Supplemental Methods and Figures

Synergistic Targeting of *FLT3* Mutations in AML via Combined Menin-MLL and FLT3 Inhibition

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Supplemental Material and Methods

Small molecule inhibitors

Menin-MLL1 inhibitor (MI-503) and FLT3 inhibitors Crenolanib and Gilteritinib were purchased from Selleckchem, FLT3 inhibitors Quizartinib and Ponatinib were purchased from LC Laboratories. A sample of VTP-50469 was kindly provided by Syndax. For *in vitro* studies, MI-503, VTP-50469 and FLT3 inhibitors were dissolved in DMSO, aliquoted and stored at -20°C. For *in vivo* applications, MI-503 was dissolved in DMSO and added freshly to 50% PBS/25% PEG400 prior to intraperitoneal injections. Quizartinib was dissolved in 22% 2-hydroxyl-beta-cyclodextrin (freshly prepared every 7 days) and applied via oral gavage.

Cell Culture and Cell Lines

The human AML cell lines MV411, MOLM13, OCI-AML3, NB4, and HL-60 were cultured in RPMI-1640 media (Gibco) and OCI-AML2 in alpha-MEM media (Gibco), all supplemented with 10% fetal bovine serum (FBS, Gibco), 1% Penicillin/Streptomycin (PS, Sigma) and 1% L-Glutamine (L-Glu, Sigma). HEK293T and HS27 cells were cultured in DMEM with 10% FBS, 1% PS and 1% L-Glu. Murine leukemia cells were cultured in DMEM supplemented with 15% FBS, 1% PS, and cytokines (SCF 100 ng/mL, IL-3 20 ng/mL, IL-6 20 ng/mL, all from PeproTech).

Primary AML blast cells and co-culture assay

Treatment of primary human *NPM1*^{mut}*FLT3*^{ITD} and *NPM1*^{WT}*FLT3*^{WT} AML samples was performed in co-culture with irradiated HS27 stromal cells for 7 days for cell viability assay. Co-culture was maintained in StemSpan media (Stem Cell Technologies) supplemented with 1 µM StemRegenin (Stemcell Technologies), 0.1 mM 2-Mercaptoethanol (Sigma), 100 ng/mL SCF, 20 ng/mL IL-3, 20 ng/mL G-SCF and 50 ng/mL FLT3L (all from PeproTech). Cells were treated with single drug (2 µM MI-503 or 6 nM Quizartinib), their combination (2 µM MI-503 and 6 nM Quizartinib) or vehicle (DMSO). Treatment of each individual AML sample was performed in three replicates. To exclude HS27 contamination, primary AML blast cells were stained with anti-human CD45-FITC antibody (Biolegend) and cell number was determined by flow-cytometry.

***In vitro* cell viability assay and drug synergism calculation**

Trypan-excluding cells were plated at a density of 20,000 cells per well in 150 µL media in 96 well plates. Cells were split and replated in fresh media every 3-4 days if they were treated for longer than 4 days. For IC_{50} determination, cells were treated with menin-MLL1 inhibitors and FLT3 inhibitors sequentially diluted 2-fold for a total of 9 concentrations plus vehicle control (DMSO). Combinatorial treatment of serial dilutions of MI-503 and Quizartinib, Ponatinib and Gilteritinib were performed using constant ratios of 833:1, 25:1 and 6.25:1, respectively. VTP-50469 was combined with Quizartinib, Ponatinib or Gilteritinib using constant ratios of 33:1, 1:1 or 1:0.25, respectively. The highest concentration of MI-503, VTP-50469, Quizartinib, Ponatinib and Gilteritinib was 2.5 µM, 100 nM, 3 nM, 100 nM and 400 nM, respectively. Cell viability was assessed by flow-cytometry using DAPI (Roth, 1 µg/mL) staining. Drug synergism was assessed using the CompuSyn software tool for Chou-Talalay method-based calculations.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation was performed as described previously[1]. In short, after four days of treatment with DMSO or MI-503, crosslinking was performed with 1% formaldehyde (Sigma) for 10 min at room temperature and subsequent quenching with Glycine (125 mM, pH 8.0) for 5 min. Cells were lysed in SDS buffer and sonicated to fragment DNA. The following antibodies were used for precipitation: anti-Menin (A300-105A, Bethyl), anti-MLL1 (A300-086A, Bethyl) and normal rabbit IgG as negative control (12-370, Merck Milipore). Eluted DNA fragments were analyzed by qPCR using the following primers:

Human ChIP-qPCR primers:		
MEIS1	F: CCGCACACAGCTCATACCAA	R: AAGAGGGAAGCGTTGAGTCT
MEIS1 prom	F: CCTTAGCAGAGGCTTTCCCA	R: TATTGGGGTCTGCCAGTGTT
MEIS1 TSS	F: CCGGGTTCTAGCATTCTGGT	R: TGCTGCACTGGAAGGAGATTAG
SOX2	F: CAGGAGTTGTCAAGGCAGAGA	R: CCGCCGCCGATGATTGTTA
Murine ChIP-qPCR primers:		
Meis1	F: TGCAAGGGCTTGGGAAAT	R: CCTCGGTCAATGACGCTTTA
Meis1 prom	F: GCGTGTGTAAAGTGTGTGTTG	R: TGTAAGACGCGACCTGTTATG
Meis1 TSS	F: CCTCAGACCCAACTACCAAATC	R: GACAGAACGGACGATCATTCA
Sox2	F: CCCTGAGCAGCGTGAATAA	R: TCTGATTTGGCGCAGTATCTC

Analysis of apoptotic cell death by Annexin V staining

Cells treated with small molecule inhibitors at the indicated concentrations or vehicle control were stained with Annexin V antibody (APC-conjugated, eBioscience, 1:25) and DAPI (Roth, 1 µg/mL). The number of apoptotic cells, defined as the sum of Annexin V-positive/DAPI-negative and Annexin V-positive/DAPI-positive cells, was determined by flow-cytometry.

Morphological analysis

100,000 cells were harvested in 150 µL PBS. Cytospins and Giemsa staining were performed by the Cytology laboratory within the Department of Hematology and Medical Oncology of the University Medical Center Mainz. Images were captured on an EVOS M5000 imaging system (ThermoFisher) at 100x amplification.

RNA purification, cDNA synthesis and qRT-PCR

RNA of $1x10^6$ trypan-excluding cells was isolated using the RNeasy Mini Kit (Qiagen) and cDNA synthesis was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher). Quantative real-time polymerase chain reactions (qRT-PCR) were carried out using SYBR Green 1 Mastermix (Roche) for human sequences or TaqMan MasterMix (ThermoFisher) for murine sequences, and run on LightCycler®480 (Roche) or QuantStudio3 (Thermo Fisher) cyclers, respectively. Relative gene expression was determined by the Δ/ΔCt value method[2] and normalized to the internal control*, β-2 Microglobulin* or *GAPDH*. Primer sequences are available on request.

Western blot

Cells treated with drug(s) and vehicle were lysed in RIPA buffer ($5x10^6$ cells/40 µl lysis buffer), and the protein content was quantified by Bradford assays (Protein Assay Dye Reagent Concentrate, Bio-Rad). Identical amounts of protein of each sample were loaded on a 8% Bis-Tris gel (Thermo Fisher), electrophoretically sparated and transferred to Nitrocellulose membranes (Noves) using an iBlot 2 Transfer machine (Thermo Fisher) according to the manufacturer's protocol. Each lysate was loaded twice to detect total and phospho-proteins in parallel without stripping and reprobing the membrane. Membranes were cut to allow

independent incubation with antibodies. Following antibodies were used: anti-ACTIN (2A3, EMD Millipore), anti-ERK1/2 (#9102, Cell Signaling), anti-phospho-ERK1/2 (Thr202/Tyr204, #9101, Cell Signaling), anti-FLT3 (S-18, Santa Cruz), anti-phospho-FLT3 (Tyr591, 54H1, Cell Signaling), anti-STAT5 (A-9, Santa Cruz) and anti-phospho-STAT5 (Tyr694, Cell Signaling). The results for FLT3/pFLT3 detection were confirmed in separate experiments using a stripping and reprobing protocol: Anti-pFLT3-probed membranes were incubated with Restore Western Blot Stripping Buffer (ThermoFisher) according to the manufacturer´s instructions and reprobed with FLT3 antibody. Western blot signals were quantified by densitometry using the ImageJ software tool.

