RXRA DT448/9PP generates a dominant active variant capable of inducing maturation in acute myeloid leukemia cells

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Supplemental methods

Reagents and constructs. Bexarotene was from LC Laboratories. Anti-RXRA antibody (H-10) and GAPDH Antibody (FL-335) were from Santa Cruz Biotechnology, anti-RXRA antibody (5388) was from CellSignaling. HX531, UVI3003, GSK2033, GW6471, T0070907 and BMS493 were from Tocris. The VP16-RXRA construct was a gift from Mitchell Lazar, University of Pennsylvania. The pBABE-RXRA, PPRE-Luciferase and RARE-Luciferase plasmids were gifts from Vivek Arora, Washington University. The ApoA1-Luciferase plasmid was a gift from Christopher Glass, University of California at San Diego. *MSCV-RXRA* DT448/9PP-*IRES-mCherry*, *MSCV-RXRA*-*IRES-mCherry, MSCV*-3x*Flag*-Gal4-*RXRA* DT448/9PP LBD-*IRES-mCherry,* MSCV-3x*Flag*-Gal4-*RXRA LBD-IRES-mCherry, MSCV-Flag-RXRA* R316A/L326A/DT448/9PP-*IRES-mCherry and MSCV-RXRA S427F-IRES-mCherry* retroviruses were generated in our laboratory. The *Flag-RXRA* R316A/L326A/DT448/9PP-*IRES-mCherry* contained a Flag tag simply as a matter of cloning history. PGC1α and PGC1α L2/3A were from Addgene. Cytokines were purchased from R&D Systems. Methylcellulose complete media was purchased from R&D. The following antibodies were used for flow cytometry: CD11b (BD Biosciences, Clone M1/70), CD14 (eBioscience, Clone Sa2-8), CD64 (BD Biosciences, Clone X54-5/7.1), CD115 (BioLegend, Clone AFS98), F4/80 (eBioscience, Clone BM8), Gr1 (eBioscience, Clone RB6-8C5), and Ly6C (BD Biosciences, Clone AL-21). Fluorescence was detected on a ZE5 Cell Analyzer (Biorad).

Colony forming and cell proliferation analysis. Colony forming was evaluated in methylcellulose with complete cytokines (R&D Systems HSC007). 3,000 cells were plated in replicate in 1.1 mls and colonies assessed after 7 days. Cell proliferation was assessed using a CFSE based method using the FxCycle-Violet (Biolabs) dye. The Invitrogen CellTrace CFSE kit is used to monitor distinct generations of proliferating cells by dye dilution. Live cells are covalently labeled with the violet dye. The proliferation rate was analyzed on ZE5 Flow Cytometer (Biorad)

at the indicated time points and every generation of cells appears as a different peak on a flow cytometry histogram.

Immunoblot. Total protein extracts were lysed in RIPA buffer (Cell Signaling) including 1× cocktails of protease and phosphatase inhibitors (Sigma Aldrich). Gel electrophoresis was performed in a SDS polyacrylamide gel and proteins transferred to a Hybond-P membrane (Millipore). Binding of each antibody was visualized using the ECL detection system (Thermofisher). All images were acquired by myECL Imager (Thermofisher). Detection of endogenous Rxra was possible in KMT2A-MLLT3 leukemia cells using some, but not all commercial antibodies (endogenous Rxra was detected only by 5388 anti-RXRA from CellSignaling which showed a lower intensity of the truncated 36 kDa fragment than what observed with H-10 anti-RXRA from SantaCruz Biotech).

UAS/Gal4 assay. Bone marrow cells from UAS-GFP mice (11) were transduced with retroviruses *MSCV-Gal4* (DNA binding domain, DBD) – *RXRA* (ligand binding domain, LBD) – *IRES* – *mCherry* or *MSCV-Gal4* (DBD) – *RXRA* DT448/9PP (LBD) – *IRES* – *mCherry.* Gal4 is a yeast transcription factor and the UAS sequence is not recognized by mammalian transcription factors. Cells were treated, and after 48 hours, GFP measured by flow cytometry.

Mammalian two-hybrid assay. 293Tcells were co-transfected using Lipofectamine 2000 (Invitrogen) with plasmids encoding the reporter: *UAS-GFP;* “bait”: *Gal4* (DBD)-*PGC1A*; and “prey”: *VP16-RXRA* (LBD) or *VP16-RXRA* DT448/9PP (LBD). The percentage of GFP+ cells and the GFP MFI was assessed 48 hours after transfection by flow cytometry.

Luciferase detection. 293T cells were transfected using Lipofectamine 2000 (Invitrogen). Six hours after transfection, the cells were collected and plated into a 48 well plate in 1% BSA media in biological triplicates and treated with compounds. After 40h incubation, the cells were

harvested and assayed for luciferase (Luc Assay System with Reporter Lysis Buffer, Promega) in a Beckman Coulter LD400 plate reader.

Microscope images. Images were acquired using a Olympus TH4-100 fluorescent microscope and Q-Capture-pro 7 software.

Immunophenotyping. KMT2A-MLLT3 leukemia cells were transduced with *MSCV-RXRA-IRES mCherry* or *MSCV-RXRA* DT448/9PP-*IRES-mCherry* and treated with or without 250 nM bexarotene. Non-transduced KMT2A-MLLT3 cells and RAW 264.7 cells (ATCC) cells provided staining controls. After 72 hours, cells were washed, stained, for 1 hour, washed, and analyzed on a ThermoFisher Attune flow cytometer (Thermo Fisher Scientific) and using FlowJo v10 software (BD Biosciences).

