SUPPLEMENTAL METHODS

Cell culture

RMF and cancer associated fibroblast (CAF9 and CAF7J) were generously provided by Dr. Melissa Troester (UNC-CH, USA). RMF was maintained in RPMI (Cellgro, Manassas, VA) with 10%FBS and 100 units/mL penicillin/streptomycin. CAF9 and CAF7J cells were isolated from luminal and basal breast tumors respectively and were maintained in DMEM/F12 (Cellgro) with 10%FBS and 100 units/mL penicillin/streptomycin.

Western blot

Cells were washed twice with PBS and harvested in RIPA lysis buffer. Protein concentration was determined with Bio-Rad protein assay and 20 µg of whole cell lysates were used for SDS-PAGE. Membranes were blocked with 5% milk or BSA diluted in 1X TBST for 1 h and incubated at 4°C overnight with the following primary antibodies: Anti-SV40 T Antigen (Calbiochem, DP02, 1:200), EpoR (M-20, Santa Cruz, sc-697), p-ERK (T202/Y204, Cell signaling, cat # 9106), ERK (Cell signaling, cat # 9102), Ku80 (GeneTex, cat # GTX70485).

Immunohistochemisty, Ki67 and MVD quantitation

Upon necropsy tumors were excised, formalin fixed and paraffin embedded. Tumors were sectioned (5 µm) and slides were stained for either CD31 (Abcam, Cat # ab28364, 1:100) or Ki67 (BD Pharmingen, Cat# 550609, 1:50) using an automated

staining system (Leica Bondmax). All staining was performed by the UNC translational pathology laboratory core facility. Microvessel density was determined by averaging the number of CD31+ vessels per 20x field over 10 random, non-adjacent fields. Ki67 positive nuclei were quantitated in an automated fashion. Data was analyzed by unpaired student's t-test in GraphPad Prism 4.0 software (GraphPad Software Inc.)

Gene Set Enrichment Analysis

The datasets (GSE18229 and 26338) were log2 transformed and median centered. EPO levels were rank ordered and divided into tertiles. GSEA was performed using JAK/STAT associated gene lists (Figure 5B) comparing the top 2 tertiles (EPO high) and the bottom tertile (EPO low) (1, 2).

SUPPLEMENTAL REFERENCES

1. Subramanian A et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. *Proc Natl Acad Sci USA*. 2005;102(43):15545–15550.

2. Mootha VK et al. PGC-1alpha-responsive genes involved in oxidative phosphorylation are coordinately downregulated in human diabetes. *Nat Genet*. 2003;34(3):267–273.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. Exogenous EPO does not increase JAK/STAT signaling in EPOR expressing breast cancer cell lines. (A) Indicated breast cancer cell lines were immunoblotted with the anti-EPOR antibody (M-20). **(B)** Indicated cell lines were cultured in serum free media overnight and treated with EPO (1IU/ml or 10IU/ml) or PBS for 15 min. Cell lysis were immunoblotted with the indicated antibodies.

Supplemental Figure 2. CBC and tumor free survival of breast cancer GEMMs. (A) C(3)Tag mice treated with PBS or EPO for four weeks were bled and complete blood counts (CBC) were performed showing that EPO does not cause significant polycythemia. **(B)** Kaplan Meir tumor free survival curves of MMTV-Neu mice. Mice were randomized to EPO (500 IU/kg IP twice per week) or saline injections and followed for tumor development. **(C)** Kaplan Meir tumor free survival curves of C(3)Tag mice.

Supplemental Figure 3. EPO does not alter proliferation or microvessel density of GEMM tumors. Tumors from orthotopic *MMTV-Neu or C(3)Tag* mice were formalin fixed and paraffin embedded. Sections were stained with antibodies specific to **(A)** Ki67 or **(B)** CD31. Random high powered fields were quantified for percent of **(A)** Ki67 positive cells or **(B)** Number of microvessels per high powered field by a blinded reviewer.

Supplemental Figure 4. Validation of GEMM-derived cell lines. (A) Cell lysates from

the tumor derived C(3)Tag cell line were immunoblotted for SV40 large T antigen. **(B)** C(3)Tag–luc and NT2 cells were immunoblotted for EpoR. 293T cell transfected with pcDNA3-EpoR plasmid was used as a positive control.