Flow-Cytometry

Cells treated with drug(s) or vehicle were centrifuged and washed twice with cold 1 x phosphate buffered saline (PBS). Cells were stained with anti-FLT3 APC-conjugated antibody (BV10A4H2, Biolegend, dilution 1:25) for 30 min at 4°C in the dark or with phosphorylated protein-specific antibodies (anti-phospho(Thr202/Tyr204)-ERK1/2-APC, MILAN8R, eBioscience; anti-phospho(Tyr591)-FLT3-FITC, Biorbyt; anti-phospho(Tyr694)-STAT5-APC, SRBCZX, Thermo Fisher) for 1h at room temperature in the dark. Stained cells were washed with 1 x PBS and stained with DAPI (Roth, 1 µg/mL). As a positive control for pFLT3 staining, cells were stimulated with FLT3 ligand (100 ng/ml, Peprotech) for 15 min. Flow-cytometry was performed on a FACSCanto II Flow Cytometer (BD Biosciences). Data were analyzed using FlowJo Version 10 software.

Colony-forming assay

Murine leukemia cells were plated at a density of 20,000 cells/1 mL in MethoCult (M3234, Stem cell Technologies) with additionally added mouse cytokines (10 ng/mL IL-3, 10 ng/mL IL-6 and 50 ng/mL SCF, all from PeproTech) and primary patient samples were plated at a density of 100,000 cells/1 ml in MethoCult™ H4435 Enriched (Stem cell Technologies). Cells were cultured for 7 days at 37°C and 5% CO_2 and colonies (defined as a collection of > 10 cells) were counted. Cells were replated and cultured for another 7 days under the same conditions in secondary plating experiments. Colonies were captured using an EVOS M5000 imaging system (ThermoFisher) at 20x amplification.

RNA sequencing

To control the results from the RNA-sequencing analysis for potential bias from standard normalization in the context of global transcriptional changes, $1x10^6$ cells were harvested and 2 µL of 1:100 ERCC RNA Spike-in mix (Thermo Fisher) were added to the cell lysis buffer of each sample for normalization. RNA was isolated using the RNeasy Mini Kit (Qiagen) including on column DNAse digestion. RNA was quantified on an Agilent 2100 Bioanalyzer using the Quant-IT RiboGreen RNA Assay Kit (Thermo Fisher). Sequencing of mRNA (50 base pair paired-end sequencing) was performed on an Illumina HiSeq 4000 Next Generation machine. Each library yielded 30-40 Million reads. For gene expression analysis, reads were mapped to the human genome (Hg38). Read counts were normalized to ERCC spike-in controls. Differentially expressed genes between the treatment groups were calculated using DESeq version 2. Gene Set Enrichment Analysis (GSEA) was performed using publically available software (http://software.broadinstitute.org/gsea/index.jsp.)

Retroviral overexpression of *Meis1* and *Meis1*/*HoxA9*

For production of retroviral particles, 50-70% confluent HEK293T cells were co-transfected with 10 µg of gag-pol[3], 3 µg of pHCMV-EcoEnv[4] and 8 µg of MSCVneo_*Meis1* or MSCVneo_*Meis1*/*Hoxa9*[5] per 10 cm cell culture dish. Retroviral particles were harvested 24 h and 48 h post transfection, filtered (0.45-micron filter) and concentrated by adding 1:5 v/v 50% PEG in PBS (Sigma P3640). After overnight incubation, the viral precipitate was centrifuged at 2,500 rpm for 20 min at 4°C and the pellet was resuspended in 500 µL of ice cold PBS, aliquoted and kept at -80°C.

For transduction, 6-well plates were coated with 25 µg/mL Retronectin (Takara) for 2h at room temperature and subsequently blocked with 2% bovine serum albumine (PAN Biotech) for 30 min. Retroviral particles were mixed with polybrene (Sigma, final concentration 8 µg/mL) and added to $1x10^6$ cells in 2 mL of media per well of a retronectin-coated 6-well plate. Spinfection was performed at 2,500 rpm and 37°C for 1.5 h, followed by 3-4 h of incubation at 37°C and 5% CO_2. Afterwards, cells were replated in fresh media in non-treated 6-well plates. Successfully transduced cells were selected in neomycin-containing media (G418, Merck, 1 µg/mL) for 14 days.

Knockdown using shRNAs

Stable cell lines with knockdown of *MEN1* were created using shRNA directed against *MEN1* and control shRNA directed against *LUC*. shRNAs cloned into hairpin pLKO.1 lentiviral vector (TRCN0000040141, TRCN0000338331, MFCD07785395) were purchased from Sigma Aldrich. Cells were co-transfected with lentiviral expression vectors (pLKO.1), gag-pol viral packaging (Addgene) and pMD2.G envelope (Addgene) plasmids. Spin infection was performed with 0.8 µg/ml polybrene at 2,000 rpm at 37°C for 30 min. Afterwards, cells were replated in fresh media in 6-well plates. After selection with 1 µg/ml puromycin (Sigma) for 48 hours, transduced cells were counted and collected for gene expression analysis. In parallel, cells were plated at a density of 20,000 cells per well in 150 µL media containing 1 µg/ml puromycin in 96 well plates, followed by treatment with Quizartinib sequentially diluted 2-fold for a total of 9 concentrations plus vehicle control (DMSO) for 24h.

Data Analysis and Statistical Methods

Statistics were calculated using Prism, Version 7 software (GraphPad). Student t test was used to assess significance in *in vitro* assays, and the Kaplan-Meier Log-Rank was used for survival analysis. P values < 0.05 were considered significant with *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. All *in vitro* experiments were performed in at least two independent experiments, each performed in three technical replicates.

Supplemental References

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Supplemental Figure Legends

Supplemental-Figure 1: Differentiation effects of Menin-MLL-inhibition in *NPM1*^{mut} and *MLL*-rearranged leukemia cells.

A-C) Dose-response curves from cell-viability assays after 4 (A), 7 (B) and 11 (C) days of treatment with MI-503 in murine (left) and human (right) leukemia cells. Viable (DAPI-negative) cells were assessed by flow-cytometry. The corresponding IC_{50} values for 11 days of treatment with MI-503 are shown in Figure 1A-B. D) Giemsa-stained cytospins showing murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ and human MV411 and MOLM13 cells after treatment with DMSO or MI-503 (2.5 µM) for 11 days. E) List of the top 5 Gene Ontology Biological Process 2018 terms gained in MOLM13 (upper panel) and MV411 (lower panel) cells following MI-503 treatment (2.5 µM) for 4 and 3 days, respectively. Input was the full list of significantly increased transcripts in MOLM13 and MV411 cells after MI-503 treatment. Graphs were computed with Enrichr and shown in the order from the mostly significant enrichment (top bar) to the less significant (bottom), while the numbers on the right represent the significance (p-value). F) Gene-set-enrichment-analysis (GSEA) of gene expression changes in MOLM13 and MV411 cells treated with MI-503 (2.5 µM, 4 and 3 days, respectively) and DMSO compared to neutrophil activation involved in immune response (GO:0002283).

Supplemental-Figure 2: Effects of Menin-MLL-inhibition in *NPM1*^{mut} and *MLL*-rearranged leukemia cells.

A) Volcano plots of RNA-seq data obtained from OCI-AML3, MOLM13 and MV411 cells treated with MI-503 (2.5 µM, 4 days for OCI-AML3 and MOLM13 cells and 3 days for MV411 cells). *HOX* genes are labeled. B) *FLT3* mRNA expression in MOLM13 and MV411 cells following four days of MI-503 treatment (2.5 µM), as assessed by qRT-PCR. Individually designed primers that bind to both non-mutant and *FLT3*-ITD or specifically to *FLT3*-ITD sequences were used. C-D) Chromatin immunoprecipitation (ChIP) in MOLM13, MV411 and murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells using anti-Menin (C) or anti-MLL1 (D) antibodies and IgG as negative control. ChIP was followed by qPCR detecting sequences of the *MEIS1* promoter (prom), *MEIS1* transcriptional start site (TSS) and *SOX2* as negative control. Error bars represent standard deviation (SD) of three independent experiments, each performed in three technical replicates.

