). The clinically tested BETi, OTX015 was combined with a panel of inhibitors targeting RTK-dependent signaling pathways including cobimetinib or trametinib (MEK inhibitors), copanlisib (PI3K inhibitor), patritumab (anti-HER3 antibody) and lapatinib (HER2/EGFR inhibitor). Only weak cooperativity was observed between BETi and partritumab or BETi/lapatinib (HER2/EGFR inhibitor) (Supplementary Fig. S7A, EOB generally <1). A more pronounced effect was seen between copanlisib and BETi, (Supplementary Fig. S7B, EOB generally between 0-10).

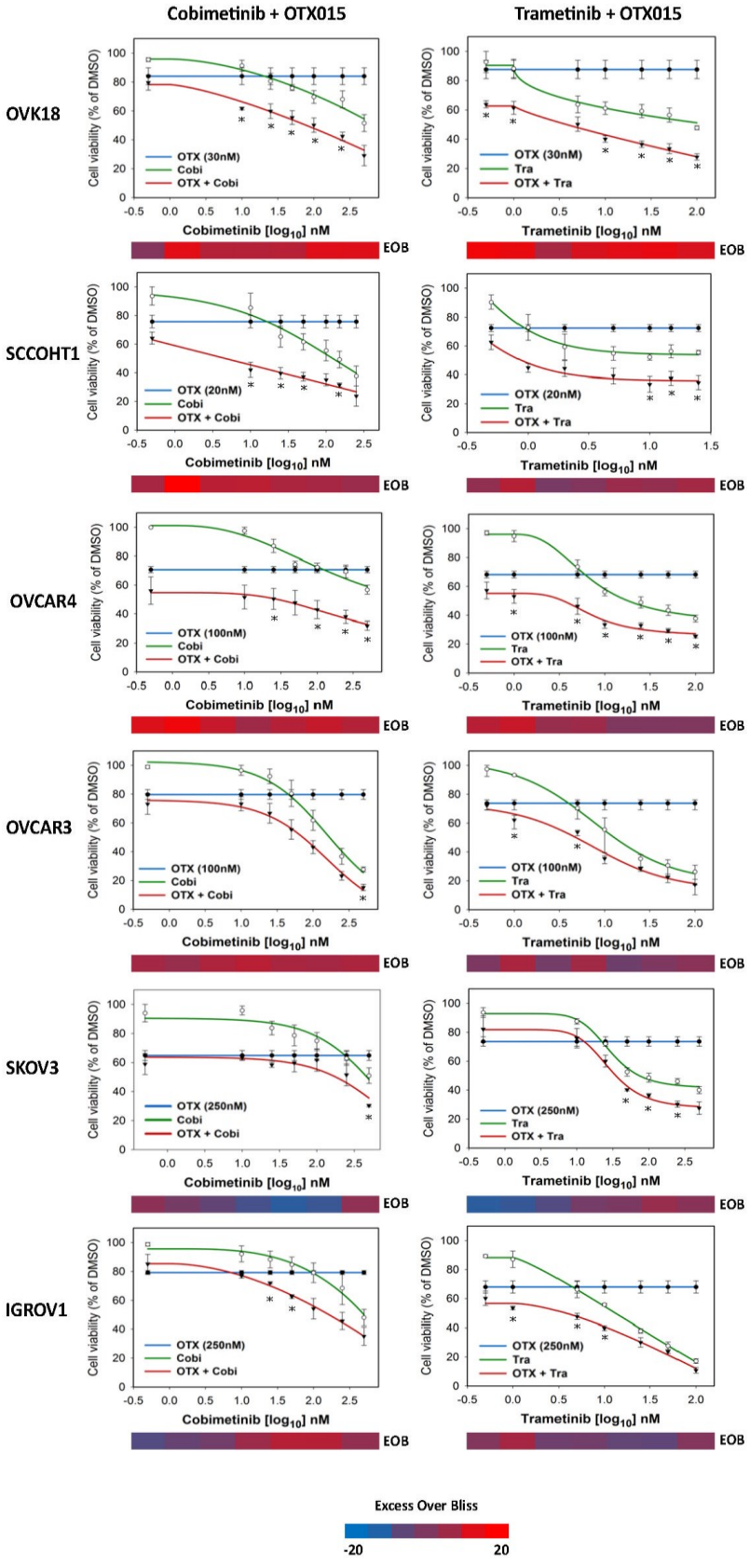

Cobimetinib + OTX015
Trametinib + OTX015
OVK18
SCCOHT1
OVCAR4
OVCAR3
SKOV3
IGROV1
Cell viability (% of DMSO)
OTX (30nM)
OTX (20nM)
OTX (100nM)
OTX (250nM)
Cobi
Tra
OTX + Cobi
OTX + Tra
Cobimetinib [log10] nM
Trametinib [log10] nM
EOB
Excess Over Bliss
-20
20

Figure 16. Synergy between BETi/MEKi across ovarian cancer cell types. Cell viability assays for SCCOHT and ovarian adenocarcinoma cells treated with OTX015 (at 20-250nM concentrations) and cobimetinib or trametinib (at 0.5-500nM concentrations), either alone or in combination, for a period of 5 days. OVK18, SCCOHT1: SCCOHT cell lines; OVCAR4, OVCAR3, SKOV3, IGROV1: ovarian adenocarcinoma cells; OTX015: BETi; cobimetinib, trametinib: MEKi; EOB: Excess Over Bliss (n=3, error bars: SEM, two-tailed Student t test; $^{*}P \leq 0.05$).

However, of the tested combinations, the most marked synergy was detected between the MEK inhibitors cobimetinib and trametinib with OTX015 (Fig.16, EOB observed between 5-20). Surprisingly, this response was also observed in the SMARCA4-expressing ovarian adenocarcinoma cancer models OVCAR4, OVCAR3, but to a lesser degree in IGROV1 and SKOV3. This supports the concept that targeting the MAPK pathway represents a means to overcome intrinsic resistance to BETi.

4.4.2 Combination of OTX015 and cobimetinib reduces tumor growth in ovarian tumor xenograft models

To investigate whether the combination of OTX015/cobimetinib has *in vivo* relevance, we tested the combination at suboptimal doses against orthotopic ovarian xenograft models of SMARCA4-deficient SCCOHT (OVK18) and SMARCA4-expressing ovarian adenocarcinoma (OVCAR4) (Fig.17). For these studies we used suboptimal doses of both drugs, with OTX015 being given at 20 mg/kg/day and cobimetinib at 5 mg/kg/day, each administered 5 days per week. 3 weeks post cell implantation, mice received either vehicle, OTX015, cobimetinib or OTX015/cobimetinib in combination for 3 weeks. These concentrations are less than half the drug concentrations typically employed in preclinical studies (342,343). At the experimental endpoint, tumors were obtained, and volume measured. We employed OVK18 as a model of SCCOHT because of its relative resistance to BETi *in vivo* compared to other SCCOHT models which show acute sensitivity at the concentrations currently employed (172). This relative resistance is likely due to an activating A59G *KRAS* mutation in OVK18 that may impart partial resistance *in vivo*. We observed that the OTX015/cobimetinib combination nearly eradicated tumor growth in the SCCOHT model. We previously found that ovarian adenocarcinomas generally harbor intrinsic resistance to BETi (172).

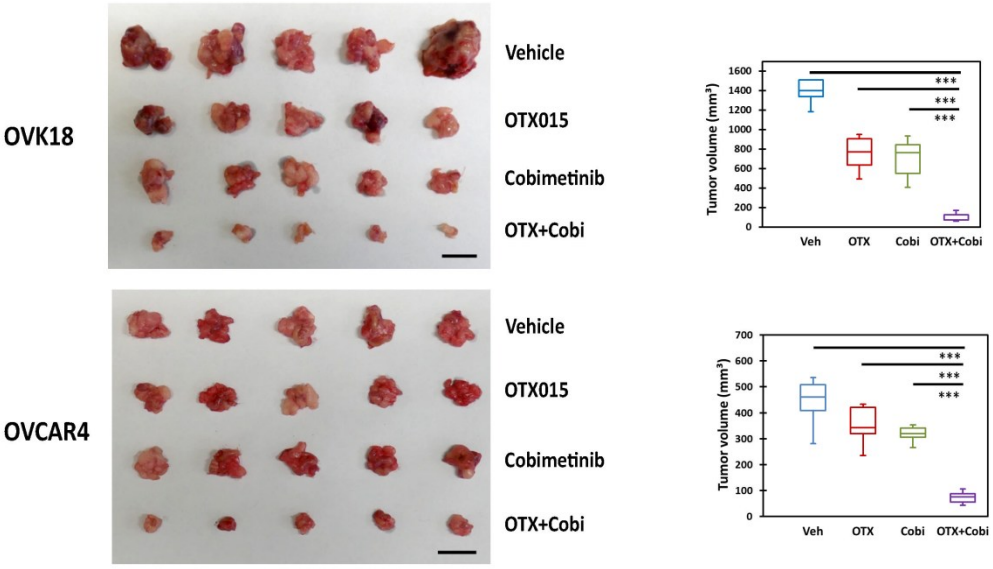


Figure 17. OTX015/cobimetinib combination reduces tumor growth in ovarian cancer models. Left, photos showing tumors after 3 weeks of treatment with OTX015 (at 20mg/kg/day) and cobimetinib (at 5mg/kg/day) alone or in combination (scale bars, 1cm). Right, box plots showing tumor volume in response to the treatments Vehicle: control; OTX015: BETi; cobimetinib: MEKi; OVK18: SCCOHT; OVCAR4: ovarian adenocarcinoma (n=5; two-tailed Student t test; $^{***}P \leq 0.001$).

However, the data from these xenograft experiments revealed drastically stronger effects of OTX015/cobimetinib combination against OVCAR4 xenografts, as compared to single drug treatments (Fig.17). This demonstrates that resistance to BETi can be overcome by combining them with MEKi (Fig.17). Both compounds have been reported to be associated with strong toxicities (279,280,299,344). Thus, the combinatorial approach might be promising from a clinical perspective due to the suboptimal doses required to prohibit tumor growth.

4.4.3 OTX015/cobimetinib combination down-regulates the expression of proteins involved in nucleotide synthesis

To date, most studies aiming to define the downstream effectors of BETi response have focused on RNA sequencing. However, mRNA expression does not necessarily coincide with, and accurately reflect, protein expression (345). In order to find potential drivers of the response to the combination of BETi/MEKi we conducted SILAC mass spectrometry experiments on OVK18 cells treated with DMSO, OTX015, cobimetinib or OTX015/cobimetinib combination for 24 hours (Fig.18A,B). This method employs stable isotope labeling of cells for six days prior

to drug exposure, allowing us to quantify abundance ratios between proteins from treated and untreated conditions. Surprisingly, under these conditions, of the 1797 detected proteins, only a few were identified as undergoing significant changes in expression of either drug individually. In contrast, 27 proteins underwent significant changes in the combination setting after 24 hours of drug exposure. This relatively early time point was chosen to identify potential effectors of BETi/MEKi that might initiate the anti-proliferative effects later observed in these cells. Even though a small number of target proteins were identified, Gene Ontology (GO) term pathway analysis of proteins undergoing altered expression revealed an enrichment for proteins involved in nucleotide metabolism (Fig.18C, $p<0.05$). Importantly, among the key down-regulated targets, we discovered proteins involved in DNA synthesis such as TYMS (Thymidylate synthase), DUT (dUTPase) and RRM1 (Ribonucleotide Reductase, subunit 1) (346,347). TYMS and DUT are enzymes catalyzing thymidine DNA synthesis (346). RRM1 is one of the subunits of the ribonucleotide reductase (RNR) complex which participates in DNA synthesis by converting ribonucleotides to deoxyribonucleotides (dNTPs) (347). Notably, all three enzymes have been reported overexpressed in several cancers and are considered as attractive anti-cancer therapeutic targets (347-349).