Data analysis. Statistical analysis was performed using Prism (Graphpad). T-test and ANOVA tests were performed, as appropriate. Error bars represent standard deviation. Data points without error bars have standard deviations below Graphpad's limit to display. Crystal structure data from PDB: 4K4J was visualized in Pymol (Schrodinger, Inc).

Supplemental Figure 1. RXRA Activation associated with differentiated immunophenotype. KMT2A-MLLT3 leukemia cells were transduced with *MSCV-RXRA-IRES-mCherry* or *MSCV-RXRA* DT448/9PP-*IRES-mCherry* and cultured for 72 hours with or without 250 nM bexarotene and assessed by flow cytometry. Controls of RAW 264.7 cells were included. Mean fluorescence intensity (MFI) of results from Figure 1G. A. CD115, B. F4/80, C. CD15, D. CD64, E. CD11b, F. Ly6C, and G. Gr1 are shown. N=3 for all samples. Comparison's were done by one-way ANOVA with Tukey's multiple comparison tests, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Supplemental Figure 2. **Plastic adherence induced by *MSCV-RXRA* DT448/9PP-*IRES-mCherry* in AML cell lines**. A, C, and D. Plastic adherence induced by *MSCV-RXRA* DT448/9PP-*IRES-mCherry* in THP1, Monomac-6, and K562 cells at indicated time points. B *MSCV-RXRA-IRES-mCherry* in THP1 cells.

Supplemental Figure 3. Effects of *RXRA* DT448/9PP in Kit+ bone marrow cells. A - B. Kit+ bone marrow cells from BL6 wild type mice were transduced with *MSCV-RXRA-IRES-mCherry* or *MSCV-RXRA* DT448/9PP-*IRES-mCherry* and evaluated by fluorescent microscopy at indicated time points. C. Kit+ bone marrow cells from UAS-GFP mice were transduced with *MSCV-Gal4-RXRA-IRES-mCherry* or *MSCV-Gal4-RXRA* DT448/9PP-*IRES-mCherry,* treated as indicated, and GFP and mCherry assessed after 48 hours in triplicate. T-test, ** $p < 0.01$. NS: not significant.

Supplemental Figure 4. Resistance of *MSCV-RXRA* DT448/9PP-*IRES-mCherry* in KMT2A-MLLT3 leukemia cells to RXRA antagonists. A. KMT2A-MLLT3 leukemia cells were transduced with *MSCV-RXRA* DT448/9PP-*IRES-mCherry* and treated with indicated antagonists for 72 hours and imaged by fluorescent microscopy. B. The experiment was repeated, and cells were treated with FxCycle Violet and assessed for cell divisions at 72 hours in mCherry+ vs. mCherry- cells in triplicate. T-test, *** $p < 0.001$. C. KMT2A-MLLT3 leukemia cells derived from UAS-GFP bone marrow were transduced with retrovirus expressing Gal4-RXRA DT448/9PP, treated as indicated, and GFP assessed 48 hours later. Two-way ANOVA comparison identified significant variance ($p < 0.001$) associated with both drug treatment and mCherry status, although 99% of the variance was associated with mCherry status.

Supplemental Figure 5. Resistance of *MSCV-RXRA* DT448/9PP-*IRES-mCherry* to nuclear receptor antagonists. A - B. KMT2A-MLLT3 leukemia cells were transduced with *MSCV-RXRA* DT448/9PP-*IRES-mCherry* and treated with indicated antagonists for 48 or 72 hours and imaged by fluorescent microscopy. C-D. The experiment was repeated, and cells were treated with FxCycle Violet and assessed for cell divisions at 72 hours in mCherry+ vs. mCherry- cells in triplicate. T-test, *** $p < 0.001$. Two-way ANOVA comparison identified significant variance ($p < 0.001$) associated with both drug treatment and mCherry status, although 95% of the variance was associated with mCherry status in panel C, and 99% in panel D. GSK2033: LXR antagonist. GW6471: PPARA antagonist. T0070907: PPARG antagonist. BMS493: RAR antagonist.

