**Supplementary Information**

# RANK pathway inhibition sensitizes triple-negative breast cancer to CDK4/6 inhibitors and enhances immune response

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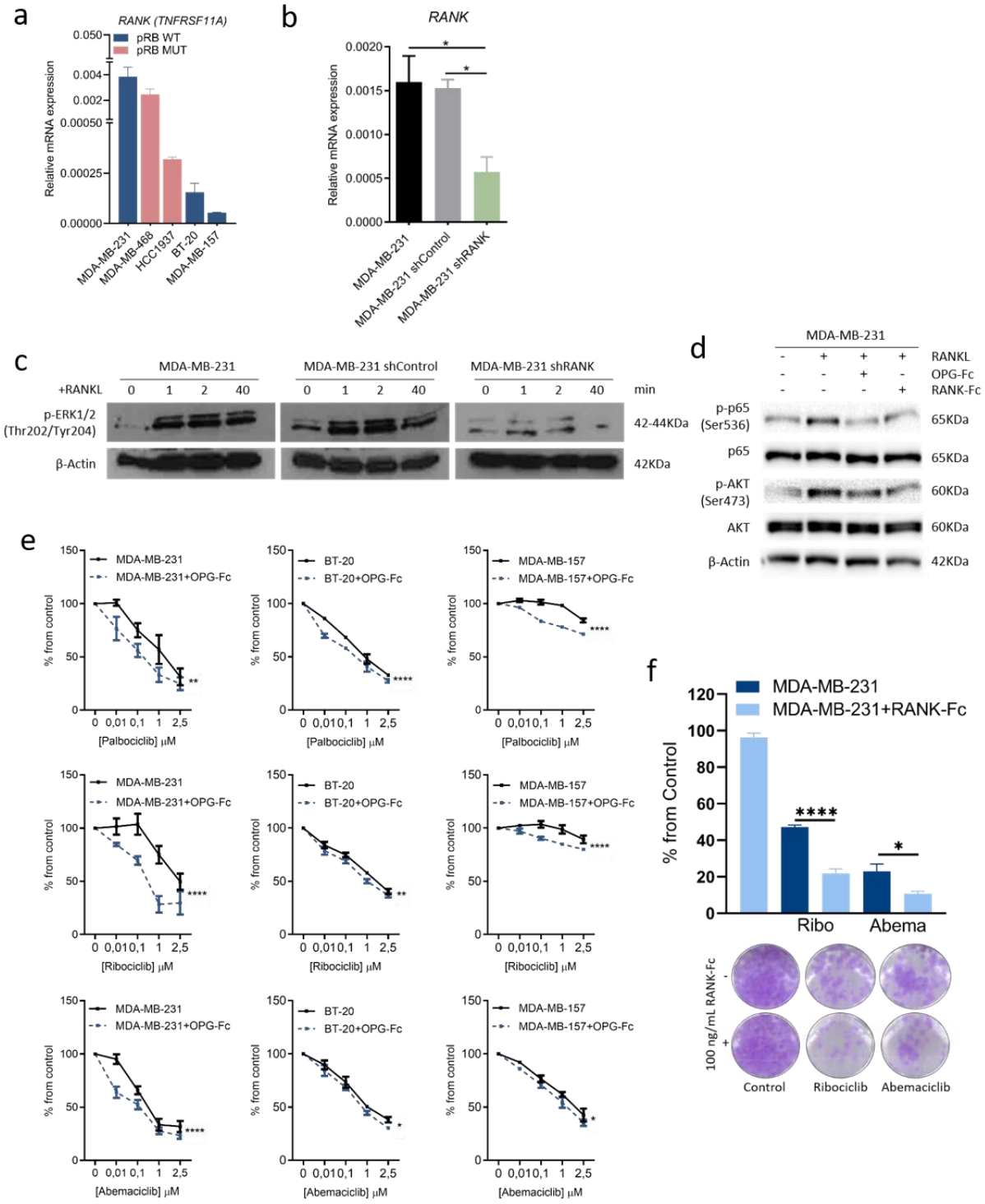
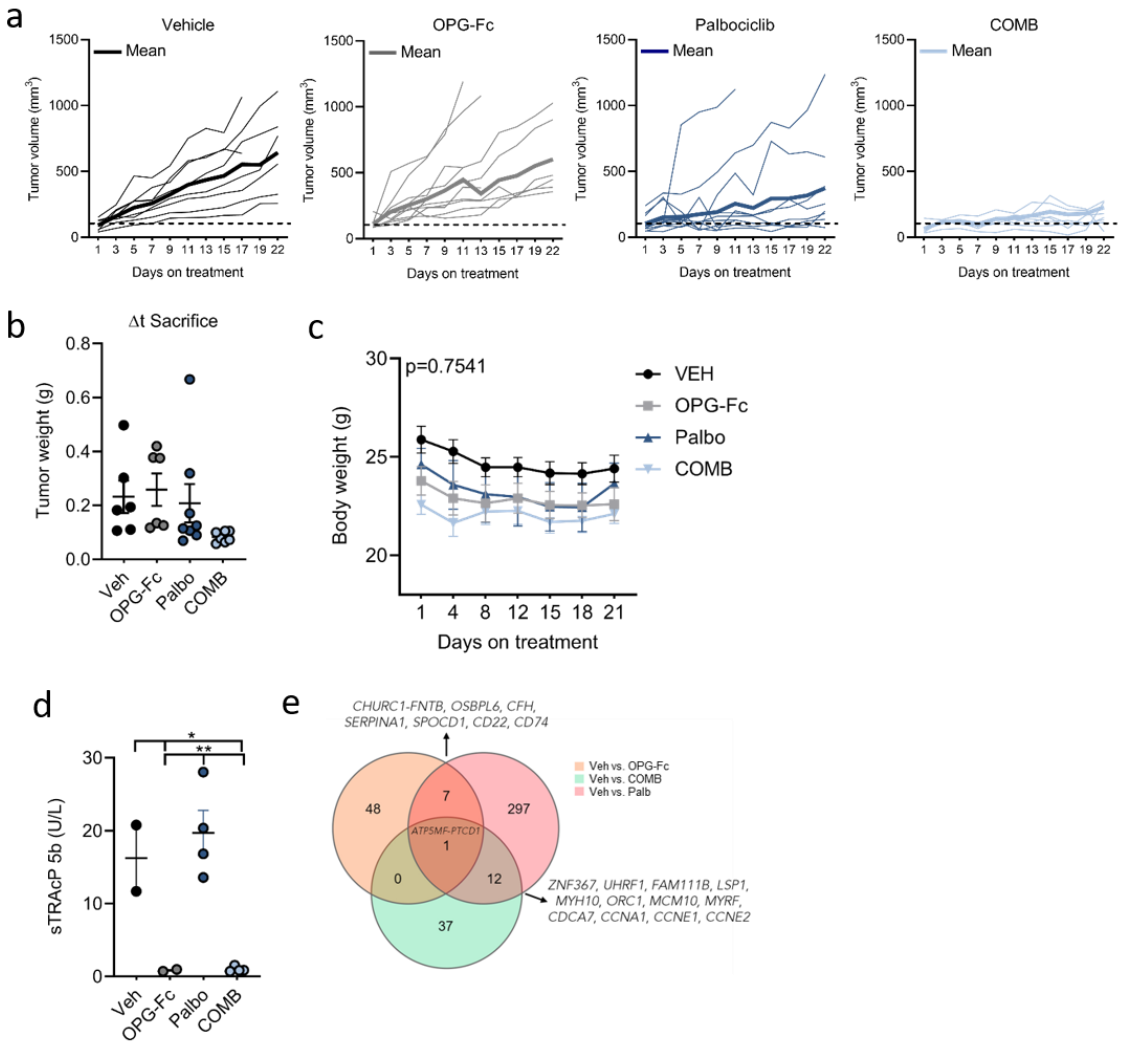
Supplementary information includes three (3) supplementary figures, five (5) tables, and one (1) supplementary reference.

