Supplementary Material

Pregnancy-Associated Plasma Protein-A (PAPP-A) in Ewing sarcoma: Role in Tumor Growth and Immune Evasion

Sabine Heitzeneder, Elena Sotillo, Jack F. Shern, Sivasish Sindiri, Peng Xu, Robert Jones, Michael Pollak, Pernille R. Noer, Julie Lorette, Ladan Fazli, Anya Alag, Paul Meltzer, Ching Lau, Cheryl A. Conover, Claus Oxvig, Poul H. Sorensen, John M. Maris, Javed Khan, and Crystal L. Mackall

Supplementary Methods

Tissue processing and RNA sequencing of pediatric solid tumors and normal tissue samples and differential gene expression analysis in Ewing sarcoma compared to normal tissues

PolyA selected RNA libraries were prepared for sequenced on the Illumina HiSeq2000 using TruSeq v3 chemistry according to the manufacturer's protocol (Illumina). RNA sequencing was performed with an average yield of 18.6 Gb per sample. Raw reads were mapped to UCSC human reference genome (hg19) using STAR/2.4.0k and subsequently genes and transcripts expression was quantified using edgeR and ENSEMBL gene annotation. The transcripts per million (TPM) gene expression values of all genes interrogated (n= 18539) on tumors and a panel of NCI in-house generated normal tissue samples was log transformed and standardized (Z-score) to calculate the difference in expression distribution for any gene between the two groups. EdgeR was used [1] to generate a list of differentially expressed genes between the two groups (n= 5890).

LogFC (fold-change) > 1 and $p < 0.01$ was used to filter for genes significantly overexpressed on Ewing sarcoma (n=584). Genes were further filtered by their cellular compartment annotation. Therefore, as part of the St. Baldrick's/SU2C consortium, a database was generated to annotate gene products that were most likely to be expressed on the cell surface. Eleven annotation datasets were compiled from commercially generated databases (Compendia, n=5), from the academic resources (Mike Jensen Lab, Seattle Children's, n=4), and from the public databases (Gene Ontology – Intrinsic or Extrinsic plasma membrane, n=2). In total, 341 genes of the 584 genes significantly overexpressed in Ewing sarcoma carried evidence of membrane annotation in at least 6 out of the 11 datasets (evidence filter >/=6). The likelihood of a gene product's association with the cell membrane was assumed to increase with the number of datasets it was observed in, whereby a score of 11 indicated those genes with the strongest evidence of being on the cell

surface. Overexpression of *PAPPA* on EWS samples was defined by values above the upper 95% confidence interval of the mean of the normal samples (upper 95% CI of mean =1.552, n=42).

For bioinformatics analysis of osteosarcoma samples, Salmon, a lightweight method for gene expression quantification (doi: 10.1038/nmeth.4197, version 0.8.1) used unstranded paired-end fastq files to produce gene-summarized transcript-per-million (TPM) values for the Gencode version 25 gene models. Gene expression values for *PAPPA* according to the Human Protein Atlas were queried from Uhlen et. al [2].

Cells and culture conditions

Ewings sarcoma cell lines used in this study included NCI-EWS-925, NCI-EWS-95, NCI-EWS-021, NCI-EWS-981 and NCI-EWS-022, were generated after Informed Consent from patients treated at Pediatric Oncology Branch, Center for Cancer Research, NCI (Bethesda, MD) between 1997 and 2002 and previously reported cell lines TC71 and 5838 [3]. EW8, TC32, RD-ES, 6647 and CHLA258 were kindly provided by L. Helman (NCI, Bethesda) and A673 and SKNMC by A. Sweet-Codero (Stanford, Palo Alto). The neuroblastoma cell line Kelly was provided by C. Thiele (NCI, NIH). 293T cells were obtained from the American Type Culture Collection (ATCC, Manassas). The 293T cell line was cultured in DMEM (Life Technologies). All other cell lines were cultured in RPMI-1640. DMEM and RPMI-1640 were supplemented with 10% heat-inactivated FBS (Gibco, Life Technologies), 10mM HEPES, 100U/mL penicillin, 100 μg/ml streptomycin and 2mM L-glutamine (Gibco, Life technologies). All cell lines used were fingerprinted annually and regularly tested negative for Mycoplasm by luminescence (MycoAlert™ Mycoplasma Detection Kit, Lonza).

Flow Cytometry

Flow Cytometry experiments were carried out according to standard protocols. In brief, data was collected with an LSR Fortessa or LSR Fortessa X-20 (BD Bioscience) and analyzed using the FlowJo software. Cells were washed twice with PBS and labelled with fixable Viability Dye eFluor® 450 (ebioscience #65-0863-14) at $1x10^6$ cells/mL in PBS, followed by two washes with FACS buffer (PBS supplemented with 2% FBS and 0.4% 0.5M EDTA). PAPP-A antigen expression was assessed by staining of $0.5x10^6$ cells with unconjugated monoclonal anti-PAPP-A antibody (#ABS 006-01-02, Thermofisher) which exhibits specificity for PAPP-A membrane immunoreactivity of placental tissue according to the HPA (www.humanproteinatlas.org) or isotype control antibody (#400102 MOPC-21 Mouse IgG1, κ) for 30 min on ice in the dark and detection with the secondary goat-anti mouse IgG Alexa Fluor® 647 (#405322, Biolegend). Cells were washed 3 times with FACS buffer after each incubation step. Cells were gated on viable cells and singlet discrimination (FSC-A/FSC-H) was performed before assessment of antigen expression. Mean fluorescent intensity (MFI) was calculated by subtraction of isotype geometric means from samples run in triplicates.

Enzyme Linked Immunosorbent Assay

All ELISA kits used for analysis of cell culture supernatants as well as patient serum and plasma samples were obtained from Ansh Labs (Webster, TX). The following ELISA kits were used in this study: picoPAPP-A (AL-101), bioactive IGF-I (AL-122), IGF-I (AL-121), total IGFBP-4 (AL-126) and intact IGFBP-4 (AL-128). Assays were performed according to the manufacturer's protocol and levels were calculated using OD values measured in triplicates or quadruplicates for samples and standards at 450nm with background wavelength correction at 630nm. For picoPAPP-A, levels were converted from ng/mL to μIU/mL according to manufacturer's instructions (1ng/mL = 2.7 μIU/mL). Cell culture supernatants were analyzed after incubation of $1x10^5$ cells

for 24 hours at 37°. Serum and Plasma samples from healthy control donors and Ewing sarcoma patients and Non-EWS patients were obtained from the National Institutes of Health Blood Bank (Bethesda, MD) between April and June 2015 after informed consent on National Institutes of Health Institutional Review Board–approved protocols. Diagnoses of Non-EWS patients were Rhabdomyosarcoma (n=6), Desmoplastic Small Round Cell Tumor (n=3), Osteosarcoma (n=2), Melanoma (n=1) and Synovial Cell Sarcoma (n=1). Samples were stored at -80ºC prior to assessment of PAPP-A levels.

Tissue microarray

TMA staining was performed at the BC Cancer Agency, Vancouver, Canada and analyzed according to standard methodology. To semi-quantitatively evaluate protein expression in TMA cores, H-scores were calculated adding the sum of the percentage of positive cells (0-100%) and the staining intensity (range 0-3+) according to the formula [1x (% of cells 1+) + 2x (% of cells 2+) + 3x (% of cells 3+)]. Normal tissue TMA was derived from the CHTN (Cooperative Human Tissue Network), the small Round blue cell TMA from CHOP (Children's Hospital of Philadelphia, PA), the osteosarcoma TMA from Nationwide Children's Hospital (OH) and the Ewing sarcoma from the COG (Children's Oncology Group, PA). All IHC staining for PAPP-A (ABS 006-01, Thermo Fisher) was performed on the Venatana Discovery XT automated stainer. After standard antigen retrieval using CC1 (Cell Conditioning 1, Ventana, AZ), primary antibody incubation was performed for 1 hour at room temperature at a dilution of 1:200. For dilution SignalStain enhancer (#8112 Cell signaling Technology, MA) was used. Incubation with Ultramap Ms HRP secondary antibody for 16 min was followed by by Chromomap DAB detection. A light hematoxylin counterstain was applied prior to coverslipping.

CRISPR/Cas9 gene editing

Reagents used for gene editing of the *PAPPA* locus were obtained from Genecopoeia (Rockville, MD). Expression of Cas9 was achieved by lentiviral transduction (#CP-LvC9NU-01, Genecopoeia, Rockville, MD) of $0.5x10^6$ cells in the presence of 10μg/mL of protamine sulfate and subsequent antibiotic selection with neomycin (500 μg/mL for EW8). Individual delivery of three different sgRNA's and expression of an mCherry reporter was done by a second lentiviral transduction (#HCP212189-LvSG02-3-B, Genecopoeia, Rockville, MD). After 48 hours, cells were sorted for expression of the reporter and underwent single cell cloning by serial dilution into 96-well plates. Whole wells were consecutively imaged for formation of colonies derived by single cells using the IncuCyte Live Cell Analysis System (IncuCyte ZOOM, Essen Bioscience) at a 4x objective. Efficient PAPP-A knockout was confirmed by ELISA of cell culture supernatants after incubation of $1x10^5$ cells for 24 hours at 37°.

