Supplemental Data

Cell Culture. Cell culture media contained 50 µg/mL gentamycin and 5 µg/mL ciprofloxacin to prevent bacterial contamination. HCT116 cells were obtained from Dr. Bert Vogelstein (Johns Hopkins University, MD) and grown in McCoy's 5A supplemented with 10% FBS. DPL cells were generated in the lab of Dr. Ronald Rodriguez and grown in DMEM supplemented with 10% FBS (*1*). PC3-CAR cells were obtained from Dr. J.T. Hsieh (University of Texas Southwestern Medical School, TX) and grown in RPMI 1640 medium supplemented with 10% FBS and 300 µg/mL G418.

Cloning of Adenoviral Vectors. Viruses were generated using the AdEasy vector system (*2*). PSMA was sub-cloned from pDonor-CMV-PSMA into pAd-Track-CMV using BglII and NotI. hNIS was sub-cloned from pDonor-CMV-hNIS into pAd-Track-CMV using BglII and NotI. The CMV promoter was sub-cloned by PCR using primers 5'-CMV (ACGAAGATCTTAATAGTAATCAATTACGGGG) and 3'-CMV (TCCGGAATTCCTAGCGGATCTGACGGTTCAC) upstream of sr39tk-GFP in the shuttle plasmid pGal-mNLS-sr39tk-EGFP using BglII and EcoRI restriction sites. The expression cassette from pCMV-mNLS-sr39tk-EGFP was then cloned into RpSWC1 using NotI and SalI restriction sites. The resulting plasmids, pAd-Track-CMV-PSMA, pAd-Track-CMV-NIS, or RpS-CMV-mNLS-sr39tk-EGFP, were recombined with pAdEasy-1 vector in BJ5183 *Escherichia coli* and selected for kanamycin resistance. Each resulting viral genome was linearized with Pac I and transfected into DPL cells for virus production. The resulting adenoviruses were column-purified using Adenopure adenovirus purification kit and titered in HEK293 cells by GFP and Hexon staining with Adeno-X rapid titer kit (Clonetech) 36 h after infection.

Preparation of J591 Antibody Conjugated to IRDye 800CW. The J591 antibody was labeled by using IRDye® 800CW NHS Ester (LI-COR). Briefly, 20 µL of 3 mM IRDye® 800CW NHS Ester (~10 molar fold excess in DMSO) were added to 200 µL of J591 antibody (5 mg/mL in PBS) and kept 1 h at ambient temperature. The unreacted dye was removed by passing through a Zeba desalting column (Pierce). The conjugation of dye was confirmed by measuring Absorbance at 280 and 750 nm and by SDS-PAGE.

Western Blot. Cells were washed with 1× PBS, resuspended in RIPA lysis buffer supplemented with protease inhibitor cocktail (Roche), incubated on ice for 30 min, and then centrifuged for 10 min at 4°C. Total protein concentration was measured using the Micro BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's protocol. Equal amounts of proteins were separated by SDS–PAGE and transferred to PVDF membrane, blocked with 5% nonfat dry milk in PBS for 1 h at room temperature, and then incubated with primary antibody (mouse monoclonal anti-hNIS from ABCAM [SPM186], mouse monoclonal anti-PSMA J591, rabbit polyclonal anti-HSV TK obtained from Dr. William Summers [Yale University]) overnight at 4°C. The membrane was then probed with antimouse IR800 conjugated secondary antibody or antirabbit IR680 conjugated secondary antibody for 1 h and scanned on the Odyssey Infrared Imager (LI-COR) using the manufacturer's protocol.

Cell Uptake by GCV. Cells infected with a control or reporter adenovirus were plated in 6-well plates and allowed to adhere for at least 12 h. GFP expression was measured in a FLUOstar Optima FL plate reader (BMG LABTECH, GmbH) to normalize for adenovirus infection. The culture medium was removed, the cells were washed once with PBS, and

fresh culture media containing 1 μCi ^{3}H-GCV was added with cells allowed to incubate for 1, 2, 3, and 4 h in a cell culture incubator (37°C 5% CO_2). Media containing ^{3}H-GCV was then removed and cells washed twice with PBS. Aspirated media containing ^{3}H-GCV and associated PBS washes were collected for quantification. Cells were then harvested in cold RIPA lysis buffer and incubated on ice for 30 min. Following lysis, the amount of ^{3}H-GCV retained in the cells was quantified by scintillation counting (Microbeta Wallac). Uptake was calculated as amount of radioactivity in the cells divided by radioactivity in the media and normalized by GFP to take into account the efficiency of adenovirus infection.