Supplementary Fig. 1 (related to Fig. 1)



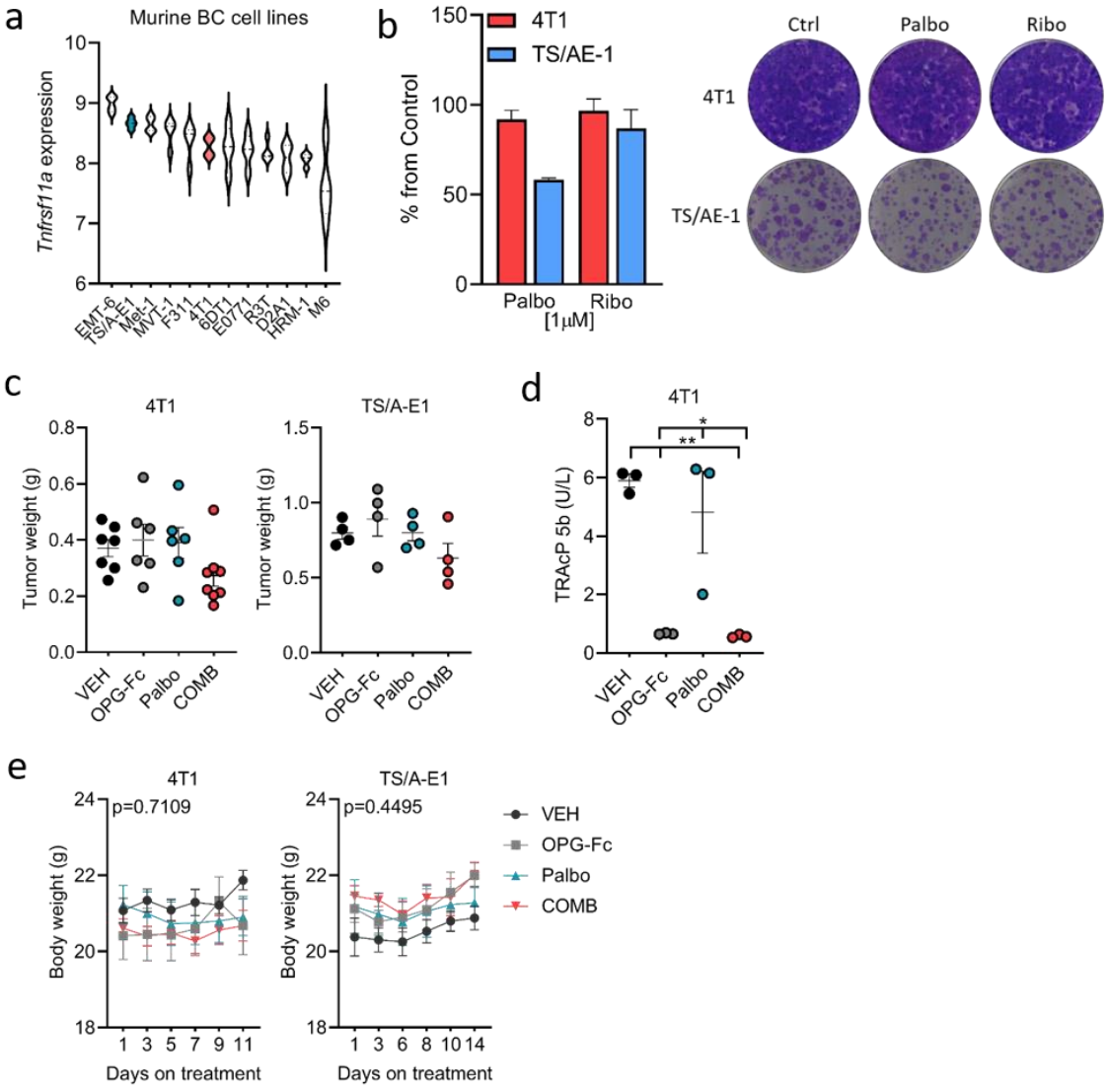
**Supplementary Fig. 1 (related to Fig. 1). RANK pathway inhibition sensitizes TNBC cells to CDK4/6i. a.** RT–qPCR analysis of *RANK* in TNBC cell lines (n=2-3 biological replicates). **b.** RT–qPCR analysis of *RANK* in parental, shControl and shRANK MDA-MB-231 cells (n=3 biological replicates). Western blot analysis of **c.** p-ERK1/2 or **d.** p-p65 and p-AKT after 15 min of stimulation with 1 μg/ml RANKL neutralized or not with 2.5 μg/ml RANKLi (OPG-Fc or RANK-Fc), with β-actin as a loading control. **e.** Viability of the indicated cell lines cultured for 7 days in the presence of increasing concentrations of CDK4/6i ± 100 ng/ml OPG-Fc, as measured by the Alamar blue assay (n=3-4 independent experiments). **f.** Proliferation of the indicated cell lines cultured in the presence of 1 μM CDK4/6i ± 100 ng/ml RANK-Fc for 7 days and in drug-free media for an additional 7 days, as measured by clonogenic assay (n=3-6 independent experiments). The results are presented as the means ± SEMs. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$.

