Combined PI3K and MLL inhibition synergize in models of HR+ breast cancer

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Abstract:

PI3K pathway hyperactivation, including mutational activation of the PIK3CA isoform that encodes p110α, is a common occurrence in hormone receptor-positive (HR+) breast cancer and has led to the clinical approval of the p110α-selective inhibitor alpelisib. However, PI3K inhibition as a monotherapy lacks durability, at least in part due to aberrant downstream activation of histone H3 lysine 4 (H3K4) methylation. Here we show that inhibition of the H3K4 methyltransferase MLL1 in combination with PI3K inhibition reduces HR+ breast cancer clonogenicity and cell proliferation. While combined PI3K/MLL1 inhibition reduces AKT effector signaling and H3K4 methylation, MLL monotherapy may lead to PI3K effector hyperactivation, suggesting a feedback loop between MLL activity and AKT activation. We additionally show that combined PI3K and MLL1 inhibition synergizes to cause cell death in *in vitro* models of HR+ breast cancer, which is enhanced by the additional genetic ablation of the H3K4-directed methyltransferase MLL4. Together, our data provide evidence of a feedback mechanism connecting histone methylation with AKT and support the preclinical development and testing of pan-MLL inhibitors.

Introduction:

The Phosphatidylinositol-3-kinase (PI3K) signaling pathway coordinates cellular response to external stimuli, supporting cellular processes including cell growth, metabolism, proliferation, and survival, processes that are essential to cancer development, progression, and can dictate therapeutic response[1]. Class IA PI3Ks are heterodimeric protein complexes that consist of a catalytic p110 isoform and a regulatory p85 isoform[2]. The genes PIK3CA, PIK3CB, and PIK3CD encode the catalytic subunit of the p110α, p110β, and p110δ PI3Ks, respectively, which complex with regulatory p85 isoforms[1]. Activated PI3Ks catalyze PIP_2 into PIP_3, which leads to activation of AKT and other downstream effectors. While PI3K activity supports biological processes mediated independently of AKT, AKT is considered a critical and principal PI3K effector[3]. With more than 200 identified substrates, the PI3K effector AKT is a critical signaling amplifier that integrates upstream PI3K signaling with downstream biological functions through substrate phosphorylation[3]. Aberrant PI3K/AKT hyperactivation is a common event in human cancers and occurs via multiple distinct genomic alterations, e.g., receptor tyrosine kinase (RTK) mutation or amplification, PTEN functional inactivation via deletion or mutation, PIK3R1 or AKT1 mutation. Collectively, PIK3CA mutational activation is among the most common oncogenic driver events in breast cancer[1,4]. PIK3CA mutation renders the p110α protein product constitutively active, leading to AKT hyperactivation[5].

The importance of cancer-associated genomic alterations, including PIK3CA mutation in the PI3K pathway and its subsequent dysregulation, has prompted the development and clinical testing of numerous PI3K and AKT inhibitors[6]. Given the divergent functions of PI3K isoforms, isoform-selective PI3K inhibitors exhibit enhanced preclinical and clinical success compared to pan-PI3K agents due to the on-target selectivity and has resulted in the FDA approval of the several isoform-selective PI3K inhibitors: idelalisib[7], copanlisib[8], and alpelisib[9]. Alpelisib is a p110α-selective inhibitor, approved for the treatment of metastatic PIK3CA-mutant, HR+ breast cancers in combination with the estrogen receptor antagonist fulvestrant[9]. Despite this success, preclinical and clinical studies utilizing PI3K inhibitors including alpelisib demonstrate a lack of durability when used as monotherapies, and resistance remains a common occurrence. Thus, achieving a durable response to PI3K inhibition remains an unmet clinical need.

Histones, including H3, serve an integral role downstream of signaling pathways as they support a variety of posttranslational modifications (PTMs) that include phosphorylation, acetylation, and methylation[10]. The precise combination of histone PTMs contribute to chromatin state and ultimately gene expression. Evidence suggests that some enzymes responsible for the reading, writing, and erasure of histone PTMs are effectors of signaling cascades including PI3K. AKT has been shown to phosphorylate the H3K27 histone methyltransferase EZH2[11] and the histone acetyltransferase p300[12]. Breast cancer-associated redundant phosphorylation of the H3K4 demethylase KDM5A[13] and the H3K4 methyltransferase MLL4/KMT2D[14] by AKT augments H3K4 methylation and enhances gene expression to support pro-oncogenic growth and therapeutic resistance, indicating that H3K4 methylation and associated epigenomic regulation may be a critical downstream effector of aberrant PI3K/AKT signaling in breast and other cancers. Clinical studies including meta-analyses demonstrate that aggressive breast and other cancers are characterized by elevated H3K4 methylation and that H3K4 trimethylation (H3K4me3) is an indicator of poor patient prognosis[15-17].

Here, we demonstrate that combined therapeutic targeting of PI3K/AKT and the H3K4 methyltransferase MLL1 reduces PI3K effector phosphorylation and H3K4 methylation in PIK3CA-mutated HR+ breast cancer cell lines. Dual PI3K and MLL1 inhibition reduces breast cancer cell clonogenicity and synergizes to reduce cell viability and increase apoptosis, effects that are further enhanced following genetic loss of MLL4. We also uncover a previously uncharacterized feedback loop between MLL and AKT, demonstrating that pharmacological MLL inhibition relieves PI3K repression and elevates AKT activity, and combined PI3K and MLL1 inhibition abrogate aberrant AKT and effector activation.

Materials and Methods:

Cell lines and inhibitors:

MCF7, T47D, and 293T cells were maintained at 37C and 5% CO_2 in RPMI or DMEM + 10% FBS and 1% Pen-Strep. GDC-0941, BYL719, GDC-0032, GDC-0068 were purchased from Selleck, and MI-136, MI-503, and MM-102 were purchased from Cayman.

Cell lysis and immunoblotting:

Cells were lysed in IP buffer (20 mM TrisHCl [pH 7.5], 150 mM NaCl, 5 mM MgCl2, 1% NP-40), supplemented with protease and phosphatase inhibitors. Histones were acid extracted by lysing in Triton extraction buffer (TEB; PBS, 0.5% Triton X-100) supplemented with protease inhibitors. Lysates were centrifuged 6,500 x g and histones were acid extracted from the resulting pellet with 1:1 TEB:0.8 M HCl. Histones were centrifuged and the supernatant precipitated with the addition of an equal volume of 50% TCA, then 12,000 x g centrifugation. Histones were washed one time in ice cold 0.3 M HCl in acetone and two times ice cold in 100% acetone before drying and resuspended in 20 mM Tris-HCl (pH 8.0), supplemented with protease inhibitors. Whole cell lysate or acid-extracted proteins were separated using SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blocked in TBST+5% milk. Proteins of interest were visualized and quantified after primary antibody incubation (Odyssey, Li-Cor). Primary antibodies used were as follows: AKT, AKTS473, AKTT308, S6, S6S240/44, (CST), β-actin (Millipore), H3, H3K4me3, H3K4me2, H3K4me, (Abcam).