In vitro proliferation assay and IncuCyte experiments

Cells ($0.05\ x10^6$) were plated and after 1 day cultured for 72 hrs in the presence of monoclonal anti-PAPP-A antibody 1/41 [Ansh Laboratories (Webster, TX) or IgG2a control mAB (#BE0085, BioXCell, West Lebanon, NH) at a concentration of 20 μg/mL. After 72 hrs, cells were counted with a hemocytometer. All measurements were performed in quadruplicate, and mean values are presented. Experiments determining cell growth were carried out using the IncuCyte Live Cell Analysis System (IncuCyte ZOOM, Essen Bioscience). Cells were plated in triplicates at $10\ x10^3$ cells/well in 96-well plates (day 0) and allowed to adhere. Confluency of cells was monitored starting on day 1, every 2-4 hours over the course of a total of 90-140 hours in the presence of monoclonal anti-PAPP-A antibody 1/41 [Ansh Laboratories (Webster, TX) or IgG2a control mAB (#BE0085, BioXCell, West Lebanon, NH), anti-IGF1R h7C10 (Merck, Kenilworth, NJ) or human IgG1 Isotype CTRL (#C0001, Crown Bioscience, Santa Clara, CA) at a concentration of 10 μg/mL

for proliferation assays and in the presence or absence of 60 ng/mL native soluble PAPP-A (#AG-301-BP040, AnshLabs TX, purified heterotetrameric PAPP-A from pregnancy serum, >80% purity) for rescue experiments. Means of percentage of phase object confluency was graphed over time. Alloreactivity of activated T-cells against PAPPA.KO and CTRL clones was assessed by measuring Average Green Object Mean Intensity (GCU) on GFP-transduced cell lines. Isolated T-cells from normal blood donors were activated with CD3/CD28 Dynabeads (Dynabeads Human T-activator CD3/CD28, Thermofisher, #Cat.NO 111.32D) and cultured in AIM-V media (Thermofisher, #12055083), supplemented with 5% heat-inactivated FBS (Gibco, Life Technologies), 10mM HEPES, 100U/mL penicillin, 100 μg/ml streptomycin and 2mM L-glutamine (Gibco, Life technologies) and 100 IU/mL IL-2. Dynabeads were removed on day 4 after activation. For co-culture experiments day 7 T-cells were used and all cells were cultured in RPMI complete media as described above.

Proteinase cleavage assay of ^{125}I-labeled IGFBP-5

SDS-PAGE experiments were carried out as previously described[4]. In brief, cell culture supernatant (24hrs) from EW8.PAPPA.KO or CTRL cell lines was diluted 2.5x in reaction buffer (50 mM Tris, 100 mM NaCl, 1 mM CaCl2, pH 8.0) with either polyclonal rabbit anti PAPP-A (1:200) or irrelevant polyclonal rabbit IgG (rabit anti-cow serum) (1:450, DAKO). The proteolytic reaction was initiated by adding 10nM radiolabeled IGFBP5 to the reaction mix. Cleavage reactions was stopped at 60 minutes by adding SDS loading buffer supplemented with 25 mM EDTA. Cleaved IGFBP5 was separated from intact by non-reducing SDS-PAGE, and bands were visualized by autoradiography using a storage phosphor screen (GE Healthcare).

In vivo experiments

Male 6-8 weeks old NSG mice were xenografted with $2x10^6$ EW8 cells in 100 µl PBS into the right M. gastrocnemius (4-5 mice/group/experiment). Tumor cells were washed twice with PBS before injection. Mice received weekly intraperitoneal injections with 800 µg/mouse PAPP-A mAB 1/41 (AnshLabs, Webster, TX) or IgG2a control mAB (#BE0085, BioXCell, West Lebanon, NH), 333 µg/mouse anti-IGF1R h7C10 (Merck, Kenilworth, NJ) or PBS/ human IgG1 Isotype CTRL (#C0001, Crown Bioscience, Santa Clara, CA) or in combination starting at day 3-4 after tumor engraftment. Combined data of n=3 individual experiments is presented (n=14-15 mice/group). Baseline measurement of legs were taken before engraftment and tumor growth was measured two times a week with digital calipers in two dimensions. Maximum tumor size was defined as or exceeding 20mm in its longest dimension. All animal care was in accordance with NIH guidelines.

RNAsequencing of PAPP-A targeted knockout clones

After RNA extraction, cDNA library Generation and bar-coding of samples was done using a KAPA Stranded mRNA-Seq Kit, with KAPA mRNA Capture Beads (#KK8420, Kapa Biosystems). A paired-end pooled high-throughput sequencing run (Illumina NextSeq 2x75 high-output, 400M total reads) was performed at the Stanford Functional Genomics Facility. NextSeq fastq files were aligned onto GRCh38 using STAR aligner (**DOI:** https://doi.org/10.1093/bioinformatics/bts635). RNA counts were generated with HTSeq (**DOI:** https://doi.org/10.1093/bioinformatics/btu638) on Homo_sapiens.GRCh38.84.gtf. Sample B-H8 was excluded due to loss of gene expression in location (Chr21: 39,387,421-41,778,490) and the samples were divided into two groups, PAPP-A knockout (KO) and control (CTRL). Genes with greater than 10 counts in either all KO or CTRL samples were retained. From this set, genes with greater than FPKM>1 in either all KO or CTRL samples were retained. Differential genes

expression analysis on this set was performed with DESeq2 (**DOI**: https://doi.org/10.1186/s13059-014-0550-8). Genes with adjusted p-value of < 0.5 were retained. The remaining list of 7,457 genes and their FPKM expression values were submitted to the GSEA (http://software.broadinstitute.org/gsea/msigdb/index.jsp) website for analysis against the Molecular Signatures Database v5.2.

HLA class I expression in EWS cell lines

EWS cell lines EW8 or TC32 were grown for 4 days in RPMI complete media the presence of 10 μg/mL of anti-PAPP-A mAB 1/41 and/or anti-IGF1R mAB h7C10 or according isotype control antibodies as described above in six replicates. Media supplemented with antibodies was changed once after 48hrs. Adherent cells were detached using non-enzymatic solutions (Cellstripper #25-056-CI, Corning, NY), and processed for Flow Cytometry as described above. Staining was performed in triplicates with HLA-ABC (#311410, Biolegend, San Diego, CA) or isotype control (#400220, Biolegend, San Diego, CA). Mean fluorescent intensity (MFI) of viable, singlet populations were calculated by subtraction of isotypes from samples. EW8.PAPP-A.KO and CTRL samples were also stained with these antibodies.

Gene set enrichment analysis of immune cell infiltration

Custom GSEA analysis was performed utilizing gene expression signatures for various immune cell types as previously published [5]. To identify gene signatures, that were skewed due to overlapping gene expression by EWS tumors, normalized immune scores for each subset were calculated and analyzed in both cell lines (n=45) and primary tumor samples (n=75). Immune cell gene signatures with >/= 5 genes, in which scores were negative in cell lines were used as a basis for GSEA analysis and included T-cells, B-cells, Tem, Th1, TFH, CD8 T-cells, Cytotoxic cells, NK cells, NKCD56bright, DC, iDC, aDC, Eosinophils, Macrophages, Mast cells and Neutrophils.

Statistical methods

Graphs were generated using GraphPad Prism software version 7.0 and values represent mean ± SD (as indicated) of biological replicates unless otherwise stated.

All statistical tests were two-sided and a p-value of >0.05 was considered statistically significant. TPM gene expression values of all genes (n= 18539) in tumors (n=120) and NCI in-house generated normal tissue samples (n=42) were log transformed and standardized (Z-score) to calculate the difference in expression distribution for any gene between the two groups (n= 5890). EdgeR was used to generate a list of differentially expressed genes between the two groups. Differences in PAPP-A levels in patients and HLA-B/HLA-C expression in EWS vs. normal were calculated using a Wilcoxon–Mann–Whitney test. Kaplan-Meier survival curves of vivo experiments were analyzed using log-rank. Deaths of mice occurring before the establishment of tumor were censored from survival analysis. Comparison of tumor growth curves of in vitro and in vivo experiments were calculated using repeated-measures Analysis of variance (Two-way RM-ANOVA). All other experiments were evaluated for statistical significance using a student's t-test. P-values of enrichment analysis were calculated using GSEA software. All figures represent three replicates unless otherwise stated.

References

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Supplementary Table 1. Most differentially expressed cell surface genes in EWS

