Experimental Section

Tissue culture

A431, MCF7, and MDA-MB-468 cells were purchased from ATCC (Manassas, VA) and MDA-MB-435 cells were obtained from Dr. Konstantin V. Sokolov's lab at the University of Texas at Austin. A431, MDA-MB-435, and MDA-MB-468 cells were cultured in DMEM (ATCC) supplemented with 10% FBS (Invitrogen, Carlsbad, CA), and MCF7 cells were cultured in RMPI-1640 (ATCC) supplemented with 10% FBS.

In vitro selection of anti-EGFR aptamers

The DNA library for selection was synthesized on a DNA synthesizer (Expedite, Applied Biosystems, Foster City, CA) in our laboratory. The library consisted of a 62-nucleotide random region flanked by two constant regions: 5'-ga<u>taatacgactcactata</u>ggcgctccgaccttagtctctg-N_{62}-gaaccgtgtagcacagcaga-3' (the T7 RNA polymerase promoter is underlined). The initial RNA pool was generated by transcribing some 10^{14} DNA templates using an Ampliscribe kit (Epicentre, Madison, WI) followed by DNase treatment and PAGE purification. About 2 nmole of RNA ($1.2x10^{15}$ molecules) and 100 pmole of hEGFR protein (R&D Systems, Minneapolis, MN) were used for each round of selection in a reaction volume of 100 µL. RNA was first denatured and refolded in 100 µL Selection Buffer (1x DPBS (Invitrogen) and 5 mM $MgCl^{2+}$), and was then passed through a 0.45 µm modified cellulose disk (Millipore, Bedford, MA) to remove non-specific binding species. Human EGFR (R&D systems, Minneapolis, MN) was added to the filtered RNA solution. After incubation at 25º C for 30 min the binding reaction was filtered through a 0.45 µm modified cellulose disk and washed with 1 mL Selection Buffer. The modified cellulose filter was then boiled at 95ºC for 5 min in

Elution Buffer (200 mM NaCl, 25 mM EDTA, and 8 M urea) to release bound RNA. The RNA was washed twice with 150 µL water on M30 filters (Millipore), recovered by inversion and centrifugation., reverse transcribed, and PCR amplified. The DNA pool from Round 12 of the selection was cloned and sequenced.

Binding assays

The K_d of the aptamer:protein complexes were determined by assaying binding as a function of protein concentration, as previously described[1].

To assay the formation of monomeric hEGFR, about 0.3 µg of protein was incubated in Selection Buffer with 5 mM DTT for 10 min at 25º C. The solution was then mixed with 4x loading dye (Invitrogen) and loaded onto a 4-12% NuPAGE gel with 1x MOPS running buffer (Invitrogen) alongside a SeeBlue® Plus2 standard (Invitrogen). After running at 200 V for 1 hour, the gel was stained with Simply Blue Safe Stain (Invitrogen).

Aptamer J18's binding affinity for dimeric and monomeric hEGFR was assayed by incubating 10 nM of [α-^{32}P]-ATP-labeled Aptamer J18 with 100 nM hEGFR (with or without DTT treatment) for 30 min at 25º C. To internally label RNA 1µL of [α-^{32}P]-ATP (3000Ci/mmol, 10mCi/ml, Perkin Elmer, Waltham, MA) was added for a 20µL reaction. About 10 nM of [α-^{32}P]-ATP-labeled Aptamer J18 was also incubated with 100 nM hIgG, hErbB2, and mEGFR (with and without DTT treatment) for 30 min at 25º C. All proteins were from R&D Systems. The percentage of bound RNA was analyzed and calculated as previously described.[1]

Assaying cell surface binding by flow cytometry

A431 and MDA-MB-435 cells were grown to 70% confluence, trypisinized, and washed 3 times with 100 µL Selection Buffer. Aptamer J18 and the unselected N62 pool RNA were labeled with PE (1:1) (Phycoerythrin, Prozyme, San Leandro, CA) as described previously.[2] Briefly, Aptamer J18 and the unselected N62 pool RNA were extended with a 24 nt sequence, hybridized to a biotinylated oligonucleotide, and incubated with streptavidin-PE. Phycoerythrin-labeled unselected N62 pool RNA (10 nM), Aptamer J18 (10 nM), IgG (0.3 µg/mL, ca. 0.1 nM, BD Bioscience, San Jose, CA) , and anti-EGFR antibody (0.3 µg/mL, ca. 0.1 nM, BD Bioscience, San Jose, CA) were added to 200,000 cells. In a separate experiment, phycoerythrin-labeled Aptamer J18 and phycoerythrin-labeled anti-EGFR antibody were also mixed with 300 nM antibody 225 (EGFR Ab-2, Thermo Fisher Scientific, Fremont, CA) before being applied to cells. After incubation at 25º C for 30 min cells were washed 3 times with 100 µL Selection Buffer and then suspended in 300 µL Selection Buffer. For each sample, 10,000 events were collected and analyzed using FACSCalibur with a FL2-H detector (Becton Dickinson, San Jose, CA).[2]

