SMARCA4/2-loss is synthetic lethal with MCL1 inhibition in cancers

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ABSTRACT

SMARCA4 (BRG1) and SMARCA2 (BRM) are the two mutually exclusive ATPase subunits of the SWI/SNF chromatin remodeling complexes often altered in cancers. SMARCA4 is frequently inactivated by mutations whereas SMARCA2 is rarely mutated but often epigenetically silenced. Concurrent loss of SMARCA4/2 characterizes small cell carcinoma of the ovary, hypercalcemic type (SCCOHT), a rare but lethal ovarian cancer affecting young women, and occurs also in other aggressive cancers including non-small cell lung cancer (NSCLC), associated with resistance to conventionally chemotherapy. Since SWI/SNF loss is not directly druggable, SMARCA4/2-deficient cancers lack effective targeted treatment options. Using a synthetic lethal approach, we identified MCL1, an anti-apoptotic protein of the BCL-2 family, whose inhibition is synthetic lethal with SMARCA4/2 loss. We showed that MCL1 inhibition by RNAi or a small molecule inhibitor, S63845, selectively induce apoptosis in SMARCA4/2-deficient SCCOHT and NSCLC cells but not in proficient controls. Mechanistically, we found that SMARCA4/2 directly promote transcription of *BCL-xL*, encoding another key anti-apoptotic protein of the BCL-2 family; SMACRA4/2 loss results in downregulation of BCL-xL leading to their dependency on MCL1 to suppress apoptosis in these cancer cells. We also showed that treatment of the MCL1 inhibitor S63845 resulted in significant suppression of tumor growth in patient derived xenografts of SMARCA4/2-deficient NSCLC and SCCOHT. Collectively, our work uncovered MCL1 as a novel druggable target in SMARCA4/2-deficient lung and ovarian cancers and suggest that MCL1 inhibitors may be considered for the treatment of these hard-to-treat cancers.

RÉSUMÉ

SMARCA4 (BRG1) et SMARCA2 (BRM) sont les deux ATPases mutuellement exclusives des complexes de remodelage de la chromatine SWI/SNF fréquemment perdus dans les cancers. SMARCA4 est fréquemment inactivé par des mutations alors que SMARCA2 est rarement muté, mais est souvent réprimé épigénétiquement dans les tumeurs. La perte concomitante de SMARCA4/2 caractérise le carcinome à petites cellules de l'ovaire de type hypercalcémique (SCCOHT), un cancer de l'ovaire rare mais mortel affectant les jeunes femmes, et se produit également dans d'autres cancers agressifs, notamment le cancer du poumon non à petites cellules (NSCLC), associé à résistance à la chimiothérapie conventionnelle et mauvais résultats pour les patients. Étant donné que la perte de SMARCA4/2 n'est pas directement médicamenteuse, nous avons utilisé une approche létale synthétique pour identifier MCL1, une protéine anti-apoptotique de la famille BCL-2, comme un candidat dont l'inhibition létale synthétique avec la perte de SMARCA4/2. En validant cela, nous avons montré que l'inhibition de MCL1 par l'ARNi ou un inhibiteur à petite molécule, le S63845, induisait sélectivement l'apoptose dans les cellules SCCOHT et NSCLC déficientes en SMARCA4/2, mais pas chez les témoins compétents. Mécaniquement, la perte de SMARCA4/2 dans ces cellules cancéreuses entraîne une profonde insuffisance de BCL-xL, une autre protéine anti-apoptotique clé de la famille BCL-2, conduisant à leur dépendance à MCL1 qui peut être sauvée par l'expression ectopique de BCL-xL. De plus, la restauration de SMARCA4 dans les cellules cancéreuses déficientes en SMARCA4/2 a augmenté l'expression de BCL-xl en favorisant sa transcription et les a sauvées de l'inhibition de MCL1 ; cela a été inversé par l'ajout d'un inhibiteur sélectif de BCL-xL WHEI-539, suggérant que le déficit en BCL-xL est exclusivement responsable de cette susceptibilité. Enfin, nous avons montré que le traitement de l'inhibiteur de MCL1 S63845 entraînait une suppression significative de la croissance tumorale dans des modèles de xénogreffe dérivés de patients de NSCLC et SCCOHT mutant SMARCA4/2. Collectivement, nos travaux ont révélé que MCL1 était une nouvelle cible médicamenteuse pour les cancers du poumon et de l'ovaire déficients en SMARCA4/2 et suggèrent une option de traitement potentielle pour aider à améliorer les résultats pour les patients.

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CONTRIBUTION OF AUTHORS

J.J. analyzed the data and wrote the thesis. S.H. provided substantial feedback and editorial help on the thesis. J.J, X.Z. and S.H. designed the experiments. X.Z. and S.H. provided substantial direction on the project. X.Z. performed data analysis in **Figure 1A-D**, Z.F. performed short-term cell viability assay in **Figure 3H-I**. J.J. performed all the experiments except those stated above.

LIST OF ABBREVIATIONS

AML	Acute myeloid leukemia
ATAC-seq	Assay for Transposase-Accessible Chromatin with high-throughput sequencing
ATP	Adenosine Triphosphate
AURKA	Aurora kinase A
BAF	Canonical brahma-related gene 1/brahma-associated factor
BCL-2	B-Cell Lymphoma 2
BCL2L1	BCL2 Like 1
BETi	Bromodomain and extraterminal motif protein inhibitors
BH	BCL-2 homology
CDK4/6	Cyclin dependent kinases 4 and 6
ChIP	Chromatin immunoprecipitation
ChIP-seq	Chromatin immunoprecipitation sequencing
DISC	Death-inducing signaling complex
DR	Death receptors
FADD	Fas-associated protein with death domain
FDA	Food and Drug Administration
HGSC	High-grade serous ovarian carcinoma
IC_{50}	Half-maximal inhibitory concentration
IP	Intraperitoneal injection
MCL1	Myeloid cell leukemia 1
MM	Multiple myeloma
MOMP	Mitochondrial outer membrane permeabilization
mSWI/SNF	Mammalian switch/sucrose non-fermentable
ncBAF	Defined non-canonical BAF
NSCLC	Non-small cell lung cancer
OXPHOS	Oxidative phosphorylation pathway
PBAF	Polybromo-associated BAF
PDX	Mouse patient derived xenograft
PRC1	Polycomb repressive complex 1
RNA-seq	RNA sequencing
SCCOHT	Small cell carcinoma of the ovary, hypercalcemic type

TGF- β	Transforming growth factor β
TNF	Tumor necrosis factor
TRAIL	TNF related apoptosis-inducing ligand
WT	Wild type

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INTRODUCTION

SWI/SNF complexes

The switch/sucrose non-fermentable (SWI/SNF) complexes are ATP-dependent chromatin remodelers which utilize the energy from ATP hydrolysis to control gene expression by regulating chromatin organization (1) **(Figure 1A)**. In mammalian cells, there are the three types of mature SWI/SNF complexes including the canonical brahma-related gene 1/brahma (BRG1/BRM)-associated factor (BAF) complex, the polybromo-associated BAF (PBAF) complex, and the newly defined non-canonical BAF (ncBAF) complex. Each of these SWI/SNF complexes consists of approximately 15 protein subunits, with several isoforms existing for many of these subunits. Although differing in subunit composition, they share a common SWI/SNF core module of ATPase subunit conferring catalytic activity (2). SMARCA4 and SMARCA2 are two mutually exclusive SWI/SNF ATPase subunits, utilizing chemical energy to remodel nucleosome conformation and induce accessibility to the transcriptional machinery (3). Besides, there are subunits including SMARCB1, SMARCC1 and SMARCC2 known as important modules of SWI/SNF for structural integrity and chromatin recruitment (4). In addition to control gene transcription, SWI/SNF complexes also directly participate in other various important cellular processes, such as DNA repair (5), chromosomal stability, and centromere function (6).

SMARCA4/2 inactivation in cancers

Cancer genome-sequencing efforts have revealed mutations in different SWI/SNF subunits in more than 20% of human cancers, across a broad range of tumor types with a tissue specificity pattern (7,8). *SMARCA4* is one of the most frequently altered SWI/SNF subunits with a frequency up to 16% in human cancers, such as in ~10% of non-small cell lung cancers (NSCLC)s and ~100% of small cell carcinoma of the ovary, hypercalcemic type (SCCOHT), a rare but lethal ovarian cancer affecting young women (9,10). In contrast, *SMARCA2* is rarely mutated or deleted, but is lost by epigenetic silencing, which is thought to cooperate with

SMARCA4 loss in SMARCA4/2-deficient NSCLC and SCCOHT for cancer development (11).

NSCLC constitutes 80–85% of all lung cancers, mainly classified into adenocarcinoma, squamous cell carcinoma and large cell carcinoma (12). NSCLC is molecularly heterogeneous, where *KRAS* and *EGFR* are the most commonly mutated genes at 3-32% and 9-27%, respectively (13). *SMARCA4*-inactivating mutations are also found in ~10% NSCLC patients (as described above) and 15-35% of NSCLC cell lines (14,15). Approximately 20% of *SMARCA4* mutations in NSCLCs co-occurred with *KRAS* mutations, but the remaining 80% cases lack other known druggable oncogenic mutations. Furthermore, concomitant loss of SMARCA4/2 protein expression occurring in a subset of NSCLC is associated with poor prognosis (16).

SCCOHT is a rare and aggressive cancer of young females, representing less than 0.01% of overall ovarian malignancies (17). ~100% of SCCOHT is caused by inactivating mutations in *SMARCA4*, considered as the sole genetic driver event (10,18,19). In addition, SCCOHT is characterized by concomitant loss of SMARCA4 and SMARCA2 protein expression, while re-expression of SMARCA4 or SMARCA2 using experimental approaches efficiently repressed SCCOHT growth (20,21). In addition to NSCLC and SCCOHT, concurrent loss of SMARCA4/2 also occurs in undifferentiated thoracic sarcoma (22,23), undifferentiated uterine sarcoma (24), and dedifferentiated/undifferentiated carcinoma of various organs (25-28), representing a significant cancer subset that remain hard to treat.

Therapeutic strategies for treating SMARCA4/2-deficient tumors

SCCOHT is known as a lethal cancer with a long-term survival reported as only 10-20% overall (9). SMARCA4-deficient NSCLCs are highly resistant to conventional chemotherapies and present poor prognosis (14,29). Since loss of protein function is not directly targetable, synthetic lethality is often exploited to identify druggable dependencies of tumor suppressor loss (30,31). For example, SWI/SNF complexes are known to oppose polycomb repressor function in regulating gene expression (1,32). Thus, SWI/SNF loss leads to elevated polycomb

repressor activity, which in turns may be targeted. Indeed, SMARCA4-deficient cancer cells are sensitive to suppression of EZH2 (33), the catalytic subunit of polycomb repressor complex 2 (PRC2). However, this genetic interaction is also dependent on a non-catalytic role of EZH2 for stabilizing the PRC2 complex, which is not targetable by current EZH2 inhibitors (33). Nevertheless, this dependency of EZH2 has also been demonstrated in SMARCA4/2-deficient ovarian cancers including SCCOHT (34,35). Currently, there are ongoing clinical studies testing tazemetostat (an EZH2 inhibitor) for treating SMARCA4-deficient cancers (36).

