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Supplemental Information

17-DMAG dually inhibits Hsp90 and histone lysine demethylases in alveolar rhabdomyosarcoma

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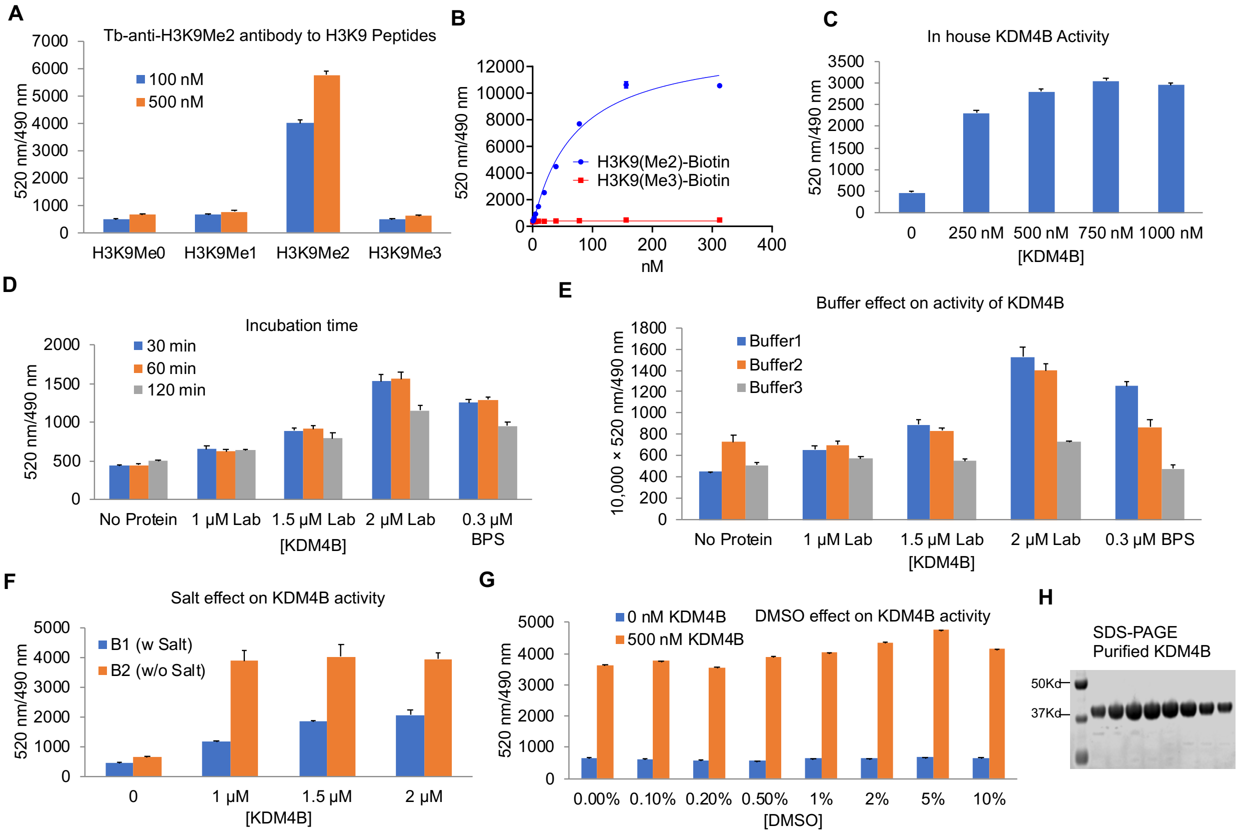
Supplemental Information



Supplementary Figure 1. Optimization of the TR-FRET assay. Related to Figure 1.

(**A**) The Tb-anti-H3K9me2 antibody is very specific to the Biotin-H3K9Me2 peptide. Specificity test of the Tb-anti-H3K9me2 antibody against Biotin-H3K9me0, Biotin-H3K9me1, Biotin-H3K9me2, or Biotin H3K9me3 at 100 nM and 500 nM, respectively. (**B**) The Tb-anti-H3K9Me2 antibody is very specific to the product peptide Biotin-H3K9me2 (Blue curve), but not to the substrate peptide Biotin H3K9me3 (Red curve) at a wide concentration range tested.(**C**) The in-house KDM4B activity tested with the TR-FRET assay at selected concentrations of 0 nM, 250 nM, 500 nM, 750 nM and 1000 nM. (**D**) The effect of various incubation times on KDM4B activity in the TR-FRET assay. No KDM4B protein group (Background group), lab produced KDM4B protein at 1 µM (1 µM Lab), 1.5 µM (1.5 µM Lab), 2 µM (2 µM Lab), or BPS Bioscience KDM4B protein at 0.3 µM ((0.3 µM BPS) was tested for comparison.(**E**) The effect of 3 representative buffers on KDM4B activity in the TR-FRET assay. No KDM4B protein group (Background group), lab produced KDM4B protein at 1 µM (1 µM Lab), 1.5 µM (1.5 µM Lab), 2 µM (2 µM Lab), or BPS Bioscience KDM4B protein at 0.3 µM ((0.3 µM BPS) was tested for comparison. Buffer 1: 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 1 mM α-ketoglutarate, 80 µM $FeSO_4$, 2 mM ascorbic acid, 0.01% BSA.

Buffer 2: 50 mM HEPES (pH 7.5), 1 mM α-ketoglutarate, 2 mM L-ascorbic acid, 50 μM $FeSO_4$, 0.01% BSA.

Buffer 3: 50 mM HEPES (pH 7.5), 50 mM KCl, 10 mM $MgCl_2$, 1 mM α-ketoglutarate, 2 mM L-ascorbic acid, 50 μM $FeSO_4$, 0.01% BSA.

(**F**) The effect of salt on KDM4B activity in the TR-FRET assay. No KDM4B protein group (Background group), lab produced KDM4B protein at 1 μM, 1.5 μM, or 2 μM was tested under indicated buffer condition for comparison.