Confocal microscopy

A431 cells were cultured in an 8-chamber slide (Thermo Fisher Scientific) for 12 hours prior to analysis. A431 cells were washed 3 times with 150 µL Selection Buffer and then incubated with 150 µL of 100 nM PE-labeled Aptamer J18 or PE-labeled unselected N62 pool RNA. In addition, 100 nM Aptamer J18 was mixed with 1 µM EGF (R&D systems), FGF (R&D systems), antibody 225, or anti-transferrin antibody (Abcam, Cambridge, MA) before being added to the A431 cells. After incubation at 37º C for 30 min A431 cells were washed 3 times with 150 µL Selection Buffer. Fluorescent images

were obtained with a Leica SP2 AOBS confocal microscope (Leica Microsystems, Mannheim, Germany) with excitation at 543 nm and emission between 550 to 600 nm under a 63x oil objective.

Flow cytometry-based internalization assay

A431 cells were trypsinized and washed 3 times with 100 µL Selection Buffer. Phycoerythrin-labeled Aptamer J18 and unselected N62 pool RNA were incubated with A431 cells for 30 min either on ice or at 37º C. The cells incubated with Aptamer J18 were then treated with 0.01 u/µL Riboshredder (Epicentre Biotechnologies, Madison, WI) for 10 min at 25º C followed by washing 3 times with 100 µL Selection Buffer. Samples were analyzed by flow cytometry as described above and the amount of internalized Aptamer J18 was calculated using the following equation:

$$\text{RNA Internalized} = \frac{\text{F2-F0}}{\text{F1-F0}} \qquad (1)$$

where F0, F1, and F2 represent fluorescence of unselected N62 pool RNA-labeled cells, fluorescence of Aptamer J18-labeled cells, and fluorescence of Aptamer J18-labeled cells after Riboshredder treatment, respectively.

Imaging Aptamer J18 conjugated to gold nanoparticles

Gold nanoparticles (GNP, 20 nm) were purchased from Ted Pella (Redding, CA). Conjugation of the aptamers to GNP was achieved by hybridizing a biotin- and thiol-dual labeled capture oligonucleotide (IDT, Coralville, IA) to an extension sequence at the 3' end of Aptamer J18, essentially as previously described.[3] In the current instance, there

was also an 18-atom spacer following the 5' thiol modification on the capture oligonucleotide: 5' - /THIOMC6-D/SP18/CTGGTCATGGCGGGCATTTAATTC/BIOTEG/ - 3'. In detail, the dual-labeled DNA oligonucleotide was first deprotected on an Illustra NAP-5 Column (GE Healthcare, Piscataway, NJ). Two 1.5 mL Eppendorf tubes were heated to 80º C with one containing 1 mL of GNP and the other containing 180 µL of water, 16 µL of 25x DPBS, 2 µL of 0.5 M buffered TCEP (tris(2-carboxyethyl)phosphine hydrochloride, Sigma-Aldrich, St. Louis, MO) and 2 nmoles of deprotected oligonucleotide. GNPs were rapidly added to the oligonucleotide solution, and after 5 min 60 µL of 25x DPBS was added. The particles were allowed to cool to 25º C and then washed twice with 1x DPBS. Particles were centrifuged at 16,060 x g for 30 minutes to remove any unreacted capture oligonucleotide. Afterward, 300 pmoles of extended Aptamer J18 or unselected N62 pool RNA were hybridized to 1 mL of DNA oligonucleotide-coated GNP by mixing and heating to 80º C for 5 min. The RNA-GNP conjugate was spun down, resuspended in 1 mL 1x DPBS, and incubated with 25 µL of 6.7 µM streptavidin-PE for 10 min at 25º C. The RNA-GNP conjugate was washed another time with 1x DPBS to remove unreacted RNA and streptavidin-PE, and then resuspended in 1 mL of Binding Buffer. GNPs were disaggregated with a sonicator as necessary (Covaris S2 system, Applied Biosystems, Foster City, CA).

