Supplemental Figures and Methods

Inhibition of MEK and ATR is effective in a B-ALL model driven by *Mll-Af4* and activated *Ras*

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Supplemental Figure Legends

Figure S1. Retroviral transduction of *Mll-Af4* BM with mutant *N-Ras*^{G12D} generates aggressive, serially transplantable B-ALL

A second independent *Mll-Af4* donor (*Mll-Af4*-2) bone marrow was transduced with both retroviruses expressing Cre (Tomato) and mutant *N-Ras*^{G12D} (GFP) as previously described. Pre-leukemic cells double positive for cre and *N-Ras*^{G12D} were confirmed to be cytokine independent and have the same pro-B immunophenotype described in Figure 1 ($B220^{+}CD43^{+}IgM^{-}$ and $Mac1^{-}Gr1^{-}$ as well as $Cd19^{+}Cd24^{+}Flt3^{+}Cd25^{-}IgD^{-}$).

Figure S2. Immunophenotyping of *Mll-Af4*/*N-Ras*^{G12D} primary and secondary proB-ALLs

Representative flow cytometry plots of cell surface expression of *Mll-Af4/N-Ras*^{G12D} primary and secondary leukemias derived from independent donors (*Mll-Af4-1 and Mll-Af4-2*). Primary and secondary leukemias were found to express cell surface markers consistent with those in pre-leukemic cultures (Figure 1 and Figure S1) and those found on normal proB cells ($B220^{+}Cd43^{+}Cd19^{+}Cd24^{+}Flt3^{+}cKit^{+}IgM^{-}IgD^{-}Cd25^{-}$).

Figure S3. *Mll-Af4/Nras*^{G12D} B-ALL gene expression resemble glucocorticoid insensitive human B-ALL gene expression and have low *Hoxa* cluster and *Meis1* expression

RNA-Sequencing was conducted on primary *Mll-Af4/Nras*^{G12D} B-ALLs and compared to wildtype pro-B cells ($B220^{+}CD43^{+}IgM^{-}$). Ranked lists of differential expression were calculated and GSEA analysis conducted against datasets in MSigDB (A) Significant positive enrichment of gene signatures was observed for a gene signature of persistent B-ALLs that had been treated with glucocorticoids[20] (B) Ct values and of quantitative real-time PCR, of *Hoxa9, Hoxa10, Hoxa7, Meis1* and housekeeping genes *Gapdh* and *Actb* were conducted on independent primary and resultant secondary leukemias (indicated by primary leukemia identifier number followed by a number) using Taqman probes (LifeTechnologies). Ct values for wildtype (WT) pro B cells ($B220^{+}CD43^{+}IgM^{-}$) were also calculated.

Figure S4. Prolonged treatment with Trametinib insufficient to prevent leukemic progression. Mice treated for more than 7 days with either 0.5 mg/kg (A) or 1 mg/kg (B) Trametinib were sacrificed when mice appeared sick and hematopoietic tissues analyzed for leukemic involvement. High levels of $GFP^{+}Tomato^{+}$ leukemic cells were present in all tissues assessed.

Figure S5 GSEA of RNA-Sequencing of *Mll-Af4*/*N-Ras*G12D B-ALL compared to normal pro-B cells

RNA-Sequencing was conducted on primary *Mll-Af4/Nras*^{G12D} B-ALLs and compared to wildtype pro-B cells ($B220^{+}CD43^{+}IgM^{-}$). Ranked lists of differential expression were calculated and GSEA analysis conducted against MSigDB. Significant negative enrichment of gene signatures for the ATR and G2/M cell cycle checkpoint pathways was observed in primary B-ALLs for (A) Reactome Activation of ATR in Response to Replication Stress, (B) PID ATR Pathway, (C) Whitfield Cell Cycle G2-M, (D) Hallmark G2-M Checkpoint, and (E) Reactome G2-M Checkpoints gene expression signatures.

Figure S6. Gene expression of ATR pathway members in human patient samples of *MLL*-r B-ALL compared to normal pre-B cells

Heatmaps and meta-gene expression boxplots for the (A) PID_ATR pathway or (B) Reactome Activation of ATR in response to Replication Stress gene sets from MSigDB was selected for gene expression analysis. Human *MLL-AF4* infant B-ALL patient Affymetrix Human Genome U133 Plus 2.0 Array data from GEO data sets GSE79450, GSE19475 and GSE77416 were normalized and the relative expression of genes determined. Heatmap includes also expression data from HSCs derived from cord blood (CD34+CD38-CD19-CD33-), pre-B (CD34+CD19+CD33-) and myeloid HSPCs (CD34+CD33+CD19-) from GEO data set GSE79450.

Figure S7. ATR inhibition induces DNA damage in *Mll-r* B-ALL with already elevated basal replicative stress and DNA damage levels and an intact p53 pathway

(A) Phospho-γH2AX foci detected in *Mll-Af4/N-Ras*^{G12D} pre-leukemic cells by immunofluorescence and confocal microscopy. Foci were quantified by Image J analysis. (B) Phospho-γH2AX levels of *Mll-Af9* AMLs and *Mll-Af4* B-ALLs treated for

24 hours with 0, 1, 3 μM AZ20. (C) *Mll-Af4/N-Ras*^{G12D} pre-leukemic cells were treated with 10μM Nutlin-3 for 24 hours. Cell viability as compared to DMSO controls was measured by DAPI staining by flow cytometry and (D) Fold increase of the percentage of Annexin V^+ cells as a ratio to DMSO controls. Data represented as an average of 4 independent treatment experiments.

Figure S8. *In vivo* treatment of *Mll-Af4*/*N-Ras*^{G12D} ALL with Trametinib, AZ20 and combination.

Replicate *in vivo* treatment of B-ALL model as described in Figure 5 with *Mll-Af4*/*N-Ras*^{G12D} leukemia. Survival (A), spleen weights (B), leukemic infiltration (C) and apoptosis (D) measured in hematopoietic tissues as indicated. (*** $p< 0.001$) (** $p< 0.01$) (* $p <0.05$)

Figure S9. *In vivo* treatment of *Mll-Af4*/*N-Ras*^{G12D} ALL with Trametinib, AZ20 and combination.

Replicate *in vivo* treatment of B-ALL model as described in Figure 5 with *Mll-Af4*-2/*N-Ras*^{G12D} leukemia. Leukemic infiltration (A), spleen weight (B) and apoptosis (C) measured in hematopoietic tissues as indicated and similar to those seen in *Mll-Af4*/*N-Ras*^{G12D} leukemia described in Figure 5 and Supplemental Figure 8. (*** $p< 0.001$) (** $p< 0.01$) (* $p <0.05$)

Figure S10. Histopathological resolution of leukemic infiltration in BM and spleen of treated mice. H&E staining of bone marrow from formalin preserved spine sections (A) and spleen (B) from vehicle and combination Trametinib+AZ20 treated mice injected with secondary leukemia cells from Figure 5. Black bar denotes 250 μm scale.

Figure S11. VAF of mutant *RAS* in PDXs harboring MLL-AF4 translocations after treatment.

Mutational status of *N/K-RAS* for (A) PDX#83 and (B) PDX #24 treated with vehicle, single agent trametinib or AZ20 or the combination. Frequencies relative to reference sequence and VAF of relevant activating *RAS* mutations plotted at appropriate nucleotide positions plotted. Where there were variants from the reference sequence, plots of all alternate /variant nucleotides at those nucleotide positions and a summary plot of VAFs upon treatment are included with initial PDX sample VAF indicated with dotted blue line.

Table S1. Genetic characteristics of PDXs harboring *MLL-AF4* translocation and mutant *N-RAS*. Mutational status of *MLL* and *N-RAS* of patient 042.D1 (#83) and 072.D1 (#24) and PDX samples (042.D1.m88BM and 072.D1.CD3-.m189BM) used in Figure 6.

Supplemental Methods

D-J rearrangements

D-J_H rearrangements in the IgH locus were detected by a PCR strategy employing two upstream degenerate primers binding 50 of the DFL/DSP element or the DQ52 element, DJHF1 (5'-ACGTCGACTTTTGTSAAGGGATCTACTACTGT-3') and (5'-ACGTCGACGCGGASSACCACAGTGCAACTG-3'). The reverse primer was complementary to a binding site downstream of the JH4 segment (5'-GGGTCTAGACTCTCAGCCGGCTCCCTCAGGG-3')

All three primers were used in a single PCR reaction in a multiplex PCR (94 °C, 1 minute followed by 35 cycles of 94 °C for 60 sec, 60°C for 30 sec, 72 °C for 105 seconds and 72 °C for 10 minutes).