To validate the key targets from our SILAC screen, we carried out time course treatments with OTX015 and cobimetinib followed by RTqPCR and Western blotting analysis of putative targets. We did not observe major changes in mRNA levels for TYMS, DUT and RRM1 after BETi/MEKi combination treatment as compared to control or single treatments (Fig.18D) suggesting that the decrease to these proteins revealed by mass spectrometry is not transcriptionally regulated. As expected, Western blotting validated that the expression levels for all the three proteins were significantly down-regulated in both OVK18 and OVCAR4 cell lines (Fig.18E).

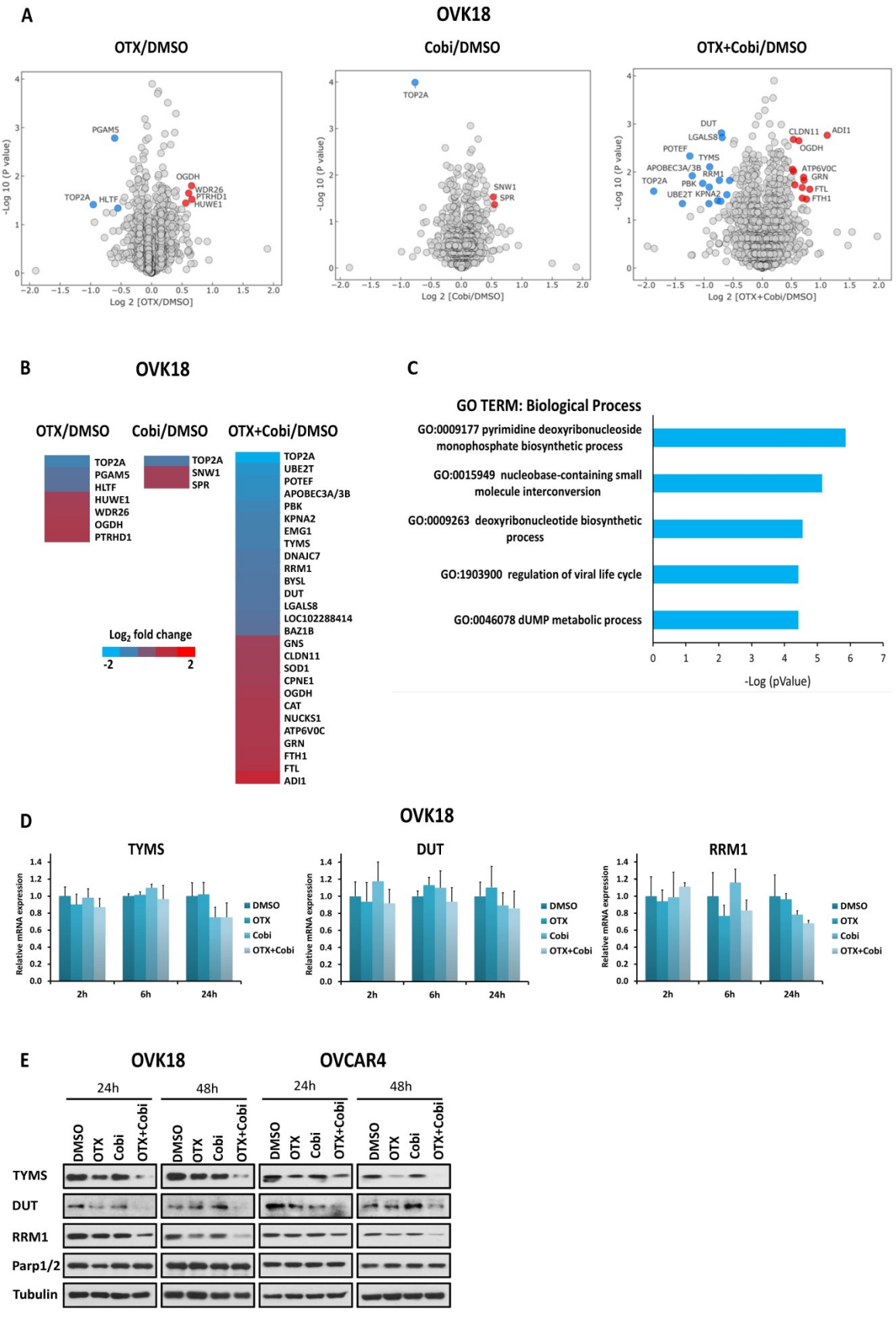

A
OVK18
OTX/DMSO
Cobi/DMSO
OTX+Cobi/DMSO
-Log 10 (P value)
Log 2 [OTX/DMSO]
Log 2 [Cobi/DMSO]
Log 2 [OTX+Cobi/DMSO]
PGAM5
TOP2A
HLTF
OGDH
WDR26
PTRHD1
HUWE1
SNW1
SPR
DUT
LGALS8
POTEF
TYMS
APOBEC3A/3B
PBK
RRM1
UBE2T
KPNA2
CLDN11
ADI1
OGDH
ATP6V0C
GRN
FTL
FTH1
B
OVK18
OTX/DMSO
Cobi/DMSO
OTX+Cobi/DMSO
TOP2A
PGAM5
HLTF
HUWE1
WDR26
OGDH
PTRHD1
TOP2A
SNW1
SPR
TOP2A
UBE2T
POTEF
APOBEC3A/3B
PBK
KPNA2
EMG1
TYMS
DNAJC7
RRM1
BYSL
DUT
LGALS8
LOC102288414
BAZ1B
GNS
CLDN11
SOD1
CPNE1
OGDH
CAT
NUCKS1
ATP6V0C
GRN
FTH1
FTL
ADI1
Log2 fold change
-2
2
C
GO TERM: Biological Process
GO:0009177 pyrimidine deoxyribonucleoside monophosphate biosynthetic process
GO:0015949 nucleobase-containing small molecule interconversion
GO:0009263 deoxyribonucleotide biosynthetic process
GO:1903900 regulation of viral life cycle
GO:0046078 dUMP metabolic process
-Log (pValue)
D
OVK18
TYMS
DUT
RRM1
Relative mRNA expression
DMSO
OTX
Cobi
OTX+Cobi
2h
6h
24h
E
OVK18
OVCAR4
24h
48h
DMSO
OTX
Cobi
OTX+Cobi
TYMS
DUT
RRM1
Parp1/2
Tubulin

Figure 18. OTX015/cobimetinib combination represses protein expression of TYMS, DUT and RRM1. A, SILAC mass spectrometry analysis of OVK18 ovarian cancer cells exposed to OTX015 (at 200nM) and cobimetinib (at 200nM) alone or in combination for 24h. Volcano plots demonstrating protein expression for each treatment condition compared with DMSO control (log_2-fold change >0.5, P≤0.05). **B,** Heat maps representation of SILAC experiment showing differential protein expression profile for OVK18 cells treated with single OTX015 or cobimetinib and OTX015/cobimetinib treatments for 24h (log_2-fold change >0.5, P≤0.05). **C,** GO term pathway analysis for proteins changed in response to OTX015/cobimetinib treatment in OVK18 cell line (FDR<0.05). **D,** Quantitative PCR analysis of TYMS, DUT and RRM1 expression in OVK18 cell line treated with DMSO, OTX015 (at 200nM), cobimetinib (at 200nM) or OTX015/cobimetinib for 2h, 6h and 24h. The relative mRNA expression was normalized to DMSO (n=3, error bars: SEM). **E,** Western blotting showing TYMS, DUT and RRM1 protein expression decrease in ovarian cancer cells treated with DMSO, OTX015 (at 200nM), cobimetinib (at 200nM) or OTX015/cobimetinib for 24 and 48h. OVK18: SCCOHT cells; OVCAR4: ovarian adenocarcinoma cells.

Our data indicates that BETi and MEKi cooperate to promote reduced protein levels of key enzymes involved in proliferation and survival. Previous proteomic studies (available at PhosphoSitePlus (350)) identified TYMS as a target for ubiquitylation and thus, it is a likely candidate for proteasome-mediated degradation (351,352). Toward this end, we treated OVK18 cells with proteasome inhibitor MG132 and subsequently exposed the cells to OTX015, cobimetinib alone or in combination (Supplementary Fig.S8). Interestingly, we observed that the MG132 treatment abrogated protein down-regulation stabilizing TYMS levels. These data indicate that BETi/MEKi act to promote proteasome-mediated degradation of TYMS, potentially impacting nucleotide synthesis.

To substantiate that downregulation of these targets might be responsible for the anti-proliferative activity of BETi/MEKi *in vivo*, we probed for DUT, TYMS and RRM1, using immunohistochemistry, within tumors after drug exposure. Consistent with our *in vitro* findings, we observed a striking synergy in the reduction of TYMS, DUT and RRM1 within tumors from mice treated with the OTX015/cobimetinib combination (Fig. 19).

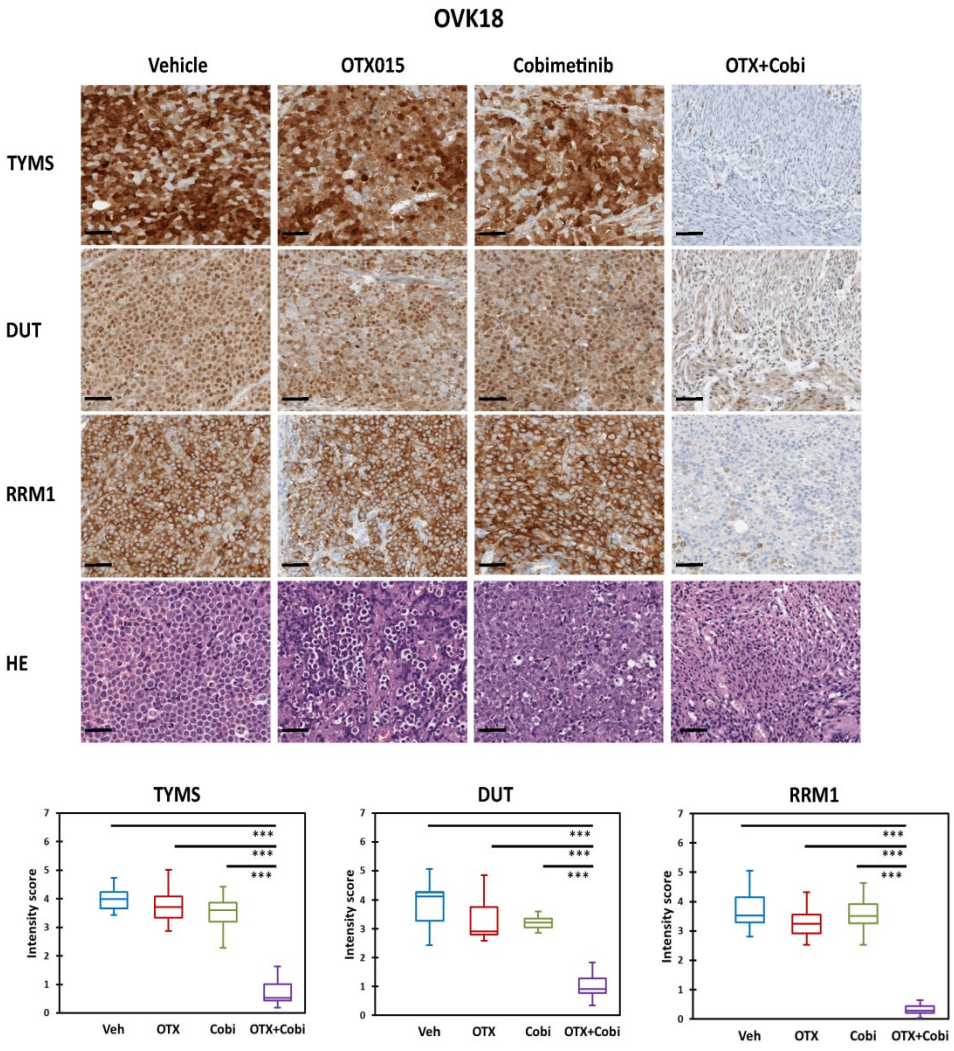


Figure 19. OTX015/cobimetinib combination decreases TYMS, DUT and RRM1 expression in ovarian tumor xenografts. Top, photos showing immunohistochemistry analysis of OVK18 tumors for TYMS, DUT and RRM1 expression upon 3 weeks of treatment with vehicle, OTX015 (20mg/kg/day), cobimetinib (5mg/kg/day) and OTX015/cobimetinib (scale bars, 50uM). HE: hematoxylin and eosin staining. Bottom, box plots representing intensity score of protein expression (n=3, two-tailed Student t test, P≤0.001).