Cell Viability, Apoptosis and Clonogenic assays:

For resazurin viability assays, 3,000 cells/well were seeded in 96-well plates. The next day, serially diluted inhibitors were added and incubated for 5 d, after which resazurin (sigma) was added to a final concentration of 0.1 mg/ml and incubated for 5 h prior to measuring the excitation/emission (544/590) (Biotek). To assay for apoptosis, cells were seeded and treated with the indicated inhibitors for 5 d, after which cells were detached using accutase (Biolegend) and stained using Annexin V/PI following manufacturer's protocol (BioLegend). Cells were kept in the dark until assessment and were analyzed by flow cytometer (BDFACSymphony A3) within 1 h. To measure cell clonogenicity, 500 cells/well were seeded in a 6-well plate, and treated the following day with the designated inhibitors, and incubated for a total of 19 d with media and inhibitor change every 3-4 d, after which cells were fixed and crystal violet stained. Plates were imaged, destained, and crystal violet quantitated at OD 595 (Biotek).

Synergy Calculation:

Bliss and Loewe synergy scores were calculated in Python using the *synergy* package: https://pypi.org/project/synergy; source code https://github.com/djwooten/synergy[18].

Statistical analysis:

All experiments including western blot, resazurin viability, clonogenic, and annexin V assays, were performed in three independent experiments unless otherwise noted. Mean ± SEM are reported. Statistical significance ($p < 0.05$) of differences between two groups was determined by Student's t test.

Results:

Combined PI3K/MLL1 inhibition reduces breast cancer cell line clonogenicity through on-target activity

Previous reports demonstrate that aberrant PI3K/AKT signaling regulates H3K4 methylation through the activity of H3K4-enzymes in hormone receptor-positive (HR+) breast cancer[13,14] and high H3K4 methylation is associated with poor prognosis in breast and other cancers[15]. We thus postulated that combined PI3K and MLL inhibition may provide additional therapeutic benefit in PI3K pathway activated, HR+ breast cancer. To assess the effects of combined PI3K/MLL1 inhibition on cell growth, the PI3K-activated (PIK3CA^{H1047R}-mutant), HR+ breast cancer cell line MCF7 was treated with the PI3K inhibitor alpelisib, the MLL1 inhibitor MI-503, or the combination. Combined PI3K and MLL1 inhibition reduced the clonogenicity and cell proliferation compared to either monotherapy (Fig 1A), which was observed at multiple doses (Fig S1A). MI-503 is a small-molecule MLL1 inhibitor that disrupts the association between MLL1 and its co-factor Men1, thereby disrupting target gene expresion[19]. Cells treated with the MLL-Men1 inhibitor MI-136[20] in combination with PI3K inhibitors also exhibit impaired clonogenicity (Fig 1A). A reduction in clonogenicity was also observed in cells treated with MLL1 inhibitors in combination with the p110β-sparing PI3K inhibitor taselisib[21] (Fig 1B) or the pan-PI3K inhibitor pictilisib[22] (Fig S1B). Combined PI3K and MLL1 inhibition also decreased clonogenicity in the HR+, PIK3CA^{H1047R}-mutant cell line T47D (Fig 1C, D, Fig S1C). These data suggest that combined PI3K and MLL1 inhibition impairs the clonogenicity in PI3K-activated, HR+, breast cancer models.

We next determined whether the biological activity of PI3K and MLL1 inhibitors observed (Fig 1) is a result of on-target enzymatic inhibition. MCF7 cells were treated with PI3K and/or MLL1 inhibitors, and PI3K inhibition reduced effector signaling as indicated by a decrease in phosphorylated AKT and S6 (Fig 2A, C) and MLL inhibition modestly reduced H3K4 methylation (Fig 2B, D, Fig S2C, D). Combined PI3K and MLL1 inhibition reduced H3K4 methylation and was detectable as early as 72h (Fig 2C, D). Robust MLL1-inhibitor monotherapy - directed loss of H3K4 methylation was detected at later time points such as 96-120h (Fig S2E), which is consistent for detecting global changes to histone PTMs in response to therapeutic inhibition[13]. Unexpectedly, we found that MLL inhibition in PI3K-activated cancer cell line models increases PI3K effector signaling as indicated by increased AKT and S6 phosphorylation, which was abrogated in the combination treatment modalities (Fig 2A, B, Fig S2A, B). The detected increase in PI3K effector signaling following MLL1 monotherapy suggests the presence of a feedback loop between MLL and AKT. Similar MLL1 inhibitor-induced PI3K effector activity was detected in the PTEN-null prostate cancer cell line LNCAP, which is also characterized by PI3K pathway hyperactivation (data not shown). We found that MLL1 inhibition increased AKT phosphorylation as early as 5min

post inhibition, which was sustained for at least 24h (Fig 2E). To test whether the ability of MLL1 inhibition to enhance PI3K effector activity is due to on-target inhibit activity, we treated T47D cells with a panel of MLL1 inhibitors that function through disparate mechanisms of action and found that MLL1/Men1 (MI-503 and MI-136) and MLL/WDR5 (MM102) inhibitors increased AKT phosphorylation (Fig 2F). Collectively, these data suggest a functional relationship between PI3K/AKT signaling and MLL methyltransferases which may support their combined inhibition.