Immunofluorescence staining

Cytospins of 100 000 cells were prepared at 500 rpm for 5 minutes, permeabilized and fixed in 4% paraformaldehyde/PBS at 4^{o}C for 10 minutes. Phospho-γH2AX (Abcam) primary antibody was used at 1:400 and incubated at 4^{o}C overnight. Secondary antibody Anti-rabbit Alexa647 (CST) was applied for 30 minutes at room temperature. DAPI (1 μg/ml) was applied for 5 minutes at room temperature, and the samples were covered with Prolong Gold Antifade Reagent (ThermoFisher). Slides were analyzed by confocal microscopy (Leica TCS SP5) (Leica) and foci were quantified using Image J software (NIH).

VAF determination of PDX samples

Genomic DNA was isolated from hCD45 enriched bone marrow from PDX mice treated with vehicle, single agent trametinib or AZ20 or combination and PCR-amplified for a 236-246bp amplicon which was submitted for next generation sequencing. >50,000 reads of 150bp paired-end reads were obtained on an Illumina MiSeq platform and aligned to hg19 for allele frequency detection. Primers for amplification were as follows: *NRAS*G12 Fwd: 5'-TAGATGTGGCTCGCCAATTA-3', *NRAS*G12 Rev: 5'-TGGGTAAAGATGATCCGACA-3', *KRAS*G12 Fwd 5'-GGTGGAGTATTTGATAGTGTATTAACC-3', *KRAS*G12 Rev: 5'-AGAATGGTCCTGCACCAGTAA-3'
*NRAS*Q61 Fwd: 5'-CACCCCCAGGATTCTTACAG-3', *NRAS*Q61 Rev:5'-CCTCATTTCCCCATAAAGATTCAGA-3'

RNA-sequencing and human gene expression analysis

For RNA-sequencing, reads were quality and adapter trimmed using 'trim_galore' before aligning to mouse assembly mm9 with STAR v2.4 and assessed using the Picard tool (http://broadinstitute.github.io/picard/). Read count tables were created using HTSeq v0.6.1. Normalization and expression dynamics were evaluated with DESeq2 using the default parameters.

fRMA normalization uses pre-computed estimates of probe specific effects and variances to permit samples to be processed in batches[18]. The probe set with maximum variance was used with genes with multiple probe sets. Heat maps were produced with the

bioconductor package pheatmap in R using row scaling of log2 data. Meta-genes for the box plots were found by taking the mean of the log2 expression for all genes in the signature for each sample. The probe set with maximum variance was used with genes with multiple probe sets. Heat maps were produced with the bioconductor package pheatmap in R using row scaling of log2 data. Meta-genes for the box plots were found by taking the mean of the log2 expression for all genes in the signature for each sample.

Histology

Tissues were fixed in 4% paraformaldehyde for 24 hours and then put into graded alcohol from 75% to 100% to dehydrate. After transparent in xylene, the tissues were embedded in paraffin. 4 μm section were sliced from the paraffin blocks and then kept in 65^{o}C oven for two hours. The slides were de-paraffinized in xylene and rehydrated in graded alcohol from 100% to 75%.

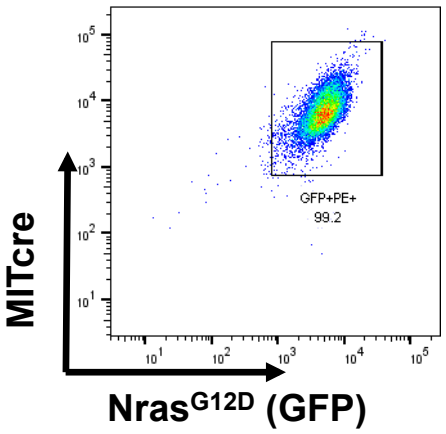
De-paraffinized sections were stained in hematoxylin solution (Servicebio, G1005-1) for 5 minutes. After washed in water, the slides were differentiated in 1% acid alcohol for 30 seconds and then stained in 0.2% ammonium hydroxide for several seconds. After washed in water, the slides were dehydrated through 85% alcohol and 95% alcohol respectively and then stained in Eosin Y solution (Servicebio, G1005-2) for 3 minutes. After dehydration in three absolute alcohol solutions and transparent in xylene, the slides were covered with a neutral resin mounting medium.

Whole tissues on slides were scanned by the digital slides scanner (3D Histech, MIDI) and then viewed with Caseviewer (3D Histech).

Statistical analysis

p values for standard comparisons of two groups were calculated using unpaired, two-tailed, Student's t-test. For multivariate analysis, 1-way ANOVA analyses were conducted to determine significance. p values for survival studies were calculated using log-rank (Mantel-Cox) test. For all p values, (*) $p<0.05$; (**) $P<0.01$, (***) $p<0.001$.

***Mll-Af4-2/Nras^{G12D}* pre-leukemic cells:**



Gating on GFP^{+}MITcre^{+} cells:

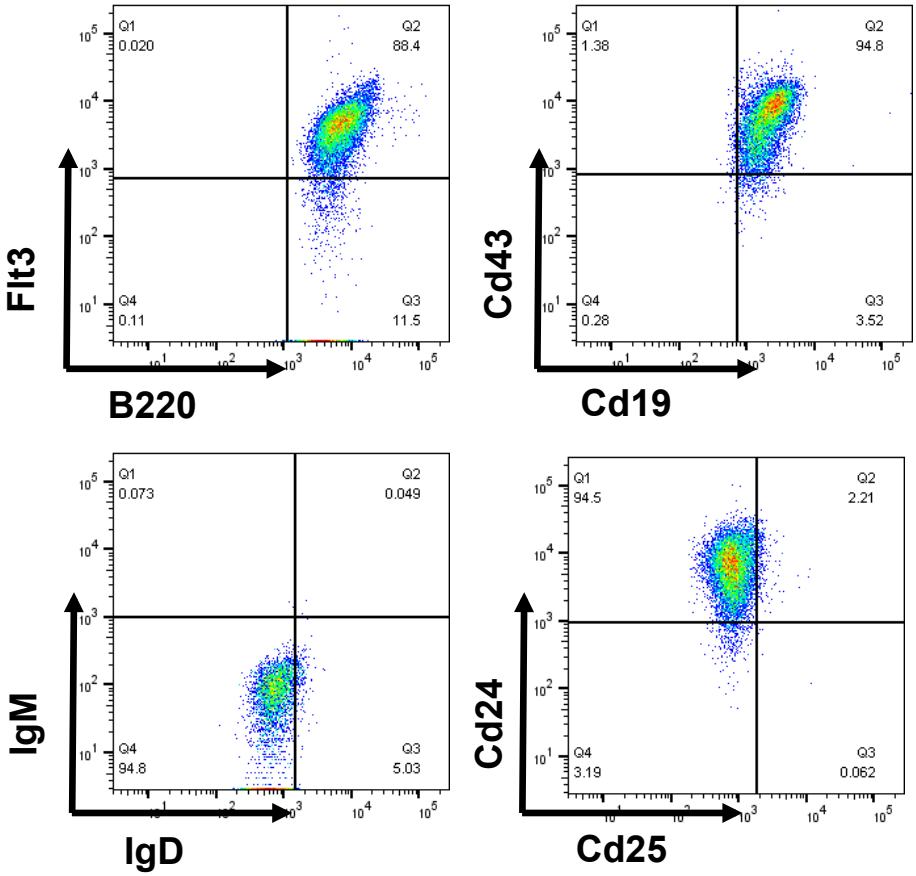


Figure S1

Gating on leukemic cells ($GFP^+MITcre^+$):