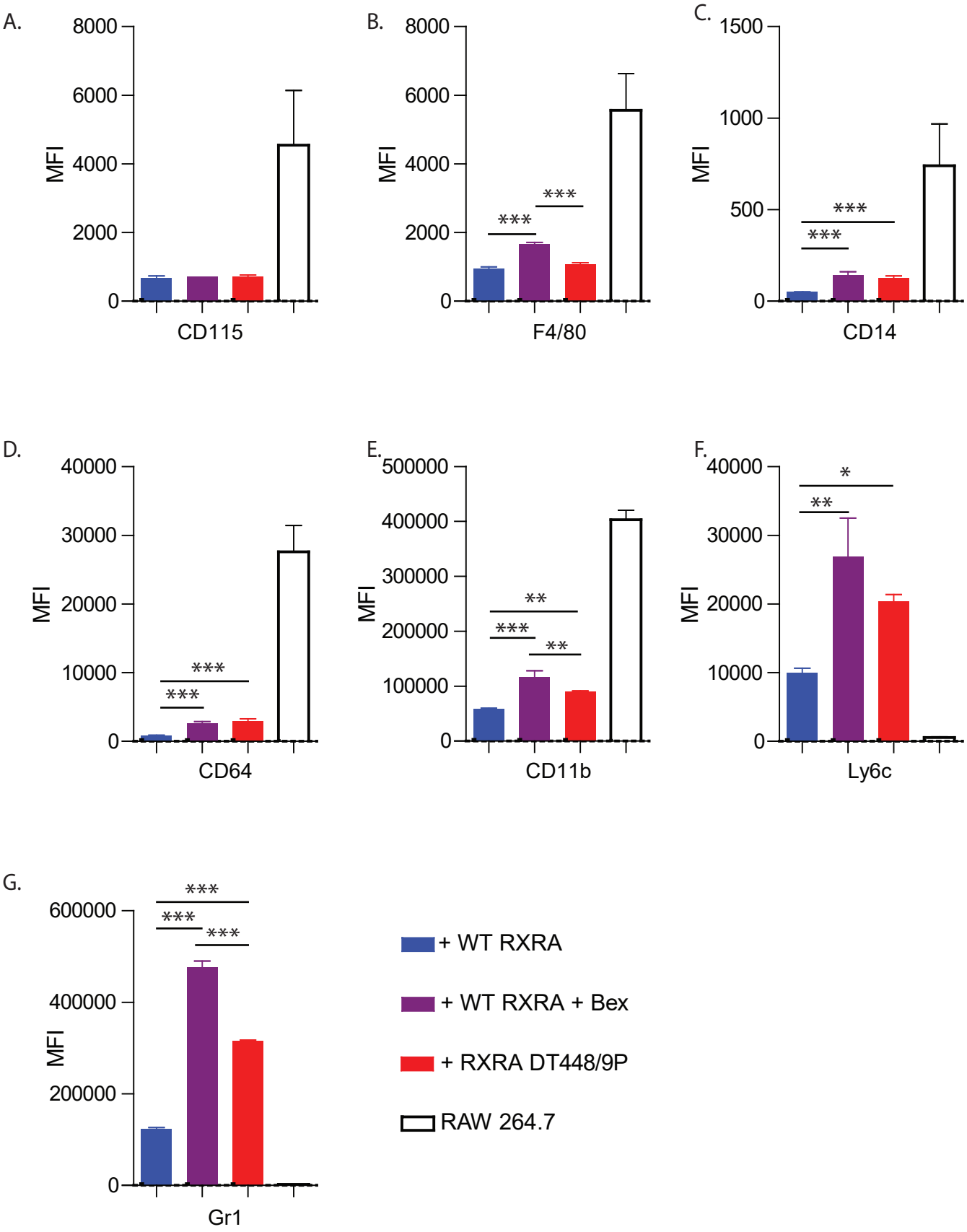
Supplemental Figure 6. Spleen weight of RXRA WT vs RXRA DT448/9PP. A. Spleen weight (g) of moribund KMT2A-MLLT3 leukemia mice (transformed with *MSCV-RXRA-IRES-mCherry* or *MSCV-RXRA* DT448/9PP-*IRES-mCherry)* upon their sacrifice.

Supplemental Figure 7. RXRA activation leads to upregulation of cell surface markers of the complement system and integrin family. KMT2A-MLLT3 cells were treated as shown in Figure 5A and assessed by RNA Seq. A. Heatmap of the DEGs associated with cell surface proteins, 67 genes total. B and C. 27 of the cell surface DEGs had increased expression from WT+Bex to DT448/9PP. D-F. 40 of the cell surface DEGs had equivalent or decreased expression from WT + Bex to DT448/9PP. For B-F, transcripts are separated into high, medium and low expression to allow better visualization and expression levels are shown in transcripts per million (TPM).

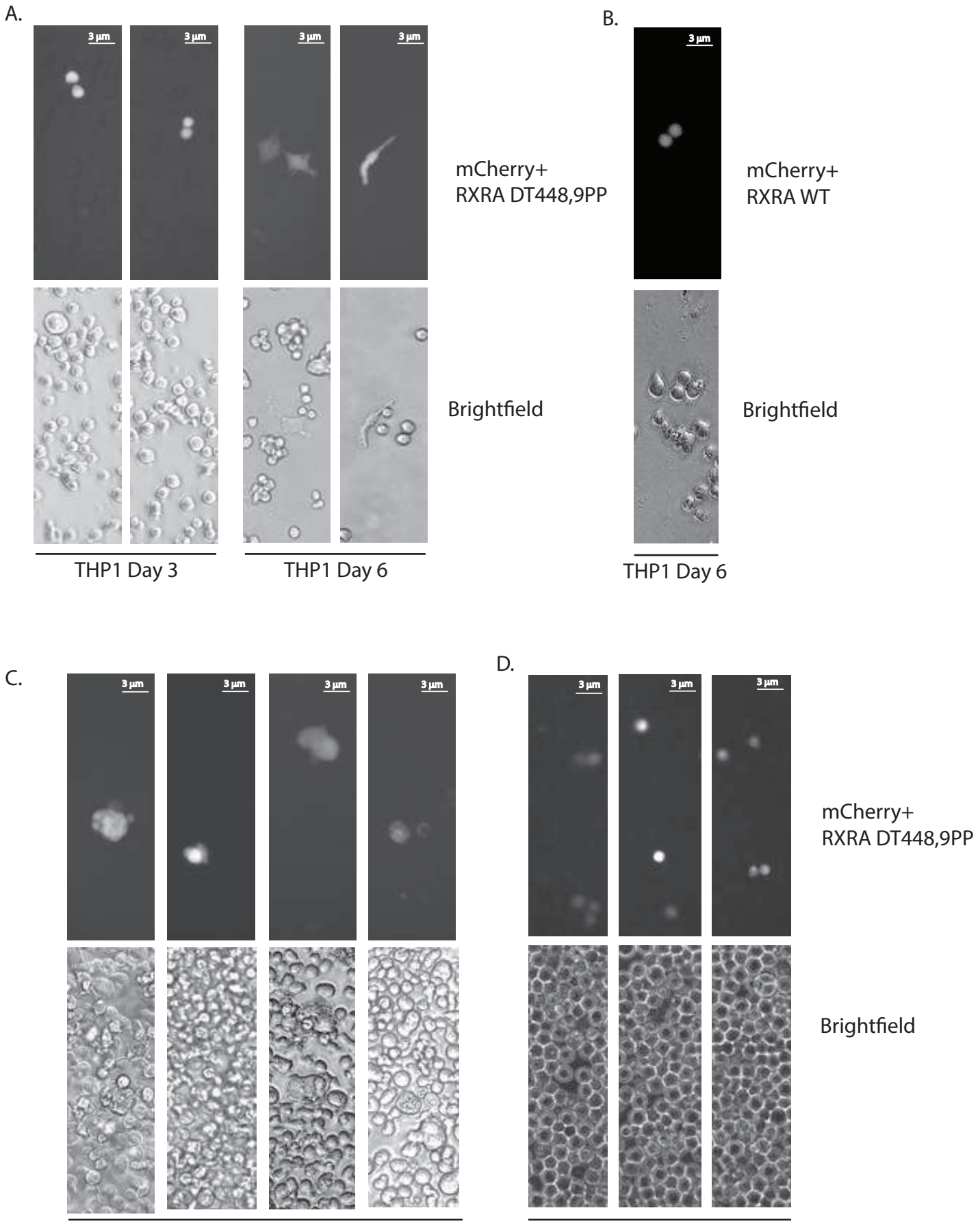
Supplemental Figure 8. Heatmap comparing RXRA WT + bexarotene vs. RXRA DT448/9PP A. Heatmap with 11 DEGs comparing RXRA WT + bexarotene vs. RXRA DT448/9PP. B-C. Among the 11 DEGs identified between WT+Bex and DT448/9PP, 5 show an enhancement of the canonical response to RXRA ligand (4 genes upregulated, 1 gene downregulated) and 6 show a non-canonical response (5 upregulated with DT448/9PP, 1 downregulated with DT448/9PP). Heatmaps are shown as row z-scores with color key below panel A and expression levels in B and C are shown in transcripts per million.