Combined PI3K/MLL1 inhibition synergizes to reduce viability by enhancing apoptosis

A major limitation of clinical PI3K inhibition is the lack of apoptotic induction that leads to tumor clearance, as PI3K monotherapy is primarily associated with a cytostatic effect induced by G1 arrest[23]. To determine whether combined PI3K/MLL1 inhibition overcomes PI3K inhibitor induced cytostasis, we treated PI3K-activated, HR+ breast cancer cells with the PI3K inhibitor alpelisib, the MLL1 inhibitor MI-503, or the combination, and cellular viability was evaluated. Combined PI3K and MLL inhibition reduced cell viability in multiple models of breast cancer (Fig 3A, B), which was confirmed with additional PI3K and MLL1 inhibitors (Fig 3A, B, Fig S3A). We next aimed to determine whether PI3K and MLL1 combination therapy is synergistic, as synergistic regimens potentially maximize therapeutic effects while minimizing off-target effects[24]. Drug synergy was calculated using both Loewe and Bliss, which measure synergy within the same pathway or among distinct pathways, respectively. Bliss and Loewe calculations/scores separately defined the combination of low-dose PI3K and MLL1 inhibition as synergistic (Fig 3C)[25]. While PI3K or MLL1 monotherapies do not yield apoptotic induction, we found that the combination of PI3K and MLL1 inhibition increased apoptosis (Fig 3D), which is consistent with the metabolism-based cell viability assays (Fig 3A). To assess whether the detected loss of viability is due to on-target effects of MLL1 inhibition, we utilized the MLL1 inhibitor MM102. While MM102 is not as potent as MI-503 or MI-136, the peptidomimetic MM102 inhibits MLL1 function through the disruption of the MLL1-WDR5 protein-protein interaction, which is essential for MLL1 histone methyltransferase activity[26]. Combined PI3K inhibition with MM102 reduces cell viability and proliferation in models of PI3K-activated breast cancers (Fig S3B). These data demonstrate that combined PI3K and MLL inhibition synergize to reduce viability in models of PI3K activated, HR+, breast cancer.

MLL4/KMT2D genetic ablation enhances PI3K/MLL1 inhibitor-driven apoptosis

The majority of MLL inhibitors in preclinical and clinical investigation specifically target the MLL1-containing COMPASS complex, through the loss of MLL1 association with essential complex members MEN1 or WDR5[19,20,26]. Because H3K4 methylation is elevated in some cancer types and is associated with a poor patient prognosis[15], MLL1 inhibition may function as a viable therapeutic target. However, published reports suggest that the MLL enzyme MLL4/KMT2D is an AKT substrate, and its enzymatic activity is associated with resistance to the PI3K inhibitor alpelisib in HR+ breast cancer via enhancing estrogen receptor signaling[14]. To address whether the loss of MLL4 enhances the observed synergy with dual PI3K and MLL1 inhibition, we utilized siRNA to knock down MLL4. Combined PI3K/MLL1 inhibition with MLL4 knockdown further enhanced apoptosis compared to all single- and double-agent treatment groups (Fig 4A, S4A, B) in the MCF7 cell line. These data suggest that enzymatic inhibition of MLL1 and MLL4, when combined with PI3K inhibition, is a promising therapeutic modality to induce cell death and produce a durable therapeutic response in PI3K-activated cancers.

Discussion:

Of the more than 70% of breast cancers that are hormone receptor-positive, 40% are characterized by aberrant PI3K pathway activity, which has prompted the clinical testing of pan- and isoform-selective PI3K inhibitors in this patient population. However, the utility of PI3K-targeted therapies is limited as inhibitors of PI3K enzymes lack efficacy and durability as monotherapies. Thus, research to define novel mechanisms by which PI3K/AKT contributes to oncogenesis and exploiting this knowledge to develop and test viable combination therapies for the treatment of PI3K pathway-activated cancers is critical. Here, we build upon previous studies demonstrating the PI3K effector AKT regulates cellular H3K4 methylation through the phosphorylation of the H3K4 methyltransferase MLL4/KMT2D[14] and the H3K4 demethylase KDM5A[13] in breast cancer. AKT-mediated KDM5A phosphorylation redistributes KDM5A from the nucleus into the cytoplasm, rendering it ineffective at H3K4me2/3 demethylation[13]. In this context, elevated promoter H3K4me3 supports the transcription and ultimately the expression of cell cycle promoting genes, encouraging proliferation and oncogenesis. In contrast, AKT- or SGK2-driven phosphorylation of the H3K4 methyltransferase MLL4 attenuates its activity[14,27]. PI3K inhibition enhances MLL4 activity, further driving H3K4 methylation and promoting an open chromatin conformation, primarily at promoters containing estrogen receptor binding motifs. The open chromatin conformation supports the binding of pioneer transcription factors such as FOXA1, which then recruits ER to initiate the transcription of ER-regulated genes. These disparate mechanisms of action drive biologically distinct and separable H3K4 methylation under conditions of either PI3K pathway hyperactivation or inhibition, suggesting that H3K4 methylation is downstream of the PI3K/AKT pathway, and that combined PI3K and MLL inhibition may provide therapeutic benefit. While the data presented here focuses on the potential utility of combined PI3K/MLL inhibition in HR+, PIK3CA-mutant breast cancers, we speculate that benefit may be derived in other cancers characterized by PI3K pathway activation. PI3K signaling antagonizes the activity of ER[28] and other nuclear hormone receptors such as androgen receptor (AR)[29], suggesting a similar therapeutic opportunity in AR-dependent, PI3K pathway hyperactivated prostate cancers given AKT-mediated regulation of MLL4/KMT2D and KDM5A in this context. However, previous reports demonstrating that AKT dysregulates H3K4 methylation through KDM5A phosphorylation in cell lines representing all molecular subtypes of breast cancer[13] suggest potential utility of dual PI3K/MLL inhibition in hormone receptor-independent cancers. Further studies are necessary to test this hypothesis.

Since a significant limitation of PI3K inhibitors is their cytostatic nature[23], a major attribute in defining effective therapies to pair with PI3K inhibitors is robust apoptotic induction. Herein, we demonstrate that the combined inhibition of p110α and MLL1 synergizes to induce apoptosis of PIK3CA-mutant, HR+, breast cancer cell lines (Fig 3). Dual PI3K and MLL1 inhibition reduces the clonogenicity of PIK3CA-mutant breast cancer cell lines (Fig 1), while also decreasing their proliferation (Fig 3). This synergy is enhanced by genetic ablation of MLL4. Further, we identify a compensatory feed-back loop between MLL methyltransferases and the PI3K pathway. While additional experiments are needed to understand the mechanism(s) that underlie the observed MLL feedback loop, our results suggest that MLL inhibition hyperactivates PI3K signaling upstream of AKT. AKT hyperactivation occurs relatively quickly and is conserved amongst MLL inhibitors that exhibit divergent scaffolding and thus function through divergent mechanisms – through MLL1/WDR interaction, or via MLL1/Men1 binding. While the possibility of MLL-inhibitor off-target effects cannot be eliminated, AKT hyperactivation following the use of multiple MLL inhibitors suggests that compensatory AKT activation is the result of on-target MLL

inhibition. The placement of MLL both upstream and downstream of AKT in the PI3K signaling network highlights the importance of dual inhibition of these enzymes as a mechanism to durably suppress PI3K pathway activation.