			EXPRESSION (AVG log2[TPM+1])		EWS vs. NORMAL		
NO	**GeneID**	**Gene Name**	**Normal (n=42)**	**EWS (n=120)**	**P-val (*)**	**logFC**	**FDR**
1	ENSG00000171873	*ADRA1D*	1.734	6.806	$4.54x10^{-55}$	5.745	$3.54x10^{-53}$
2	ENSG00000182752	*PAPPA*	1.741	5.729	$8.31x10^{-42}$	4.600	$2.48x10^{-40}$
3	ENSG00000221977	*OR4E2*	0.013	2.236	$3.04x10^{-29}$	8.537	$3.81x10^{-28}$
4	ENSG00000186867	*QRFPR*	0.730	5.148	$4.78x10^{-28}$	7.506	$5.43x10^{-27}$
5	ENSG00000187323	*DCC*	0.774	4.931	$7.98x10^{-27}$	4.897	$8.33x10^{-26}$
6	ENSG00000147145	*LPAR4*	1.196	3.703	$6.40x10^{-26}$	3.330	$6.27x10^{-25}$
7	ENSG00000100593	*ISM2*	0.464	3.167	$3.23x10^{-25}$	4.871	$2.98x10^{-24}$
8	ENSG00000142513	*ACPT*	0.547	2.628	$3.97x10^{-24}$	3.129	$3.39x10^{-23}$
9	ENSG00000150551	*LYPD1*	1.199	4.718	$4.74x10^{-23}$	3.875	$3.74x10^{-22}$
10	ENSG00000188778	*ADRB3*	0.948	5.305	$5.56x10^{-23}$	5.741	$4.37x10^{-22}$
11	ENSG00000180537	*RNF182*	1.544	5.656	$1.44x10^{-22}$	3.778	$1.10x10^{-21}$
12	ENSG00000179520	*SLC17A8*	0.459	4.193	$1.50x10^{-22}$	8.526	$1.14x10^{-21}$
13	ENSG00000162631	*NTNG1*	1.624	5.127	$4.39x10^{-22}$	3.691	$3.22x10^{-21}$
14	ENSG00000143590	*EFNA3*	1.772	4.141	$6.80x10^{-22}$	2.462	$4.93x10^{-21}$
15	ENSG00000102290	*PCDH11X*	0.463	3.370	$1.70x10^{-21}$	4.800	$1.19x10^{-20}$
16	ENSG00000150394	*CDH8*	0.889	4.504	$1.86x10^{-21}$	3.842	$1.30x10^{-20}$
17	ENSG00000171094	*ALK*	0.786	3.503	$4.51x10^{-21}$	3.874	$3.07x10^{-20}$
18	ENSG00000105642	*KCNN1*	1.108	5.536	$6.18x10^{-21}$	3.831	$4.17x10^{-20}$
19	ENSG00000165349	*SLC7A3*	0.884	3.496	$3.20x10^{-20}$	4.293	$2.06x10^{-19}$
20	ENSG00000164082	*GRM2*	0.965	4.556	$1.43x10^{-19}$	4.656	$8.68x10^{-19}$
21	ENSG00000165323	*FAT3*	1.159	4.015	$8.57x10^{-19}$	3.428	$4.90x10^{-18}$
22	ENSG00000137571	*SLCO5A1*	1.443	5.584	$1.25x10^{-17}$	3.711	$6.52x10^{-17}$
23	ENSG00000136099	*PCDH8*	1.039	5.516	$1.94x10^{-17}$	5.092	$9.97x10^{-17}$
24	ENSG00000082684	*SEMA5B*	1.871	3.667	$4.35x10^{-14}$	2.835	$1.73x10^{-13}$
25	ENSG00000188389	*PDCD1*	1.470	3.788	$7.23x10^{-14}$	4.759	$2.84x10^{-13}$
26	ENSG00000105464	*GRIN2D*	1.047	2.565	$1.27x10^{-13}$	2.398	$4.90x10^{-13}$
27	ENSG00000006283	*CACNA1G*	1.268	4.556	$1.83x10^{-13}$	2.817	$6.97x10^{-13}$
28	ENSG00000179242	*CDH4*	1.624	3.814	$1.90x10^{-13}$	3.319	$7.23x10^{-13}$
29	ENSG00000101188	*NTSR1*	1.021	3.425	$1.35x10^{-12}$	3.364	$4.77x10^{-12}$
30	ENSG00000187714	*SLC18A3*	0.635	3.950	$1.53x10^{-12}$	4.537	$5.39x10^{-12}$
31	ENSG00000166828	*SCNN1G*	1.629	6.549	$2.67x10^{-12}$	4.077	$9.27x10^{-12}$
32	ENSG00000151012	*SLC7A11*	1.199	3.504	$2.99x10^{-12}$	3.450	$1.03x10^{-11}$
33	ENSG00000117009	*KMO*	1.306	4.949	$3.15x10^{-12}$	3.036	$1.09x10^{-11}$
34	ENSG00000197410	*DCHS2*	1.362	3.177	$4.97x10^{-12}$	3.970	$1.69x10^{-11}$
35	ENSG00000090932	*DLL3*	0.525	2.199	$5.49x10^{-12}$	2.973	$1.85x10^{-11}$
36	ENSG00000157168	*NRG1*	1.451	3.025	$9.02x10^{-12}$	2.582	$2.99x10^{-11}$

37	ENSG00000184986	*TMEM121*	1.992	3.197	$9.36x10^{-12}$	1.573	$3.10x10^{-11}$
38	ENSG00000119915	*ELOVL3*	1.000	2.222	$2.11x10^{-11}$	2.567	$6.80x10^{-11}$
39	ENSG00000121904	*CSMD2*	0.646	2.116	$3.53x10^{-11}$	3.021	$1.11x10^{-10}$
40	ENSG00000114646	*CSPG5*	1.668	4.960	$4.04x10^{-11}$	2.803	$1.27x10^{-10}$
41	ENSG00000147138	*GPR174*	1.671	4.336	$7.65x10^{-11}$	3.655	$2.36x10^{-10}$
42	ENSG00000125864	*BFSP1*	1.806	2.878	$1.78x10^{-10}$	1.407	$5.31x10^{-10}$
43	ENSG00000105376	*ICAM5*	1.345	3.929	$1.62x10^{-09}$	2.600	$4.48x10^{-09}$
44	ENSG00000142449	*FBN3*	0.712	2.197	$1.81x10^{-09}$	2.910	$5.00x10^{-09}$
45	ENSG00000089558	*KCNH4*	0.666	2.015	$2.66x10^{-09}$	2.718	$7.23x10^{-09}$
46	ENSG00000174482	*LINGO2*	1.153	2.890	$3.12x10^{-09}$	2.537	$8.44x10^{-09}$
47	ENSG00000116031	*CD207*	1.156	2.358	$9.43x10^{-09}$	1.828	$2.44x10^{-08}$
48	ENSG00000100346	*CACNA1I*	0.825	2.618	$1.24x10^{-08}$	2.902	$3.17x10^{-08}$
49	ENSG00000144339	*TMEFF2*	1.704	4.144	$1.58x10^{-08}$	2.547	$4.02x10^{-08}$
50	ENSG00000111981	*ULBP1*	0.868	2.757	$1.76x10^{-08}$	2.508	$4.46x10^{-08}$
51	ENSG00000108556	*CHRNE*	1.368	2.307	$4.98x10^{-08}$	1.574	$1.21x10^{-07}$
52	ENSG00000135119	*RNFT2*	1.259	2.719	$5.52x10^{-08}$	1.870	$1.34x10^{-07}$
53	ENSG00000168702	*LRP1B*	1.182	2.572	$7.63x10^{-08}$	2.249	$1.83x10^{-07}$
54	ENSG00000113763	*UNC5A*	1.237	4.291	$9.76x10^{-08}$	2.851	$2.32x10^{-07}$
55	ENSG00000167083	*GNGT2*	1.952	3.060	$1.14x10^{-07}$	1.573	$2.69x10^{-07}$
56	ENSG00000171462	*DLK2*	1.687	2.938	$2.38x10^{-07}$	1.150	$5.46x10^{-07}$
57	ENSG00000164220	*F2RL2*	1.362	2.298	$2.71x10^{-07}$	2.843	$6.19x10^{-07}$
58	ENSG00000128011	*LRFN1*	1.652	2.759	$3.84x10^{-07}$	1.363	$8.67x10^{-07}$
59	ENSG00000158089	*GALNT14*	1.817	3.884	$4.69x10^{-07}$	1.665	$1.05x10^{-06}$
60	ENSG00000183840	*GPR39*	0.995	2.277	$5.11x10^{-07}$	2.119	$1.14x10^{-06}$
61	ENSG00000185561	*TLCD2*	1.835	2.776	$6.40x10^{-07}$	1.014	$1.42x10^{-06}$
62	ENSG00000179603	*GRM8*	1.043	2.301	$6.54x10^{-07}$	2.000	$1.45x10^{-06}$
63	ENSG00000197406	*DIO3*	1.418	2.245	$6.72x10^{-07}$	3.528	$1.48x10^{-06}$
64	ENSG00000188517	*COL25A1*	1.794	2.681	$9.59x10^{-07}$	2.045	$2.08x10^{-06}$
65	ENSG00000139364	*TMEM132B*	1.338	2.808	$1.00x10^{-06}$	2.504	$2.17x10^{-06}$
66	ENSG00000100427	*MLC1*	1.621	5.647	$1.50x10^{-06}$	2.159	$3.20x10^{-06}$
67	ENSG00000133107	*TRPC4*	1.815	2.939	$2.55x10^{-06}$	2.039	$5.34x10^{-06}$
68	ENSG00000183780	*SLC35F3*	1.435	3.099	$3.54x10^{-06}$	2.185	$7.34x10^{-06}$
69	ENSG00000149571	*KIRREL3*	1.450	2.878	$4.71x10^{-06}$	2.041	$9.67x10^{-06}$
70	ENSG00000125510	*OPRL1*	1.919	3.087	$5.11x10^{-06}$	1.136	$1.05x10^{-05}$
71	ENSG00000134207	*SYT6*	1.040	2.048	$5.37x10^{-06}$	2.506	$1.10x10^{-05}$
72	ENSG00000198400	*NTRK1*	1.592	2.408	$6.56x10^{-06}$	1.767	$1.33x10^{-05}$
73	ENSG00000125657	*TNFSF9*	1.885	3.521	$8.36x10^{-06}$	1.294	$1.68x10^{-05}$
74	ENSG00000007129	*CEACAM21*	1.723	2.566	$1.10x10^{-05}$	1.866	$2.19x10^{-05}$
75	ENSG00000156564	*LRFN2*	0.881	2.233	$2.26x10^{-05}$	1.901	$4.36x10^{-05}$
76	ENSG00000072041	*SLC6A15*	1.031	2.693	0.00013	2.684	0.00023

77	ENSG00000185008	*ROBO2*	1.718	2.706	0.00015	1.452	0.00027
78	ENSG00000121871	*SLITRK3*	1.400	2.775	0.00016	1.836	0.00028
79	ENSG00000196990	*FAM163B*	0.938	2.533	0.00017	2.420	0.00029
80	ENSG00000176956	*LY6H*	1.681	3.618	0.00022	2.039	0.00038
81	ENSG00000171864	*PRND*	0.953	3.537	0.00023	2.071	0.00041
82	ENSG00000196811	*CHRNG*	0.536	2.093	0.00025	1.760	0.00043
83	ENSG00000095970	*TREM2*	1.712	2.445	0.00044	2.051	0.00076
84	ENSG00000237515	*SHISA9*	1.303	2.426	0.00078	1.675	0.00129
85	ENSG00000148848	*ADAM12*	1.328	2.091	0.00124	1.490	0.00202
86	ENSG00000184845	*DRD1*	1.397	2.093	0.00152	1.461	0.00244
87	ENSG00000120088	*CRHR1*	0.610	2.061	0.00252	1.571	0.00395
88	ENSG00000086548	*CEACAM6*	1.106	2.742	0.00270	2.118	0.00421
89	ENSG00000145428	*RNF175*	1.532	2.264	0.00365	1.191	0.00561
90	ENSG00000145242	*EPHA5*	1.154	2.161	0.00510	1.210	0.00771
91	ENSG00000142408	*CACNG8*	0.903	3.026	0.00740	1.199	0.01096

(*) p-values were calculated using EdgeR. Statistical test was two-sided.