Supplemental Figure 5. Orthotopically injected C(3)Tag cells are responsive to EPO. (A) Tumor volumes calculated from the caliper measurements of orthotopically injected C(3)Tag cells into mice randomized to receive PBS or EPO injections. **(B)** The percent change in tumor volume of orthotopically injected tumors of C(3)Tag mice was calculated based on the change in tumor volume from the time of detection until 14 days later. **(C)** Kaplan Meir curves of percent living of mice with C(3)Tag orthotopic tumors treated with PBS or EPO. (* $p \leq 0.05$)

Supplemental Figure 6. Sample FACS plots of enrichment for SUM149 TICs. SUM149 cells were stained with the indicated antibodies and sorted for distinct populations.

Supplemental Figure 7. Sample FACS plots of TIC evaluation for *MMTV-Wnt1* orthotopic tumors. (A) *MMTV-Wnt1* cells were stained with the indicated antibodies and gated for distinct populations. **(B)** Sample FACS plots of the final TICs populations from MMTV-Wnt1 orthotopic tumors treated with PBS or EPO.

Supplemental Figure 8. SUM149 TICs have higher level of EPO-R than nonTICs. SUM149 cells were stained with antibodies to isolate TICs and non-TIC fractions as well as the anti-EPO-R antibody (M-20:1:200). EPO-R expression was analyzed with the secondary antibody (Dylight 405 Donkey anti-rabbit IgG, Biolegend, Cat # 406409) by gating on both TICs (red rectangel) and nonTICs (blue rectangle).

Supplemental Figure 9. pERK induction by EPO is abrogated by EPO and EPO-R neutralizing antibodies. SKBR3 cells were serum starved overnight followed by pre-treament for 1 hr with HA, EPO or EPO-R antibodies as indicated. The cells were then treated with either DMSO or EPO (1 IU/mL) for 15 min. Whole cell lysates were then immunoblotted with the indicated antibodies.

Supplemental Figure 10. Primary breast tumors with high EPO gene expression demonstrate enrichment for a STAT5 gene signature. Gene Set Enrichment Analysis was performed on patient samples from two independent datasets (GSE18229 and GSE26338) comparing groups with high and low EPO mRNA expression (as defined in Figure 6A). The EPO high group in both datasets were significantly enriched for STAT5 related gene expression.

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Supplemental Table 1. Table of antibodies used for breast TIC FACS.

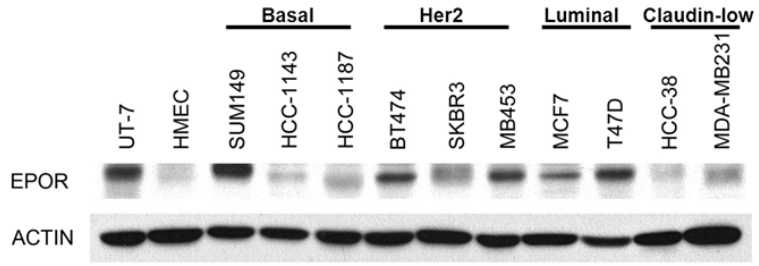
Table 1

Fluorescence Conjugated Antibodies for Flow Analysis of Breast Cancer Stem Cells

Antibodies	Clone	Supplier
For flow analysis of cancer stem cells in MMTV-Wnt-1 murine breast tumors		
CD24-PE	30-F1	eBioscience
Thy1.1-APC	HIS51	eBioscience
CD45-PE-Cy5	30-F11	BD Pharmingen
CD31 (PECAM-1)-FITC	390	BD Pharmingen
CD24-PE	30-F1	eBioscience
For flow analysis of tumor initiating cells in human breast cancer cells		
CD44-APC	G44-26	BD Pharmingen
CD24-PE	ML5	BD Pharmingen
EpCAM-FITC	VU-1D9	Stem Cell Technologies

Supplemental Figure 1.

A



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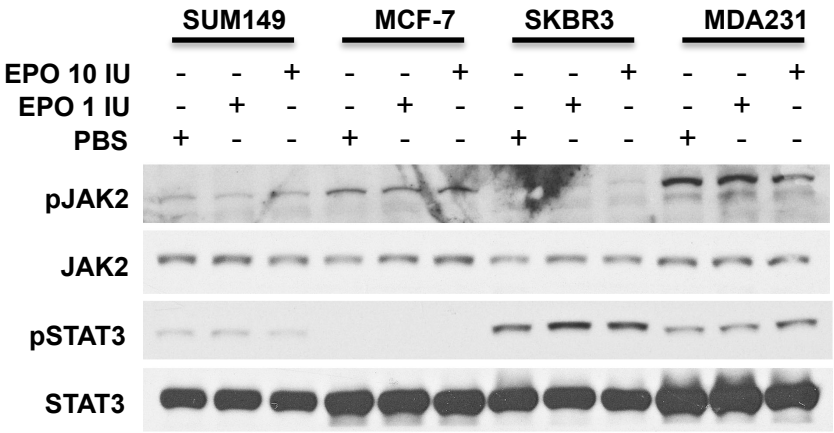