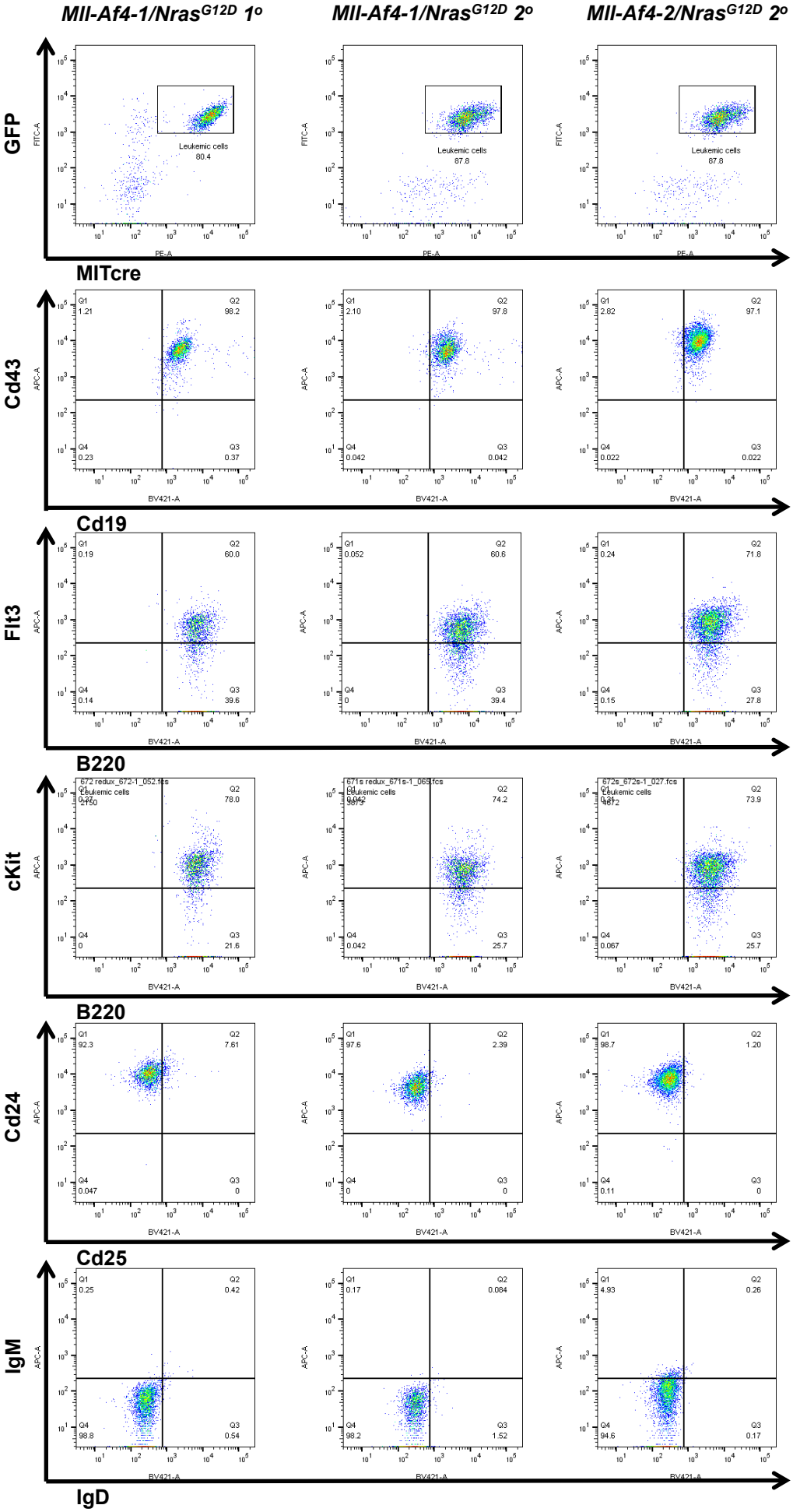
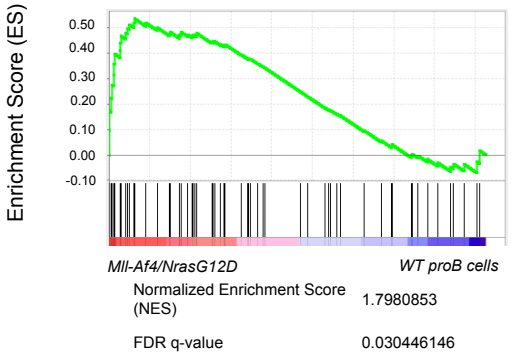


Figure S2

A



B

	HoxA9	stdev	HoxA10	stdev	Meis1	stdev	Actb	Stdev		
Primary Mll-Af4/Nras leukemia										
72	32.539	0.222	34.637	0.346	28.492	0.238	21.162	0.045		
672	31.669	0.011	35.059	0.820	32.631	0.175	18.676	0.218		
668	32.980	0.152	Undetermined	N/A	34.053	0.212	20.682	0.170		
N1	33.428	1.752	34.819	0.856	32.948	0.660	21.038	0.101		
671	30.719	0.191	34.031	2.277	31.858	0.769	18.841	0.341		
Secondary Mll-Af4/Nras leukemia										
672-1	32.534	0.008	Undetermined	N/A	32.446	0.285	17.231	0.081		
668-1	33.064	0.069	37.090	N/A	35.677	0.209	20.401	0.436		
672-2	31.143	0.839	34.437	0.354	31.727	0.233	17.451	0.103		
668-2	32.836	0.001	35.882	0.129	35.217	0.422	19.913	0.261		
672-3	32.427	0.470	34.758	N/A	31.510	0.231	16.726	0.066		
668-3	32.929	0.895	36.001	N/A	34.135	0.359	19.981	0.052		
668-4	33.103	0.001	33.502	0.056	34.764	0.291	19.530	0.421		
N1-1	32.939	0.473	34.669	N/A	33.572	0.074	19.736	0.287		
668-5	32.674	0.057	Undetermined	N/A	35.178	0.096	19.710	0.226		
N1-2	35.248	0.180	Undetermined	N/A	36.120	0.620	21.736	0.463		
N1-3	33.386	0.276	34.775	N/A	34.505	0.290	18.813	0.112		
671-1	33.144	0.190	Undetermined	N/A	33.734	0.243	18.062	0.141		
N1-4	31.571	0.155	34.867	0.813	35.282	0.282	19.561	0.241		
671-2	33.200	0.286	34.486	N/A	34.083	0.068	18.449	0.274		
671-3	34.545	1.066	35.225	0.742	36.269	0.105	19.061	0.170		
671-4	34.670	0.363	35.769	N/A	36.147	1.190	20.833	0.114		
WT proB cells	31.409	0.155	37.790	0.380	33.022	0.033	19.409	0.006		
	HoxA9	**stdev**	**HoxA10**	**stdev**	**HoxA7**	**stdev**	**Meis1**	**stdev**	**Gapdh**	**stdev**
WT proB cells	32.999	0.053	35.640	0.086	33.015	0.038	32.465	0.117	20.962	0.037
J601 primary	32.515	0.276	31.127	0.295	34.713	0.154	31.666	0.148	21.316	0.109
J602 primary	32.716	0.014	32.102	0.239	35.746	0.144	30.882	0.129	21.118	0.052
J603 primary	31.520	0.237	31.635	0.141	34.327	0.114	30.139	0.085	21.597	0.133

