ONLINE METHODS

This project was approved by the Institutional Animal Care and Utilization Committee (IACUC) of the University of Iowa.

Chemicals were purchased from Sigma-Aldrich. Enzymes were obtained from New England BioLabs (NEB). Cell culture products were purchased from GIBCO BRL/Life Technologies (Invitrogen Corp.). Antibodies were purchased from the following manufacturers: Plk1 (Zymed/Invitrogen); Erk1 K-23 (sc-94); PSMA (Biodesign); β-actin (Sigma-Aldrich); HRP-labeled rabbit anti-mouse IgG secondary antibody (Zymed/Invitrogen).

siRNA sequences. Control siRNA target sequence: 5′-AATTCTCCGAA CGTGTCACGT-3′.

Plk1 siRNA target sequence: 5′-AAGGGCGGCTTTGCCAAGTGC-3′.

Aptamer-siRNA chimera sequences. In italics are the 2′ fluoropyrimidine–modified nucleotides. In the G-U chimera the C in the long RNA strand was mutated to a **U** (bold/underline).

A10-Plk1 chimera. A10-Plk1 sense strand (modified with 2′ fluoropyrimidines): 5′-GGGAGGA*C*GA*U*G*C*GGA*U*CAG*CCAUGUUUACGUCACUCCU UGUCAA*U*CC*U*CA*U*CGGCAGACGA*C*U*C*GC*CC*CGAAAGGG*C*GG*CUUU*G *CC*AAG*U*G*C*-3′

Plk1 Antisense siRNA (unmodified RNA): 5′-GCACUUGGCAAAGCCG CCCUU-3′

Blunt chimera. Blunt RNA sense strand (modified with 2′ fluoropyrimidines):

5′-GGGAGGA*C*GA*U*G*C*GGA*U*CAG*CCAUGUUUACGUCACUCCU*AAA AGGG*C*GG*CUUUGCC*AAG*U*G*C*-3′

Plk1 antisense siRNA (unmodified RNA): 5′-GCACUUGGCAAAGCCG CCCUU-3′

*OVH chimera.*OVH RNA sense strand: (modified with 2′ fluoropyrimidines)

5′-GGGAGGA*C*GA*U*G*C*GGA*U*CAG*CCAUGUUUACGUCACUCCU*AAA AGGG*C*GG*CUUUGCC*AAG*U*G*CUU*-3′

Plk1 antisense siRNA (unmodified RNA): 5′-GCACUUGGCAAAGCCG CCCUU-3′

G-U chimera's-U RNA sense strand (modified with 2′ fluoropyrimidines):

5′-GGGAGGA*C*GA*U*G*C*GGA*U*CAG*CCAUGUUUACGUCACUCCU*AAA AGGG*C*GG*CUUUGCC*AAG*UG**U***UU*-3′

Plk1 antisense siRNA: (unmodified RNA) 5′-GCACUUGGCAAAGCCG CCCUU-3′

Swap chimera. Swap RNA sense strand (modified with 2′ fluoropyrimidines): 5′-GGGAGGA*C*GA*U*G*C*GGA*U*CAG*CCAUGUUUACGUCACUCCU* AAAAGCA*CUU*GGCAAAG*CCGCCCUU*-3′

Plk1 sense siRNA (unmodified RNA): 5′-GGGCGGCUUUGCCAAGUG CUU-3′

Swap-2′F chimera. Swap RNA sense strand (modified with 2′ fluoropyrimidines): 5′-GGGAGGA*C*GA*U*G*C*GGA*U*CAG*CCAUGUUUACGUCACUCCU* AAAAGCA*CUU*GGCAAAG*CCGCCCUU*-3′

Plk1 sense siRNA: (modified with 2′ fluoropyrimidines) 5′-GGGCGGC *UUUGCCAAGUGCUU*-3′

Swap-2′F-PEG chimera. Swap RNA sense strand (modified with 2′ fluoropyrimidines): 5′-GGGAGGA*C*GA*U*G*C*GGA*U*CAG*CCAUGUUUACGUCAC UCCU*AAAAGCA*CUU*GGCAAAG*CCGCCCUU*-3′

Plk1 sense siRNA: (modified with 2′ fluoropyrimidines)