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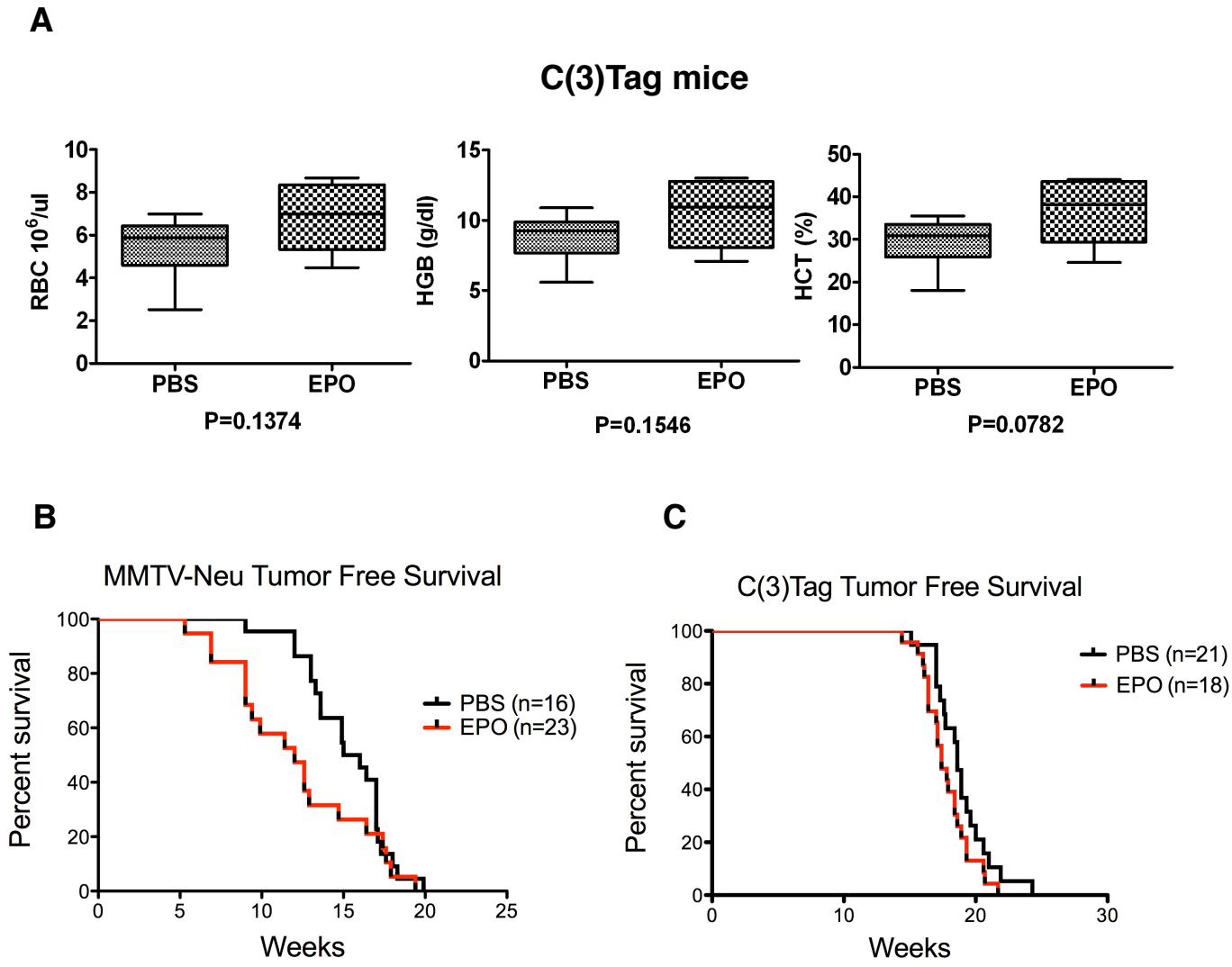