B1 (w Salt): 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 1 mM α-ketoglutarate, 80 μM $FeSO_4$, 2 mM ascorbic acid, 0.01% BSA

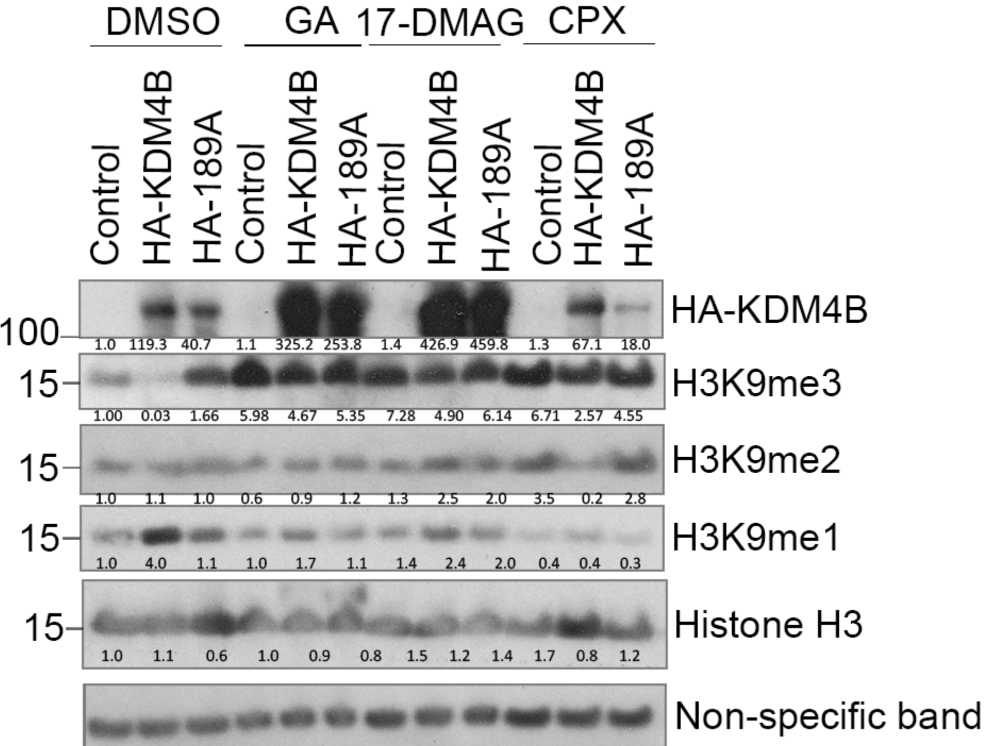
B2 (w/o Salt): 50 mM Tris-HCl (pH 8.0), 1 mM α-ketoglutarate, 80 μM $FeSO_4$, 2 mM ascorbic acid, 0.01% BSA

(**G**) The effect of DMSO on KDM4B activity in the TR-FRET assay. No KDM4B protein group (Background group) or lab produced KDM4B protein at 500 nM was tested for comparison at indicated DMSO concentration.

(**H**) High purity and active catalytic domain of KDM4B that is expressed and purified from bacteria. Photo was taken after SDS-PAGE following Coomassie Blue R-250 Staining.

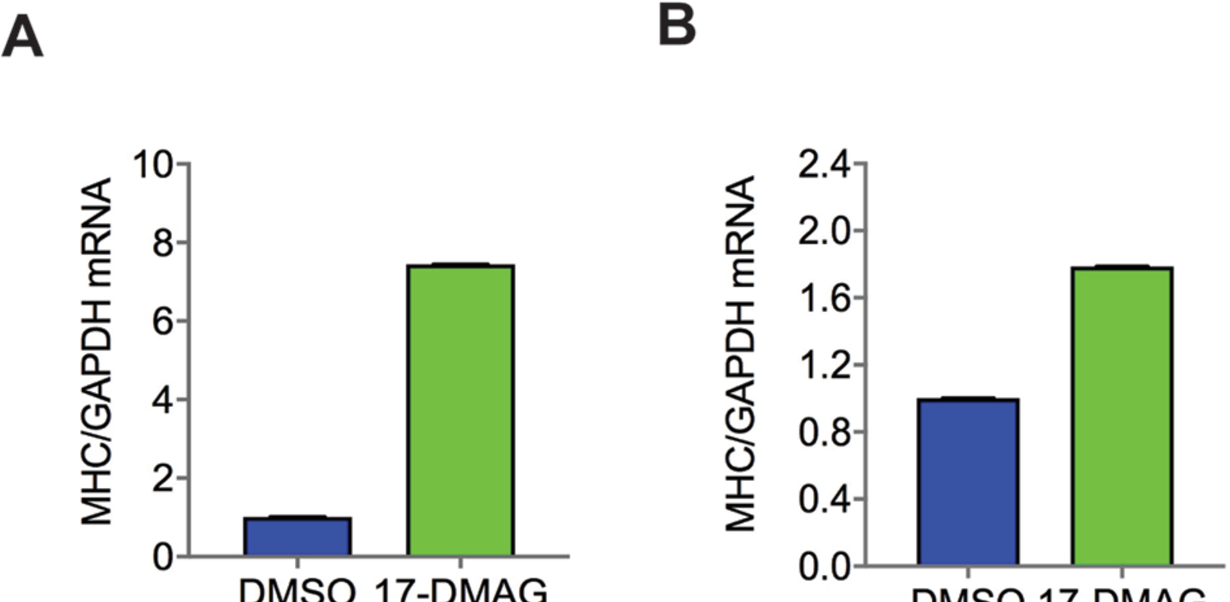
HSP90i	Company	Structure	TR-FRET activity (@15μM)
Geldanamycin			90%
Ganetespib	Synta Pharmaceuticals		12%
KW2478	Kyowa Hakko Kirin		4%
SNX-5422	Serenex/Pfizer		3%
AT13387	Astex		2%
NVP-AUY922	Novartis		-7%
STA-4783	Synta Pharmaceuticals		-7%
XL888	Daiichi Sankyo Inc		-107%

Supplementary Figure 2. KDM4B inhibiting activity of different chemotypes of HSP90 inhibitors assessed by the TR-FRET assay. Related to Figure 1.

