



Discovery of SOX10 as a Key Pharmacodynamic Marker of BRM/BRG1 Inhibition in Uveal Melanoma

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Discovery of SOX10 as a Key Pharmacodynamic Marker of BRM/BRG1 Inhibition
in Uveal Melanoma

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A Thesis in the Field of Biology
for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

Epigenetic dysregulation plays a fundamental role in the development of cancer. Chromatin remodeling complexes are major epigenetic modifiers, and defects in its activity has emerged as an important feature causing aberrant regulation of transcription in many cancers. BRG1, a catalytic subunit of SWI/SNF chromatin remodeling complex, is mutated in more than 20% of cancers, and synthetic lethal relationship with its paralog BRM points to the potential of BRM and BRG1 as therapeutic targets. Here we demonstrate that a small molecule BRM and BRG1 dual inhibitor Compound-FHT induces growth defect and apoptosis in uveal melanoma models. RNA-seq revealed *SOX10* as a gene significantly downregulated in response to BRM and BRG1 inhibition, and genetic studies have revealed its key role in mediating Compound-FHT induced growth defect. Together, these results provide promise of pharmacological inhibition of BRM and BRG1 as a therapeutic approach for uveal melanoma, and *SOX10* as a biomarker of pharmacodynamic response.

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Chapter I

Introduction

Evolution of cancer therapy

Cancer is a complex collection of distinct genetic diseases (Luo *et al.*, 2009). There are more than 100 distinct types of cancer, but all have defects in regulatory circuits that govern normal cell proliferation and homeostasis (Hannahan and Weinberg, 2000). As normal cells evolve progressively into a neoplastic state, they acquire a succession of hallmarks in a multi-step process. The six hallmarks of cancer include: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. These functions are acquired in different tumor types via distinct mechanisms during the course of tumorigenesis (Hannahan and Weinberg, 2011). Many of these phenotypic traits can manifest as a result of genetic alterations that involve the gain-of-function mutations such as amplification and overexpression of key oncogenes, or the loss-of-function mutations such as deletion and epigenetic silencing of key tumor suppressors (Luo *et al.*, 2009).

The first historical record of cancer date back to around 3000 BC, however, from the Egyptian period to the end of nineteenth century, cancer treatment was primarily

based on the adoption of a healthy diet, cautery, surgery, and palliative pain therapies. The use of chemotherapy to treat cancer began in the 1940s with the use of nitrogen mustards and antifolate drugs (Falzone *et al.*, 2018). Despite limited activity in cancer patients, introduction of these chemical agents provided a stimulus for the synthesis of other drugs. 5-fluorouracil (5-FU) was discovered in 1957 as a chemical agent with activity against a range of solid tumors, and still to this day, it remains as a key component of the colorectal cancer treatment (DeVita and Chu, 2008).

Cancer therapy has evolved significantly in the past 30 years with the introduction of targeted therapy. Targeted therapy refers to a new generation of cancer drugs designed to interfere with specific molecular target that is believed to have a critical role in tumor growth or progression (Sawyers, 2004). Knowledge of cancer at the molecular level has catalyzed a shift toward using targeted therapy as a cancer therapeutic (Sawyers, 2008). Herceptin, a humanized monoclonal antibody against human epidermal growth factor 2 (HER2) in HER2+ breast cancer, provides an excellent example of early success in the development of targeted cancer therapeutics (Baselga, 2001). Imatinib, targeting BCR-ABL (break point cluster and Abelson tyrosine kinase) fusion protein in chronic myeloid leukemia (CML) and receptor tyrosine kinase KIT in gastrointestinal stromal tumors (GIST), has established a paradigm for the treatment of tumors whose growth is dependent on specific kinase activities (Lyseng-Williamson and Jarvis, 2001; Croom and Perry, 2003; Sawyers, 2004). Monoclonal antibodies and kinase inhibitors have revolutionized the field, followed by a more recent advancement in the immunotherapy field. Cancer immunotherapy attempts to enhance the immune response against tumor cells, and the success of checkpoint inhibitors and chimeric antigen receptor T-cells

(CAR-T) have provided an avenue for potential cure in patients suffering from late stage cancers (Kuehn, 2017).

Cancer and epigenetics

In recent years, there has been an increased interest in the impact of epigenetics on cancer biology. Epigenetic changes are heritable changes in gene expressions without alterations in DNA sequences (Berger *et al.*, 2009). Epigenetic programming is essential in mammalian development, and majority of differentiation processes are initiated or maintained through epigenetic modifications (Lund and van Lohuizen, 2004). Genome-wide sequencing has revealed that nearly all cancers harbor epigenetic changes, and findings suggests that epigenetic dysregulation plays a fundamental role in the development of cancer (Kadoch and Crabtree, 2015).

Changes in DNA methylation patterns are an important characteristic of cancer. A link between DNA methylation and cancer was first reported in 1983, when a study revealed that cancer cell genomes are hypomethylated compared to normal cells (Brucerud *et al.*, 2007). While global DNA hypomethylation is linked with oncogene activation and tumorigenesis, site specific DNA hypermethylation at cytosine-phosphate-guanine (CpG) island promoter region causes transcriptional inactivation of genes involved in DNA repair such as *breast cancer susceptibility type 1 (BRCA1)* and genes involved in apoptosis such as *protein 53 (p53)*, both of which are well characterized tumor suppressors (Kanwal and Gupta, 2013).

Histone modifications are also altered in certain cancers, with high levels of histone H3 lysine 4 trimethylation (H3K4me3), histone H3 lysine 27 acetylation (H3K27ac) and histone H4 lysine 20 methylation (H4K20me1) found in the promoter region of actively transcribed genes (Karlic *et al.*, 2010). Enhancer of zeste homolog 2 (EZH2) is a catalytic component of the polycomb repressive complex 2 (PRC2), one of the two classes of polycomb group proteins (PcG) responsible for the methylation of histone H3 lysine residue 27 (H3K27). Gain of function mutations in *EZH2* have been characterized in cancer, resulting in aberrant H3K27 methylation and transcriptional repression of tumor suppressors (Huet *et al.*, 2017; Zhang *et al.*, 2019). Loss of function mutation in *EZH2* have also been associated with some myeloid malignancies (Morin *et al.*, 2010; Nikoloski *et al.*, 2010), thus characterizing *EZH2* as both an oncogene and a tumor suppressor, depending on the disease context (Dawson and Kouzarides, 2012).

Defects in chromatin remodeling activity have also emerged as an important feature of many cancers. A large number of genomic loci repressed in normal human physiology are activated by aberrant activity of chromatin remodelers in cancerous cells, in response to deregulated oncogenic signals (Luo and Elledge, 2009; Nair and Kumar, 2012).

Chromatin remodeling

Chromatin is a densely packaged form of DNA, and nucleosome is the basic functional unit of chromosome. Each nucleosome consists of DNA molecule wrapped

around an octameric protein complex of core histones H3, H4, H2A, and H2B (Dawson and Kouzarides, 2012). Post-translational histone modifications play key roles in gene expression, by altering chromatin structure (Bannister and Kouzarides, 2011). Chromatin can be subdivided into two major regions: heterochromatin, which is highly condensed and primarily contains inactive genes, and euchromatin, which is relatively open and contains most of the active genes. Chromatin provides both a means to accommodate a large amount of genetic material in a small space and a means to partition the genome into transcriptionally active and transcriptionally repressed regions (Narlikar *et al.*, 2013).

Cells are defined by the set of genes transcribed, and chromatin remodeling complexes facilitate the maintenance of different gene expression states (Bradner *et al.*, 2017). Adenosine triphosphate (ATP)-dependent chromatin remodeling complexes utilizes ATP hydrolysis to mobilize nucleosomes to change the packaging state of chromosome, and to provide transcription apparatus and its regulators an access to DNA for gene expression (Varga-Weisz, 2001; Lee and Young, 2013). Mammalian chromatin remodeling complexes can be subdivided into 4 major families: the switching and sucrose nonfermenting (SWI/SNF) family, the imitation SWI (ISWI) family, the nucleosome remodeling and deacetylation (NuRD)/Mi-2/chromodomain helicase DNA-binding (CHD) family, and the inositol requiring 80 (INO80) family. Classifications are based on differences in catalytic ATPases and associated subunits (Dawson and Kouzarides, 2012; Clapier *et al.*, 2017).

SWI/SNF chromatin remodeling complex

SWI/SNF is an evolutionally conserved multi-subunit chromatin remodeling complex composed of approximately 15 protein subunits (Alver *et al.*, 2017). There are about 29 genes encoding mammalian SWI/SNF subunits, and these are assembled into three distinct complexes: canonical brahma related gene (BRG)/brahma (BRM) associated factor (cBAF), polybromo-associated BAF (PBAF), and noncanonical BAF (ncBAF). Specific subunits characterize these complexes, such as AT-rich interaction domain (ARID) 1A/1B, double PHD fingers 2 (DPF2) in cBAF, polybromo 1 (PBRM1), ARID2, and bromodomain containing (BRD) 7 in PBAF, and glioma tumor suppressor candidate region gene (GLTSCR) 1 and BRD9 in ncBAF (Michel *et al.*, 2018). All three complexes contain the same core subunits, including SWI/SNF related matrix associated actin dependent regulator of chromatin (SMARC) subfamily C member 1/2 (SMARCC1/2), SMARC subfamily member D1 (SMARCD1), and SMARC subfamily A2 or A4 (SMARCA2 or SMARCA4) (Mittal and Roberts, 2020). Mammalian SWI/SNF complexes play diverse roles in transcriptional regulation, including cell differentiation and lineage specification. SWI/SNF complexes are often enriched in the enhancer regions, modulating accessibility required for transcription factors to activate gene expression related to development and differentiation (Alver *et al.*, 2017). SWI/SNF complexes have also been implicated in DNA damage repair (DDR), from modifying chromatin structures to recruiting DDR proteins to sites of DNA damage (Mittal and Roberts, 2020). The function of mammalian SWI/SNF is highly context dependent, and

its enzymatic activity that generate changes in chromatin accessibility can either negatively or positively affect transcription (Muntean and Hess, 2009; Wu *et al.*, 2017).