Supplementary Table 2. Expression of *PAPPA* in TCGA tumor and matched normal tissue samples.

		TUMOR SAMPLES					
Abbr.	Description	total (n=)	n.d. (n=)	mean log2[TPM]	SD	samples (n=) log2[TPM] >2	% log2[TPM]>2
ACC	ADRENOCORTICAL CARCINOMA	77	5	-3.81	2.21	0	0
BLCA	BLADDER UROTHELIAL CARCINOMA	503	4	-2.12	1.95	10	2
BRCA	BREAST INVASIVE CARCINOMA	1139	0	-1.39	1.46	11	1
CESC	CERVICAL SQUAMOUS CELL CARCINOMA AND ENDOCERVICAL ADENOCARCINOMA	306	4	-2.57	1.93	5	2
CHOL	CHOLANGIOCARCINOMA	36	0	-1.69	2.02	0	0
COAD	COLON ADENOCARCINOMA	311	0	-1.57	1.57	2	1
DLBC	LYMPHOID NEOPLASM DIFFUSE LARGE B-CELL LYMPHOMA	47	4	-4.12	1.89	0	0
ESCA	ESOPHAGEAL CARCINOMA	182	0	0.81	1.48	32	18
GBM	GLIOBLASTOMA MULTIFORME	168	2	-2.64	1.78	1	1
HNSC	HEAD AND NECK SQUAMOUS CELL CARCINOMA	520	0	0.07	1.66	60	12
KICH	KIDNEY CHROMOPHOBE	66	0	-2.49	1.53	1	2
KIRC	KIDNEY RENAL CLEAR CELL CARCINOMA	543	0	-1.31	1.62	13	2
KIRP	KIDNEY RENAL PAPILLARY CELL CARCINOMA	289	0	1.29	1.69	90	31
LAML	ACUTE MYELOID LEUKEMIA	173	15	-3.39	1.93	1	1
LGG	BRAIN LOWER GRADE GLIOMA	527	1	-1.94	1.59	4	1
LIHC	LIVER HEPATOCELLULAR CARCINOMA	372	43	-4.10	1.73	0	0
LUAD	LUNG ADENOCARCINOMA	539	1	-1.23	1.85	25	5
LUSC	LUNG SQUAMOUS CELL CARCINOMA	500	0	-1.16	1.80	18	4
MESO	MESOTHELIOMA	87	0	0.18	1.70	11	13
OV	OVARIAN SEROUS CYSTADENOCARCINOMA	428	11	-3.17	1.79	1	0
PAAD	PANCREATIC ADENOCARCINOMA	179	0	-0.11	1.42	6	3
PCPG	PHEOCHROMOCYTOMA AND PARAGANGLIOMA	182	0	-0.85	1.66	11	6
PRAD	PROSTATE ADENOCARCINOMA	504	2	-2.31	1.30	2	0
READ	RECTUM ADENOCARCINOMA	93	0	-1.48	1.43	0	0
SARC	SARCOMA	262	2	0.03	2.31	52	20
SKCM	SKIN CUTANEOUS MELANOMA	469	6	-3.12	2.03	12	3
STAD	STOMACH ADENOCARCINOMA	415	0	0.22	1.33	33	8
TGCT	TESTICULAR GERM CELL TUMORS	154	4	-2.57	1.91	0	0
THCA	THYROID CARCINOMA	512	0	-1.23	1.61	7	1
THYM	THYMOMA	119	3	-2.23	1.94	2	2
UCEC	UTERINE CORPUS ENDOMETRIOID CARCINOMA	185	9	-3.10	1.82	0	0
UCS	UTERINE CARCINOSARCOMA	57	1	-2.54	1.67	0	0
UVM	UVEAL MELANOMA	79	6	-3.65	1.63	0	0

		MATCHED NORMAL TISSUE SAMPLES					
Abbr.	**Description**	**total (n=)**	**n.d. (n=)**	**mean log2[TPM]**	**SD**	**samples (n=) log2[TPM] >2**	**% log2[TPM]>2**
ACC	ADRENOCORTICAL CARCINOMA	n.a.	n.a.	n.a.		n.a.	n.a.
BLCA	BLADDER UROTHELIAL CARCINOMA	19	0	-0.55	1.48	1	5
BRCA	BREAST INVASIVE CARCINOMA	114	0	-0.79	0.95	0	0
CESC	CERVICAL SQUAMOUS CELL CARCINOMA AND ENDOCERVICAL ADENOCARCINOMA	3	0	0.40	1.36	0	0
CHOL	CHOLANGIOCARCINOMA	9	0	-4.68	1.49	0	0
COAD	COLON ADENOCARCINOMA	41	0	-1.29	0.84	0	0
DLBC	LYMPHOID NEOPLASM DIFFUSE LARGE B-CELL LYMPHOMA	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
ESCA	ESOPHAGEAL CARCINOMA	13	0	-0.07	1.17	1	8
GBM	GLIOBLASTOMA MULTIFORME	5	0	-2.95	1.85	0	0
HNSC	HEAD AND NECK SQUAMOUS CELL CARCINOMA	44	0	-0.60	1.84	1	2
KICH	KIDNEY CHROMOPHOBE	25	0	2.44	0.98	17	68
KIRC	KIDNEY RENAL CLEAR CELL CARCINOMA	72	0	2.66	0.97	54	75
KIRP	KIDNEY RENAL PAPILLARY CELL CARCINOMA	32	0	2.31	0.77	22	69
LAML	ACUTE MYELOID LEUKEMIA	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
LGG	BRAIN LOWER GRADE GLIOMA	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
LIHC	LIVER HEPATOCELLULAR CARCINOMA	50	2	-4.31	1.18	0	0
LUAD	LUNG ADENOCARCINOMA	59	0	-1.20	0.82	0	0
LUSC	LUNG SQUAMOUS CELL CARCINOMA	50	0	-0.70	1.03	1	2
MESO	MESOTHELIOMA	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
OV	OVARIAN SEROUS CYSTADENOCARCINOMA	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
PAAD	PANCREATIC ADENOCARCINOMA	4	0	0.07	0.57	0	0
PCPG	PHEOCHROMOCYTOMA AND PARAGANGLIOMA	3	0	-1.44	1.24	0	0
PRAD	PROSTATE ADENOCARCINOMA	52	0	-1.20	1.13	0	0
READ	RECTUM ADENOCARCINOMA	10	0	-1.51	1.21	0	0
SARC	SARCOMA	2	0	-0.39	4.37	1	n.a.
SKCM	SKIN CUTANEOUS MELANOMA	1	0	-1.43	n.a.	0	n.a.
STAD	STOMACH ADENOCARCINOMA	36	0	-0.22	0.98	0	0
TGCT	TESTICULAR GERM CELL TUMORS	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
THCA	THYROID CARCINOMA	59	0	-1.65	1.04	0	0
THYM	THYMOMA	2	0	-0.21	1.28	0	n.a.
UCEC	UTERINE CORPUS ENDOMETRIOID CARCINOMA	23	0	0.56	1.18	2	9
UCS	UTERINE CARCINOSARCOMA	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
UVM	UVEAL MELANOMA	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Supplementary Table 3. Characteristics of patients analyzed for PAPP-A levels.

	demographics			sample type			PAPP-A (µIU/mL)		
Group	**F/M**	**age range**	**median age**	**serum**	**plasma**	**total**	**range (min-max)**	**median**	**p-val. (*)**
EWS patients	19/37	7-40	19	7	49	56	0.12 - 126.48	1.94	n.a.
CTRL donors	20/34	18-30	25	6	47	53	0.04 - 3.99	1.37	0.01
Non-EWS patients	5/8	5-32	17	9	4	13	0.56 - 4.62	1.40	0.04

(*) p-value indicates level of significance of the EWS group compared to the other groups according to two-sided Wilcoxon–Mann–Whitney test.
n.a.: not applicable

Supplementary Table 4. Statistics of GSEA analysis of hallmark pathways enriched in EW8.PAPPA.KO compared to EW8.PAPPA.CTRL

	SIZE	ES	NES	NOM	FDR	FWER	RANK
HALLMARK PATHWAY NAME				**p-val (*)**	**q-val**	**p-val**	**AT MAX**
INTERFERON_ALPHA_RESPONSE	92	0.656	2.329	>0.001	0.000	0.000	5767
HYPOXIA	195	0.518	2.033	>0.001	0.000	0.000	6645
INTERFERON_GAMMA_RESPONSE	189	0.504	1.972	>0.001	0.000	0.000	7924
TNFA_SIGNALING_VIA_NFKB	196	0.452	1.784	>0.001	0.001	0.004	7183
INFLAMMATORY_RESPONSE	190	0.445	1.747	>0.001	0.001	0.008	6962
KRAS_SIGNALING_DN	183	0.426	1.685	>0.001	0.002	0.018	5812
APICAL_SURFACE	44	0.514	1.652	0.001	0.004	0.031	4868
IL6_JAK_STAT3_SIGNALING	85	0.460	1.641	>0.001	0.004	0.038	6653
P53_PATHWAY	199	0.410	1.606	>0.001	0.006	0.063	6270
COAGULATION	130	0.396	1.481	0.006	0.026	0.275	7946
BILE_ACID_METABOLISM	107	0.397	1.459	0.006	0.030	0.334	7311
HEME_METABOLISM	191	0.366	1.444	0.002	0.032	0.375	6607
KRAS_SIGNALING_UP	193	0.357	1.403	0.005	0.045	0.515	6407
ALLOGRAFT_REJECTION	183	0.357	1.396	0.005	0.046	0.557	6145
MYOGENESIS	197	0.354	1.394	0.005	0.044	0.561	7632
IL2_STAT5_SIGNALING	198	0.345	1.368	0.008	0.054	0.663	6546
APOPTOSIS	158	0.351	1.354	0.02	0.059	0.716	5233
COMPLEMENT	192	0.337	1.326	0.03	0.073	0.803	7007
E2F_TARGETS	199	-0.691	-3.249	>0.001	0.000	0.000	4814
MYC_TARGETS_V1	199	-0.653	-3.066	>0.001	0.000	0.000	4359
G2M_CHECKPOINT	198	-0.650	-3.031	>0.001	0.000	0.000	5126
MYC_TARGETS_V2	58	-0.718	-2.795	>0.001	0.000	0.000	3493
MTORC1_SIGNALING	198	-0.478	-2.249	>0.001	0.000	0.000	4410
UNFOLDED_PROTEIN_RESPONSE	110	-0.490	-2.098	>0.001	0.000	0.000	5886
MITOTIC_SPINDLE	198	-0.377	-1.765	>0.001	0.001	0.004	6852
DNA_REPAIR	143	-0.343	-1.546	>0.001	0.011	0.045	5990
UV_RESPONSE_UP	153	-0.309	-1.384	>0.001	0.038	0.166	4859
PI3K_AKT_MTOR_SIGNALING	105	-0.320	-1.363	0.02	0.038	0.197	5430
REACTIVE_OXIGEN_SPECIES_PATHWAY	47	-0.375	-1.369	0.04	0.038	0.182	3529