Supplementary Fig. 2 (related to Fig. 2)




**Supplementary Fig. 2 (related to Fig. 2). Palbociclib in combination with OPG-Fc restrains tumor growth *in vivo*. a.** Single tumor growth curves (all treated animals independently of treatment completion, n=4-5 animals, n=8-10 tumors). Tumors were measured externally with a caliper at the indicated time points, and Tvol = 1/2 (length × width$^2$) was calculated. **b.** Tumor weight at sacrifice (n=6-8 tumors). **c.** Mouse body weight during treatment (n=4-5 animals). **d.** Quantification of TRAcP5b in mouse serum by ELISA (n=2-4 samples/group). **f.** Venn diagram depicting the overlap between DEGs of MDA-MB-231 tumors treated with OPG-Fc (n=2), palbociclib (Palbo, n=2) or palbociclib + OPG-Fc (COMB, combination, n=3) in comparison with vehicle (Veh, n=3). The results are presented as the means ± SEMs. *p < 0.05, **p < 0.01.

Supplementary Fig. 3 (related to Fig. 3)




**Supplementary Fig. 3 (related to Fig. 3). Palbociclib in combination with OPG-Fc decreases the growth of palbociclib-resistant tumors *in vivo* and induces an inflammatory response. a.** *Rank* expression in murine BC cell lines retrieved from CCLE (n=4). **b.** Proliferation of the indicated cell lines cultured in the presence of CDK4/6i for 7 days and in drug-free media for an additional 7 days, as measured by a clonogenic assay (n=3-4 independent experiments). **c.** Tumor weight at sacrifice (n=4-8 tumors). **d.** Quantification of TRAcP5b in mouse serum by ELISA (n=3 samples/group). **e.** Mouse body weight during treatment (n=4-5 animals). The results are presented as the means ± SEMs. *p < 0.05, **p < 0.01.

