

## **Supplementary Figures and Tables**

### **GM-CSF signaling is critical for HER2+ breast leptomeningeal carcinomatosis**

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**Supplementary Table 1: Proteins commonly present in media from mono-cultured OPCs and OPCs co-cultured with Lepto1 cells.** Secreted proteins are shown in green.

| Enrichment Rank | Gene          | Protein Name  | Enables  | Function  | Localization                             |
|-----------------|---------------|---|--|---|--|
| 1               | <i>CGREF1</i> | Cell growth regulator with EF hand domain protein 1 isoform         | Calcium binding, metal ion binding   | Cell cycle arrest, cell adhesion, negative regulation of cell proliferation                                   | Extracellular region                     |
| 2               | <i>ENO1</i>   | $\alpha$ -Enolase isoform 1   |  |   | Cytosol, extracellular exosomes,         |
|                 | <i>CLSTN1</i> | Calsyntenin-1 isoform X1  | Calcium protein binding,   | Regulation of cell growth, cell adhesion  | ER, Cell membrane, extracellular exosome |
| 3               | <i>PTPRZ1</i> | Receptor-type tyrosine-protein phosphatase zeta isoform 1 precursor | Protein tyrosine phosphatase activity, protein binding, hydrolase, phosphatase | Hematopoietic progenitor cell differentiation, protein dephosphorylation, cytokine-mediated signaling pathway | Extracellular matrix                     |
| 4               | <i>TRRAP</i>  | Transformation/transcription domain-associated protein isoform 1    |  |   |  |
| 5               | <i>NIPBL</i>  | Nipped-B-like protein isoform A                                     |  |   |  |
| 6               | <i>CENPF</i>  | Centromere protein F  |  |   |  |
| 7               | <i>SPARC</i>  | SPARC isoform 1 precursor   | Protein binding, Calcium binding, metal ion binding,                           | Negative regulation of cell proliferation, signal transduction  | Extracellular region, basement membrane  |
| 8               | <i>SEZ6L</i>  | Seizure 6-like protein isoform 4 precursors                         |  | Regulation of protein kinase C signaling  | ER                                       |
| 9               |               | Utrophin isoform X1   |  |   | Cytoskeleton                             |
| 10              | <i>MAP1B</i>  | Microtubule-associated protein 1B                                   |  |   | Cytoskeleton                             |
| 11              | <i>NCAM2</i>  | Neural cell adhesion  | Protein binding  | Cell adhesion   | Cell membrane                            |

|    |                |  |                   |  |  |
|----|----------------|--|-------------------|--|--|
|    |                | molecule 2 precursors                                |                   |  |  |
| 12 | <i>SPTBN1</i>  | Spectrin beta chain, non-erythrocytic isoform 1      | Protein binding   | MAPK cascade   | Cell membrane, extracellular exosomes                            |
| 13 | <i>CMYA5</i>   | Cardiomyopathy-associated protein 5                  |                   |  | cytoplasm, nucleus   |
| 14 | <i>EPHA4</i>   | Ephrin type-A receptor isoform a precursor           |                   |  | Cell membrane  |
| 15 | <i>PHF14</i>   | PHD finger protein isoforms X1                       | Metal ion binding | Negative regulation of cell proliferation, negative regulation of mesenchymal cell proliferation | Nucleus  |
| 16 | <i>PPP1R9A</i> | Neurabin-1 isoform X1                                |                   |  |  |
| 17 | <i>TUBB2B</i>  | Tubulin beta-2B chain                                |                   |  |  |
| 18 | <i>NEGR1</i>   | Neuronal growth regulator precursor                  |                   |  | Extracellular (EC) region, Extracellular exosomes, Cell membrane |
| 19 | <i>PLXDC2</i>  | Plexin domain-containing protein isoform 1 precursor |                   |  | EC exosome   |
| 20 | <i>GFRA2</i>   | GDNF family receptor alpha-2 isoform X2              |                   |  | plasma membrane  |
| 21 | <i>LSS</i>     | Lanosterol synthase isoform 2                        |                   |  | ER, membrane   |
| 22 | <i>COL4A1</i>  | Collagen alpha-1(IV) chain preproprotein             |                   |  | ER, lumen  |
| 23 | <i>GDII</i>    | Rab GDP dissociation inhibitor alpha                 |                   |  | Cytosol, extracellular exosomes, membrane                        |
| 24 | <i>CKB</i>     | Creatine kinase B-type                               |                   |  | Cytosol, extracellular exosomes,                                 |
| 25 | <i>GPLD1</i>   | Phosphatidylinositol-glycan-specific                 |                   |  | Extracellular region, extracellular exosomes,                    |