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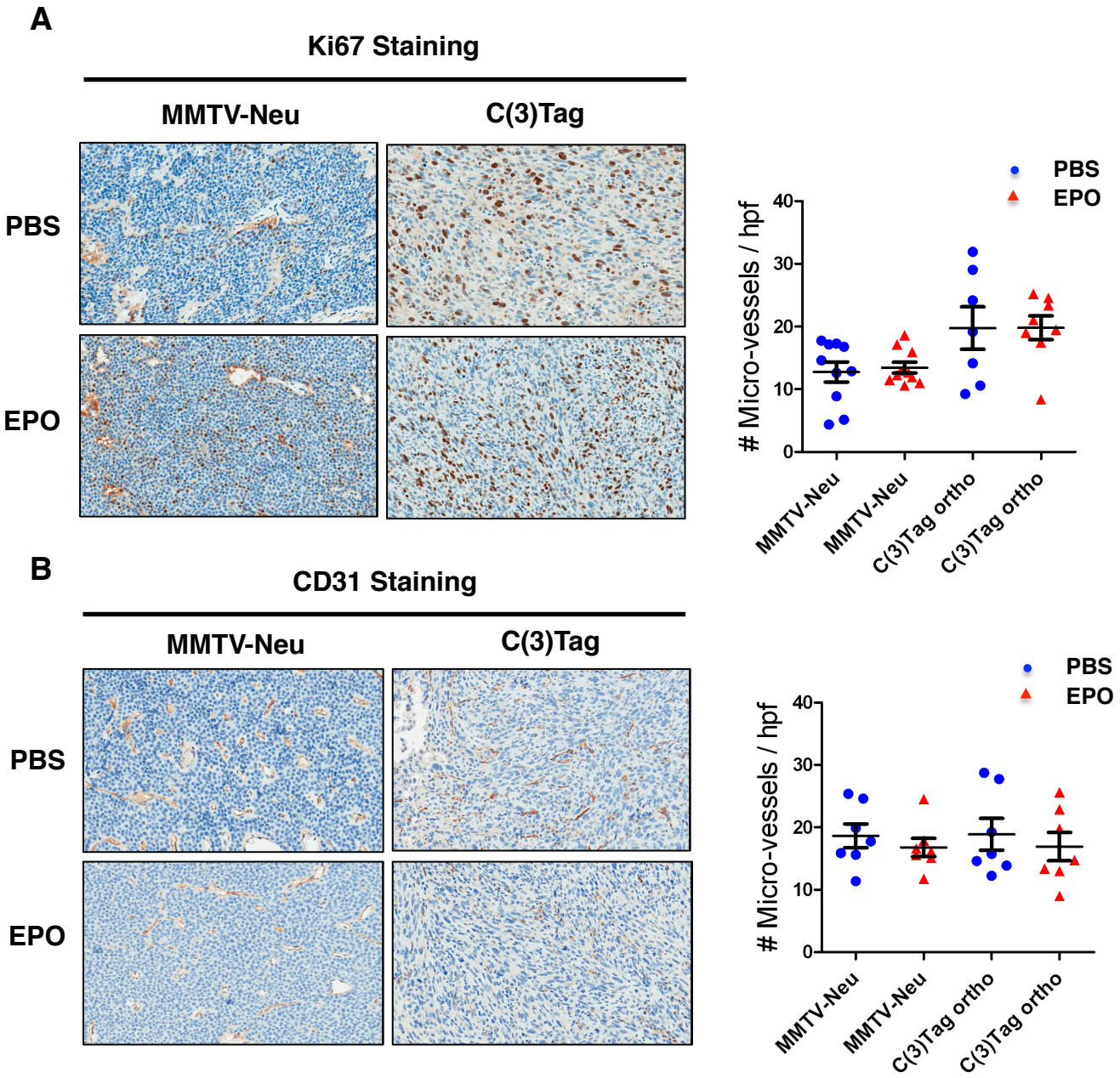

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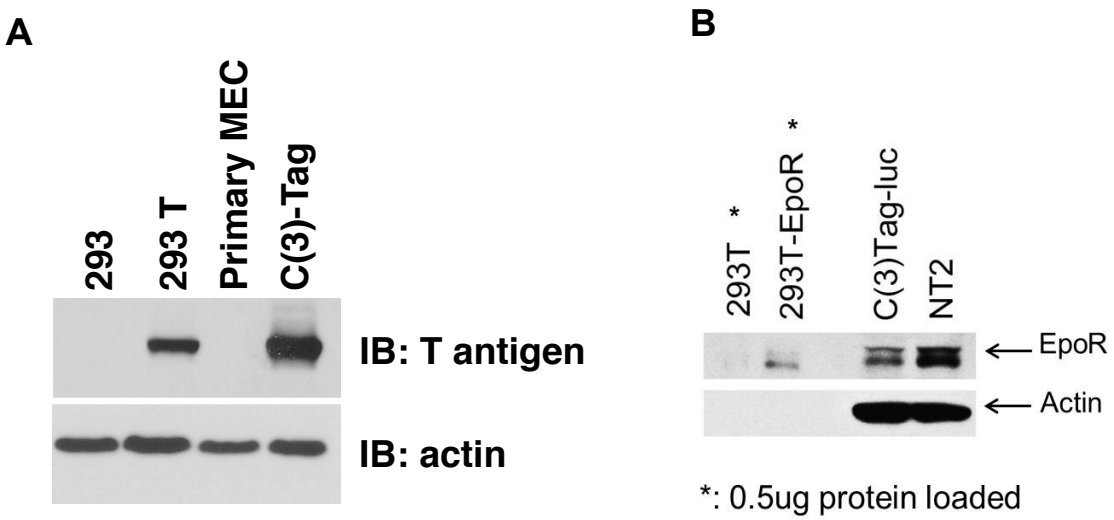