Figure S3

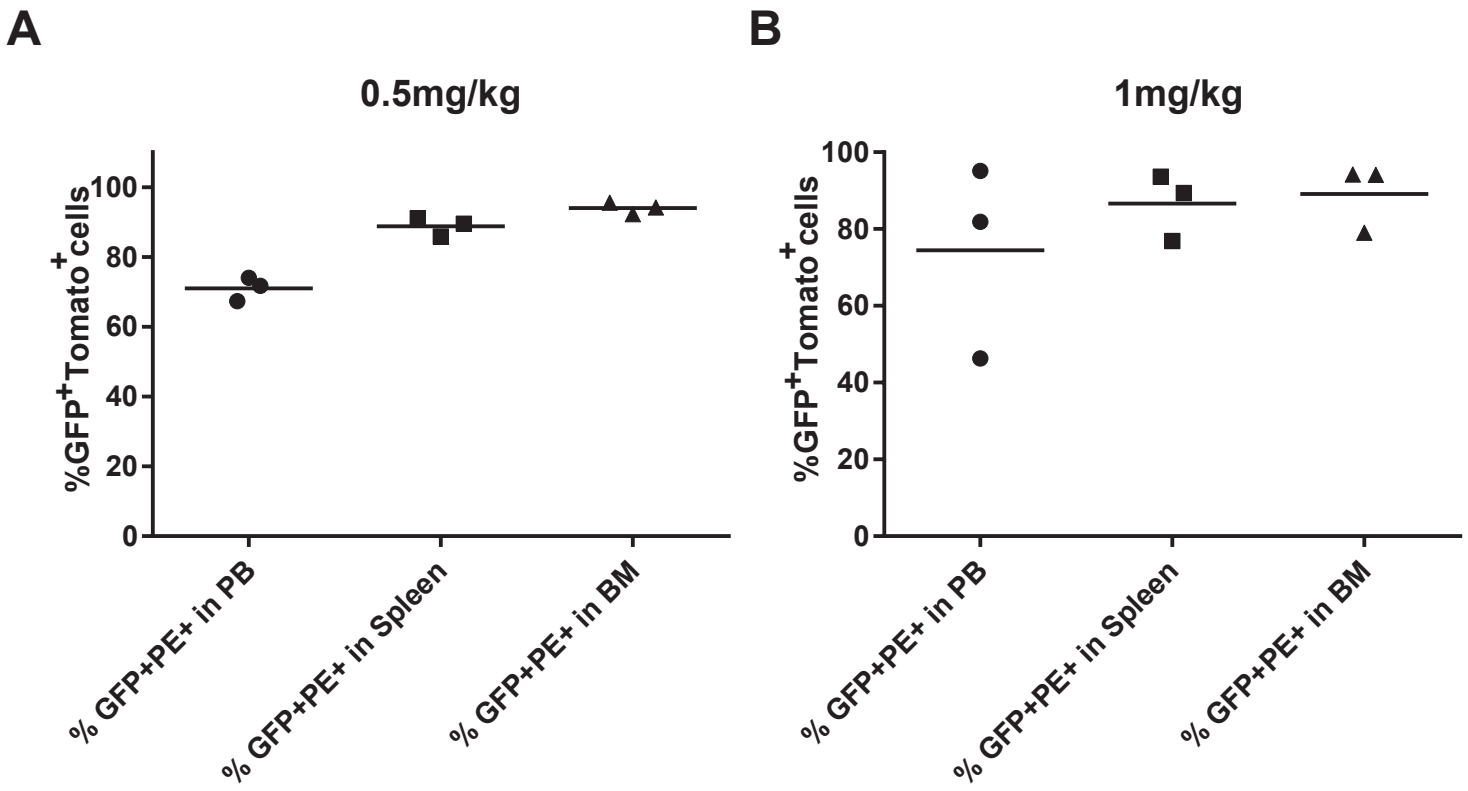
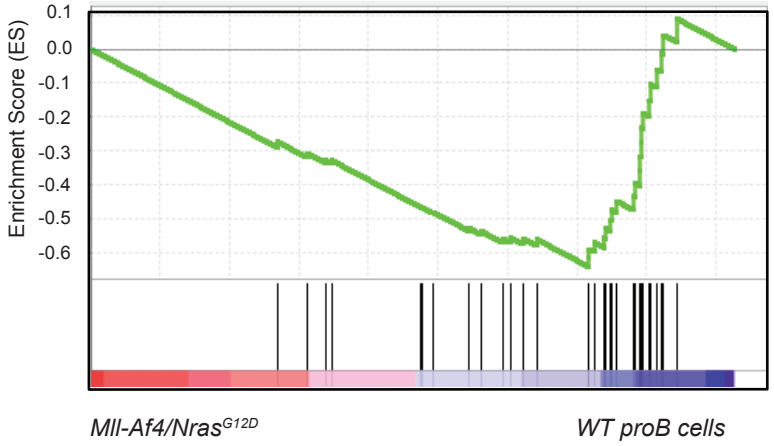

A
0.5mg/kg
%GFP+Tomato+cells
100
80
60
40
20
0
% GFP+PE+ in PB
% GFP+PE+ in Spleen
% GFP+PE+ in BM
B
1mg/kg
%GFP+Tomato+cells
100
80
60
40
20
0
% GFP+PE+ in PB
% GFP+PE+ in Spleen
% GFP+PE+ in BM

Figure S4

A

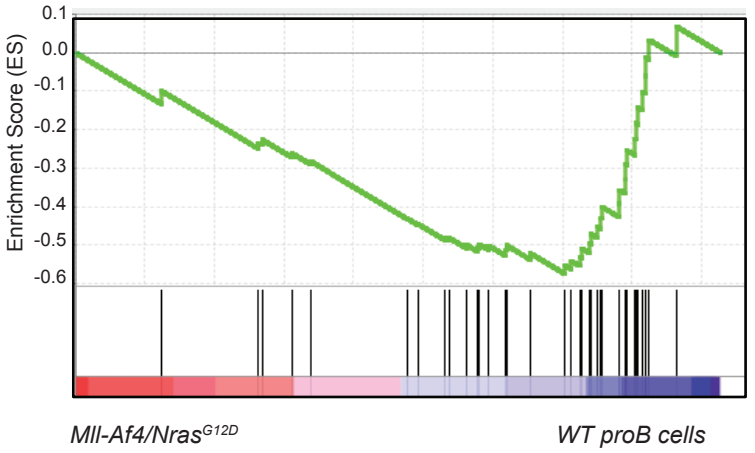
Reactome Activation of ATR in Response to Replication Stress