|    |                 |   |  |                                       |  |
|----|-----------------|---|--|---------------------------------------|--|
|    |                 | phospholipase D precursor                                     |  |                                       |  |
| 26 | <i>YWHAQ</i>    | 14-3-3 protein theta  |  |                                       | Cytosol, extracellular exosomes                            |
| 27 | <i>ZNF75D</i>   | Zinc finger protein 75D isoform 2                             |  |                                       |  |
| 28 | <i>NTRK2</i>    | BDNF/NT-3 growth factors receptor isoform c precursor         |  |                                       | Membrane, cytosol  |
| 29 | <i>TPP1</i>     | Tripeptidyl-peptidase 1 preproprotein                         |  |                                       | Extracellular exosomes                                     |
| 31 |                 | Agrin isoform X1  |  |                                       | Plasma membrane  |
| 32 | <i>SOD3</i>     | Extracellular superoxide dismutase [Cu-Zn] precursor          | Protein zinc heparin binding           | Oxidation-reduction process           | Extracellular matrix, extracellular exosome                |
| 33 | <i>VIM</i>      | Vimentin isoform X1   | Protein binding                        | cytokine-mediated signaling           | Cytosol  |
| 34 | <i>LRP2</i>     | Low-density lipoprotein receptor-related protein 2 precursors | protein, metal SH3 domain binding      | Endocytosis                           | Cell membrane, ER  |
| 35 | <i>NEGR1</i>    | Neuronal growth regulator 1 precursor                         | Protein binding                        | Cell-cell adhesion                    | Cell membrane, extracellular region, extracellular exosome |
| 36 | <i>HIST1H1D</i> | Histone H1.3  |  |                                       |  |
| 37 | <i>HNRNPA B</i> | Heterogeneous nuclear ribonucleoprotein A/B isoform b         |  |                                       |  |
| 38 | <i>DPYSL3</i>   | Dihydropyrimidinase-related protein 3 isoforms 1              | SH3 domain binding, hydrolase activity | Negative regulation of cell migration | Cytosol  |

**Supplementary Table 2: Summary of survival analyses of all the *in vivo* experiments with Lepto1 lines**

| Figure | Number of mice | Survival in months | Treatments            | Injection site  | Cell lines tested | No. of Lepto1 injected on Day 0 | Number of cells injected (Day 7 or 14) |
|--------|----------------|--------------------|-----------------------|-----------------|-------------------|---------------------------------|--|
| 1C     | 12             | 25                 | NA                    | Cisterna magna  | Lepto1            | 100K                            | NA                                     |
| 1C     | 12             | 44                 | NA                    | Cisterna magna  | 231-BR            | 100K                            | NA                                     |
| 1F     | 12             | 30                 | NA                    | Cerebral cortex | Lepto1            | 100K                            | NA                                     |
| 1F     | 12             | >50                | NA                    | Brain stem      | Lepto1            | 100K                            | NA                                     |
| 2G     | 6              | 28                 | HEK293 Control        | Cisterna magna  | Lepto1            | 100K                            | NA                                     |
| 2G     | 6              | 42                 | OPCs                  | Cisterna magna  | Lepto1            | 100K                            | 100K, OPCs on D7                       |
| 2G     | 6              | >56                | OPCs                  | Cisterna magna  | Lepto1            | 100K                            | OPCs on D14                            |
| 3I     | 6              | 28                 | Control               | Cisterna magna  | Lepto1            | 100K                            | NA                                     |
| 3I     | 6              | 44                 | Anti-GM-CSF           | Cisterna magna  | Lepto1            | 100K                            | NA                                     |
| 4I     | 6              | 26                 | Control               | Cisterna magna  | Lepto1            | 100K                            | NA                                     |
| 4I     | 6              | 48                 | OPCs                  | Cisterna magna  | Lepto1            | 100K (D7)                       | 100K, OPCs on D7                       |
| 4I     | 6              | 60                 | OPCs                  | Cisterna magna  | Lepto1            | 200K (D7)                       | 200K, OPCs on D7                       |
| 5I     | 9              | 26                 | Control               | Cisterna magna  | Lepto1            | 100K                            | NA                                     |
| 5I     | 9              | 36                 | Anti-GM-CSF           | Cisterna magna  | Lepto1            | 100K                            | NA                                     |
| 5I     | 9              | 49                 | Anti-GM-CSF+CCT137690 | Cisterna magna  | Lepto1            | 100K                            | NA                                     |