Our findings suggest that while PI3K and MLL1 inhibition is an effective therapeutic strategy in PIK3CA-mutant, HR+ breast cancer cell lines, this combination can be further improved. Current MLL1 inhibitors specifically target MLL1 through protein-protein interactions with essential MLL1-specific COMPASS complex members WDR5 or MEN1. MLL1 methyltransferase activity is dependent on its association with WDR5; high-affinity peptidomimetic inhibitors including MM102 bind WDR5 to prevent association with MLL1[26]. Other MLL1 inhibitors including MI-136 and MI-503 share a thienopyrimidine scaffolding and function via abrogation of the MLL1/MEN1 association to robustly induce cell death in models of MLL1-translocated leukemias and AR-dependent prostate cancer[20,30]. While we demonstrate that these MLL1 inhibitors synergize with PI3K inhibitors in PIK3CA-mutant breast cancer, this combination does not effectively target MLL4/KMT2D, which is a downstream PI3K effector that is essential in mediating resistance to ER-degraders such as fulvestrant[14]. Because MLL4 or pan-MLL inhibitors that target the enzymatic activity of the SET-domain are currently unavailable, we genetically ablated MLL4 in combination with PI3K/MLL1 dual inhibition and found that loss of MLL4 further enhances the PI3K/MLL1-driven apoptosis (Fig 4). The results presented here suggest a therapeutic opportunity to design and test MLL4 or pan-MLL inhibitors in PI3K-activated breast cancers.

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Author Contributions:
J.F., R.B.J., M.Z., and J.M.S. designed the research. J.F., R.B.J., K.P., M.A., M.Z., R.S.L., and J.M.S. performed the research and analyzed the results. J.M.S. supervised the studies. J.M.S. wrote the manuscript with input from J.F., R.B.J., K.P., and M.A.

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Figures and Figure Legends:

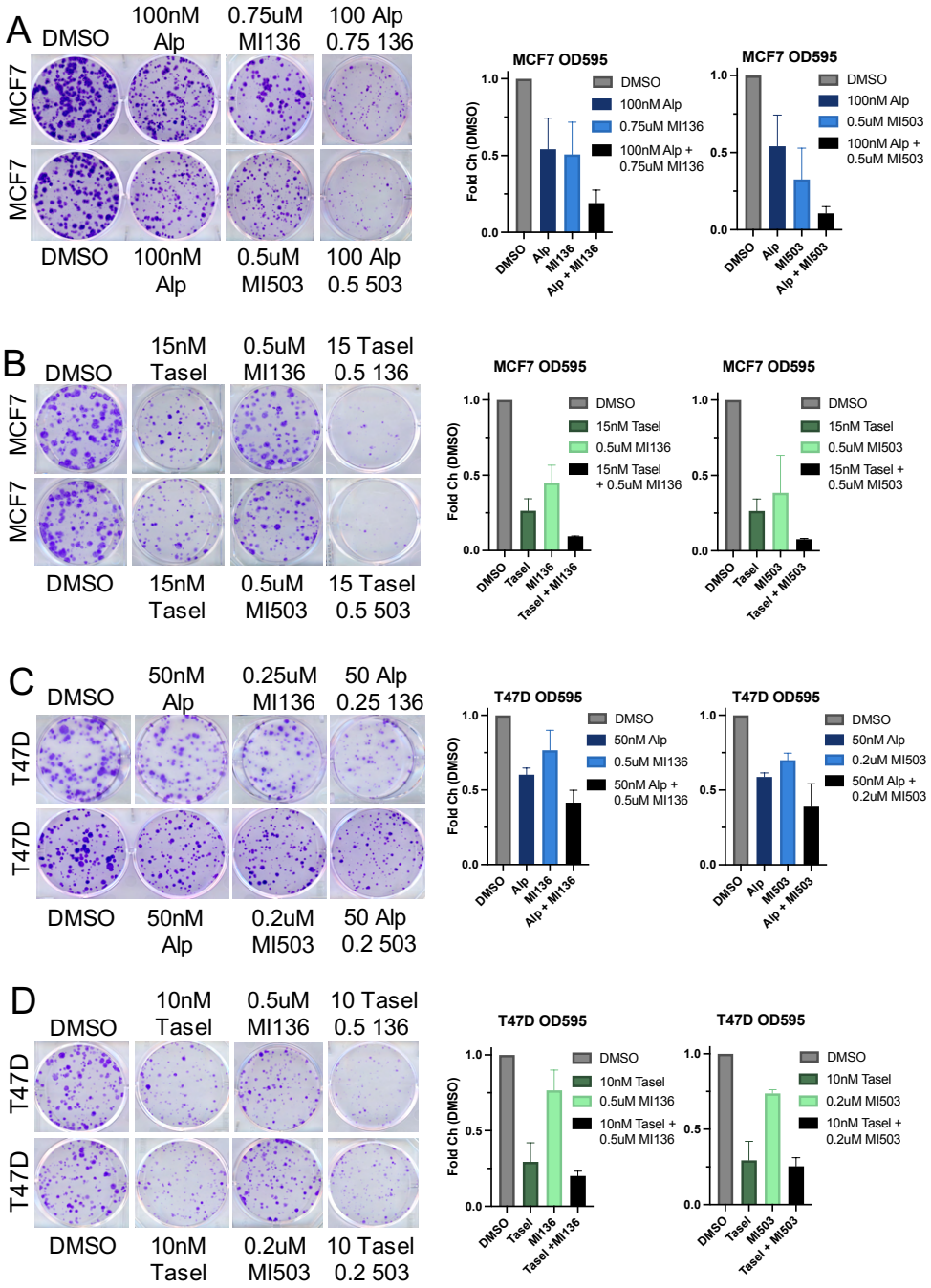


Figure 1. Combined PI3K and MLL1 inhibition reduces clonogenicity of HR+, PIK3CA-mutant, breast cancers. (A) MCF7 breast cancer cells treated with the indicated concentrations of alpelisib, MI-136, MI-503, or DMSO for 19 d before fixation and crystal violet staining. (B) MCF7 breast cancer cells treated with the indicated concentrations of taselisib, MI-136, MI-503, or DMSO for 19 d before fixation and crystal violet staining. (C) T47D breast cancer cells treated with the indicated concentrations of alpelisib, MI-136, MI-503, or DMSO for 19 d before fixation and crystal violet staining. (D) T47D breast cancer cells treated with the indicated concentrations of taselisib, MI-136, MI-503, or DMSO for 19 d before fixation and crystal violet staining. For all experiments, representative images from at least 3 independent experiments are shown. Data are shown as mean ± SEM.