**Supplementary Table 1.** Molecular characteristics of the human cell lines used in this study (from[1]).

| Cell line | pRB status | p53 status | BRCA1 status | PI3K pathway | Other features |
|---|---|---|---|---|---|
| MDA-MB-231 | WT | **Mut** | WT | WT | **KRAS Mut** |
| MDA-MB-468 | **Mut** | **Mut** | WT | **PTEN deletion** | **Amplified EGFR** |
| MDA-MB-157 | WT | **Mut** | WT | WT | - |
| BT-20 | WT | **Mut** | WT | **PIK3CA Mut** | **Amplified EGFR** |
| HCC1937 | **Mut** | **Mut** | **Mut** | **PTEN deletion** | - |

**Supplementary Table 2.** Differentially expressed genes (DEGs) between treated (OPG-Fc n=2; Palbo n=2; COMB n=3) and untreated (Vehicle, n=3) MDA-MB-231 xenografts, ranked according to Log2FC (log2>1 or <-1; FDR q value<0.1). (.xls file)

**Supplementary Table 3.** KEGG pathway and GO_P enrichment analysis of DEGs (NOM p value<0.05; FDR q value<0.25).

| **COMB vs Vehicle** | **Rich Ratio** | **P-val** | **Q-val** |
|---|---|---|---|
| GO_P_DNA replication initiation | 0.2059 | 0.0000 | 0.0000 |
| GO_P_regulation of transcription involved in G1/S | 0.1351 | 0.0000 | 0.0000 |
| GO_P_G1/S transition of mitotic cell cycle | 0.0813 | 0.0000 | 0.0000 |
| KEGG_DNA replication | 0.0612 | 0.0002 | 0.0041 |
| KEGG_Cell cycle | 0.0559 | 0.0000 | 0.0000 |
| GO_P_DNA replication | 0.0529 | 0.0000 | 0.0000 |
| KEGG_Hippo signaling pathway - fly | 0.0349 | 0.0009 | 0.0159 |
| KEGG_Cellular senescence | 0.0209 | 0.0000 | 0.0013 |
| KEGG_Hepatitis B | 0.0192 | 0.0011 | 0.0159 |
| GO_P_cell cycle | 0.0172 | 0.0000 | 0.0000 |
| **Palbo vs Vehicle** | **Rich Ratio** | **P-val** | **Q-val** |
| GO_P_ossification | 0.0756 | 0.0000 | 0.0065 |
| GO_P_positive regulation of protein phosphorylation | 0.0522 | 0.0000 | 0.0087 |
| KEGG_Proteoglycans in cancer | 0.0500 | 0.0000 | 0.0029 |
| KEGG_Platelet activation | 0.0497 | 0.0010 | 0.0391 |
| GO_P_positive regulation of ERK1 and ERK2 cascade | 0.0476 | 0.0000 | 0.0042 |
| KEGG_Hippo signaling pathway | 0.0469 | 0.0007 | 0.0391 |
| KEGG_Oxytocin signaling pathway | 0.0464 | 0.0008 | 0.0391 |
| KEGG_Rap1 signaling pathway | 0.0440 | 0.0002 | 0.0208 |
| GO_P_positive regulation of gene expression | 0.0380 | 0.0000 | 0.0042 |
| GO_P_cell adhesion | 0.0360 | 0.0000 | 0.0004 |
| **OPG-Fc vs Vehicle** | **Rich Ratio** | **P-val** | **Q-val** |
| GO_P_cell-cell junction organization | 0.0667 | 0.0021 | 0.0685 |
| GO_P_heart morphogenesis | 0.0484 | 0.0004 | 0.0685 |
| KEGG_Mucin type O-glycan biosynthesis | 0.0444 | 0.0039 | 0.0760 |
| KEGG_Lysine degradation | 0.0294 | 0.0012 | 0.0475 |
| GO_P_response to wounding | 0.0268 | 0.0021 | 0.0685 |
| KEGG_Complement and coagulation cascades | 0.0254 | 0.0019 | 0.0481 |
| KEGG_Protein digestion and absorption | 0.0190 | 0.0202 | 0.2310 |
| GO_P_leukocyte migration | 0.0190 | 0.0013 | 0.0685 |
| KEGG_Cell adhesion molecules (CAMs) | 0.0179 | 0.0003 | 0.0201 |
| GO_P_cell adhesion | 0.0090 | 0.0018 | 0.0685 |