PEG (20 kDa)-5′-GGGC*GGCUUUGCCAAGUGCUU*-3′ (obtained from TriLink Biotechnologies)

Stem loop chimera (fully modified with 2′ fluoropyrimidines): 5′-GGG*C*G G*CUUUGCCAAGUGCUU*GGGAGGA*C*GA*U*G*C*GGA*U*CAG*CCAUGUUUA C*GUCACUCCU*AAG*CACUUGGCAAAG*CCGCCCUU*-3′

A10-3.2-Con chimera. A10-3.2-Con RNA sense strand (modified with 2′ fluoropyrimidines): 5′-GGGAGGA*C*GA*U*G*C*GGA*U*CAG*CCAUGUUUACG UCACUCCU*AA*UU*CU*CC*GAA*C*G*UG*UCACG*UUU*-3′

Con siRNA antisense (unmodified RNA): 5′-ACGUGACACGUUC GGAGAAUU-3′

Generating individual chimeras. Double-stranded DNA templates were generated by PCR as described[25]. Briefly, templates and primers for generating the individual chimeras are listed: PSMA template (5′-GGGAGGACGATGCGG ATCAGCCATGTTTACGTCACTCCTTGTCAATCCTCATCGGCAGACGACT CGCCCGA-3′) was used to generate A10-Plk1, blunt, OVH, G-U, swap, and G-U swap chimeras. The 5′ primer (5′pr) was common to all above chimeras (5′pr: 5′-TAATACGACTCACTATAGGGAGGACGATGCGG-3′)

The 3′primers used to generate each individual chimera are listed:

A10-Plk1 (3′pr: 5′-GCACTTGGCAAAGCCGCCCTTTCGGGCGAGTCG TCTG-3′)

Blunt (3′pr: 5′-GCACTTGGCAAAGCCGCCCTTTTAGGAGTGACGTAA AC-3′)

OVH (3′pr: 5′-AAGCACTTGGCAAAGCCGCCCTTTTAGGAGTGACGT AAAC-3′)

G-U (3′pr: 5′-AAACACTTGGCAAAGCCGCCCTTTTAGGAGTGACGTA AAC-3′)

Swap (3′pr: 5′-AAGGGCGGCTTTGCCAAGTGCTTTTAGGAGTGACGT AAAC-3′)

A10-3.2-Con (3′pr: 5′-AAACGTGACACGTTCGGAGAATTAGGAGTGA CGTAAAC-3′)

The stem loop chimera was generated with the stem loop template oligo (SL-oligo) (5′-AAGTGCTTGGGAGGACGATGCGGATCAGCCATGTTTACG TCACTCCT-3′)

SL 5′ primer: 5′-TAATACGACTCACTATAGGGCGGCTTTGCCAAGTGC TTGGGAGGA

SL 3′ primer: 5′-AAGGGCGGCTTTGCCAAGTGCTTAGGAGTGACGTA AAC

DNA templates were purified with Qiagen DNA purification columns and used in *in vitro* transcription reactions as described[25] to make individual RNA aptamers. All RNAs generated by *in vitro* transcription were produced with 2′ fluoro-modified pyrimidines to render the RNAs resistant to nuclease degradation. With the exception of the stem loop chimera, the 2′ fluoro-modified RNAs generated by transcription for all the other chimeras were annealed to the respective chemically synthesized sense or antisense Plk1 siRNA oligos. The RNAs were annealed at a ratio of 1:4 (RNA oligo:siRNA oligo) in a final concentration of the RNA oligo of 1 μM in DPBS including calcium and magnesium. For the annealing step, the RNA/siRNA mixtures were incubated at 65 °C for 10 min and then allowed to cool slowly at 25 °C for 30 min. Excess siRNA oligo was removed based on size exclusion with a 30 kDa cutoff Amicon spin filter (Millipore).