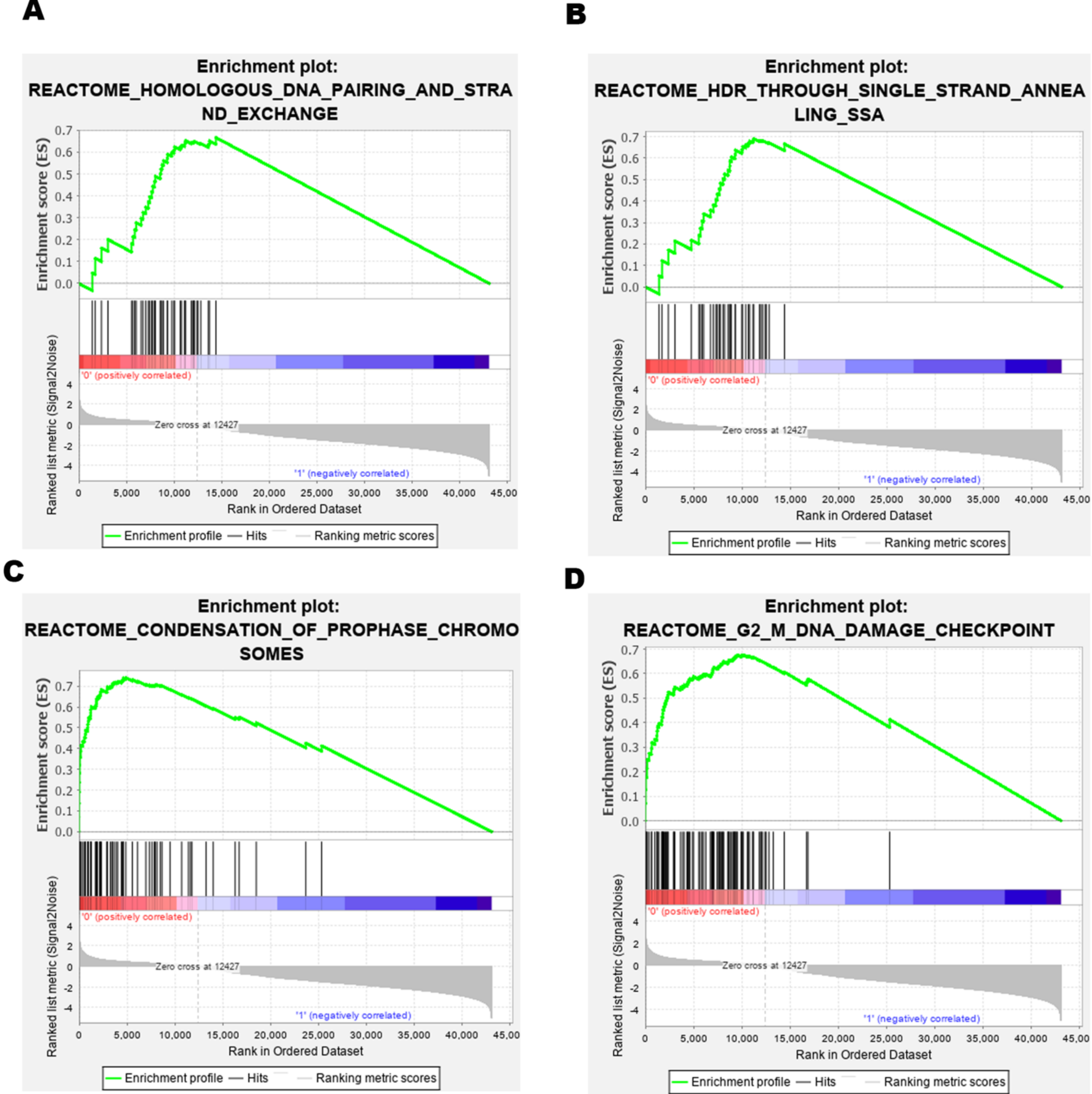


Supplementary Figure 3. Geldanamycin and 17-DMAG inhibit KDM4B enzymatic activity in cells. Related to Figure 2.

293T cells were transiently transfected with pCMV-HA-KDM4B and pCMV-HA-KDM4B(H189A). Eight hours later following transfection, cells were treated with 1 M of geldanamycin, 1 M of 17-DMAG and 5 M of ciclopirox (CPX) for 24 hours. Whole cell lysates were harvested for western blotting with indicated antibodies.