**Supplementary Table 4.** HALLMARK gene set enrichment analysis (H-GSEA) of mouse xenografts (NES>1 or <-1; NOM p value<0.05; FDR q value<0.25).

| NAME (GSEA_MSigDB_H) | NES | NOM p-val | FDR q-val |
|---|---|---|---|
| **OPG-Fc vs Vehicle** | | | |
| MYOGENESIS | 1.8486 | 0.0000 | 0.0222 |
| OXIDATIVE_PHOSPHORYLATION | 1.7588 | 0.0032 | 0.0225 |
| MITOTIC_SPINDLE | -2.0415 | 0.0000 | 0.0000 |
| UV_RESPONSE_DN | -1.7068 | 0.0000 | 0.0166 |
| G2M_CHECKPOINT | -1.5505 | 0.0000 | 0.0441 |
| EPITHELIAL_MESENCHYMAL_TRANSITION | -1.6935 | 0.0015 | 0.0134 |
| TGF_BETA_SIGNALING | -1.6273 | 0.0062 | 0.0230 |
| INTERFERON_GAMMA_RESPONSE | -1.3436 | 0.0334 | 0.1724 |
| COMPLEMENT | -1.3503 | 0.0374 | 0.1827 |
| PROTEIN_SECRETION | -1.3835 | 0.0541 | 0.1590 |
| NOTCH_SIGNALING | -1.3841 | 0.0794 | 0.1849 |
| **Palbo vs Vehicle** | | | |
| KRAS_SIGNALING_DN | 1.6648 | 0.0052 | 0.0693 |
| INTERFERON_ALPHA_RESPONSE | 1.6323 | 0.0013 | 0.0486 |
| INTERFERON_GAMMA_RESPONSE | 1.4923 | 0.0083 | 0.1374 |
| MYOGENESIS | -2.5068 | 0.0000 | 0.0000 |
| EPITHELIAL_MESENCHYMAL_TRANSITION | -2.0044 | 0.0000 | 0.0000 |
| G2M_CHECKPOINT | -1.9188 | 0.0000 | 0.0032 |
| UV_RESPONSE_DN | -1.8758 | 0.0000 | 0.0041 |
| MTORC1_SIGNALING | -1.8582 | 0.0000 | 0.0043 |
| MITOTIC_SPINDLE | -1.8412 | 0.0000 | 0.0045 |
| HYPOXIA | -1.6111 | 0.0000 | 0.0224 |
| E2F_TARGETS | -1.5513 | 0.0000 | 0.0303 |
| MYC_TARGETS_V2 | -1.7942 | 0.0035 | 0.0057 |
| COMPLEMENT | -1.4712 | 0.0048 | 0.0477 |
| APICAL_JUNCTION | -1.4415 | 0.0066 | 0.0538 |
| ANDROGEN_RESPONSE | -1.5107 | 0.0122 | 0.0386 |
| ANGIOGENESIS | -1.6985 | 0.0137 | 0.0118 |
| MYC_TARGETS_V1 | -1.3243 | 0.0204 | 0.1112 |
| GLYCOLYSIS | -1.3085 | 0.0231 | 0.1171 |
| PROTEIN_SECRETION | -1.3771 | 0.0386 | 0.0814 |
| TNFA_SIGNALING_VIA_NFKB | -1.3006 | 0.0473 | 0.1161 |
| **COMB vs Vehicle** | | | |
| TNFA_SIGNALING_VIA_NFKB | 2.2571 | 0.0000 | 0.0000 |
| IL6_JAK_STAT3_SIGNALING | 1.9851 | 0.0000 | 0.0000 |
| INFLAMMATORY_RESPONSE | 1.8247 | 0.0000 | 0.0018 |
| KRAS_SIGNALING_UP | 1.6661 | 0.0021 | 0.0077 |
| INTERFERON_ALPHA_RESPONSE | 1.5295 | 0.0044 | 0.0240 |
| INTERFERON_GAMMA_RESPONSE | 1.5281 | 0.0000 | 0.0201 |
| EPITHELIAL_MESENCHYMAL_TRANSITION | 1.4724 | 0.0045 | 0.0297 |
| E2F_TARGETS | -3.3527 | 0.0000 | 0.0000 |
| G2M_CHECKPOINT | -3.2092 | 0.0000 | 0.0000 |
| MYC_TARGETS_V1 | -2.5682 | 0.0000 | 0.0000 |
| MYC_TARGETS_V2 | -2.4079 | 0.0000 | 0.0000 |