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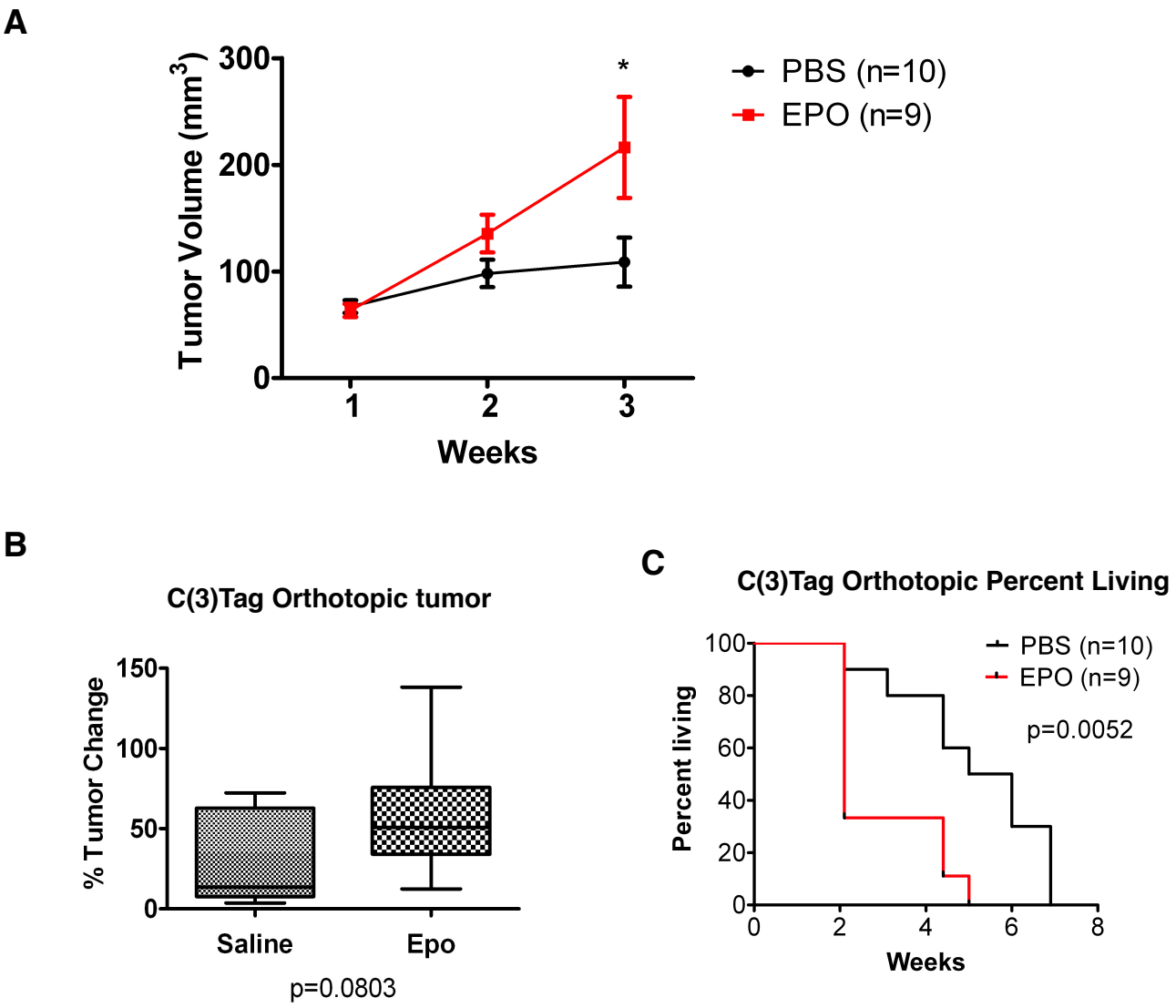