(*) nominal p-values were calculated using GSEA software, which is based on a two-sided permutation-based Kolmogorov-Smirnov-like test

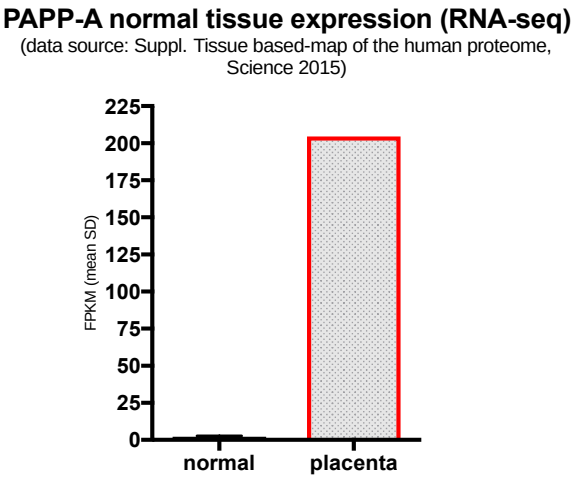
Supplementary Table 5. Statistics of GSEA analysis of hallmark pathways in primary EWS tumors in in-house generated dataset and GEO dataset GSE34620

DATA SET	HALLMARK PATHWAY NAME	SIZE	ES	NES	NOM p-val (*)	FDR q-val	FWER p-val	RANK AT MAX
in-house generated	ALLOGRAFT_REJECTION	200	0.411	2.379	<0.001	<0.0001	<0.0001	4078
	INFLAMMATORY_RESPONSE	197	0.374	2.081	<0.001	<0.0001	<0.0001	4396
	IL6_JAK_STAT3_SIGNALING	87	0.419	2.018	<0.001	<0.0001	<0.0001	3956
	COMPLEMENT	195	0.354	1.979	<0.001	<0.0001	<0.0001	3333
	TNFA_SIGNALING_VIA_NFKB	198	0.298	1.649	<0.001	0.01	0.03	4310
	INTERFERON_GAMMA_RESPONSE	197	0.241	1.361	<0.001	0.05	0.22	3211
GEO dataset GSE34620	ALLOGRAFT_REJECTION	192	0.702	2.361	<0.001	<0.0001	<0.0001	2156
	INFLAMMATORY_RESPONSE	187	0.682	2.291	<0.001	<0.0001	<0.0001	2666
	INTERFERON_GAMMA_RESPONSE	188	0.663	2.240	<0.001	<0.0001	<0.0001	4063
	COMPLEMENT	185	0.662	2.232	<0.001	<0.0001	<0.0001	2900
	IL6_JAK_STAT3_SIGNALING	86	0.716	2.200	<0.001	<0.0001	<0.0001	2270
	TNFA_SIGNALING_VIA_NFKB	190	0.608	2.053	<0.001	<0.0001	<0.0001	3328
	APOPTOSIS	156	0.586	1.933	<0.001	<0.0001	<0.0001	3345
	INTERFERON_ALPHA_RESPONSE	87	0.563	1.743	<0.001	<0.0001	0.003	3231

(*) nominal p-values were calculated using GSEA software, which is based on a two-sided permutation-based Kolmogorov-Smirnov-like test

Supplementary Figure 1

A



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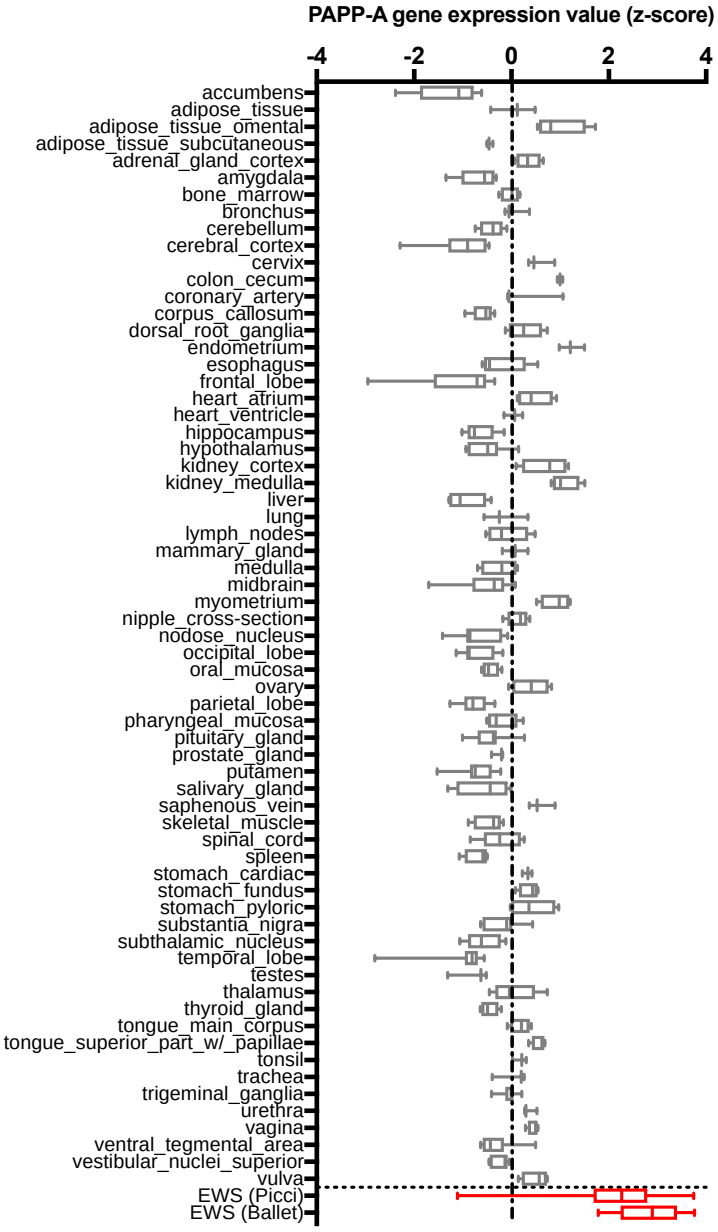