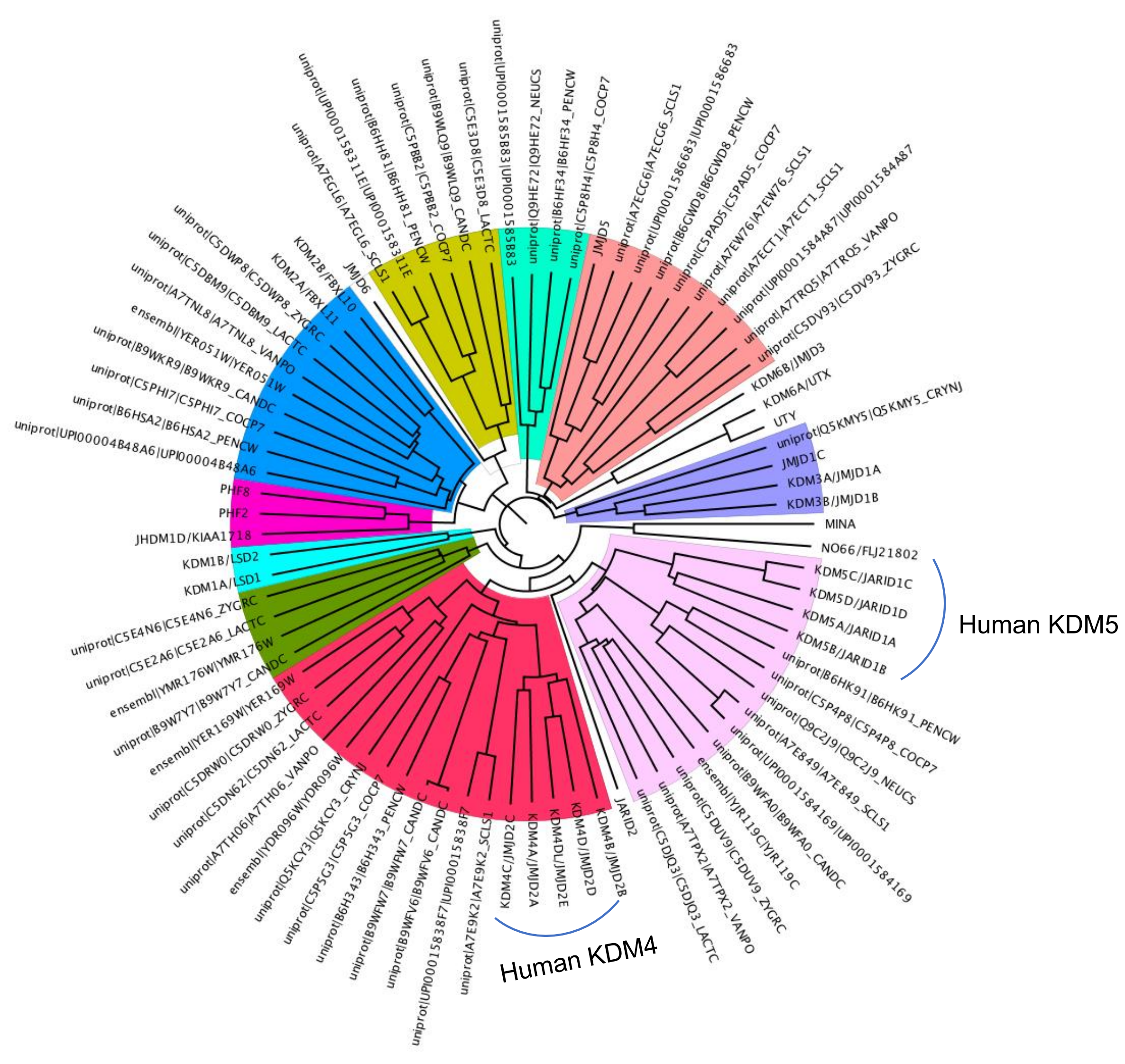


Supplementary Figure 4. 17-DMAG induces RMS cell differentiation. Related to Figure 6. Induction of differentiation marker, muscle heavy chain (*MHC*) by 17-DMAG. 10^5 of Rh30 (A) or Rh41 (B) cells were seeded in a 6-well plate in 2 ml of complete RMPI medium. 24h later, 17-DMAG was added to a final concentration of 200nM, for 72h incubation. RNA was extracted and cDNA was synthesized. Quantitative PCR was performed to assess the differentiation marker gene *MHC* and the control *GAPDH* for normalization. The experiment was performed in biological triplicates. Data are represented as mean +/- SEM.



Supplementary Figure 5. 17-DMAG inhibits the gene signatures of DNA repair and cell cycle. Related to Figure 6.

GSEA analysis reveals that 17-DMAG downregulates the genes involved in homologous DNA paring and strand exchange (**A**), homologous DNA repairing through single strand annealing (**B**), condensation of prophase chromosomes (**C**), and G2/M phase DNA damage checkpoint (**D**).



Supplementary Figure 6. Phylogenetic tree of JmjC domains of KDM in human and fungus. Related to Figure 9.

TRANSPARENT METHODS

Cell lines and reagents

Cell lines 293T, U2OS, Rh30, Rh41 cell lines were purchased from ATCC and validated by short tandem repeat (STR) using Promega PowerPlex 16 HS System once per month. PCR-based method was used for detection of Mycoplasma with LookOut Mycoplasma PCR Detection Kit (Sigma) and JumpStart *Taq* DNA Polymerase (Sigma) once per month to ensure cells were mycoplasma negative.

Compounds. 17-DMAG, 17-AAG, geldanamycin, AT13387, Ganetespib, GSK-J4 were purchased from Selleckchem. 17-AG was purchased from Sata Cruz Biotechnology. MG132 and Ciclopirox were purchased from Sigma. Vincristine, Irinotecan were purchased from MedChem Express (MCE). KDM5-C70 was purchased from Xcessbio.

Antibodies. The anti-PAX3-FOXO1 mouse monoclonal antibody was provided by Dr. Liang Cao (NCI). The anti-KDM4B antibody (A301-478A) was purchased from Bethyl Laboratories. The anti-Actin antibody (A2228) was purchased from Sigma. Anti-H3K4me3 (07-473) and Anti-normal mouse IgG antibody (12-371) was purchased from Millipore. The anti-total H3 (4499), anti-H3K9me3 (13969), anti-H3K36me3 (9763), anti-H3K4me3 (9751), H3K27me3 (9733) antibodies were purchased from Cell Signaling Technology (CST). Anti-FOXO1 antibody (2880) that recognizes the fusion protein PAX3-FOXO1 was purchased from CST. Anti-Hsp90 antibody (F-8) (sc-13119), anti-Hsp70 antibody (C92F3A-5)(sc-66048) and p53 antibody (DO-1) were purchased from Santa Cruz Biotechnology. Secondary horseradish peroxidase(HRP)-conjugated goat anti-mouse (31430) and goat anti-rabbit (31460) antibodies were purchased from Thermo Fisher Scientific.

Plasmids. MSCV-KDM4B(wt)-RFP and MSCV-KDM4B(H189A)-RFP constructs were generated by PCR of the full length of wild type and mutant KDM4B following by subcloning into MSCV-IRES-RFP plasmid, and standard retroviral packaging. U2OS cells were transduced with retroviral particles for high-content image screen. pCMV-HA-KDM4B was obtained from Addgene (24181). The catalytic domain of KDM4B(1-348) was subcloned into pET28a to produce histidine tagged KDM4B protein by Protein Production Facility at St Jude for TR-FRET and MALDI-FTICR screening.