Recently, using a synthetic lethal screening approach, our group uncovered that SMARCA4 loss induces cyclin D1 deficiency which limits cyclin-dependent kinases 4/6 (CDK4/6) activity in SCCOHT cells leading to susceptibility to CDK4/6 inhibitors (37); this druggable vulnerability is also conserved in lung cancer (38), suggesting that CDK4/6 inhibitors may be effective to treat *SMARCA4*-mutant cancers. CDK4/6 inhibitors including palbociclib, ribociclib and abemaciclib have been approved by the U. S. Food and Drug Administration (FDA) for treating patients with estrogen receptor-positive (ER^+), human epidermal growth factor receptor 2-negative ($HER2^-$) advanced/metastatic breast cancers (39-44). Our studies have led to a new study arm of the Canadian Profiling and Targeted Agent Utilization Trial (CAPTUR) testing palbociclib to treat *SMARCA4*-mutant cancers (NCT03297606) (45).

Despite these above encouraging advances leading to ongoing clinical studies repurposing approved agents, these drugs mostly suppress cancer cell proliferation and unlikely eradicate cancer cells completely when used alone. Furthermore, drug resistance is expected to arise as seen in other single-agent therapeutics. Therefore, it remains important to uncovered additional potential druggable targets in SMARCA4-deficient cancers.

To this end, other studies have also identified potential druggable targets in SMARCA4-deficient cancers. For example, SMARCA2 knockdown was shown to be synthetic lethal with SMARCA4 loss in NSCLC cells (46), likely driven by paralogous subunit compensation. This synthetic lethal interaction was also identified in a shRNA screen across 58 cancer cell lines of diverse origins using an epigenome-focused library (47). This has led to the recent development

of ATPase inhibitors and targeted protein degradation (PROTAC) targeting SMARCA2 (34,35). However, as discussed above, concurrent loss of SMARCA4/2 is found in ~100% of SCCOHT and a subset of NSCLC and also occurs in other highly aggressive human malignancies, which will not respond to SMARCA2 inhibition.

Furthermore, it has been reported that Aurora kinase A (AURKA) activity is required for SMARCA4 deficiency in lung cancer cells (48). However, treatment with the AURKA inhibitor VX-680 in a xenograft model appeared to only delay tumor growth (48). Moreover, hyperactivation of the oxidative phosphorylation pathway (OXPHOS) is observed in SMARCA4-deficient NSCLC tumors, leading to their vulnerabilities to OXPHOS inhibition with a selective complex I inhibitor (49). However, it is not clear if these findings could be extended to other SMARCA4-deficient cancers beyond NSCLC. Similarly, it has been shown that SMARCA4/2-deficient ovarian cancers are responsive to inhibitors targeting histone deacetylases and the bromodomain-containing protein 4 (50-52), receptor tyrosine kinases (53), and arginine (54), but their applications in other SMARCA4/2-deficient cancers remain to be examined.

Dysregulation of Apoptosis and BCL-2 Family in cancer

Resisting cell death is one of the well-recognized hallmarks of cancer (55). Evasion of apoptosis, a typical form of cell death, is regarded as the most aggressive and lethal aberration during tumorigenesis (55). Dysregulated apoptosis is widely associated with unhampered cell growth and drug resistance in diverse cancers (56,57).

Apoptosis is a mode of programmed cell death leading to the orderly removal of impaired or potentially harmful cells, which is induced by two core signaling processes: intrinsic and extrinsic pathways **(Figure 1B)**. Extrinsic pathway triggers apoptosis through a typical ligand-cell-surface-receptor interaction (58). The death receptors (DR) family comprises a diversity of cell surface receptors including tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) receptors, TNF receptors, and CD95 (Fas/Apo1). In the context of extrinsic

pathway, recruitment of adaptor molecules such as the Fas-associated protein with death domain (FADD) binds to the corresponding DR on the cell surface, forming death-inducing signaling complex (DISC), and initiate downstream caspase cascade to induce apoptosis event through activation of caspase-8 or caspase-10 proteins (58).

In contrast, intrinsic pathway is usually induced in a cell-autonomous apoptosis mechanism which is the most typical in vertebrates (59). Mitochondrial outer membrane permeabilization (MOMP) is the crucial step of intrinsic pathway, resulting from the formation of pores in the mitochondrial outer membrane, which enables protein diffusion from mitochondrial into the cytosol and activates downstream caspase signal (59,60). Generally, interactions between the pro-apoptotic and anti-apoptotic proteins determine the fate of cell apoptosis (61). Once the mitochondrial permeability transition pore forms, sequestered pro-apoptotic proteins including cytochrome c, Smac/DIABLO and HtrA2/Omi are released from intermembrane space into the cytosol (62). Cytochrome c binds and activates Apaf-1, then proteins caspase-9 can be recruited and activated, forming the 'apoptosome' to trigger apoptotic cell death through activating caspase-3 and caspase-7 signaling (63).

In addition to the pores inducing MOMP, mitochondrial Ca^{2+} overload can induce mitochondria swelling leading to perturbation or rupture of the outer membrane, which in turn releases these mitochondrial apoptotic factors into the cytosol to induce apoptosis (64). Indeed, altered Ca^{2+} homeostasis had been shown to directly contribute to the tumorigenesis through suppression of apoptosis driven by loss of major tumor suppressors PTEN, BAP1 and PML (65-67). In addition, our group recently showed that SMARCA4/2 dual loss in SCCOHT and NSCLC inhibits apoptosis by restricting IP3R3-mediated Ca^{2+} flux to mitochondria, underlying the chemotherapy resistance in these aggressive cancers (68).

In the case of MOMP control, B-cell lymphoma–2 (BCL-2) family are the accountable proteins regulating these events. Thirty members of this family have been identified so far and are classified as three groups according to their composition of BCL-2 homology (BH) domains and functional characters: (1) Pro-apoptotic BH3-only proteins (BID, BAD, BIM, BIK, HRK, BMF, PUMA, NOXA, etc). (2) Pro-apoptotic pore-formers (BAX, BOK, BAK). (3) Anti-

apoptotic proteins (BCL-xL, MCL1, BCL-2, BFL-1/A1, BCL-W) (69). Among BCL-2 family, BCL2, BCL-W and BCL-xL have 4 complete BH domains (BH1-BH4), while MCL1, BCL-B and BFL1 do not contain BH4 domain (70). The BH domains enable the family proteins' interactions with each other to exert pro- or anti-apoptotic function (71). Both pore-forming and anti-apoptotic proteins have BH domains and maintain a hydrophobic groove structure to bind BH3 domains of other BCL-2 family members as a receptor (72). Dysregulation of certain BCL-2 family proteins is a common event causing resistance to apoptosis driving tumorigenesis. Therefore, selective inhibitor against a few of anti-apoptotic proteins emerge as potential drugs with promising therapeutic value (to be discussed further below) (57).

MCL1, myeloid cell leukemia sequence 1, is commonly expressed in various tissues (73) and located in the mitochondria, inserting into the outer mitochondrial membrane with a hydrophobic tail (74). MCL1 has complete BH1-3 domains without BH4; it also contains a PEST domain, a unique structure of MCL1 enriched in proline, glutamic acid, serine and threonine, which plays a pivotal role of post-translationally regulation on MCL1 degradation through phosphorylation (75,76). MCL1 sequesters the proapoptotic proteins BAK/BAX via its hydrophobic groove in BH3 domain (77). Of note, in addition to anti-apoptotic activity, MCL1 also involves in other important biological processes, including maintaining mitochondrial homeostasis and bioenergetics (78) , inhibiting autophagy in neurons through the interaction with BECLIIN1 (79,80), suppression senescence which is an irreversible growth arrest (81-83), and regulating DNA damage response (84,85).

BCL2 Like 1 (BCL2L1), also known as BCL-x, is encoded by the *BCL2L1* gene. There are two major isoforms of *BCL2L1* mRNAs generated after alternative splicing, *BCL-xL* and *BCL-xS*. The long isoform containing 4 exons (86) is translated in to BCL-xL protein with 233 amino acid residues in length (87), which contains BH1-BH3 domains forming a hydrophobic pocket to accommodate BH3 domain of other pro-apoptotic protein to trigger oligomerization. In addition, its BH4 domain plays a critical role in its anti-apoptotic function by inhibiting the activation of BAX (88,89). Besides its anti-apoptotic activity, BCL-xL is also involved in the regulation of other crucial cellular processes, such as autophagy, neural growth, synaptic

plasticity, neuroprotection, Ca^{2+} signaling, ATP synthesis, autoimmune diseases, and aging (90). The short isoform, termed as BCL-xS, is a pro-apoptotic protein of 170 amino acids lacking the BH1 and BH2 domains, but remaining the hydrophobic tail and BH3 domain (88). BCL-xS directly binds to BCL-xL by forming heterodimers, causing the release of the pro-apoptotic BAK (91). In general, BCL-xL is known as the most abundant Bcl-x protein. However, altered splicing event can cause perturbation of BCL-xL/BCL-xS balance which has also been reported in some cancers (87).

MCL1 and BCL-xL coordinate in regulating BAK/BAX-dependent apoptosis and cell survival (92). Development is perturbed by loss of either, but much deeper influences emerge with the loss of both proteins (93,94). MCL1 and BCL-xL overexpression is widely found in hematologic cancer and solid tumors, meanwhile reported to be associated with worse prognosis and therapy resistance (90,95,96).

Therefore, small molecule inhibitors targeting BCL-2 family have been developed. Clinical evaluation of various MCL1 inhibitors is currently underway (97). Recently, S63845, a novel small molecule, was discovered as an MCL1 competitive inhibitor with great affinity to its BH3-binding groove. As a single agent, it has been reported to show potent tumor cytotoxicity in hematological malignancies (97). Besides, S63845 exerted synergy on diverse cancers as it was combined with another drug (98). In terms of BCL-xL–selective inhibitors, WEHI-539 was discovered from a high-throughput chemical screen as a BH3 mimetic compound. It showed high affinity and selectivity for BCL-xL by competitively occupying BCL-xL's fourth hydrophobic pocket. It has been showed WEHI-539 potently killed MCL1-deficient mouse embryonic fibroblast model cells *in vitro* by selectively antagonizing the anti-apoptotic activity of BCL-xL (99). Also, WEHI-539 was reported to be synergistic with carboplatin in ovarian cancer cells (100). However, the application of MCL1 and BCL-xL inhibitors has not been reported in SMARCA4/2-deficient NSCLC and SCCOHT.