4.4.4 Concomitant DUT and TYMS knockdown is synthetically lethal in ovarian cancer cells

Our study revealed that DUT and TYMS are likely involved in the anti-tumor response to the combination of OTX015/cobimetinib. TYMS catalyzes thymidine DNA synthesis through conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP)

(353-355). TYMS is found over-expressed in some cancers and appears to promote proliferation and may be correlated with poor clinical outcomes (356,357). The inhibition of TYMS promotes metabolic imbalance leading to aberrant incorporation of nucleotides during DNA replication resulting in cell cycle arrest (358). TYMS is a target of the potent, and widely used, chemotherapeutic 5-fluorouracil and targeted TYMS inhibitors have also been explored clinically (359,360). The enzyme DUT is responsible for the hydrolysis of dUTP to dUMP which is subsequently further metabolised by TYMS (361). It has been shown that DUT overexpression conferred resistance to TYMS inhibitors (349). Thus, concomitant inhibition of both DUT and TYMS might represent a logical approach to anti-cancer treatment because this will lead to DNA replication arrest and ultimately cell death. Indeed, this approach was found to be synthetically lethal in NSCLC models by using siRNAs (346). Additionally, a new DUT inhibitor, TAS-114 also showed synergy with TYMS inhibitor, 5-FU in a number of cancer cell lines (362).

To validate whether synthetic lethality between TYMS and DUT represent a potential mechanism whereby OTX015/cobimetinib acts in synergy, we utilized two independent lentiviral shRNAs targeting DUT and TYMS either alone, or in combination, and carried out cell viability analysis for 5 days in OVK18 cells (Fig.20A). Consistent with the studies mentioned above, we observed a significant synergy between shDUT and shTYMS using all four combinations. Notably, knockdown of DUT or TYMS alone did not exert substantial anti-proliferative effects in this setting, highlighting the limitations of targeting either of these enzymes as monotherapy in ovarian cancer. This study confirms that DUT and TYMS down-regulation is at least partially responsible for the anti-proliferative effects of OTX015/cobimetinib combination.

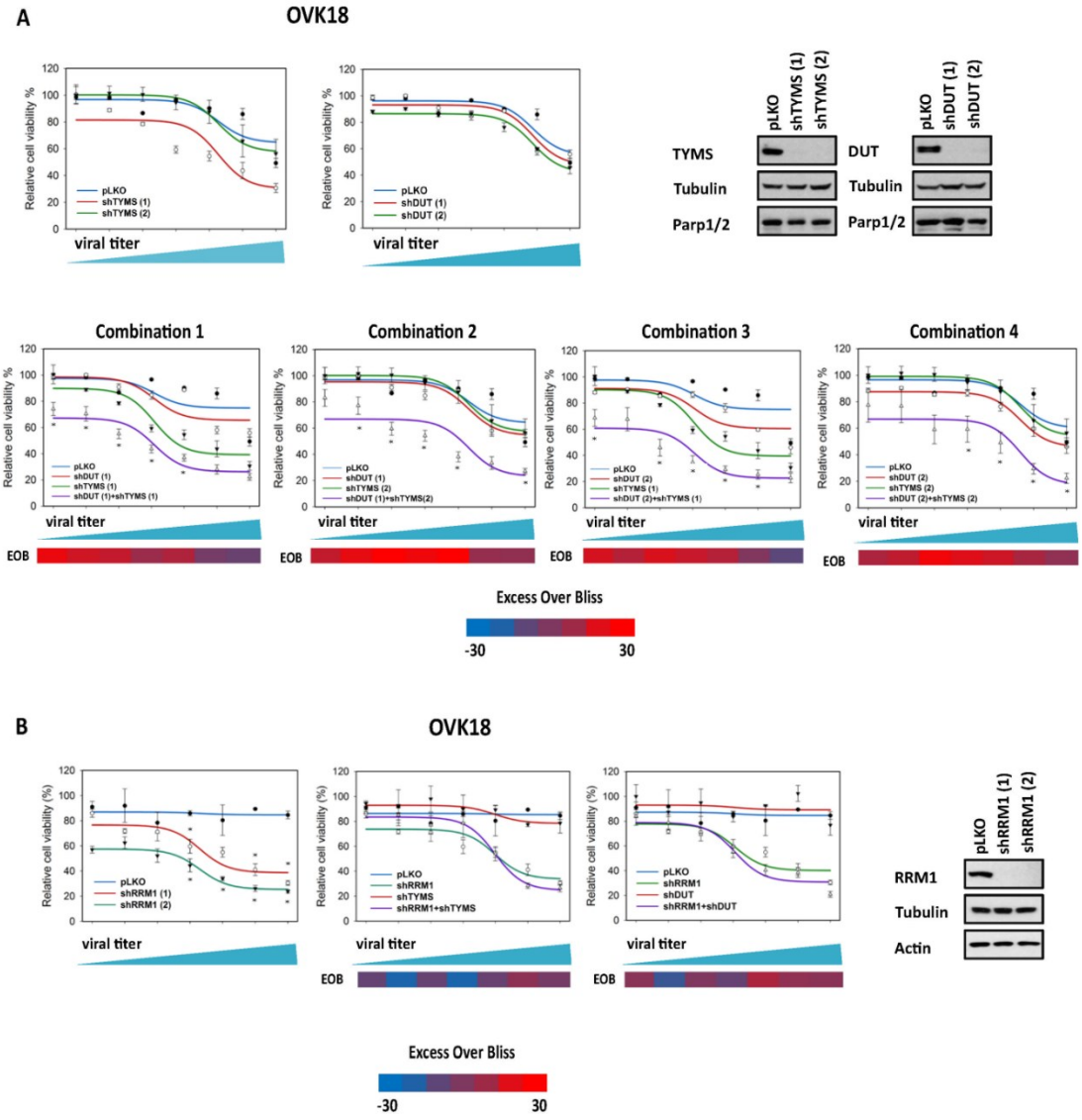


Figure 20. Knockdown studies of TYMS, DUT and RRM1 in ovarian cancer cells. A, Top right, Western blotting analysis of TYMS and DUT expression after shRNA knockdown. Top left, cell viability assay in response to 5 days of TYMS and DUT knockdown using increasing viral titer. Bottom, cell viability analysis of 5 days for knockdown combinations of TYMS and DUT (n=3, error bars: SEM, two-tailed Student t test, *P≤0.05). OVK18: SCCOHT cells; pLKO: control vector; shTYMS, shDUT: shRNAs targeting TYMS and DUT; EOB: excess over bliss synergistic efficiency. **B,** Right, Western blotting analysis showing RRM1 depletion in OVK18 cells upon RRM1 knockdown. Left, cell viability analysis of cells after 5 days of RRM1 knockdown alone or in combinations with either shTYMS or shDUT at increasing viral titer. (n=3, error bars: SEM, two-tailed Student t test, *P≤0.05). pLKO: control vector; shRRM1, shTYMS, shDUT: shRNAs targeting RRM1, TYMS and DUT; EOB: excess over bliss

Another potential mediator of the anti-proliferative activity of BETi/MEKi identified from our mass spectrometry experiments is RRM1. This is the largest subunit of the ribonucleotide complex (RNR), formed with the smaller subunit, RRM2 (347). RRM1 participates in DNA synthesis by catalyzing the production of mature deoxyribonucleotides (dNTPs) from ribonucleotides. High RRM1 expression is associated with dismal prognosis in lung and pancreatic cancer (347,363,364), underscoring its potential as an anti-cancer target. We again employed a lentiviral shRNA approach to target RRM1 and we observed that 5 days of RRM1 depletion effectively reduced the viability of OVK18 cells without the depletion of additional factors (Fig.20B). Next, we performed concomitant knockdown for either TYMS and RRM1 or DUT and RRM1. However, we did not observe a synergy between these shRNA combinations, likely due to effects of RRM1 alone being quite strong. Again, these data suggest that altered nucleotide metabolism plays a critical role in compromising cell proliferation after exposure to BETi/MEKi.

4.4.5 BETi/MEKi combination induces cell cycle arrest and depletes the pool of nucleotide precursors

The enzymes DUT, TYMS and RRM1 are responsible for nucleotide synthesis and potentiate DNA replication and progression through S-phase of cell cycle. To examine the impact of OTX015/cobimetinib treatment on cell cycle profiles we treated OVK18 cells with DMSO, OTX015, cobimetinib alone or in combination for 3 days and analyzed the cell cycle using two complementary techniques, PI staining and BrdU incorporation (Fig.21A,B). Both approaches showed a decrease in S-phase in the combination setting. BrdU incorporation offered a more refined S phase profile and suggested that the reduced number of cells detected in S phase is primarily due to a lack of progression to late S-phase. This supports a model where DNA replication is initiated, but quickly stalls due to either the misincorporation of nucleotides or lack of available nucleotides.