TR-FRET demethylation functional assay

Stock compound solutions (10 mM compound in DMSO) or DMSO only (vehicle control) were transferred to the individual wells in low volume black 384-well assay plates containing 1.5 μM

biotin-H3K9me3 in 10 µL assay buffer [50 mM Tris-HCl (pH 8.0), 1 mM α-ketoglutarate, 80 µM $FeSO_4$, 2 mM ascorbic acid, 0.01% BSA] by using a V&P 384-well pintool (V&P Scientific, San Diego, CA) at 30 nL/well. KDM4B protein (750 nM) or buffer only was then dispensed (5 µL/well). After a brief spin down and shake, the plates were incubated at room temperature for 30 min. Detection reagent (5 µL/well) of 8 nM Tb-anti-H3K9me2 antibody and 8 nM AF488-streptavidin was dispensed, followed by a brief spin down, shake and 15 min room temperature incubation. The TR-FRET signal (fluorescence emission ratio of 10,000 × 520 nm/490 nm) from each well was collected with a PHERAstar *FS* plate reader (BMG LABTECH Inc., Cary, NC). The final tested compound concentration was 20 µM and the final DMSO concentration was 0.2% for all wells in the primary screening. The DMSO control wells with KDM4B protein and those without KDM4B protein were used as negative (0% inhibition) and positive (100% inhibition) controls, respectively. The individual compound activities were normalized to those of negative and positive controls. Compounds with %Inhibition ≥ 30% were selected as hits for DR analysis (10 concentrations, following a 1:3 serial dilution scheme; final concentration range 4.7 nM to 93.3 µM, in triplicates) under similar assay condition as the primary screening, with the exception of the final DMSO concentration at 0.93% for all assay wells. The activity data for individual chemicals were normalized to that of positive and negative controls and fit into sigmoidal DR equation, if applicable, to derive DR curves and IC_{50} values with GraphPad Prism 8.0.

High-content immunofluorescence imaging assay

1000 U2OS-KDM4B expressing cells in 25 µl of media were plated into each well of a poly-D-lysine coated Perkin Elmer 384-well View plates (Perkin Elmer 6007710) with a Thermo Scientific Wellmate. The cells were then grown for 18 hours overnight before they were drugged using a VP scientific pintool with S100 pins. The cells were then treated with compound for a twenty-four hours. Following treatment, the cells were fixed with 4% formaldehyde for 20 minutes at 37°C and permeabilized with 0.1% Triton-X 100 for 15 minutes at 25°C. Fixative was removed and each well washed with PBS. Cells were blocked using 1% BSA in PBS for 1 hour at 25°C. The primary antibody against trimethyl-histone H3 at Lys9 (Millipore 07-442) was used at 1/400 dilution in 1% BSA in PBS. This mixture was added to each well before incubation overnight at 4°C. Each well was then washed 3 times with PBS using a Biotek plate washer, and incubated for 1 hour at 25°C with a solution containing 1/400 goat α-rabbit-Alexa-488 (Cell Signaling 4412S) and 1 µM Hoechst 34580 to detect nuclear material (H21486 Molecular Probes.) Two images were captured of each well at 10X using a GE Healthcare InCell 6000 at 405 to detect nuclear staining and 488 nM to detect H3K9me3. The number of nuclear objects in each well, as detected through Hoechst

staining, was compared to the number of cells in each well expressing a minimum amount of H3K9me3 as determined by Alexa-488 fluorescence (1.5 million counts total intensity), to identify the percentage of cells in each considered “H3K9me3 Positive.” Averages shown are the result of eight replicate measurements per data point.

Dose response HTRF demethylation functional assay

Those selected hits with %Inhibition ≥ 30% from the TR-FRET demethylation functional assay were subjected to a HTRF demethylation functional assay with condition similar to the TR-FRET assay, except that the AF488-streptavidin was replaced with AF647-strepavidin. For data normalization, the DMSO control wells with KDM4B protein and those without KDM4B protein were defined as negative (0% inhibition) and positive (100% inhibition) controls, respectively. The individual compound activities were normalized to that of negative and positive controls and fit into sigmoidal DR equation, if applicable, to derive DR curves and IC_{50} values with GraphPad Prism 8.0.

MALDI-FTICR mass spectrometry-based demethylation assay

To assess the inhibition potency of geldanamycin and its analogs, we used a MALDI-TOF MS-based platform developed by our group. A truncated version of KDM4B that contains only the JmjC catalytic domain, KDM4B(1-348) was used. KDM4B(1-348), to a final concentration of 250 nM (in 50 mM Tris base, pH 7.3), was incubated with different concentrations of each compound (10 concentrations in total) for 1 hour at room temperature. The final concentration of DMSO in each well was 1 %. As negative control, KDM4B(1-348) was incubated with 1 % DMSO. Positive control wells contained 10 % formic acid. Reactions were initiated upon adding the “substrate mixture” (200 μM α-ketoglutarate, 100 μM ascorbate, 10 μM NH4Fe(SO4)2, and 10 μM H3K9me3(1-15) peptide) to each well. Reactions were incubated for 90 min at room temperature (to achieve ~ 20 % Turnover) prior to quenching with 10 % formic acid. Assays were performed in triplicates (n = 3).

Two microliters from each well were mixed with 18 μL of MALDI matrix solution (20 mg/mL of 2,5 dihydroxybenzoic acid dissolved in 95 % methanol), from which 1 μL was spotted on a 384 AnchorChip® MALDI target plate. Crystalized samples were then analyzed using a 7 T Solarix XR Fourier Transform Ion Cyclotron Resonance (FT-ICR) mass spectrometer (Bruker Co., MA, USA). The MALDI-FTICR parameters were optimized as follows: laser power = 20 % at 200 shots, laser shot frequency = 800 Hz, isolated Q1 m/z = 1530 ± 20. The mass of two species were

detected: m/z = 1530.87 (substrate), and m/z = 1516.85 (dimethylated product). The following formula was used to calculate % Turnover: $\% \text{ Turnover} = (\frac{I_P}{I_P + I_S}) \times 100$

Where IP and IS are the ion intensities of product and substrate, respectively. Values for % Turnover were then normalized based on negative and positive controls.