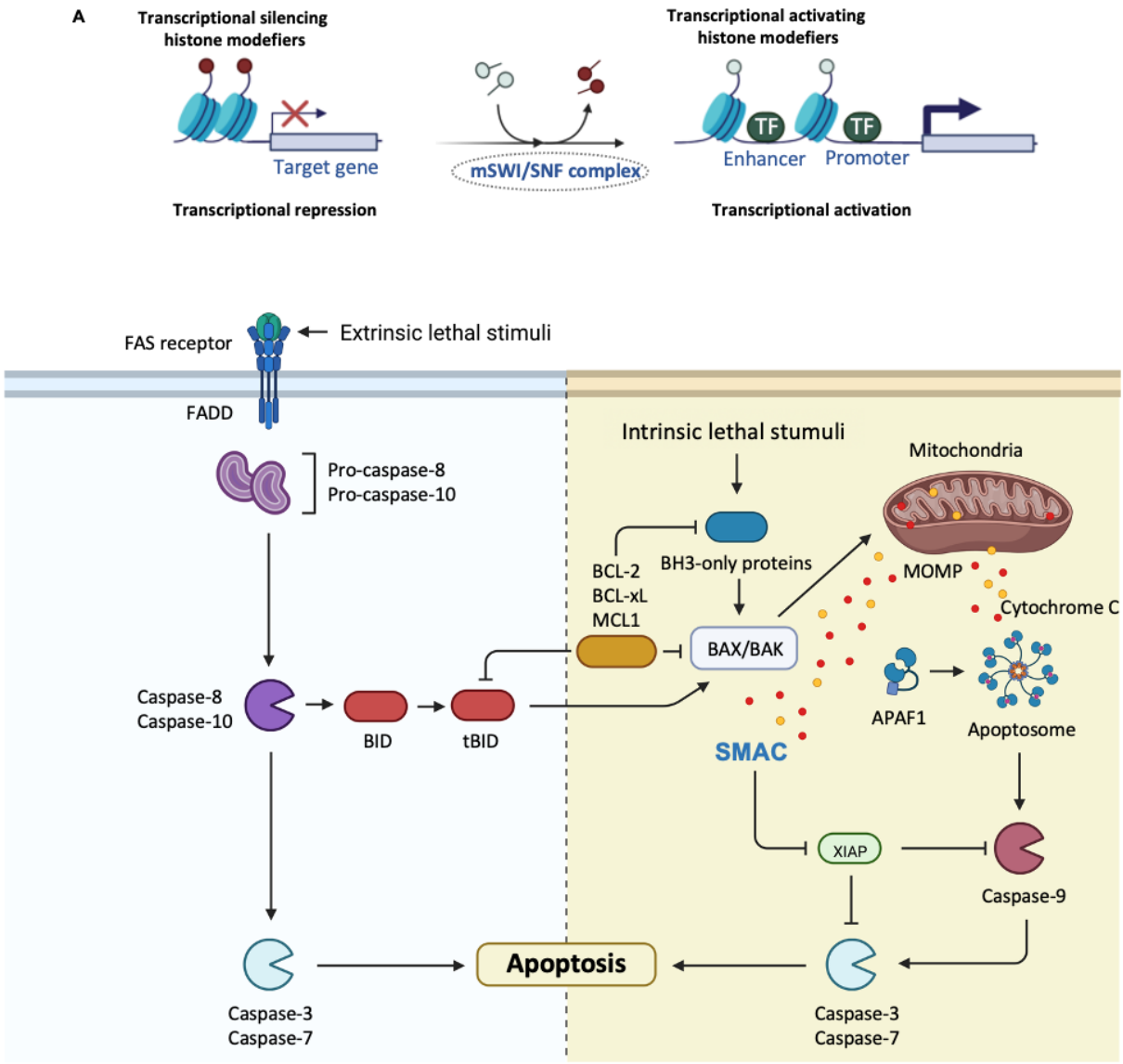


Figure 1. Background knowledge of SWI/SNF complex and apoptosis.
(**A**) Schematic overview of the SWI/SNF complex and transcription regulation. (**B**) Schematic overview of apoptosis pathways. Created by Biorender (https://biorender.com).

AIMS

In this study, I sought to 1) uncover potential targetable candidates whose inhibition is synthetic lethal with SMARCA4 loss in NSCLC and SCCOHT, 2) validate identified synthetic lethal targets and investigate the underlying mechanisms, and 3) establish in vivo relevance using available clinical agents.

METHODS

Cell culture and viral transduction

293T was cultured in Dulbecco's modified Eagle medium (Thermo Fisher Scientific, Cat# 11995-065). All other cell lines were cultured in Roswell Park Memorial Institute 1640 Medium (Thermo Fisher Scientific, Cat# 11875-093) with 7% fetal bovine serum (Sigma, Cat# F1051), 2 mM L-glutamine (Thermo Fisher Scientific, Cat# 25030-081), and 1% penicillin/streptomycin (Thermo Fisher Scientific, Cat# 15140-122). Cells were maintained at 5% CO_2 and 37 °C and regularly tested for Mycoplasma by Mycoalert Detection Kit (Lonza, Cat # LT07-318). All cell lines came directly from ATCC or have been validated by short tandem repeat (STR) profiling, except H1703B11 as a single cell clone (B11) with SMARCA4 restoration in H1703.

Lentivirus production and infection.

All experiments with ectopic expression, CRISPR single guide RNA (sgRNA) knockout and shRNA knockdown were performed using lentiviral constructs. For lentivirus production, 2.5×10^6 293T cells were plated in 2 mL of DMEM medium per well in a six-well plate and transfected after ~8 h with lentiviral constructs, the packaging (psPAX2), and envelope plasmid (pMD2.G) by $CaCl_2$. Virus containing medium were harvested at 24 and 36 h after transfection before use or stored at −80 °C. For infection, ~5 × 105 target cells were plated the day before and infected with virus for ~8 h. Infected cells (20~30 hours post-infection) were selected with 2 μg/mL puromycin or 20 μg/mL blasticidin for 2–4 days and harvested for the experiments.

Compounds and antibodies.

S63845 (HY-100741) and WEHI-539 **(**HY-15607) were purchased from MedChemExpress (Monmouth Junction, New Jersey, USA). Antibodies against β-Actin (Cat# sc-47778), HSP90 (Cat# sc-13119) were from Santa Cruz Biotechnology; antibodies against SMARCA2 (Cat# 11996), MCL1 (Cat# 94296S), BCL-xL (Cat# 2764), cleaved PARP (Cat# 5625) and cleaved caspase 3 (Cat# 9664) were from Cell Signaling; antibody against

SMARCA4 (Cat# A300- 813A) was from Bethyl Laboratories. All antibodies were used with 1:1000 dilution except SMARCA4 with 1:5000 dilution.

Plasmids

Individual ORF and shRNA vectors used were from the Mission TRC library (Sigma) provided by Genetic Perturbation Service (GPS) of Goodman Cancer Research Center and Biochemistry at McGill University: Individual shRNA vectors used were from the Mission TRC library (Sigma) provided by McGill Platform for Cellular Perturbation (MPCP) of Rosalind and Morris Goodman Cancer Research Centre and Biochemistry at McGill University: sh*MCL1*#1 (TRCN0000196390); sh*MCL1*#2 (TRCN0000197024), sh*SMARCA2* (TRCN0000358828). For shRNA experiments, pLKO vector control was used. Additional sgRNA (GCTGGCCGAG- GAGTTCCGCCC) targeting *SMARCA4* was cloned into pLentiCRISPRv2. pReceiver-*Lv105* control and pReceiver-*SMARCA4* were purchased from GeneCopoeia. pLX304-*MCL1* (ccsbBroad304_00985) pLX304-*BCL-xL*, and pLX304-*GFP* control (ccsbBroad304_07515) were from Transomic provided by MPCP.

CRISPR/Cas9 editing

Plasmid-based CRISPR/Cas9 editing was employed to generate *SMRCA4* knockout in FT190 and H1437 cells by standard lentiviral delivery followed by single-cell cloning. Single clones were generated by manually plating of 0.5 cells/well into a 96-well plate upon through a cell strainer.

Colony-formation assays

Considering variable proliferation rates and sizes of different cell lines, we optimized plating densities for each line to allow about 2 weeks of drug treatment, before cells grow to 90% confluency in 6-well plates. Single-cell suspensions of all cell lines were then counted and seeded into 6-well plates with the densities predetermined (2–8 × 10^4 cells/well). Cells were treated with vehicle control or drugs on the next day and culture medium was refreshed every 3 days for 10–14 days in total. At the endpoints of colony-formation assays, cells were

fixed with 3.75% formaldehyde, stained with crystal violet (0.1%w/v), and photographed. All relevant assays were performed independently at least three times.

Cell viability assays.

Cultured cells were seeded into 96-well plates (1,000–6,000 cells per well). Serial dilutions of compounds were added to cells 24 hours after seeding. Cells were then incubated for another 4 days, and cell viability was measured using the CellTiter-Blue Viability Assay (Promega) by measuring the fluorescence (560/590 nm) in a microplate reader. Relative survival in the presence of drugs was normalized to the untreated controls after background subtraction.

Protein lysate preparation and immunoblots

Cells were first seeded in 6-well plates. After 24 hours, cells were washed with cold PBS, lysed with protein sample buffer and collected. For drug assays, the medium was replaced with media containing inhibitors 24 hours post-seeding and collected 24 hours post-treatment. After being washed by cold PBS and lysed with protein loading buffer, samples were processed with Novex® NuPAGE® Gel Electrophoresis Systems (Thermo Fisher Scientific). β-Actin and HSP90 served as loading controls.

Transcriptome analysis

Cell lines. There were 5 sets of transcriptome data used in this study, including SMARCA4 restoration in SCCOHT-1, COV434, A427 (GSE151026, RNA-seq), SMARCA4/2 restoration in BIN-67 cells (GSE117735, RNA-seq and ChIP-seq), SMARCA4 restoration in H1703 (GSE121755, ChIP-seq and ATAC-seq), SMARCA4 knock out in H358 (GSE162611, RNA-seq) and unpublished RNA-seq data of SMARCA4 restoration in H1703. For GSE117735 and GSE121755, sequencing files were downloaded from sequence read archive and mapped to reference human genome sequence (hg19) with STAR (2.6.1c) (101). Gene expression counts were calculated by featureCounts (v1.6.4) (102) with UCSC hg19 gene annotation GTF file.

Patient tumors. RNA-seq data of 13 SCCOHT patient tumors were obtained from two previous studies (23,68). RNA-seq read counts of 379 ovarian cancer tumors were obtained from UCSC Xena (http://xena.ucsc.edu/) which followed the exact same pipeline. The fragments per kilobase of transcript per million mapped reads (FPKM) for each gene was calculated as

$$FPKM = (RCg \times 10^9) / (RCpc \times L)$$

in which RCg is the number of reads mapped to the gene; RCpc is the number of reads mapped to protein-coding genes; and L is mean of lengths of the gene isoforms. Volcano plot, violin plot and bubble plot were generated with gglot2 (Version 3.3.3) (103).

Mouse patient derived xenograft (PDX) and *in vivo* drug studies

SCCOHT PDX NRTO-1 mouse model was established and viably preserved at Goodman Cancer Research Institute of McGill University, and SMARCA4/2-deficient NSCLC PDX model was obtained from The Jackson Laboratory (TM01563). Tumors were cut into pieces and then inserted into a pocket in the subcutaneous space of NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG) mice. Animal experiments were performed according to standards outlined in the Canadian Council on Animal Care Standards (CCAC) and the Animals for Research Act, R.S.O. 1990, Chapter c. A.22, and by following internationally recognized guidelines on animal welfare. All animal procedures (Animal Use Protocol) were approved by the Institutional Animal Care Committee according to guidelines of the CCAC. All animal experiments were carried out at the Goodman Cancer Research Center of McGill University.

For *in vivo* drug studies, S63845 (MedChemExpress) was formulated extemporaneously in 25 mM HCl, 20% hydroxypropyl-β-cyclodextrin (Sigma). The reagent is stored at −20 °C.

Mice were randomly allocated to control (carrier, n=4), treatment (25 mg/kg S63845, twice a week, intraperitoneal injection n=5) groups in SCCOHT PDXs and control (carrier, n=5), treatment (25 mg/kg S63845, twice a week, n=5) groups in SCCOHT PDXs. Control and treatment groups were both matched for tumor size on day 0 of treatment. Carrier and S63845

were given by intraperitoneal injection. Tumor progression was monitored and measurements using digital calipers (VWR) were recorded twice weekly. The persons who performed all the tumor measurements were blinded to the treatment information.