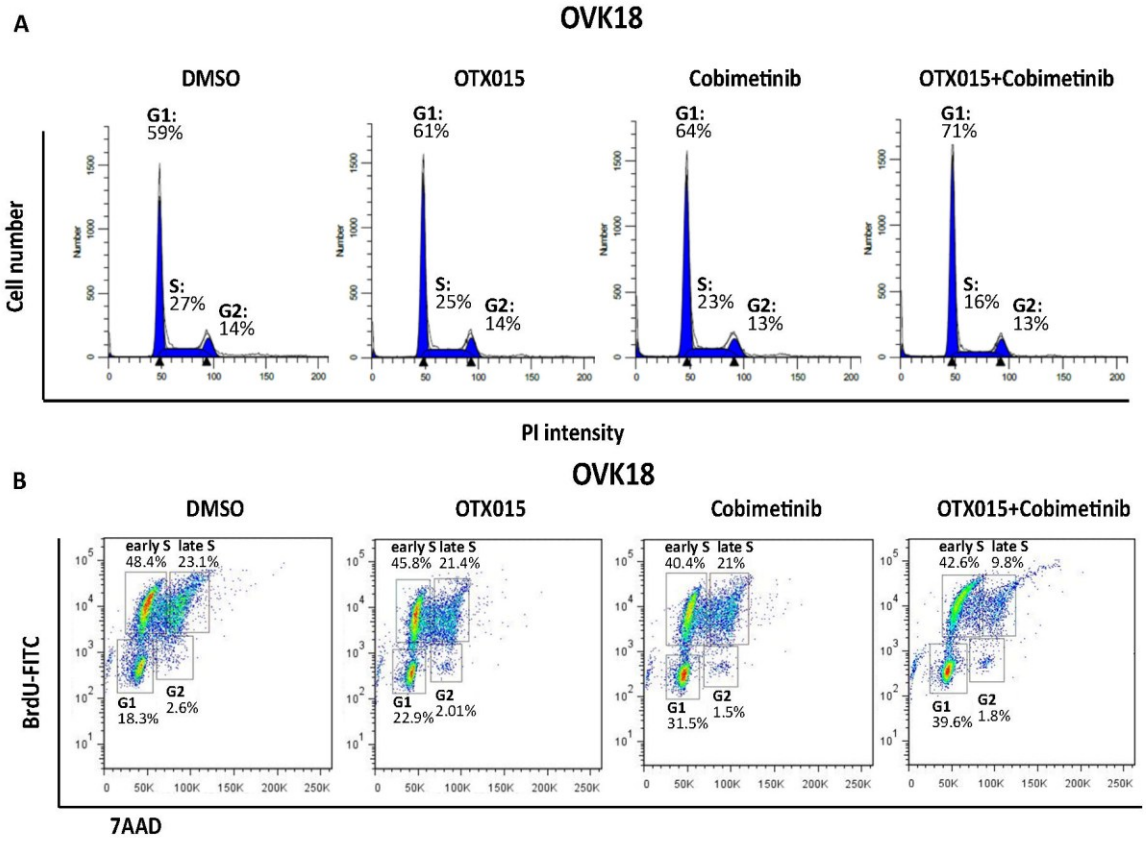

A
OVK18
DMSO
OTX015
Cobimetinib
OTX015+Cobimetinib
G1: 59%
S: 27%
G2: 14%
G1: 61%
S: 25%
G2: 14%
G1: 64%
S: 23%
G2: 13%
G1: 71%
S: 16%
G2: 13%
Cell number
PI intensity
B
OVK18
DMSO
OTX015
Cobimetinib
OTX015+Cobimetinib
early S late S
48.4% 23.1%
G1 18.3%
G2 2.6%
early S late S
45.8% 21.4%
G1 22.9%
G2 2.01%
early S late S
40.4% 21%
G1 31.5%
G2 1.5%
early S late S
42.6% 9.8%
G1 39.6%
G2 1.8%
BrdU-FITC
7AAD
C
OVK18
OTX+Cobi/DMSO
Log2 fold change
-6
-2
0
2
6
Guanine***
Guanosine*
Adenosine**
Cytidine*
Cytosine**
Uridine*
2'-Deoxyadenosine***
2'-Deoxyguanosine**
GDP**
GMP*
ADP***
AMP***
CDP**
UMP**
UDP**
IMP*
dGMP**
dTMP
2'-Deoxycytidine
dUMP
TTP
FAD
UDP-N-Acetyl Glucosamine
Creatiine
GTP
CTP
cAMP
dATP
dCTP
dGTP
dTTP
NAD
NADP
ATP**
UTP**
D
Enrichment overview (top 30)
Purine Metabolism
Pyrimidine Metabolism
Lactose Synthesis
Thiamine Metabolism
Selenoamino Acid Metabolism
Aspartate Metabolism
Alanine Metabolism
Pyruvate Metabolism
Riboflavin Metabolism
Glutamate Metabolism
Pantothenate and CoA Biosynthesis
Cysteine Metabolism
Pentose Phosphate Pathway
Urea Cycle
Starch and Sucrose Metabolism
Ammonia Recycling
Citric Acid Cycle
Amino Sugar Metabolism
Gluconeogenesis
Nicotinate and Nicotinamide Metabolism
Galactose Metabolism
Sphingolipid Metabolism
Phenylacetate Metabolism
Lactose Degradation
Propanoate Metabolism
Methionine Metabolism
Histidine Metabolism
Trehalose Degradation
Phosphatidylethanolamine Biosynthesis
Arginine and Proline Metabolism
Phosphatidylcholine Biosynthesis
0 2 4 6 8 10 12 14
Fold Enrichment
P value
1e-08
2e-01
3e-01

Figure 21. BETi/MEKi combination induces inhibition of S-phase of cell cycle and down-regulates nucleotide precursors pool. A, Cell cycle analysis by PI staining of OVK18 cells after 3 days of exposure with DMSO, OTX015 (at 100nM), cobimetinib (at 100nM) and OTX015/cobimetinib. **B,** Cell cycle analysis by BrdU-FITC in OVK18 cells treated with DMSO, OTX015 (at 100nM), cobimetinib (at 100nM) and OTX015/cobimetinib for 3 days. **C,** Heatmap demonstrating results from LC-MS metabolomics profiling of OVK18 metabolites after treatment for 48 hours with DMSO or OTX015/cobimetinib combination at 200nM concentration for each compound (n=3, log_2-fold change >0.5, two-tailed Student t test, *P≤0.05, **P≤0.01, ***P≤0.001). **D,** Metaboanalyst pathway analysis for metabolites changed in response to OTX015/cobimetinib treatment in OVK18 cell line (P value<0.05).

Our cell cycle and protein profiling data strongly suggested that nucleotide pools might be altered within the cells in response to combination treatment. To explore this hypothesis, we utilized LC/MS metabolomics profiling to examine the steady state levels of 35 metabolites, including nucleotides, nucleosides and their metabolic precursors in OVK18 cells treated with DMSO, or OTX015/cobimetinib, for 48 hours (Fig.21C). This analysis revealed a marked decrease to a spectrum of both pyrimidines and purines, beyond the expected changes such as reduced dTMP, a product of TYMS activity. DUT, TYMS and RRM1 are all involved in *de novo* nucleotide synthesis and the profile we observed likely reflects an attempted compensation through the nucleotide salvage pathway at this early time point (see discussion). Metaboanalyst Pathway analysis of significantly regulated metabolites also predicted the enrichment for changes in purine and pyrimidine metabolism (Fig.21D). These data strengthen the concept that the anti-proliferative effects of OTX015/cobimetinib in combination are mediated through extensive metabolic reprogramming and down-regulation of nucleotide synthesis.

4.5 Discussion

Small Cell Carcinoma of the Ovary, Hypercalcemic Type (SCCOHT) is an extremely aggressive cancer where conventional therapy has proved to be largely ineffective, with only about one-third of those diagnosed at an early stage experiencing long term survival.(33). Recent studies of SCCOHT resulted in the discovery that it is a virtually monogenic disorder, with germline and/or somatic mutation in the *SMARCA4* gene accounting for nearly all cases (38). These mutations are usually deleterious leading to a complete loss of the protein detected by IHC analysis in almost 100% of cases (48). Coupled with this loss, the SMARCA4 paralogue, SMARCA2, is invariably lost as well. This unique genetic signature of SCCOHT led to the rational

development of new approaches to treat SCOOHT in preclinical studies. We previously found that SCCOHT and NSCLC SMARCA4/A2-deficient tumors are highly sensitive to epigenetic drugs targeting bromodomain proteins (BETi) (172). In parallel with the significant anti-tumor BETi response observed in the SMARCA4-deficient cancer models, we also found that SMARCA4-expressing ovarian and lung cancer cells are intrinsically resistant to BETi.

One of the anti-proliferative signatures of BETi response in sensitive cells, based on our RNAseq data, was the downregulation of RTK-dependent PI3K-AKT and RAS-MAPK signaling. In particular, inactivation of the MAPK pathway was strongly repressed by BETi in sensitive cells (172), but not observed in the SMARCA4-expressing, BETi resistant cells. Thus, MAPK repression may act as a pharmacodynamic biomarker predicting sensitivity to BETi and constitutive MAPK activity may represent a possible mechanism whereby cells resist the anti-proliferative effects of BETi. These findings are consistent with additional reports that indicate signaling through PI3K and MAPK impart both intrinsic and acquired resistance to BETi (284,285,288).

BET inhibitors have been widely studied in preclinical settings in numerous types of cancer, but the drug concentrations used in many of these studies were far above the nanomolar concentrations required to inhibit BRD4 functions. (242,273). As a result, the biomarkers identified in preclinical studies have not proven robust in a clinical setting and these clinical trials have not met expectations (279,280,299). Thus, there is an urgent need to find biomarkers predicting sensitivity to BETi and to uncover therapeutic avenues to overcome intrinsic resistance to BETi. Combination treatment of BETi with inhibitors of RTK-dependent signaling potentially represents a rational approach to more effectively switch off oncogenic signaling and overcome resistance to BETi. Here, amongst all the tested inhibitors of RTK, PI3K and MAPK pathways, we found the most significant synergy between BETi and MEK inhibitors in both SMARCA4-deficient and SMARCA4-expressing *in vitro* and *in vivo* ovarian tumor models. Our results are consistent with previous reports showing synergistic efficiency between these types of compounds in *NRAS*-mutant melanoma (287). Thus, our data, and those of others, highlight that this approach might hold a broad clinical relevance for diverse, aggressive cancers.

Currently, the mechanism through which BETi and MEKi act in synergy is uncertain. Our SILAC-based mass spectrometry experiments pinpointed rate limiting enzymes involved in nucleotide metabolism including TYMS, DUT and RRM1 being strongly repressed by BETi/MEKi. These targets are necessary for DNA replication and their depletion is expected to prevent progression through S phase due to a lack of available nucleotides or misincorporation of nucleotides, both leading to DNA damage and stalled replication forks (361,365-368). Our data suggests TYMS may be regulated through proteasomal degradation. TYMS is known to be a target for ubiquitylation (352,353), which we suggest is precipitated by the combination therapy. Future work will aim to identify the ubiquitin ligase targeting TYMS after co-treatment with BETi/MEKi, but a candidate ligase of TYMS, UBE2K has already been identified (369).

Clinically approved pyrimidine analogues that inhibit TYMS, such as 5-fluorouracil (5-FU), are being used in the treatment of different types of cancer (370-373). However, overexpression of TYMS and DUT results in the resistance to this therapy (348,361). Because DUT and TYMS function within the same essential pathway, dual TYMS/DUT inhibition might hold promise for anti-cancer treatments through effective dampening of nucleotide synthesis. Consistent with this, it has been shown that concomitant down-regulation of TYMS and DUT leads to a synthetic lethality in NSCLC (346). Recently, a new DUT inhibitor, TAS-114 in combination with a novel TYMS inhibitor, capecitabine which is selectively converted to 5-FU in tumors, entered to clinical trials against solid tumors (374). These results are consistent with our data, where OTX015/cobimetinib down-regulates TYMS/DUT expression and decreases cancer cell proliferation. This is supported by dual knockdown experiments showing synergy between TYMS and DUT depletion in ovarian cells. Our data also indicates that BETi or MEKi might be combined with already clinically approved TYMS inhibitors or novel DUT inhibitors in order to elicit anti-tumorigenic effects. RRM1 represents another repressed target of OTX015/cobimetinib that participates in DNA synthesis (347). The significant loss of proliferation upon depletion of RRM1 suggests RRM1 inhibitors hold great potential as anti-cancer therapy, either as solo agents, or perhaps in combination with DUT or TYMS inhibitor. It has been shown that ERK inhibition along with the mTOR inhibitor Evrolimus overcame resistance in renal cell carcinoma by reducing nucleotide pools as a result of RRM1 inhibition (375). This consistent with our data indicating that nucleotide reprogramming is critical

determinant of the growth inhibitory properties of BETi/MEKi.