Fluorescent Cellular Immunoassay. Control cells or cells infected with reporter virus were plated on 96-well plates. When 90% confluent, cells were washed with 1× PBS and fixed with 4% paraformaldehyde for 30 min at room temperature. After fixation, cells were washed with 1× PBS, blocked with 1% BSA in PBS for 1 h at room temperature, and incubated with primary antibodies described above at 37°C for 1 h. Cells were then probed with secondary antibody conjugated with IR800 for 1 h and scanned on the Odyssey Infrared Imager using the manufacturer's protocol.

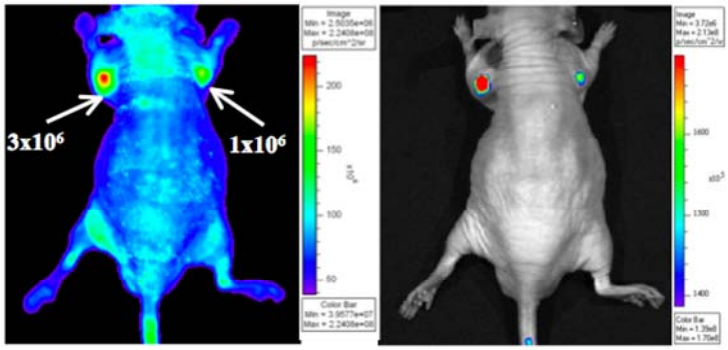
References

1. Hoti N, Li Y, Chen CL, et al. Androgen receptor attenuation of Ad5 replication: implications for the development of conditionally replication competent adenoviruses. *Mol Ther.* 2007;15:1495–1503.

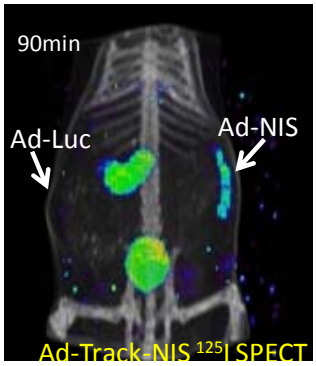
2. Luo J, Deng ZL, Luo X, et al. A protocol for rapid generation of recombinant adenoviruses using the AdEasy system. *Nature Protocols.* 2007;2:1236–1247.

Supplemental Figure 1

Day 2 Optical Imaging



Day 3 Nuclear Imaging



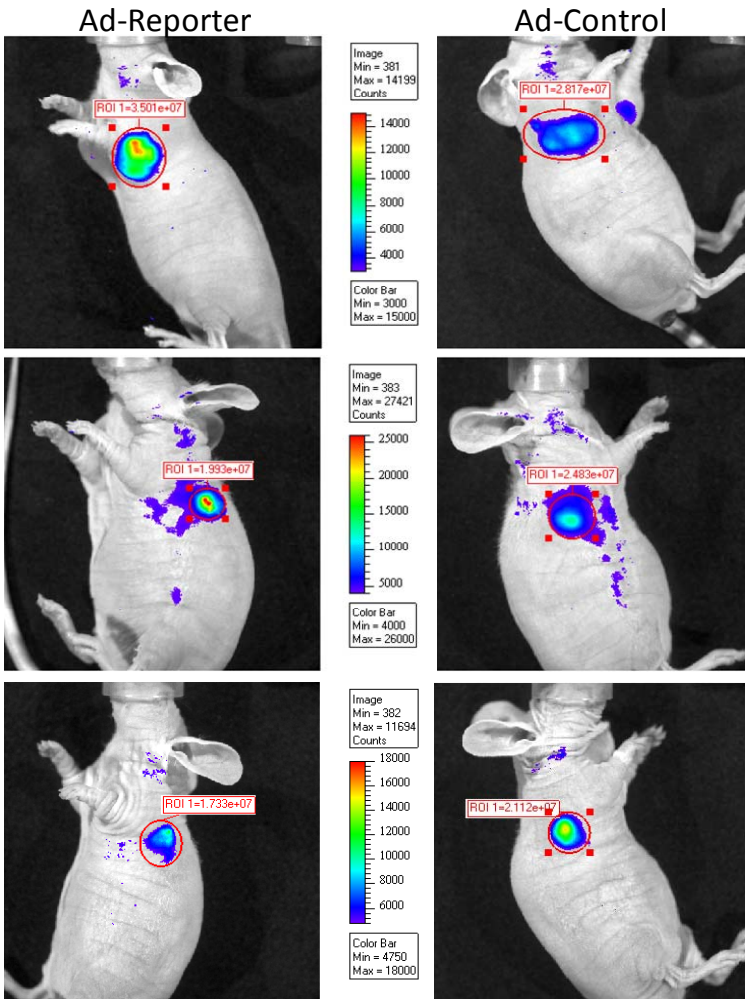
PC3-CAR cells were infected in vitro with equal MOI of a PET imaging reporter or control adenovirus (Ad-Track-Luciferase), and then each population of infected cells was separately mixed with Matrigel and implanted contralaterally into the upper flanks of a nude mouse (24 h after infection). Twenty-four hours after implantation, in vivo GFP imaging was performed to ensure that a similar level of control and reporter-infected cells remained at the sites of implantation.