Statistical analysis

Statistical significance was calculated by one-way ANOVA, Dunnett's multiple comparison test. Prism 9 software was used to generate graphs and statistical analyses. Error bars represent mean ± standard deviation (SD). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Data availability

Original CRISPR/Cas9 knockout screening data was from Depmap Public 21Q2 dataset (https://depmap.org/portal/). The *SMARCA4/2* expression and mutation background of DepMap cell lines were available from the Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle). Cell lines were defined as *SMARCA4/2*-dual deficient according to literature references (SCCOHT-1, COV434, BIN-67, TOV112D, OVK18, H1703, A427, H522, H23) (15) or if the cell lines displayed damaging mutations on *SMARCA4* and low *SMARCA2* expression (Log_2(TPM+1) <3). Dependency score (CERES score), a score expressing how vital a particular gene is, in terms of how lethal the knockout/knockdown of that gene is on a target cell line, was calculated to compare the differential essentiality of genes between *SMARCA4/2*-dual deficient cell lines versus proficient cell lines. Unpaired two-tailed t-test was used to assess significance. IC_{50} of drugs are available from Genomics of Drug Sensitivity in Cancer (GDSC) (https:// www.cancerrxgene.org/). mRNA expression data of *MCl1*, *BCL-xL* and *SMARCA4/2* are available from the Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle) for cell lines and from UCSC Xena (https://xenabrowser.net/datapages/) for TCGA tumors of lung and ovarian cancer patients. RNA-seq data of 13 SCCOHT patient tumors were obtained from two previous studies (23) (68). Source data for RNA-seq, ChIP-seq, and ATAC-seq can be found using the accession number GSE151026 (104), GSE117735 (105), GSE121755 (38), GSE162611 (106).

RESULTS

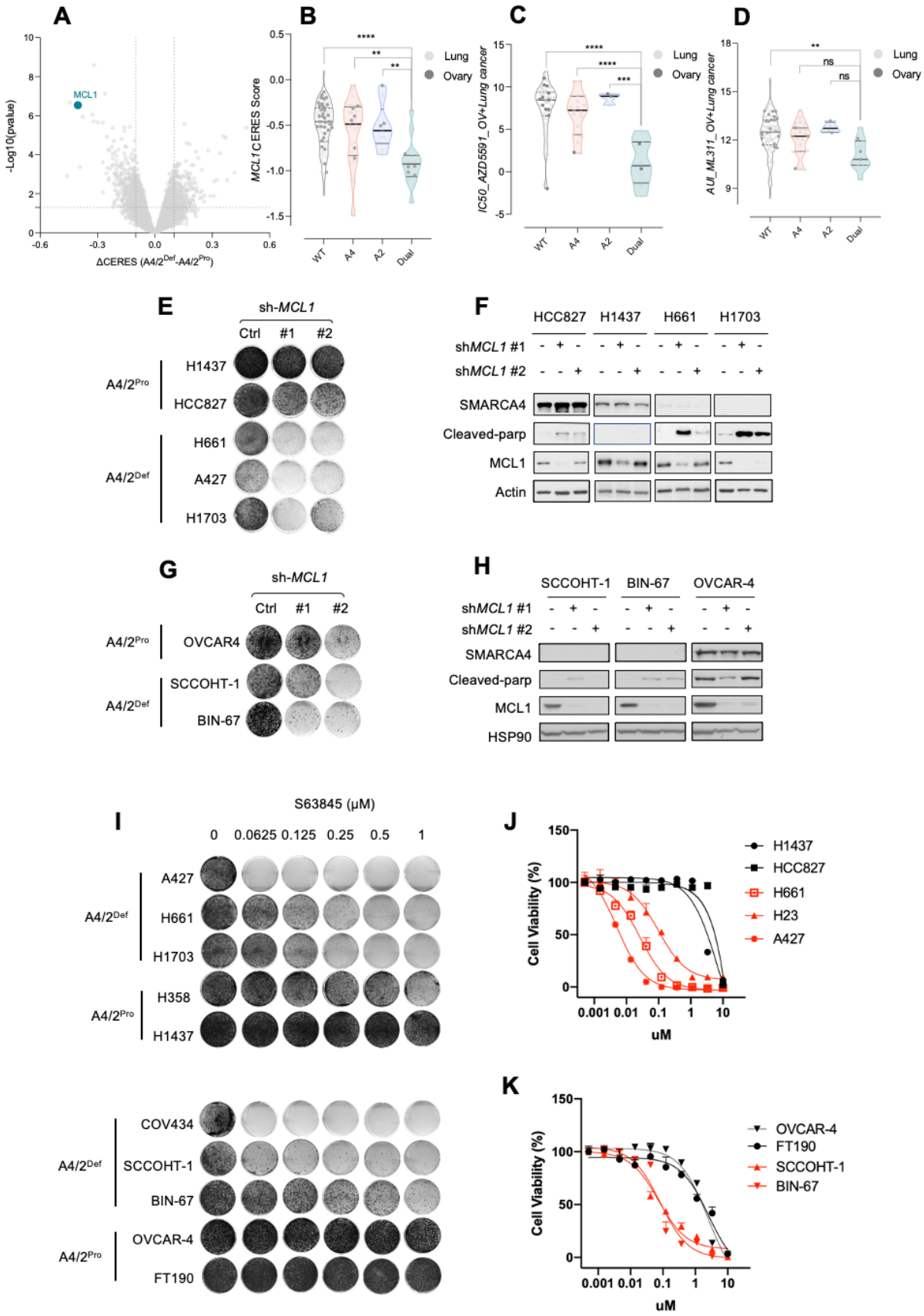
SMARCA4/2-deficient NSCLC and SCCOHT cells are selectively sensitive to MCL1 inhibition

To systematically uncover genetic dependencies of SMARCA4/2 loss, we analyzed the genome-wide CRISPR/Cas9 knockout screens from the Cancer Dependency Map (DepMap, https://depmap.org) across 114 ovarian and lung cancer cell lines: 14 are SMARCA4/2-deficient, including 3 SCCOHT cell lines (BIN-67, SCCOHT-1, COV434) (21,107), 2 dedifferentiated ovarian cancer cell lines (TOV-112D, OVK18) (52,107) and 9 NSCLC cell lines (15), while the remaining 100 are SMARCA4/2-proficient. As shown in **Figure 2A** and **Supplemental Table 1**, *MARCH5* and *MCL1* were the top 2 ranked candidates identified from this analysis (ΔCERES<-0.4, $-\log_{10}$(p-value)>6.5). MARCH5, a mitochondrial E3 ubiquitin ligase, is known to control mitochondria fission and its knockout was shown to cause mitochondrial fragmentation (108). Hereby the dependency of *MARCH5* was expected based on our other independent study revealing that SMARCA4/2-deficient cells were highly dependent on mitochondrial function (109). Notably, MCL1, but not other BCL-2 family members, was also identified among the top-ranked candidate genes, suggesting a unique role of MCL1 in SMARCA4/2-deficient cancers. Therefore, we chose to focus on MCL1 for this study.

To extend this finding, we integrated DepMap data with publicly available RNA sequencing (RNA-seq) data from Cancer Cell Line Encyclopedia (CCLE) (110,111) and stratified a total 159 ovarian and lung cancer cell lines into 4 groups based on *SMARCA4* mutation status and *SMARCA2* mutation or expression status: SMARCA4/2 wild type (n=100), SMARCA4-deficient (*SMARCA4* mutations only, n=36), SMARCA2-deficient (*SMARCA2* mutations only; n=9) and SMARCA4/2-dual deficient (*SMARCA4* mutations and *SMARCA2* low, see Methods; n=14). We found that SMARCA4/2-dual deficient cancer cells showed the strongest essentiality scores for *MCL1* than cells that are deficient only in SMARCA4 or SMARCA2 (**Figure 2B**). This is consistent with the redundant function of these two paralogous

ATPases. Further supporting this, we analysed the CCLE RNA-seq data and drug sensitivity data from Genomics of Drug Sensitivity in Cancer (GDSC) (112) using the same cell line stratification and found that SMARCA4/2-dual deficient cell lines have the lower half maximal inhibitory concentration (IC_{50}) for two MCL1 inhibitors (AZD5591 , AUI_ML311) compared to other groups (**Figure 2C-D**). Together, these results suggest that MCL1 is a synthetic lethal target in SMARCA4/2-deficient ovarian and lung cancer cells.

Validating this, knockdown of MCL1 using two independent shRNAs strongly suppressed growth in long term colony formation assay in SMARCA4/2-deficient NSCLC cells (A427, H1703, and H661 (15)) but had little effect on SMARCA4/2-proficient NSCLC cells (H1437, HCC827) (**Figure 2E-F**). As expected, MCL1 knockdown increased apoptosis as indicated by elevated cleaved PARP (**Figure 2F**). We also obtained similar results in ovarian cancer cells where SCCOHT cells (SCCOHT-1, BIN-67) were more sensitive to knock down of MCL1 compared to SMARCA4-proficient OVCAR4 high-grade serous carcinoma (HGSC) cells (**Figure 2G-H**). Consistent with the data with shRNA-mediated MCL1 suppression, SMARCA4/2-deficient NSCLC and SCCOHT cells were highly sensitive to the treatment of S63845, a highly selective MCL1 inhibitor (113), compared to SMARCA4-proficient cancer (NSCLC: H358, H1437; ovarian: OVCAR4) or non-transformed fallopian control cells (FT190) in both colony-formation **(Figure 2I)** and short-term cell viability assays **(Figure 2J-K)**. These functional data establish that SMARCA4/2-deficient cancer cells are selectively sensitive to MCL1 inhibition.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B
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MCL1
-Log10(pvalue)
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MCL1 CERES Score
IC50_AZD5591_OV+Lung cancer
AUI_ML311_OV+Lung cancer
Lung
Ovary
WT
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A2
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H1437
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SMARCA4
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MCL1
Actin
G
OVCAR4
SCCOHT-1
BIN-67
H
SCCOHT-1
BIN-67
OVCAR-4
HSP90
I
S63845 (μM)
0
0.0625
0.125
0.25
0.5
1
H358
COV434
FT190
J
Cell Viability (%)
uM
K