Supplemental-Figure 3: Synergistic effects of combined Menin-MLL- and FLT3-inhibition in *NPM1*^{mut} and *MLL*-rearranged leukemia cells.

A) *FLT3*-ITD positive MOLM13 (left) and MV411 (right) cells were treated with various FLT3-inhibitors for 48h. IC_{50} values were graphically determined using GraphPad Prism. B) *FLT3*-ITD-positive and *FLT3* wildtype human leukemia cell lines were treated with Quizartinib for 72h. IC_{50} values were determined using the GraphPad Prism software tool. C) Dose-response curves from cell-viability assays after 48h and 72h of treatment with Quizartinib in *FLT3*-ITD-positive and *FLT3* wildtype human leukemia cell lines. D) Murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells were treated with various FLT3-inhibitors for 24h. IC_{50} values were graphically calculated using GraphPad Prism. E) Dose-response curves from cell-viability assays after 24h treatment with various FLT3 inhibitors in murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells. F) Dose-response curves from cell-viability assays after 48h of treatment with Ponatinib in murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ and *Hoxa9–Meis1*-transformed cells. G) Summary of IC_{50} values comparing MI-503 (2.5 µM, 4 days for MOLM13 and 3 days for MV411 cells) and Quizartinib (3 nM, 24h) in human MOLM13 and MV411 cells or MI-503 (2.5 µM, 4 days) and Ponatinib (100 nM, 24h) or Gilteritinib (400 nM, 24h) in murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells. H) Isobolograms corresponding to dose-response curves of MOLM13 and MV411 cells treated with MI-503 (4 days for MOLM13 and 3 days for MV411), Quizartinib (24h) and their combination as shown in Figure 2F. Combination indices (CI)<1 indicate synergism. CI values were calculated using the CompuSyn software tool basing on the Chou-Talalay algorithm. I) Isobologram corresponding to the dose-response curve of murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells treated with MI-503 (4 days), Ponatinib (24h) or Gilteritinib (24h) and their combination as shown in Figure 2G. J) Dose-response curves from cell viability assays of HL-60 and NB4 cells comparing MI-503 (MI, 4 days), Quizartinib (Qz, 24h), and combinatorial MI-503 and Quizartinib treatment. Dashed lines indicate IC_{50} values. K) Dose-response curves from cell viability assays of *Hoxa9–Meis1*-transformed cells comparing MI-503 (MI, 4 days), Ponatinib (Po, 24h), and combinatorial MI-503 and Ponatinib treatment. Error bars represent SD of three independent experiments, each performed in three technical replicates.

Supplemental-Figure 4: Effects of single and combined Menin-MLL- and FLT3-inhibition on apoptosis induction.

A) Effect of MI-503 (2.5 µM), Ponatinib (100 nM) and combinatorial treatment (2.5 µM and 100 nM) on total and blast-like colony-forming units after secondary plating in murine

Npm1$^{CA/+}$*Flt3*$^{ITD/+}$ cells, normalized to DMSO. B) Percentage of apoptotic (Annexin V) and dead (DAPI) cells after single and combinatorial treatment with MI-503 (2.5 µM) and Quizartinib (3 nM) in human MV411 (upper panel) and MOLM13 (middle panel) or with MI-503 (2.5 µM) and Ponatinib (100 nM) in murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells (lower panel). Representative dot plots of three independent experiments are shown.

Supplemental-Figure 5: Effects of combined Menin-MLL- and FLT3-inhibition on phosphorylated FLT3 protein levels.

A) Immunoblotting of FLT3 and phosphorylated (p)FLT3 in MV411 cells (left) and MOLM13 cells (right) upon treatment with 2.5 µM of MI-503 (for 3 and 4 days in MV411 and MOLM-13, respectively) Quizartinib (3 nM, 24h) or their combination. pFLT3 blots were stripped and reprobed for FLT3 detection. Numbers indicate the DMSO-normalized quantification of western blot signals, relative to the loading control, performed by densitometry using the ImageJ software tool. B) Immunoblotting of ERK and phosphorylated (p)ERK in MV411 cells (left) and MOLM13 cells (right) upon treatment with 2.5 µM of MI-503 (for 3 and 4 days in MV411 and MOLM-13, respectively) Quizartinib (3 nM, 24h) or their combination. Numbers indicate the DMSO-normalized quantification of western blot signals, relative to the loading control, performed by densitometry using the ImageJ software tool. C and D) pFLT3 protein expression in C) human MOLM13, MV411 and D) murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells following treatment with MI-503 (2.5 µM, 4 days for MOLM13 and *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells and 3 days for MV411 cells), FLT3-inhibitor (Quizartinib: 3 nM and Ponatinib: 100 nM, 24 hours) or their combination, as assessed by flow-cytometry. Bar graphs represent the mean of three independent experiments. Error bars represent SD.

E) pFLT3 protein expression in MOLM13, MV411 and murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells with or without previous stimulation with FLT3 ligand (100 ng/ml, 15 min), assessed by flow-cytometry. For each cell model, one representative histogram of three independent experiments is shown. Bar graphs represent three independent experiments with pFLT3 expression normalized to the non-stimulated (untreated) control. F-G) pSTAT5 protein expression in F) human MOLM13, MV411 and G) murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells following treatment with MI-503 (2.5 µM, 4 days for MOLM13 and *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells and 3 days for MV411 cells), FLT3-inhibitor (Quizartinib: 3 nM and Ponatinib: 100 nM, 24 hours) or their combination, as assessed by flow-cytometry. H-I) pERK protein expression in H) murine

Npm1$^{CA/+}$*Flt3*$^{ITD/+}$ cells and I) human MOLM13, MV411 cells following treatment with MI-503 (2.5 µM, 4 days for MOLM13 and *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells and 3 days for MV411 cells), FLT3-inhibitor (Quizartinib: 3 nM and Ponatinib: 100 nM, 24 hours) or their combination, as assessed by flow-cytometry. Bar graphs in F-J represent mean and SD of three independent experiments. J) pERK protein expression in human MOLM13, MV411 and murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells following treatment with MI-503 (2.5 µM, 4 days for MOLM13 and *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells and 3 days for MV411 cells), FLT3-inhibitor (Quizartinib: 3 nM and Ponatinib: 100 nM, 24 hours) or their combination, as assessed by flow-cytometry. One representative histogram of three independent experiments is shown. The percentage of pERK-positive cells is indicated within the histograms.

Supplemental-Figure 6: Expression of STAT5A target genes upon Menin-MLL-inhibition.

A) Volcano plots of RNA-seq data obtained from MOLM13 (left) and OCI-AML3 (right) cells treated with MI-503 (2.5 µM, 4 days). Target genes of the STAT5A signaling pathway are labeled. B) Heatmap of differentially expressed genes (log2 fold change >1 and <-1, respectively, and adjusted p-value<0.05) in MOLM13 (left) and OCI-AML3 (right) cells following MI-503 (2.5 µM, 4 days) treatment. Target genes of the STAT5A signaling pathway are labeled.

Supplemental-Figure 7: Effects of ectopic *Meis1* expression in murine *Npm1*^{mut} *Flt3*-ITD positive leukemias.

A) Relative mRNA expression of *Meis1*, *Hoxa9* and *Flt3* in murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells ectopically expressing *Meis1* or both *Meis1* and *Hoxa9,* normalized to cells with just endogenous *Meis1* and *Hoxa9* expression. B) Dose-response curves from cell viability assays after 11 days of MI-503 treatment comparing *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells versus *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells overexpressing *Meis1* or *Meis1-Hoxa9*. Dashed lines indicate the shift of IC_{50} values. C) Dose-response curves from cell viability assays after combinatorial treatment with MI-503 (MI, 6 days) and Ponatinib (Po, 72 hours) comparing *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells versus *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells overexpressing *Meis1* or *Meis1-Hoxa9*. Dashed lines indicate the shift of IC_{50} values. Cells with ectopic *Meis1* and *Hoxa9* expression in panel A), B) and C) were each obtained from two different clones after retroviral transduction, shown are the averaged

results from three different experiments with clone #2, all performed in triplicates. Error bars represent SD.

Supplemental-Figure 8: Effects of ectopic *Meis1* expression on colony-forming potential in murine *Npm1*^{mut} *Flt3*-ITD positive leukemias.