Extended Aptamer J18 or unselected N62 pool RNA and capture oligonucleotides were removed from GNPs for gel analysis by incubating 10 µL of the RNA-GNP conjugates with 10 µL of 2x loading dye (20 mM EDTA, 0.05% bromophenol blue, prepared in 8 M urea) containing 5 mM DTT at 70º C for 10 min. To determine the

sensitivity of RNA-GNP conjugates to Riboshredder, RNA-GNP was treated with 0.01 u/µL Riboshredder for 10 min at 25º C before the addition of 2x loading dye. All samples were loaded onto a 8% denaturing PAGE. The amount of eluted RNA or capture oligonucleotide was assessed by comparison with standards.

To monitor the binding and internalization of RNA-GNP conjugates to cells, A431 and MDA-MB-435 cells were trypsinized and washed as described before. RNA-GNP (500 µL) was then incubated with 200,000 cells (100 µL) in Binding Buffer for 30 min either on ice or at 37º C. Some samples were furthered treated with Riboshredder as described above. After washing 3 times with Binding Buffer, cells were resuspended in 300 µL Binding Buffer and bound fluorescent signals were determined by flow cytometry. The remaining samples were transferred to 8-chamber slides, cells were allowed to settle for 5 min, and cells were imaged by confocal microscopy.

References

1 Li, N., Y. Wang, A. Pothukuchy, A. Syrett, N. Husain, S. Gopalakrisha, P. Kosaraju, and A.D. Ellington, *Nucleic Acids Res.* **2008**, *36*, 6739-6751.

2 N. Li, J. N. Ebright, G. M. Stovall, X. Chen, H. H. Nguyen, A. Singh, A. Syrett and A. D. Ellington, *J. Proteome. Res*. **2009**, *8*, 2438-2448.

3 D. J. Javier, N. Nitin, M. Levy, A. Ellington and R. Richards-Kortum, *Bioconjug. Chem*. **2008**, *19*, 1309-1312.

Supplementary Figures

A

GGCGCUCCGACCUUAGUCUCUGCAAGAUAAACCGUGCUAUUGACCACCCUC
AACACACUUAUUUAAUGUAUUGAACGGACCUACGAACCGUGUAGCACAGC
AGA

B

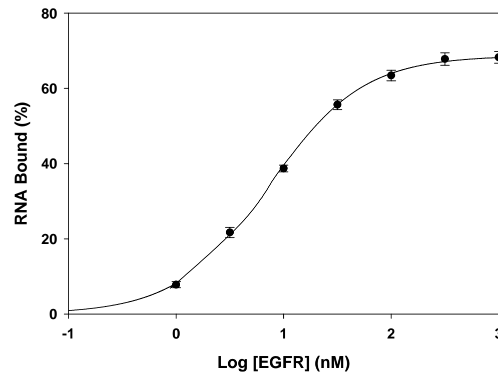

Figure S1. Anti-EGFR aptamer. A) Sequence of selected anti-EGFR aptamer J18. B) Binding isotherm for Aptamer J18:EGFR complexes. Kd values were derived by fitting the percentage of bound RNA versus the protein concentration to the equation $Y=B_{max}X/(K_d+X)$**,** where B_{max} is the extrapolated maximal amount of RNA:protein complex bound.