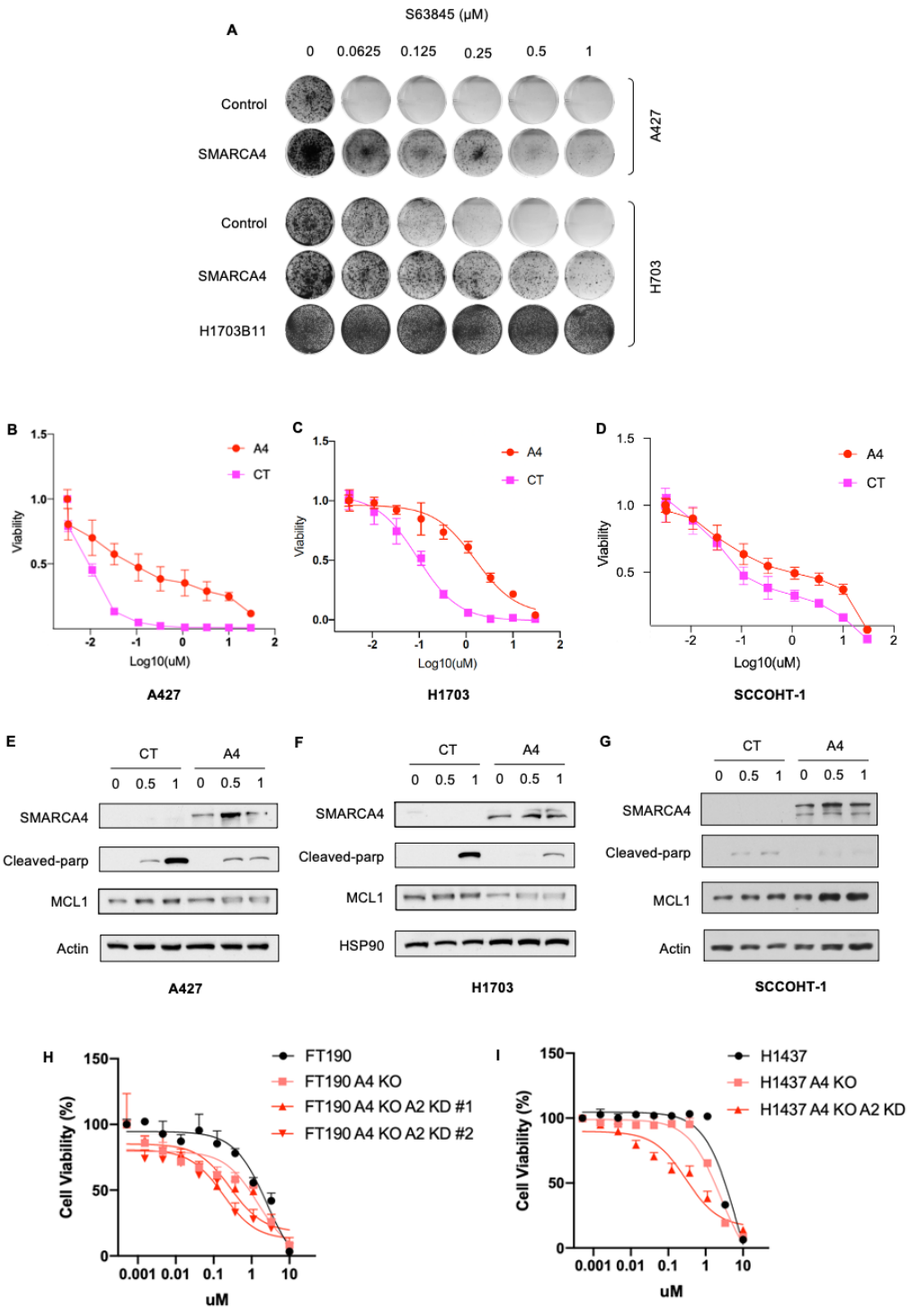
Figure 2. MCL1 inhibition is synthetic lethal with SMARCA4/2 loss in NSCLC and SCCOHT.

(A) Volcano plot showing the differential genetic dependency of genes between SMARCA4/2-deficient (A4/2^{Def}; n=14) and proficient (A4/2^{Pro}; n=100) NSCLC and ovarian cancer cell lines. The genetic dependency was calculated using the CERES score data from DepMap genome-wide CRISPR-Cas9-based screens. Each dot represents a gene. **(B)** Violin plot showing CERES score in cell lines with distinct genetical alteration. WT: SMARCA4/2 wild type, A4: SMARCA4-deficient, A2: SMARCA2-deficient, Dual: SMARCA4/2 dual deficient. Violin plot showing IC_{50} of MCL-1 inhibitors **(C)** AZD5591 and **(D)** AUI_ML311 in cell lines with distinct genetical alteration. WT: SMARCA4/2 wild type, A4: SMARCA4-deficient, A2: SMARCA2-deficient, Dual: SMARCA4/2 dual deficient. **(E-H)** MCL1 knockdown selectively suppressed SMARCA4/2 deficient cancer cells. Colony-formation assay of the NSCLC (**E**) and ovarian cancer (**G**) cell lines expressing pLKO control or shRNAs targeting MCL1 after 10–15 days of culturing. For each cell line, all dishes were fixed at the same time. Western blot analysis of NSCLC (**F**) and ovarian cancer (**H**) cell lines expressing pLKO control or shRNAs targeting MCL1 using antibodies against SMARCA4, cleaved PARP and MCL1. HSP90/Actin were used as loading controls. **(I-K)** MCL1 inhibitor selectively suppressed SMARCA4/2 deficient cancer cells. **(I)** Colony-formation assay of the NSCLC and ovarian cancer cell lines cultured in medium containing different dose of MCL1 inhibitor S63845. Cell viability assay of NSCLC (**J**) and ovarian cancer (**K**) cell lines treated with different dose of MCL1 inhibitor S63845 for 5 days. Error bars: mean ± standard deviation (s.d.).

SMARCA4/2-loss causes the selective sensitivity to MCL1 inhibition

Further above correlation between SMARCA4/2 status and sensitivities to MCL1 inhibition, forced polyclonal expression of SMARCA4 in SMARCA4/2 deficient A427 and H1703 NCSLC cells conferred resistance to S63845 in both long-term colony formation and short-term cell viability assays (**Figure 3A-C**). The resistant phenotype was even more pronounced in a single cell clone of H1703 expressing higher levels of SMARCA4 (**Figure 3A**). Consistently, ectopic expression of SMARCA4 suppressed the apoptosis induction caused by S63845 as indicated by reduced induction of cleaved PARP expression compared to control cells (**Figure 3E-F**). In the case of SCCOHT, both our earlier study (37) and previous report (21) showed that enforced SMARCA4 expression alone leads to strong growth repression in the SCCOHT cell lines. Hence, it was not feasible to perform long-term assays in this context. Nevertheless, the short-term cell viability assay also showed that ectopic SMARCA4 expression also alleviated cytotoxicity and apoptosis induced by MCL1 inhibitor **(Figure 3D,**

G). Conversely, while CRISPR/Cas9-mediated *SMARCA4* knockout in SMARCA4/2-proficient H1437 NSCLC cancer cells marginally enhanced their sensitivity to S63845, knockdown of *SMARCA2* in these $A4^{KO}$ cells led to significant increased sensitivity, indicated by decreased cell viability upon S63845 treatment (**Figure 3H**). Similar results were also obtained in FT190 cells (**Figure 3I**). Taken together, these data indicate that SMARAC4/2 loss in ovarian and lung cancer cells results in selective sensitivity to MCL1 inhibition, at least in part



through apoptosis induction.

Figure 3. SMARCA4 restoration confers resistance to MCL-1 inhibitor in NSCLC and SCCOHT cell lines. (A) Colony-formation assay of A427 and H1703 cells, ± SMARCA4 re-expression, treated with MCL1 inhibitor (100 nM) for 14 days. H1703B11: a single clone of H1703 cell line with stable restoration of SMARCA4. Cell viability assay of A427 (**B**), H1703 (**C**) and SCCOHT1 (**D**) cells, ± SMARCA4 re-expression, treated with different dose of MCL1 inhibitor for 5 days. CT: control, A4: SMARCA4. Western blot analysis assay of A427 (**E**), H1703 (**F**) and SCCOHT-1 (**G**) cells, ± SMARCA4 re-expression, treated with different dose of MCL1 inhibitor for 24 hours. Cell viability in FT190 **(H)** and H1437 **(I)** cells with indicated SMARCA4/2 perturbations treated with different dose of MCL1 inhibitor for 5 days. CT: control, A4: SMARCA4. KO: knockout, KD: knockdown. Error bars: mean ± standard deviation (s.d.).

SMARCA4/2 loss results in BCL-xL deficiency

Next, we investigated the mechanism underlying this selective sensitivity to MCL1 inhibition due to SMARCA4/2 loss. Our above data indicate that this sensitivity to MCL1 inhibition is associated with apoptosis induction, we reasoned that SMARCA4/2 loss may results in either aberrant MCL1 expression or dysregulation of other BCL-2 members leading to dependency of MCL1. To examinate this, we analyzed gene expression of BCL-2 family members in published RNA-seq data sets in three SCCOHT cell lines SCCOHT-1 (37) , COV434 (104), BIN-67 (104) before and after restoration of SMARCA4 or SMARCA2. As shown in **Figure 4A**, among the 12 key BCL-2 members presenting both pro-apoptotic and anti-apoptotic groups, *BCL2L1* was the only one that was strongly upregulated upon restoration of SMARCA4 or SMARCA2 in all three SCCOHT cell lines while *MCL1* remained largely unaffected. Similarly, *BCL2L1* was the only BCL-2 family member whose expression was consistently induced upon restoration of SMARCA4 in SMARCA4/2-deficient NSCLC A427 (104) and H1703 (our unpublished data) and reduced when SMARCA4 was knock downed in SMARCA4/2-profoicient H358 cells (106) (**Figure 4B**).

BCL2L1 encodes BCL-xL and BCL-xS that are results of alternative splicing and have opposition functions in inhibiting and promoting apoptosis, respectively (87). Closer

examination of the RNA-seq data showed that the dominant isoform in BIN-67 cells was *BCL-xL* which was upregulated upon SMARACA4 restoration whereas *BCL-xS* was ~40 fold less abundant than *BCL-xL* and was not regulated by SMARCA4 (**Supplemental Figure 1**). *BCL-2*, another important anti-apoptotic protein, was expressed at a very low baseline among all cell lines. In line with these cell line data, analysis of the RNA-seq datasets of SCCOHT(114) and TCGA high-grade serous ovarian carcinoma (HGSC) (115) showed that SCCOHT expressed significantly lower *BCL2L1* than HGSCs (**Figure 4C**). These observations suggest that SMARCA4/2 loss may cause reduced BCL-xL expression leading to dependency on MCL1 on these cancer cells to suppress apoptosis.

Confirming the transcriptional regulation of BCL-xL by SMARCA4/2, ectopic expression of SMARCA4 in SMARCA4/2-deficent SCCOHT and NSCLC cell lines (SCCOHT-1, COV434, BIN-67, A427, H1703) upregulated BCL-xL protein expression (**Figure 4D**). This is further supported by the correlation between SMARCA4/2 status and BCL-xL protein expression in a panel of 21 NSCLC cell lines. As shown in **Figure 4E**, SMARCA4/2-deficient NSCLC cells indeed expressed the lowest levels of BCL-xL and compared to all other groups including SMARCA4-deficient cells with intact SMARCA2; among SMARCA4-deficient cell lines, there was a notable positive correlation between BCL-xL and SMARCA2 expression levels. These observations are in line with the redundancy of SMARCA4/2 in promoting BCL-xL expression as shown above (**Figure 4A**, BIN-67 cells). Consistent with above RNA-seq data, MCL1 expression was not associated with SMARCA4/2 status. Together, these results support that SMARCA4/2 loss results in reduced *BCL2L1* expression leading to BCL-xL deficiency.