Supplemental Table 1. Gene Ontology Pathways Enriched in the Genes Upregulated in WT + Bex and DT448/9PP Cells. Upregulated genes were analyzed using Panther GO Analysis software. Shown are pathways with fold enrichment >/= 2 and p-value <0.05.

Supplemental Table 2. Gene Ontology Pathways Enriched in the Genes Downregulated in WT + Bex and DT448/9PP Cells. Downregulated genes were analyzed using Panther GO Analysis software. Shown are pathways with fold enrichment >/= 2 and p-value <0.05.

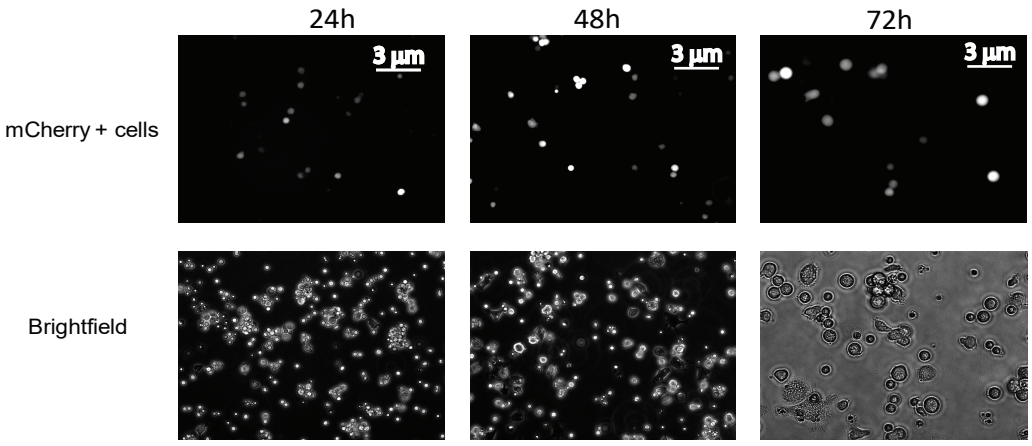


Supplemental Figure 1



Supplemental Figure 2.

A.



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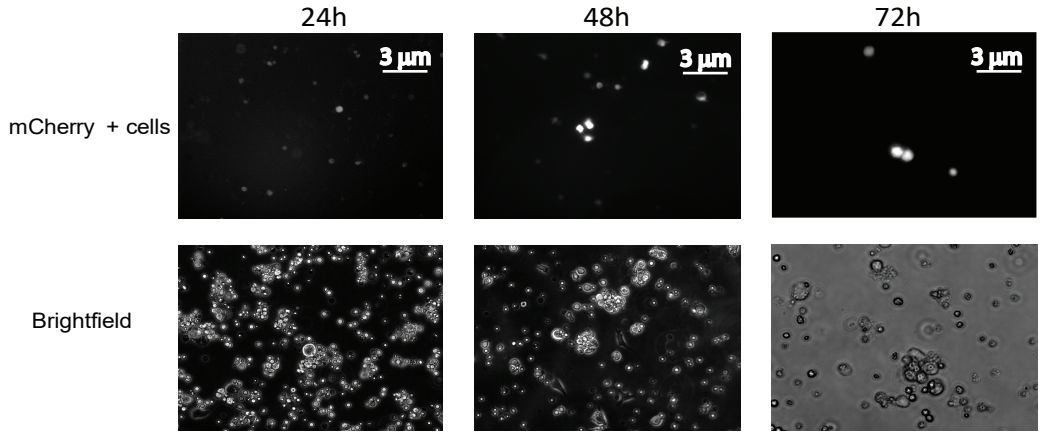