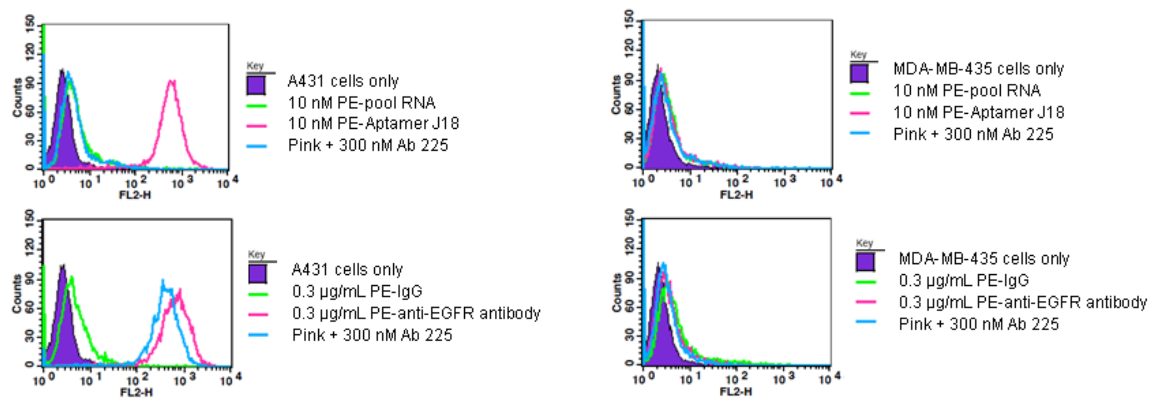

Figure S2. Cell surface binding of Aptamer J18. Phycoerythrin-labeled, unselected N62 pool RNA (10 nM), Aptamer J18 (10 nM), IgG (0.3 µg/mL), and an anti-EGFR Ab (0.3 µg/mL) were incubated with A431 cells (left) and MDA-MB435 cells (right) in the absence or presence of anti-EGFR antibody 225 in Selection Buffer for 30 min at 25ºC. Bound aptamer:PE and other conjugates were detected and quantitated on the FL2-H channel of a FACSCalibur. Green line: PE-labeled unselected N62 pool RNA (upper) or IgG (lower). Pink line: PE-labeled Aptamer J18 (upper) or anti-EGFR antibody (lower). Blue line: Either PE-labeled Aptamer J18 (left) or anti-EGFR antibody (right) together with antibody 225.