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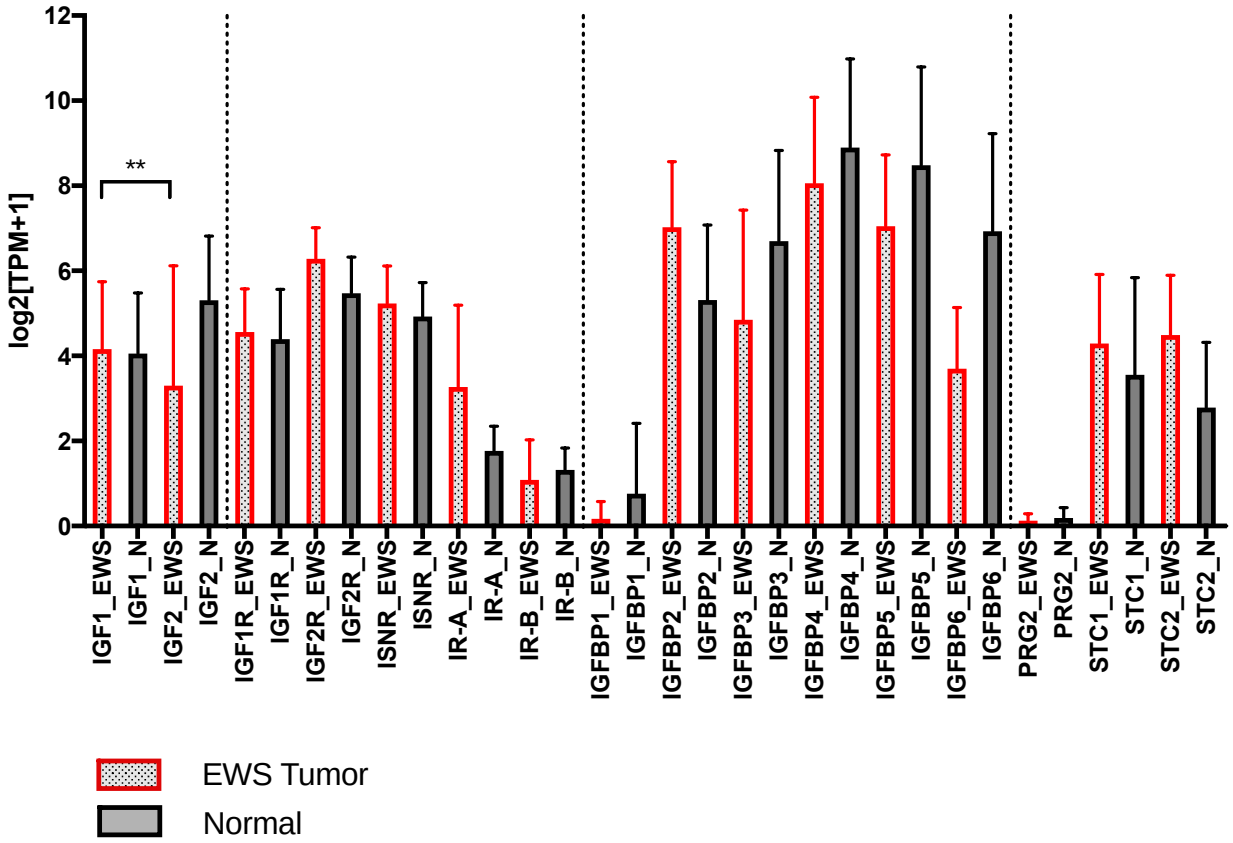
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6
4
2
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IGF1R_N
IGF2R_EWS
IGF2R_N
ISNR_EWS
ISNR_N
IR-A_EWS
IR-A_N
IR-B_EWS
IR-B_N
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IGFBP4_N
IGFBP5_EWS
IGFBP5_N
IGFBP6_EWS
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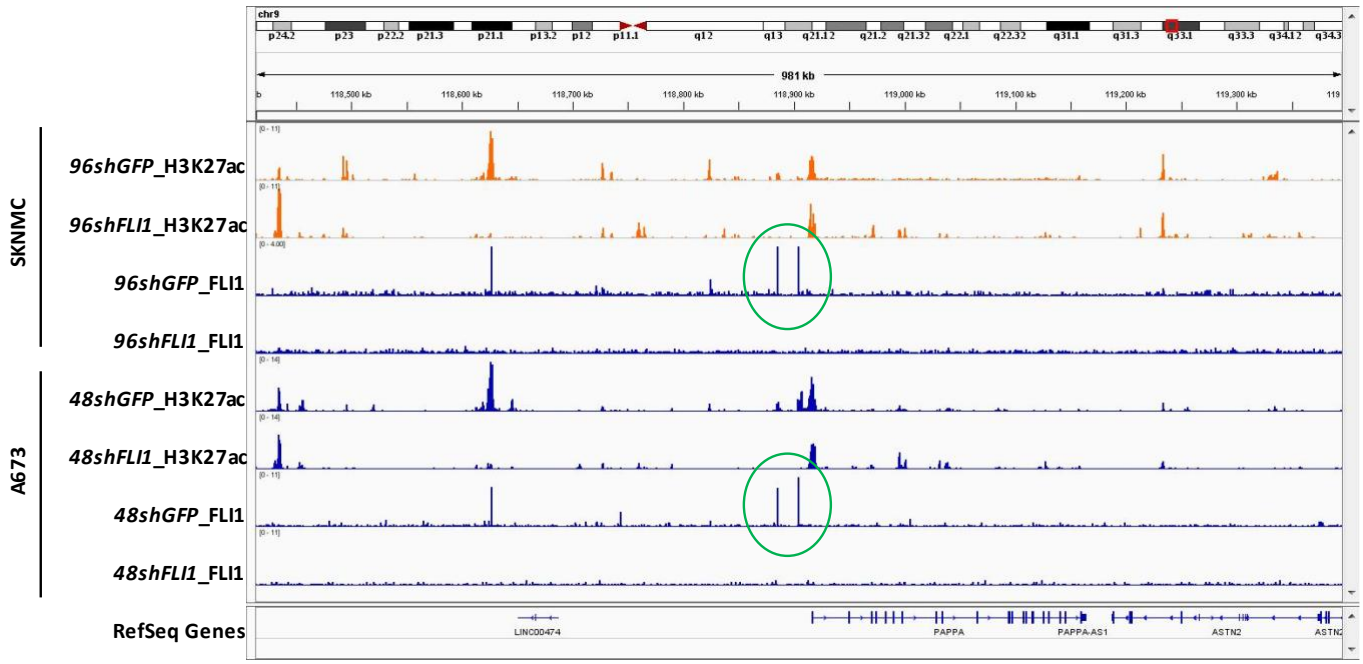
EWS Tumor
Normal

D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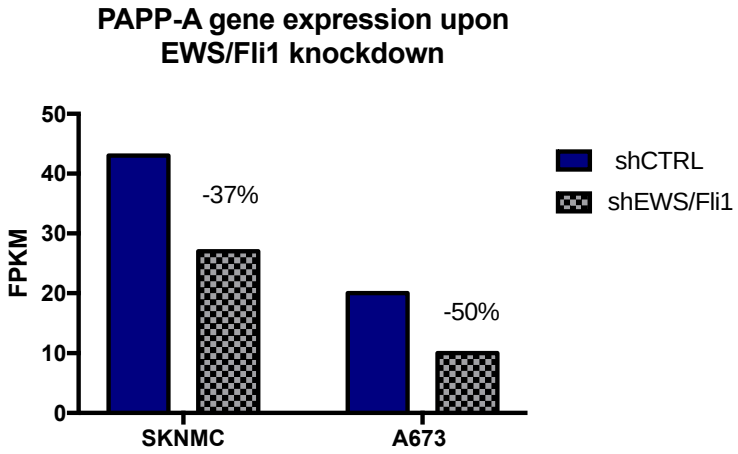
ASTN2

E

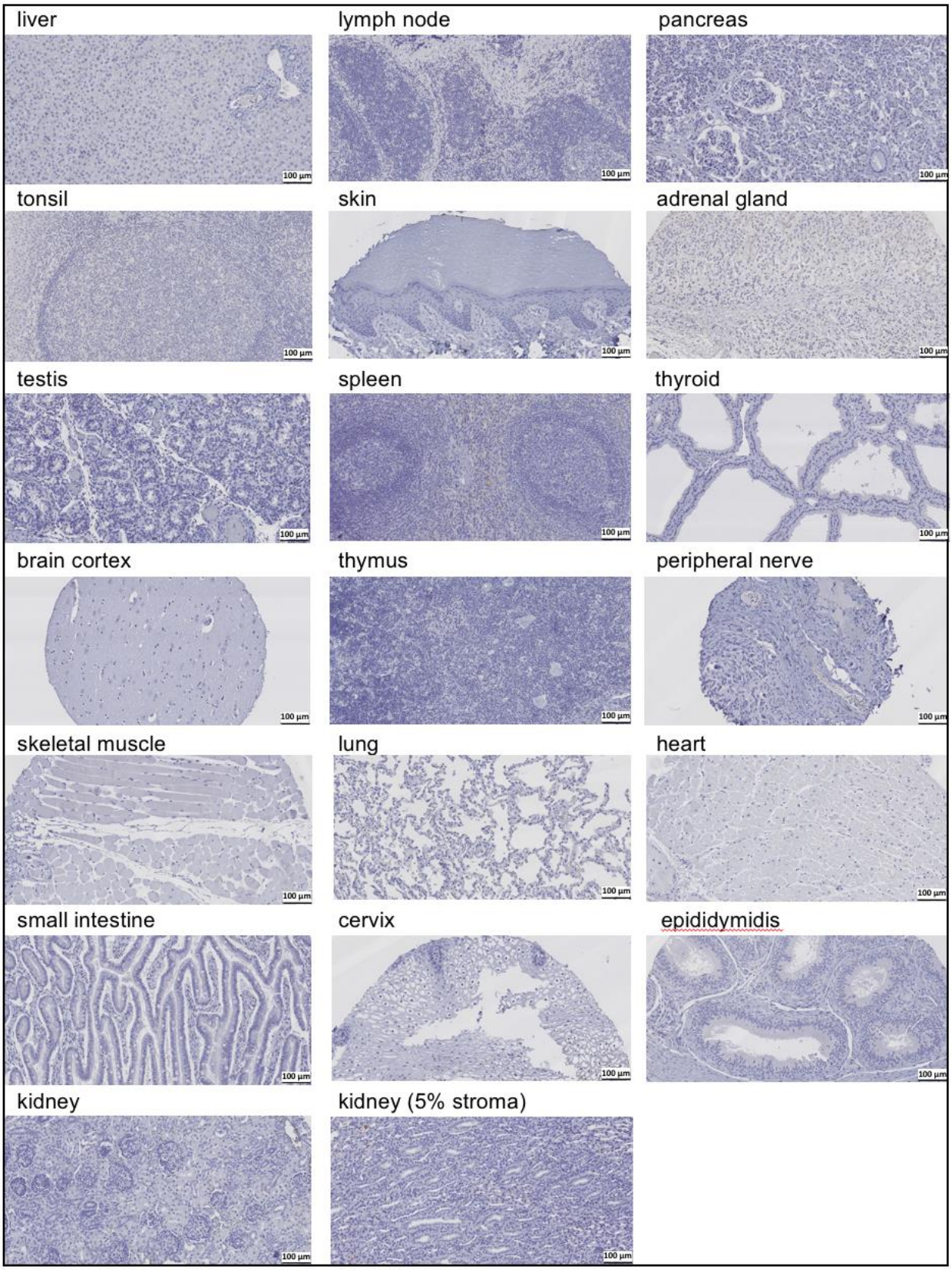


Supplementary Figure 1. Expression of *PAPPA* in the placenta, normal tissues and EWS, expression of genes involved in autocrine IGF signaling loop and role of EWS-Fli1 on *PAPPA* expression

(A) Tissue-enhanced FPKM expression values of *PAPPA* in the placenta (n=1) compared to normal tissue samples (n=31 tissues) according to RNAseq data queried from the Human Protein Atlas [2]. (B) Differential expression of *PAPPA* in EWS compared to normal tissue in 2 Affymetrix datasets derived from the Pediatric cBioPortal for Integrated Childhood Cancer Genomics (Picci et.al, n=37, GSE12102 and Ballet et.al, n=19, GSE37371), values represent z-scores. (C) Gene expression of *IGF/PAPPA* related genes according to in-house generated RNAseq dataset in Normal Tissue (n=42) and EWS (n=120) samples. Values represent log2[TPM+1]±SD. (D) ChIP-seq data queried from Riggi et. al [6] show direct EWS-Fli1 binding site in the putative *PAPPA* promoter identified in A673 and SKNMC cell lines. (E) Gene expression of *PAPPA* (RNAseq) upon shRNA silencing of EWS-Fli1 in both cell lines according to Riggi et.al. Values represent FPKM.

