

## Supplementary Materials and Methods

### Viability assay

Cell lines were cultured in their respective culture conditions prior to harvest in the exponential growth phase. Cells were seeded at  $2 \times 10^3$  cells per well in 96-well white bottom plates (Greiner Bio-One; Kremsmünster, Austria), and incubated at  $37^\circ\text{C}$ , 5%  $\text{CO}_2$  for 24 hours. Cells were subsequently treated with test compound or vehicle control for 48 hours. The concentration of DMSO (vehicle) was 1% for all treatments. Viability was determined using the CellTiter-Blue<sup>®</sup> Cell Viability Assay (Promega; Madison, WI, USA) according to the manufacturer's instructions. Briefly, 20  $\mu\text{L}$  of the CellTiter-Blue reagent was added to each well containing 100  $\mu\text{L}$  medium and incubated for 4 h at  $37^\circ\text{C}$ , and fluorescence was measured at 560ex/590em using a plate reader. Values normalized to the vehicle control were used to calculate  $\text{IC}_{50}$  values and CI values were calculated using the Chou and Talalay method (CompuSyn).

### Synergy analysis and combination index (CI)

The synergy analysis and calculation of combination index for the JAK2 and SMO inhibitor combinations was done using CompuSyn. The initial design of these experiments followed that of Chou and Talalay<sup>1</sup> wherein the inhibitors were set at their  $\text{IC}_{50}$  for the inhibitor combinations for each specific cell line. Upon further testing, we modified the ratio of the inhibitors to those presented in **Fig. 1B**.

### Flow cytometry analysis

Flow cytometry to assess the  $\text{CD44}^{\text{high}}/\text{CD24}^{\text{low}}$  MDA-231 population was performed as previously described<sup>2</sup>. MDA-231 or BT-20 cells were seeded in T75 flasks and treated with vehicle or test compounds overnight. Collected cells were stained for CD44 (Cat# 103012, BioLegend; San Diego, CA, USA) and CD24 (Cat# 11024742, eBioscience; San Diego, CA, USA) expression or the respective isotype controls (APC Rat IgG2b  $\kappa$ , Cat# 400612, Biolegend; FITC Mouse IgG1  $\kappa$ , Cat# 11471482, eBioscience) for 20 minutes at room temperature and fixed on ice in 1% paraformaldehyde. A fixable Live/Dead stain (Cat# L23105, Invitrogen) was used to exclude dead cells from analysis. For each samples, 10 000 events were acquired and cell populations were gated using control IgG and stem cell population was determined by percentage of cells positive for CD44 and negative for CD24 ( $\text{CD44}^{\text{high}}/\text{CD24}^{\text{low}}$ ) using a BD Fortessa X20 instrument.

### Mammosphere assay

Adherent BT20, BT474-TtzmR, or MDA-468 cells were harvested and seeded at a density of  $2 \times 10^3$  cells per well in 24-well ultra-low attachment plates (Corning Life Sciences; Corning, NY, USA) with Dulbecco's modified Eagle's medium/F12 (Gibco) containing 2% B27 (Gibco), 20 ng/mL rEGF (Millipore Sigma; Burlington, MA, USA), 4  $\mu\text{g}/\text{mL}$  insulin (Millipore Sigma), and 100 ng/mL human recombinant Sonic hedgehog protein (Millipore Sigma). Beginning 24 hours after seeding, mammospheres were treated with vehicle (1% DMSO) or test compound. Mammospheres were cultured for 7-14 days and supplemented with 100  $\mu\text{L}$  of fresh treatment prepared in mammosphere medium every 48 hours. The number of spheres per well was counted under 5x objective.

### Mouse study with mammary fat pad (orthotopic) implantation

Female nude mice 6-7 weeks of age (Athymic Nude-Foxn1nu, Charles River) were housed in a pathogen-free facility of the Animal Research Program at Wake Forest School of Medicine (WFSM) under a 12:12-hour light/dark cycle and fed irradiated rodent chow *ad libitum*. Animal handling and procedures were approved by the WFSM Institutional Animal Care and Use Committee (IACUC). Anesthetized mice were subjected to mammary fat pad implantation of  $5 \times 10^6$  actively growing BT474-TtzmR cells or  $1 \times 10^5$  MDA-231 cells expressing luciferase resuspended in 100  $\mu\text{L}$  Matrigel (Corning). Successful inoculations were confirmed by visualization of bioluminescent signal localized to the mammary fat pad within 1 hr post-inoculation; otherwise, mice were immediately sacrificed. Caliper measurements were taken twice weekly to monitor tumor progression and tumor volume was estimated using the following equation:

$$Volume (mm^3) = (Width)^2 \times \frac{Length}{2}$$

where width and length are the shortest and longest diameters of the tumor, respectively. Metastasis was monitored by twice weekly BLI. Once average tumor size reached 90-100 mm<sup>3</sup>, mice were randomized into vehicle (90% PEG-300, 10% dimethylacetamide v/v), 7.5 mg/kg pacritinib, 20 mg/kg sonidegib, or the combination of pacritinib and sonidegib treatment groups such that tumor volumes were approximately equal across the groups. IP treatments were administered three times per week.

