Supplemental Methods

Cell Culture Methods

All cell lines were cultured in 75cm2 cell culture flasks (430641U; Corning) or Nunc™ EasyFill™ Cell Factory™ System 2528 cm2 (140360; Nunc) in a 50:50 mixture of Dulbecco's modified essential medium and Ham's F-12 medium (DMEM/F12, Gibco) supplemented with 10% fetal bovine serum (FBS, HyClone), 1 mM sodium pyruvate (Gibco), 2 mM L-Glutamine (Gibco), and 1% penicillin-streptomycin (Gibco). Cells were grown in a humidified incubator maintained at 37°C and 5% CO2 and propagated a maximum of 25 times to qualify for use in any experiment. PC-3, PC3-GFP-AR-V7, 22RV1, and MCF7 cells were split 1:10 at 80-90% confluency, typically once a week. LNCaP and LNCaP C4-2 cells were split 1:5 at 80-90% confluency, typically once a week.

Compound Library Storage and Handling

A set of 145 small molecule compounds assembled by the Center for Translational Cancer Research at the Texas A&M Institute of Bioscience and Technology was used for screening. The library consists of compounds to a wide variety of targets. Fifty-seven percent of the compounds are currently used in the clinic for the treatment of various forms of cancer and 37% are in clinical trials. Compounds stock solutions at 10 mM in dimethyl sulfoxide (DMSO) were stored and frozen at -20°C under low humidity nitrogen enriched conditions in 96-well format. Daughter plates for primary screening and follow-up studies were prepared and stored in an identical manner. DMSO stock solutions for follow-up studies were retrieved directly from replicate daughter plates of

the library. For screening purposes, the compound solutions were transferred to 96 well working dilution plates from which plates containing concentrations ranging from 10 μM to 100 pM were prepared in growth media containing 10% stripped FBS in columns 2-11. Columns 1 and 12 were prepared with 0.5% DMSO and 20 nM dihydrotestosterone (DHT) controls in growth media with 10% stripped FBS. All liquid transfers were handled using a BioMek FX platform (Beckman Coulter).

Small Molecule Screening

Small molecule screening was accomplished using PC3-GFP-AR-V7 and HeLa-GFP-AR cells seeded in DMEM/F12 media supplemented with 10% stripped FBS onto optical polymer bottom 384 well plates (Aurora Microplates) at a density of 3,000 cells per well. Cells were allowed to grow for 24 hours in a humidified 37°C incubator prior to compound treatment. Immediately prior to compound treatment, the stock compound solutions were transferred from a thawed daughter plate to 96 well working dilution plates from which plates containing concentrations ranging from 10 μM to 100 pM were prepared in growth media containing 10% stripped FBS in columns 2-11. Columns 1 and 12 were prepared with 0.5% DMSO and 20 nM dihydrotestosterone (DHT) controls in growth media with 10% stripped FBS. Working dilutions of compounds were added in quadruplicate to cells in a 1:1 mix with plating media resulting in a final concentration range of 50 pM to 5 μM. Cells were incubated with compounds for 48 hours.

Human Kinome siRNA Library Storage and Handling

The Stealth RNAi Human Kinome Collection (Invitrogen) contains 636 human kinae targes with three individual non-overlapping Stealth RNAi duplexes per target. The

library is a collection of 208 validated targets proven to knockdown and 486 Modified Stealth RNAi duplexes with advanced specificity alignment applied for reduction of off-target effects. The library consists of twenty-four 96-well plates containing 20 uM solutions stored at -20°C under low humidity nitrogen enriched conditions. Library plates were thawed and siRNA wre printed into 384 well optical bottom plates in quadruplicate (AURORA) using a BioMek FX (Beckman Coulter). To reduce screening size, target replicates A,B, and C were printed into the same well. Printed plates were sealed with foil under nitrogen and stored at -80°C until use.

Human Kinome siRNA Library Screening

Screening plates were thawed and siRNA was complexed by addition of 20 ul of diluted XtremeGene (Roche) in OptiMEM (Invitrogen) using a µFill (BioTek) followed by incubation for 30 minutes at room temperature. PC3-GFP-AR-V7 cells were trypsinized and resuspended in growth media without penicillin-streptomycin and added at a concentration of 1500 cells/well using µFill. Cells were placed into a 37°C/5% CO2 humidified incubator for 72 hours.