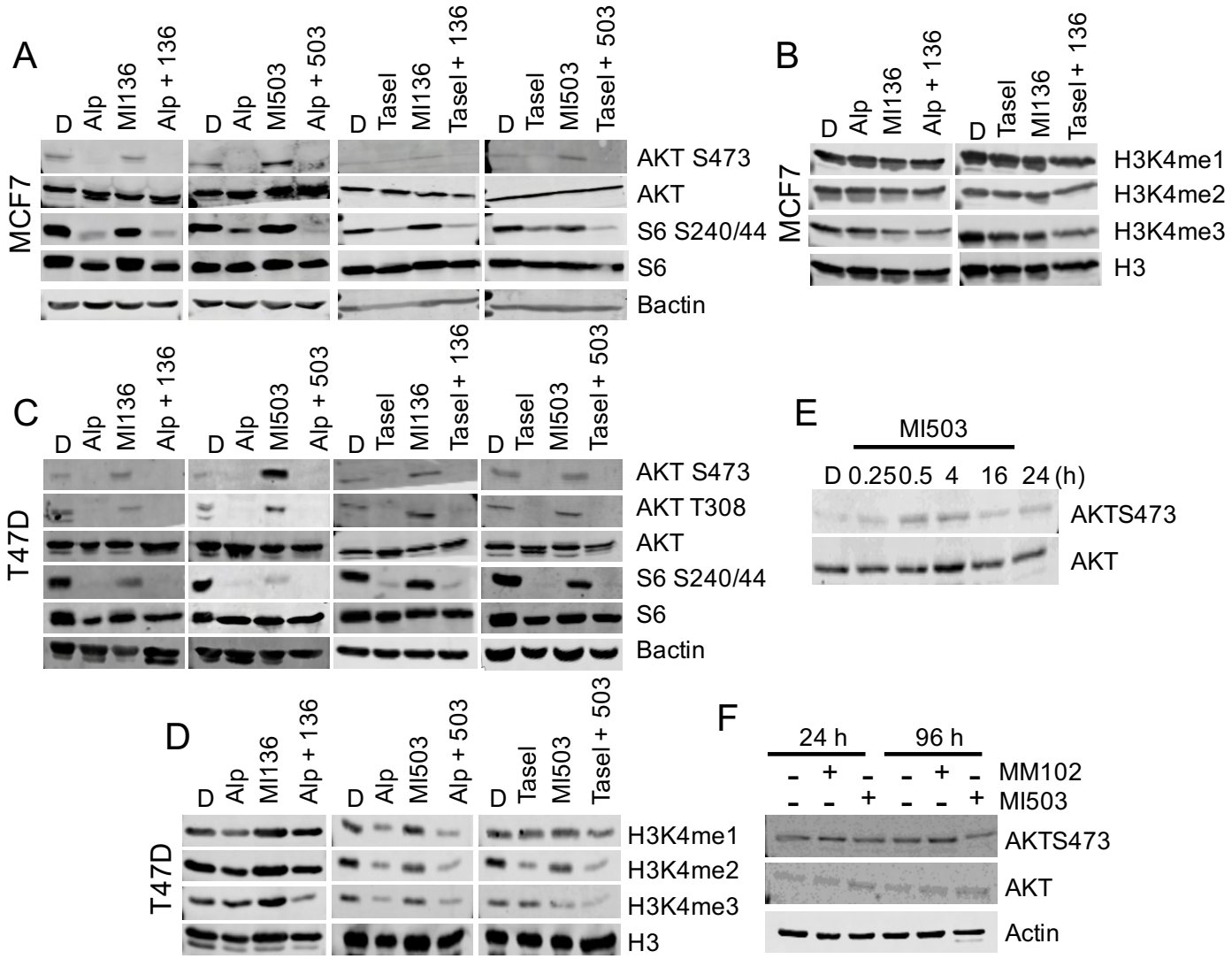


Figure 2: MLL1 inhibition hyperactivates AKT through a feedback loop. (A) MCF7 breast cancer cells were treated with alpelisib (1 uM), taselisib (0.5 uM), MI-136 (4 uM), MI-503 (4 uM) or DMSO for 24h prior to lysis. Lysates were immunoblotted for the indicated antibodies. (B) MCF7 cells were treated with alpelisib (1 uM), taselisib (0.5 uM), MI-136 (4 uM), MI-503 (4 uM) or DMSO for 72h and then lysates acid extracted. Lysates were immunoblotted for the indicated antibodies. (C) T47D breast cancer cells were treated with alpelisib (1 uM), taselisib (0.5 uM), MI-136 (4 uM), MI-503 (4 uM) or DMSO for 24h prior to lysis. Lysates were immunoblotted for the indicated antibodies. (D) T47D cells were treated with alpelisib (1 uM), taselisib (0.5 uM), MI-136 (4 uM), MI-503 (4 uM) or DMSO for 72h and then lysates acid extracted. Lysates were immunoblotted for the indicated antibodies. (E) T47D cells treated with the MI-503 (4 uM) for the indicated time and then lysates prepared. Lysates were immunoblotted for the indicated antibodies. (F) T47D cells treated with MI-503 (4 uM) or MM102 (6 uM) for the indicated time and then lysates prepared. Lysates were immunoblotted for the indicated antibodies.