Cell culture. Normal human foreskin fibroblasts cells (obtained from A. Klingelhutz) were maintained in NuAire water-jacketed CO2 incubators at 37 °C and 5% CO_2 (NuAire) in DMEM supplemented with 10% FBS. Prostate cancer cell lines LNCaP (ATCC) were maintained in Ham's F12-K medium supplemented with 10% FBS. PC-3 and 22Rv1(1.7) luciferase-expressing cells (kindly provided by M. Henry) were grown in RPMI 1640 medium (GIBCO) supplemented with 10% FBS (Hyclone), 1 mM nonessential amino acids (GIBCO) and 100 μg/ml G-418.

^{32}P binding assays. PC-3 or LNCaP and 22Rv1(1.7) cell lines were used for these experiments. For the experiment in **Figure 2a**: 50,000 PC-3 or LNCaP cells (500 cells/μl) in DPBS (plus Ca^{+2} and Mg^{+2}) were blocked with 100 μg/ml tRNA and poly I:C for 15 min. Blocked cells were incubated at 37 °C for 30 min with 500,000–1 million c.p.m. of γ-^{32}P end-labeled A10 aptamer or truncated versions of A10 (A10-3; A10-3.2) in block solution. Cells were then washed profusely with DPBS (plus Ca^{+2} and Mg^{+2}) and bound and/or internalized RNA measured by scintillation counter. Percent aptamer bound was calculated based on input counts. This experiment was performed in triplicate. For determining the relative affinity of the PSMA aptamer and truncated PSMA aptamers, LNCaP cells were fixed in 1% formaldehyde in PBS for 20 min at 25 °C. Fixed cells were washed several times after which cells were diluted and blocked as above. Cells were incubated with serial dilutions of γ-^{32}P end-labeled RNAs ranging from 2 nM to 0 nM at 37 °C for 10 min. The amount of bound RNA was determined by filter binding assay as described in McNamara *et al.*[48] For assessing binding efficacy and specificity of the individual optimized PSMA chimeras, PC-3, LNCaP and 22Rv1(1.7) cells were prepped as for the experiment in **Figure 2a**. Cells were then incubated with 500,000 c.p.m. of γ-^{32}P end-labeled chimeras for 30 min at 37 °C. After several washes with DPBS (plus Ca^{+2} and Mg^{+2}), the amount of bound/internalized

RNA was determined by scintillation counter. The percent of RNA bound was calculated based on input counts.

Silencing assay and quantitative PCR. 22Rv1(1.7) cells were transfected with increasing amounts (4, 40 or 400 nM) of the individual optimized chimeras using Superfect (Qiagen) for 6h (**Fig. 3a**). Alternatively, cells were treated with increasing amounts (4, 40 or 400 nM) of the individual optimized chimeras in the absence of transfection reagent (**Fig. 3b**). 24–48 h (**Fig. 3a**) or 4d (**Fig. 3b**) after treatment, cells were processed for total RNA using RNeasy Kit (Qiagen). For the *in vivo* experiments in **Figure 6f**, tumors from mice treated with the various chimeras were excised and processed for total RNA followed by mRNA extraction as recommended by the manufacturer (RNeasy and Oligotext; Qiagen). Gene silencing was assessed by either qRT-PCR. Real-time PCR was performed on mRNA (50 ng) from 22Rv1(1.7) cells or tumors treated with the various siRNAs or chimeras using iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad) using an Eppendorf Realplex Mastercycler. All reactions were done in a 50 µl volume in triplicate. Primers for human *GAPDH* and *PLK1* are:

GAPDH forward: 5′-TCGCTCTCTGCTCCTCCTGTTC-3′; *GAPDH* reverse: 5′-CGCCCAATACGACCAAATCC-3′; *PLK1* forward: 5′-GACAA GTACGGCCTTGGGTA-3′; *PLK1* reverse: 5′-GTGCCGTCACGCTCTATGTA-3′. PCR parameters were as follows: 50 °C for 30 min, 5 min of *Taq* activation at 95 °C, followed by 45 cycles of PCR at 95 °C × 30s, 57 °C × 30s, 72 °C × 30s. Standard curves were generated with a serially diluted PCR product as template and the relative amount of target gene mRNA was normalized to *GAPDH* mRNA. Specificity was verified by melt curve analysis and agarose gel electrophoresis. Percent Plk1 mRNA expression in treated cells was determined relative to untransfected/untreated control sample, which was set to 100%.

