ESTABLISHING THE CELLULAR AND MOLECULAR IMPACTS OF THE DUAL BRM/BRG1 (SMARCA2/SMARCA4) INHIBITOR FHD-286 ON PRE-CLINICAL MODELS OF NON-SMALL CELL LUNG CANCER (NSCLC)

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Abstract

The BRG/BRM-associated factor (BAF) complex plays critical roles in chromatin regulation. Within this complex, the two mutually exclusive ATPases BRM and BRG1 (*SMARCA2* and *SMARCA4*, respectively) drive chromatin remodeling activity. Genetic alterations in the *SMARCA4* gene, encoding BRG1, are found in approximately 10% of patients with non-small cell lung cancer (NSCLC), leaving this subset of BRG1-deficient tumors highly susceptible to BRM inhibition. In addition, BRG1 has oncogenic activity in NSCLC, rationalizing the use of a dual BRM/BRG1 ATPase inhibitor such as the highly potent clinical stage compound FHD-286 in NSCLC patients, regardless of *SMARCA4* status. Here, we demonstrate that a panel of NSCLC cell lines respond to dual BAF ATPase inhibition in both in vitro and in vivo settings, irrespective of *SMARCA4* mutation status. Interestingly, dual BRM/BRG1 inhibition in cell lines reduces chromatin accessibility at motifs normally bound by AP-1 transcription factors, which play tumorigenic roles in NSCLC. We also find that expression of the stemness-associated glycoprotein CD44 is decreased by FHD-286, suggesting that dual BRM/BRG1 inhibition impacts the differentiation state of NSCLC. Moreover, transcriptional analyses of NSCLC xenograft models reveal that a basal cell gene expression signature, which is associated with a less differentiated cell state, is downregulated by FHD-286 treatment. Finally, genome-wide CRISPR knockout screening with FHD-286 in two NSCLC lines further elucidates mechanisms of action and molecular vulnerabilities induced by dual BAF ATPase inhibition. Collectively, these results indicate a path forward for the development of FHD-286 for the treatment of NSCLC patients.

NSCLC cell lines are sensitive to dual BRM/BRG1 inhibition, regardless of BRG1 status

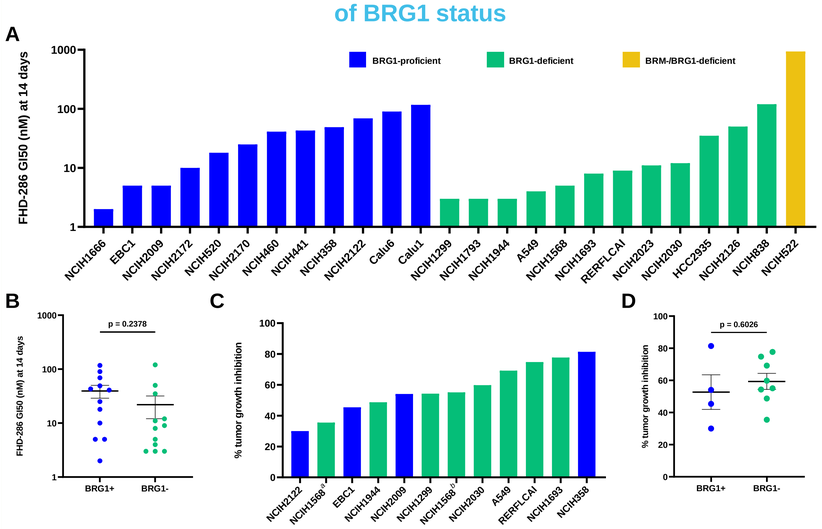

Figure 1. NSCLC models demonstrate a broad range of responses to FHD-286. A) 25 NSCLC cell lines were treated with a serial dilution of FHD-286 for 14 days to calculate GI50. **B)** No statistically significant difference in response to FHD-286 was observed between BRG1-proficient and –deficient lines *in vitro*. **C)** NSCLC cell line-derived xenograft (CDX) models were treated with 15 or 25 mg/kg FHT-2256, a dual BAF ATPase inhibitor and tool compound, and final tumor growth inhibition relative to vehicle was determined. **D)** BRG1-proficient and –deficient CDX tumor models demonstrated similar responses to dual BAF ATPase inhibition *in vivo*.