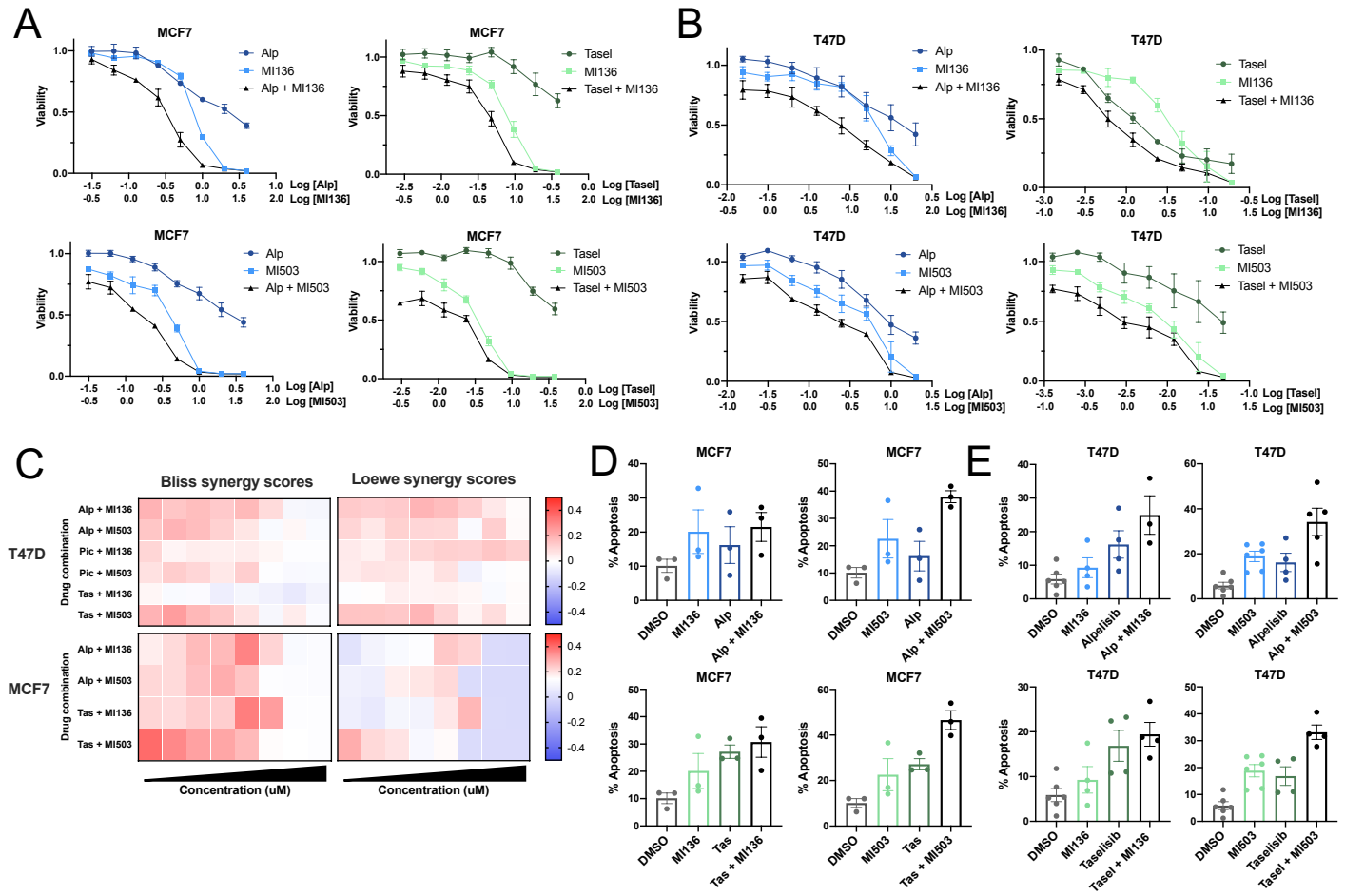


Figure 3: PI3K and MLL1 combination therapy synergizes to reduce viability and enhance apoptosis. (A) Cell viability curves in MCF7 breast cancer cells treated with an 8-point range of DMSO, alpelisib, taselisib, MI-503, and/or MI-136 for 120h. Results shown are representative of at least 3 independent experiments. Data are shown as mean ± SEM. (B) Cell viability curves in T47D breast cancer cells treated with an 8-point range of DMSO, alpelisib, taselisib, MI-503, and/or MI-136 for 120h. Results shown are representative of at least 3 independent experiments. Data are shown as mean ± SEM. (C) Bliss (left) and Loewe (right) synergy calculated from datapoints in (A) and (B). (D) Annexin V staining in MCF7 cells treated with DMSO, alpelisib (1 uM), taselisib (0.5 uM), MI-136 (4 uM), MI-503 (4 uM) for 120h. Results shown are representative of at least 3 independent experiments. Data are shown as mean ± SEM. (E) Annexin V staining in MCF7 cells treated with DMSO, alpelisib (1 uM), taselisib (0.5 uM), MI-136 (4 uM), MI-503 (4 uM) for 120h. Results shown are representative of at least 3 independent experiments. Data are shown as mean ± SEM.