ALPHA Screen demethylation assay

All reagents including KDM proteins were provided by BPS Bioscience company. All of the enzymatic reactions were conducted in duplicate at room temperature for 60 minutes in a 10 µl mixture containing assay buffer, histone H3 peptide substrate, demethylase enzyme, and the test compounds. These 10 µl reactions were carried out in wells of 384-well Optiplate (PerkinElmer). The dilution of the compounds was first performed in 100% DMSO with the highest concentration at 0.5mM. Each intermediate compound dilution (in 100% DMSO) will then get directly diluted 30x fold into assay buffer for 3.3x conc (DMSO). Enzyme only and blank only wells have a final DMSO concentration of 1%. From this intermediate step, 3 µl of compound is added to 4 µl of demethylase enzyme dilution is incubated for 30 minutes at room temperature. After this incubation, 3 µl of peptide substrate is added. The final DMSO concentration is 1%. After enzymatic reactions, 5 µl of anti-Mouse Acceptor beads (PerkinElmer, diluted 1:500 with 1x detection buffer) or 5 µl of anti-Rabbit Acceptor beads (PerkinElmer, diluted 1:500 with 1x detection buffer) and 5 µl of Primary antibody (BPS, diluted 1:200 with 1x detection buffer) were added to the reaction mix. After brief shaking, plate was incubated for 30 minutes. Finally, 10 µl of AlphaScreen Streptavidin-conjugated donor beads (Perkin, diluted 1:125 with 1x detection buffer) were added. In 30 minutes, the samples were measured in AlphaScreen microplate reader (EnSpire Alpha 2390 Multilabel Reader, PerkinElmer). Enzyme activity assays were performed in duplicates at each concentration. The A-screen intensity data were analyzed and compared. In the absence of the compound, the intensity (Ce) in each data set was defined as 100% activity. In the absence of enzyme, the intensity (C0) in each data set was defined as 0% activity. The percent activity in the presence of each compound was calculated according to the following equation: %activity = (C-C0)/(Ce-C0), where C= the A-screen intensity in the presence of the compound.

The values of % activity versus a series of compound concentrations were plotted using non-linear regression analysis of Sigmoidal dose-response curve generated with the equation Y=B+(T-B)/1+10((LogEC50-X)×Hill Slope), where Y=percent activity, B=minimum percent activity, T=maximum percent activity, X= logarithm of compound and Hill Slope=slope factor or

Hill coefficient. The IC50 value was determined by the concentration causing a half-maximal percent activity.

Microscale Thermophoresis assay

KDM4B was labeled with a cysteine reactive fluorescent dye (Protein Labeling Kit RED-MALEIMIDE, Nanotemper GmbH). A serial dilution of titrant was prepared in MST buffer containing 10 mM HEPES (pH 7.5), 200 mM NaCl, 2 mM NiCl2, 5% DMSO and 0.05% Tween 20. An equal volume of diluted titrant and the constant concentration of labeled KDM4B were added and loaded in a standard treated capillaries (Nanotemper GmbH). Binding measurements were performed on a Monolith NT.115 Blue/Red instrument (Nanotemper GmbH) at 40 % LED power and 40 % MST power. The data were analyzed using MO Affinity Analysis software (Nanotemper GmbH).

Phylogenetic tree analysis

Amino acid sequences of KDM domains of histone lysine demethylases were aligned with Clustal Omega program (https://www.ebi.ac.uk/Tools/msa/clustalo/) and the phylogenetic tree was generated using neighbor-joining method, which was shown using iTOL program (https://itol.embl.de).

RNA-seq

Total RNA was extracted from xenograft tissues by RNeasy Mini Kit (cat. # 74104) from QIAGEN. Paired-end sequencing was performed using the High-Seq platform with 100bp read length. Reads were aligned to the human GRCh37-lite using SJCRH's Strongarm pipeline. Counts per gene were obtained using htseq-count version 0.6.1 with Gencode vM5 level 1and 2 gene annotations. Counts were normalized with VOOM and analyzed with LIMMA within the R statistical environment. Significance was defined as having a false discovery rate (FDR) <0.05. VOOM normalized counts were analyzed with Gene Set Enrichment Analysis (GSEA)[79].

RNA-extraction and RT PCR

The RNA extraction and RT PCR were performed as we previously described. Briefly, RNA was extracted using the RNeasy Mini Kit from Qiagen according to the manul. cDNA was synthesized using SuperScript™ II (Thermo Fisher). RT-PCR was performed using an Applied Biosystems 7500 Real-Time PCR system. The results were analyzed using ΔΔCT methods. Fold-differences calculated using the ΔΔCT method are expressed as a range by incorporating the standard deviation of the ΔΔCT value into the fold- difference calculation according to the manufacture's

PCR Guide instructions (Applied Biosystem). The PCR primer sequences for -actin (forward primer 5-CCAACCGCGAGAAGATGA-3; reverse primer 5-CCAGAGGCGTACAGGGATAG-3), Muscle heavy chain (MHC) gene (forward primer 5-TCCTGGATGCCAGTGAGC-3; reverse primer 5-CCAGCTTCTTCTTGGTGTTGA-3), Pax3-Foxo1 (forward primer CATGGATTTTCCAGCTATACAGAC; reverse primer 5-GTTCCTTCATTCTGCACACG-3), GAPDH (forward primer 5-AACGGGAAGCTTGTCATCAATGGAAA-3; Reverse primer 5-GCATCAGCAGAGGGGGCAGAG-3).

Western blot

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and then directly lysed on ice with 2X sample loading buffer (0.1 M Tris HCl [pH 6.8], 200 mM dithiothreitol [DTT], 0.01% bromphenol blue, 4% sodium dodecyl sulfate [SDS], 20% glycerol). On ice, cell lysates were briefly sonicated once for 5 seconds at 40% amplitude output followed by 25 minutes heating at 95 °C. Afterwards, cell lysates were briefly centrifuged at 13,000 × g at room temperature for 1 minute. Then, 20 µl of cell lysates were separated on 4-12% tris-glycine SDS-polyacrylamide gel electrophoresis (SDS-PAGE) from Invitrogen, and transferred to methanol-soaked polyvinylidene difluoride (PVDF) membranes from Millipore. Membranes were blocked in PBS buffer supplemented with 0.1% TWEEN 20 and 5% skim milk (PBS-T), and incubated for 1 hour at room temperature under gentle horizontal shaking. Afterwards, membranes were incubated overnight at 4°C with the primary antibodies under gentle horizontal shaking. The primary antibodies were prepared in PBS-T with the following dilutions: anti-KDM4B (1:1000), anti-PAX3-FOXO1 (1:200), anti-FOXO1 (1:1000), anti-Hsp90 (1:1,000), anti-actin (1:5,000), anti-total H3 (1:2,000), anti-H3K4me3 (1:2,000), anti-H3K9me3 (1:2,000) and anti-H3K36me3 (1:2,000). Next day, membranes were washed 3 times (each wash for 5 minutes) with PBS-T at room temperature. Protected from light, membranes were then incubated with goat anti-mouse or goat anti-rabbit HRP-conjugated secondary antibodies (1:5,000) for 1 hour at room temperature. Then, membranes were washed 3 times (each wash for 5 minutes) with PBS-T at room temperature. Lastly, membranes were incubated for 1 minute at room temperature with SuperSignal West Pico PLUS Chemiluminescent Substrate (34580, Thermo Fisher Scientific), and the bound antigen-antibody complexes were visualized using Odyssey Fc Imaging System (LI-COR Corp., Lincoln, NE).