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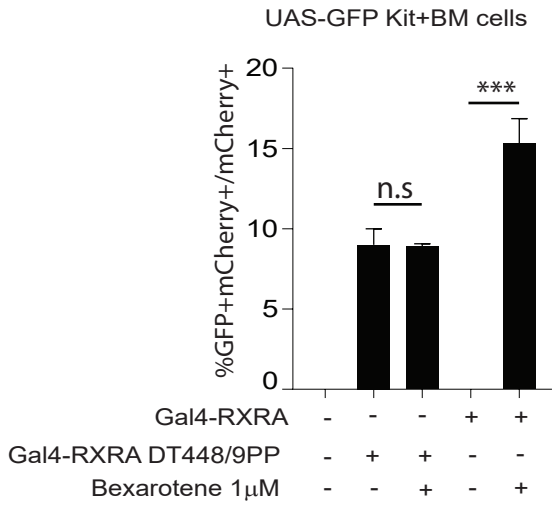

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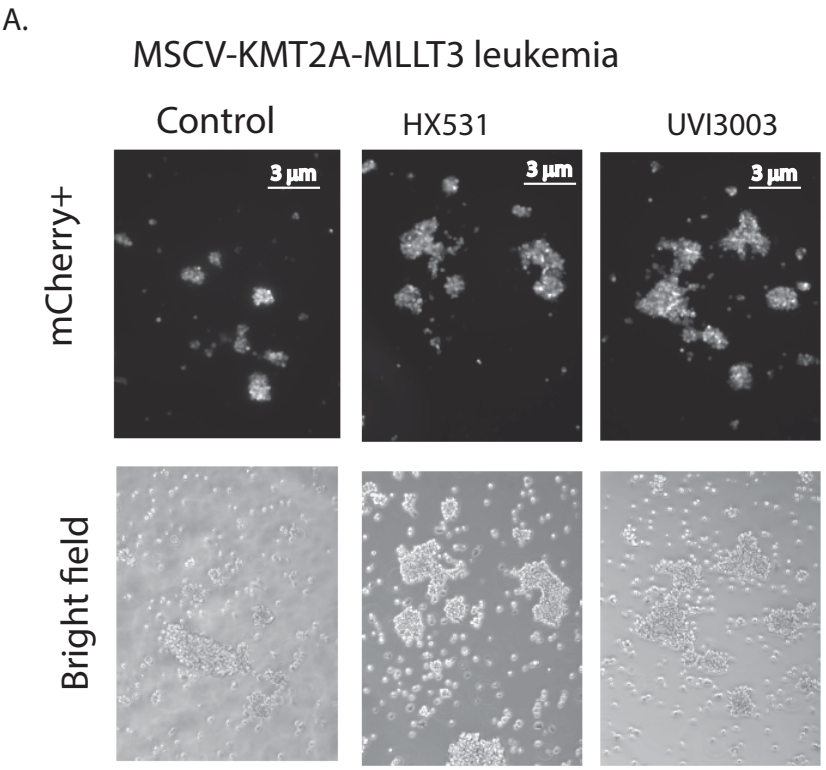
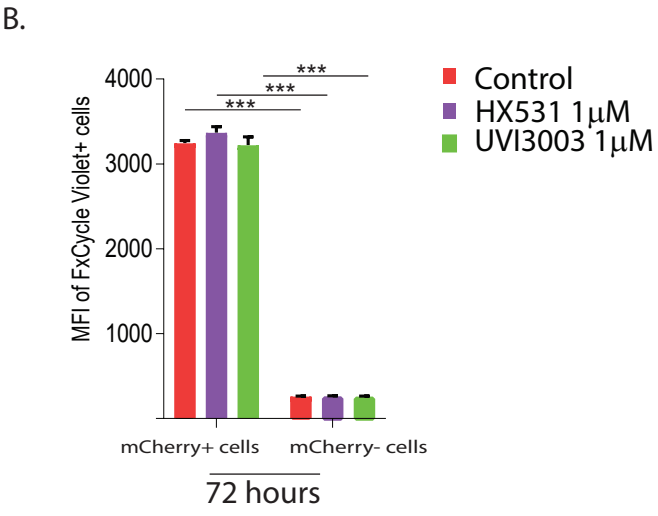