Nucleotides for DNA synthesis may be formed through either *de novo* synthesis or the nucleoside salvage pathway (376,377). For the *de novo* pathway, glucose is converted to phosphoribosyl diphosphate (PRPP) which subsequently leads to the synthesis of purine and pyrimidine precursors, IMP and UMP respectively. This leads to NDP (ribonucleotides) production, that are in turn converted by RRM1 to dNDPs utilized for the synthesis of dNTPs. All three enzymes, RRM1, DUT and TYMS are involved in *de novo* DNA synthesis (346,347,361). The salvage pathway utilizes early precursor nucleosides (dNs) as substrates to produce DNA through multiple enzymatic reactions. These two pathways are interconnected and the salvage pathway has been shown to compensate for the *de novo* pathway in case of its inhibition (378). As TYMS and DUT were down-regulated in the response to combination therapy, we expected to see a substantial depletion of dUMP and dTMP, the products of TYMS and DUT dependent activity. At the timepoint we chose, there is indeed a tendency toward dUMP and dTMP decrease though they are not as significant as the changes observed for the early precursors. This suggests that the *de novo* pathway might be inhibited but a later time-point would be required to see the fully executed metabolomic reprogramming. It is likely that flux through the salvage pathway is enhanced to compensate for supressed *de novo* DNA synthesis. In this situation, cells are slowly depleting their nucleotide precursors to maintain dNTP levels, resulting in the stark loss of the precursor pools that we observed in our metabolic analysis.

Overall, this work demonstrates that the synergy between BETi/MEKi in ovarian cancer models is mediated by the down-regulation of multiple, critical regulators of nucleotide metabolism. Considering the sub-optimal concentrations of drugs required to elicit anti-tumor effects, this combination appears to hold great promise for the treatment of poor prognosis cancers.

4.6 Materials and methods

4.6.1 Cell culture

The OVK18 cell line was received from the RIKEN cell bank. The SCCOHT1 cell line was a gift from Dr. Ralf Hass (Hannover Medical School, Hannover, Germany). OVCAR4, OVCAR3, SKOV3, IGROV1 and HEK293T were purchased from the ATCC. The cell lines were grown in RPMI-1640 medium supplemented with 10% FBS. The culture medium for HEK293T cells was DMEM with 10% FBS. The cell lines were cultured for a maximum of three weeks. The cell lines were maintained at 37^0C in an atmosphere of 5% CO_2.

4.6.2 Lentiviral production and cells transduction

Lentiviral production was carried out in HEK293T cells. $5.5x10^6$ cells were seeded in 100mm plate. On the next day, the cells were transfected with 5ug of Pax2 packaging vector, 2ug of MD2G envelop vector and 7ug of plasmid DNA. Plasmids were incubated with transfection agent PEI at 1mg/mL for 10-15min and added to the cells. In 3 days, the viruses were collected and used for experiments. For TYMS, DUT and RRM1 knockdown experiments, $0.5x10^6$ of OVK18 cells were seeded in 6 well-plate. Cells were incubated with 0.5mL of virus and 2.5mL of media with polybrene at 5ug/mL. The selection with 1ug/mL of puromycin was carried out after 48 hours of viral exposure and the media was changed. After selection and 5 days of knockdown, the cells were used for protein expression analysis. KRAS (G12D) and BRAF (V600E) stable OVK18 and SCCOHT1 cell lines were generated by the incubation with respective viral suspensions at 1:3 ratio (1mL of virus/3mL of media) and polybrene. KRAS stable cells were elected with 50ug/mL of hygromycin. GFP-positive BRAF expressing cells were selected by FACSAria Fusion cell sorting.

4.6.3 Plasmids

For ectopic expression of KRAS, pLenti-PGK-KRAS4B(G12D) (Addgene, #35633) was employed. BRAF plasmid was a gift from Ian Watson (Goodman Cancer Center, McGill University) and was generated by subcloning BRAF(V600E)-HA tagged into backbone vector pHAGE-EF1a. pReciever-LV120 (GeneCopeia, #EX-EGFP-Lv120) was used as a control

vector. shRNA plasmids against DUT, TYMS and RRM1 were purchased from Sigma (Supplementary Table S4).

4.6.4 Compounds

OTX015, cobimetinib, trametinib, copanlisib, lapatinib, patritumab and cisplatin were purchased accordingly: MedChem Express (#HY-15743), Selleckchem (#S8041), Selleckchem (#S2673), Selleckchem (#S2802), Selleckchem (#S2111), Creative Biolabs (#TAB-189) and Accord (#DIN 02355183).

4.6.5 Cell viability assay

Ovarian cancer cells were seeded in 48-well plates at 500-1000 cells per well and exposed for 5 days to indicated concentrations of OTX015, cobimetinib, trametinib, copanlisib, patritumab, lapatinib either alone or in combination. 0.01% DMSO was used as a control. For knockdown studies OVK18 cells were plated and transduced with shDUT, shTYMS, shRRM1 either separately or in combination at increasing viral titer (virus-media: 1:20, 1:15, 1:10, 1:8, 1:6, 1:4, 1:3). The next day, selection with puromycin was performed and cell viability analysis was carried out at day 5 after transduction. pLKO was used as a control vector. For ectopic expression studies, OVK18 and SCCOHT cell lines with stable expression of LV120 (control vector), KRAS (G12D) and BRAF (V600E), were plated and treated for 5 days with a 5-500nM range of concentrations of OTX015. For cell viability analysis, cells were fixed with 4% formaldehyde/1xPBS for 10min and stained with 0.1% crystal violet/10% ethanol solution for 30min. The de-staining step was carried out with 10% acetic acid and absorbance was measured with spectrophotometer Perkin Almer EnSpire.

4.6.6 RNA extraction and Reverse Transcription

RNA extraction was performed with Gene Elute Mammalian Total RNA kit (Sigma, cat #RTN350). 1ug of RNA was used for reverse transcription reaction carried out with 5X All-In-One RT MasterMix (ABM cat #G490) according commercial guidelines. qPCR reaction was carried out with GoTaq® qPCR Master Mix (Promega, cat #A6002) following to commercial

protocol. Amplification of PCR product was performed with specific primers listed in Supplementary Table S5. mRNA expression was normalized to DMSO control and expression of human 36B4, RPLP0, RPL4 and HSPCB.

4.6.7 SILAC mass spectrometry

OVK18 cells were cultured for six passages in light (R0K0), medium (R6K4) and heavy (R10K8) isotope containing media provided by François-Michel Boisvert (Department of Anatomy and Cell Biology, Université de Sherbrooke). Next, the cells were exposed to 0.01% DMSO, OTX015 (200nM), cobimetinib (200nM) alone or in combination. After 24 hours of treatment, the cell pellets were frozen in liquid nitrogen and send for the SILAC analysis to the Department of Anatomy and Cell Biology, Université de Sherbrooke. The analysis was performed precisely as previously described (379). Perseus program was used for statistical analysis. The data was obtained from 3 replicates with $\log_2$-fold change >0.5 and P value ≤0.05. Volcano plots were generated with Instant Clue. The data are available at the PRIDE database (accession #PXD017581).

4.6.8 Western blotting

Whole-Cell Lysis buffer (20mM Tris pH 7.5, 420mM NACl, 2mM $MgCI_2$, 1mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 0.5% Triton, fresh 1mM DTT, protease and phosphatase inhibitor cocktail, Beta Glycerol Phosphate, NaF) was added to cell pellets. Samples were kept on ice for 30min and centrifuged at maximum speed for 15min at 4^0C. Lysates were collected and protein concentration was analyzed by Bradford assay. 30ug of protein were loaded into 8% polyacrylamide gel. Proteins were transferred to membrane overnight at 30 volts at 4^0C. Washes of membrane were performed 3 times (5, 10, 15 min) in TBST (20mM Tris base, 137mM NaCl, 0.1% Tween 20). 5% milk/TBST was used to block membranes for 1 hour at room temperature. Membrane was incubated with primary antibody overnight at 4^0C and washed 3 times as mentioned above. Incubation with secondary antibody was carried out for 1 hour at room temperature. Membrane was analyzed with a Clarity Western ECL kit (Biorad).

4.6.9 In vivo experiments

Animal experiments were performed following guidelines of the Canadian Council of Animal Care and approved by the Animal Resources Centre (ARC) at McGill University. 5-week-old female NOD/SCID mice were injected with $5x10^6$ of OVK18 cells or $1x10^7$ of OVCAR4 cells in 1xPBS into left ovary. Treatments with vehicle, 20mg/kg/day of OTX015 and 5mg/kg/day of cobimetinib alone or in combination were initiated after 3 weeks of tumor cells injection. Each treatment group contained 5 mice. The compounds were administered by oral gavage for a period of 3 weeks. After this period of time, mice were euthanized and tumors were collected and measured. The calculation of tumor volume was performed by using following formula: $½ \times ((\text{length in mm}) \times (\text{width in mm})^2)$. Immunohistochemistry analysis was performed at the Segal Cancer Centre Research Pathology Facility (Jewish General Hospital) as previously described (331). Xenograft tumor sections were incubated with TYMS (at 1:50 dilution), DUT (at 1:25 dilution) and RRM1 (at 1:25 dilution) rabbit antibodies. Slides were scanned in low power field to choose the most stained area. TYMS and DUT protein expression was distributed homogeneously within the tumor tissues. RRM1 expression was mostly localized at the tumor boarder representing ≈10% of the tissue. Sections were analyzed and the final score was obtained by normalizing to the average of the total intensity. The staining intensity was quantified by ImageJ and assigned by using five-tiered system (0=negative, 1=very weak, 2=weak, 3=moderate, 4=strong, 5=very strong staining).

4.6.10 Cell cycle analysis

OVK18 cells were seeded in 6-well plates and exposed to DMSO, OTX015 (200nM), cobimetinib (200nM) and OTX015/cobimetinib for 3 days. The cell cycle analysis was performed via propidium iodide staining as described previously (328). For BrdU proliferation assay, the cells were incubated with 7uM of BrdU for 24 hours prior the analysis. The BrdU analysis was assessed by using commercial kit (BD FITC BrdU Flow kit, #51-2354AK). Early and late S-phases were sub-divided by low and high 7AAD content. ModFit and FlowJoe were used for the analysis.

4.6.11 MG132 protein degradation experiments

OVK18 cells were plated and treated with 7uM of MG132 protease inhibitor for a period of 3, 7 and 12 hours. Next, the cells were exposed for 24h to DMSO, OTX015 (200nM), cobimetinib (200nM) and OTX015/cobimetinib combination. Protein expression was analyzed by Western Blotting as described above.