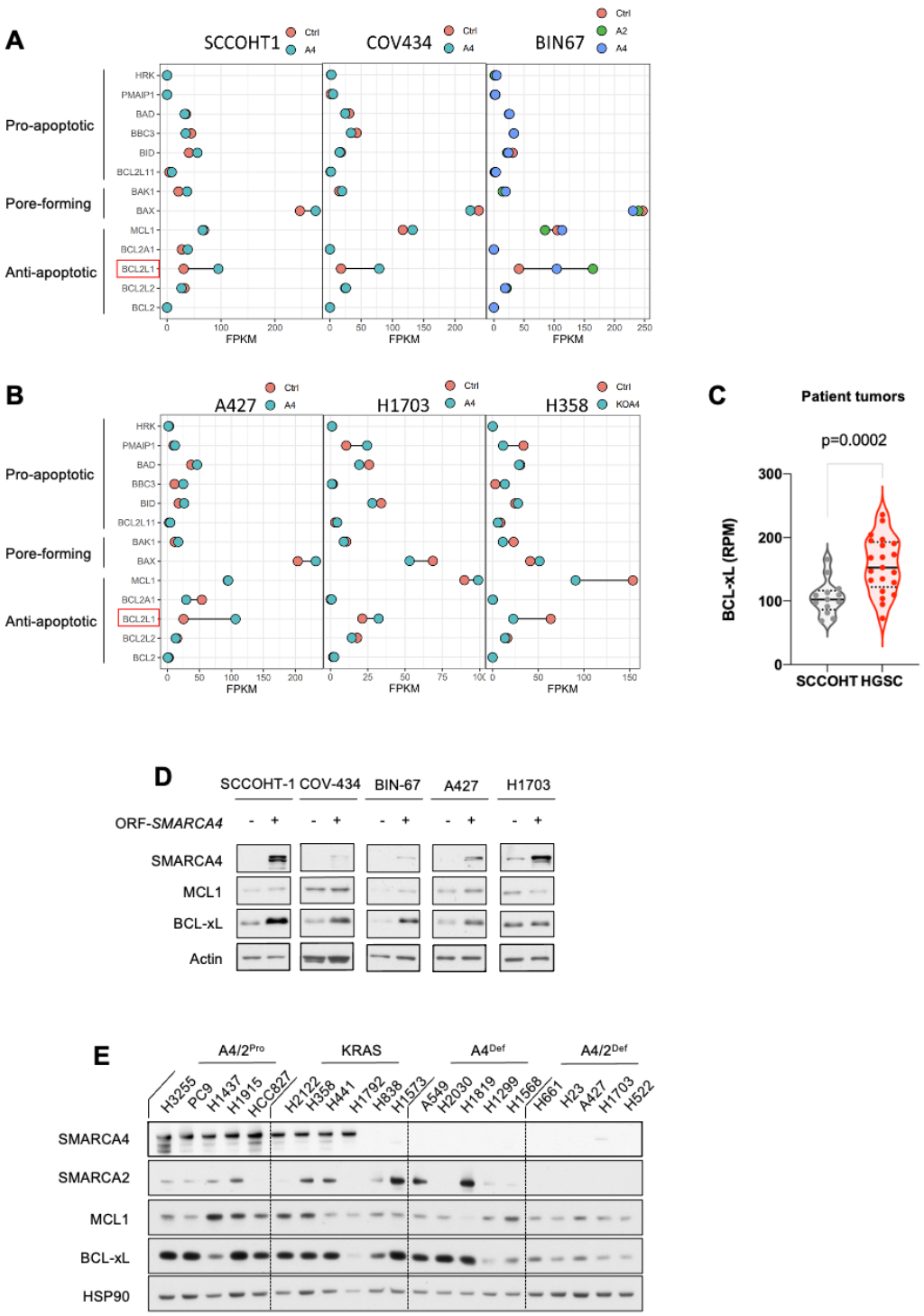


Figure 4. BCL-xL deficiency underlies the vulnerability to MCL1 inhibition in SMARCA4/2-deficient cancer cells. Expression levels of apoptosis associated genes in ovarian **(A)** and lung **(B)** cancer cell lines ± SMARCA4/2 restoration or SMARCA4 knockout. Each dot represents a gene. **(C)** *BCL-xL* mRNA levels in SCCOHT and HGSC patient tumors samples. **(D)** Immunoblots analysis in indicated cell lines ± SMARCA4 restoration measuring MCL1 proteins levels. **(E)** Immunoblots of indicated cell line panels using antibodies against SMACA4, SMARCA2, MCL1 and BCL-xL. HSP90 were used as loading controls. A4: SMARCA4, A4/2: SMARCA4/2, Pro: proficient, Def: deficient, KRAS: KRAS mutation.

SMARCA4/2 directly controls chromatin accessibility of the *BCL2L1* locus

Given the chromatin remodeling role of SWI/SNF, we next examined the chromatin architecture of the *BCL2L1* locus and its potential regulation by SMARCA4/2. Indeed, SMARCA4 occupancy was observed at the *BCL2L1* promoter and regulatory regions in ChIP-seq data of BIN-67 (104) and H1703 (38) cells upon SMARCA4 restoration (**Figure 5A-B**), suggesting that SMARCA4 directly regulates *BCL2L1* expression. Supporting this, we found that ChIP-seq signals of H3K27Ac, a chromatin mark associated with active promoter and enhancer, were elevated at these regions where SMARCA4 bound to in both BIN-67 and H1703 cells. In addition, the Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) peaks at these genomic regions were also elevated upon SMARCA4/2 restoration in BIN-67 and H1703 cells (**Figure 5A-B**), indicating an enhanced chromatin accessibility at the *BCL2L1* locus when SMARCA4/2 were present. These data suggest that SMARCA4/2 activate *BCL2L1* transcription by directly remodeling chromatin structure at its gene locus.

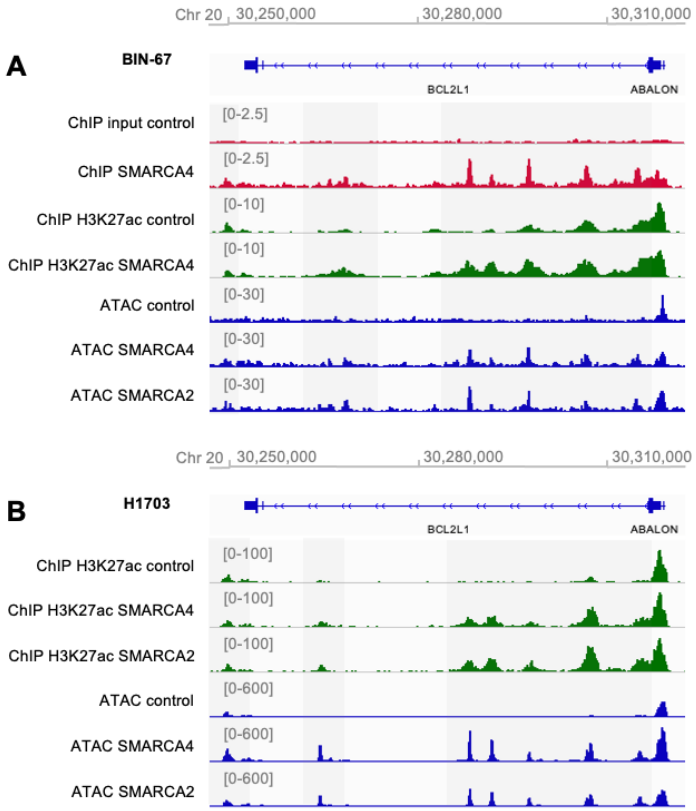
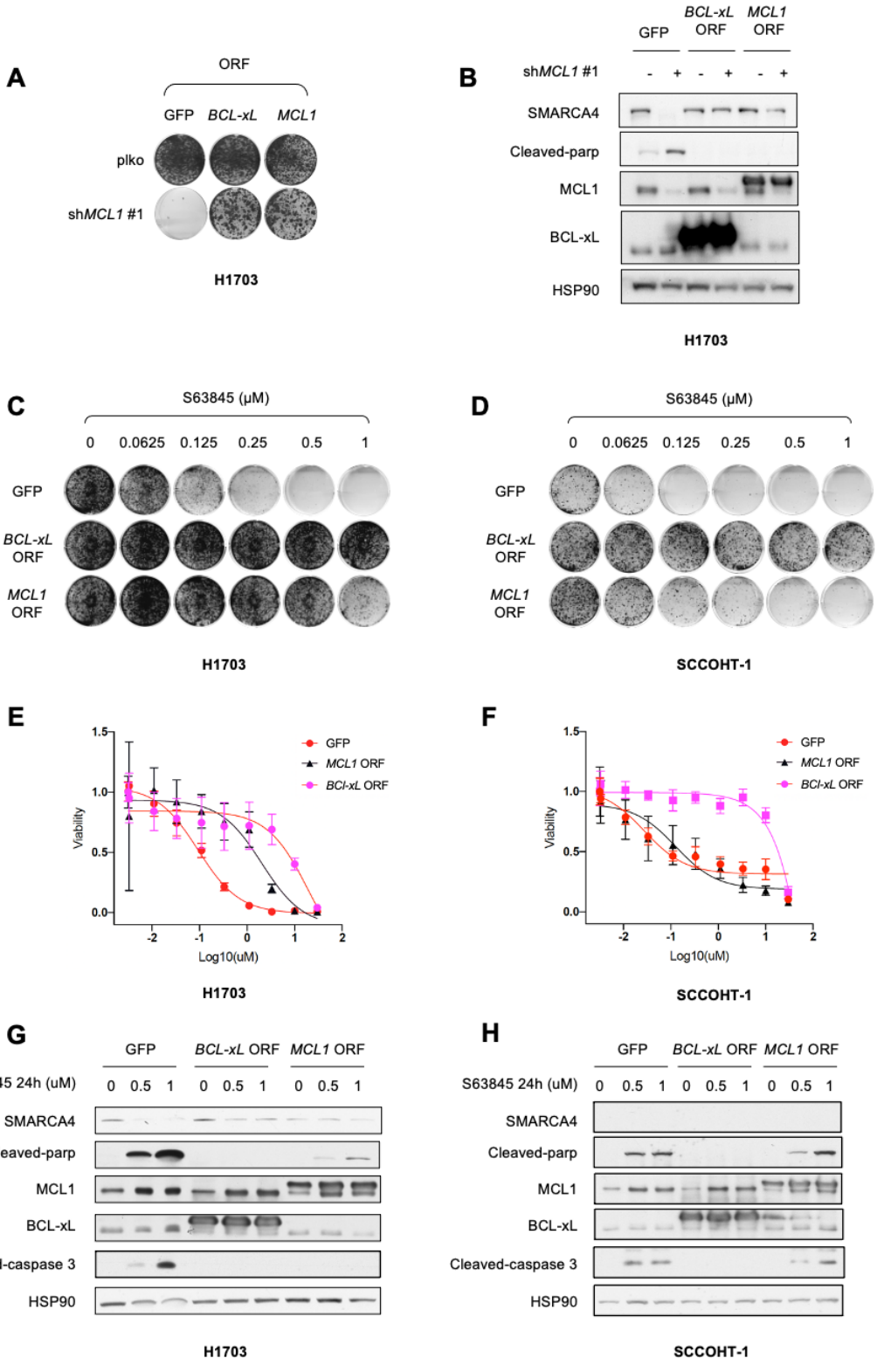


Figure 5. SMARCA4/2 control the chromatin accessibility to the *BCL2L1* locus. Representative browser track of SMARCA4/2, H3K27Ac ChIP-Seq and ATAC-Seq peak on the MCL1 genomic locus in BIN-67 **(A)** and H1703 **(B)** cells re-expressing SMARCA4/2. (38,104-106)

BCL-xL deficiency underlies the synthetic lethal interaction between MCL1 inhibition and SMARCA4/2 loss

Our data thus far suggest that SMARCA4/2 loss leads to reduced BCL-xL expression which limits the total anti-apoptotic capacity in SMARCA4/2-deficient cancer cells and therefore renders their sensitivities to MCL1 inhibition. If this hypothesis is correct, we would expect that elevation of BCL-xL should confer resistance to MCL1 inhibition. Indeed, ectopic expression of BCL-xL protected H1703 cells from the growth inhibition and apoptosis induction caused by a shRNA targeting 3' UTR of *MCL1* (**Figure 6A-B**). As a control, ectopic expression of an *MCL1* cDNA lacking 3' UTR also rescued these shRNA-mediated phenotypes. Consistently, overexpression of BCL-xL conferred resistance to S63845 treatment in H1703 and SCCOHT-1 cells in both colony-formation and cell viability assays (**Figure 6C-F**) and suppressed apoptosis induction (**Figure 6G-H**). MCL1 overexpression also yielded similar phenotype although less pronounced when higher concentrations of S63845 were applied. This is expected as S63845 directly binds to BH3 domain of MCL1 to inhibits its function and hence higher concentration of S63845 may saturate the available MCL1 pool.