Supplementary Figure 2

A



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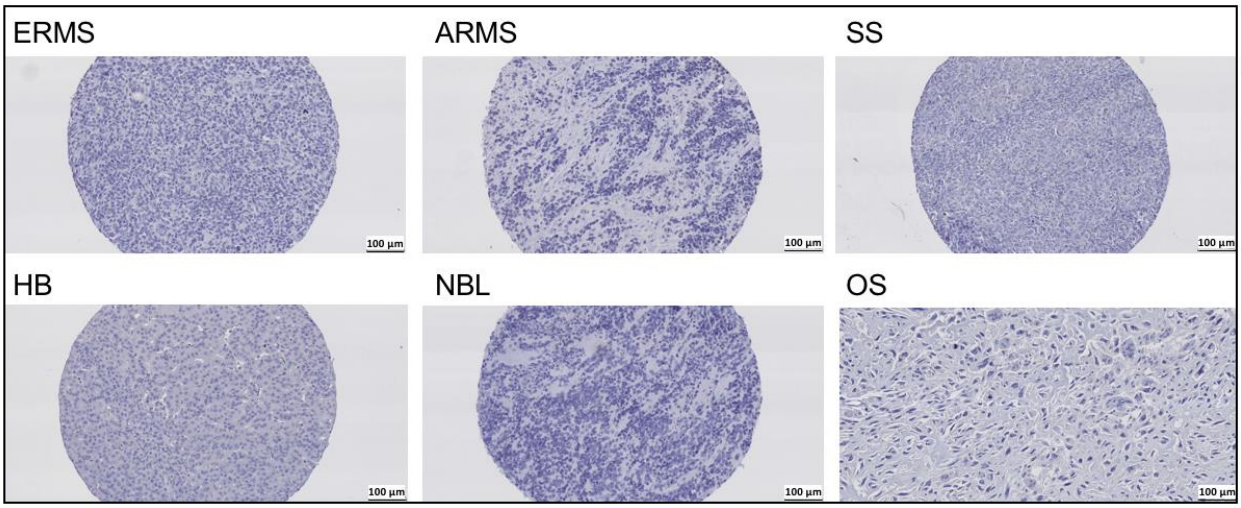

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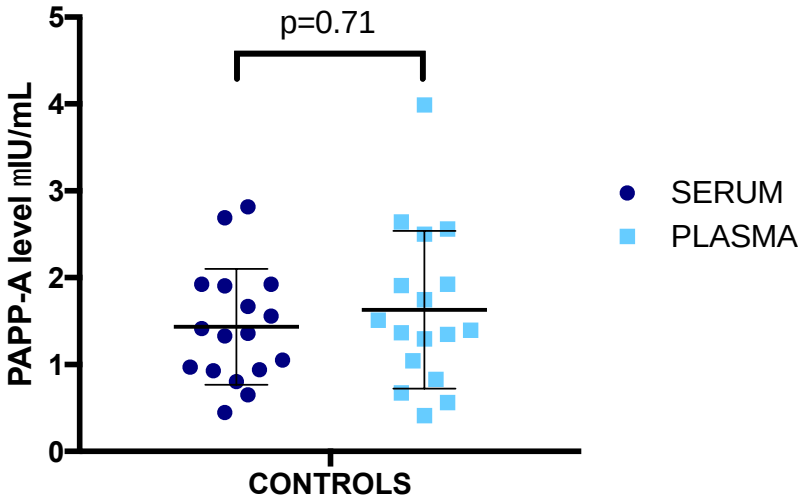
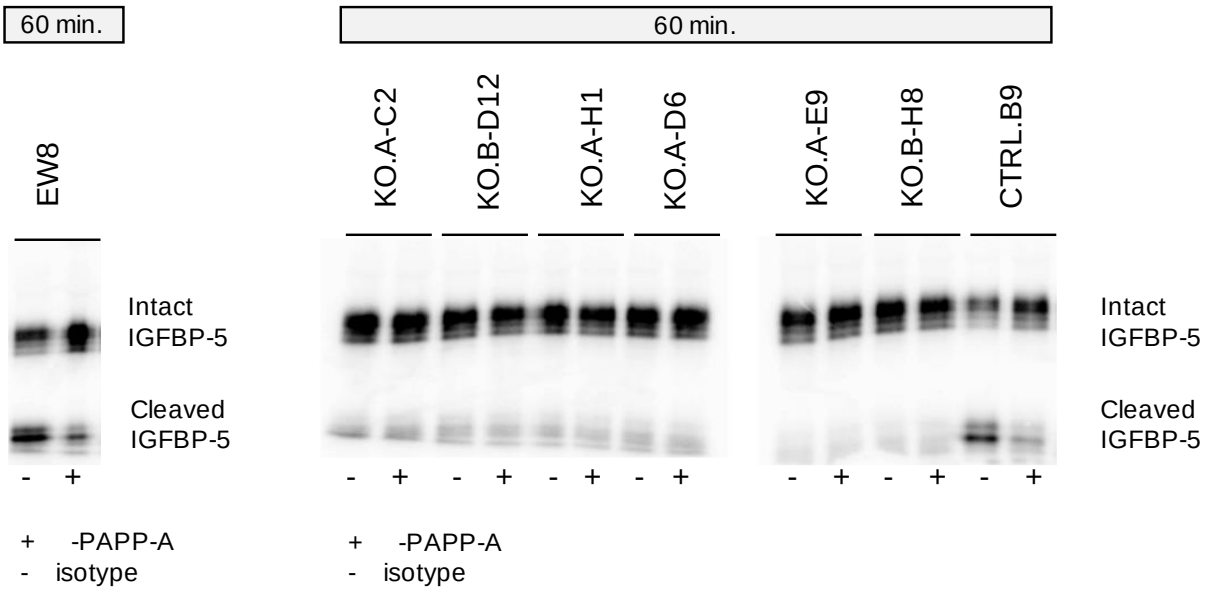


Supplementary Figure 2. PAPP-A tissue expression in pediatric solid tumors and normal tissue and circulating PAPP-A levels in serum compared to plasma.

(A) Representative images of absent PAPP-A staining of pediatric solid tumor TMA's and (B) normal tissue TMA's. Scale bars represent 100 µm. PAPP-A IHC staining (ABS 006-01, Thermo Fisher) was performed on the Venatana Discovery XT automated stainer. (C) Simultanous measurement of PAPP-A serum and plasma levels of normal control donors (n=17) from the same blood draw using picoPAPP-A (AL-101) ELISA kits from AnshLabs (Webster, TX). Values represent mean±SD, p-value according to two-sided Wilcoxon Mann-Whitney U test.

Supplementary Figure 3

A



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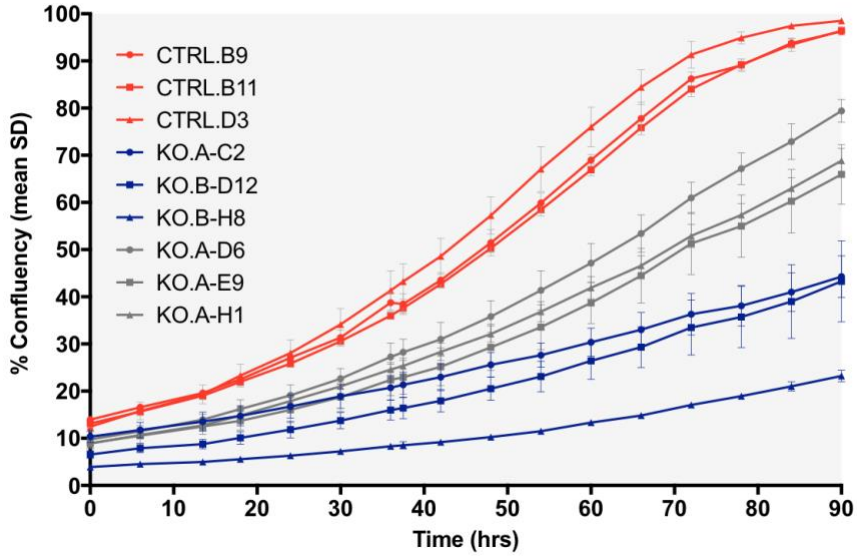