Various SWI/SNF subunits are mutated or lost at high frequencies, and mutations in *SMARCA4* accounts for more than 20% of human malignancies (Kadoch *et al.*, 2017). *SMARCA2* and *SMARCA4*, also referred to as BRM and BRG1, are mutually exclusive catalytic subunits with 92% identity (Papillon *et al.* 2018). A large shRNA-based genetic screening (Achilles, Broad Institute) has revealed a synthetic lethal relationship between the loss of *SMARCA2* and *SMARCA4* in human cancer (Kadoch and Crabtree, 2015). Cancer cells deficient in *SMARCA4*, such as through homozygous loss of function mutations, are dependent on its paralog *SMARCA2* for growth (Papillon *et al.* 2018). Likewise, cancer cells with low *SMARCA2* expression seems to depend on *SMARCA4* for growth and proliferation in certain contexts (Ehrenhofer-Wolfer *et al.* 2019). These findings suggest that a loss of SWI/SNF subunit is partially compensated by a paralog, which then becomes the vulnerability (Michel *et al.*, 2018). While underlying mechanisms leading to cancer remain unclear, this synthetic lethality points to the potential of *SMARCA2* and *SMARCA4*, or BRM and BRG1 as therapeutic targets.

Research aims

High throughput screening to explore the potential of BRM and BRG1 ATPase inhibition has led to the discovery of a novel chemical matter that potently and selectively inhibits both BRM and BRG1. This research aims to understand the viability effect of BRM/BRG1 dual inhibition using Compound-FHT as a tool, and to identify key mediator of pharmacodynamic response in disease model system.

Chapter II

Materials and Methods

Cell culture and compounds

Cell lines were purchased from ATCC (MP38, MP41, MP46, 293T) or Millipore-Sigma (92-1, Mel-202) and cultured according to supplier's recommendations. 92-1 and Mel-202 were cultured in Roswell Park Memorial Institute (RPMI) 1640 Medium (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco); MP38, MP41, and MP46 were cultured in RPMI 1640 supplemented with 20% heat-inactivated FBS; and 293T was cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX (Gibco) supplemented with 10% heat-inactivated FBS. Cells were authenticated by short tandem repeat (STR) profiling and confirmed mycoplasma-free by testing performed at Genetica (Burlington, NC).

Commercially available compounds were purchased from MedChem Express (IDE-196) and Selleck Chemicals (Paclitaxel). Compound-FHT was synthesized at Foghorn Therapeutics (Cambridge, MA). IDE-196, a Protein Kinase C (PKC) inhibitor, was included as a positive control inducing growth inhibition for its known impact on cancers harboring *GNAQ/GNA11* mutations (Croce *et al.*, 2019), and Paclitaxel was included as a positive control for the induction of apoptosis (Wang *et al.*, 2000).

Proliferation and apoptosis assays

For proliferation assays, 5,000 cells/well were seeded in 96 well plates and incubated at 37°C with a dose titration of compounds for 72 hours. After incubation, 50µL of Cell-Titer Glo 2.0 (Promega) reagent was added to each well, and luminescence signal was measured using EnVision plate reader (PerkinElmer). Growth relative to dimethyl sulfoxide (DMSO) control was measured, curves were fit using a 4-point non-linear regression model, and absolute IC₅₀ values were calculated in GraphPad Prism. Percent growth inhibition was calculated as follows:

If $\text{signal}_{72} > \text{signal}_0$,

$$\% \text{ growth inhibition} = 100 \times (\text{signal}_{72} - \text{signal}_0) / (\text{DMSO}_{72} - \text{signal}_0).$$

If $\text{signal}_{72} < \text{signal}_0$,

$$\% \text{ growth inhibition} = 100 \times (\text{signal}_{72} - \text{signal}_0) / (\text{signal}_0).$$

Signal₀ = day 0 signal and signal₇₂ = endpoint signal.

For apoptosis assays, 5,000 cells/well were seeded in 96 well plates and incubated at 37°C with a dose titration of compounds and 6 µM CellEvent Caspase3/7 Detection Reagent (Life Technologies). Real-time fluorescence signal was measured using Incucyte Zoom (Sartorius), and time-course graphs were generated in GraphPad Prism.

RNA sequencing and analysis

92-1, MP41, and Mel-202 cells were incubated with 100 nM Compound-FHT or DMSO for 4 or 24 hours in 6 well plates. Cells were harvested, pelleted and submitted for RNA extraction and sequencing at Novogene (Sacramento, CA). RNA-seq data was generated using the Illumina RNA Sequencing platform. Sequencing reads were aligned to version hg38 of the human genome using gencode v30 gene annotations. Differential gene expression was calculated using Limma-Voom R package (Bioconductor).

Western blot

Uveal melanoma cell lines (92-1, Mel-202, MP38, MP41, MP46) were treated with 100 nM of Compound-FHT or DMSO for 4, 6, 24, or 48 hours in 6 well plates at 37°C. After incubation, cells were rinsed with ice cold PBS and placed on ice. Cells were lysed directly in sample buffer (RIPA buffer, 1X NuPAGE LDS sample buffer, 1X NuPAGE sample reducing agent, 1X Halt protease and phosphatase inhibitor cocktail) and heated at 95°C for 5 minutes. Samples were resolved on 4-20% Criterion TGX precast gels (Bio-Rad), and proteins were transferred onto nitrocellulose membrane using the iBlot 2 dry blotting system (Life Technologies). Blot was blocked for 1 hour in Odyssey Blocking Buffer (LI-COR) and probed overnight on a shaker at 4°C with SRY-related HMG-box 10 (SOX10) (D5V9L) rabbit monoclonal antibody, microphthalmia-associated transcription factor (MITF) (D5G7V) rabbit monoclonal antibody, and β -Actin

(8H10D10) mouse monoclonal antibody (Cell Signaling Technology) diluted 1:1000 in blocking buffer. After washing three times with 1X PBS-T, blot was probed with IRDye 800CW donkey anti-rabbit IgG secondary antibody and IRDye 680RD donkey anti-mouse IgG secondary antibody diluted 1:10,000 in blocking buffer for 1 hour at room temperature with shaking. Blot was washed three times with 1X PBS-T, then imaged with the Odyssey CLx Imaging System (LI-COR).

For dose-response experiment, 92-1 and MP38 cells were incubated with a dose-titration of Compound-FHT for 24 hours at 37°C. Western blotting was performed as described above for SOX10 and β -Actin.

For apoptosis experiment, 92-1 and MP41 cells were incubated with 100 nM of Compound-FHT for 4 hours and 24 hours at 37°C. Western blotting was performed as described above using the following primary antibodies: myeloid cell leukemia 1 (MCL-1) (clone 542808, R&D Systems), B-cell lymphoma 2 (BCL-2) (clone 124, Cell Signaling Technology), B-cell lymphoma extra-large (BCL-XL) (clone E18, abcam), Phorbol-12-myristate-13-acetate-induced protein 1 (Noxa) (clone 114C307, abcam), p53 upregulated modulator of apoptosis (Puma) (clone D30C10, Cell Signaling Technology), BH3-interacting domain death agonist (BID) (clone 3C5, Cell Signaling Technology), BMF (abcam), and Bcl-2 interacting mediator of cell death extra-long, long, and short (BIM_{EL,L,S}) (clone C34C5, Cell Signaling Technology).

CRISPR knockout experiment

To generate a stable clustered regularly interspaced short palindromic repeats (CRISPR) associated protein 9 (Cas9) expressing cell line, 92-1 cells were transduced with lentiviral particles expressing Cas9 construct. 50 μ L of the Cas9 lentiviral particles were added drop-wise to 92-1 cells seeded in 6 well plates with 1X protamine sulfate (Sigma-Aldrich), then centrifuged for 60 minutes at 30°C and 2500 revolutions per minute (rpm). After 24 hours of incubation at 37°C, media containing viral particles were removed and replaced with fresh media containing 20 μ g/mL Blasticidin (Gibco). Transduced cells were grown in the presence of selection antibiotics for 2 weeks, and Cas9 expression was confirmed by western blot. Western blotting was performed as described above using Cas9 antibody (clone 7A9-3A3, Cell Signaling Technology).

Lentiviral particles expressing pLentiGuide-SOX10-Puro (guide RNA sequence: CCATGTTGGACATTACCTCG), pLentiGuide-MITF-Puro (guide RNA sequence: TAAGCGTAAGCATAGCCATG), pLentiGuide-SMARCA2-Puro (guide RNA sequence: CCGTGGA ACTAAAAGCACTT), and pLentiGuide-SMARCA4-Puro (guide RNA sequence: GCAGCAGACAGACGAGTACG) sequences (Genscript) were generated by transfecting 293T cells seeded in 10cm dishes. Single guide RNA (sgRNA) sequences were designed using an online tool CRISPRko (BROAD Institute). 8 μ g of the packaging mix containing packaging plasmid psPAX2 and VSV-G envelope plasmid pMD2.G (Cellecta) were combined with 6 μ g of sgRNA and TransIT-Lenti (Mirus) diluted in Opti-MEM (Gibco), then added drop-wise to 293T cells. Viral particles in the supernatant were collected 72 hours post-transfection.

To edit target DNA sequences, 92-1 cells stably expressing Cas9 were transduced with lentiviral particles expressing sgRNA constructs. 150 μ L of the lentiviral particles were added drop-wise to 92-1_Cas9 cells seeded in 6 well plates with 1X protamine sulfate, then centrifuged for 60 minutes at 30°C and 2500 rpm. After 24 hours of incubation at 37°C, media containing viral particles were removed and replaced with fresh media containing 20 μ g/mL Blasticidin and 0.5 μ g/mL Puromycin (Gibco). Transduced cells were cultured in the presence of selection antibiotics for 3-4 days. Knock-out was confirmed by western blot using antibodies targeting SOX10 (clone D5V9L), MITF (clone D5G7V), BRM (clone D9E8B), and BRG1 (clone E8V5B) (Cell Signaling Technology).

For colony formation assay, transduced cells were seeded in a 24 well plate immediately after removing lentiviral particles. Cells were cultured with 20 μ g/mL Blasticidin and 0.5 μ g/mL Puromycin for 10 days, until the mock transduced cells reached confluency. Cells were then rinsed with PBS and incubated with crystal violet solution (Sigma-Aldrich) for an hour at room temperature. After removing crystal violet solution, cells were washed with water then air-dried at room temperature. Image was captured on Odyssey CLx Imaging System.

SOX10 rescue experiment

Lentiviral particles expressing pLenti-SOX10-Myc-DDK-P2A-Puro (Origene) were generated by transfecting 293T cells seeded in 10 cm dishes. 8 μ g of the packaging

mix containing packaging plasmid psPAX2 and VSV-G envelope plasmid pMD2.G were combined with 6 µg of constructs and TransIT-Lenti diluted in Opti-MEM, then added drop-wise to 293T cells. Viral particles in the supernatant were collected 48 hours post-transfection.