NES -1.74183
FDR q value: 0.02333

B

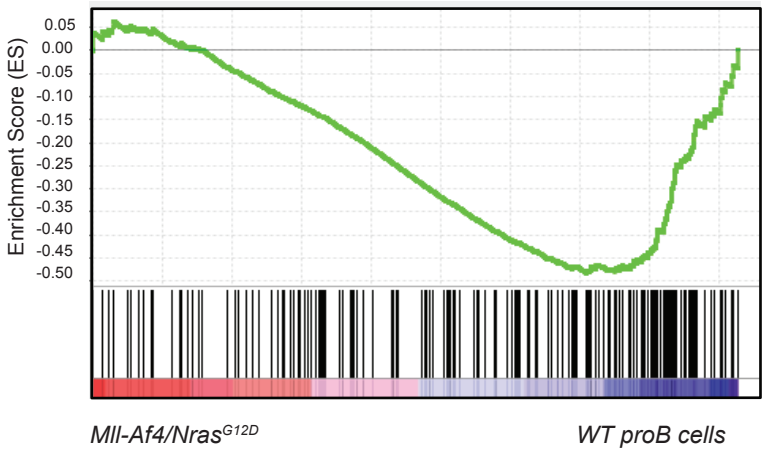
PID ATR Pathway



NES -1.5832285
FDR q value: 0.08237424

C

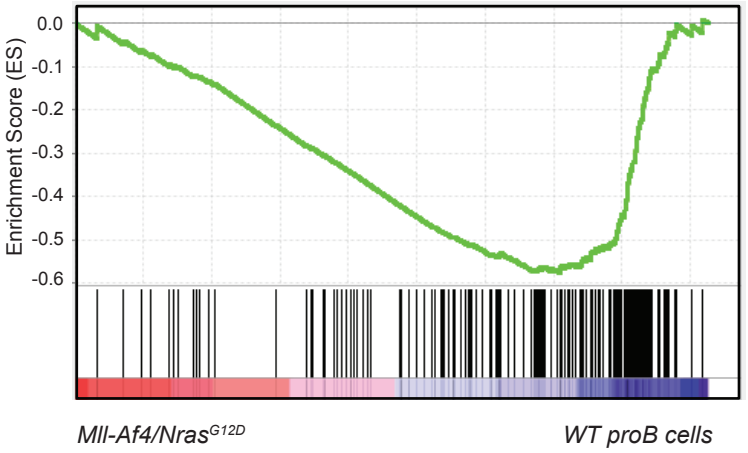
Whitfield Cell Cycle G2-M



NES -1.692854
FDR q value: 0.03722845

D

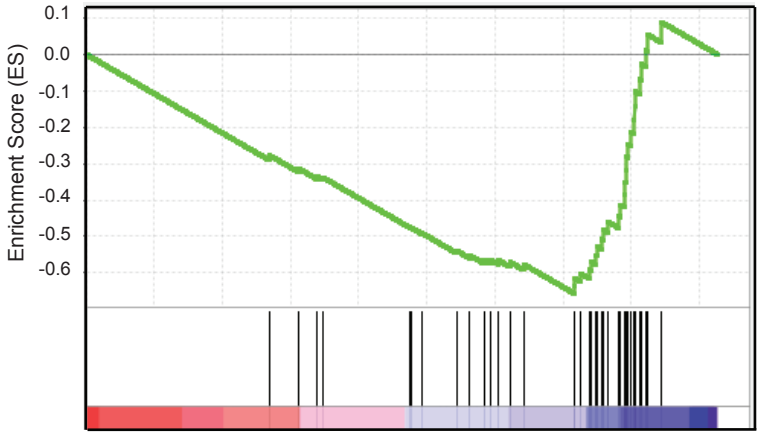
Hallmark G2-M Checkpoint



NES -2.0076184
FDR q value: 2.8699753E-4

E

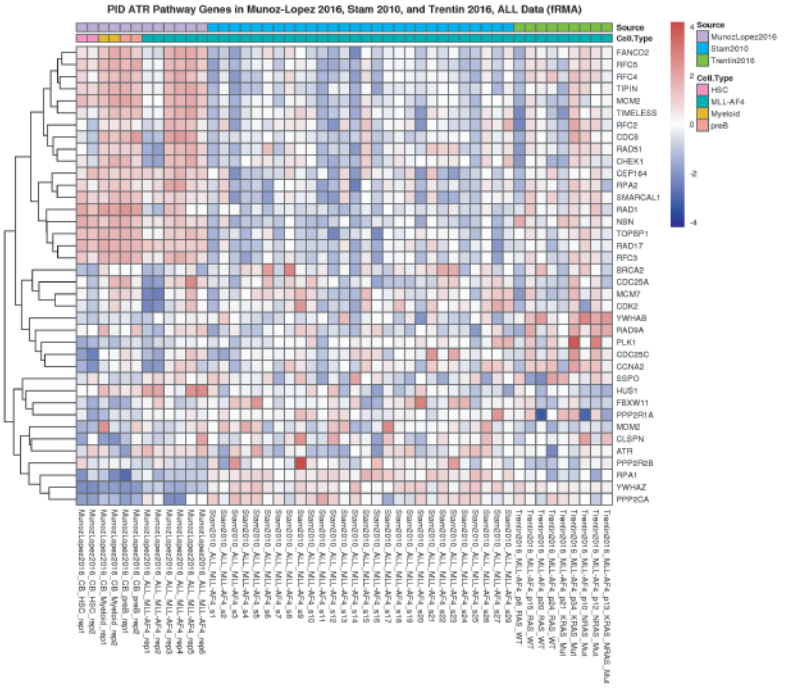
Reactome G2-M Checkpoints



NES -1.8267856
FDR q value: 0.008583433

Figure S5

A


PID ATR Pathway Genes in Munoz-Lopez 2016, Stam 2010, and Trentin 2016, ALL Data (fRMA)
Source
Cell.Type
MunozLopez2016
Stam2010
Trentin2016
HSC
MLL-AF4
Myeloid
preB
FANCD2
RFC5
RFC4
TIPIN
MCM2
TIMELESS
RFC2
CDC6
RAD51
CHEK1
CEP164
RPA2
SMARCAL1
RAD1
NBN
TOPBP1
RAD17
RFC3
BRCA2
CDC25A
MCM7
CDK2
YWHAB
RAD9A
PLK1
CDC25C
CCNA2
SSPO
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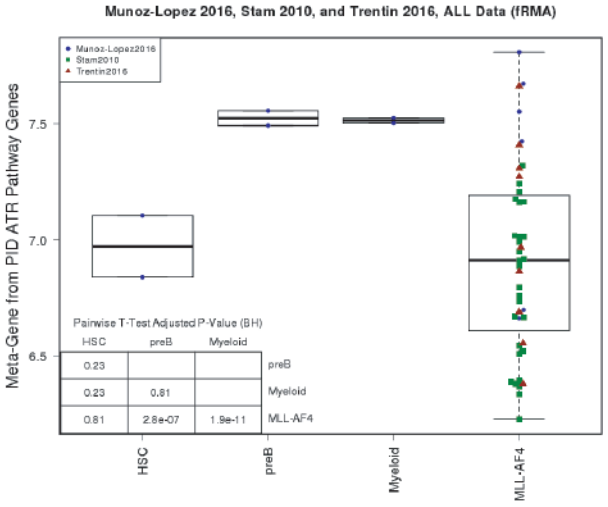
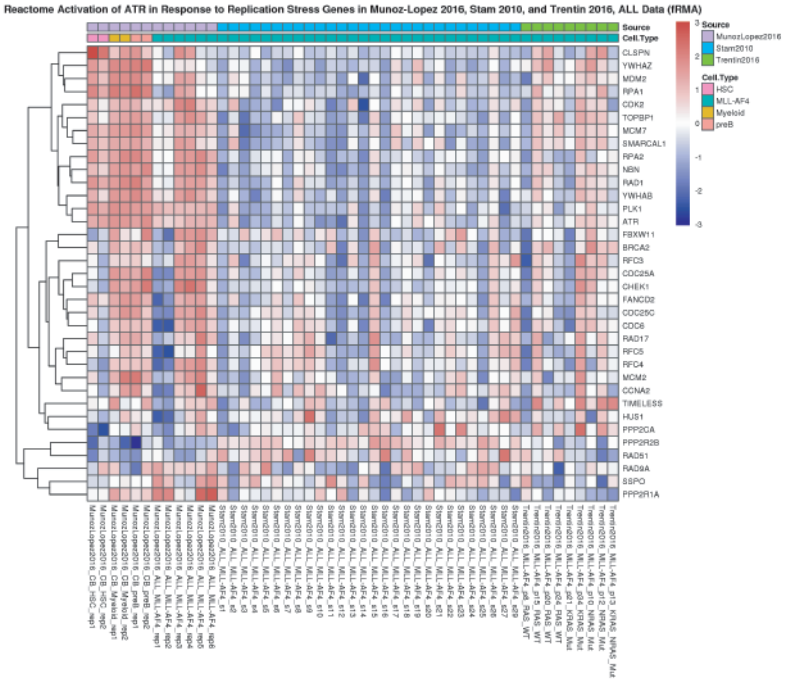
CLSPN
ATR
PPP2R2B
RPA1
YWHAZ
PPP2CA


Munoz-Lopez 2016, Stam 2010, and Trentin 2016, ALL Data (fRMA)
Munoz-Lopez2016
Stam2010
Trentin2016
Meta-Gene from PID ATR Pathway Genes
7.5
7.0
6.5
Pairwise T-Test Adjusted P-Value (BH)
HSC
preB
Myeloid
MLL-AF4
0.23
0.81
2.8e-07
1.9e-11
HSC
preB
Myeloid
MLL-AF4

B


Reactome Activation of ATR in Response to Replication Stress Genes in Munoz-Lopez 2016, Stam 2010, and Trentin 2016, ALL Data (fRMA)
Source
Cell.Type
MunozLopez2016
Stam2010
Trentin2016
HSC
MLL-AF4
Myeloid
preB
CLSPN
YWHAZ
MDM2
RPA1
CDK2
TOPBP1
MCM7
SMARCAL1
RPA2
NBN
RAD1
YWHAB
PLK1
ATR
FBXW11
BRCA2
RFC3
CDC25A
CHEK1
FANCD2
CDC25C
CDC6
RAD17
RFC5
RFC4
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PPP2R2B
RAD51
RAD9A
SSPO
PPP2R1A

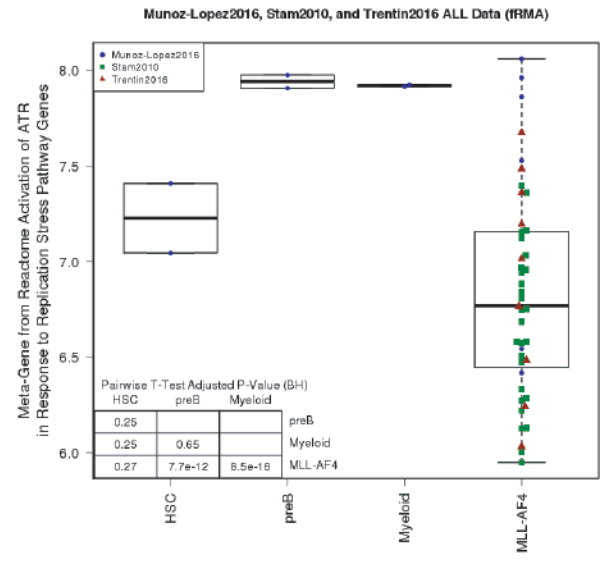

Munoz-Lopez2016, Stam2010, and Trentin2016 ALL Data (fRMA)
Munoz-Lopez2016
Stam2010
Trentin2016
Meta-Gene from Reactome Activation of ATR in Response to Replication Stress Pathway Genes
8.0
7.5
7.0
6.5
6.0
Pairwise T-Test Adjusted P-Value (BH)
HSC
preB
Myeloid
MLL-AF4
0.25
0.25
0.65
0.27
7.7e-12
8.5e-16
HSC
preB
Myeloid
MLL-AF4

