MATERIALS AND METHODS – Supplement

aCGH. The Agilent 244K aCGH platform (Agilent Technologies, Santa Clara, CA) was used to determine gene copy losses and gains. Genomic tumor DNA and patient-matched germ-line DNA or an anonymous female germ-line reference DNA sample and cell line DNAs were used for co-hybridization experiments. Methods for data acquisition and analysis were described previously (1). The data were rendered for visualization in Java Treeview (2).

Transcriptional Profiling. The Agilent 1x44K Whole Human Genome microarray was used to measure gene expression in tumor samples collected prior to endocrine therapy. Total RNAs from both a tumor sample and a common reference sample was amplified and either a Cy5 (tumor) or Cy3 (reference) label was incorporated during in vitro transcription using Agilent's Low Input Linear Amplification Kit. Differentially labeled tumor and reference samples were co-hybridized to the array, washed and dried according to Agilent. Fluorescent signals were obtained using an Agilent scanner and Feature Extraction software. The data was lowess normalized and retrieved as log2 ratios representing tumor versus reference expression levels. Intrinsic subtypes (luminal A and luminal B) were identified using the PAM50 classification methodology (3).

Gene Re-sequencing. *PIK3CB* mutation status of 22 POL tumor samples, TMA samples, and cell lines were assessed by sequencing *PIK3CB* cDNA (Accession No. NM_006219) encoding the helical (nucleotides 1503–2208) and kinase domains (nucleotides 2359–3213). Overlapping PCR products generated from cDNA were sequenced using BigDye reagent (Applied Biosystems, Foster City, CA) and analyzed with a capillary sequencer. *PIK3CA* mutation status

for 72 POL breast tumor samples and the HCC712 cell line was assessed either by genomic DNA sequencing of exons 9 and 20 or cDNA sequencing the helical (nucleotides 1280–2017) and kinase domains (nucleotides 2600–3422) of *PIK3CA* (Accession No. NM_006218). PCR products were sequenced using Big Dye reagent and a capillary sequencer. Traces for *PIK3CA* and *PIK3CB* sequencing were assembled and analyzed for mutations using Mutation Surveyor software (SoftGenetics, State College, PA).

Protein Extracts. To prepare lysates for the breast cancer cell line panel, cells were washed with cold PBS and extracted with lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10% glycerol, 1 mM EDTA, 1X Complete EDTA-free protease inhibitor (Roche Diagnostics), 1 mM NaPPi, 10 mM NaF, 1 mM Na_3VO_4). Lysates were cleared by centrifugation and protein concentration was determined by Bradford assay (Bio-rad, Hercules, CA). Lysates were mixed with 2x reducing sample buffer, boiled and stored at -80^{o}C. Lysates for siRNA gene knockdowns were prepared by depriving transfected cells in serum-free medium for 3–4 h, treating cells ± 20% FBS for 15 min, and extracts were prepared as above. For pharmacological experiments cells were deprived of serum for 3–4 hr, pre-treated with BEZ235 (Axon Medchem BV, Groningen, The Netherlands), LY294002 (BIOMOL International, Plymouth Meeting, PA) or rapamycin (Sigma-Aldrich) for 20 min , treated ± 20% FBS for 15 min, and extracts were prepared.

Immunoblotting. Protein lysates were separated by SDA-PAGE in NuPAGE gels (Invitrogen), transferred to nitrocellulose membranes, blocked with 5% nonfat dry milk (NFDM)/ TBST (10 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20) and incubated in primary antibodies diluted in

blocking buffer overnight at 4°C. Blots were washed with TBST and incubated with horseradish peroxidase-conjugated secondary antibodies (1:20,000, Jackson Immmunoresearch Laboratories, West Grove, PA) in blocking buffer. Blots were washed, developed with SuperSignal substrate (Pierce, Rockford, IL) and exposed to film. Primary antibodies for immunodetection included: p110β (04-400, Millipore), ER (RM-9101, Fisher Scientific, Fremont, CA), HER2 (A0485, Dako, Carpinteria, CA) and actin (sc-1616, Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies for detecting p110α (#4255), Akt (#4685), phospho-Ser473 Akt (#4051), S6 (#2317) and phospho-S6 (#4857) were from Cell Signaling Technology (Danvers, MA).

Cell Growth Assay. For RNAi experiments, cells cultured in phenol red-free RPM1 1640 containing 5% CSS (CSS medium) were reverse transfected with 10 nM siRNAs (single gene knockdowns) or 20 nM siRNAs (dual gene knockdowns) in 96-well Optilux dishes (Becton Dickinson, Franklin Lakes, NJ). The day after transfection, cells were treated as indicated with E_2, Fulvestrant (Tocris Bioscience, Ellisville, MO), BEZ235 or LY294002. For pharmacological experiments, cells growing in CSS medium were plated in 96-well Optilux dishes and treated with the indicated agents the day after plating. Medium was replenished every 3–4 d and cell growth was assessed after 10 d by measuring Alamar Blue reduction ($555\lambda_{Ex}$/ $585\lambda_{Em}$) with a fluorescent microplate reader.

Cell Death Assay. Cells growing in CSS medium were plated (for pharmacological experiments) or reverse transfected with duplex siRNAs in 6-well dishes. Cells were treated as indicated the day after transfection or plating and medium and pharmacological agents were replenished 3–4 d after the beginning of treatment. Floating and adherent cells were collected

after 7d of treatment and fixed with 2% paraformaldehyde. For the detection of apoptosis, cells were labeled using the APO-BrdU TUNEL Assay kit (Invitrogen) and counterstained with Hoechst 33,342 (single gene knockdown experiments) or were stained with Hoechst only (dual gene knockdowns, BEZ235 treatments). Cells were then mounted on glass slides and sealed under coverslips with Prolong Gold Antifade (Invitrogen). A minimum of 300 nuclei from 4–8 randomly selected fields were counted in each experiment and assessed for apoptosis. Cells with TUNEL positive or pyknotic /fragmented Hoechst stained nuclei were counted as apoptotic.

***PIK3CB* FISH.** FISH analysis for *PIK3CB* was performed on 6µm FFPE sections taken from POL biopsies and 6µm FFPE breast TMA sections. The *PIK3CB* probes were created from DNA isolated from the BAC clones RP11-112O24 and RP11-579O13 (BACPAC Resources Centre, Children's Hospital Oakland Research Institute). Spectrum Orange labeled chromosome 3 centromeric probe, CEP3 (Vysis, Downer's Grove, IL) was used for chromosome normalization. Methods for FFPE tissue preparation, probe hybridization, and automated scoring were described previously (1). Cases were considered amplified when the *PIK3CB* / CEP3 ratio was ≥2 and cases in which the *PIK3CB*/ CEP3 ratio was ≥1.5<2 were considered to contain a copy number gain. *PIK3CB* FISH signals from approximately 100 nuclei in POL samples were subjected to both automated and manual scoring. Informative *PIK3CB* FISH results were obtained in 81/111 cases on the breast tissue microarray and in 12 of the POL cases with paired aCGH.

REFERENCES

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3. Parker JS, Mullins M, Cheang MC, et al. Supervised Risk Predictor of Breast Cancer Based on Intrinsic Subtypes. J Clin Oncol 2009.