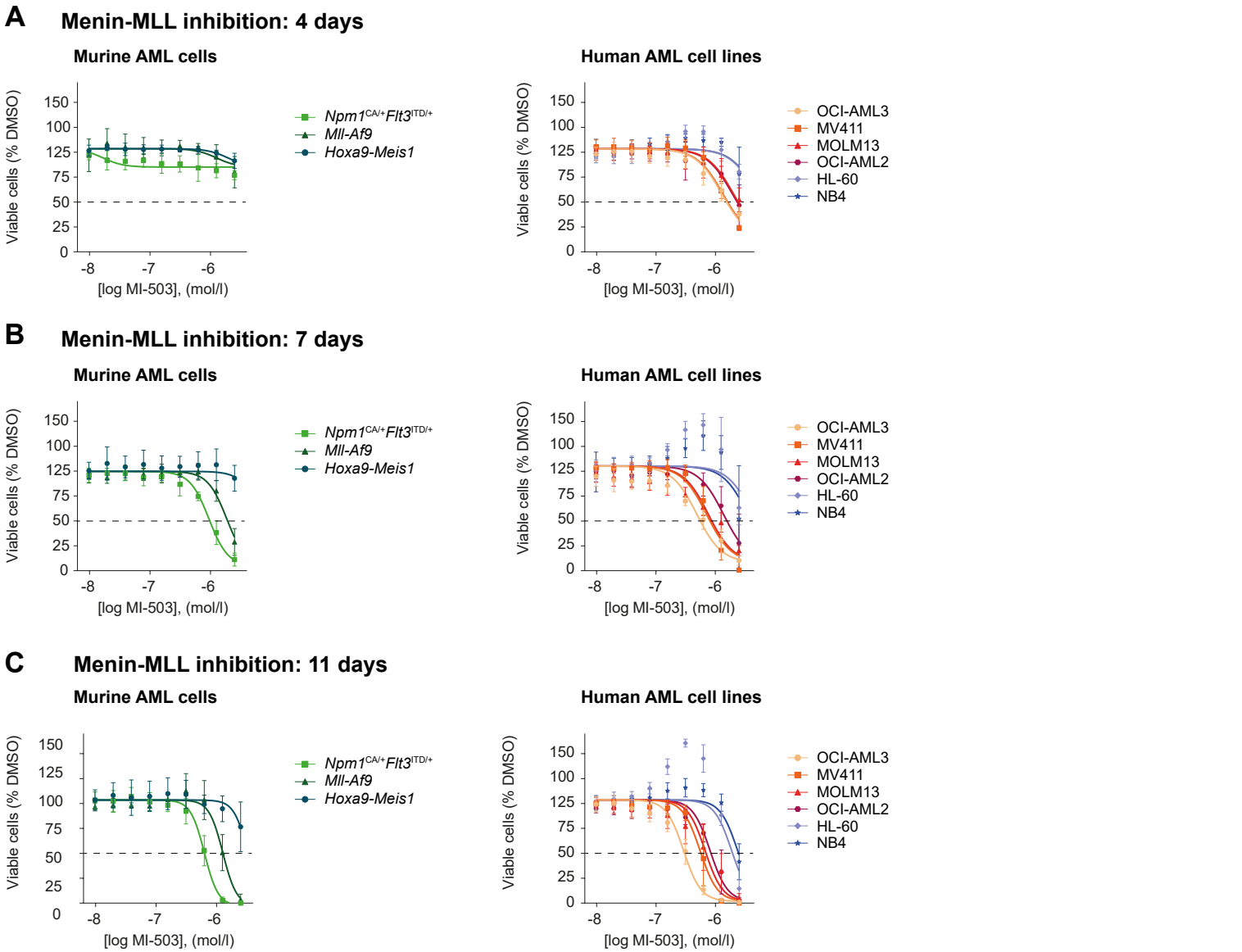
A) Relative mRNA expression of *Flt3* and *Meis1* in murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells ectopically expressing *Meis1* or both *Meis1* and *Hoxa9*, following MI-503 (2.5 µM, 4 days), Ponatinib (100 nM, 24h) and combinatorial treatment. Cells with ectopic *Meis1* and *Hoxa9* expression were obtained from two different clones after retroviral transduction (#1, upper panel, and #2, lower panel). B) Effects of MI-503 (2.5 µM), Ponatinib (100 nM) and combinatorial treatment (2.5 µM and 100 nM) on blast colony-forming units in murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells ectopically expressing *Meis1* or both *Meis1* and *Hoxa9* (clone #1, upper panel, and clone #2, lower panel), normalized to DMSO and assessed on day 7 of treatment. Bar graphs represent the mean and standard deviation of three (A) and two (B) independent experiments, each performed in three replicates. Representative photos of two independent experiments are shown.

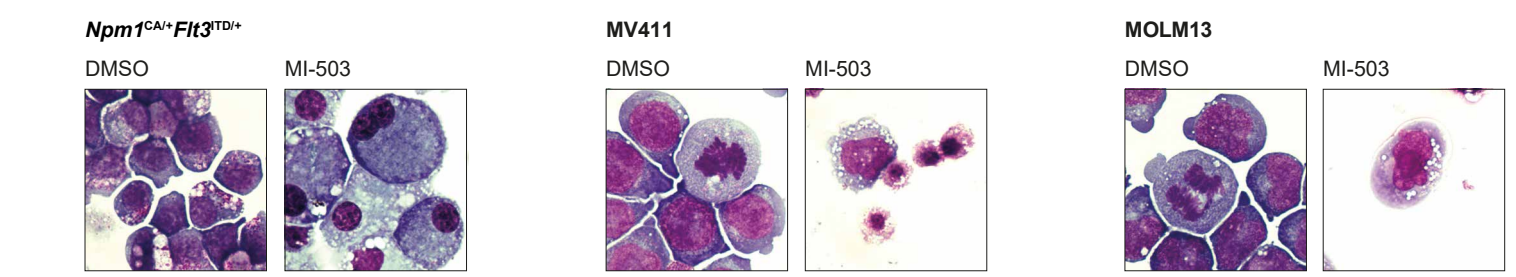
Supplemental-Figure 9: Growth inhibition of leukemic cells upon single drug treatment with VTP50469 or in combination with FLT3 inhibitors

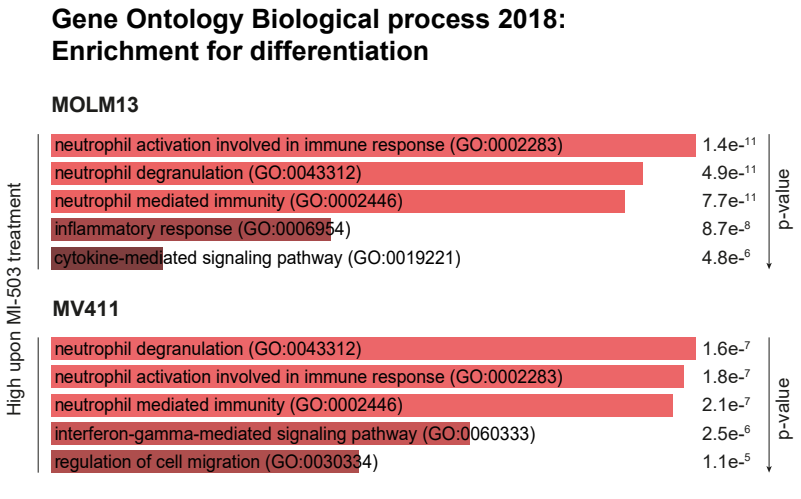
A) Human (left) and murine (right) AML cells were treated for 7 days with VTP-50469. Viable (DAPI-negative) cells were assessed by flow-cytometry and IC_{50} values were graphically calculated using GraphPad Prism. B) Dose-response curves from cell-viability assays after 4 days of treatment with VTP-50469 in human (left) and murine (right) leukemia cells. Viable (DAPI-negative) cells were assessed by flow-cytometry. C-D) Isobolograms corresponding to dose-response curves of MOLM13 and MV411 cells treated with VTP-50469 (4 days for MOLM13 and 3 days for MV411), Quizartinib (24h, C) or GIlteritinib (24h, D) and their combination as shown in Figure 6D-E. Combination indices (CI)<1 indicate synergism. CI values were calculated using the CompuSyn software tool basing on the Chou-Talalay algorithm. E) Isobologram corresponding to the dose-response curve of murine *Npm1*$^{CA/+}$*Flt3*$^{ITD/+}$ cells treated with VTP-50469 (6 days), Ponatinib or Gilteritinib (24h) and their combination as shown in Figure 6F. Combination indices (CI)<1 indicate synergism. CI values were calculated using the CompuSyn software tool basing on the Chou-Talalay algorithm. F) Dose-response