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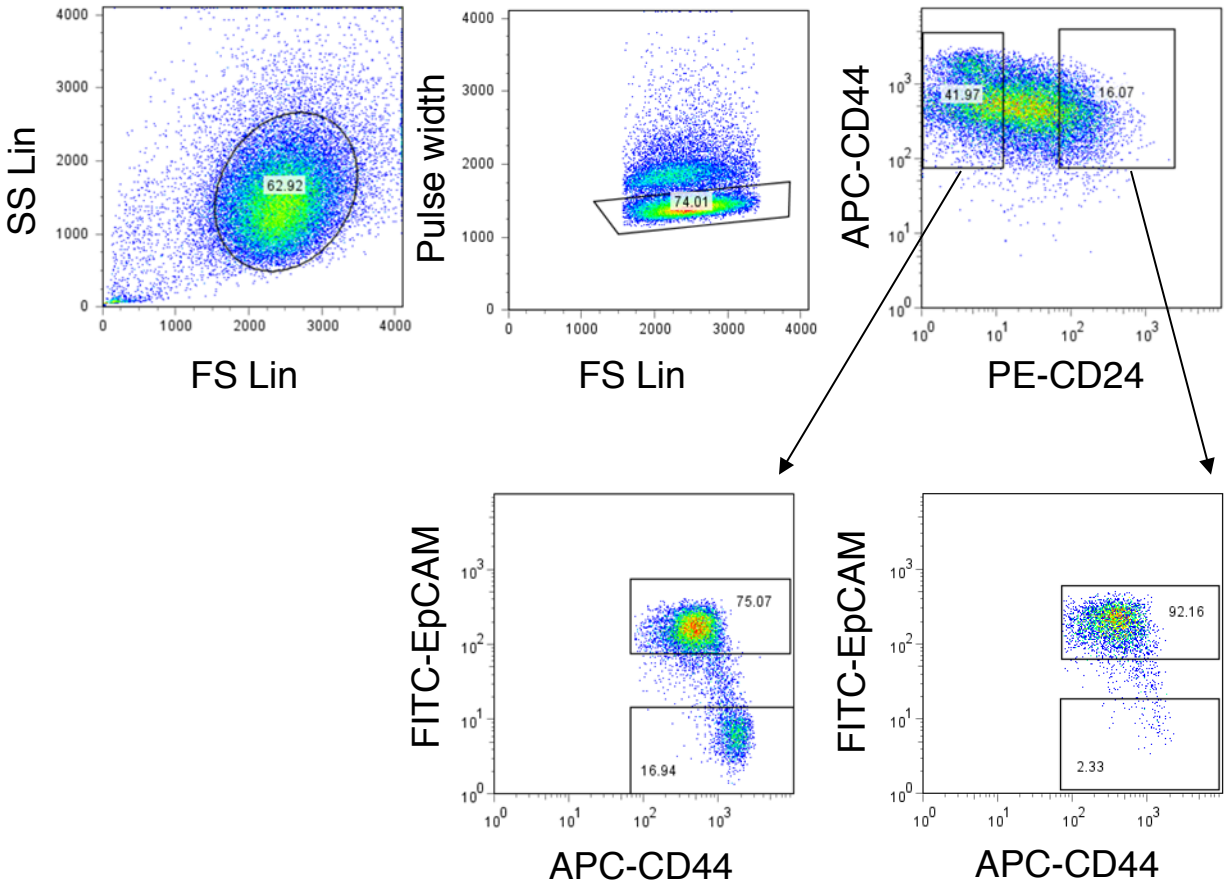

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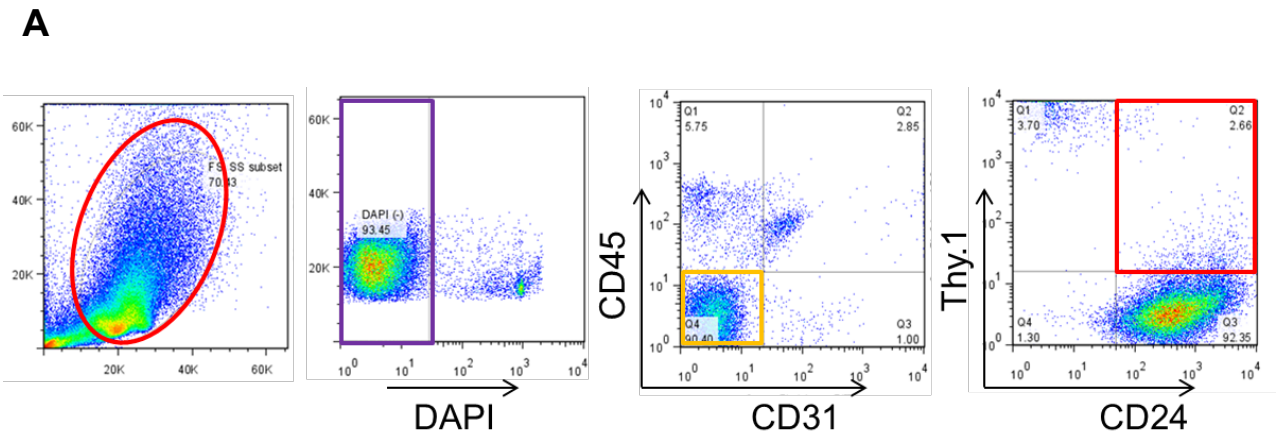