### **Intracardiac inoculation study as a mouse model of TNBC metastasis**

All colonies of female nude mice 7-8 weeks of age (Athymic Nude-Foxn1nu, Charles River; Wilmington, MA, USA) were housed in a pathogen-free facility of the Animal Research Program at Wake Forest School of Medicine (WFSM) under a 12:12-hour light/dark cycle and fed irradiated rodent chow *ad libitum*. Animal handling and procedures were approved by the WFSM Institutional Animal Care and Use Committee (IACUC). Mice were inoculated with 2x10<sup>5</sup> exponentially growing MDA-231 cells expressing luciferase in 100 µL ice-cold PBS into the left ventricle. Successful inoculations were confirmed by visualization of brain bioluminescent signal 1 hr post-inoculation; otherwise, mice were immediately sacrificed. Tumor progression was monitored with biweekly bioluminescent imaging (BLI) in which xenograft-bearing mice were intraperitoneally (IP) injected with 100 mg/kg d-luciferin (Perkin Elmer; Waltham, MA, USA) and imaged using the IVIS Lumina LT Series III imager (Perkin Elmer). Mice having received successful inoculations were randomized into vehicle (90% PEG-300, 10% dimethylacetamide v/v), 20 mg/kg sonidegib, 7.5 mg/kg pacritinib, or the combination of sonidegib and pacritinib treatment groups and treatments were administered three times per week until study termination. Tumor burden was analyzed by quantifying BLI signal in each region-of-interest measured in total flux (p/s) with the Living Image software version 4.7.2 (Perkin Elmer).

### **Alanine transaminase assay**

The Alanine Transaminase Colorimetric Assay kit (Cayman Chemical; Ann Arbor, MI, USA) was used to determine alanine transaminase activity according to the manufacturer's instructions. At the time of sacrifice, mice were exsanguinated by intracardiac bleed. Serum was collected by allowing the blood to clot at room temperature for 30 minutes. The clot was removed via centrifugation at 2 000 x g for 15 minutes at 4°C and the supernatant collected. Serum samples and kit components were equilibrated to room temperature before assembling the assay plate with 20 µL positive control or sample according to the manufacturer's instructions. Absorbance at 340 nm was measured every minute for 10 minutes at 37°C using a SpectraMax iD3 (Molecular Devices; San Jose, CA, USA). The change in absorbance ( $\Delta A_{340}$ ) per minute was determined for each sample. ALT activity (U/L) was then determined with the following equation:

$$ALT \text{ activity (U/L)} = \left[ \frac{\Delta A_{340}/min \times 0.21 \text{ mL}}{4.11 \text{ mM}^{-1} \times 0.02 \text{ mL}} \right] \times 1000$$

Samples were run in technical triplicate and four samples per treatment group were assayed.

### **Quantitative RT-PCR**

RNeasy Mini Kit (Qiagen 74104) was used to isolate total RNA and cDNA was produced from 1 µg total RNA using the Superscript III First-Strand cDNA synthesis system (Cat# 18080044, Invitrogen). Quantitative PCR was carried out as previously described<sup>2-4</sup> using primers described in Supplementary Table 1.

### **Immunohistochemistry**

Immunohistochemistry (IHC) was performed as previously described<sup>2</sup>. IHC antibodies included Ki-67 (Cat# RB-9043-R7, Lab Vision; Fremont, CA, USA), p-STAT3 (Y705) (Cat# SAB4300033, Sigma-Aldrich, MO,

USA), mCD31 (Cat# 550274, BD Biosciences; NJ, USA), Bcl-2 (Cat# MA126233, Invitrogen), and VEGF-A (A-20) (Cat# sc-152, Santa Cruz; TX USA).

#### **Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay**

Tumors from the mammary fat pad studies were embedded in OCT medium and sectioned using a cryostat. OCT-embedded slides were subjected to TUNEL assay using the Click-iT™ Plus TUNEL Assay (Invitrogen; Carlsbad, CA, USA) and counterstained with DAPI (Invitrogen). The percentage of TUNEL-positive cells was quantified for at least 5 fields of view per animal (n = 3-5 mice per group).

#### **Immunoprecipitation western blot analysis**

Immunoprecipitation and western blot analyses were performed as previously described<sup>4-6</sup>. Cells were transfected with either flag-tagged GLI1 or tGLI1, and STAT3, treated with vehicle (1% DMSO), 0.04 μM pacritinib, 0.2 μM sonidegib, or the combination and stimulated with 100 ng/mL EGF and 100 ng/mL SHH for 24 h, washed, fractionated, and precleared with Mouse IgG-Agarose (Cat# A0919, Sigma). Precleared lysates were incubated with anti-flag M2 affinity gel or mouse immunoglobulin G at 4 °C overnight with gentle agitation. Pellets were collected, washed and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis and WB analysis. GLI1 and tGLI1 immunoblotting was performed using a GLI1 antibody (Cat# 2643, Cell Signaling) that recognizes the COOH-terminal end and, therefore, detects both GLI1 and tGLI1. Total STAT3 was detected using a STAT3 antibody from Cell Signaling (Cat# 3538).

#### **Statistical analysis**

Data were analyzed and graphed using CompuSyn (ComboSyn, Inc.; Paramus, NJ, USA), Prism 8 (GraphPad Software; San Diego, CA, USA), and Excel 2016 (Microsoft; Redmond, WA, USA). Descriptive statistics are presented as mean ± SEM. One-way ANOVA and survival analyses were performed using Prism 8.

#### **References**

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