To overexpress SOX10 in a cell line, 92-1 cells were transduced with lentiviral particles expressing empty vector (pLenti-C-Myc-DDK-P2A-Puro) (Origene) or pLenti-SOX10-Myc-DDK-P2A-Puro. This vector contains a cytomegalovirus (CMV) promoter, a highly efficient promoter that can drive constitutive expression of a cloned Open Reading Frame (ORF). 150 µL of the lentiviral particles were added drop-wise to 92-1 cells seeded in 6 well plates with 1X protamine sulfate, then centrifuged for 60 minutes at 30°C and 2500 rpm. After 24 hours of incubation at 37°C, media containing viral particles were removed and cells were further incubated for 24 hours in fresh media. Two days after transduction, 0.5 µg/mL Puromycin was added to culture medium for selection. After 5 days of selection, cells were harvested and SOX10-Myc-DDK protein overexpression was confirmed by western blot.

To measure growth of transduced cells, cells were seeded in 96 wells with a dose-titration of Compound-FHT or IDE196 for 72 hours. Cell-Titer Glo reagent was added to cells, and luminescence signal was measured using Envision plate-reader. Growth relative to a DMSO-control was measured and plotted in GraphPad Prism. Caspase activity was measured using CellEvent Caspase3/7 Detection Reagent incubated together with compounds. Real-time fluorescence signal was measured using Incucyte Zoom.

Chapter III

Results

BRM/BRG1 inhibition induces growth defect in uveal melanoma cells

Compound-FHT was identified as a potent and selective inhibitor of BRM and BRG1 ATPases at Foghorn Therapeutics (Cambridge, MA). To find cancer lineages sensitive to Compound-FHT, we tested a small panel of cancer cell lines in a 3 day growth inhibition assay (Figure 1). Only a subset of cell lines responded to treatment at this time point, and based on the uniform high sensitivity demonstrated, we decided to focus on uveal melanoma as a target indication for this compound.

To confirm the growth inhibitory effect of Compound-FHT on uveal melanoma cell lines, five commercially available cell lines (92-1, Mel-202, MP38, MP41, and MP46) (Table 1) were screened for changes in viability by 72 hour proliferation assay. Relative growth was measured using an ATP-based Cell-Titer Glo assay, and luminescence signals were normalized to DMSO and day 0 controls to calculate growth inhibition (Figure 2).

In all 5 cell lines, Compound-FHT induced robust growth inhibition with half-maximal growth inhibitory concentration (GI_{50}) of about 10 nM or less. Interestingly, the effect of Compound-FHT was more potent compared to IDE-196, a PKC inhibitor

currently under clinical evaluation for the treatment of metastatic uveal melanoma (ClinicalTrials.gov, NCT03947385). IDE-196 induced growth inhibition with GI₅₀ values in the range of 100-1,000 nM in uveal melanoma cell lines tested. MP46 was the most sensitive cell line in this experiment with Compound-FHT GI₅₀ of about 1 nM and IDE-196 GI₅₀ of about 50 nM, however the 10 to 100 folds difference in potency between the two compounds remained consistent.

In 92-1, Mel-202, and MP41, Compound-FHT induced max growth inhibition of about 100%, resulting in 0% growth. In MP38 and MP46, max growth inhibition was about 150%, resulting in -50% growth. Zero percent growth suggests cytostasis, as there were no changes in signal relative to day 0 when compound was added to the cells. Negative percent growth indicates cell death, as signals were lower relative to day 0 measurements. There are limitations to these assumptions, as Cell-Titer Glo reagent simply measures the level of ATP molecules present in cells as a surrogate marker for cell viability. Interestingly, MP38 and MP46 are both characterized by slower growth with doubling time of about 80 hours and 110 hours, respectively (Table 1). Perhaps slower cell growth contributed to a clear cell death phenotype, whereas in fast growing cells, the balance between the speed of proliferation and the speed of cell death may contributed to 0% growth. Overall, these results demonstrate the ability of Compound-FHT to potently inhibit growth of uveal melanoma cells in a dose-dependent manner.

BRM/BRG1 inhibition induces apoptosis in uveal melanoma cells

Next, to investigate whether the growth defect is due to Compound-FHT inducing apoptosis, 92-1, Mel-202, MP38, MP41, and MP46 uveal melanoma cells were screened for changes in caspase 3 and caspase 7 activity. Caspase 3/7 are activated in response to cell death signal and its activation serves as a marker for apoptosis. Cells treated with 111 nM of Compound-FHT were incubated with Caspase3/7 activity detection reagent, and real-time fluorescence signal was measured using IncuCyte Zoom.

In 92-1, Compound-FHT induced robust caspase 3/7 activity, with over 12-fold increase in signal compared to DMSO control (Figure 3, Table 2). This was substantially higher compared to the 3-fold increase in caspase 3/7 activity induced by Paclitaxel, a known apoptosis-inducing agent, at this concentration (Wang *et al.*, 2000). Clear increase in caspase 3/7 activity with Compound-FHT treatment was also observed in MP41 and MP38 cells, with 20 and 6-fold increase over DMSO, respectively. This was a clear contrast to IDE-196, where 111 nM of compound treatment did not induce substantial increase in caspase 3/7 activity over DMSO. Results were ambiguous in Mel-202 and MP46, where caspase 3/7 activity was induced by both Compound-FHT and IDE-196, as well as by control DMSO in MP46. In the case of MP46, cells may be sensitive to seeding density or culture conditions, resulting in caspase 3/7 activity in every treatment conditions. Nevertheless, difference in Compound-FHT induced caspase 3/7 activity is noticeable over DMSO control in all 5 cell lines, suggesting a link between Compound-FHT treatment and apoptosis.

To confirm whether Compound-FHT induced BRM/BRG1 inhibition induces apoptosis in uveal melanoma cells, 92-1 and MP41 cells were subjected to Compound-FHT treatment for 4 or 24 hours, and the expression of pro-survival members of the apoptosis-related proteins such as BCL-2, MCL-1, BCL-XL, as well as pro-apoptotic Bcl-2 homology 3 (BH3) proteins such as BIM, BID, Noxa, and Puma were analyzed by western blot. Maximum growth inhibition can be achieved with 100 nM of Compound-FHT in both 92-1 and MP41 (Figure 2), and 111 nM of compound-FHT has shown to induce exponential increase in caspase 3/7 activity between 24 to 36 hours of compound incubation (Figure 3). As caspase 3/7 activation and cell death occurs as a result of changes in BCL-2 and BH3 protein interactions (Letai, 2017), we decided to look at changes in protein expression after 4 and 24 hours. In 92-1, pro-survival BCL-2 and MCL-1 expressions were downregulated, while pro-apoptotic BIM extra-long (BIM_{EL}) expression was upregulated after 24 hours of compound treatment (Figure 4). While changes in MCL-1 expression was unclear, BCL-2 downregulation and BIM_{EL} upregulation was also observed in MP41. These changes seem to align with caspase 3/7 activity, however pro-apoptotic Noxa, BID, and BIM short (BIM_S) expressions were also downregulated in 92-1 in response to Compound-FHT treatment. A clear reduction in BCL-2 (and MCL-1 in 92-1) protein expression could be shifting the balance of apoptosis regulators in favor of pro-apoptotic activity, yet it is not clear whether these are sufficient to support BCL-2 antagonist/killer (BAK), BCL-2 related ovarian killer (BOK), or BCL-2 associated X (BAX) induced release of cytochrome c (Figure 5) (Giam *et al.*, 2009).

While the interpretation of changes in BCL-2 and BH3 protein expressions are complex, caspase 3/7 activity is a clear indicator of apoptosis. Overall, these results

suggest that uveal melanoma cells undergo apoptosis in response to Compound-FHT treatment. Further studies are necessary to understand the mechanism of apoptosis, and to investigate whether all uveal melanoma cells undergo apoptosis in response to BRM/BRG1 inhibition.

BRM/BRG1 inhibition downregulates SOX10 in uveal melanoma

To understand the effects of Compound-FHT induced BRM/BRG1 inhibition at the transcript level and to identify genes mediating the growth defect phenotype, 92-1 cells were sequenced following 4 hours of Compound-FHT treatment. RNA-seq result revealed 833 genes downregulated by 2-folds or more, and 386 genes upregulated by 2-folds or more in response to BRM/BRG1 inhibition (Figure 6). Of all the genes with significant changes in expression, *SOX10* stood out, with 5-fold downregulation and a p-value of less than 10^{-12} .

Next, 92-1, MP41, and Mel-202 cells were sequenced following 4 and 24 hours of Compound-FHT treatment to further understand the changes in gene the expression. Heatmap representing RNA-seq result revealed *SOX10* as a gene consistently modulated by Compound-FHT treatment across uveal melanoma cell lines, at both 4 and 24 hour time points (Figure 7). While there are additional genes consistently modulated across cell lines, *SOX10* was of a particular interest, as significant downregulation at an earlier time point suggests direct link to BRM/BRG1 inhibition, and stability of response point to the potential as a response marker.

To confirm gene expression changes observed by RNA-seq, cells were treated with Compound-FHT and SOX10 protein expression was analyzed by western blot. In 92-1, MP41, and Mel-202, SOX10 expression decreased significantly after 24 hours of compound incubation (Figure 8). In MP46 and MP38, SOX10 expression decreased, but there were still detectable amounts of protein present after 24 to 48 hours of compound incubation. We also looked for changes in MITF levels, as SOX10 is known to regulate MITF expression (Hou *et al.*, 2006). MITF downregulation was consistent with SOX10, however changes in protein expression was challenging to capture in some cell lines, due to the existence of multiple isoforms and post-translational modifications (Ngeou *et al.*, 2018).

To test whether changes in SOX10 expression are dose-dependent, 92-1 and MP38 cells were treated with a dose-titration of Compound-FHT for 24 hours. Western blot analysis demonstrated that the change in SOX10 expression is in fact dose-dependent (Figure 9).

Overall, SOX10 is clearly downregulated in all 5 uveal melanoma cell lines, indicating its potential role in the mechanism of Compound-FHT induced growth defect. Additionally, Dose-dependent changes in expression suggests that SOX10 could serve as a good pharmacodynamic (PD) marker.