Immunoprecipitation

Cells were washed twice with ice-cold PBS and then directly lysed on ice with co immunoprecipitation (co-IP) buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], 1% nonyl phenoxypolyethoxylethanol [NP40], 5% glycerol) supplemented with phosphatase (Roche) and protease (Roche) inhibitor cocktails. The cell lysate was transferred to a 2-ml Eppendorf tube, and incubated on ice for 15 minutes, and vortexed every 5 minutes. Then, the cell lysate was centrifuged at 13,000 × g at 4 °C for 15 minutes. The pre-cleared supernatant was incubated with rotation at 4 °C overnight with 4 µg of anti-HSP90 and 4 µg of anti-normal IgG as a negative control. Next day, 50 µl of protein A/G magnetic beads (88802, Thermo Scientific Fisher) were washed 3 times at room temperature with the co-IP buffer, and then added to each pre-cleared supernatant for 1 hour incubation with rotation at 4 °C. Afterwards, the supernatant (flow-through) was discarded; the beads were washed 3 times with co-IP buffer, eluted with 50 µl of the 2X sample loading buffer and heated for 10 mins at 95 °C. Input lysate was heated for 25 mins at 95 °C. 20 µl of co-IP and 20 µl of input reactions were subjected to SDS-PAGE and immunoblotting with anti-Hsp90 and anti-PAX3-FOXO1 antibodies (as described above).

Immunohistochemistry

Xenografts were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 µm, stained with hematoxylin and eosin and reviewed by light microscopy using an upright Nikon Eclipse Ni microscope (Nikon Instruments, Inc.). Immunohistochemistry was performed on 4 µm thick tissue sections mounted on positively charged glass slides (Superfrost Plus; Thermo Fisher Scientific, Waltham, MA), and dried at 60°C for 20 minutes. The procedures for immunohistochemistry were performed using a Ventana DISCOVERY ULTRA autostainer (Roche). Heat induced epitope retrieval was applied for 1 hour using cell conditioning 1 buffer (CC1, Roche, #950-500) followed by the application of anti-CD31 (Histobiotec, DIA-310, 1:50) or anti-Cleaved Caspase 3 (BioCare Medical, CP229C, 1:500) for 32 minutes. The following reagents were used for visualization: DISCOVERY OmniMap anti-Rat HRP (Roche,760-4457) for CD31 or OmniMap anti-Rabbit HRP (Roche, 760-4311) and the DISCOVERY ChromoMap DAB kit (Roche, 760-159), which was applied for 8 min at room temperature. Tissues were counterstained with Hematoxylin II (Roche, 790-228) for 12 min and Bluing reagent (Roche, 760-2037) for 4 min as a post-counterstain procedure. TUNEL was performed using the *In situ* Cell Death Detection Kit (Roche, 11684817910) according to the manufacturer's instructions. The quantification of Caspase 3, TUNEL and CD31 was performed using ImageJ IHC tool box (for

Caspase 3 and TUNEL) and Vessel Analysis plug in program (for CD31), and unpaired student t test was used to compare the difference between vehicle and treatment.

Chromatin immunoprecipitation

$1x10^7$ of RH30 cells treated with DMSO control or 1µM of Geldanamycin for 24 h were cross-linked with fresh prepared 1% formaldehyde (final concentration) for 10 min and quenched with 125 mM glycine for 5 min at room temperature. Cells were washed once with Dulbecco's Phosphate-Buffered Saline (DPBS). 5-8 ml of cold Cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40 with 1 tablet protease inhibitor cocktail) were added to plate after removing DPBS. The cells were scraped with a cell scraper and transfer into 50-ml conical tubes on ice. Cell pellets were collected by centrifuging at 2,000 rpm for 5 minutes at 4°C. The pellets were resuspended in cell lysis buffer and incubated on ice for 10 min. Cells were passed through a 20-gauge needle 20 times. The nuclei were collected by centrifuging at 2,000 rpm at 4°C for 5 minutes. The nuclear pellets were resuspended and lysed with 250 µl RIPA buffer (1X PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS with 1 tablet protease inhibitor cocktail). The lysates were sonicated for at least 30 min (30 sec on / 30 sec off) using Bioruptor Pico (Diagenode, Inc., Denville, NJ) and centrifuged at 14,000 rpm in a microfuge for 15 minutes at 4°C. The supernatant was collected and the sonicated DNA fragments were examined by electrophoresis on a 1% agarose gel. The sonicated samples with enrichment of fragments between the 100 to 500 bp range were used for the chromatin immunoprecipitation (ChIP) after removing 50 µL from each sample as input. The primary antibodies H3K9me3 (Millipore, 07-442) and H3K36me3 (RevMab, 31-1051-00) were coupled with 25 µL magnetic beads (Dynabeads M-280 Sheep Anti-Rabbit IgG, Invitrogen 11203D) overnight in the cold room at 4°C, washed three times with PBS/BSA (1XPBS / 5 mg/ml BSA (fraction V)) at 4°C, then incubated with sonicated DNA chromatin samples overnight in the cold room at 4°C. The beads containing immuno-bound chromatin were collected by placing the microfuge tube on a magnet rack. The beads were extensively washed with LiCl wash buffer (100 mM Tris pH 7.5 / 500 mM LiCl / 1% NP-40 / 1% sodium deoxycholate) 5 times and TE Buffer (10 mM Tris-HCl pH 7.5 / 0.1 mM Na2EDTA) once. Bound chromatin was eluted by IP Elution Buffer (1 % SDS / 0.1 M NaHCO3) and reverse-crosslinked at 65°C overnight. DNAs were purified using Min-Elute PCR purification kit (Qiagen, Valencia, CA) after the treatment of RNase A and proteinase K. ChIP enrichment DNAs were submitted to library preparation and sequencing. ChIP-seq library preparation and sequencing were carried out by the Hartwell Center at St Jude Children's Research Hospital. Briefly, 5-10 ng of DNA was used to prepare libraries by the NEBNext ChIP-Seq Library Prep Reagent Set for