4.6.12 LC/MS metabolomic studies

$2.5x10^6$ of OVK18 cells were plated in 10cm petri dishes and treated for 48 hours with DMSO or OTX015/cobimetinib combination at 200nM concentration for each compound. Media was washed from adherent cells (4.3 million per plate) using ice cold 150 mM ammonium formate pH 7.4. Cells were then scraped into 380 µL of 50% methanol/water to which 220 µL of ice-cold acetonitrile was added. Cells were then subjected to bead beating for 2 min at 30 Hz (Eppendorf Tissue-lyser). Lipids were partitioned through the addition 600 µL of cold dichloromethane and 300 µL of cold H_2O. The upper aqueous layer was then removed and dried using a vacuum centrifuge with sample temperature maintained at -4°C (LabConco). Samples were resuspended in 25 µL of water and subjected to LC-MS analysis. For nucleotide analysis, a 10x dilution was prepared by adding 3 µL of sample to 27 µL of water. The relative concentrations of the targeted nucleotides and deoxynucleotides were measured using a triple quadrupole mass spectrometer (QQQ 6470) equipped with a 1290 ultra high-pressure liquid chromatography system (Agilent Technologies, Santa Clara, California, USA). Chromatographic separation was achieved using a Scherzo SM-C18 column 3 µm, 3.0×150 mm (Imtakt Corp, JAPAN). The chromatographic gradient started at 100% mobile phase A (5 mM ammonium acetate in water) with a 5 min gradient to 100% B (200 mM ammonium acetate in 20% ACN / 80% water) at a flow rate of 0.4 mL/min. This was followed by a 5 min hold time at 100% mobile phase B and a subsequent re-equilibration time (6 min) before next injection. In order to ensure proper instrumental duty cycle, samples were injected twice: nucleotide analysis followed by deoxynucleotide analysis. A sample volume of 5 µL was injected for each run. Multiple reaction monitoring (MRM) transitions were optimized on standards for each metabolite measured. MRM transitions and retention time windows are summarized in Supplementary Table S6. An Agilent JetStream™ electro-spray ionization source was used in positive ionization mode with a gas temperature and flow were set at 300°C and 5 L/min respectively, nebulizer pressure was set at 45 psi and capillary voltage was set at 3500 V. Relative concentrations were determined from external

calibration curves prepared in water. Ion suppression artifacts were not corrected; thus, the presented metabolite levels are relative to the external calibration curves and should not be considered as absolute concentrations. Data were analyzed using MassHunter Quant (Agilent Technologies). All LC-MS grade solvents and salts were purchased from Fisher (Ottawa, Ontario, Canada): water, acetonitrile (ACN), methanol (MeOH), formic acid, ammonium acetate and ammonium formate. The authentic metabolite standards were purchased from Sigma-Aldrich Co. (Oakville, Ontario, Canada).

4.6.13 Antibodies

Antibodies for Western Blotting were purchased as follows: anti-DUT (abcam, #ab229122), anti-TYMS (abcam, #ab108995), anti-RRM1 (Cell Signaling, #8637), anti-BRAF V600E (abcam, #200535), anti-KRAS G12D (Cell Signaling, #14429), anti-α-tubulin (DSHB #12G10-s1ea), anti-β-Actin (Sigma, #A5316), anti-c23-MS3-nucleolin (Santa Cruz, #sc8031), anti-AKT (Cell Signaling, #2920s), anti-pAKT (Cell Signaling, #4060s), anti-S6 (Cell Signaling, #2217s), anti-pS6 (Cell Signaling, #2215s), anti-ERK1/2 (Cell Signaling, #4695s), anti-pERK1/2 (Cell Signaling, #9101s).

4.7 Supplementary Figures and Tables

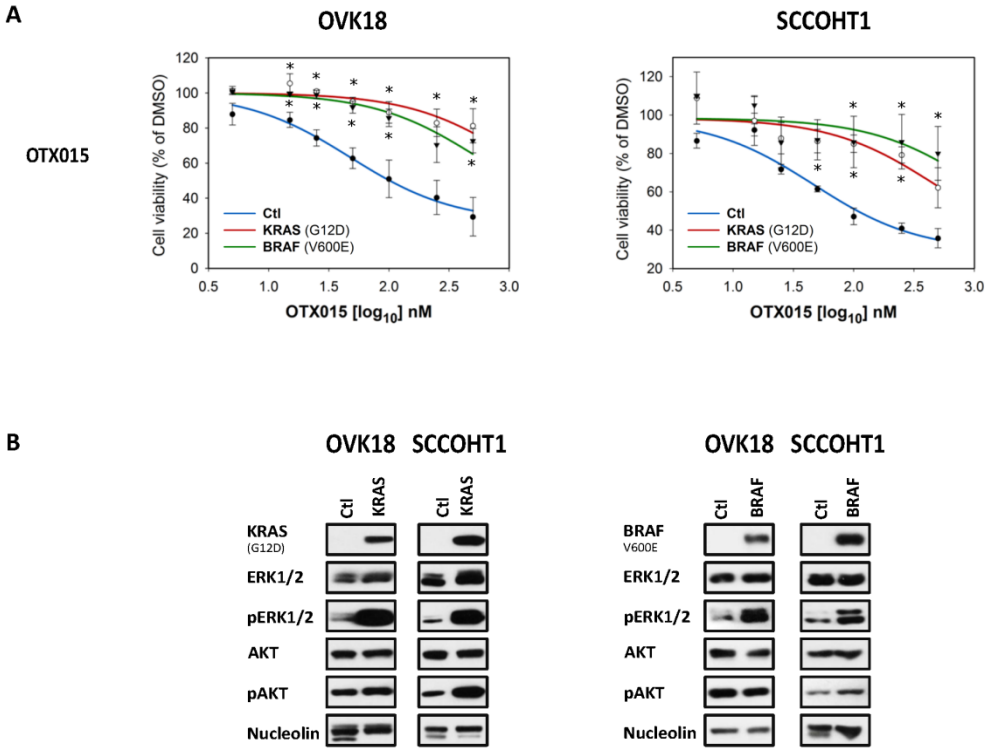
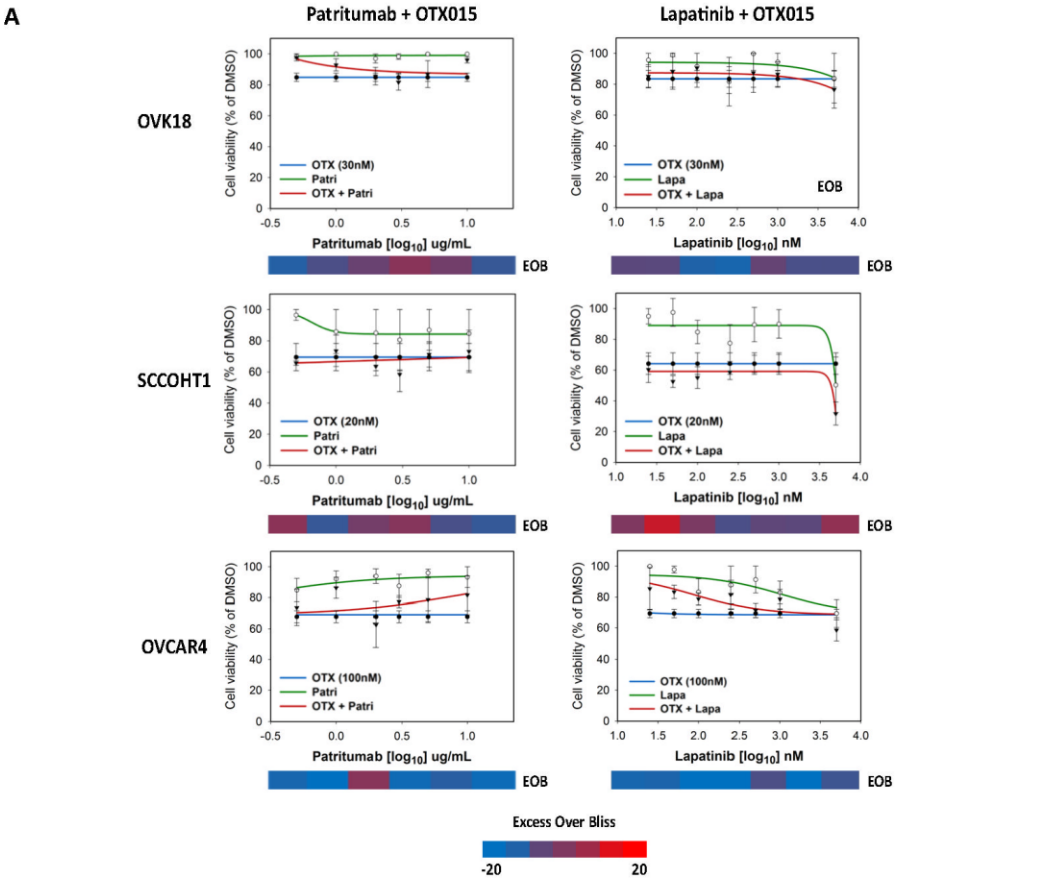


Figure S6. Re-expression of KRAS and BRAF confers resistance to BETi. A, Cell viability assay of OVK18 and SCCOHT1 cells with ectopic expression of KRAS (G12D) or BRAF (V600E) mutants and exposure to OTX015 for 5 days at 5-500nM concentrations (n=3, error bars: SEM, two-tailed Student t test, *P≤0.05). Ctl: control vector; KRAS (G12D): KRAS mutant vector; BRAF (V600E): BRAF mutant vector. **B,** Bottom, protein expression analysis of KRAS, BRAF and downstream effectors upon ectopic expression of mutant vectors in OVK18 and SCCOHT1 cells.


A
Patritumab + OTX015
Lapatinib + OTX015
OVK18
SCCOHT1
OVCAR4
Cell viability (% of DMSO)
OTX (30nM)
Patri
OTX + Patri
OTX (20nM)
OTX (100nM)
Lapa
OTX + Lapa
Patritumab [log10] ug/mL
Lapatinib [log10] nM
EOB
Excess Over Bliss
-20
20

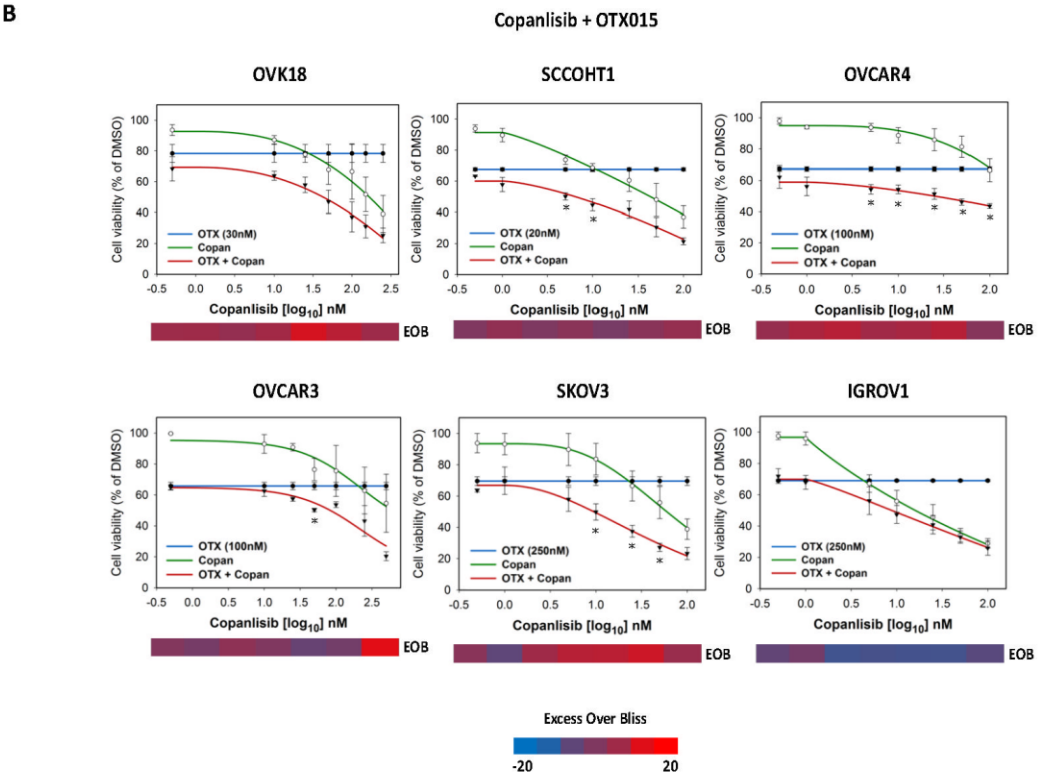

B
Copanlisib + OTX015
OVK18
SCCOHT1
OVCAR4
OVCAR3
SKOV3
IGROV1
Cell viability (% of DMSO)
OTX (30nM)
OTX (20nM)
OTX (100nM)
OTX (250nM)
Copan
OTX + Copan
Copanlisib [log10] nM
EOB
Excess Over Bliss
-20
20