AR-HCA Image Based Analysis

After incubation period was completed, cells were paraformaldehyde fixed using a 4% EM-grade formaldehyde in PEM buffer (80 mM potassium PIPES [pH 6.8], 5 mM EGTA, and 2 mM MgCl2) and quenched with 0.1 M ammonium chloride for 10 min. Cell membranes were disrupted by incubating samples with a 0.5% Triton X-100 solution for 10 minutes. Nuclei were stained using DAPI (1 µg/ml) for 10 minutes. For samples in which endogenous AR was visualized, incubation with 0.5% Triton-X was extended to

30 minutes and cells were incubated in blotto (5% milk in Tris-buffered saline/Tween 20) for 30 minutes prior to addition of primary antibody solution (Anti-AR 441, 1:2000 dilution). Primary antibody solution was incubated overnight at 4°C prior to incubation with secondary antibody (Alexa 647 conjugated Anti-Ms IgG, Molecular Probes) for 1 hour at room temperature. Nuclei were stained using DAPI (1 µg/ml) for 10 minutes. All samples were stored in PBS buffer containing calcium, magnesium, and sodium azide at 4°C prior to imaging.

Image data sets were collected twice using the INCell 6000 image cytometer (GE Healthcare) utilizing reflection-based autofocusing, initially using a 4X/0.3 Plan Apo objective for whole well imaging following by a high resolution scan with a 40X/0.90 Nikon S-fluor objective. All samples were imaged in 2D with a single plane of focus captured. Cell and nucleus segmentation and signal quantification was performed using the myImageAnalysis web application powered by Pipeline Pilot software (Biovia) as shown in Supplemental Figure X. Aggregated cells, mitotic cells, and apoptotic cells were removed using filters based on nuclear size, nuclear shape, and nuclear intensity. Degree of nuclear translocation of the GFP signal was determined by measuring the ratio of nuclear mean intensity to cytoplasmic mean intensity. Nuclear patterning of the GFP signal was measured using Haralick correlation analysis.

Data Analysis

The small molecule library and kinome siRNA library numerical data were analyzed using a standardized analysis pipeline generated using Pipeline Pilot (Biovia). This pipeline performed all necessary baseline correction, normalization, curve-fitting, and

hit-calling. All screening data were normalized to a percentile range with DHT treated positive controls set at 100%. All dose response data was attempted to be fit to one of 4 models: a constant model, a hill model, a gain-loss model, and a loss-gain model. The gain-loss and loss-gain models allows the data to either increase then decrease or decrease then increase across the concentration range. A response was labeled as significant ("hit") if the following criteria was achieved:

1) Median normalized response at a single concentration was a minimum of 2.5 deviations away from control wells.
2) Modeled curve maxima/minima was greater than a 20% change from control wells.
3) Hill, gain-loss, or loss-gain model was selected over a constant model.

To establish the response threshold, the baseline median absolute deviation (BMAD) was calculated per metric using the control wells present on each assay plate. The models were scored using the Akaike Information Criterion (AIC) with the model with the lowest AIC score selected. The model maxima/minima from the selected model was extracted.

Cytotoxicity analysis was performed using 4X whole well image sets using the toxicity analysis associated with the myImageAnalysis application. This application scores each well based on cell count, nuclear shape, nuclear size, DNA content, and DNA patterning relative to other wells in the data set. Compond-concentration pairs with an assigned toxicity index greater than 0.2 were flagged as toxicity may be a potential confounder.

Western blotting

Cell lysates were obtained from scraping cells into ice-cold NP-40 lysis buffer. Lysates were then combined with LDS Sample Buffer (Novex, Carlsbad, CA, USA) and boiled for 5 min. Samples were then loaded onto 12-well 8–12% acrylamide gels and run at 200 V at 4 °C for 90 min. Gels were then transferred onto Immobilon transfer membranes at 4 °C for 2 h at 80 V. Non-specific antibody binding was blocked by treating membranes with 5% milk in TBS-T. AR protein was detected using Anti-AR 441 antibody diluted in blotto 1:2000.

Quantitative RT-PCR

Total RNA was isolated using Trizol (Life Technologies, Carlsbad, CA, USA) as per the manufacturer's instructions. RT and qPCR were performed as previously described. Reactions were carried out in an Applied Biosystems StepOne Plus (Carlsbad, CA, USA). The fold change in expression was calculated using the ΔΔ Ct comparative threshold cycle method with the ribosomal protein 36B4 mRNA as an internal control.