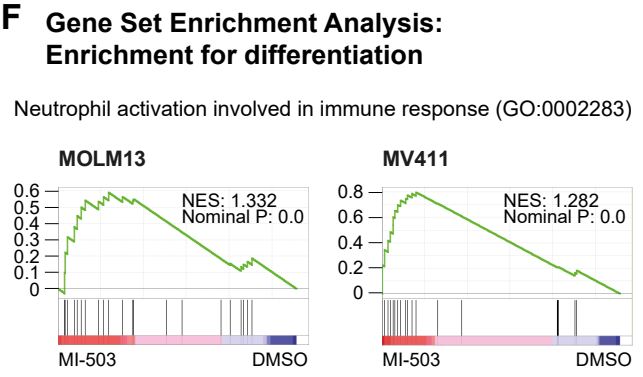
curves from cell viability assays of NB4 cells comparing VTP-50469 (VTP, 4 days), Quizartinib (Qz, 24h, left panel) and Gilteritinib (Gil, 24h, right panel), and combinatorial VTP-50469 (4 days) and FLT3-inhibitor (24h) treatment. Dashed lines indicate IC_{50} values. G) Dose-response curves from cell viability assays of *Hoxa9-Meis1*-transduced murine cells comparing VTP-50469 (VTP, 6 days), Ponatinib (Po, 24h, left panel), Gilteritinib (Gil, 24h, right panel) and combinatorial VTP-50469 (6 days) and FLT3-i (24h) treatment. Dashed lines indicate IC_{50} values.