Figure S7. Response of ovarian cancer cells to combination treatments of BETi with inhibitors of RTK-dependent signaling. A, 5 days cell viability analysis of ovarian cancer cells treated with OTX015 (at 20-100nM) alone or in combination with patritumab (anti-HER3 antibody, at 0.5-10ug/mL) or lapatinib (HER2/EGFR inhibitor, at 25nM-5uM) (n=3, error bars: SEM). OVK18, SCCOHT1: SCCOHT cells; OVCAR4: ovarian adenocarcinoma cells; EOB: excess over bliss synergistic efficiency. **B,** Cell viability analysis of ovarian cancer cells upon 5 days of exposure with OTX015 alone (at 20-100nM) or in combination with copanlisib (PI3K inhibitor, at 0.5-500nM) (n=3, error bars: SEM, two-tailed Student t test, *P≤0.05). OVK18, SCCOHT1: SCCOHT cells; OVCAR4, OVCAR3, SKOV3, IGROV1: ovarian adenocarcinoma cells; EOB: excess over bliss.

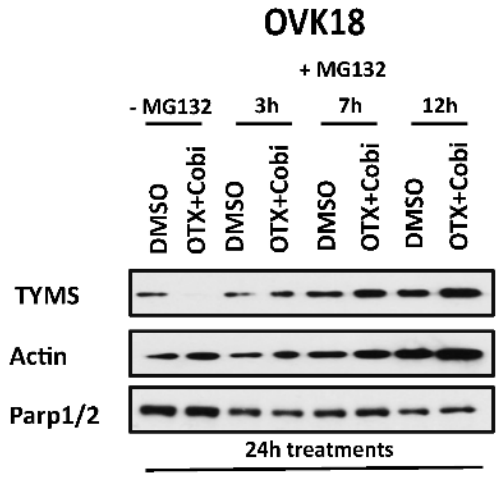


Figure S8. Protein degradation studies in OVK18 cells in response to BETi/MEKi treatments. Western blotting analysis showing protein expression for TYMS in OVK18 cells upon time course treatments with 7uM of MG132 and subsequent exposure to DMSO, OTX015 (200nM), cobimetinib (200nM) and OTX015+cobimetinib for 24 hours.

Table S4. List of shRNA vectors

Plasmid name	TRC ID	Clone Name
pLKO control vector	TRC1.5/PLKO.1	Empty vector
DUT (1)	TRCN0000431363	NM_001948.3-524s21c1
DUT (2)	TRCN0000412391	NM_001948.3-409s21c1
TYMS (1)	TRCN0000291720	NM_001071.2-358s21c1
TYMS (2)	TRCN0000045664	NM_001071.1-839s1c1
RRM1 (1)	TRCN0000038965	NM_001033.2-476s1c1
RRM1 (2)	TRCN0000038968	NM_001033.2-2471s1c1

Table S5. List of RTqPCR primers

Primer name	Sequence (5'-3')
TYMS Forward	CTCGGTGTGCCTTTCAACAT
TYMS Reverse	TGTGCATCTCCCAAAGTGTG
DUT Forward	AGCTGTTGTGAAAACGGACA
DUT Reverse	GTTTTGCAGCCAAGCCTGA
RRM1 Forward	CAGGATCGCTGTCTCTAACTT
RRM1 Reverse	AAGCATGAGTAAACCACCTCT
36B4 Forward	CGACCTGGAAGTCCAACTAC
36B4 Reverse	ATCTGCTGCATCTGCTTG
RPLP0 Forward	TTAAACCCTGCGTGGCAATCC
RPLP0 Reverse	CCACATTCCCCCGGATATGA
RPL4 Forward	GCTCTGGCCAGGGTGCTTTTG
RPL4 Reverse	ATGGCGTATCGTTTTTGGGTTGT
HSPCB Forward	AAGAGAGCAAGGCAAAGTTTGAG
HSPCB Reverse	TGGTCACAATGCAGCAAGGT

Table S6. List of targeted compounds

First Injection Compounds	Precursor ion	Quant transition	Qual transition	Approximate Retention time (min)
ADP	428	136	348	5.5
AMP	348	136	118.9	4.7
ATP	508	136	410	6.3
cAMP	330	136	118.9	6.2
CDP	404	112	69	4.8
CTP	484	112	97	5.6
FAD	786	348	439	7.3
GDP	444	152	97	5.6
GMP	364	152	97	4.1
GTP	524	152	134.9	6.2
IMP	349	137	110	4.1
NAD	664	136	428	4.6
NADP	744	136	604	5.0
dTMP	323	207	126.9	4.2
TTP	483	81	53	6.2
UDP	405	97	69	4.5
UDP-N-Acetylglucosamine	608	204	138	3.7
UMP	325	97	113	3.6
UTP	485	97	113	5.7
Second Injetion Compounds	**Precursor ion**	**Quant transition**	**Qual transition**	**Approximate Retention time (min)**
2-Deoxyadenosine	252	136	119, 117, 92	6.6
2-Deoxycytidine	228	112	95, 69	4.7
2-Deoxyguanosine/3-Deoxyguanosine	268	152	135, 110, 52	5.8
Adenosine	268	136	119, 94, 92	6.4
Cytidine	244	112	95, 69, 95, 52	4.2
Cytosine	112	95	52	3.8
dATP	492	136.1	119, 81, 53.2	6.9
dCTP	468	192	112.1, 95, 81.1	5.8
dGMP	348.1	152.1	135, 110, 81.1	5.0
dGTP	508	152.2	135, 110, 81.1	6.7
dTTP	483	81.1	53	6.2
dUTP	469	81.2	53	5.0
Guanine	152	135	110	5.4
Guanosine	284	152	135, 110, 55	5.4
Uridine	245	113	96, 70, 57	4.5

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Chapter V: General conclusion and Discussion

5.1 Discussion

The interest for this study originated from a recent report from the group of Dr. William Foulkes, and others, wherein it was discovered that SCCOHT is a monogenic disease attributable to germline and/or somatic mutations in a single gene, *SMARCA4* (38). Through whole exome sequencing (WES) of individuals from SCCOHT families, they discovered deleterious germline mutations in *SMARCA4* in all affected women. All tumours sequenced from familial cases harbored either a somatic mutation or loss of the wild-type allele in the tumour. SCCOHT are genetically uncomplicated tumors, and most tumors carry mutations/deletions exclusively within *SMARCA4*. Subsequent immunohistochemical analysis of these and additional cases showed the loss of SMARCA4 in practically all cases of SCCOHT, as well as coincidental lack of expression of the paralogue SMARCA2 (48). Thus, for my thesis SCCOHT served as an ideal model system to understand the role of SMARCA4 in tumor biology and to uncover therapeutic interventions to SMARCA4-deficient tumors, which is a common molecular abnormality across multiple tumor types including NSCLC (Non-Small Cell Lung Cancer).

SMARCA4 was predominantly considered to act as a tumor suppressor because *SMARCA4* loss-of-function mutations were found in multiple tumor types. In terms of the targeting *SMARCA4*-mutant deficient cancers, research concentrated on inhibiting the mutually exclusive paralogue SWI/SNF subunit, SMARCA2. The inhibition of SMARCA2 was found to be a synthetic lethality and preclinical models showed efficiency of this approach in some types of cancer suggesting a compensatory relationship between SMARCA4 and SMARCA2 in cancer (65,380). However, this promising approach has limitations. First, the ATPase catalytic domains of SMARCA2/4 proteins have proven difficult to target with small molecule inhibitors. The currently available SMARCA2/A4 inhibitor, PFI-3, targets the bromodomain region and has not met expectations in preclinical models. Both its potency and precision remain unclear (381). Next, many tumors show concomitant loss of both SMARCA4 and SMARCA2. In such tumors, inhibitors of these proteins would clearly be ineffective. Another targeting approach is related to Polycomb repressors. It has been shown that SMARCA4 conditional knockout contributes to

epigenetic reprogramming by inducing the enrichment of PRC1 and PRC2 occupancy at promoter regions (162). Thus, there exist antagonistic functions between SMARCA4 and PRC complexes at genes important for mediating the tumor suppressor functions of SMARCA4. Interestingly, *SMARCA4* mutations within the ATPase domain increase the occupancy of PRC1 and the accumulation of H3K27me3, a repressive mark placed by the PRC2 complex member, EZH2 (163-166). This model is consistent with the findings of efficiency of EZH2 inhibitors in preclinical SCCOHT models. While this approach holds some promise, these compounds exerted anti-proliferative effects at very high micromolar doses (332). Thus, the need for robust, tolerable compounds is highly anticipated. While the tumor suppressor role of SMARCA4 is widely studied, a growing body of evidence suggests that it may act as an oncogene by promoting cancer cell proliferation in a context dependent manner as discussed previously. Based on this concept, we explored new therapies against SMARCA4-deficient tumors.

In Chapter III, we described a novel approach for targeting SMARCA4-deficient SCCOHT and NSCLC models based on synthetic lethality between concurrent SMARCA4/A2 loss and another bromodomain protein, BRD4. Previous findings showed that esophageal *SMARCA4*-mutant cancers rely on BRD4 for cancer cell proliferation and that both SMARCA4 and BRD4 co-regulate MYC in an independent manner to promote acute leukemia (122,177). Building on these findings we explored the effect of BRD4 inhibition in SMARCA4-deficient cancers as it may completely deprive the SMARCA4/BRD4 dependent oncogenic network. These previous findings highlight the limitations of CRISPR and siRNA screens in that homologous proteins such as SMARCA2/SMARCA4 may compensate for one another. Likewise, BET family members may compensate for one another, underscoring the importance of pan-inhibitors that target multiple family members.