SOX10 is essential for uveal melanoma growth

To investigate whether SOX10 is required for the growth and proliferation of uveal melanoma cells, *SOX10* was knocked-out in 92-1 cells using CRISPR-Cas9 system. 92-1 cells stably expressing Cas9 (Figure 10) were transduced with lentivirus particles carrying sgRNAs targeting *SMARCA2*, *SMARCA4*, *SOX10*, and *MITF*. When cells express both Cas9 and sgRNA, sgRNA will guide Cas9 nuclease to a specific genomic target complementary to its sequence. On-target cleavage of the gene results in a gene knockout, and success was confirmed by western blot (Figure 11). Decrease in respective band intensities for 92-1_Cas9 cells transduced with sgSOX10, sgSMARCA2, and sgSMARCA4 confirmed the success of gene editing. Cells transduced with sgMITF survived in the presence of selection antibiotics, confirming transformation, however the lack of MITF protein downregulation suggests that sgRNA was not efficient in editing *MITF* gene.

Once gene knockouts are confirmed, transduced cells were seeded into a 24-well plate, and colony formation was monitored in the presence of selection antibiotics. After 10 days of incubation, wells containing mock transduced cells reached confluency, and cells were stained with crystal violet to compare difference in colony growth. Lack of colony formation indicates that the knockout of the target gene induces growth defect. Results were clear: confluence is significantly less in *SOX10*, *MITF*, *SMARCA2*, or *SMARCA4* knockout cells compared to mock transduced cells (Figure 12). This result is consistent with data available in the Cancer Dependency Map (BROAD Institute), where *SMARCA4* and *MITF* are included among the top 10 preferentially essential genes for 92-

1 cell line, through combined RNA interference (RNAi) loss-of-function screen. This CRISPR-Cas9 knockout data adds further insight into 92-1 genetic dependencies, identifying *SOX10* as an essential gene required for the growth and proliferation of 92-1 uveal melanoma cells.

SOX10 overexpression rescues uveal melanoma cells from Compound-FHT induced growth defects

To further confirm the requirement, SOX10 was overexpressed in 92-1 cells to test whether ectopically expressed SOX10 rescues the growth defect induced by Compound-FHT. 92-1 cells were transduced with lentiviral particles expressing empty vector (pLenti-Myc-DDK-P2A-Puro) or SOX10 expression vector (pLenti-SOX10-Myc-DDK-P2A-Puro). In this vector system, SOX10 expression is driven by cytomegalovirus (CMV) promoter, forcing transduced cells to constitutively express SOX10. Seven days post-transduction, cells were harvested and confirmed protein overexpression by western blot (Figure 13).

To understand the effect of SOX10 overexpression on growth and proliferation of uveal melanoma cells, transduced cells were subjected to a 72 hour proliferation assay with dose-titrations of compounds. SOX10 overexpression resulted in a partial rescue of growth defect induced by Compound-FHT, with only about 30 to 50% growth inhibition relative to DMSO control (Figure 14). Growth of both the parental 92-1 and 92-1 cells

transduced with empty vector were inhibited significantly by Compound-FHT, with near 100% growth inhibition with 10 nM treatment.

Interestingly, SOX10 overexpression did not rescue the growth defect induced by PKC-inhibitor IDE-196. Dose-response curves for the parental 92-1, empty vector transduced 92-1, and SOX10 overexpressing 92-1 cells were near identical, and no meaningful differences in IC₅₀ values were detected. This suggests the difference in mechanism of PKC inhibition and BRM/BRG1 inhibition, and that SOX10 is specifically involved in mediating growth defects induced by BRM/BRG1 inhibition.

To test if SOX10 overexpression rescues 92-1 cells from Compound-FHT induced apoptosis, cells were incubated with Compound-FHT and caspase 3/7 activation was monitored real time in IncuCyte for 96 hours. With 1.4 nM compound treatment, caspase 3/7 signal increased substantially between 24 to 48 hours in the control 92-1 cells, while no clear increase was detected in SOX10 overexpressed cells (Figure 15). With 111 nM compound treatment, caspase 3/7 signal increased with similar kinetics in control 92-1 cells, while no meaningful increase in SOX10 overexpressed cells for the first 72 hours. Caspase 3/7 signals did increase between 72 and 96 hours in SOX10 overexpressed cells, and difference between the two cell lines were lost after 96 hours of incubation. Multiple factors including cell confluency could have played a role in adding additional stress to the cells, resulting in more cells committing to apoptosis. Further experiments are necessary to fully understand these mechanisms.

Chapter IV

Discussion

BRM and BRG1 are promising therapeutic targets for uveal melanoma

Uveal melanoma is the most common primary tumor of the eye arising from the melanocytes of the uvea (Amaro *et al.*, 2017). Uveal melanoma is commonly seen in older age groups with overall age-adjusted incidence rate of about 5.1 cases per million (Kaliki and Shields, 2017). Currently, the most widely used treatment options are resection, radiation therapy, and enucleation. About 50% of the patients develop metastasis, and 90% of metastasis is in the liver. No effective therapy exists once tumor metastasize, and overall survival is short (Amirouchene-Angelozzi *et al.*, 2014; Kaliki and Shields, 2017). Uveal melanoma is a rare but deadly disease with high unmet needs.

Uveal melanoma is often characterized by mutually exclusive mutations in *Gq protein subunit alpha* and *G protein subunit alpha 11 (GNAQ/GNA11)* genes that activate mitogen-activated protein kinase (MAPK) pathway, which is considered a driver mutation (Van Raamsdonk *et al.*, 2008). Inactivating mutations of tumor suppressor gene *BRACA associated protein 1 (BAP1)* occurs in about 85% of the uveal melanoma cases, and it is associated with a more aggressive form of the disease (Harbour *et al.*, 2010). While little is established surrounding the role of SWI/SNF in uveal melanoma, recent

findings suggest that uveal melanoma cell lines are dependent on BRM/BRG1 (Rago *et al.*, 2020).

Here, results demonstrate the sensitivity of uveal melanoma cells to compound-FHT induced BRM/BRG1 ATPase inhibition. Growth inhibitory effects were confirmed in a panel of 5 uveal melanoma cell lines, with GI₅₀ of 10 nM or less. Robust apoptosis signals were also detected in multiple uveal melanoma cell lines, suggesting apoptosis as a mechanism. While these growth inhibitory effects are clear, these observations are limited to a panel of 5 cell lines tested in this study. Due to availability, all 5 cell lines arise from the primary tumor rather than the metastasis (Table 1), while drug interventions are intended for metastatic uveal melanoma cases. One study found that 57% of metastatic uveal melanoma cases had *GNA11* mutation, while 22% of metastatic uveal melanoma cases had *GNAQ* mutation, suggesting a link between *GNA11* mutation and a more aggressive form of the disease (Van Raamsdonk *et al.*, 2010). Of the 5 cell lines tested, only MP41 harbor *GNA11* mutation. In a study characterizing genomic landscapes of matched primary and metastatic uveal melanoma, alterations distinct from the primary tumor were found in the metastatic tumor. Furthermore, loss-of-function mutations in *PBRM1* and gain-of-function mutations in *EZH2* were found in metastatic uveal melanoma, suggesting a potential shift in balance of chromatin remodeling activity (Shain *et al.*, 2019). While frequency of such mutations and the consequences are unclear, these findings suggest distinct genomic landscapes in metastatic uveal melanoma, posing limitations to this current study conducted in primary uveal melanoma models. Nevertheless, Compound-FHT inhibits growth and induces apoptosis in uveal melanoma models, suggesting BRM/BRG1 as a promising therapeutic target.

SOX10 mediates growth defect induced by BRM/BRG1 inhibition, and is essential for the survival of uveal melanoma cells

SOX10 is a member of the SOX family transcription factors that plays a critical role in the development of neural crest and differentiation of various neural crest derivatives, including the melanocyte lineage (Haldin and LaBonne, 2010; Han *et al.*, 2018). SOX10 regulates the proliferation and survival of melanocytes through activation of its target genes such as *MITF*, *DCT*, and *TYR* (Harris *et al.*, 2010), and its expression is important for the initiation and maintenance of cutaneous melanoma (Shakhova *et al.*, 2010; Cronin *et al.*, 2013). While little is known about the role of SOX10 in uveal melanoma, it is not surprising to find similarities across melanocyte lineages.

Our RNA-seq analysis identified *SOX10* as a gene consistently modulated by Compound-FHT treatment across a panel of uveal melanoma cell lines. SOX10 is also downregulated at a protein level across a panel of 5 uveal melanoma cells in response to Compound-FHT treatment in a dose dependent manner.

Knockout experiment results demonstrate the requirement of *SOX10* for the growth and proliferation of uveal melanoma cells, and the rescue effect from the forced overexpression of SOX10, reversing the effect of Compound-FHT, suggests causal relationship between BRM/BRG1 inhibition, SOX10 downregulation, and growth defect. Interestingly, Rago and colleagues recently reported similar findings, that the overexpression of MITF rescues the effect of compound induced BRM/BRG1 inhibitions in uveal melanoma cells (Rago *et al.*, 2020). It is unclear how both SOX10 and MITF

could independently rescue BRM/BRG1 inhibition induced growth defect, but evidence suggests that SOX10 may be able to activate MITF expression through binding to its promoter site (Potterf *et al.*, 2000). SOX10 seems to cooperate with MITF and BAF in melanocyte transcription, and both are important lineage specific transcription factors regulating transcriptional programs essential for uveal melanoma cell survival (Seberg *et al.*, 2017).

Significance of this research and future directions

Pharmacodynamic (PD) or response biomarkers are critical to preclinical as well as clinical drug development, for understanding target engagement. Changes in PD biomarker expression provide insights into whether a therapeutic agent is associating with an intended target and inducing intended effects. While there may not be a clear correlation between PD response and efficacy such as tumor shrinkage, PD biomarker expression could guide research by providing confidence in dosing, and for translational proof of concept. SOX10, identified as a key mediator of response for BRM/BRG1 inhibition, has a great potential as a PD biomarker in uveal melanoma.

While we focused on uveal melanoma as a target indication for our BRM/BRG1 inhibitor, cell lines derived from other indications also showed sensitivity in the initial cell line screen (Figure 1). In addition, given the impact of BRM/BRG1 inhibition on SOX10 and MITF transcription factors, it is feasible to test Compound-FHT in cutaneous melanoma, where SOX10 and MITF play an important role. In fact, one study showed

that shRNA mediated knockdown of *SOX10* in cutaneous melanoma results in a G1 phase arrest, halting cell cycle progression (Cronin *et al.*, 2013). Additional studies are necessary to understand the dependencies of other cancer types on *SMARCA2/SMARCA4*, and mechanism mediating response in each of the different indications.