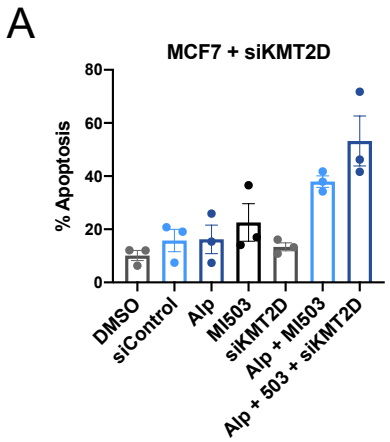
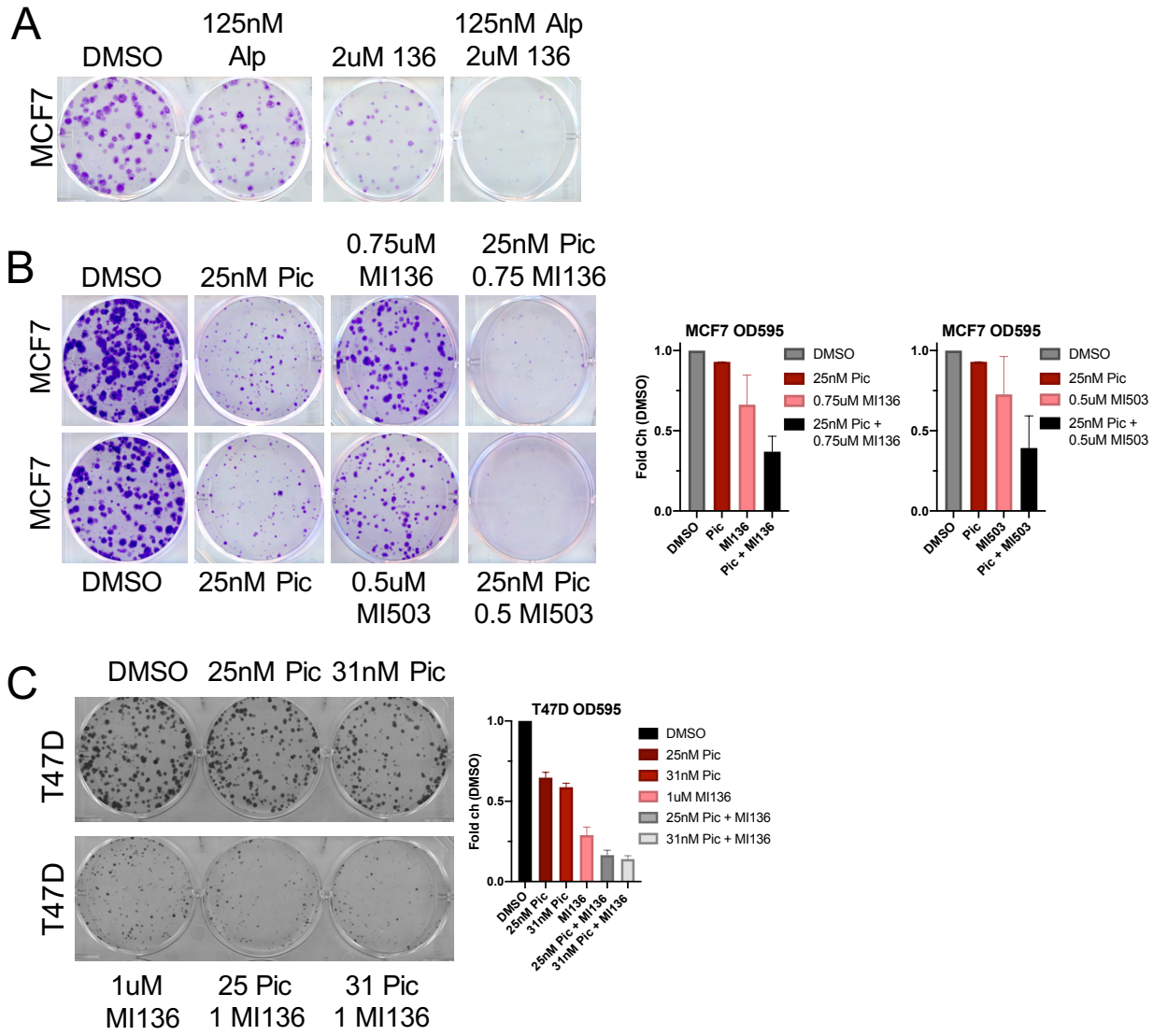
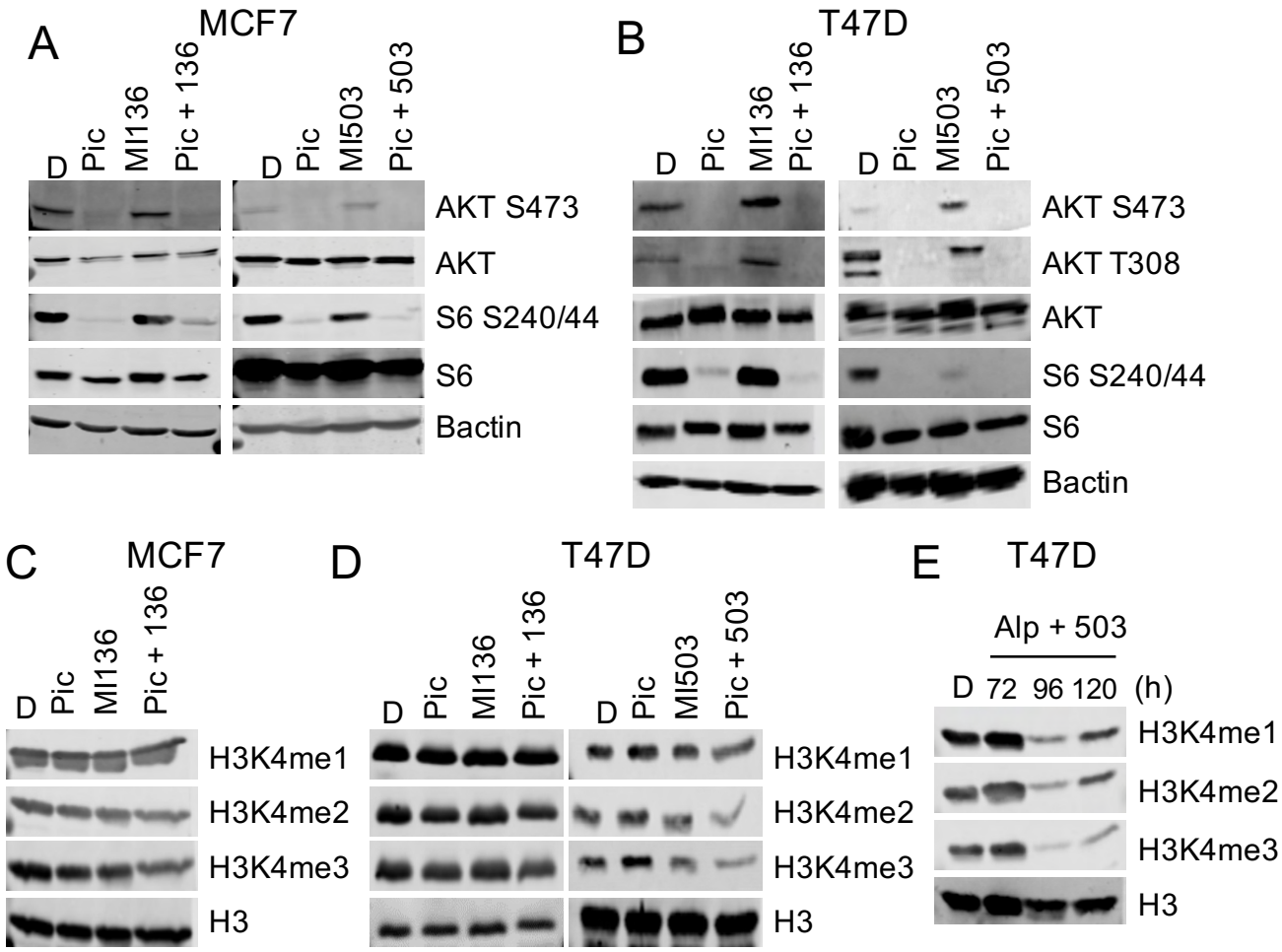