Supplemental Figure 2

ROI	Counts
Ad-Trk-NIS	3.50E+07
Ad-Trk-Luc	2.82E+07
Fold GFP Luc/NIS	0.80

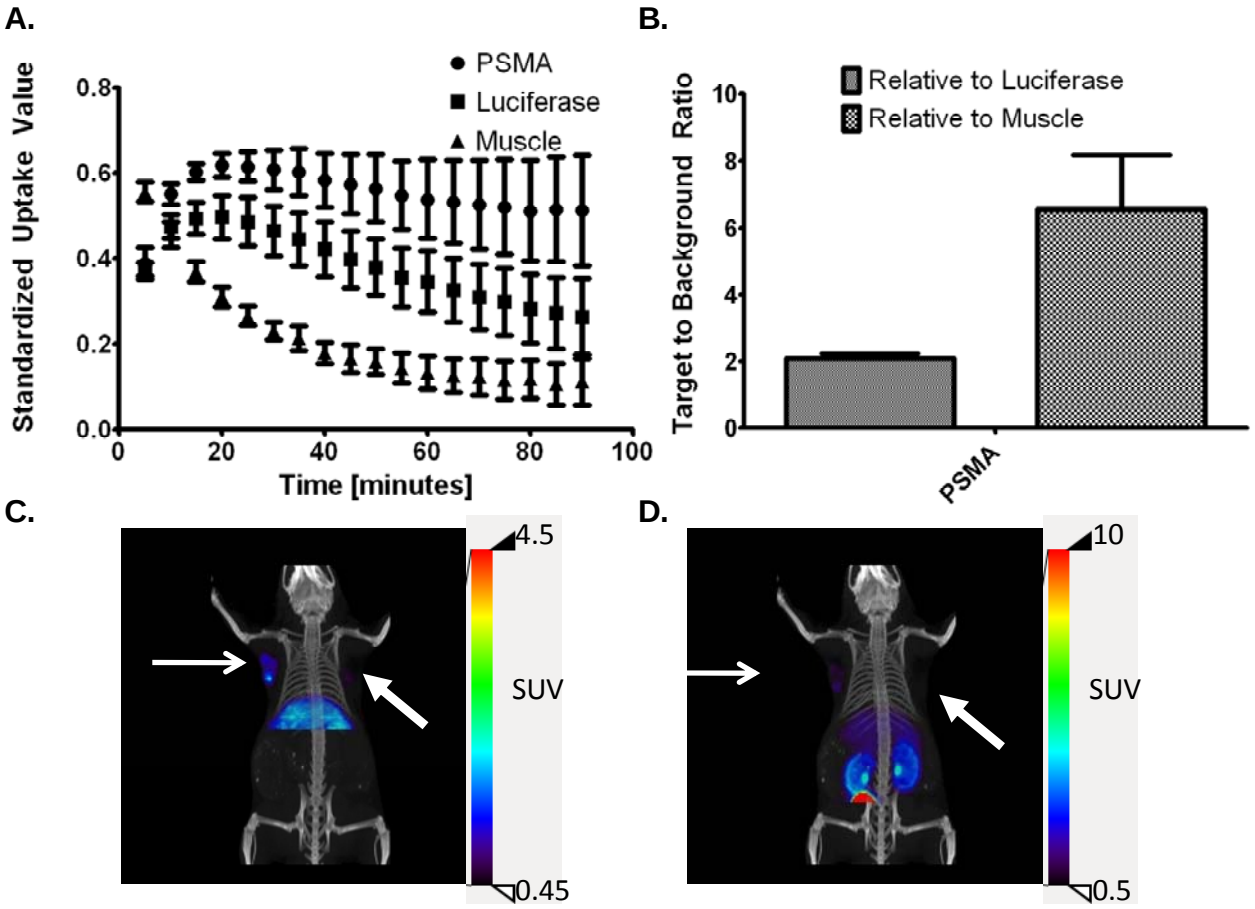
ROI	Counts
Ad-SR39TK	1.99E+07
Ad-Trk-Luc	2.48E+07
Fold GFP Luc/TK	1.25