While our above data established that reduced BCL-xL expression in SMARCA4/2-deficient cancer cells contribute to their increased dependency for MCL1 for apoptosis inhibition, it remained unclear that if this is the dominant factor since SMARCA4/2 regulate diverse genes and pathways. To test this, we took advantage of the fact that SMARCA4/2-deficient NSCLC cells can better tolerate restoration of SMARCA4 and performed the "double rescue" experiments. As shown in **Figure 6I**, while restoration of SMARCA4 in both A427 and H1703 cells conferred resistance to MCL1 inhibition by S63845, addition of WHEI-539, a selective BCL-xL inhibitor, completely reversed their sensitivities to S63845. Importantly, WHEI-539 treatment alone had no impact to their growth. These results indicate that BCL-xL deficiency in SMARCA4/2 deficient cancer cells is the predominant contributor to their dependency for MCL1.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SMARCA4
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MCL1
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HSP90
H1703
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S63845 (μM)
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GFP
BCL-xL ORF
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H1703
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S63845 (μM)
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Viability
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S63845 24h (uM)
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SMARCA4
Cleaved-parp
MCL1
BCL-xL
Cleaved-caspase 3
HSP90
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S63845 24h (uM)
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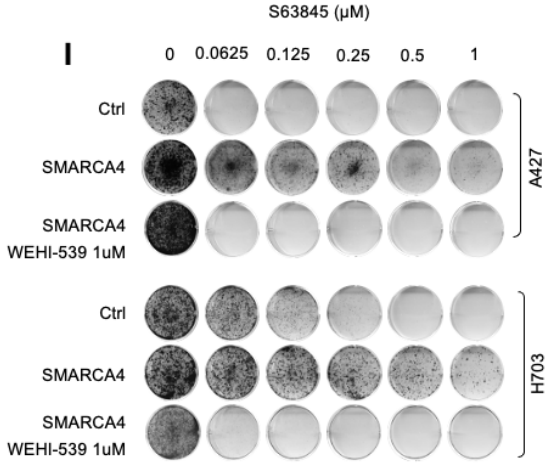


Figure 6. SMARCA4/2-loss-induced BCL-xL deficiency is the dominant contributor to MCL1 dependency in SMARCA4/2-deficient cancers.

Colony-formation assay **(A)** and Immunoblots analysis **(B)** of H1703 cells stably expressing pLX304-*GFP,* pLX304-*MCL1*, or pLX304-*BCL-xL* were infected with viruses containing pLKO control or a shRNA targeting the 3'UTR of *MCL1*. All dishes were fixed at the same time. Antibodies against SMARCA4, cleaved-parp, MCL1, BCL-xL, and HSP90 were used. Colony-formation assay of H1703 **(C)** and **(D)** SCCOHT-1 cells stably expressing pLX304-*GFP,* pLX304-*MCL1*, or pLX304-*BCL-xL* were cultured in medium containing different dose of MCL1 inhibitor for 14 days. All dishes were fixed at the same time. Cell viability assay of H1703 **(E)** and SCCOHT-1 **(F)** stably expressing pLX304-*GFP,* pLX304-*MCL1*, or pLX304-*BCL-xL* were treated with different dose of MCL1 inhibitor for 5 days. Error bars: mean ± standard deviation (s.d.). Immunoblots analysis of H1703 **(G)** and SCCOHT-1 **(H)** stably expressing pLX304-*GFP,* pLX304-*MCL1*, or pLX304-*BCL-xL* were treated with different dose of MCL1 inhibitor for 24 hours. Antibodies against SMARCA4, cleaved-parp, MCL1, BCL-xL, cleaved-caspase3 and HSP90 were used. **(I)** Colony-formation assay of A427 and H1703 cells ± SMARCA4 re-expression cultured in different dose of MCL1 inhibitor with or without 1uM WHEI-539 (BCL-xL selective inhibitor) for 14 days. All dishes were fixed at the same time.

S63845 is effective in suppressing tumor growth of SMARCA4/2-deficient SCCOHT and NSCLC

Our data show that SMARCA4/2-deficient cancer cells are selectively sensitive to MCL1 inhibitors due to their deficiency in BCL-xL. Given that MCL1 inhibitors such as S63845 are being evaluated in clinical studies (116), we sought to examine *in vivo* activity of S63845 in suppressing SMARCA4/2-deficient tumors using patient derived xenograft (PDX) models. After tumor establishment, animals were treated with vehicle or S63845 (25mg/g) by intraperitoneal injection twice a week. As shown in **Figure 7**, S63845 treatment showed significant anti-tumor activity as a single agent in both SCCOHT and NSCLC PDXs. However, we observed toxicity with drug treatment with the NSCLC PDX models and drug holiday was given to allow the animals to recover, which also resulted in regrowth of the tumors. While the effect of S63845 was not as pronounced as our *in vitro* observations, these *in vivo* data do support that S63845 may be considered to help treat SMARCA4/2-deficient tumors.

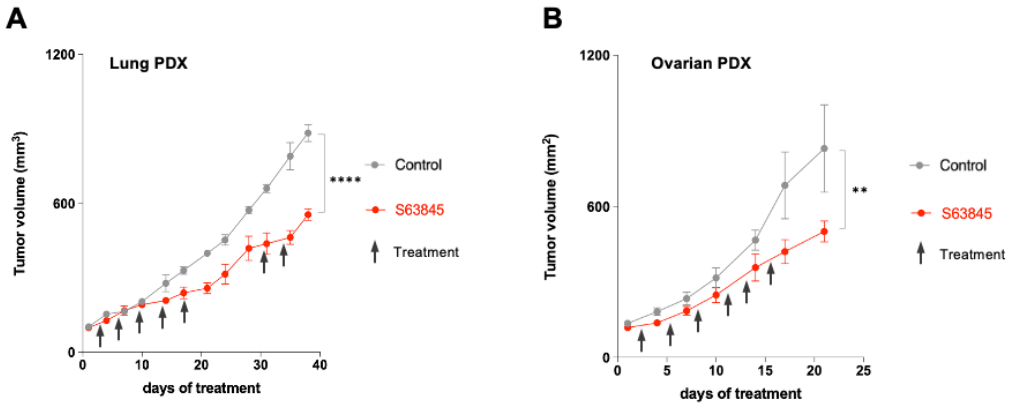


Figure 7. MCL1 inhibitor significantly suppressed growth of SMARCA4-deficient tumors *in vivo*. (A) Tumor volumes of mice bearing tumors of Lung PDX models were treated with S63845 (n=5, 25 mg per kg body weight, twice a week, 3 weeks in total) or vehicle controls (n=4) for indicated time. Two-way ANOVA, **** $p < 0.0001$. Error bars, mean ± SEM. **(B)** Tumor volumes of mice bearing tumors of SCCOHT PDX models were treated with S63845 (n=5, 25 mg/kg, twice a week, 3 weeks in total) or vehicle controls (n=5) for indicated time. Two-way ANOVA, ** $p = 0.0054$. Arrows indicate treatments. Error bars: mean ± standard deviation (s.d.).

DISCUSSION

Currently, effective treatment for patients with SMARCA4/2-deficient tumors remain as an unmet clinical challenge. These aggressive cancers are highly resistant to conventional chemotherapy and lack effective treatment options. This is partly due to that SMARCA4/2 loss is not directly druggable and this SWI/SNF deficiency rarely co-occur with other driver mutations that can be targeted. The aim of our study was to uncover novel genetic vulnerability of SMARCA4/2 loss in ovarian and lung cancers that may be exploited therapeutically. In this thesis, we identified and validated MCL1 as a novel synthetic lethal druggable target in SMARCA4/2-deficient ovarian and lung cancers.

We first took advantage of the comprehensive resource database DepMap, which contains genome-wide CRISPR/Cas9 knockout screen data sets for over 2000 cancer cell lines. By integrating with gene expression data from CCLE, we were able to select a set of 114 ovarian and lung cancer cell lines with differential SMARCA4/2 status. This large number of cell line models allowed us to confidently identify the most robust candidate synthetic lethal targets of SMARAC4/2 loss conserved in these large cell line panel. This unbiased approach led to the identification of MCL1 as a candidate synthetic lethal target of SMARCA4/2 loss.

Notably, MCL1 was the only member of BCL-2 family regulating apoptosis identified from this analysis. Relevant to this, our previous study reported that SMARCA4/2 loss inhibits apoptosis in lung and ovarian cancers, through downregulating ITPR3, a key ion channel inducing Ca^{2+} flux from ER to mitochondria required for apoptosis induction (68). Given the critical role of MCL1 in suppressing apoptosis, identification of MCL1 as a potential target of SMARCA4/2 loss is consistent with this apoptosis resistance trait of SMARCA4/2-deficient cancers. Since selective inhibitors targeting MCL1 under development (116) and promising results on MCL1 inhibition for hematologic malignancies therapy was reported (97), we focused on MCL1 with a goal to explore the feasibility using MCL1 inhibitors to target SMARCA4/2-deficient solid cancers.

We validated this synthetic lethal interaction between MCL1 inhibition and SMARCA4/2

loss using both shRNA-mediated knockdown and the selective MCL1 inhibitor S63845 in a panel of SCCOHT and SMARCA4/2-deficient NSCLC cell lines, along with proficient controls. This is further supported by our data using engineered isogenic cell pairs that differ only in SMARCA4/2 status in which we demonstrated that this selective sensitivity is caused by SMARCA4/2 loss. These findings are relevant for potential future treatment development since SMARCA4/2-deficient tumors may respond to lower doses of MCL1 inhibitors which would cause the least impact to normal cells that retain intact SMARCA4/2.

In our effort to uncover the underlying mechanism, we focused on apoptosis regulation because the well-established BCL-2 family role in apoptosis (78,117-120). MCL1, BCL-2 and BCL-xL are the 3 key anti-apoptotic BCL-2 family members. Thus, their combined expression levels play an important role in controlling cellular apoptosis response. Our transcriptomic analysis in multiple engineered isogenic cell pairs of SCCOHT and SMARCA4/2-deficient NSCLCs that differed in SMARCA4/2 status showed that only *BCL-xL* was regulated by SMARCA4/2 while *BCL-2* was very lowly expressed. This indicated to us that SMARCA4/2 loss results in BCL-xL deficiency leading to increased MCL1 dependency in these cancer cells. Indeed, ectopic expression of BCL-xL conferred resistance to MCL1 inhibition. Furthermore, forced SMARCA4 expression caused upregulation of BCL-xL and resistance to MCL1 inhibition; this was fully reserved by addition of a BCL-xL selective inhibitor. While our data do not rule out addition factors regulated by SMARCA4/2 that may also contribute to MCL1 dependency, these results do support that dominant role of BCL-xL deficiency underlying this synthetic lethal interaction between MCL1 inhibition and SMARCA4/2 loss.