Figure S6

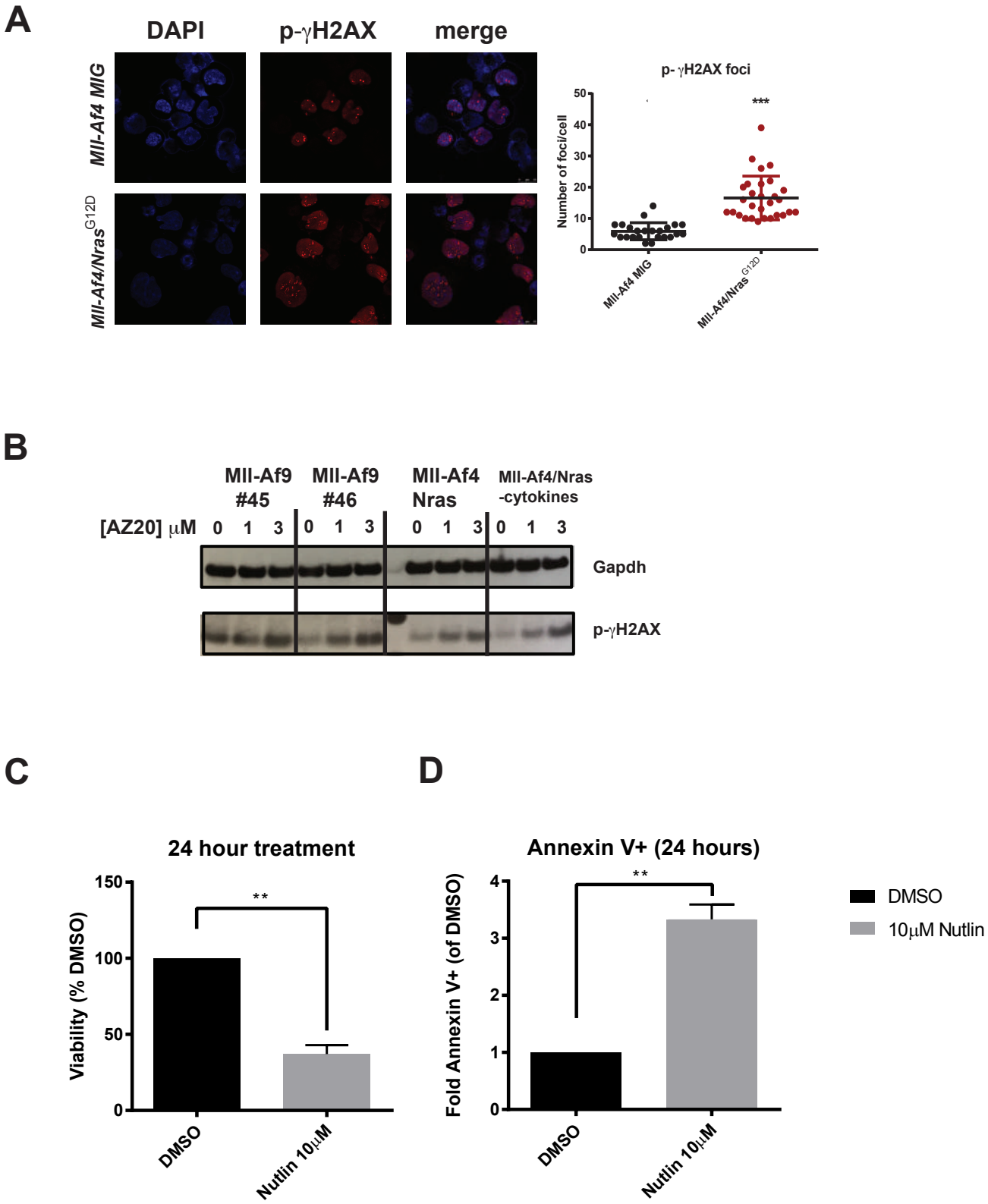

A
DAPI
p-γH2AX
merge
MII-Af4 MIG
MII-Af4/NrasG12D
p- γH2AX foci
Number of foci/cell
***
MII-Af4 MIG
MII-Af4/NrasG12D
B
MII-Af9 #45
MII-Af9 #46
MII-Af4 Nras
MII-Af4/Nras -cytokines
[AZ20] μM 0 1 3 0 1 3 0 1 3 0 1 3
Gapdh
p-γH2AX
C
24 hour treatment
Viability (% DMSO)
**
DMSO
Nutlin 10μM
D
Annexin V+ (24 hours)
Fold Annexin V+ (of DMSO)
**
DMSO
Nutlin 10μM
DMSO
10μM Nutlin

Figure S7

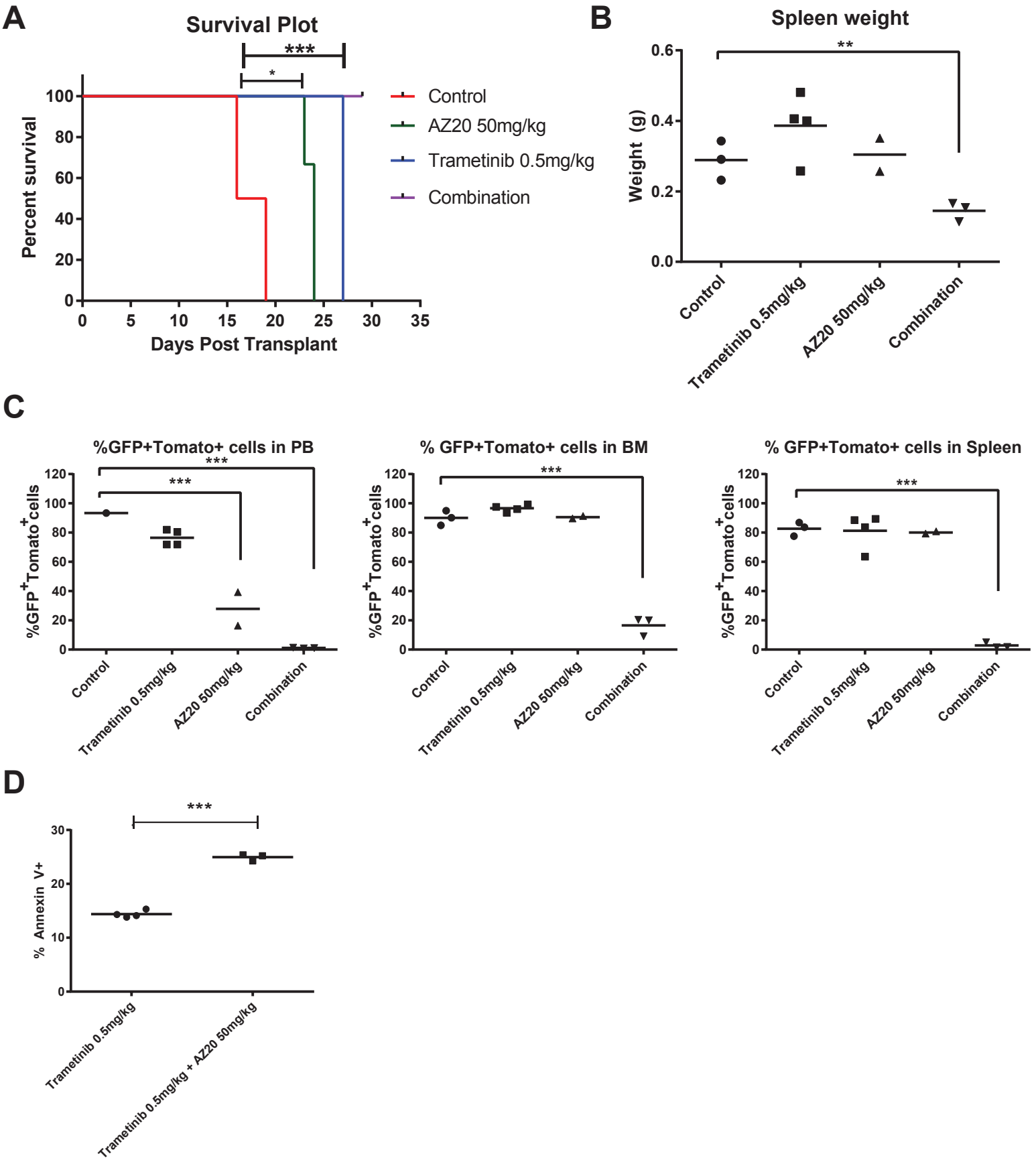

A
Survival Plot
***
*
Percent survival
Days Post Transplant
Control
AZ20 50mg/kg
Trametinib 0.5mg/kg
Combination
B
Spleen weight
**
Weight (g)
Control
Trametinib 0.5mg/kg
AZ20 50mg/kg
Combination
C
%GFP+Tomato+ cells in PB
% GFP+Tomato+ cells in BM
% GFP+Tomato+ cells in Spleen
%GFP+Tomato+ cells
D
% Annexin V+
Trametinib 0.5mg/kg
Trametinib 0.5mg/kg + AZ20 50mg/kg