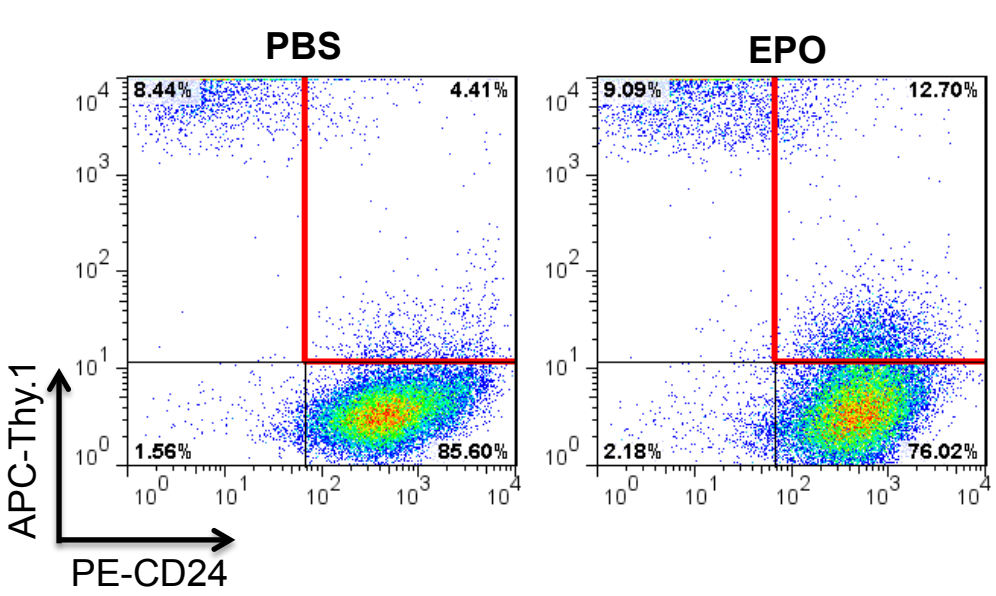

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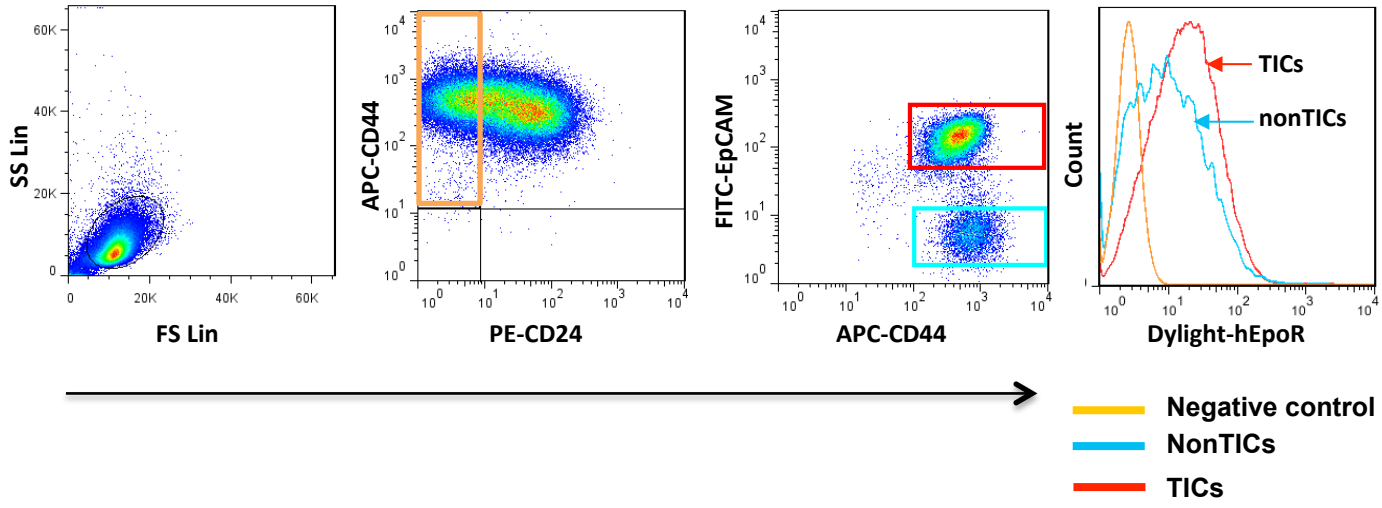