NA = not applicable

**Resource Table 1: Commercially available reagents.**

| <b>Reagent or Resource</b>           | <b>Identifier</b>  | <b>Supplier</b>                   |
|--------------------------------------|--------------------|-----------------------------------|
| <b>Compounds</b>                     |                    |                                   |
| CCT137690                            | 199596-05-9        | Sigma-Aldrich                     |
| Bosutinib                            | 28097-03-2         | Sigma-Aldrich                     |
| TPP1                                 | 33419-42-0         | Selleckchem                       |
| Doxycycline                          | 31430-18-9         | Selleckchem                       |
| <b>FACS Antibodies</b>               |                    |                                   |
| Annexin V-FITC                       | 640905             | Biolegend                         |
| DAPI                                 | D9542-1MG          | Sigma-Aldrich                     |
| EpCAM-PE                             | 324205             | Biolegend                         |
| CD49f-APC                            | 313615             | Biolegend                         |
| CD116 (GM-CSFR $\alpha$ )-PE         | 305908             | Biolegend                         |
| NG2                                  | 867701             | Biolegend                         |
| HepaCAM                              | ab236659           | Abcam                             |
| CD90                                 | 206105             | Biolegend                         |
| GALC                                 | ab240638           | Abcam                             |
| CD45                                 | 368511             | Biolegend                         |
| GM-CSFR $\alpha$                     | PA5-37358          | Thermo Fisher Scientific          |
| Anti-GM-CSF antibodies               | 404950-80-7        | Selleckchem                       |
| <b>siRNA/Assay (for human genes)</b> |                    |                                   |
| siTPP1                               | AM16708            | Thermo Fisher Scientific          |
| siGAPDH                              | AM4624             | Thermo Fisher Scientific          |
| siGFP                                | AM4626             | Thermo Fisher Scientific          |
| siLUC                                | 12935146           | Thermo Fisher Scientific          |
| X-tremeGENE                          | 4476093001         | Sigma-Aldrich                     |
| <b>Western Blot Antibodies</b>       |                    |                                   |
| Mouse Anti-GM-CSF                    | AB22258            | Abcam                             |
| Rabbit Anti- GM-CSFR $\alpha$        | Ab133678           | Abcam                             |
| Rabbit Anti-pAKT                     | Ab38449            | Abcam                             |
| AKT                                  | Ab8805             | Abcam                             |
| pSTAT5                               | Ab32364            | Abcam                             |
| STAT5                                | Ab230670           | Abcam                             |
| Tubulin                              | T9026-100UL        | Sigma-Aldrich                     |
| pmTOR                                | 09-213-25UG        | Sigma-Aldrich                     |
| mTOR                                 | SAB4501038-100UG   | Sigma-Aldrich                     |
| pro-Caspase 3 Antibody               | MA1-41163          | Thermo Fisher Scientific          |
| Goat Anti-Rabbit IgG H&L (HRP)       | Ab97051            | Abcam                             |
| Goat Anti-Mouse IgG H&L (HRP)        | Ab205719           | Abcam                             |
| <b>TaqMan Primers (Human Gene)</b>   |                    |                                   |
| TPP1 (FAM-dye)                       | Hs.PT.58.39543106  | Integrated DNA Technologies (IDT) |
| GAPDH (FAM-dye)                      | Hs.PT.39a.22214836 | IDT                               |

|                                      |                           |        |
|--------------------------------------|---------------------------|--------|
| $\beta$ -Actin (HEX-dye)             | Hs.PT.39a.22214847        | IDT    |
| GAPDH (HEX-dye)                      | Hs.PT.39a.22214836        | IDT    |
| $\beta$ -Actin (FAM-dye)             | Hs.PT.39a.22214847        | IDT    |
| RNA extraction kit                   | K1341050                  | Qiagen |
| cDNA generation kit                  | 205311                    | Qiagen |
| PrimeTime Gene Expression Master Mix | 1055770                   | IDT    |
| <b>Primary Cell Lines Generated</b>  | <b>Derivation</b>         |        |
| Lepto1                               | HER2+ LC patient 1        |        |
| Lepto2                               | HER2+ LC patient 2        |        |
| Lepto1-GM-CSF-IRES-ZSGREEN1          | DOX-inducible lentivector |        |
| Lepto1-mCherry-LUC                   | mCherry-LUC transduction  |        |
| Lepto2-mCherry-LUC                   |                           |        |

**Resource Table 2: Media cocktails.**

| <b>Lepto1 and Lepto2 (no DOX)</b>   | <b>Volume Added</b> | <b>Final Concentration</b> |
|-------------------------------------|---------------------|----------------------------|
| Advanced DMEM/F-12                  | 450 mL              |                            |
| FBS                                 | 25 mL               | 5%                         |
| 200 mM L-glutamine (100x)           | 6 mL                | 2 mM                       |
| Sodium pyruvate                     | 6 mL                | 1X                         |
| MEM-NEAA                            | 6 mL                | 1X                         |
| Insulin-transferrin-selenium (100x) | 6 mL                | 1x                         |
| 450 mmol/L 1-Thioglycerol solution  | 600 $\mu$ L         | 450 $\mu$ M                |
| 50 mg/mL Ascorbic acid solution     | 600 $\mu$ L         | 50 $\mu$ g/mL              |
| Human CSF                           | Variable            | 50%                        |
| <b>Lepto1 and Lepto2 (DOX)</b>      |                     |                            |
| Advanced DMEM/F-12                  | 450 mL              |                            |
| FBS                                 | 25 mL               | 5%                         |
| 200 mM L-glutamine (100x)           | 6 mL                | 2 mM                       |
| Insulin-transferrin-selenium (100x) | 6 mL                | 1x                         |
| Sodium pyruvate                     | 6 mL                | 1X                         |
| MEM-NEAA                            | 6 mL                | 1X                         |
| 450 mM 1-Thioglycerol solution      | 600 $\mu$ L         | 450 $\mu$ M                |
| 50 mg/mL Ascorbic acid solution     | 600 $\mu$ L         | 50 $\mu$ g/mL              |
| Human CSF                           | Variable            | 50–100%                    |
| 5 mg/mL Doxycycline solution        | 600 $\mu$ L         | 5 $\mu$ g/mL               |
| 10 mM Bosutinib                     | When needed         | 10 $\mu$ M                 |
| 10 mM CCT137690                     | When needed         | 0.5 or 1 $\mu$ M           |