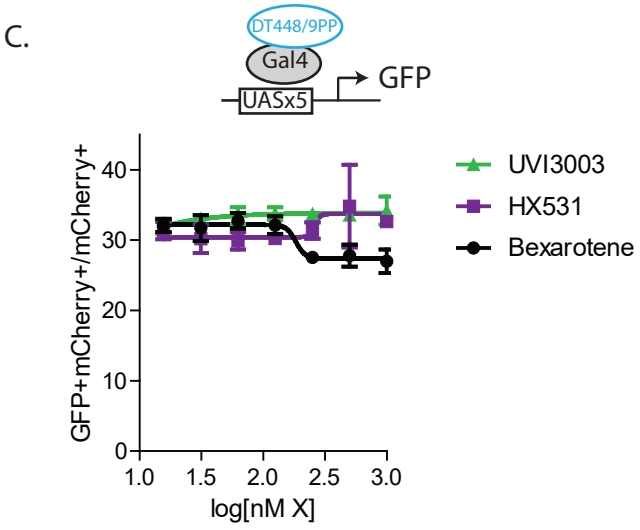
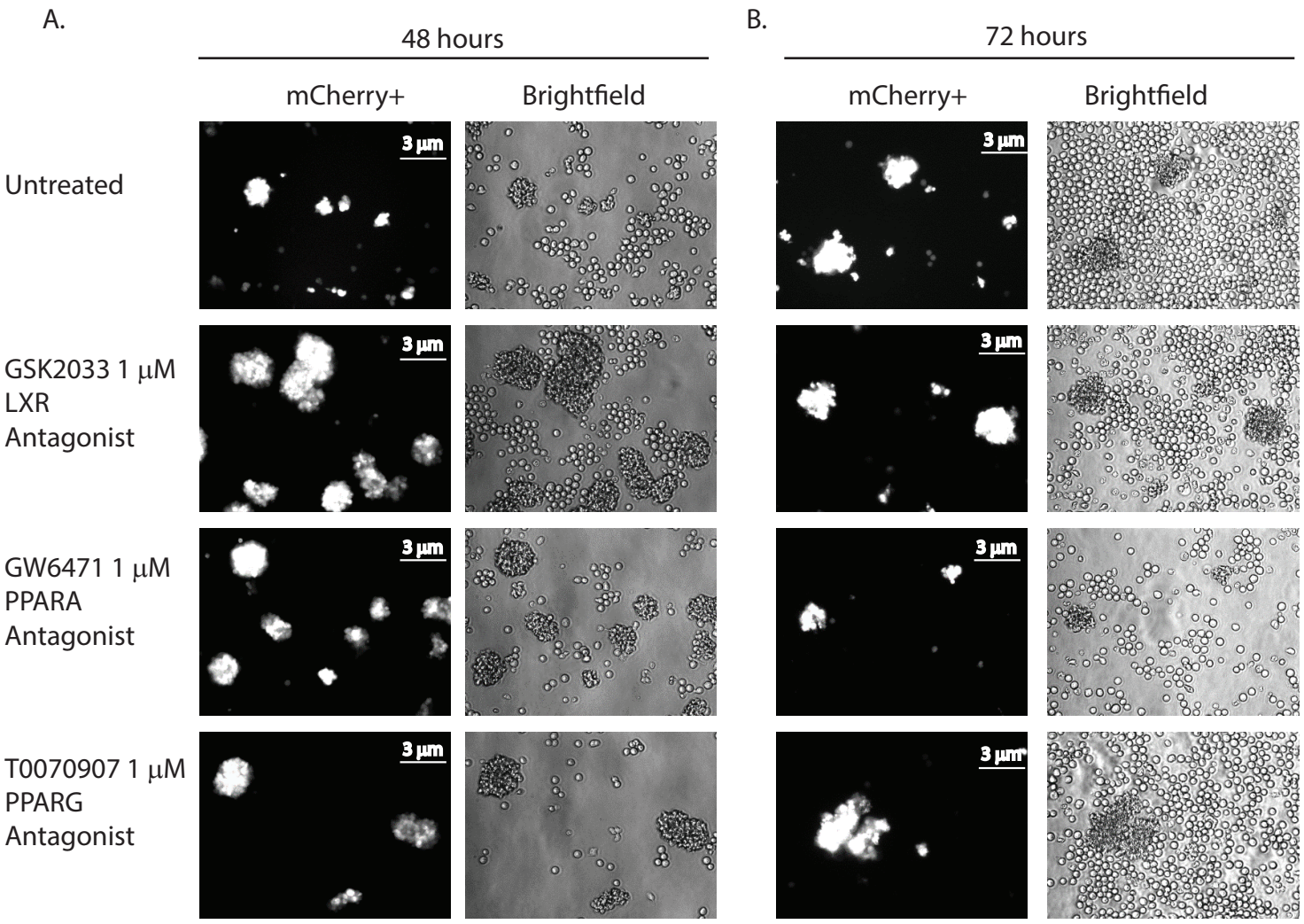
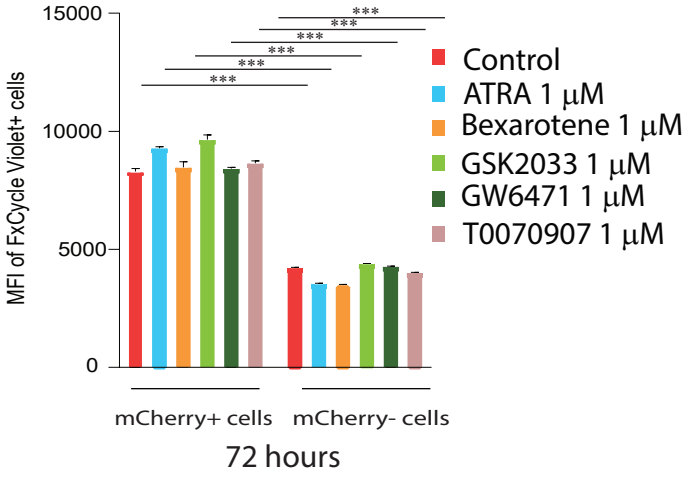


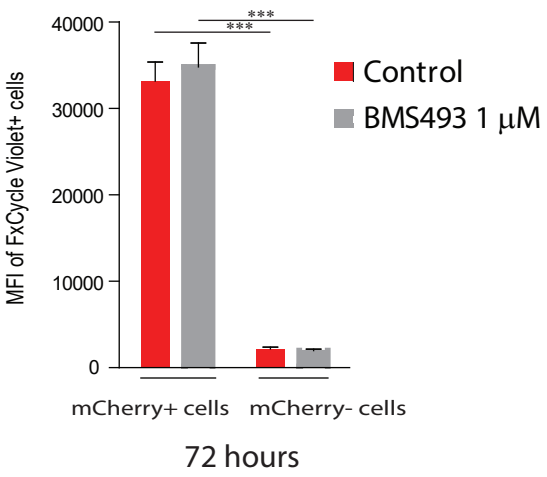




Supplemental Figure 4.