ROI	Counts
Ad-Trk-PSMA	2.11E+07
Ad-Trk-Luc	1.73E+07
Fold GFP Luc/TK	1.22



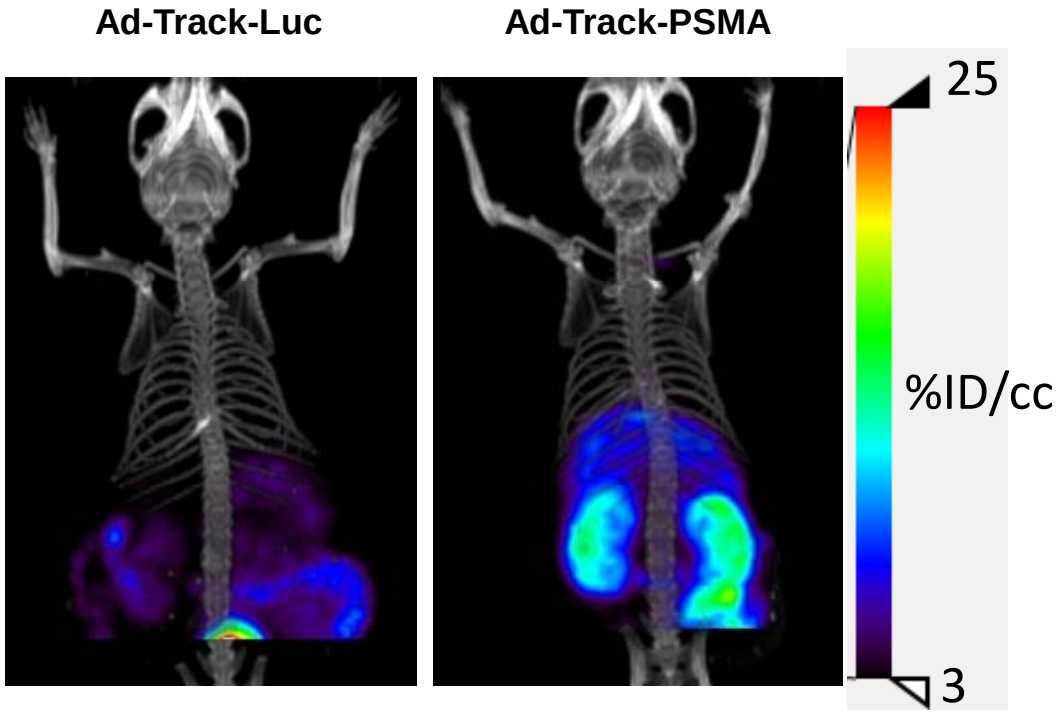
Matrigel suspension model from Supplemental Figure 1: The contralateral cell masses were generally within 30% as measured by GFP imaging in vivo.

Supplemental Figure 3



Results from DCFBC as the probe for PSMA imaging were similar to results obtained with DCFPyL, with the exception of a slower clearance from the control Matrigel suspension.

Supplemental Figure 4



Later time point (4 h) from Figure 4 in the text. Radiotracer uptake in liver of Ad-Track-PSMA remains significantly higher than that of the control, Ad-Track-Luc. Images are scaled to the same maximum value.