Supplemental-Figure 1

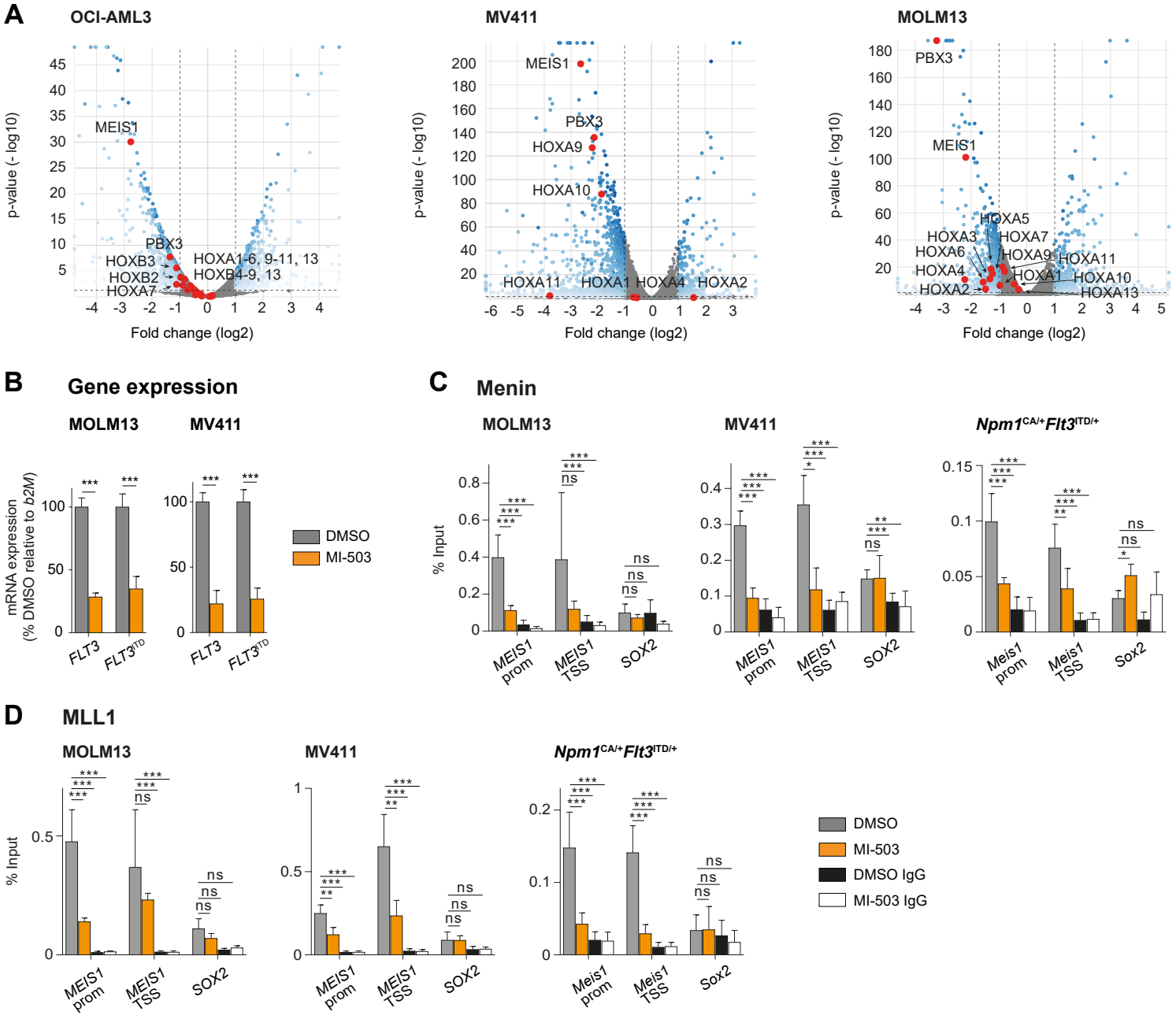




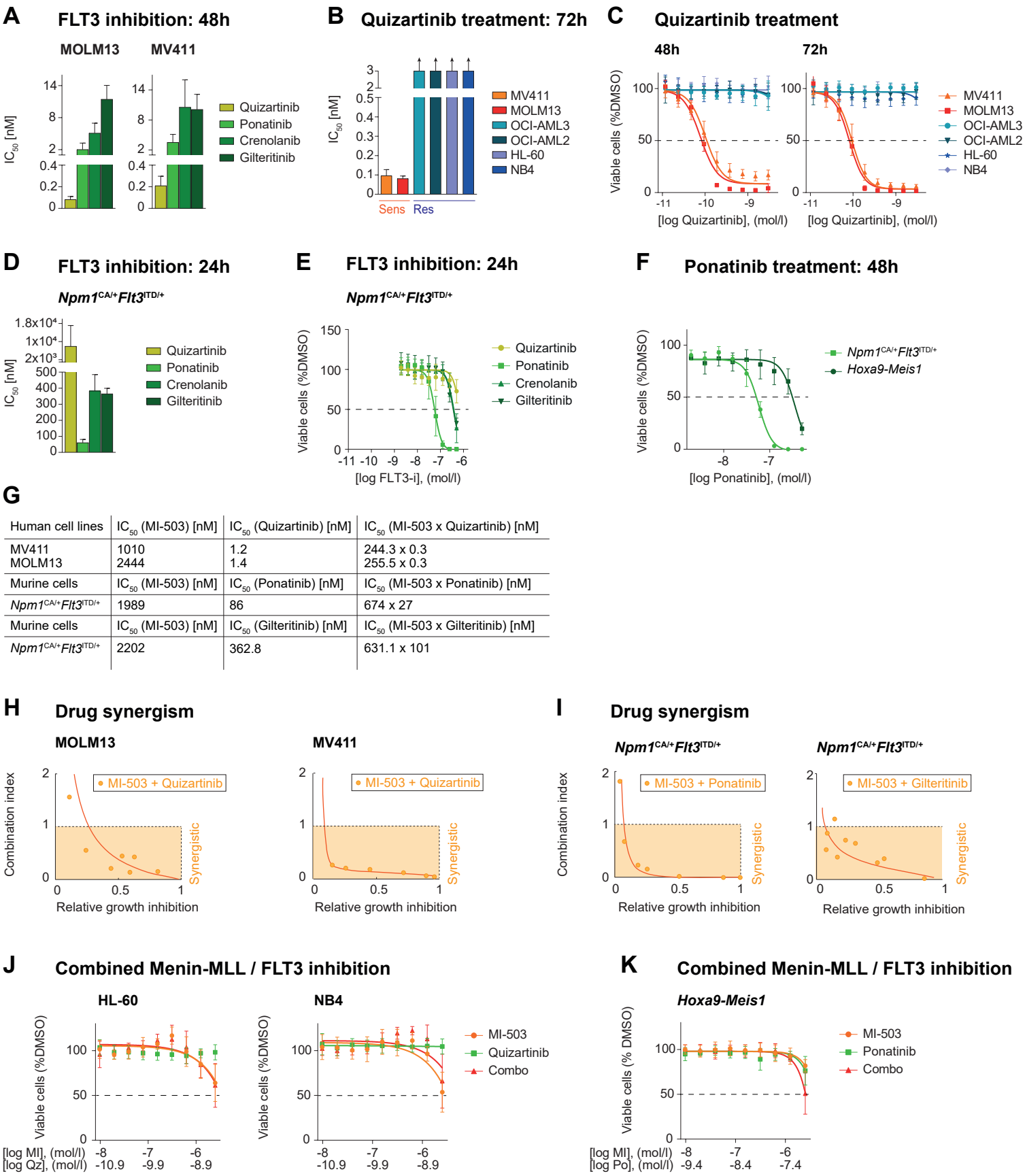




Supplemental-Figure 2



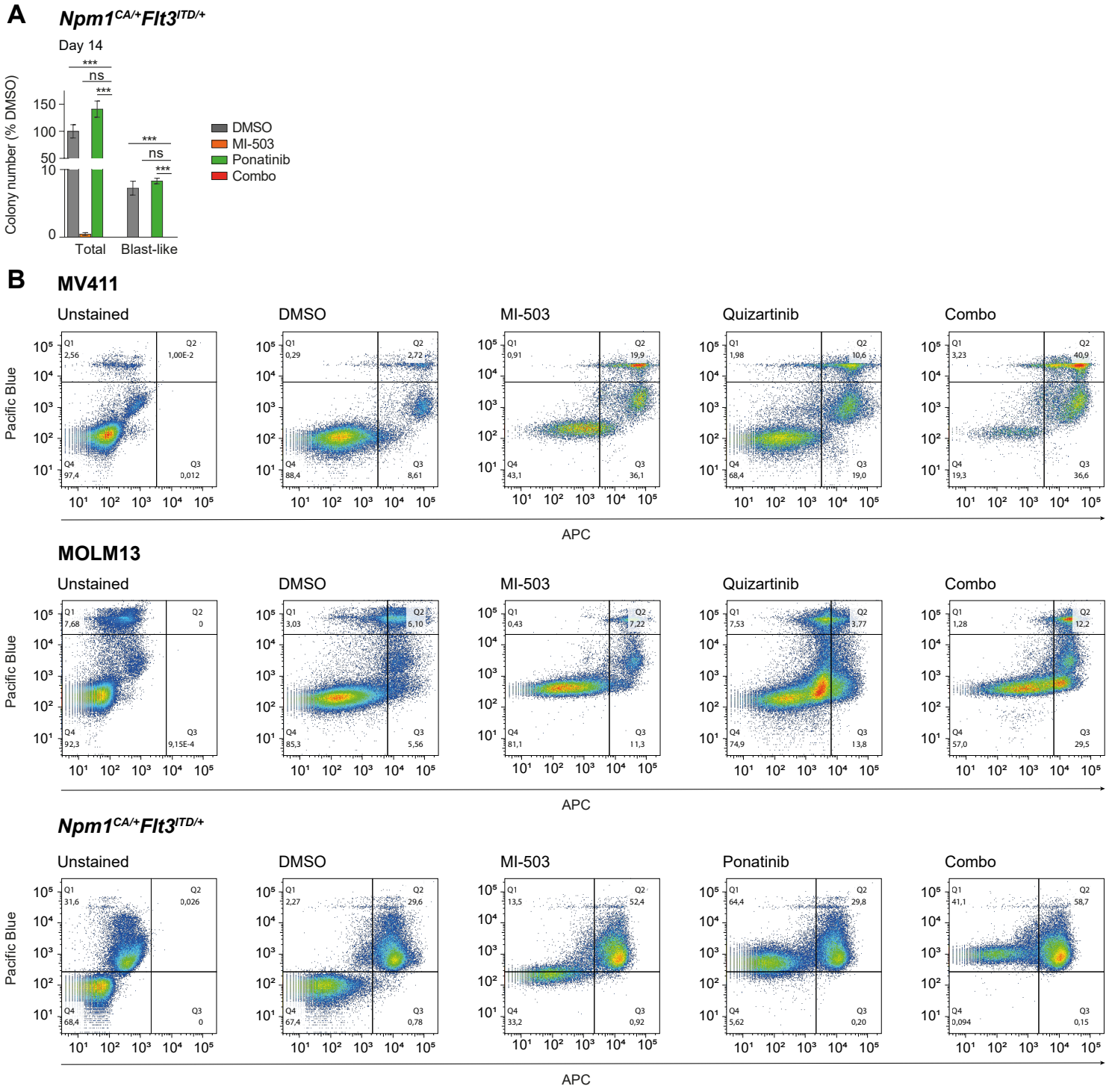
Supplemental-Figure 3



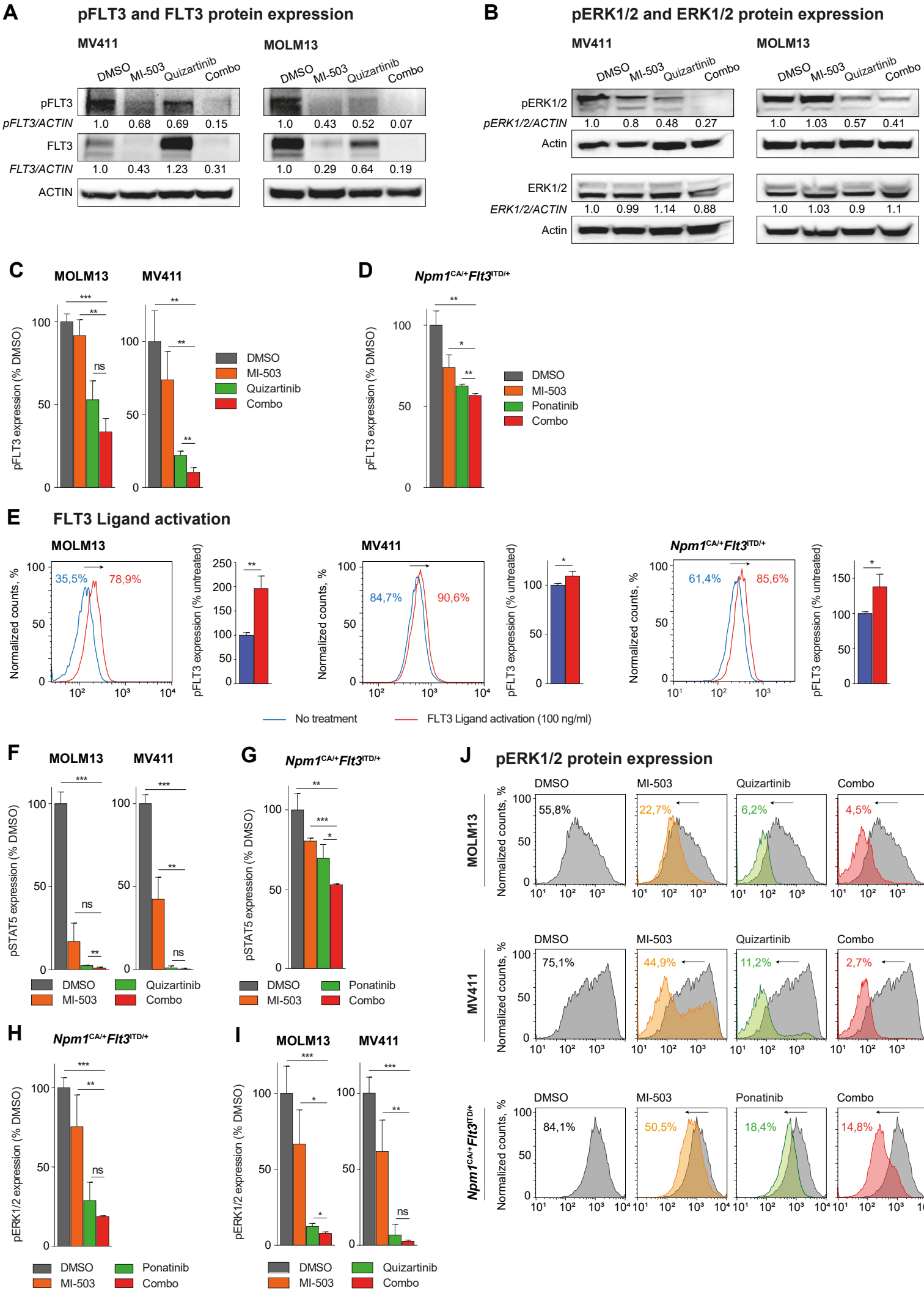
G

Human cell lines	IC_{50} (MI-503) [nM]	IC_{50} (Quizartinib) [nM]	IC_{50} (MI-503 x Quizartinib) [nM]
MV411 MOLM13	1010 2444	1.2 1.4	244.3 x 0.3 255.5 x 0.3
Murine cells	IC_{50} (MI-503) [nM]	IC_{50} (Ponatinib) [nM]	IC_{50} (MI-503 x Ponatinib) [nM]