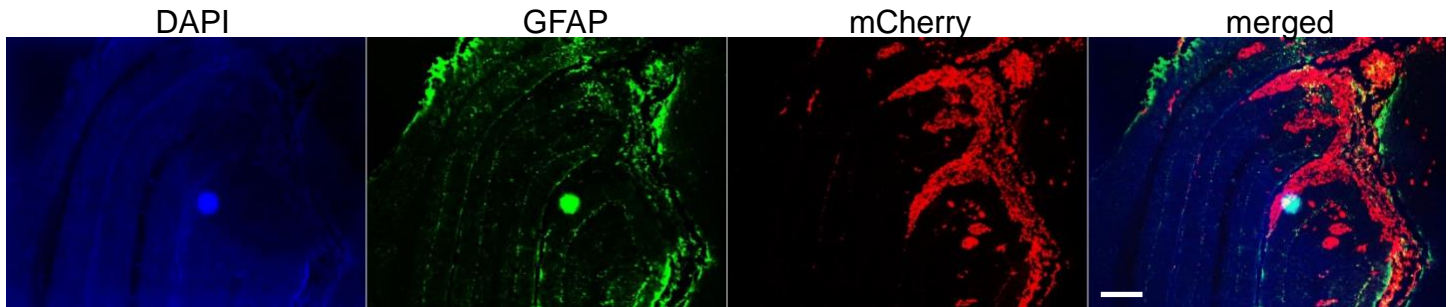
**Resource Table 3: Compounds identified through Lepto1 studies.**

| <b>Compound</b>                     | <b>Mode of Action</b>   |
|-------------------------------------|---|
| CCT137690                           | Pan selective Aurora kinase inhibitor                               |
| Bosutinib                           | SRC kinase inhibitor  |
| TPP1                                | Lysosomal serine protease activity; Proteolytically degrades GM-CSF |
| Anti-GM-CSF neutralizing antibodies | Prevents GM-CSF binding to GM-CSFR $\alpha$ , Competitive inhibitor |