***In vitro* Dicer assay.** The *in vitro* Dicer assays were performed as described previously[25] with minor modifications. Briefly, the Plk1 guide or passenger strands were end-labeled with T4 polynucleotide kinase (NEB) and γ-^{32}P-CTP. The corresponding strands of the various PSMA-Plk1 RNA aptamers were then annealed, with equimolar amounts, of the labeled siRNA strands in DPBS (plus Ca^{+2} and Mg^{+2}) to form the chimeras. The chimeras (100 pmol) were then incubated with 1 U of human recombinant Dicer enzyme at 37 °C for either 1 h or 2 h, following manufacturer's recommendations (Genlantis). Reactions were stopped with stop buffer and electrophoresed in a nondenaturing 15% polyacrylamide gel. The gels were dried and exposed to X-ray film.

Small fragment northern blots. *Transfection.* 22Rv1(1.7) PC cells were transfected with 200 pmols each of either siRNA duplex, A10-Plk1, blunt, OVH, G-U, swap, or stem loop chimeras using Superfect Reagent. After 24 h cells were processed and RNAs extracted using TRIzol extraction. Untreated cells were used as a negative control for this assay.

Probe synthesis. DNA templates complementary to the sense strand and antisense strand of the Plk1 siRNA were obtained from Integrated DNA Technologies.

Antisense probe: 5′GCACTTGGCAAAGCCGCCCTT3′.

Sense probe: 5′GGGCGGCTTTGCCAAGTGCTT3′.

U6 probe (5′-GCAGGGGCCATGCTAATCTTCTCTGTATCG-3′) was used as an internal loading control.

5 pmols of each probe was 5′ terminally modified through addition of [γ-^{32}P] (6,000 ci/mmol; 8.3 pmol) catalyzed by T4 polynucleotide kinase. Reactions were carried out for 30 min at 37 °C. Reactions were cleaned with a G25 spin columns (GE). Labeled probes were quantified by scintillation counter and equal counts were used for probing the northern blot.

Small fragment northern blot. 20 µg of RNA from each sample and 4 µl of Decade Marker System (Ambion) were heated at 95 °C for 5 min and immediately loaded onto a 15% polyacrylamide-8M urea denaturing Tris buffered saline (TBE) gel. Duplicate gels were loaded. The gels were run at 24 Watts for 3 h after which they were transferred onto a Hybond N+ nylon membrane in 1× TBE on ice for 1 h at 20 V using a semi-dry transfer apparatus. The nylon membranes were cross-linked using a Stratalinker. The membranes were pre-hybridized by incubating in Church's buffer containing 1 mg of boiled salmon sperm DNA at 37 °C for 2 h. Following the prehybridization step, the sense or antisense probes were added directly to the prehybridized blots and incubated at 37 °C overnight. The next day blots were washed 1× with 1× SSC/0.1% SDS for 20 min at 37 °C followed by three more washes with 0.5× SSC/0.1% SDS for 20 min at 37 °C. Blots were exposed overnight at −80 °C. Each blot was stripped by boiling in 0.5% SDS and reprobed for U6.

Proliferation (DNA synthesis) assay. PSMA-positive 22Rv1(1.7) cells were trypsinized and seeded in 12-well plates at ~20,000 cells/well. The next day cells were either transfected or treated in the absence of transfection reagents (data not shown) with either 400 nM or 4 nM of the various aptamer-siRNA chimeras for 24 h. Fresh media containing individual PSMA chimeras and ^{3}H-thymidine (1 µCi/ml medium) was then added to cells to monitor DNA synthesis. After 24 h incubation in the presence of media containing ^{3}H-thymidine, cells were washed twice with PBS, washed once with 5% wt/vol trichloroacetic acid (TCA) (VWR), collected in 0.5 ml of 0.5N NaOH (VWR) and placed in scintillation vials for measurement of ^{3}H-thymidine incorporation.