FHD-286 treatment decreases expression of AP-1 family transcription factors at short timepoints

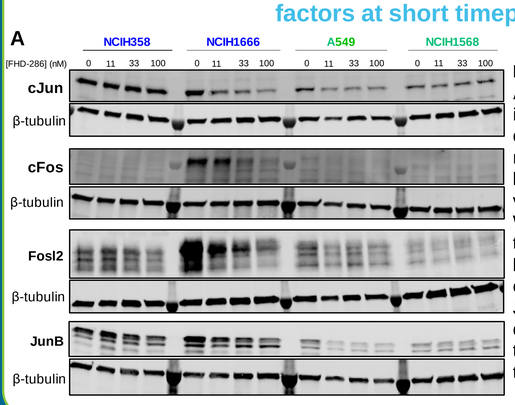

Figure 2. Components of the dimeric AP-1 transcription factor complex, including Fos and Jun proteins, are downregulated in a dose-dependent manner by FHD-286. A) NSCLC cell lines were treated for 48 hours with vehicle or 11, 33, or 100 nM FHD-286. Western blotting for AP-1 family transcription factors reveals that NSCLC lines express varying levels of AP-1 components cJun, cFos, Fosl2, and JunB at baseline. Three out of four lines demonstrate downregulation of AP-1 transcription factors upon FHD-286 treatment in a dose-dependent manner.

AP-1 transcription factor motifs are enriched at chromatin sites that lose accessibility upon FHD-286 treatment

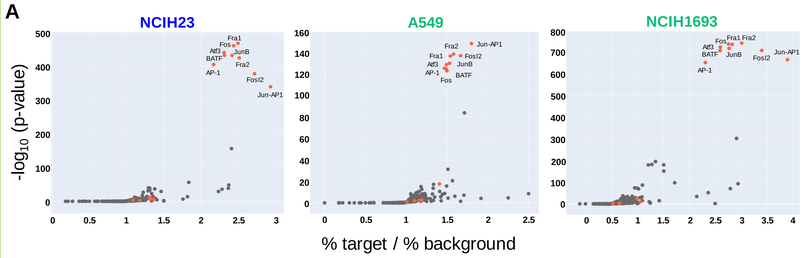

Figure 3. Homer analysis of ATAC peaks in NSCLC cell lines treated with FHD-286 for 8 hours reveals reduced chromatin accessibility at AP-1 motifs. A) A549, NCIH1693, and NCIH23 NSCLC cell lines were treated with vehicle or 100 nM FHD-286 for 8 hours. ATAC-seq analysis reveals that AP-1 transcription factor motifs are enriched within chromatin sites that lose accessibility upon FHD-286 treatment. This trend is consistent among three cell lines, suggesting that loss of AP-1 chromatin binding may be a shared molecular response to FHD-286 treatment in NSCLC.

Expression of the stem cell marker CD44 is downregulated by short-term FHD-286 treatment

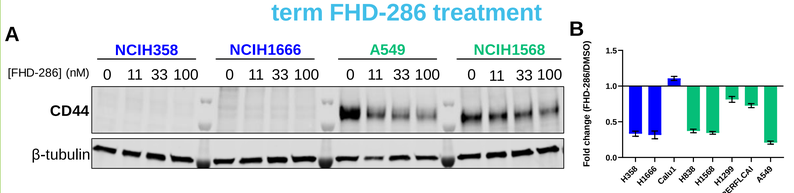

Figure 4. CD44 is downregulated at the RNA and protein level in response to 48-hour treatment with FHD-286. A) 4 NSCLC cell lines were treated with DMSO or 11, 33, or 100 nM FHD-286 for 48 hours. CD44 western blot demonstrates that CD44 downregulation is dose-dependent when protein is detectable in the vehicle-treated control. **B)** 8 NSCLC cell lines were treated with DMSO or 100 nM FHD-286 for 48 hours. Quantitative real-time PCR indicates that CD44 transcripts are downregulated in most NSCLC lines, apart from Calu1, which is notably insensitive to FHD-286 in *in vitro* proliferation assays (see Figure 1). Errors bars indicate SEM.