Figure S8

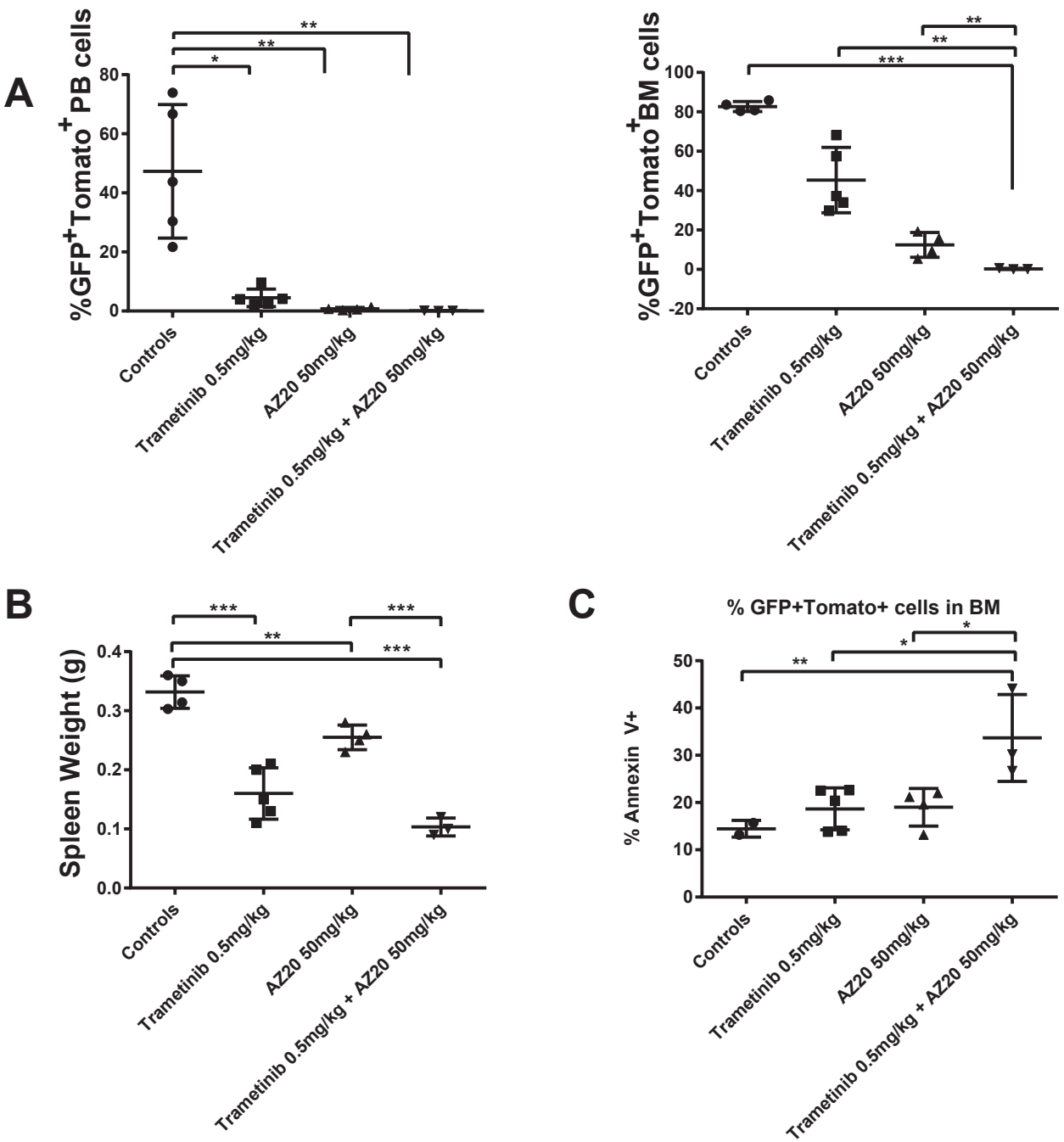
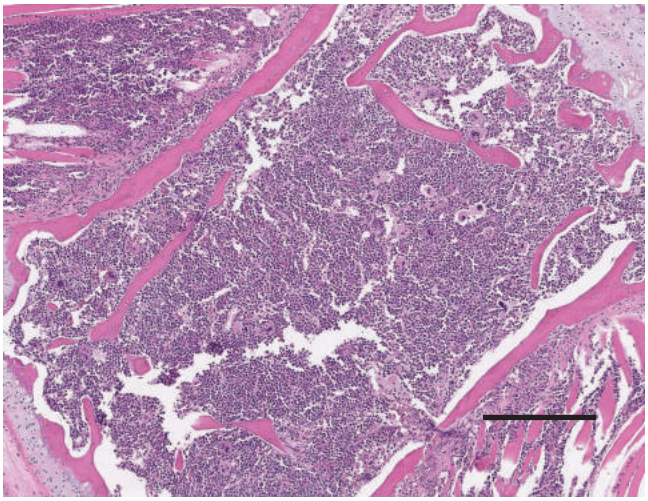

A
%GFP+Tomato+PB cells
%GFP+Tomato+BM cells
B
Spleen Weight (g)
C
% GFP+Tomato+ cells in BM
% Annexin V+
Controls
Trametinib 0.5mg/kg
AZ20 50mg/kg
Trametinib 0.5mg/kg + AZ20 50mg/kg

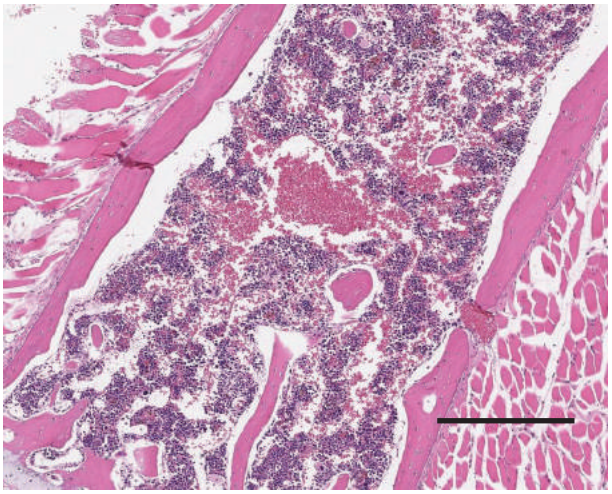
Figure S9

A

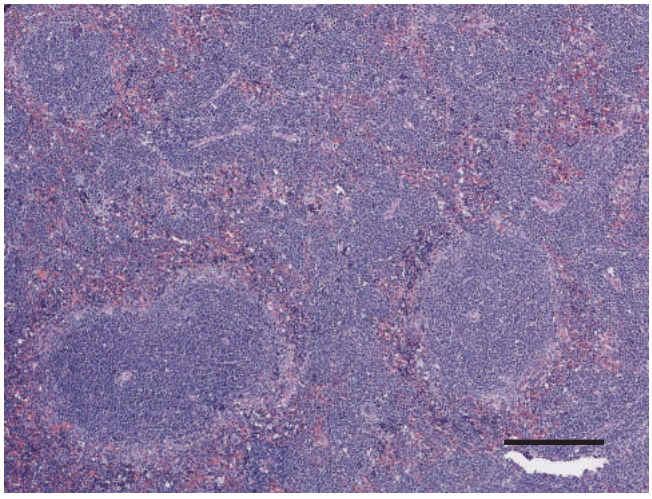
Vehicle



Combination
(AZ20+GSK212)