Cell cycle profile (PI staining). 22Rv1(1.7) cells were seeded on 60 mm plates on day 1. On day 2 cells were transfected or treated in the absence of transfection reagents (data not shown) with 4 nM of the various PSMA chimeras. Cells were processed on day 4 and DNA content measured by PI staining. Briefly, cells were trypsinized and washed several times with DPBS. Cells were then resuspended in NIM Buffer (0.5% BSA; 0.1% NP-40 in PBS) supplemented with 0.1 mg/ml RNase A (DNase free) and 5 µg/ml PI. Nocodazole (Noc) was used as a positive control to arrest cells in mitosis (G2/M phase of the cell cycle). Cells were treated with 100 ng/ml of Noc for 16 h before staining with PI. Stained cells were processed by flow cytometry to measure DNA content.

Cell viability assay (caspase 3). 22Rv1(1.7) cells were either transfected (data not shown) or treated in the absence of transfection reagent (**Table 1** and **Supplementary Fig. 2**) with either 400 nM or 4 nM of the various optimized chimeras. Cells were also treated with medium containing 2 nM cisplatin for 30 h as a positive control for apoptosis. Untreated cells were used as a negative control for the assay. Cells were then fixed and stained for active caspase 3 using a phycoerythrin (PE)-conjugated antibody specific to cleaved caspase 3 as specified in manufacturer's protocol (Pharmingen). Flow cytometric analysis was used to quantify percentage PE-positive cells as a measure of apoptosis.

Tumor implantation and monitoring tumor growth. Athymic nude male mice (nu/nu) 6–10 weeks old were obtained from Harlan Sprague Dawley and maintained in a sterile environment according to guidelines established by the US Department of Agriculture and the American Association for Accreditation of Laboratory Animal Care (AAALAC). Athymic mice were inoculated with 1×10^{6} (in 100 µl of 50% Matrigel) of either *in vitro* propagated PC-3 or 22Rv1(1.7) cells subcutaneously injected into each flank. Nonnecrotic 22Rv1(1.7) and PC-3 tumors, which exceeded 0.7 cm in diameter (average ~0.4 cm^3 in volume), were randomly divided into four groups or two groups respectively of ≥10 mice per treatment group. Mice bearing 22RV1(1.7) tumors were treated with: group 1, no treatment (DPBS); group 2, treated with blunt (1 nmol/injection × 10); group 3, treated with swap (1 nmol/injection × 10); group 4, treated with A10-3.2-Con (1 nmol/injection × 10). Mice bearing PC-3 tumors were treated with DPBS or swap (1 nmol/injection × 10); chimera only. Compounds were injected intraperitoneally in 100 µl volumes every day for a total of ten injections. Day 0 marks the first day of injection. Tumors were measured (in two dimensions) every other day with calipers. The following formula was used to calculate tumor volume: $V_T = L \times W^2/2$ (W, the shortest dimension; L, the longest dimension). The growth curves are plotted as the mean tumor volume ± s.e.m. The animals were euthanized 2–3 d after the last treatment and the tumors were excised and formalin fixed for immunohistochemistry. Slides of serial sections were stained with hematoxylin and eosin (H&E) and processed for TUNEL using the ApopTag Kit (Millipore) as a measure of apoptosis. For the PSMA-positive tumors treated with PEGylated swap chimera, athymic nude male mice (nu/nu) 6–10 weeks old were injected with 22Rv1(1.7) cells as indicated above. A total of seven mice per treatment group were injected. After ~3 weeks when tumors had reached 0.7 cm in diameter in the longest dimension, mice were divided into 3 groups: group 1 (DPBS), group 2 (250 pmols/injection swap), and group 3 (250 pmols/injection swap-PEG). Compounds were injected intraperitoneally in 100 µl volumes every other day for a total of five injections. Tumors were measured every other day on the day before the compound injection.

doi:10.1038/nbt.1560

Bioluminescence imaging. To examine tumor size after treatment, we injected luciferin intraperitoneally (50 µl of 15 mg/ml luciferin/10 g mouse body weight) using a 26-gauge needle. Following a 5 min incubation, we performed bioluminescence imaging in a Xenogen IVIS100 imaging system (Xenogen) using a 5 s exposure. Mice were imaged in a dorsal (5 min post-luciferin injection) presentation to monitor tumor growth and/or status after treatment. A mouse was euthanatized when it reached clinical endpoints such as >15% body weight loss or tumors of >2 cm in the longest diameter. We measured whole body tumor growth rates as follows. We placed a circular region of interest (ROI) around the tumor sites of each mouse and quantified total flux using Living Image Software v2.50 (Xenogen) with the units of photons/s/cm^2/sr.