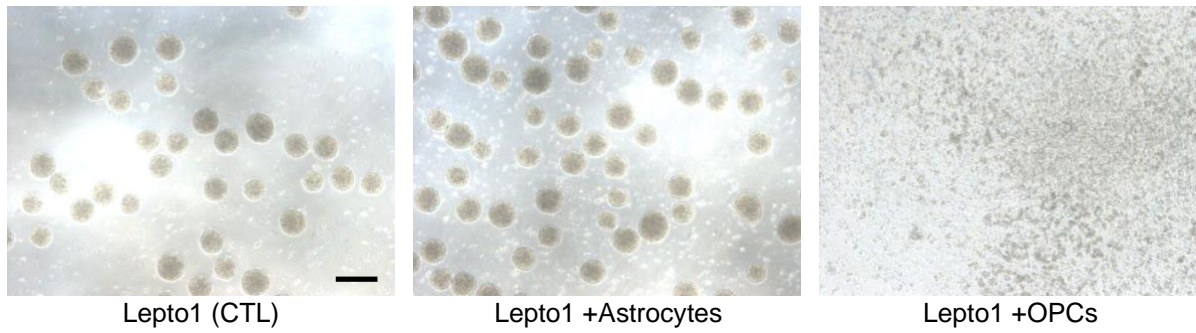


Supplementary Figures  
Supplementary Figure 1

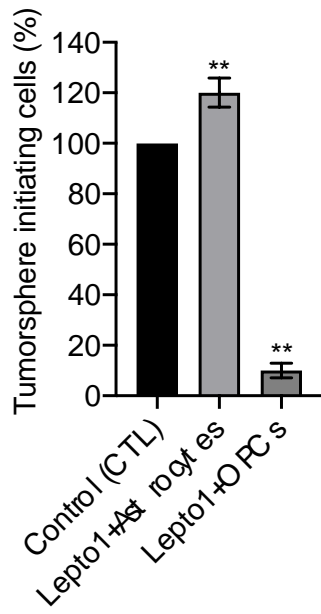
A



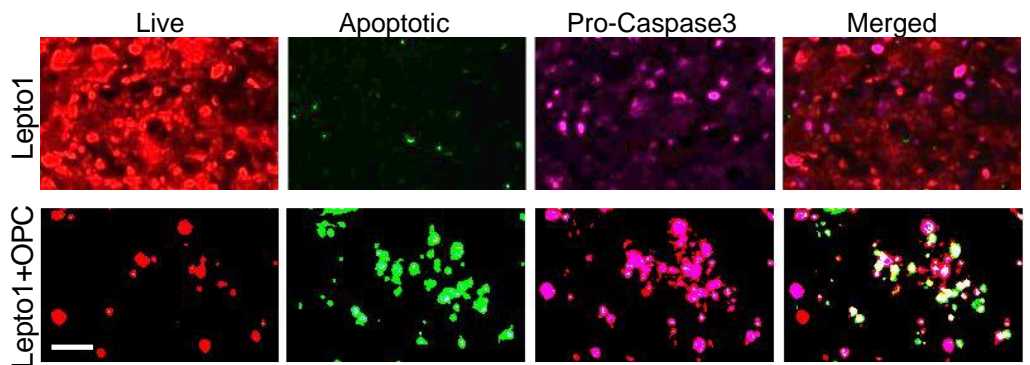
B



C

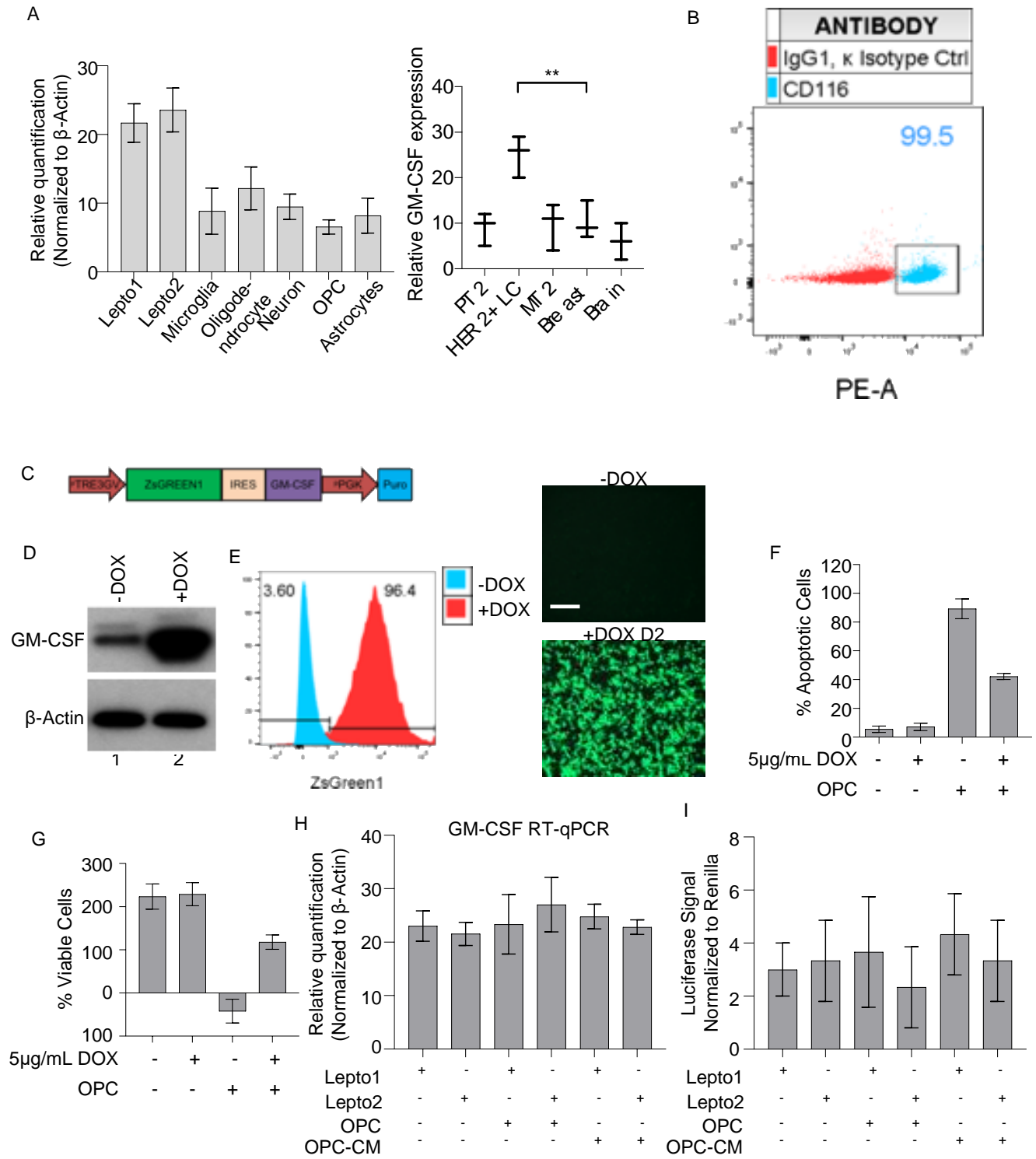


D



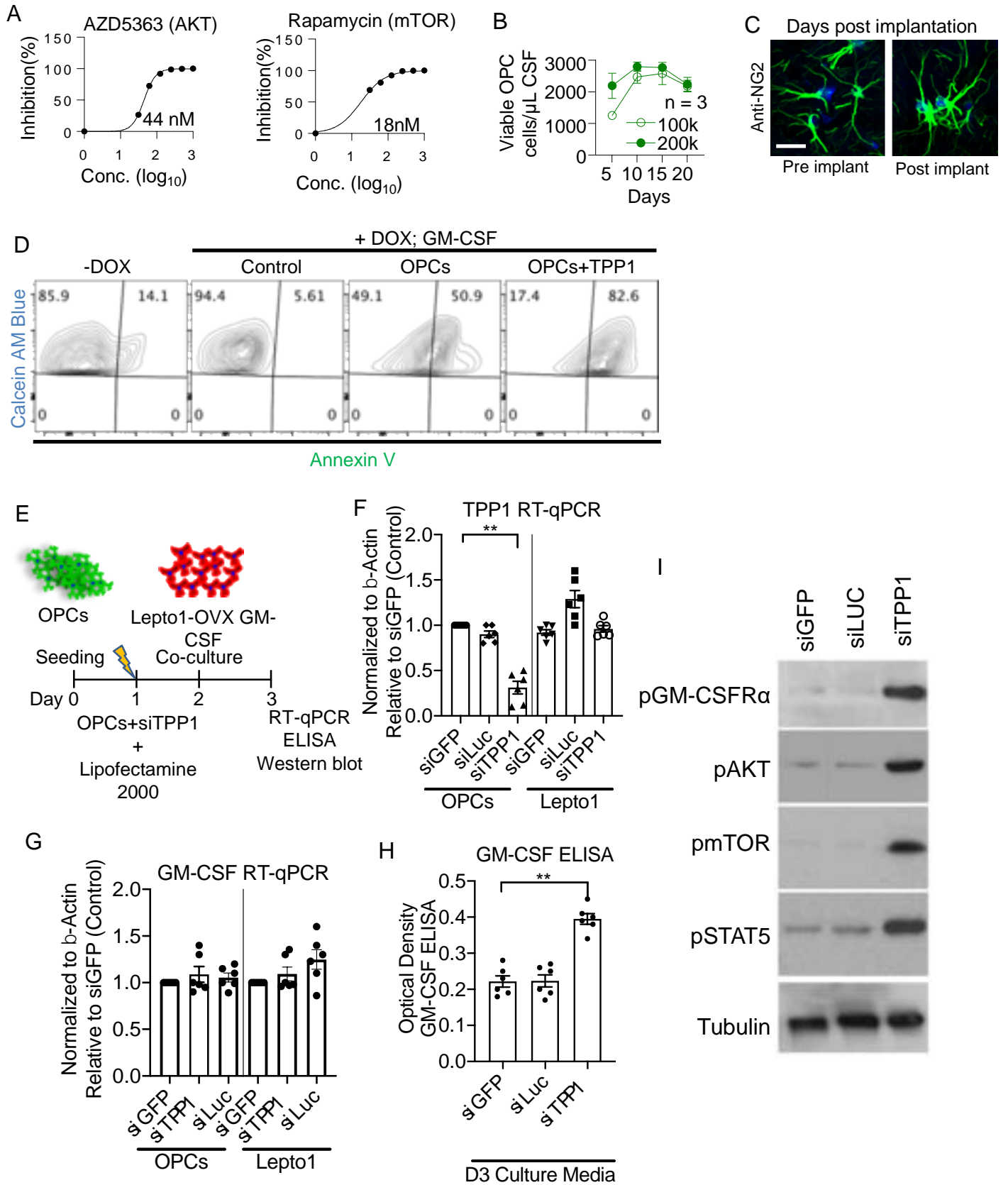
- A.)** Immunofluorescence analysis of serial brain sections taken from mice 10 days after mCherry:LUC-labeled (red) Lepto1 cell implantation and stained with markers of astrocytes (GFAP, green). The GFAP+ layer of reactive astrocytes was juxtaposed to Lepto1 cells on the surface of the brain.
- B.)** Phase contrast images of tumorspheres derived from Lepto1 cells cultured for 24 h in hCSF-supplemented media (control, CTL) or with astrocytes or OPCs, as indicated. Scale bar=50  $\mu$ m.
- C.)** Quantification of the proportion of tumorsphere-initiating cells shown in S1B, measured via CCK assays. The number of CTL cells was set to 100 (n = 3). \*\* p<0.01.
- D.)** Immunofluorescence images of Annexin V (green) and Pro-Caspase 3 (magenta) staining of mCherry:LUC-labeled (red) Lepto1 cells after 48 h of treatment with OPC-conditioned medium. Scale bar=50  $\mu$ m.