Appendix I

Tables

Table 1. Characteristics of uveal melanoma cell lines used in this study.

Cell line	UM type	Doubling time	BAP1 Mutation	BAP1 expression	GNAQ Mutation	GNA11 Mutation
92-1	primary	38h	unknown	Yes	626 A>T	no
MP41	primary	41h	no	Yes	no	626 A>T
MEL-202	primary	43h	unknown	Yes	629 G>A	no
MP38	primary	80h	68-9_72del	No	626 A>T	no
MP46	primary	110h	no	No	626 A>T	no

Amirouchene-Angelozzi et al., 2014.

Table 2. Average increase in caspase 3/7 activity in response to Compound-FHT.

92-1, 48h Caspase 3/7 activity

Treatment	Signal 1	Signal 2	Average	Fold change
Compound-FHT	4344	4563	4454	12.2
IDE-196	624	593	609	1.7
Paclitaxel	1010	1119	1064	2.9
DMSO	337	392	365	1.0

MP41, 48h Caspase 3/7 activity

Treatment	Signal 1	Signal 2	Average	Fold change
Compound-FHT	9788	10406	10097	20.4
IDE-196	450	546	498	1.0
Paclitaxel	20058	20314	20186	40.8
DMSO	443	546	495	1.0

MP38, 48h Caspase 3/7 activity

Treatment	Signal 1	Signal 2	Average	Fold change
Compound-FHT	955	1143	1049	6.4
IDE-196	198	218	208	1.3
Paclitaxel	1095	1542	1319	8.1
DMSO	140	187	163	1.0

Mel-202, 48h Caspase 3/7 activity

Treatment	Signal 1	Signal 2	Average	Fold change
Compound-FHT	1027	1501	1264	5.0
IDE-196	952	1102	1027	4.1
Paclitaxel	3792	3252	3522	14.0
DMSO	259	245	252	1.0

Fold increase in caspase 3/7 activity is calculated relative to a control DMSO signal.

Appendix II

Figures

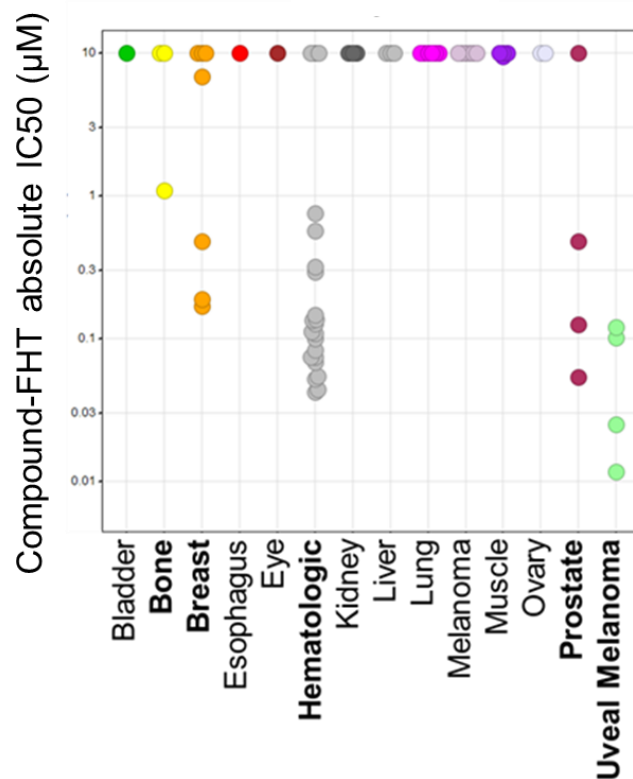


Figure 1. Comparison of cell line sensitivity to Compound-FHT.

Dot plot comparison of Compound-FHT IC₅₀ values across cancer lineages. Y-axis corresponds to an absolute IC₅₀ value derived from a dose-response curve. Each dot on the graph represents a cell line, arranged by lineages on x-axis. The lower the IC₅₀, the greater the sensitivity of the cell line to compound treatment.

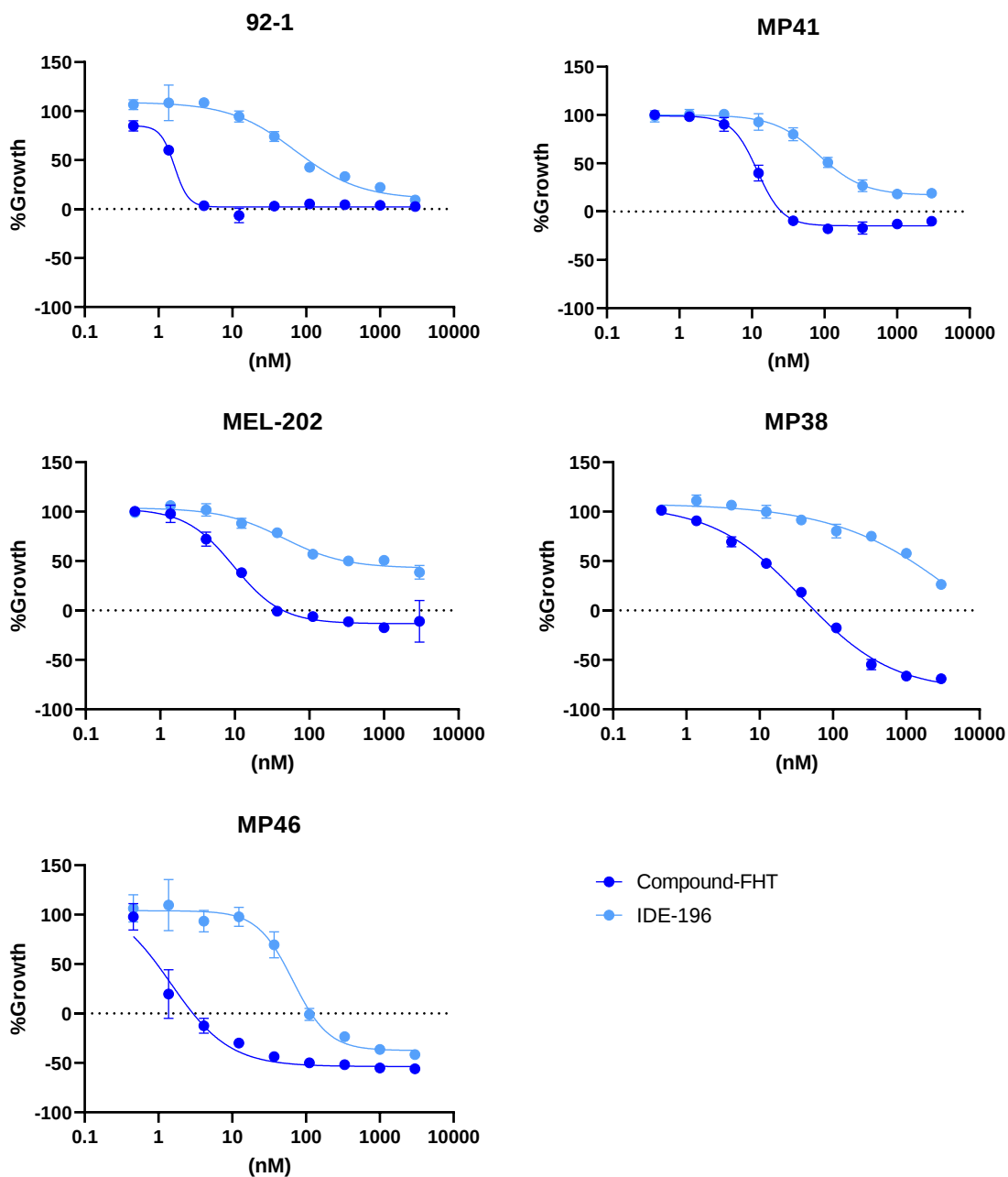


Figure 2. Growth inhibition induced by Compound-FHT or IDE-196 in UM cell lines.

72 hour growth is plotted against compound concentration on x-axis.

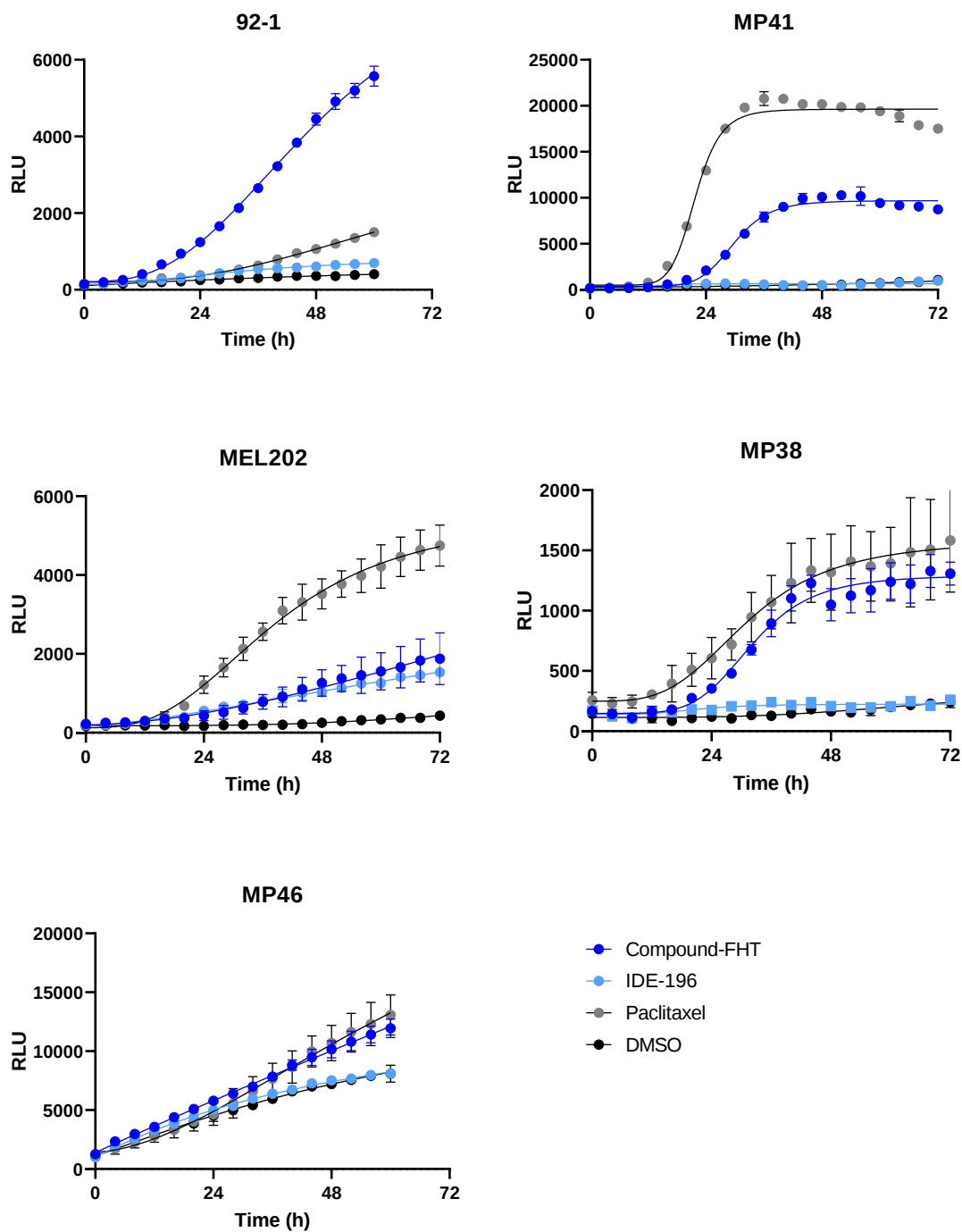


Figure 3. Apoptosis induced by Compound-FHT or IDE-196 in UM cell lines.