| | | | |
|---|---|---|---|
| MITOTIC_SPINDLE | -2.1914 | 0.0000 | 0.0000 |
| MTORC1_SIGNALING | -2.1657 | 0.0000 | 0.0000 |
| HEDGEHOG_SIGNALING | -1.8591 | 0.0000 | 0.0000 |
| CHOLESTEROL_HOMEOSTASIS | -1.8318 | 0.0000 | 0.0006 |
| DNA_REPAIR | -1.6785 | 0.0000 | 0.0036 |
| GLYCOLYSIS | -1.6315 | 0.0000 | 0.0056 |
| ESTROGEN_RESPONSE_LATE | -1.3780 | 0.0129 | 0.0830 |
| ESTROGEN_RESPONSE_EARLY | -1.2965 | 0.0357 | 0.1596 |
| **COMB vs Palbo** | | | |
| MYOGENESIS | 2.4929 | 0.0000 | 0.0000 |
| EPITHELIAL_MESENCHYMAL_TRANSITION | 2.3345 | 0.0000 | 0.0000 |
| TNFA_SIGNALING_VIA_NFKB | 2.2558 | 0.0000 | 0.0000 |
| COAGULATION | 1.9064 | 0.0000 | 0.0004 |
| UV_RESPONSE_DN | 1.8419 | 0.0000 | 0.0012 |
| ANGIOGENESIS | 1.8241 | 0.0000 | 0.0019 |
| KRAS_SIGNALING_UP | 1.7915 | 0.0000 | 0.0029 |
| INFLAMMATORY_RESPONSE | 1.7685 | 0.0000 | 0.0033 |
| HYPOXIA | 1.6459 | 0.0000 | 0.0126 |
| APICAL_JUNCTION | 1.5824 | 0.0000 | 0.0195 |
| COMPLEMENT | 1.4857 | 0.0048 | 0.0458 |
| IL6_JAK_STAT3_SIGNALING | 1.5139 | 0.0231 | 0.0380 |
| PROTEIN_SECRETION | 1.3894 | 0.0311 | 0.0923 |
| E2F_TARGETS | -1.99467 | 0 | 0 |

**Supplementary Table 5.** HALLMARK gene set enrichment analysis (GSEA_mh) of mouse allografts (NES>1.5 or <-1.5; NOM p value<0.05; FDR q value<0.25).