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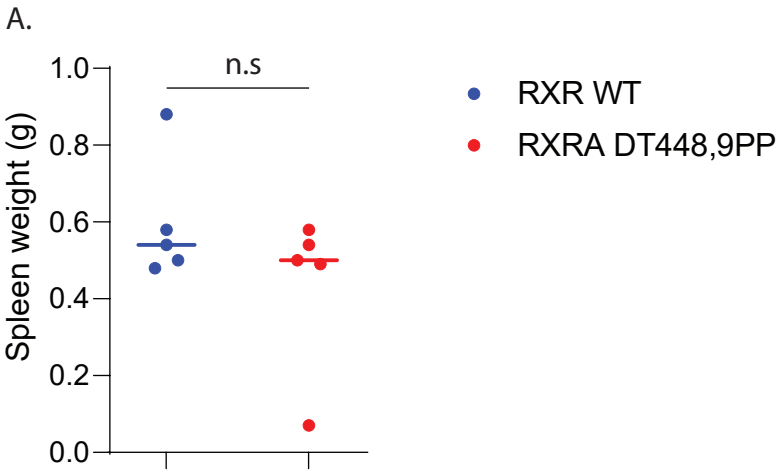

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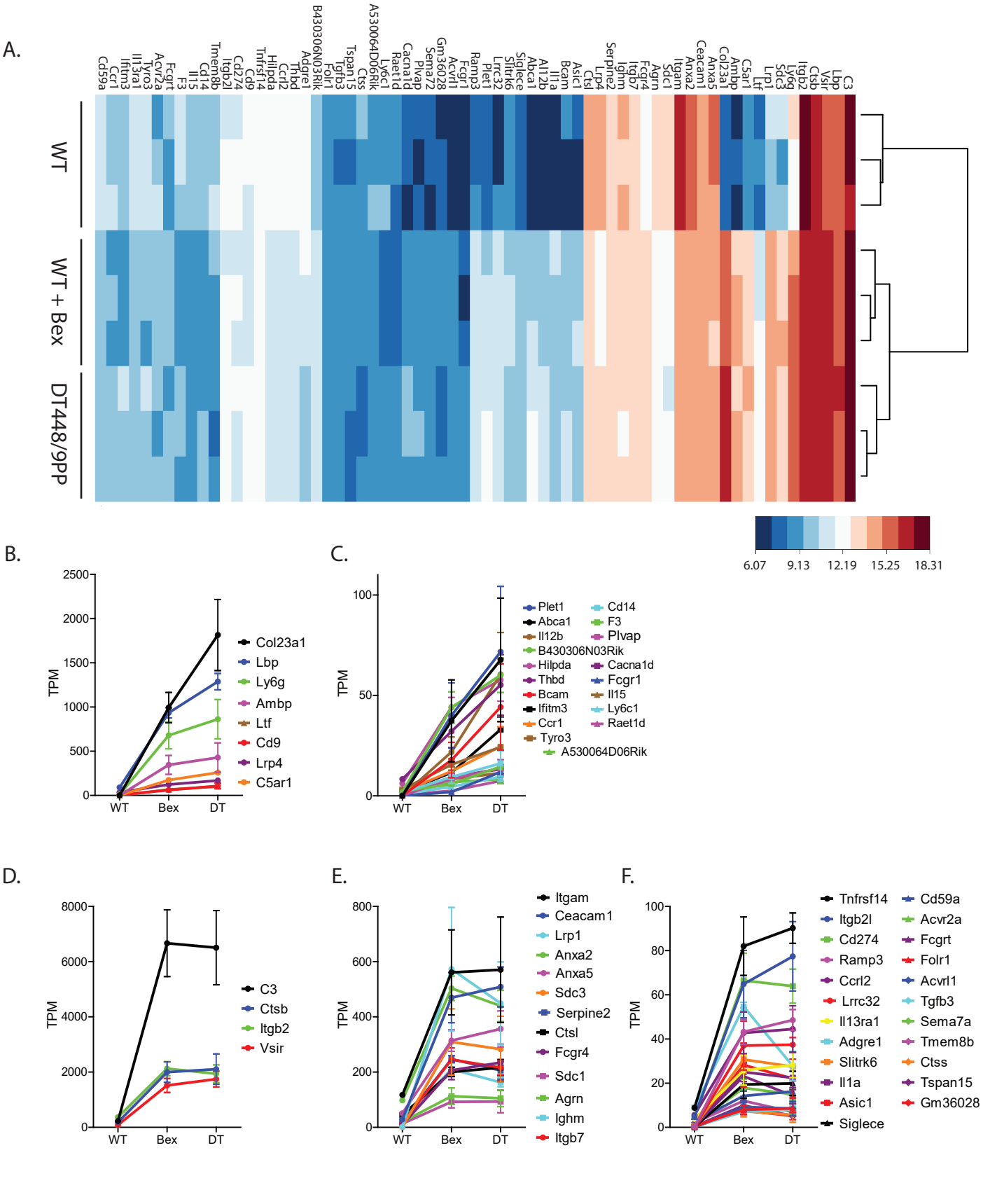
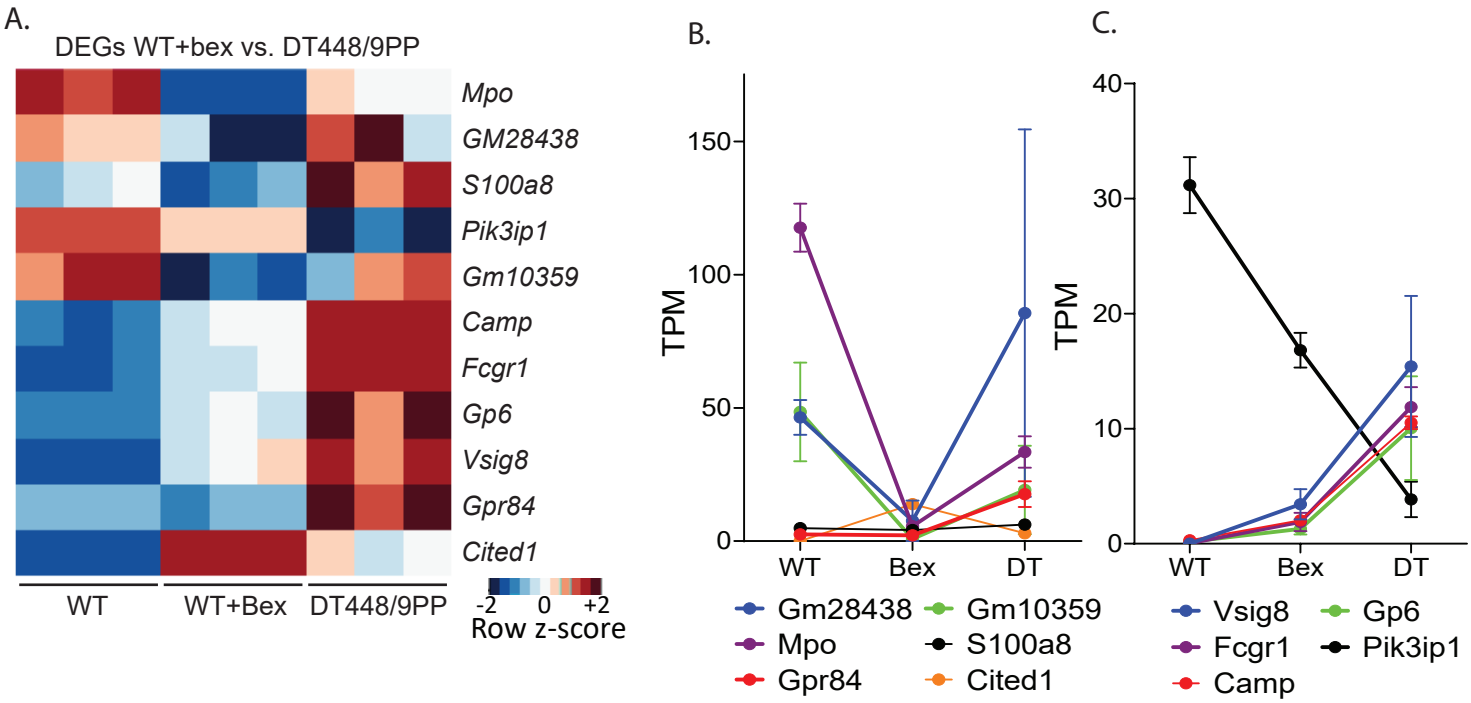