Illumina with NEBNext Q5 Hot Start HiFi PCR Master Mix according to the manufacturer's instructions (New England Biolabs). Completed ChIP-seq libraries were analyzed for insert size distribution using a 2100 BioAnalyzer High Sensitivity kit (Agilent) or Caliper LabChip GX DNA High Sensitivity Reagent Kit (Perkin Elmer). All libraries were quantified using the Quant-iT PicoGreen dsDNA assay (Life Technologies), Kapa Library Quantification kit (Kapa Biosystems). Fifty-cycle single-end sequencing was performed using an Illumlina HiSeq 2500 or HiSeq 4000.

ChIP-seq analysis

Mapping reads and visualizing data. The ChIP-seq raw reads were aligned to the human reference genome (hg19) using BWA (version 0.7.12; default parameter) to and then marked duplicated reads with Picard (version 1.65), with only nonduplicated reads kept by samtools (version 1.3.1, parameter ''-q 1 -F 1024''). To control the quality of the data and estimate the fragment size, the nonduplicated version of SPP (version 1.11) was used to calculate relative strand correlation value with support of R (version 3.5.1). To visualize ChIP-seq data on integrated genome viewer (IGV) (version 2.3.82), we used genomeCoverageBed (bedtools 2.25.0) to produce genome-wide coverage in BEDGRAPH file and then converted it to bigwig file by bedGraphToBigWig. The bigwig files were scaled to 15 million reads to allow comparison across samples.

Peak calling, annotation and motif analysis. MACS2 (version 2.1.1) was used to call narrow peaks (H3K27Ac and H3K4me3) with option "nomodel" and "extsize" defined as fragment size estimated by SPP, FDR corrected p value cutoff 0.05. For broad peaks (H3K9me3, H3K36me3, H3K27me3, and H3K4me1), SICER (version 1.1, with parameters of redundancy threshold 1, window size 200bp, effective genome fraction 0.86, gap size 600bp, FDR 0.00001 with fragment size defined above) has been used for domain calling. Enriched regions were identified by comparing the IP library file to input library file. Peak regions were defined to be the union of peak intervals in replicates from control or treated cells respectively. Promoters were defined as mouse RefSeq TSS ± 1000bp regions. Genomic feature annotation of peaks was done by annotatePeaks.pl, a program from HOMER suite (v4.8.3, http://homer.salk.edu/homer/). The HOMER software was used to perform de novo motif discovery as well as check the enrichment of known motifs in a set of given peaks.

Differential analysis. ChIP-seq raw read counts were reported for each region/each sample using bedtools 2.25.0. Raw read counts were voom normalized and statistically contrasted using the R(version 3.5.1) packages limma and edgeR (version 3.16.5) for CPM calculation and differential

analysis. An empirical Bayes fit was applied to contrast treated samples to control samples and to generate log fold changes, p values and false discovery rates for each peak region.

Animal experiments

All murine experiments were done in accordance with a protocol approved by the Institutional Animal Care and Use Committee of St. Jude Children's Research Hospital. Subcutaneous xenografts in Figure 5 and Figure 6E were established in CB17 severe combined immunodeficient mice (CB17 *scid*, Taconic) by implanting $5X10^6$ cells in Matrigel. Subcutaneous xenografts in Figure 6A - 6D were established NOD.Cg-$Prkdc^{scid}$, $Il2rg^{tm1Wjl}$/SzJ (NOD *scid* gamma, NSG) mice by implanting $5X10^6$ cells in Matrigel. Tumor measurements were done weekly using electronic calipers, and volumes calculated as width $\pi/6 \times d^3$ where *d* is the mean of two diameters taken at right angles. Subcutaneous xenografts were treated with 25 mg/kg of 17-DMAG or 50 mg/kg of 17-AAG via intraperitoneal injection twice daily, every four days. 17-DMAG was dissolved in 1% DMSO, 1% TWEEN® 80 (#P4780 from Sigma), 30% PEG300 (#202371 from Sigma) and 68% ddH_2O. 17-AAG was dissolved in 5% DMSO and 95% corn oil. Vincristine was administered in a dose of 0.38 mg/kg via IP injection once daily every week. Vincristine was dissolved in 100% saline. Irinotecan was administered in a dose of 1.25 mg/kg via IP injection once daily, for 5 days on and 2 days off schedule. Irinotecan was dissolved in 5% DMSO and 95% saline. Mice were sacrificed because of an adverse event before they had completed 14d and were removed from the data set. Tumor response: For individual mice, progressive disease (PD) was defined as < 50% regression from initial volume during the study period and > 25% increase in initial volume at the end of study period. Stable disease (SD) was defined as < 50% regression from initial volume during the study period and ≤ 25% increase in initial volume at the end of the study. Partial response (PR) was defined as a tumor volume regression ≥50% for at least one time point but with measurable tumor (≥ 0.10 cm^3). Complete response (CR) was defined as a disappearance of measurable tumor mass (< 0.10 cm^3) for at least one time point.

Statistical analysis

To determine statistical significance, the unpaired, two-tailed Student *t* test was calculated using the *t* test calculator available on GraphPad Prism 8.0 software. A *p* value of less than 0.05 was considered statistically significant. Kaplan-Meier survival analysis was calculated using log-rank (Mantel-Cox) method in GraphPad Prism 8.0 software.

The Kruskal Wallis test was utilized to determine if there was a statistically significant different among the 4 treatment groups at each time point. The exact Wilcoxon Rank Sum test was utilized

to determine if there was a statistically significant difference between receiving one treatment vs receiving the combination treatment. All statistical analyses were conducted in SAS 9.4 and a two-sided significance level of $p<0.05$ was determined a priori.