B



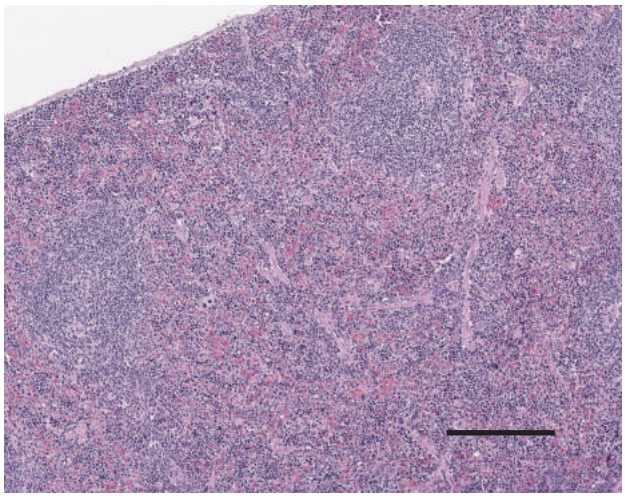
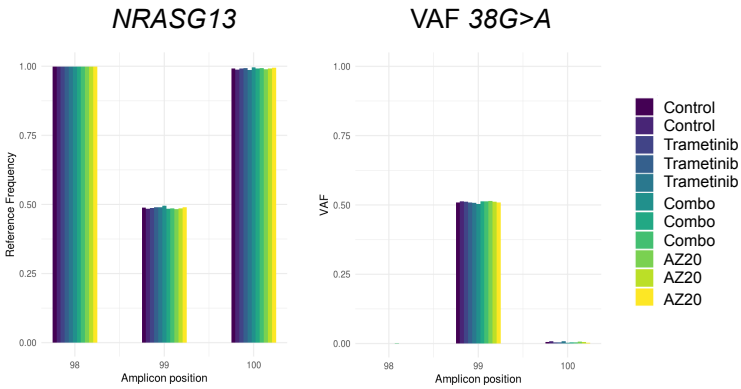
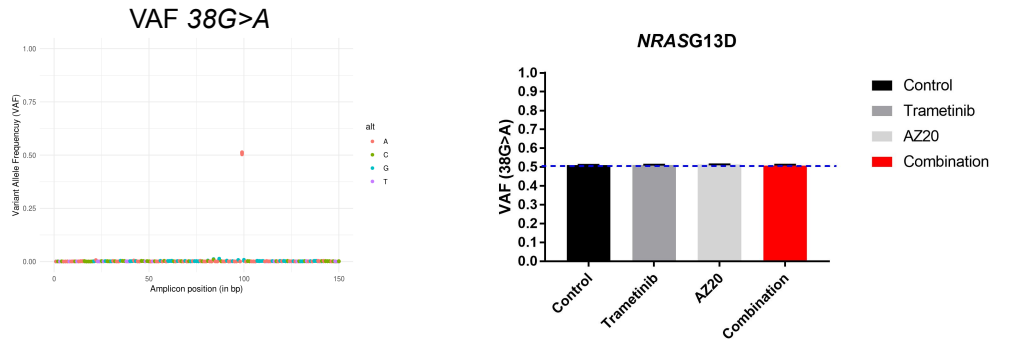


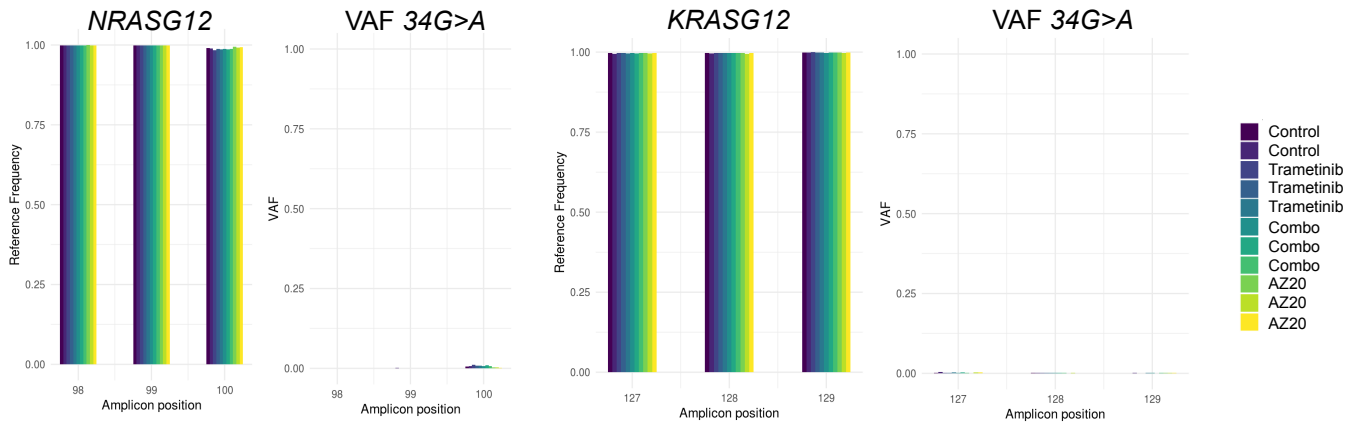
Figure S10

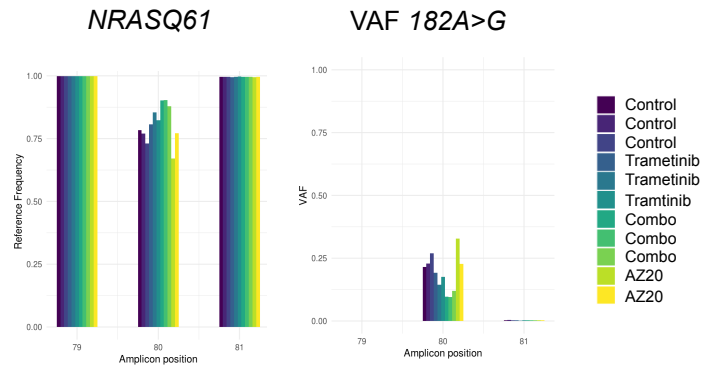
A PDX #83





B PDX #24





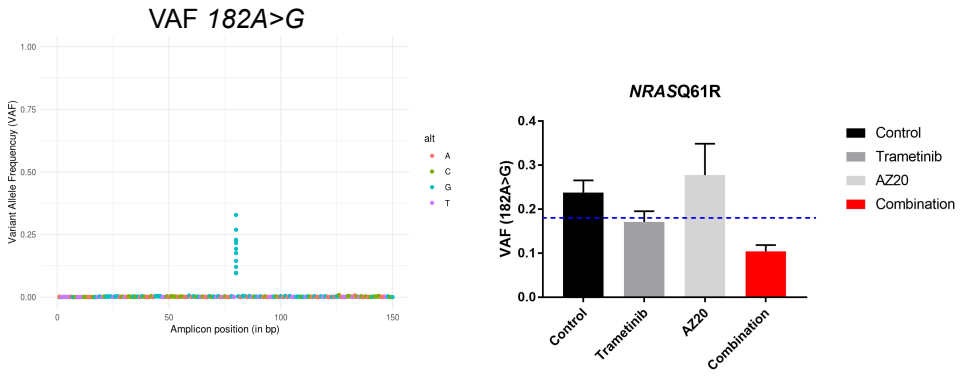


Figure S11

Sample Name	Sample ID	Source	Disease	Gene	Protein Var	nuc var	VAF	coverage	location	type of variant	Gene1, RE	Gene2, RE
042.D1	#83	PT	B-ALL	NRAS	G13D	38G>A	33	505	chr1:115258744	SNV		
				MLL1						RE	MLL1	AF4
042.D1.m88BM	#83	PDX	B-ALL	NRAS	G13D	38G>A	52	394	chr1:115258744	SNV		
				MLL1						RE	MLL1	AF4
072.D1	#24	PT	B-ALL	KRAS	G12S	34G>A	2	723	chr12:25398285	SNV		
				NRAS	G12S	34G>A	17	650	chr1:115258748	SNV		
				NRAS	Q61R	182A>G	1	668	chr1:115256529	SNV		
				MLL1						RE	MLL1	AF4
072.D1.CD3-.m189BM	#24	PDX	B-ALL	NRAS	Q61R	182A>G	18	428	chr1:115256529	SNV		
				NRAS	G12S	34G>A	2	423	chr1:115258748	SNV		
				MLL1						RE	MLL1	AF4

Table S1