Supplemental Figure 7.



Supplemental figure 8

GO biological process complete	Number of Genes in Pathway	# of Pathway Genes in DEG List	Expected	Fold Enrichment	P-value
positive regulation of multicellular organismal process (GO:0051240)	1564	65	29.38	2.21	3.62E-05
inflammatory response (GO:0006954)	480	30	9.02	3.33	2.44E-04
regulation of protein secretion (GO:0050708)	362	25	6.8	3.68	6.14E-04
negative regulation of cytokine production (GO:0001818)	263	21	4.94	4.25	7.38E-04
negative regulation of immune system process (GO:0002683)	410	26	7.7	3.38	1.62E-03
cellular lipid metabolic process (GO:0044255)	848	40	15.93	2.51	2.26E-03
response to cytokine (GO:0034097)	756	37	14.2	2.61	2.29E-03
response to oxygen-containing compound (GO:1901700)	1307	52	24.55	2.12	5.48E-03
positive regulation of signal transduction (GO:0009967)	1492	56	28.02	2	1.05E-02
regulation of response to external stimulus (GO:0032101)	901	40	16.92	2.36	1.10E-02
negative regulation of cell communication (GO:0010648)	1314	51	24.68	2.07	1.71E-02
regulation of multicellular organismal development (GO:2000026)	1435	54	26.95	2	1.72E-02
negative regulation of signaling (GO:0023057)	1318	51	24.76	2.06	1.77E-02
regulation of cell migration (GO:0030334)	933	40	17.52	2.28	2.00E-02

GO biological process complete	Number of Genes in Pathway	# of Pathway Genes in DEG List	Expected	Fold Enrichment	P-value
rRNA processing (GO:0006364)	201	17	1.17	14.53	8.80E-11
RNA modification (GO:0009451)	141	9	0.82	10.96	2.06E-03
tRNA metabolic process (GO:0006399)	168	9	0.98	9.2	8.32E-03
regulation of rRNA processing (GO:2000232)	14	4	0.08	49.08	2.76E-02
cellular nitrogen compound biosynthetic process (GO:0044271)	1075	20	6.26	3.2	4.35E-02