CD44 expression decreases over time in response to FHD-286 treatment and recovers following washout

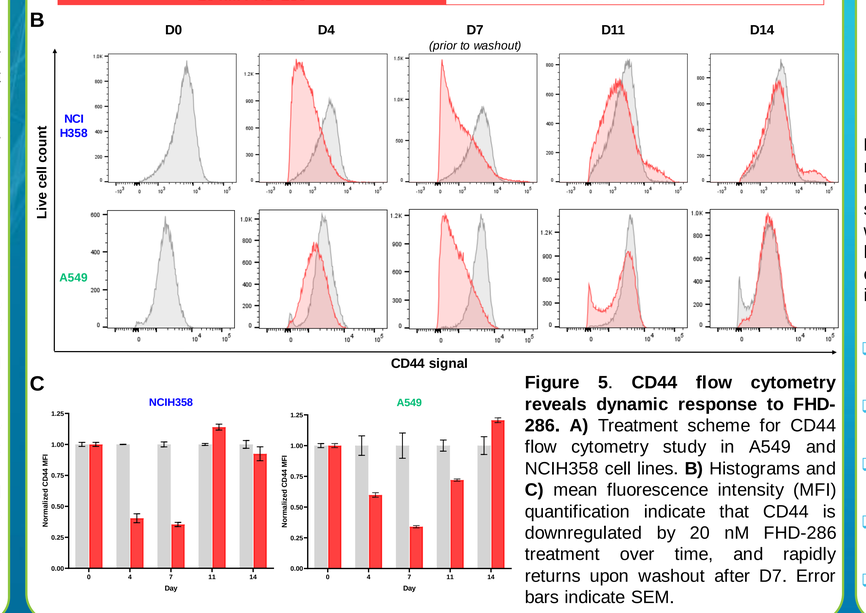

Figure 5. CD44 flow cytometry reveals dynamic response to FHD-286. A) Treatment scheme for CD44 flow cytometry study in A549 and NCIH358 cell lines. **B)** Histograms and **C)** mean fluorescence intensity (MFI) quantification indicate that CD44 is downregulated by 20 nM FHD-286 treatment over time, and rapidly returns upon washout after D7. Error bars indicate SEM.

FHD-286 treatment in NSCLC CDX models downregulates CD44 and lung basal cell genes