| NAME (GSEA_MSigDB_mh) | NES | NOM p-val | FDR q-val |
|---|---|---|---|
| **OPG-Fc vs Vehicle** | | | |
| MYOGENESIS | 3.0980 | 0.0000 | 0.0000 |
| EPITHELIAL_MESENCHYMAL_TRANSITION | 2.6930 | 0.0000 | 0.0000 |
| TGF_BETA_SIGNALING | 1.5477 | 0.0311 | 0.0697 |
| ALLOGRAFT_REJECTION | -2.2742 | 0.0000 | 0.0000 |
| INTERFERON_GAMMA_RESPONSE | -1.7050 | 0.0000 | 0.0148 |
| IL2_STAT5_SIGNALING | -1.5971 | 0.0000 | 0.0427 |
| **Palbo vs Vehicle** | | | |
| MYOGENESIS | 2.3014 | 0.0000 | 0.0000 |
| KRAS_SIGNALING_DN | 1.5724 | 0.0000 | 0.0996 |
| OXIDATIVE_PHOSPHORYLATION | 1.5554 | 0.0000 | 0.0766 |
| TGF_BETA_SIGNALING | 1.5521 | 0.0245 | 0.0575 |
| MYC_TARGETS_V1 | 1.5388 | 0.0000 | 0.0475 |
| ALLOGRAFT_REJECTION | -2.1483 | 0.0000 | 0.0000 |
| INFLAMMATORY_RESPONSE | -1.6229 | 0.0000 | 0.0366 |
| IL2_STAT5_SIGNALING | -1.5614 | 0.0010 | 0.0542 |
| **COMB vs Vehicle** | | | |
| MYOGENESIS | 2.7679 | 0.0000 | 0.0000 |
| OXIDATIVE_PHOSPHORYLATION | 2.5497 | 0.0000 | 0.0000 |
| MYC_TARGETS_V1 | 2.0595 | 0.0000 | 0.0053 |
| ALLOGRAFT_REJECTION | -2.2334 | 0.0000 | 0.0000 |
| IL2_STAT5_SIGNALING | -1.6215 | 0.0000 | 0.0480 |
| INFLAMMATORY_RESPONSE | -1.5296 | 0.0010 | 0.0927 |
| HEDGEHOG_SIGNALING | -1.5252 | 0.0310 | 0.0729 |
| **COMB vs Palbo** | | | |
| E2F_TARGETS | 2.4535 | 0.0000 | 0.0000 |
| OXIDATIVE_PHOSPHORYLATION | 2.1585 | 0.0000 | 0.0000 |
| REACTIVE_OXIGEN_SPECIES_PATHWAY | 1.8138 | 0.0016 | 0.0037 |
| G2M_CHECKPOINT | 1.8109 | 0.0000 | 0.0030 |
| ALLOGRAFT_REJECTION | 1.7786 | 0.0000 | 0.0040 |
| INFLAMMATORY_RESPONSE | 1.7529 | 0.0000 | 0.0045 |
| EPITHELIAL_MESENCHYMAL_TRANSITION | 1.6464 | 0.0014 | 0.0133 |
| DNA_REPAIR | 1.6415 | 0.0015 | 0.0119 |
| MYC_TARGETS_V1 | 1.6153 | 0.0028 | 0.0132 |
| XENOBIOTIC_METABOLISM | 1.5810 | 0.0000 | 0.0164 |
| SPERMATOGENESIS | 1.5364 | 0.0030 | 0.0234 |
| IL6_JAK_STAT3_SIGNALING | 1.5310 | 0.0166 | 0.0225 |

| | | | |
|---|---|---|---|
| KRAS_SIGNALING_UP | 1.5292 | 0.0000 | 0.0213 |
| ESTROGEN_RESPONSE_EARLY | -2.1168 | 0.0000 | 0.0000 |
| HEDGEHOG_SIGNALING | -1.8738 | 0.0000 | 0.0022 |
| UV_RESPONSE_DN | -1.8054 | 0.0000 | 0.0020 |
| TGF_BETA_SIGNALING | -1.7884 | 0.0000 | 0.0027 |
| ESTROGEN_RESPONSE_LATE | -1.7565 | 0.0000 | 0.0026 |
| WNT_BETA_CATENIN_SIGNALING | -1.6935 | 0.0052 | 0.0045 |
| APICAL_SURFACE | -1.6601 | 0.0053 | 0.0048 |
| INTERFERON_ALPHA_RESPONSE | -1.6533 | 0.0000 | 0.0046 |

## Supplementary References


1 Chavez, K. J., Garimella, S. V. & Lipkowitz, S. Triple negative breast cancer cell lines: one tool in the search for better treatment of triple negative breast cancer. *Breast disease* **32**, 35-48, doi:10.3233/bd-2010-0307 (2010).