Npm1$^{CA/+}$*Flt3*$^{ITD/+}$	1989	86	674 x 27
Murine cells	IC_{50} (MI-503) [nM]	IC_{50} (Gilteritinib) [nM]	IC_{50} (MI-503 x Gilteritinib) [nM]
Npm1$^{CA/+}$*Flt3*$^{ITD/+}$	2202	362.8	631.1 x 101

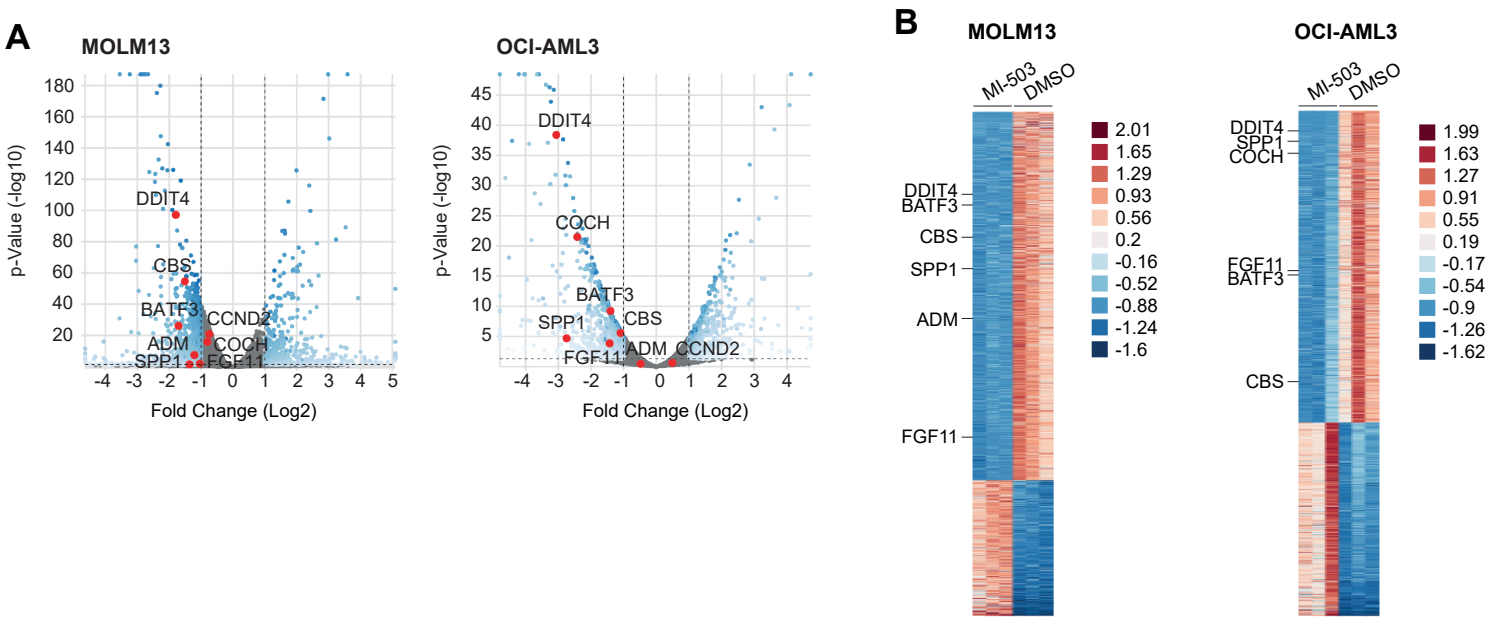
Supplemental-Figure 4



Supplemental-Figure 5

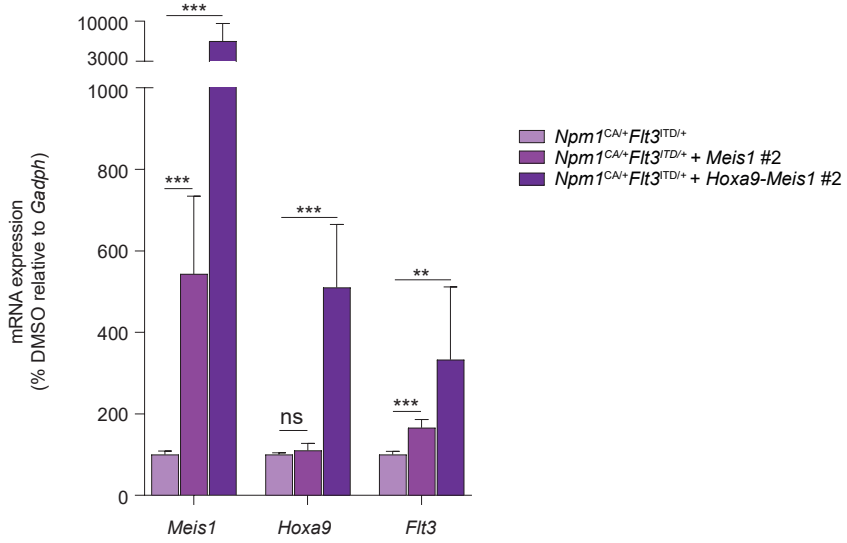


Supplemental-Figure 6

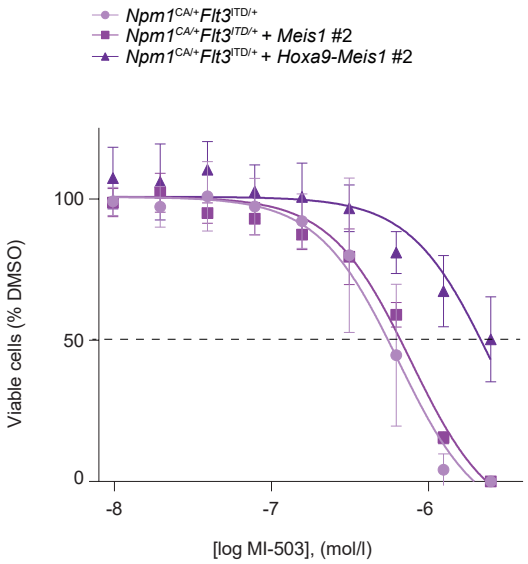


Supplemental-Figure 7

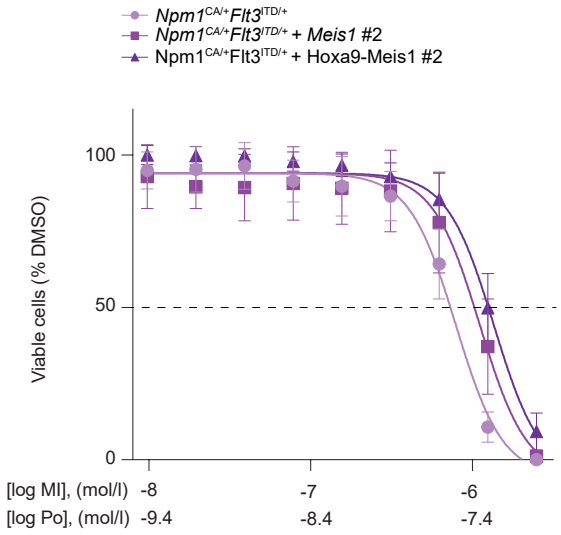
A **Ectopic *Meis1* and *Hoxa9* gene expression**