Through complementary cell viability and clonogenic assay approaches, we showed that SMARCA4-deficient SCCOHT and NSCLC cancers are hypersensitive to BET inhibitors at low nanomolar concentrations (172). These results were further extended using animal studies. We developed the first ovarian xenograft SCCOHT model and examined its sensitivity to BETi, and again, SCOOHT demonstrated high sensitivity to the treatment observed at a very low dose of 20mg/kg/day. The previously reported doses employed for the treatment of solid tumors were

significantly higher and this makes biomarker discovery challenging. A number of preclinical studies claim the down-regulation of c-MYC is the main pharmacological and predictive biomarker for BETi treatments (273,282). However, this was not always the case and other studies found that c-MYC expression, or repression, does not necessarily determine the response to BETi (327,382). This suggests other mechanisms an dpathways dictate BETi sensitivity. The lack of potential biomarkers led to clinical investigations of BETi with modest success where patients displayed severe side effects dictated by the high drug dose regiments that were utilized (279,280,299). c-MYC was not validated as a predictive biomarker to BETi. This underscores the importance of preclinical identification of BETi biomarkers using low doses in order to prevent future undesirable effects at the clinical level.

Our study also highlighted potential mechanisms of resistance to BETi. Ectopic expression of SMARCA4 resulted in resistance to BET inhibitors which again indicates that SMARCA4 loss may act as a potential biomarker for the therapeutic intervention with BETi. The down-regulation of the RTK, HER3 and PI3K-AKT, RAS-MAPK oncogenic signaling was one of the determinants to the sensitivity to BETi. Previous reports also showed that BETi downregulate effectors of PI3K and RAS signaling which supports our current investigations (284,285,288). HER3 re-expression experiments led to partial resistance to BETi. SMARCA4-expressing ovarian and lung cancer models were intrinsically resistant to BETi and did not show the inactivation of RTK-dependent signaling. This is consistent with reports indicating that the upregulation of RTK-dependent signaling is often associated with intrinsic and acquired BETi resistance (285,288). As discussed previously, while BETi showed preclinical efficiency, the majority of clinical studies were not successful raising the importance of identifying mechanisms of intrinsic resistance in order to optimize therapeutic strategies. We found that SMARCA4-expression conferred resistance and blocked the capacity of BETi to downregulate RTK oncogenic signaling. Thus, we hypothesized that possible combination therapies targeting RTK-dependent oncogenic transduction pathways might overcome this effect.

Based on these data, in Chapter IV, we focused on the combinatorial approach of the BETi, OTX015, with a panel of RTK-dependent inhibitors including PI3K and MEK inhibitors. From our cell viability screen performed on SMARCA4-deficient and SMARCA4-expressing ovarian

cancer cells, the BETi/MEKi combination displayed the most significant degree of synergy. This is consistent with our data from Chapter III where we found that the downregulation of RTK signaling went preferentially through the MAPK pathway in response to BETi treatments. Therefore, activation of the RAS-MAPK pathway may impart resistance to BETi. We also revealed that constitutively active KRAS and BRAF from the MAPK pathway confer resistance to BETi which solidifies these data. To further study this question, we analyzed not only SMARCA4-deficient SCCOHT but also SMARCA4-expressing intrinsically resistant ovarian adenocarcinoma models. Our results showed the synergistic efficiency between OTX015 and cobimetinib in both cases suggesting a strong potential for this combinatorial treatment in overcoming resistance mechanisms. Consistent with this, BETi/MEKi combination was shown efficient in *NRAS*-mutant melanoma models (287).

To uncover the mechanism of synergistic efficiency between OTX015 and cobimetinib, we conducted SILAC and metabolomics mass spectrometry experiments. The intriguing finding from this study was the downregulation of proteins involved in nucleotide metabolism and DNA synthesis, DUT, TYMS and RRM1. In the event of downregulation of enzymes involved in nucleotide synthesis, DNA replication is interrupted which leads to the cell cycle arrest in S phase. DUT, TYMS and RRM1 are considered important targets in cancer (346,375) and their inhibitors have either been evaluated, or are utilized, clinically. The most widely studied TYMS inhibitor, the nucleoside analog 5-fluoracil (5-FU), is broadly used in clinics to treat multiple types of cancer (370,371,373). While 5-FU is not a targeted inhibitor of TYMS and has many modes of action, resistance to this compound can be conferred by the overexpression of TYMS and DUT, both involved in thymidine synthesis (348,361). It has been revealed that dual inhibition of both proteins is synthetically lethal and leads to cancer cell death in NSCLC models (346). This is consistent with finding showing synergy between a specific DUT inhibitor, TAS-114, with non-specific TYMS inhibitors, is synthetically lethal and leads to cancer cell death in NSCLC models (362,374). We also showed the efficiency of this approach in ovarian cancer for the first time by concomitant knockdown of DUT and TYMS which resulted in reduced cell growth in ovarian preclinical models. These results indicate to the potential for this novel combination, or alternate combinations between BETi, cobimetinib, TYMS and DUT inhibitors. This approach might enlarge the cohort of patients who could potentially benefit from these

strategies. Again, the precise approach might take the expression of potential biomarkers into account including SMARCA4 status, RAS-MAPK pathway activation and DUT and TYMS expression.

Our data indicated that TYMS is downregulated by BETi/cobimetinib through targeting for proteasome-mediated degradation. In the future, we will employ a proteomic approach, Bio-ID, to identify ubiquitin ligases that associate with TYMS. As an alternative, we will tag the C-terminal of TYMS with GFP and carry out a CRISPR screen to search for factors whose loss prohibit the degradation of TYMS as measured by persistent GFP expression.

Another key target to the combination treatment from our screen was RRM1. RRM1 is an enzyme catalysing the reduction of both pyrimidine and purine ribonucleotides to mature "deoxy" forms. Our knock-down studies showed that RRM1 inhibition leads to decrease of cell proliferation without the knockdown of additional factors suggesting this is a potent target for anti-cancer therapy in ovarian tumors. Currently, targeted inhibitors of RRM1 are not available and this seems an important goal for future research.

In our studies, LC/MS metabolomics profiling revealed significant downregulation of DNA nucleotides, especially early precursors of both pyrimidine and purine synthesis. These data strongly suggest that the anti-cancer activity of BETi/MEKi combination is mediated through inhibition of nucleotide synthesis. Further studies to characterize the changes in nucleotide pools upon knockdown of DUT, TYMS, RRM1 alone, or in combination would help reveal which targets are most important for this profile.

Currently, ovarian cancer is one of the most lethal malignancies in women and both SCCOHT and epithelial ovarian carcinoma both have an aggressive behavior leading to poor clinical outcomes. The work described herein outlines several new strategies that might be employed to improve clinical outcomes for these deadly diseases.

5.2 General conclusion

SMARCA4 is an ATPase catalytic subunit of the SWI/SNF chromatin remodeling complex that has been found mutated in a spectrum of cancers. These mutations are variable in nature (nonsense, missense or frameshift), but generally lead to a loss of protein. Loss of function mutations of *SMARCA4* have been reported in ~100% of SCCOHT and 10% of NSCLC (38,146). Currently, both of the diseases are associated with a very poor clinical prognosis. SMARCA4 is necessary for transcriptional activation by shifting or evicting repressive nucleosomes. SMARCA4 along with a bromodomain containing protein, BRD4, independently co-regulate a transcriptional network of proliferation-related genes in a redundant manner (122). Thus, the tumors with a loss of SMARCA4 may depend solely on BRD4 for the expression of this gene network. We proposed that inactivating BRD4 with bromodomain inhibitors (BETi) is a rational therapeutic approach to target *SMARCA4*-mutant cancers because this should critically repress BRD4 dependent oncogene expression (Fig.22).

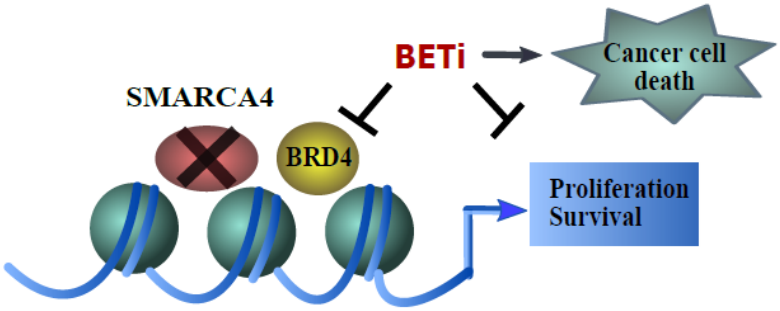

Figure 22. Schematic depiction of the proposed therapeutic application of BET inhibitors in SMARCA4-deficient cancers. SMARCA4 and BRD4 may regulate common gene networks involved in proliferation and survival. In the case of SMARCA4 loss, BRD4 promotes tumorigenesis by enhancing expression of these genes. Thus, depleting BRD4 with BETi leads to the cancer cell death.

My thesis work aimed to identify novel therapeutic options against highly aggressive SMARCA4-deficient pathologies. To achieve this, I examined a panel of SMARCA4-deficient and SMARCA4-expressing ovarian and lung cancer models and found that the SMARCA4-deficient SCCOHT and NCSLC are exquisitely sensitive to BETi *in vitro* and *in vivo* which supported our hypothesis. RNAseq and subsequent molecular studies helped to reveal target genes driving the sensitivity to this type of epigenetic targeting therapy in sensitive cells.

Amongst molecular responders to BETi we found the downregulation of oncogenic RTK-dependent signaling pathways, PI3K-AKT and RAS-MAPK. Results from this study led us to a hypothesis that BETi may cooperate with RTK, PI3K and MEK inhibitors to fully switch off the oncogenic signaling in order to potentiate anti-proliferative effect in SMARCA4-negative models and to overcome intrinsic resistance in SMARCA4-expressing tumors (Fig.23). To explore this concept, I tested a spectrum of the inhibitors of the RTK-dependent signaling and discovered a significant synergy between BETi and MEKi in both SMARCA4-deficient SCCOHT and SMARCA4-expressing ovarian adenocarcinoma models. Importantly, the effectors of the response to the combination BETi/MEKi were downregulated TYMS, DUT and RRM1 proteins involved in DNA synthesis that was detected by SILAC mass spectrometry.

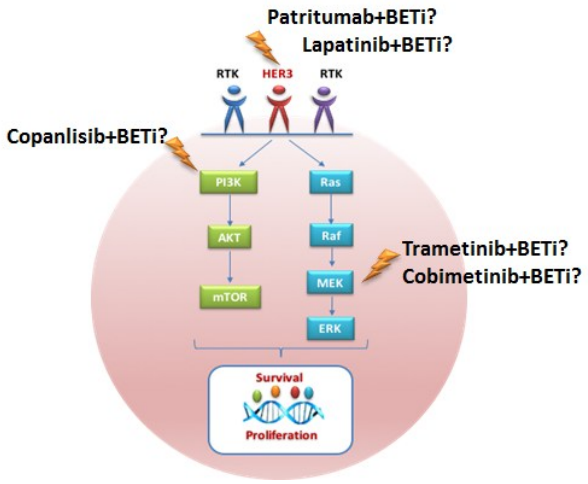

Figure 23. Combinations treatments of BETi with the inhibitors of RTK, PI3K and MAPK pathways employed in the thesis. Patritumab: HER3-antibody; Lapatinib: HER2/EGFR inhibitors; Copanlisib: PI3K inhibitor; Trametinib, Cobimetinib: MEK inhibitors. BETi: BET inhibitor

Overall, the results of this thesis indicate the potential efficiency of BETi in SMARCA4-deficient cancer models. We propose SMARCA4 loss as a predictive biomarker for the BETi treatment. We suggest that the combination strategy of BETi/MEKi represents a powerful new possibility for the therapeutic intervention of different tumor types including aggressive SCCOHT and ovarian adenocarcinoma.

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Appendix

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