Relative luminescence unit (RLU) is plotted against time for cells treated with 111nM compound.

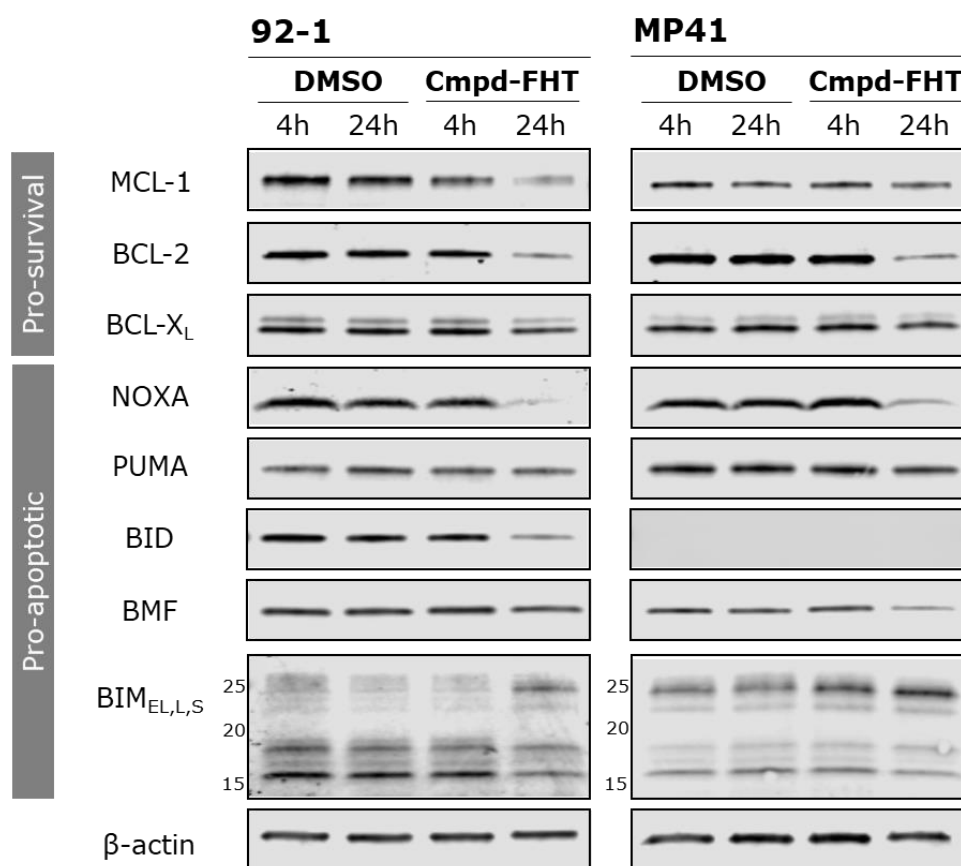


Figure 4. Changes in pro-survival and pro-apoptotic protein expressions.

Western blot analysis of 92-1 and MP41 uveal melanoma cells treated with DMSO or 100nM Compound-FHT for 4 or 24 hours. In a panel for BIM, 25, 20, and 15 indicate molecular weight (kDa), with BIM_{EL} appearing around 25 kDa. BID expression was not captured in this western blot for MP41.

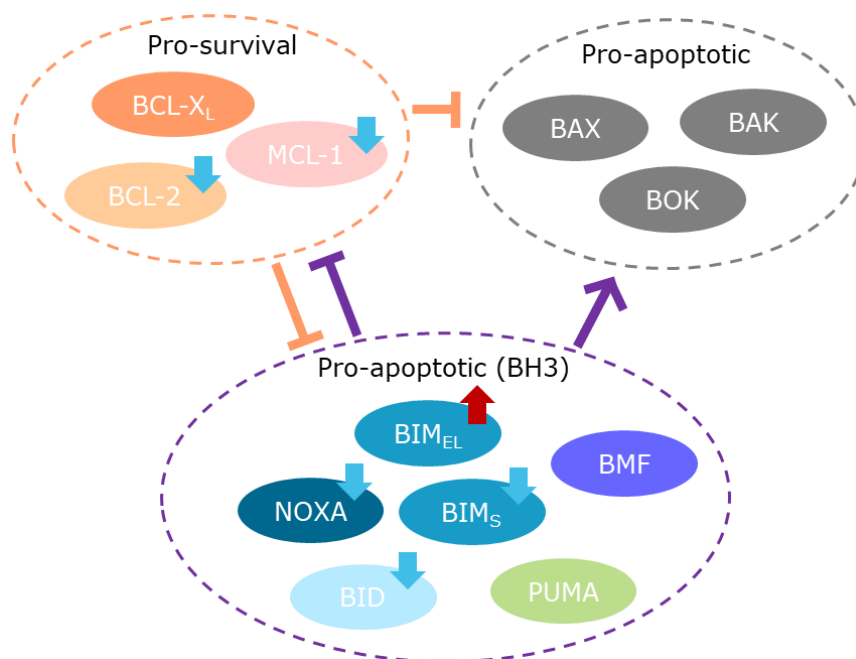


Figure 5. Summary of expected interactions between pro-survival and pro-apoptotic proteins and changes in expressions following Compound-FHT treatment.

Schematic diagram representing known interactions between pro-survival and pro-apoptotic proteins. Up or down arrows next to the proteins indicate observed up or downregulation in response to Compound-FHT treatment.

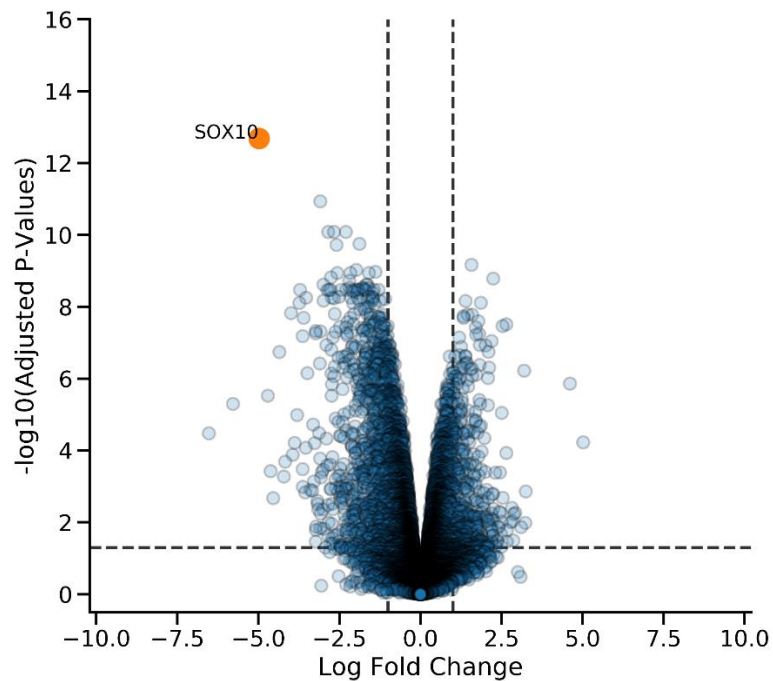


Figure 6. Gene expression changes induced by Compound-FHT in 92-1.

Volcano plot depicting results of differential gene expression analysis of 92-1 cells treated with Compound-FHT or DMSO. Vertical dashed lines indicate 2-fold expression change thresholds, and horizontal dashed line indicate adjusted p-value of 0.05. SOX10 is indicated in orange.