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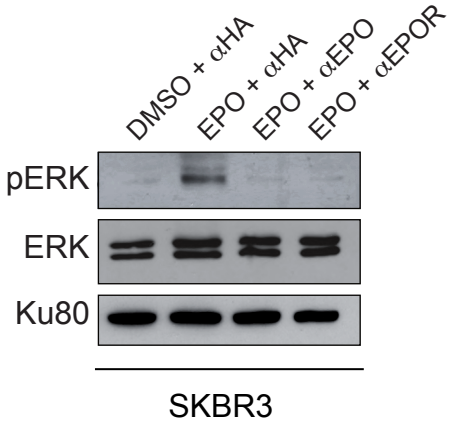

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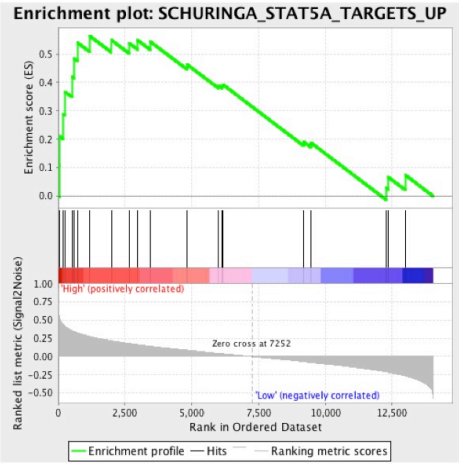
Supplemental Figure 10.

GSE18229



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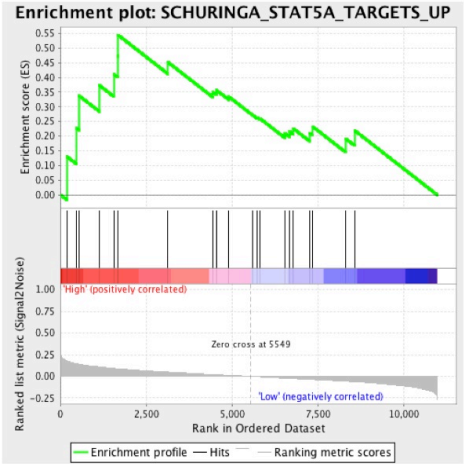
GSE26338



P = 0.01

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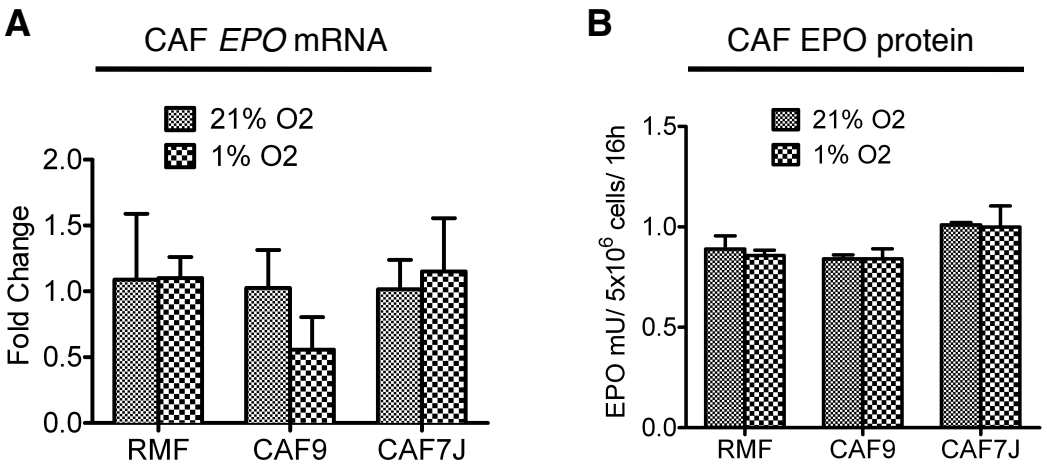

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