Our current findings are in line with a previous report showing that MCL1/BCL-xL ratio predicted the efficiency of MCL1 inhibition in NSCLC cell lines (121). However, this study did not identify a genetic event associated with this MCL1 sensitivity or the mechanism regulating MCL1/BCL-xL expression ratio. Our work demonstrated that SMARCA4/2 directly regulate *BCL-xL* transcription and SMARCA4/2 loss results in BCL-xL deficiency leading to MCL1 dependency in ovarian and NSCLC cells that are deficient in SMARCA4/2, which have not been previously reported. Notably, in contrast to our findings in these solid cancers, it was

reported that SMARCA4 loss in mantle cell lymphoma leads to increased BCL-xL expression through a different mechanism (122). In the context of mantle cell lymphoma, SMARCA4 loss causes reduced expression of the bZIP transcription factor ATF3, a direct repressor of BCL-xL transcription, and therefore leads to upregulation of BCL-xL. We did not observe significant changes in *ATF3* expression upon perturbation of SMARCA4/2 in the RNA-seq data sets of multiple SCCOHT and NSCLC cell lines (data not shown), suggesting that this SMARCA4-ATF3 relationship may be limited to mantle cell lymphoma which is consistent with well-established context-dependency of SWI/SNF in controlling gene expression.

How does SMARCA4, a highly conserved SWI/SNF chromatin remodeling core subunit, confers differentiated functions in various tissues? First, SWI/SNF complexes are known to interact with tissue-specific transcription factors, thus regulating gene expression in a context-dependent manner (1,32) and consequently different SWI/SNF subunits are mutated in different cancer types, highlighting their important roles in tumorigenesis (1,7,32). Second, some SWI/SNF subunits including SMARCA4 have tissue-type restricted variants. Indeed, we found that the 27th and 30th exon of *SMARCA4* was naturally lost in certain ovarian and lung cell lines (data not shown), which may account for the differential regulation in different tissue types as described above and requires future investigations.

Given the selective sensitivity of SMARAC4/2-deficient SCCOHT and NSCLC cells to MCL1 inhibition by RNAi or S63845 *in vitro*, we explored targeting MCL1 *in vivo* to treat PDX models of these cancers. S63845 has demonstrated potent great activity as single agent in suppressing the AMO-1 multiple myeloma and MV4-11 human acute myeloid leukaemia xenograft models (123) and was shown to overcome the regorafenib resistance in colorectal cancer (113). S64315, a derivate of S63845, is undergoing several clinical trials on hematopoietic malignancies, including acute myeloid leukaemia, multiple myeloma, lymphoma, large B-cell, diffuse, myelodysplastic syndrome (123). In our study, although S63845 as a single agent significantly suppressed tumor growth in both SMARCA4/2-deficient lung and ovarian PDX models, the effect was much less pronounced compared to its activity in cell lines. This differential *in vivo* and *in vitro* outcome could be, but not limited to: 1)

problematic drug delivery *in vivo*, resulting from metabolic inactivation in the circulation, or compactness of the solid tumor blocking drug penetration; 2) dose or treatment did not reach the effective *in vivo* concentration. Considering the toxicity observed that led to drug holiday in our experiments, it is unlikely MCL1 inhbitor as single agent would be effective to treat these solid tumors. In fact, S63845 is mostly used in combination with other drugs, such as Azacitidine (chemotherapeutics) (124) or Venetoclax (BCL-2 inhibitor) (45). Therefore, it may be more effective and safer to use lower concentration of MCL1 inhibitor in combination with other treatments to better targeting SMARCA4/2-deficient tumors.

For example, cisplatin is a commonly used chemotherapeutic in various cancers including ovarian and lung cancers. Its cytotoxicity is achieved by interacting with DNA to form DNA adducts leading to apoptosis activation. However, resistance was often observed in cancers, through dysregulation of factors preventing those cells from apoptosis, enabling them to repair DNA damages and recover to normal growth (125). As mentioned earlier, SMARCA4/2-deficient ovarian and lung cancers are resistant to cisplatin-induced apoptosis through reduced ITPR3 expression impairing the Ca^{2+} flux from the ER to the mitochondria (68). Thus, combining an MCL1 inhibitor may enhance cisplatin sensitivity in targeting these tumor cells by elevating their sensitivities to apoptosis induction exploiting their BCL-xL deficiency, which warrants further studies.

In addition to conventional chemotherapies, it may be effective to combine MCL1 inhibitor with other targeted agents that have demonstrated anti-tumor activities targeting SMARAC4/2-deficeint cancers. Inhibitors targeting EZH2 and CDK4/6 have been shown to be effective in suppressing tumor growth of preclinical models of these SWI/SNF-deficient cancers (37), leading to ongoing clinical trials testing these agents (45). However, these drugs mostly suppress cancer cell proliferation and unlikely eradicate cancer cells completely when used alone. Therefore, co-targeting MCL1 and EZH2 or CDK4/6 may be an effective approaching, by acting on two different essential processes that are selectively vulnerable in SMARCA4/2-deficient cancer cells. Of note, a previously study reported that separase-dependent cleavage of MCL1 and BCL-xL can induce apoptosis and cell death in shortened

mitosis, indicating that MCL1 and BCL-xL also participate in cell cycle regulation (126). Thus, combined MCL1 and CDK4/6 inhibitors may have a synergy targeting SMARCA4/2-deficient cancers. These combination treatment studies also require future investigations.

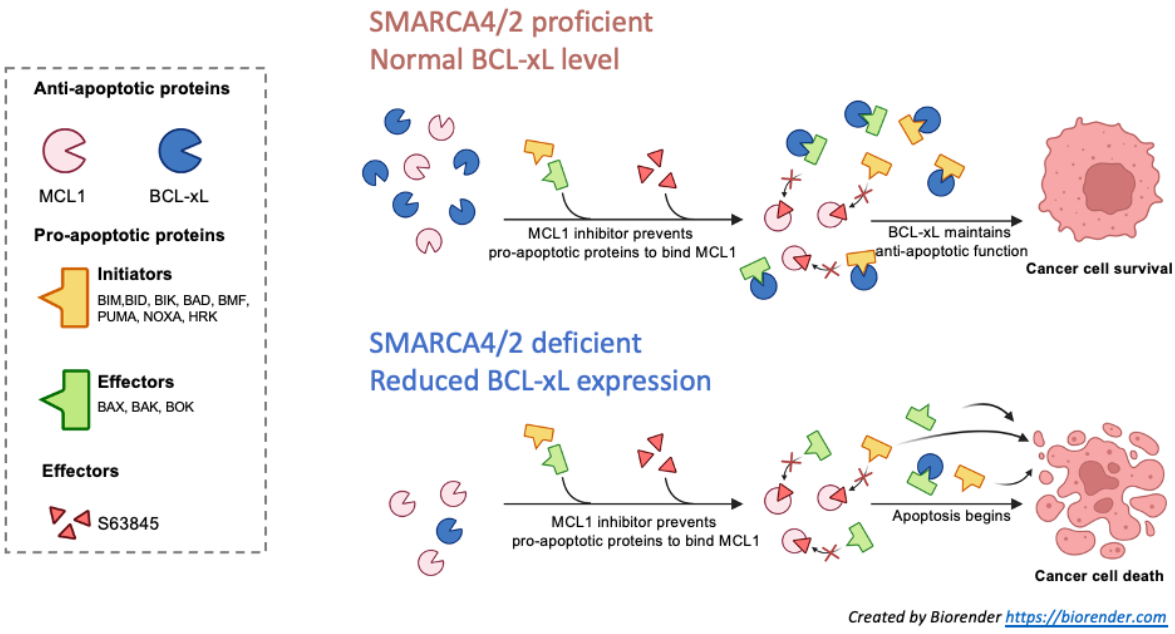
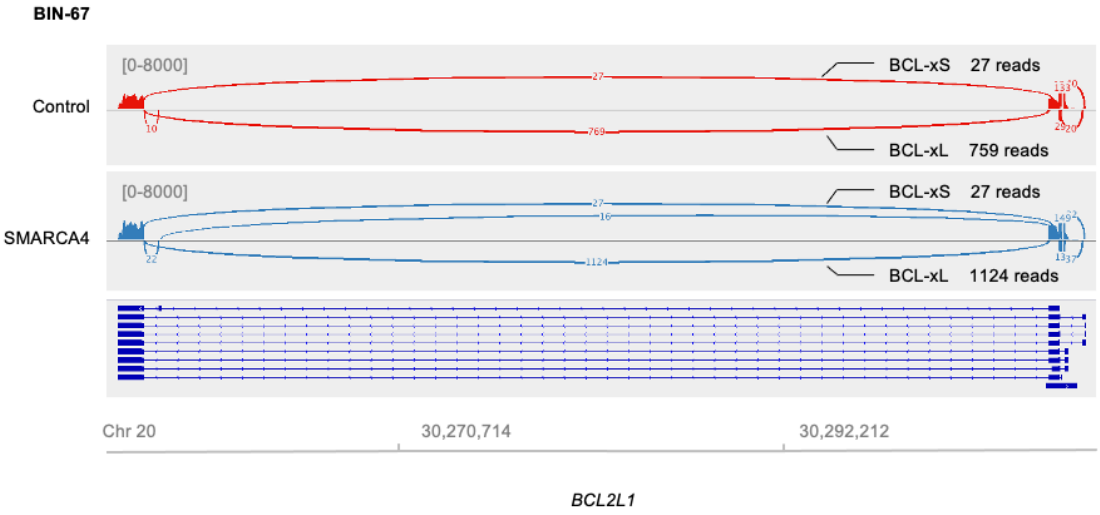


Figure 8. Proposed model for the mechanism underlying the selective MCL-1 dependency in SMARCA4/2-deficient cancer cells. SMARCA4 loss leads to reduced BCL-xL expression, resulting in vulnerability to MCL1 inhibition. Created by Biorender (https://biorender.com).



Supplemental Figure 1. BCL-xL is the dominant isoform of *BCL2L1* which is upregulated upon SMARCA4 restoration. Sashimi plot of RNA-seq data depicting density of exon-including and exon-skipping reads of *BCL2L1* in BIN-67 cells ± SMARCA4 restoration. Genomic coordinates were plotted on x-axis, per-base expression was plotted on y-axis, and mRNA isoforms of *BCL2L1* were shown on bottom. The dominant isoform of *BCL2L1* was *BCL-xL* which was upregulated upon SMARACA4 restoration, whereas *BCL-xS* was ~40 fold less abundant than *BCL-xL* and was shown not to be regulated by SMARCA4 after splicing.

CONCLUSIONS

In our study, we uncovered MCL1 as a novel candidate synthetic lethal target of SMARCA4/2 loss in lung and ovarian cancer using unbiased functional genetics approach. Experiments both *in vitro* and *in vivo* validated that SMARCA4/2-deficient cancers are selectively sensitive to MCL1 inhibition. Mechanically, we revealed that SMARCA4/2 activate *BCL2L1* transcription by directly remodeling chromatin structure at its gene locus; consequently, BCL-xL deficiency induced by SMARCA4/2 loss is the predominant contributor to the selective sensitivity to MCL1 inhibition in SMARCA4/2-defficient cancers **(Figure 8)**. Based on our findings, the therapeutical potential of S63845, the selective MCL1 inhibitor, may be further expanded from hematologic malignancies to a specific subset of SMARCA4/2-deficient NSCLC and SCCOHT, by possibly combining with current conventional chemotherapeutics or targeted agents specifically targeting SMARCA4/2-deficient cancers to improve patient outcome.

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