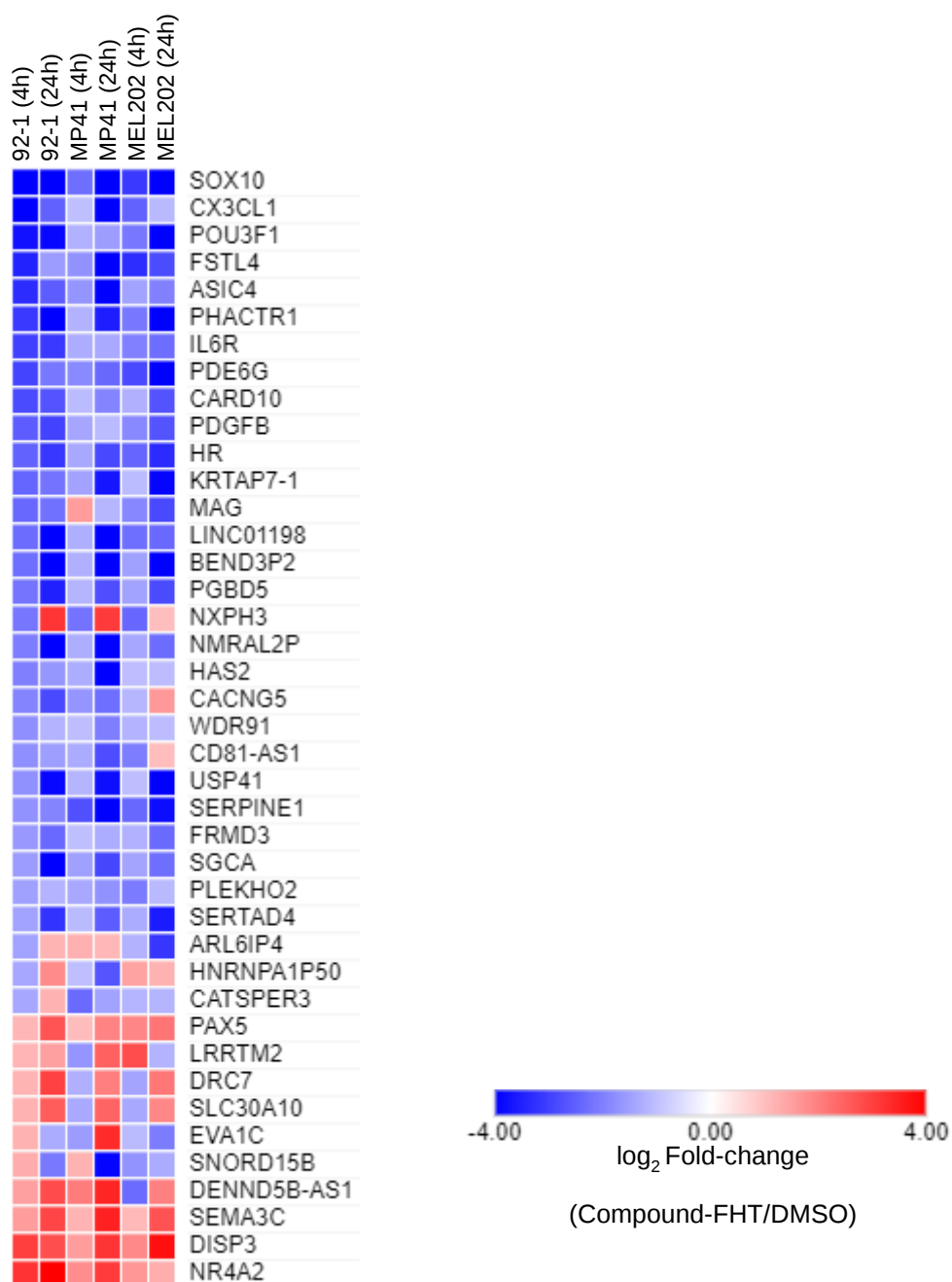


Figure 7. List of genes consistently modulated by Compound-FHT in uveal melanoma cells.

Heatmap representation of genes modulated 2-fold or more across 3 uveal melanoma cell lines. Genes are arranged by expression changes in 92-1, 4h treatment (left column).

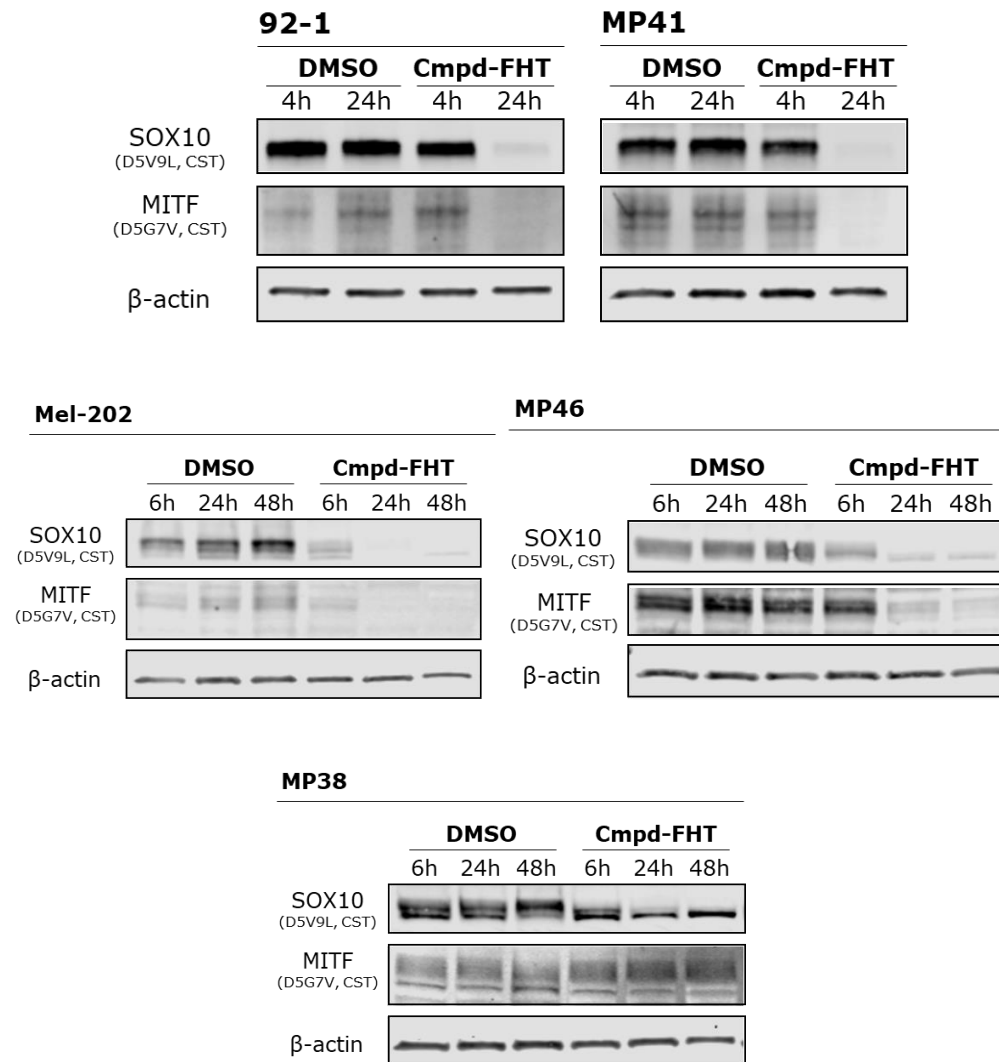
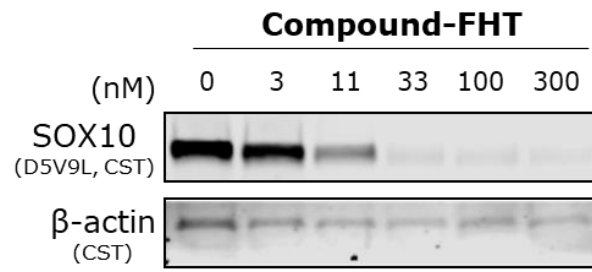


Figure 8. Compound-FHT induced changes in SOX10 protein expression.

Western blot analysis of SOX10 and MITF protein levels with DMSO or Compound-FHT at varying time points.

92-1



MP38

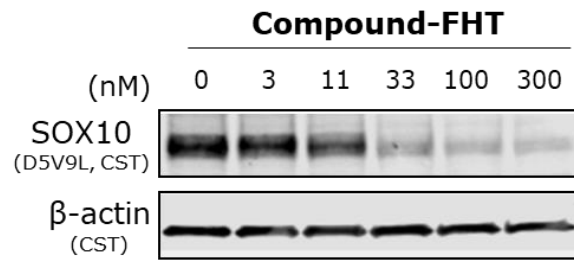


Figure 9. Dose-dependent changes in SOX10 expression with Compound-FHT treatment.

Western blot analysis of SOX10 levels in 92-1 cells (top) and MP38 cells (bottom), 24 hours after 0-300 nM Compound-FHT treatment.

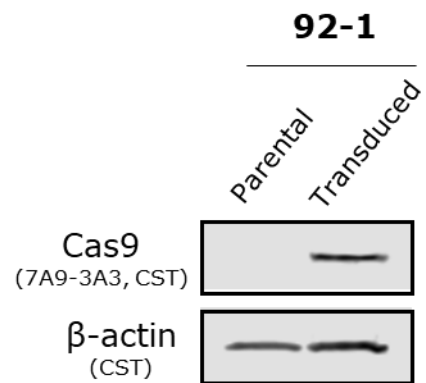


Figure 10. Validation of Cas9 expression in transduced 92-1 cells.

Western blot analysis of Cas9 protein expression in transduced 92-1 cells.

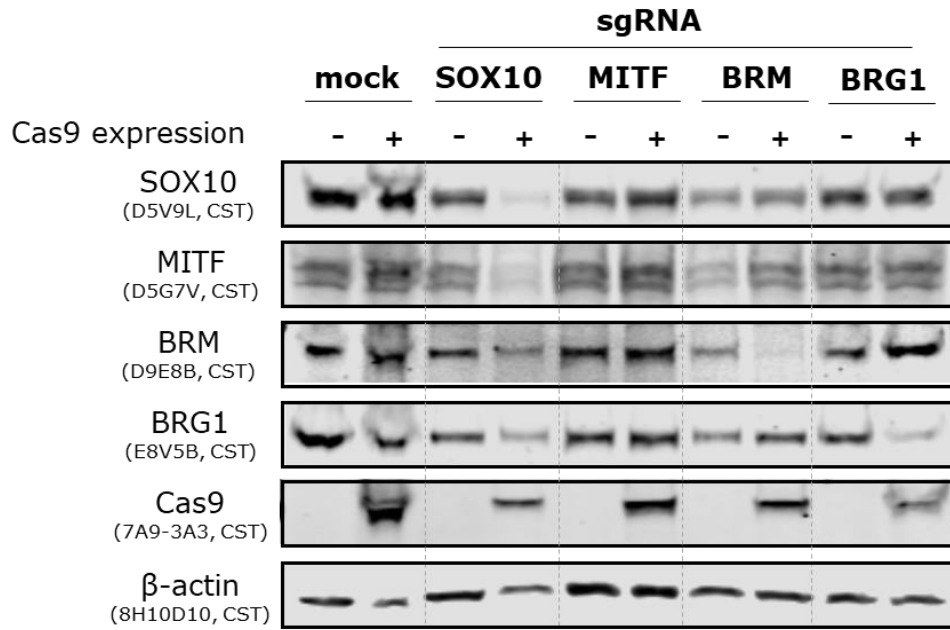


Figure 11. Confirmation of *SOX10*, *MITF*, *SMARCA2*, and *SMARCA4* knockout.

Western blot analysis of SOX10, MITF, BRM, and BRG1 protein expression in parental 92-1 or Cas9 expressing 92-1 cells transduced with sgRNA constructs. Decrease in band intensity indicate protein downregulation and a successful gene knock-out.