B **Menin-MLL inhibition: 11 days**



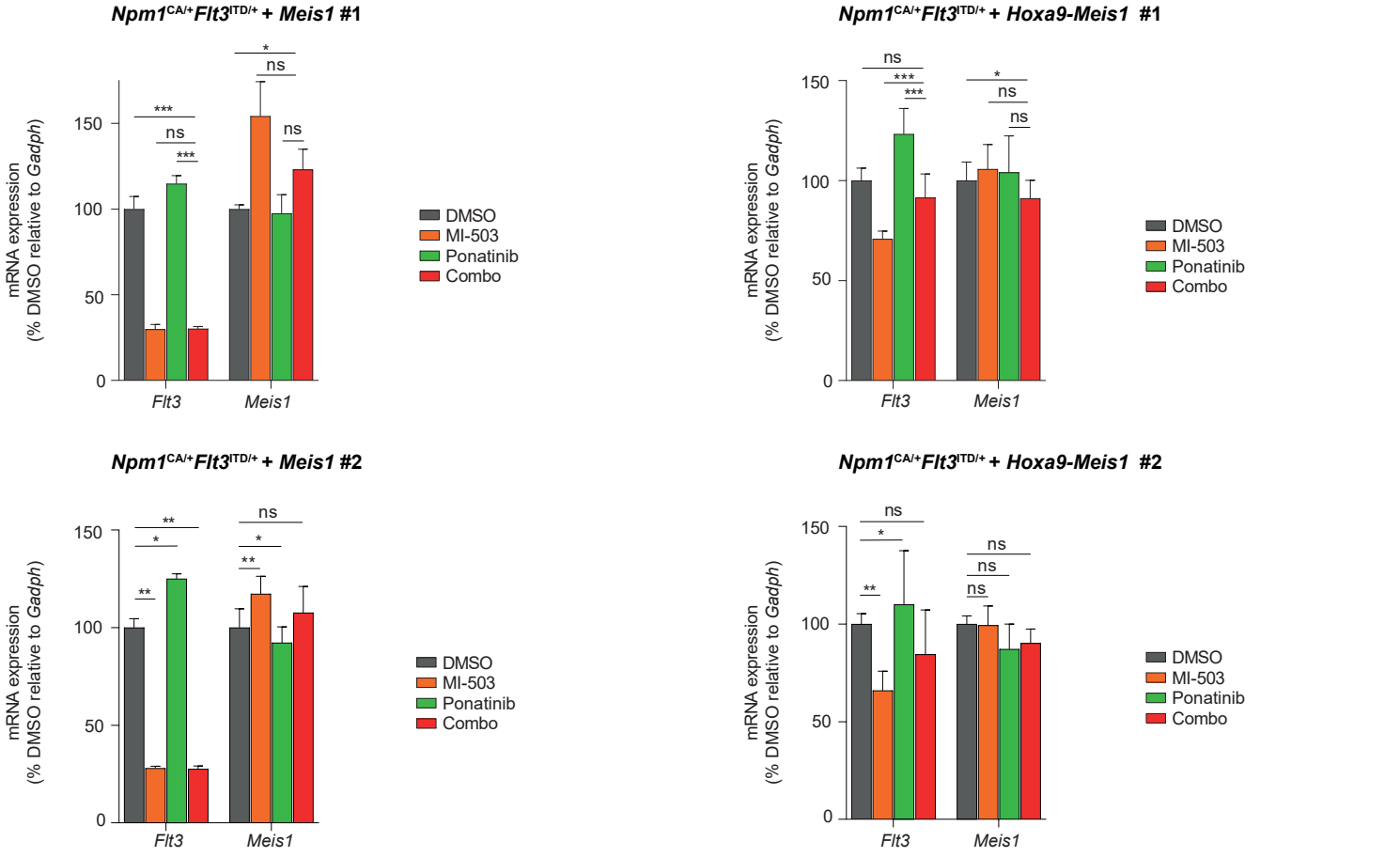
C **Combined Menin-MLL / FLT3 inhibition**



Supplemental-Figure 8

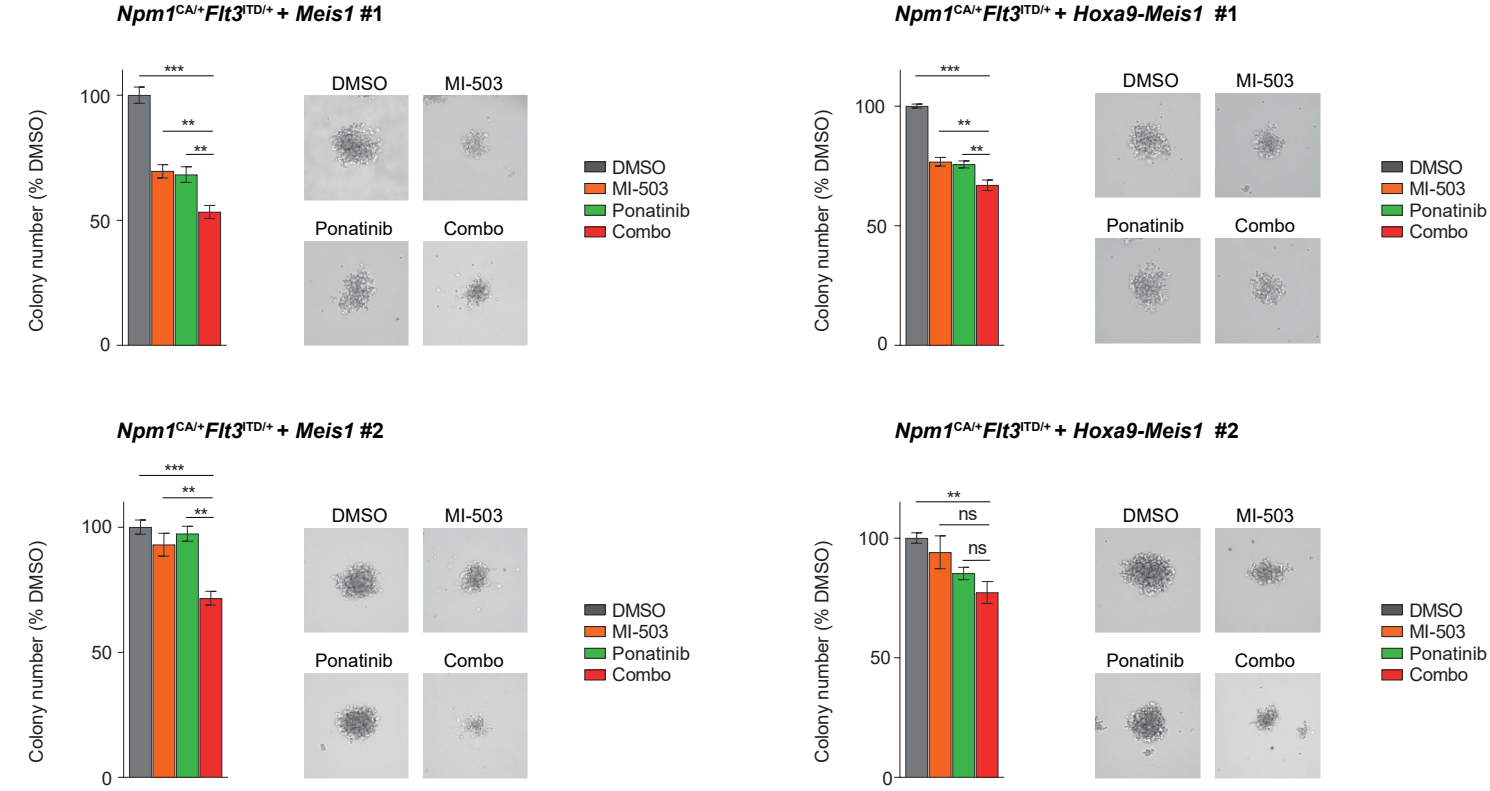
A

Gene expression



B

Colony-forming potential



Supplemental-Figure 9