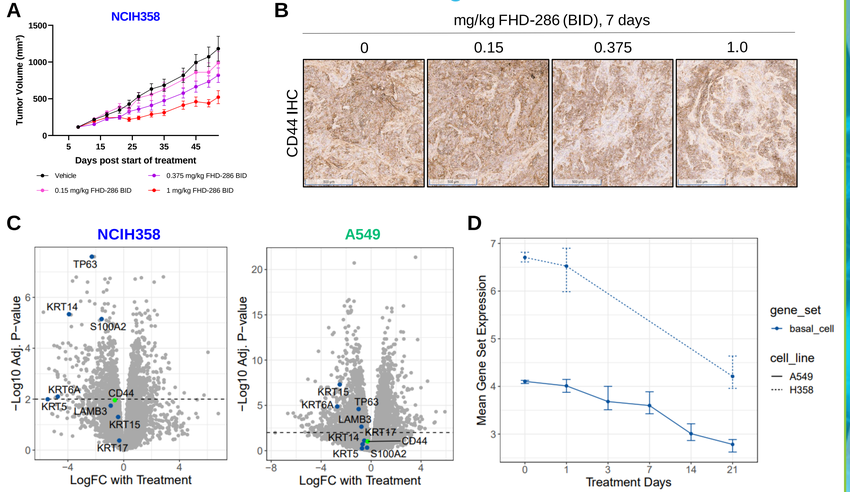

Figure 6. FHD-286 treatment in NSCLC xenograft tumor models induces downregulation of stemness-associated markers, including CD44 and lung basal cell genes. A) Tumor volume measurements for NCIH358 CDX tumors treated with FHD-286. **B)** Immunohistochemistry (IHC) for CD44 in NCIH358 tumor sections treated with FHD-28 for 7 days at doses described above demonstrates CD44 downregulation at higher doses. **C)** RNA-seq on A549 and NCIH358 CDX revealed that CD44 as well as lung basal cell identity genes are downregulated by 21-day treatment with FHD-286 (1 mg/kg, BID). **D)** The lung basal cell gene expression signature decreased over time in response to FHD-286 in both NSCLC CDX tumor models.

Genome-wide CRISPR knockout screening establishes molecular vulnerabilities induced by FHD-286 in NSCLC, including JunB loss

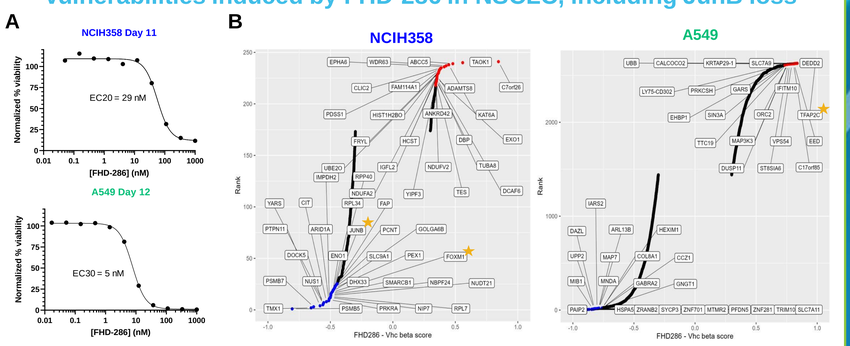

Figure 7. Genome-wide CRISPR screening indicates molecular determinants of sensitivity and resistance to FHD-286 in NSCLC. A) Viability curves used to select FHD-286 doses (EC20-EC30) to use for CRISPR screening in A549 and NCIH358 over 8-10 cell doublings. **B)** Genes ranked by beta scores (β) differences between FHD-286- and vehicle (vhc)-treated conditions. Biologically significant hits were defined as genes with ≥ 4 sgRNAs, $\beta_{diff} \geq 0.3$, and $\beta_{FHD\text{-}286} \geq 0$ for positive hits, and ≤ 0 for negative hits. MiRNAs were excluded. Positive and negative beta scores for genes reveal molecular determinants of resistance and sensitivity, respectively, to dual BRM/BRG1 inhibition. Yellow stars indicate genes involved in regulation of AP-1 activity, CD44, or stemness.

Conclusions

- NSCLC models respond to dual BRM/BRG1 ATPase inhibitor treatment *in vitro* and *in vivo*, regardless of BRG1 status
- Short-term FHD-286 treatment in NSCLC cell lines reduces chromatin accessibility at AP-1 motifs and decreases AP-1 transcription factor protein expression
- CD44, a marker of cancer stem cells, is transcriptionally and translationally downregulated by FHD-286 treatment, and CD44 levels return upon removal of treatment *in vitro*
- FHD-286 treatment in NSCLC CDX tumor models reduces expression of CD44 as well as the basal cell signature, which is also associated with stemness
- Genome-wide CRISPR KO screening in NSCLC lines suggests that impacting AP-1 activity and/or stemness regulators synergizes with FHD-286