## Supplementary Figure 2



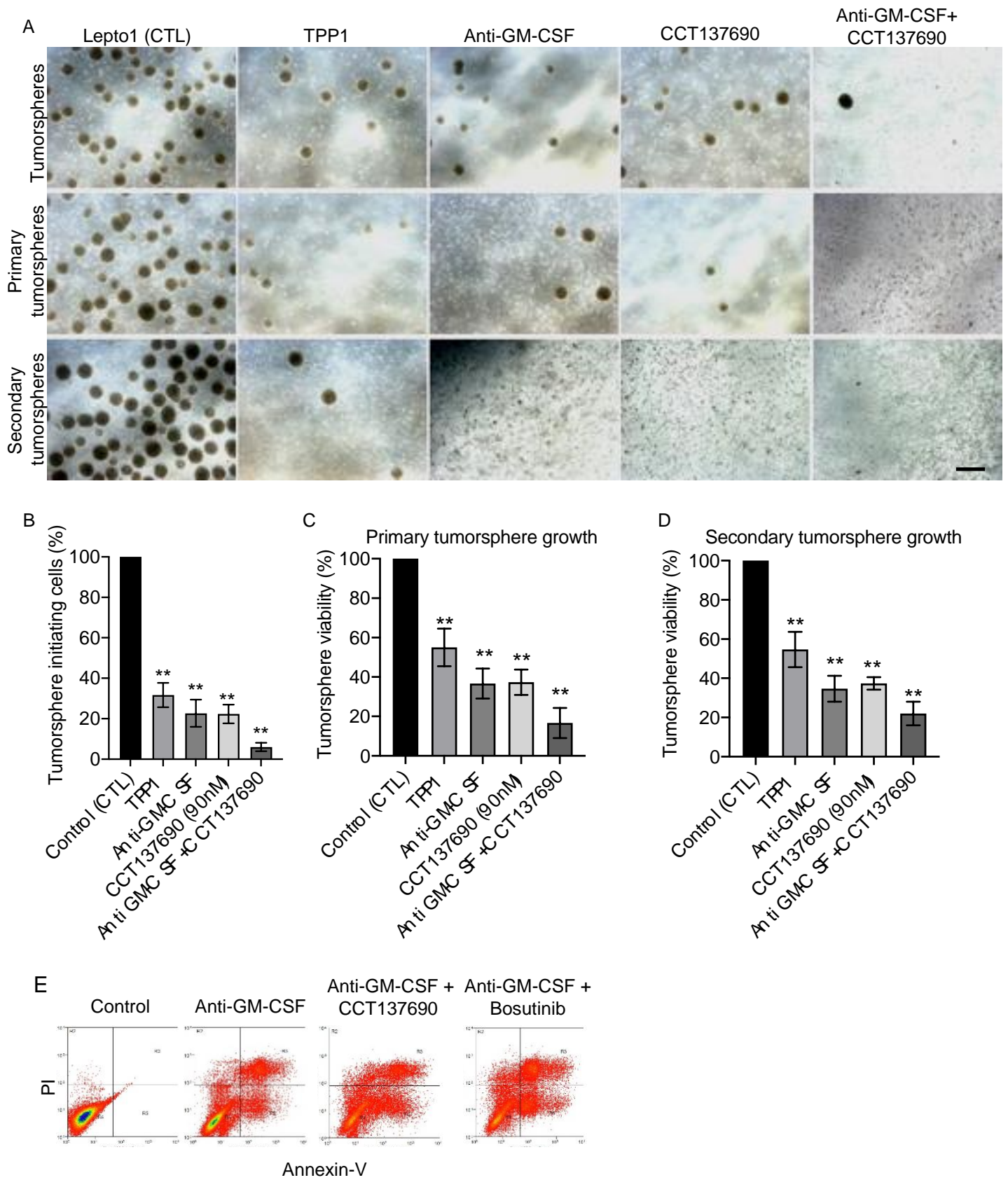
- A.)** (Left panel) RT-qPCR analysis of GM-CSF transcript levels in Lepto1 and Lepto2 cells, as well as the indicated CNS cell types. Lepto lines exhibit the highest levels among all cell types analyzed (n=3). (Right panel) RT-qPCR analysis of GM-CSF transcript levels in HER2+ LC tumor tissues, metastatic tumor tissues (MT2), primary tumor tissue (PT2), normal breast, and brain. HER2+ LC tumor tissues exhibit the highest levels among all tissues analyzed (n=3).
- B.)** FACS-based analyses showing the percentage (99.5%) of Lepto1 cells with cell surface expression of the GM-CSF receptor GM-CSFR $\alpha$  (CD116).
- C.)** Diagram showing the lentiviral Tet-On 3G inducible GM-CSF expression cassette used in this study.
- D.)** Western blot analysis of GM-CSF in the lysates of Lepto1 cells collected 48 h post 5 $\mu$ g/mL DOX administration.
- E.)** FACS-based ZsGreen1 analyses (left panel) and fluorescence imaging (right panel) of Lepto1 cells 48 hours post 5 $\mu$ g/mL DOX or vehicle (PBS) treatment showing a marked shift in the green fluorescence of Lepto1 cells, suggesting conditional overexpression of GM-CSF. Scale bar=50  $\mu$ m.
- F.)** Annexin V FACS-based analysis of apoptosis in Lepto1 cells cultured under the indicated conditions for 48 h. DOX treatment induced GM-CSF expression (n=3).
- G.)** CellTiter-Glo analysis-based assessment of Lepto1 cells grown as in S2F (n=3).
- H.)** RT-qPCR analysis of GM-CSF transcript levels in Lepto lines grown with or without exposure to OPCs or OPC-conditioned media (OPC-CM) (n=3).
- I.)** Quantification of luciferase signal in primary Lepto1 and 2 cells that cultured as in with or without exposure to OPCs or OPC-conditioned media (OPC-CM) (n=3). Lepto1 and 2 lines were transfected with luciferase reporter vector harboring the GM-CSF proximal promoter.

