Supporting Information for

A cell-permeable stapled peptide inhibitor of the estrogen receptor/coactivator interaction

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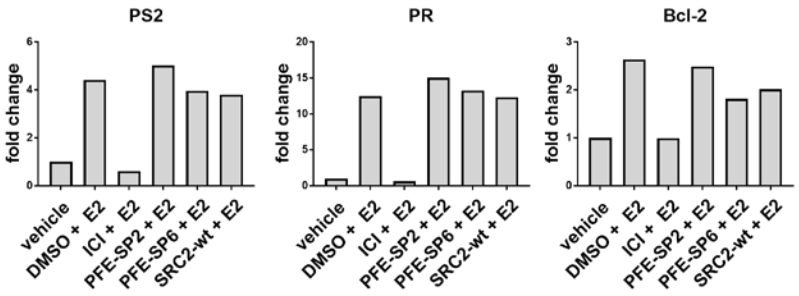

Figure S1. PFE-SP2 and PFE-SP6 do not inhibit transcription of ER-regulated native genes. mRNA levels for ER-regulated genes PS2, PR, and Bcl-2 were examined in MCF-7 cells treated for 24 hours with vehicle, 10 nM estradiol (E2) alone, 10 nM E2 + 1 μM ICI, 10 nM E2 + 50 μM PFE-SP2, 10 nM E2 + 50 μM PFE-SP6, and 10 nM E2 + 50 μM SRC2-wt. Data are normalized to beta-actin internal control and presented as fold change relative to DMSO vehicle.

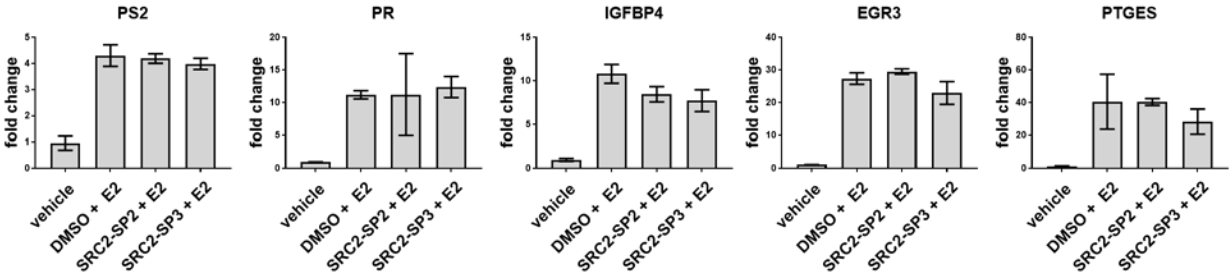

Figure S2. SRC2-SP2 and SRC-SP3 do not inhibit transcription of ER-regulated native genes. mRNA levels for ER-regulated genes PTGES, PR, PS2, EGR3, and IGFBP4 were examined in MCF-7 cells treated for 24 hours with vehicle, 10 nM estradiol (E2) alone, 10 nM E2 + 15 μM SRC2-SP2, and 10 nM E2 + 15 μM SRC2-SP3. Data are normalized to beta-actin internal control and presented as fold change relative to DMSO vehicle. Error bars represent the standard deviation from the mean. No stapled peptide treatments were significantly different from E2 treatment.