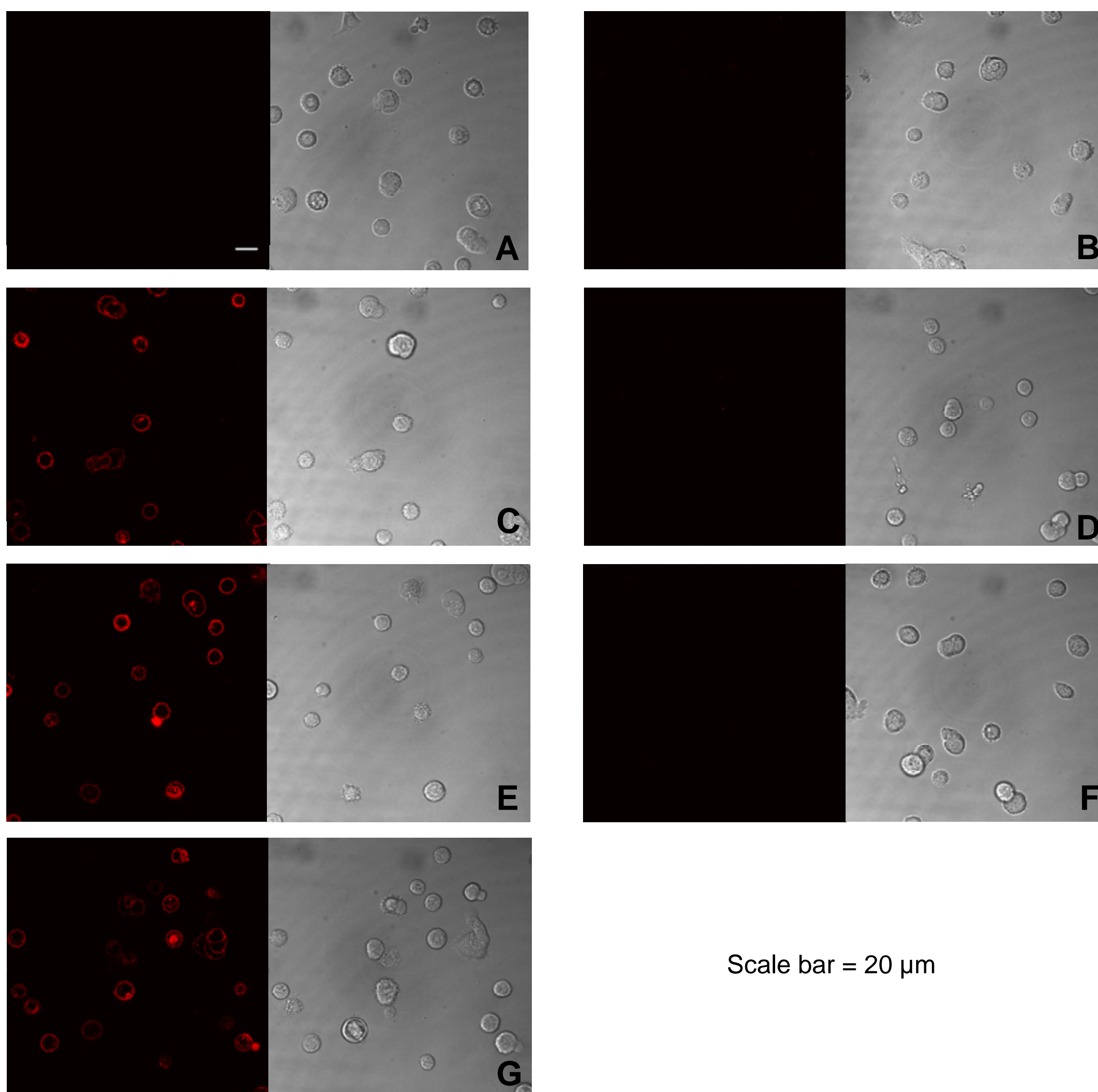

Figure S3. Microsopic detection of aptamer binding. A431 cells (A) were incubated with 100 nM PE-labeled unselected N62 pool RNA (B), 100 nM PE-labeled Aptamer J18 (C), 100 nM PE-labeled Aptamer J18 with 1 µM EGF (D), 100 nM PE-labeled Aptamer J18 with 1 uM FGF (E), 100 nM PE-labeled Aptamer J18 with 1 µM antibody 225 (F), or 100 nM PE-labeled Aptamer J18 with 1 µM anti-transferrin antibody (G) for 30 min at 37ºC. Cell-labeling was detected using a Leica confocal microscope with a 63x objective, excitation at 543 nm, and emission from 550-600 nm. Fluorescent images are on the left and bright field images on the right. Scale bar, 20 µm.

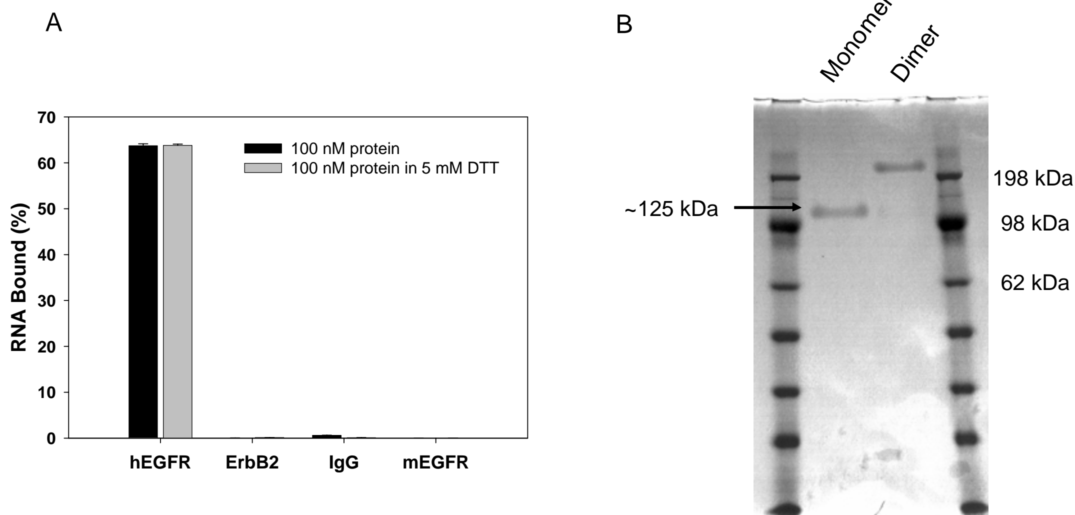

Figure S4. Specific binding to hEGFR. A) Binding assays. Different proteins (100 nM, with and without DTT treatment) were incubated with Aptamer J18 in Selection Buffer (with and without 5 mM DTT) for 30 min at 25º C. Radiolabeled, protein-bound RNA and free RNA were collected by nitrocellulose and nylon filters, respectively, and quantitated. B) Analysis of dimer disruption. EGFR was treated with 5 mM DTT ('Monomer') or untreated ('Dimer') and analyzed on a 4-12% NuPAGE relative to size standards.