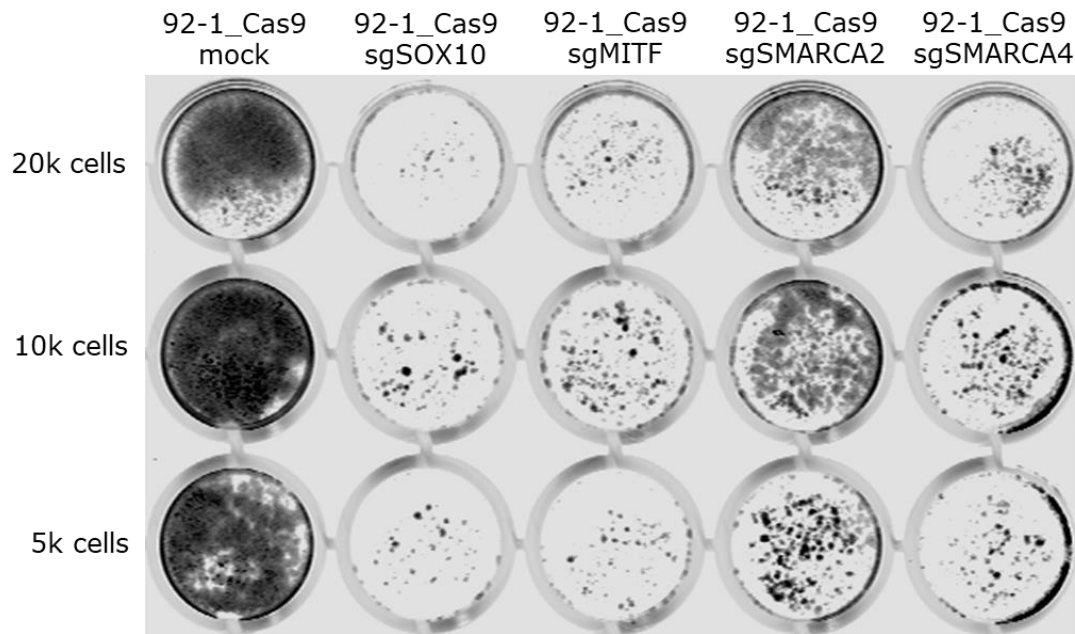


Figure 12. Requirement of *SOX10*, *MITF*, *SMARCA2*, and *SMARCA4* for the growth and proliferation of 92-1 uveal melanoma cells.

Colony formation of 92-1_Cas9 cells transduced with sgSOX10, sgMITF, sgSMARCA2, or sgSMARCA4 seeded at varying densities of 20,000 cells/ well, 10,000 cells/well, or 5,000 cells/well.

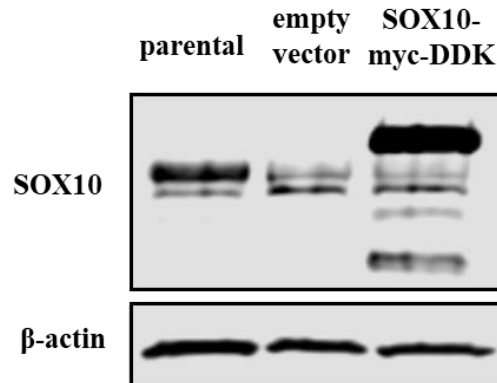


Figure 13. Validation of SOX10 protein overexpression in engineered cell line.

Western blot analysis of SOX10 protein expression in parental 92-1, empty vector transduced 92-1, and SOX10-myc-DDK transduced 92-1 cells. Thick band detected in the right most lane indicates that SOX10 protein is overexpressed in transduced cells. Ectopically expressed SOX10 protein is clearly distinguishable from endogenous protein, with a slight upward shift in molecular weight with myc-DDK tag.

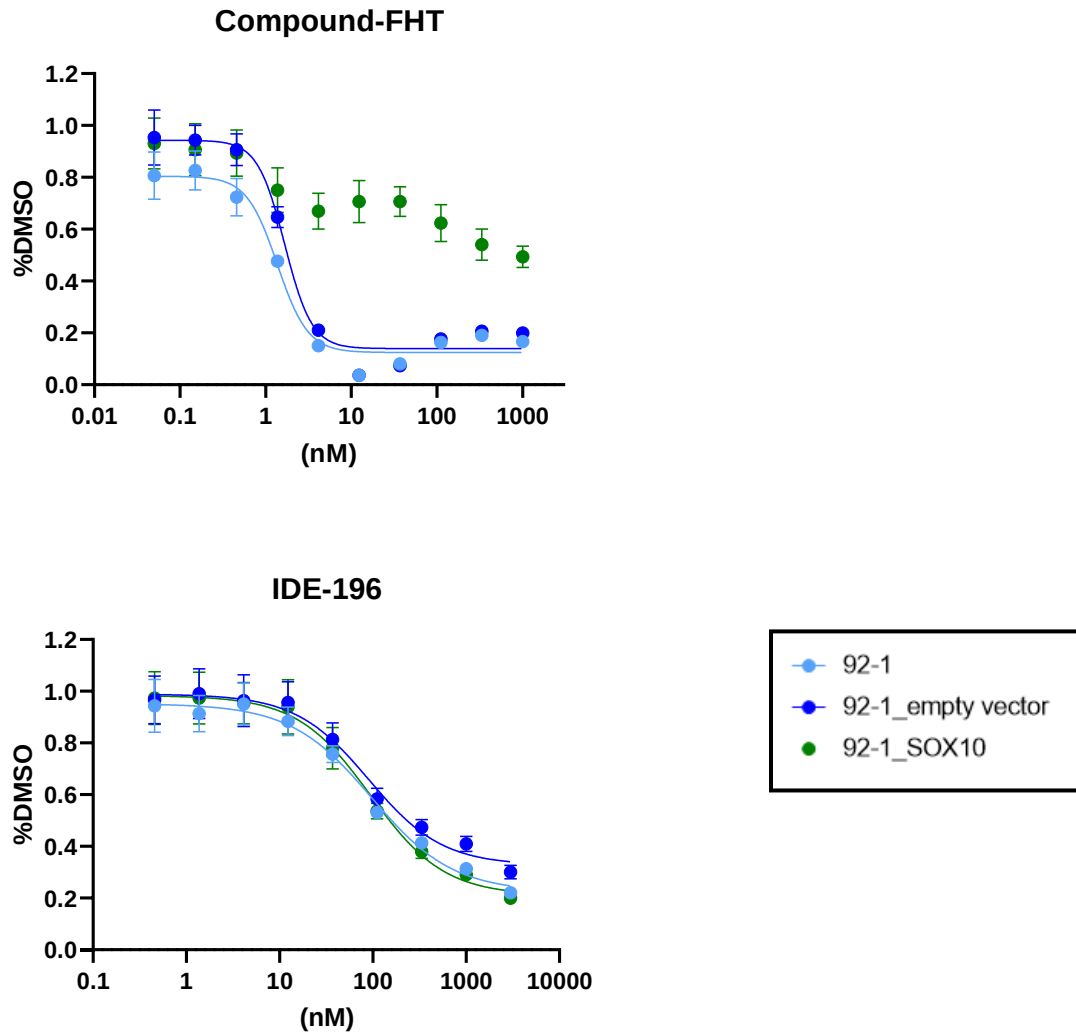


Figure 14. The impact of SOX10 overexpression on proliferation.

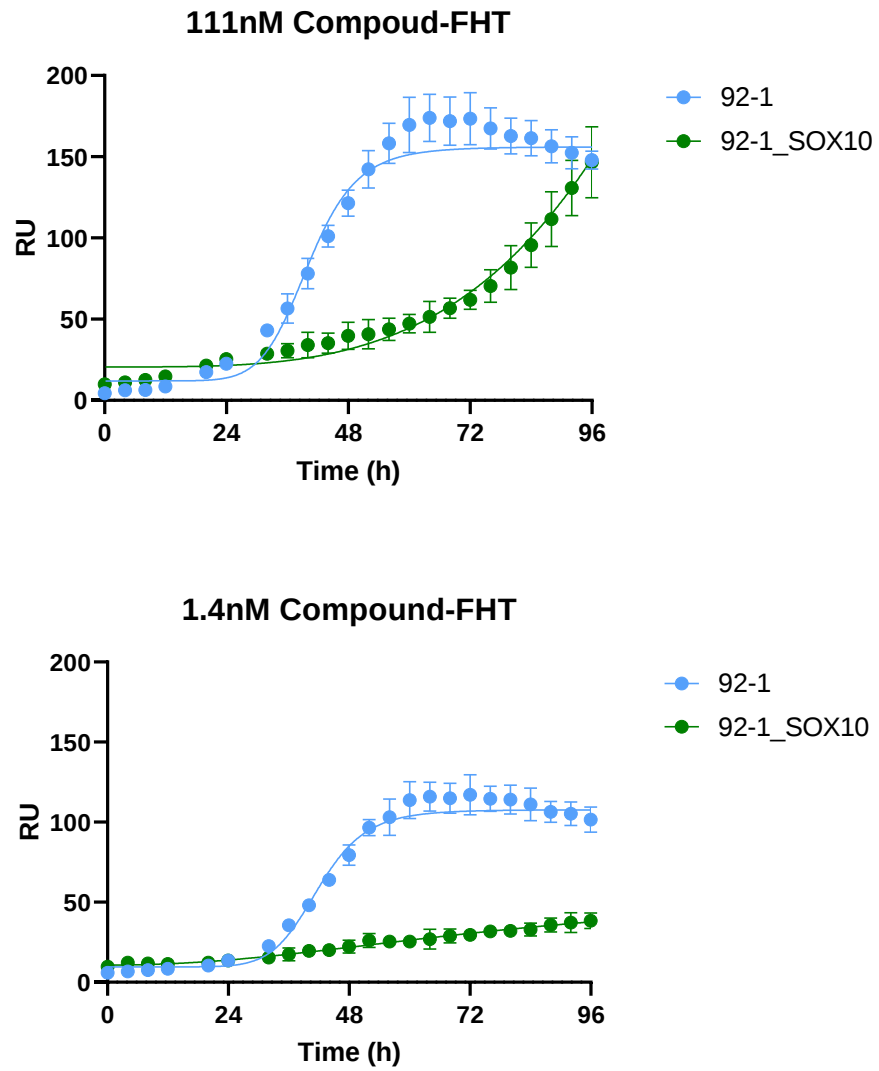


Figure 15. The impact of SOX10 overexpression on apoptosis.

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