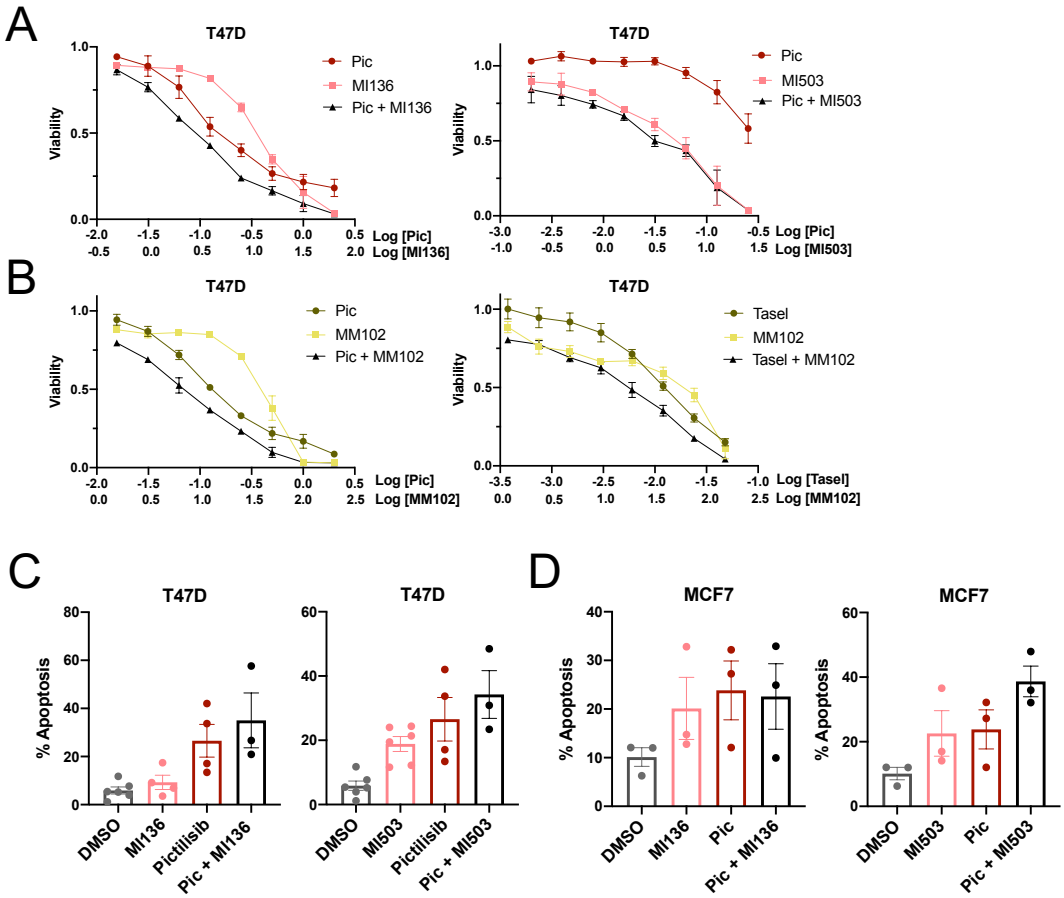
Figure 4: MLL4/KMT2D genetic ablation further enhances PI3K/MLL inhibitor-driven apoptosis. (A) Annexin V staining in MCF7 cells transfected with MLL4/KMT2D or control siRNA and treated with DMSO, alpelisib (1 uM), taselisib (1 uM), MI-136 (4 uM), MI-503 (4 uM) for 120h. Results shown are representative of at least 3 independent experiments. Data are shown as mean ± SEM.



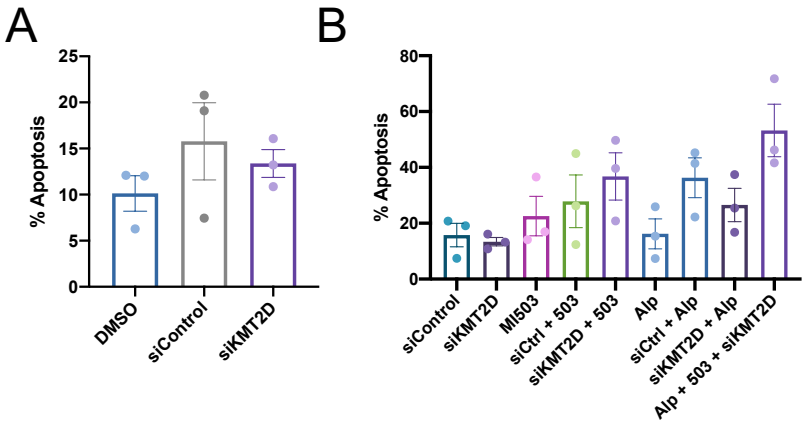
Supplementary Figure 1. Combined PI3K and MLL1 inhibition reduces clonogenicity of HR+, PIK3CA-mutant, breast cancers. (A) MCF7 breast cancer cells treated with the indicated concentrations of alpelisib, MI-136, or DMSO for 17 days before fixation and crystal violet staining. Representative images shown. (B) MCF7 breast cancer cells treated with the indicated concentrations of pictilisib, MI-136, MI-503, or DMSO for 17 days before fixation and crystal violet staining. Representative images shown. Results shown are representative of at least 3 independent experiments. Data are shown as mean ± SEM. (C) T47D breast cancer cells treated with the indicated concentrations of pictilisib, MI-136, or DMSO for 19 days before fixation and crystal violet staining. Representative images shown. Results shown are representative of at least 3 independent experiments. Data are shown as mean ± SEM.



Supplementary Figure 2: MLL1 inhibition hyperactivates AKT. (A) MCF7 or (B) T47D breast cancer cells were treated with pictilisib (1 uM), MI-136 (4 uM), MI-503 (4 uM) or DMSO for 24h prior to lysis. Lysates were immunoblotted for the indicated antibodies. (C) MCF7 or (D) T47D cells were treated with pictilisib (1 uM), MI-136 (4 uM), MI-503 (4 uM) or DMSO for 72h and then lysates acid extracted. Lysates were immunoblotted for the indicated antibodies. (E) T47D cells treated with alpelisib (1 uM) or MI503 (4 uM) for the indicated times and lysates acid extracted and immunoblotted for the indicated antibodies.



Supplementary Figure 3: PI3K and MLL1 combination therapy synergizes to reduce viability and enhance apoptosis. (A) Cell viability curves in T47D breast cancer cells treated with an 8-point range of DMSO, pictilisib, MI-503, and/or MI-136 for 120h. Results shown are representative of at least 3 independent experiments. Data are shown as mean ± SEM. (B) Cell viability curves in T47D breast cancer cells treated with an 8-point range of DMSO, pictilisib, taselisib, and/or MI-136 for 120h. Data are shown as mean ± SEM; n = 3. (C) Annexin V staining in T47D cells treated with DMSO, pictilisib (1 uM), MI-136 (4 uM), MI-503 (4 uM) for 120h. Data are shown as mean ± SEM; n = 3. (D) Annexin V staining in MCF7 cells treated with DMSO, pictilisib (1 uM), MI-136 (4 uM), MI-503 (4 uM) for 120h. Data are shown as mean ± SEM; n = 3.



Supplementary Figure 4: MLL4/KMT2D genetic loss further enhances PI3K/MLL synergy. (A) and (B) Annexin V staining in MCF7 cells transfected with MLL4/KMT2D or control siRNA and treated with DMSO, alpelisib (1 uM), taselisib (1 uM), MI-136 (4 uM), MI-503 (4 uM) for 120h. Results shown are representative of at least 3 independent experiments. Data are shown as mean ± SEM.