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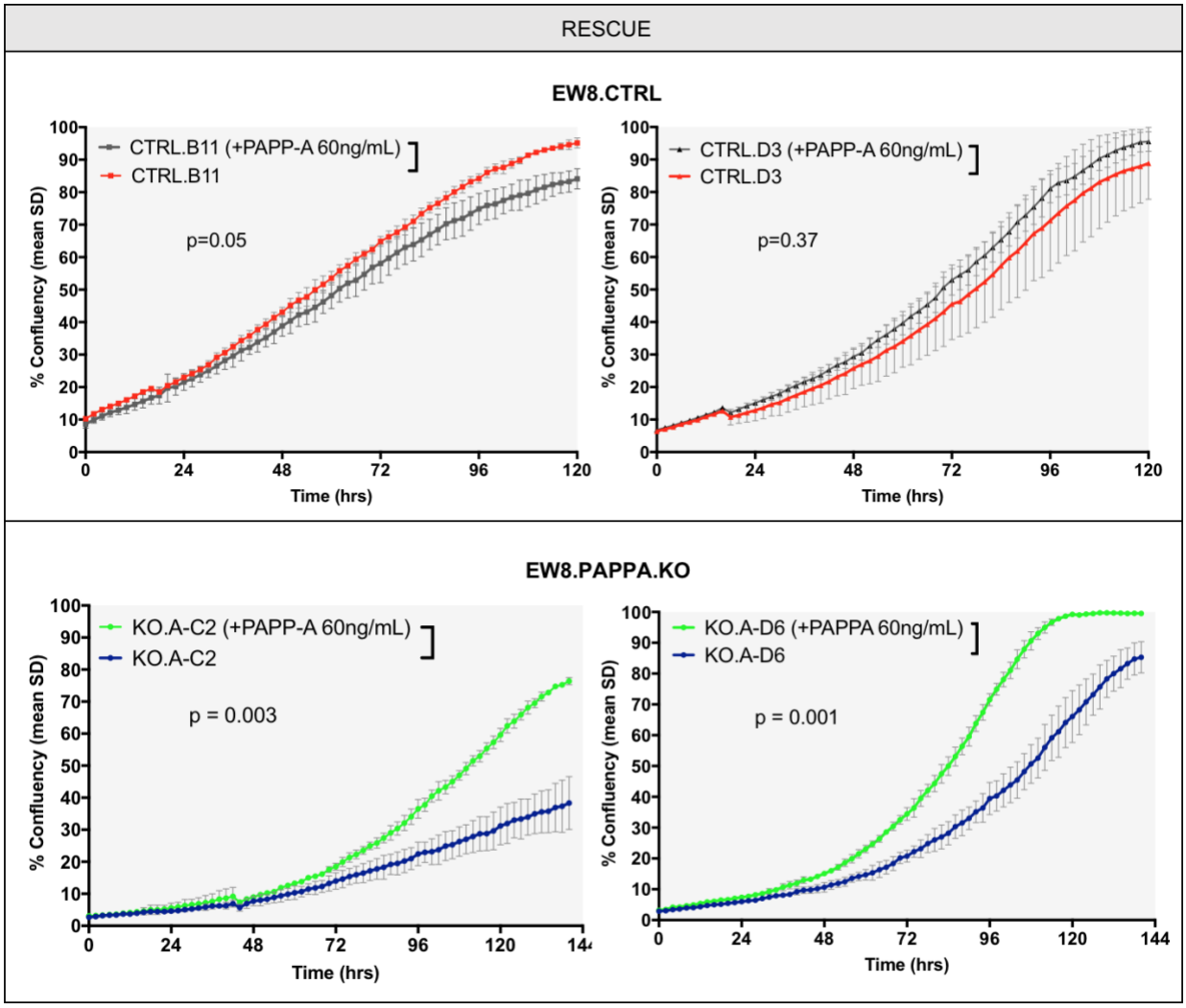
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EW8.CTRL
CTRL.B11 (+PAPP-A 60ng/mL)
CTRL.B11
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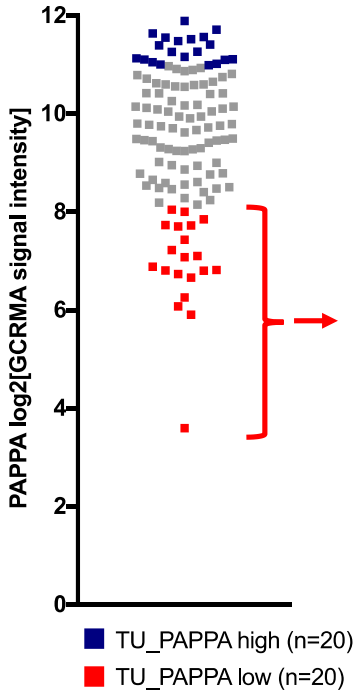
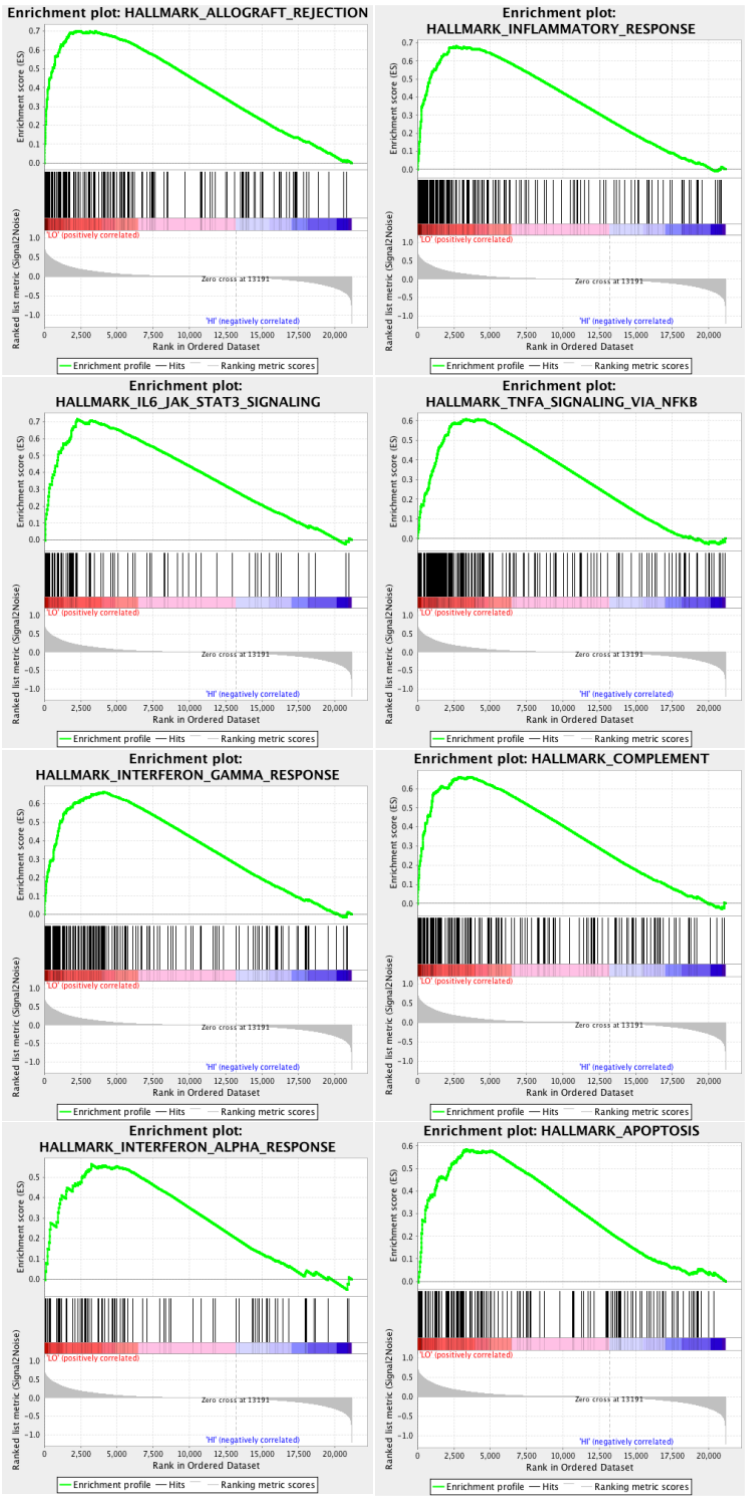
% Confluency (mean SD)
Time (hrs)
CTRL.D3 (+PAPP-A 60ng/mL)
CTRL.D3
p=0.37
% Confluency (mean SD)
Time (hrs)
EW8.PAPPA.KO
KO.A-C2 (+PAPP-A 60ng/mL)
KO.A-C2
p = 0.003
% Confluency (mean SD)
Time (hrs)
KO.A-D6 (+PAPPA 60ng/mL)
KO.A-D6
p = 0.001
% Confluency (mean SD)
Time (hrs)

D


PAPPA log2[GCRMA signal intensity]
12
10
8
6
4
2
0
TU_PAPPA high (n=20)
TU_PAPPA low (n=20)


Enrichment plot: HALLMARK_ALLOGRAFT_REJECTION
Enrichment plot: HALLMARK_INFLAMMATORY_RESPONSE
Enrichment plot: HALLMARK_IL6_JAK_STAT3_SIGNALING
Enrichment plot: HALLMARK_TNFA_SIGNALING_VIA_NFKB
Enrichment plot: HALLMARK_INTERFERON_GAMMA_RESPONSE
Enrichment plot: HALLMARK_COMPLEMENT
Enrichment plot: HALLMARK_INTERFERON_ALPHA_RESPONSE
Enrichment plot: HALLMARK_APOPTOSIS
Enrichment score (ES)
Ranked list metric (Signal2Noise)
'LO' (positively correlated)
'HI' (negatively correlated)
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores

Supplementary Figure 3. Proteolytic activity of native EW8 and EW8.PAPPA.KO/CTRL cell lines towards 125I-IGFBP-5, role of PAPPA.KO on growth of EWS cell lines ex vivo and effect of diminished *PAPPA* expression on enrichment of immune response hallmark pathways (A) Cell culture supernatant (24hrs) of native EW8 cells and EW8.PAPP-A.KO or CTRL.B9 cell lines incubated with either polyclonal rabbit anti-PAPP-A or irrelevant polyclonal rabbit IgG and assessed for proteolysis towards 10nM radiolabeled IGFBP5. Cleavage reactions was stopped at 60 minutes and cleaved IGFBP5 was separated from intact by non-reducing SDS-PAGE. (B) Growth of individual EW8.CTRL clones (n=3) and EW8.PAPPA.KO clones (n=6) over time as measured by confluency in an IncuCyte ZOOM Live Cell Analysis System. (C) Growth of individual EW8.CTRL and EW8.PAPPA.KO clones upon culture +/- 60ng/mL soluble PAPP-A. All clones were plated in triplicates/representative of three individual experiments is shown. Values represent %confluency (mean±SD), p=value for effect of treatment condition according to 2-way RM-ANOVA. (D) Gene set enrichment analysis (GSEA) against the Molecular Signatures Database Hallmark Gene Set Collection of the GEO dataset GSE34620 (Delattre et. al.) showing enriched pathways in *PAPPA*-low (n=20) compared to *PAPPA*-high (n=20) tumors.