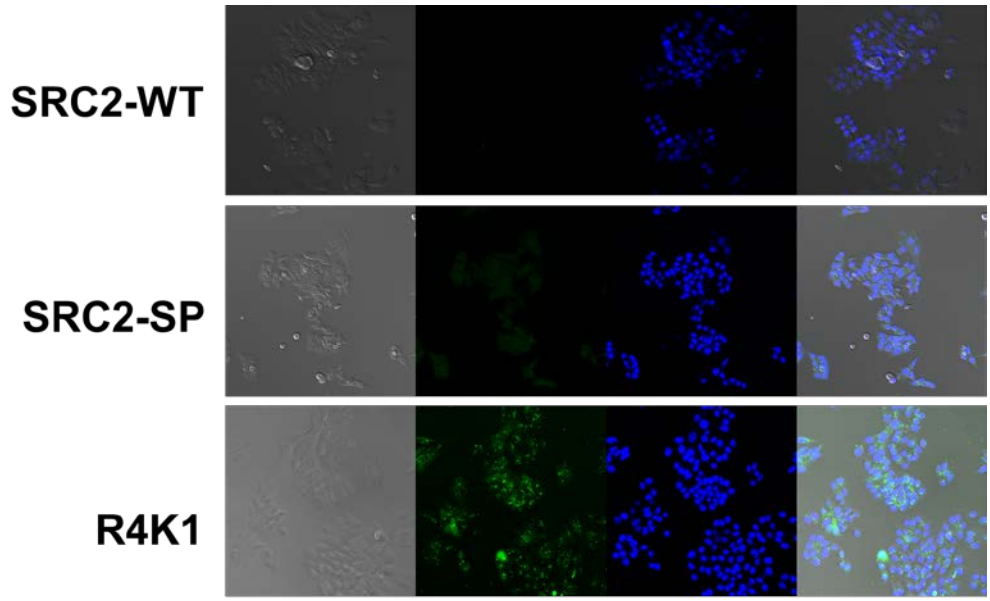

Figure S3. Uptake of FITC conjugated peptides (4 Hrs.). MCF-7 cells were treated for 4 hours with 15 μM fluorescein isothiocyanate (FITC)-labeled SRC2-WT (top), SRC2-SP (center), or R4K1 (bottom). Images from left to right include brightfield, FITC channel, Hoechst stained nucleus and FITC/Hoechst overlay at 20× magnification.

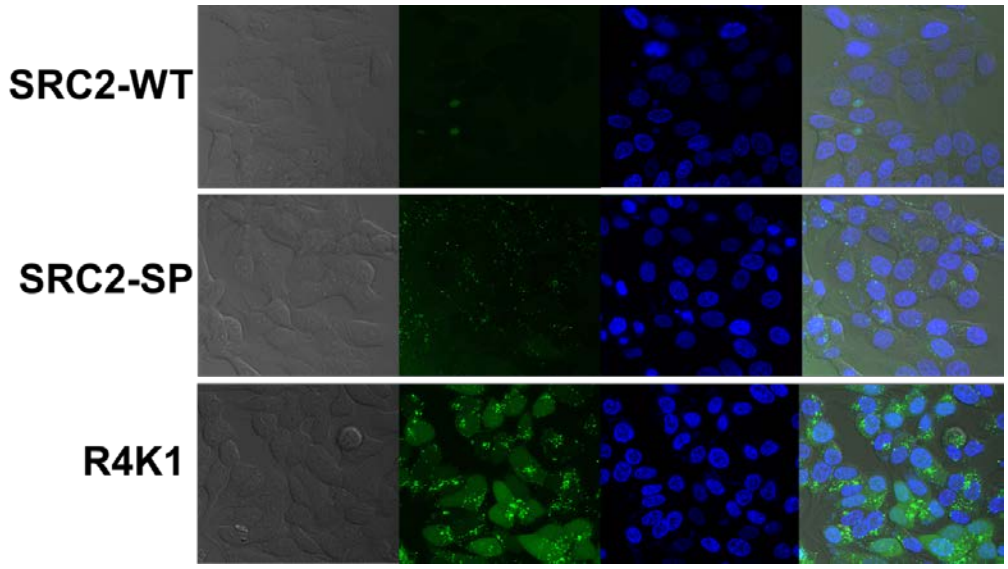

Figure S4. Uptake of FITC conjugated peptides (8 Hrs.). MCF-7 cells were treated for 8 hours with 15 μM fluorescein isothiocyanate (FITC)-labeled SRC2-WT (top), SRC2-SP (center), or R4K1 (bottom). Images from left to right include brightfield, FITC channel, Hoechst stained nucleus and FITC/Hoechst overlay at 63× magnification.

Figure S5. (**A**) MCF-7 or (**B**) T47D cells were treated for one hour with DMSO vehicle, 30 μM SRC2-WT, 30 μM SRC2-SP4, or 30 μM R4K1. Percent release of lactate dehydrogenase (LDH) was measured relative to maximum lysis with sodium dodecyl sulfate.