**Supplementary Figure 3**



- A.)** Dose-dependent inhibition of Lepto1 cell viability by an AKT inhibitor (AZD5363) and an mTOR inhibitor (Rapamycin). IC<sub>50</sub> values of the compounds are shown.
- B.)** CellTiter-Glo assay-based viability assessment of OPCs at different time points from Day 5 to Day 20 after co-implantation with Lepto1 cells into NOD/SCID mice. The OPCs did not demonstrate altered viability.
- C.)** Immunofluorescence images demonstrating that OPCs co-implanted with Lepto1 cells appropriately expressed the cell-specific marker NG2. Scale bar=50 μm.
- D.)** Annexin V FACS-based quantification of apoptotic Lepto1 cells conditionally overexpressing GM-CSF (following treatment with 5μg/mL DOX) and cultured alone (control), with OPCs, or with OPCs and TPP1. The presence of OPCs, with or without TPP1, induced Lepto1 cell apoptosis.
- E.)** Schematic showing the protocol used to analyze TPP1-depleted OPCs co-cultured with GM-CSF-overexpressing Lepto1 (Lepto1-OVX GM-CSF) cells. TPP1 and GM-CSF expression were analyzed via RT-qPCR and ELISA.
- F.)** RT-qPCR based expression analyses of TPP1 transcripts in OPCs and Lepto1 cells transduced with siTPP1 or control (siGFP or siLUC) siRNAs (n=3). \*\* p < 0.01.
- G.)** RT-qPCR analysis of GM-CSF transcripts in cells corresponding to those described in S3F (n=3).
- H.)** ELISA-based quantification of GM-CSF in the co-culture media from siRNA-transfected OPC co-cultured with GM-CSF-overexpressing Lepto1 cells (n=3). TPP1-depleted OPCs had higher levels of GM-CSF than the siGFP and siLuciferase controls.
- I.)** Western blot analysis of the indicated phosphorylated GM-CSF effector proteins in Lepto1 cells co-cultured with TPP1-depleted OPCs or control OPCs transduced with siGFP or siLUC. Tubulin served as a loading control.

**Supplementary Figure 4**



- A.)** (Top row) Phase contrast images of tumorspheres derived from cells cultured for 24 h with DMSO (control, CTL), TPP1 (100 ng/mL), anti-GM-CSF neutralizing antibodies (50 ng/mL), CCT137690 (100nM), or a combination of anti-GM-CSF neutralizing antibodies (50 ng/mL) + CCT137690 (100 nM). (Middle row) Phase contrast images of primary tumorspheres treated for 2 days as in S4A. (Bottom row) Phase contrast images of secondary tumorspheres developed from the primary tumorspheres (middle row) without drug treatment for 12 days. Scale bar=100  $\mu$ m.
- B.)** Percentages of tumorsphere-initiating cells (as shown in S4A, top row), measured via CCK assays. The number of DMSO-treated cells was set to 100 (n = 3). \*\* p < 0.01, compared to DMSO-treated cells.
- C.)** Viability of primary tumorspheres (as shown in S4A, middle row), measured via CCK assays. The number of DMSO-treated cells was set to 100 (n = 3). \*\* p < 0.01, compared to DMSO-treated tumorspheres.
- D.)** Viability of secondary tumorspheres (as show in in S4A, bottom row), measured via CCK assays. \*\* p < 0.01, compared to secondary tumorspheres from DMSO-treated primary tumorspheres.
- E.)** Annexin V FACS-based analysis of apoptotic Leptol cells treated for 48 h with anti-GM-CSF neutralizing antibodies (50 ng/mL) alone or in combination with CCT137690 (100 nM) or Bosutinib (100 nM).