ELISA. Athymic nude male mice (nu/nu) ($n = 6$) were injected with 1 nmol of either A10-3.2-Con or swap chimeras in 250 µl of saline (DPBS). As a positive control for immunostimulation, mice were injected with 200 ng of poly I:C in 100 µl saline. Mice injected with saline alone (250 µl) were used as a negative control. 18 h after injection ~300 µl of blood was drawn from each mouse. The blood was allowed to coagulate at 25 °C before centrifuging the blood samples at 17,000*g* for 10 min to remove erythrocytes and collect serum. Levels of the cytokines IL-6 (R&D Systems) and INT-α (PBL Biomedical Laboratories) in the serum of treated mice were determined by ELISA following manufacturer's recommendations.

5′-rapid amplification of cDNA ends (5′-RACE) PCR analysis. mRNA (10 ng) from tumors treated with different chimeras was ligated to a GeneRacer adaptor (Invitrogen) without prior treatment. Ligated RNA was reverse transcribed using a gene-specific primer 1 (GSP1: 5′-GAATCCTACGACGTGCTGGT-3′). To detect cleavage products, PCR was performed using primers complementary to the RNA adaptor (GR5′pr: 5′-CGACTGGAGCACGAGGACACTGA-3′) and gene-specific primer 2 (GSP2: 5′-GCTGCGGTGAATGGATATTT-3′). The amplification products were resolved by agarose gel electrophoresis and visualized by ethidium bromide staining. The identity of the specific PCR products was confirmed by sequencing of the excised bands.

Pharmacokinetics (PK measurements). C57/BL6 mice ($n = 3$ per treatment group) were injected intraperitoneally with either DPBS or 1 nmol of either swap chimera or swap chimera modified with a 20 kDa PEG group (swap-PEG). Approximately 100 µl of blood volume was retrieved from each mouse at 10 min, 1 h, 5 h, 30 h after injection with compound. The RNA chimeras in blood serum samples were extracted with phenol:chloroform and chloroform. Total RNA in samples was digested with RNase A to remove endogenous RNA and recover nuclease-resistant chimeras. Excess RNase A was removed with a subsequent phenol:chloroform extraction and the RNA chimeras were ethanol precipitated for 2 h at –80 °C by addition of 1/10 volume of sodium acetate, 5 µl of linear acrylamide and two volumes of 100% ethanol. RNA chimera pellets were resuspended in 50 µl of TE and 5 µl of the recovered RNA used for quantitative PCR analysis.

Pharmacodynamics. Athymic nude male mice (nu/nu) 6–10 weeks old were inoculated with 1×10^6 (in 100 µl of 50% Matrigel) *in vitro* propagated 22Rv1(1.7) cells subcutaneously injected into each flank. Nonnecrotic 22Rv1(1.7) tumors, which exceeded 0.7 cm in diameter (~0.4 cm^3 in volume), were randomly divided into three groups as follows: group 1, no treatment (DPBS; $n = 4$); group 2, treated with swap-2′F (1 nmol/injection) ($n = 4$); group 3, treated with swap2′F-PEG (1 nmol/injection) ($n = 4$). Mice were injected on day 1 and then again on day 2 with either DPBS, or 1 nmol each of either the swap-2′F or swap-2′F-PEG chimeras. Tumors from these mice were excised on day 3 (48 h) or on day 5 (5 d). The tumors were processed for total RNA followed by mRNA extraction as recommended by the manufacturer (RNeasy and Oligotext). Silencing of Plk1 gene expression was determined by qRT-PCR as described above.

Statistical analysis. Statistical analysis of tumor size data was conducted using a one-way ANOVA. A *P*-value of 0.05 or less was considered to indicate a significant difference. In addition, two-tailed unpaired *t*-tests were conducted to compare each treatment group to every other.

48. McNamara, J.O. *et al.* Multivalent 4-1BB binding aptamers costimulate CD8 T cells and inhibit tumor growth in mice. *J. Clin. Invest.* **118**, 376–386 (2008).