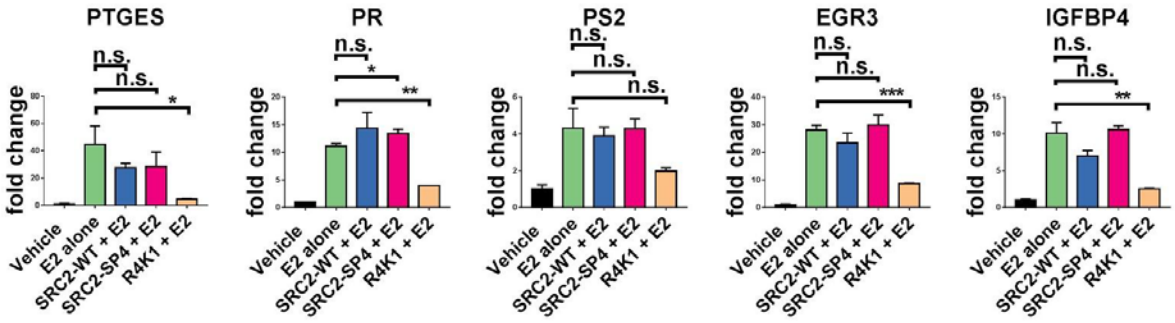

Figure S6. R4K1, but not SRC2-SP4 or SRC2-WT, inhibits transcription of ER-regulated native genes. mRNA levels for ER-regulated genes PTGES, PR, PS2, EGR3, and IGFBP4 were examined in MCF-7 cells treated for 24 hours with vehicle, 10 nM estradiol (E2) alone, 10 nM E2 + 15 μM SRC2-WT, 10 nM E2 + 15 μM SRC2-SP4, or 10 nM E2 + 15 μM R4K1. Data are normalized to beta-actin internal control and presented as fold change relative to DMSO vehicle. Error bars represent the standard deviation from the mean. n.s., not statistically significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

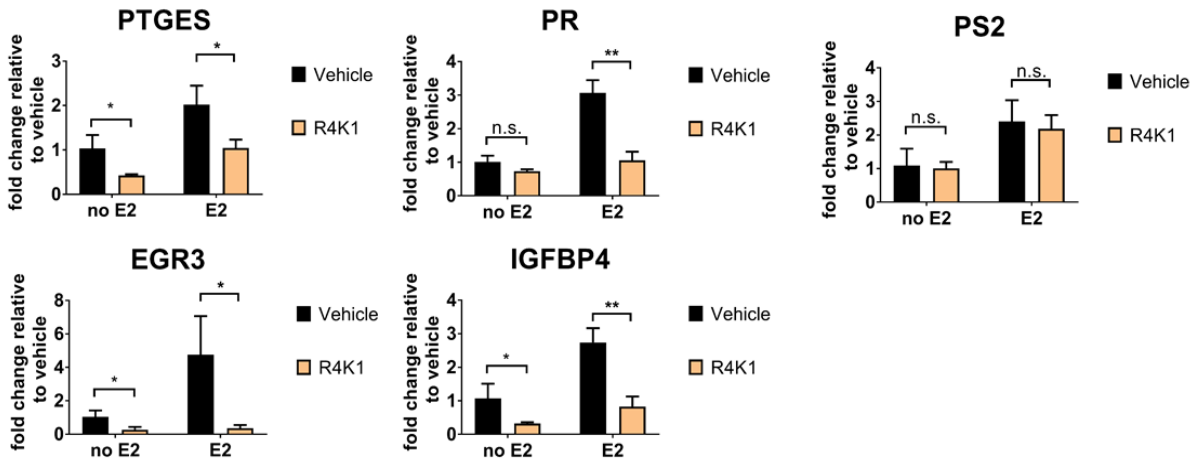

Figure S7. R4K1 inhibits transcription of ER-regulated native genes. mRNA levels for ER-regulated genes PTGES, PR, EGR3, PS2, and IGFBP4 were examined in T47D cells treated for 24 hours with vehicle, 10 nM estradiol (E2) alone, 15 μM R4K1 alone, or 10 nM E2 + 15 μM R4K1. Data are normalized to beta-actin internal control and presented as fold change relative to DMSO vehicle. Error bars represent the standard deviation from the mean. n.s., not statistically significant; *, $p<0.05$; **, $p<0.01$.

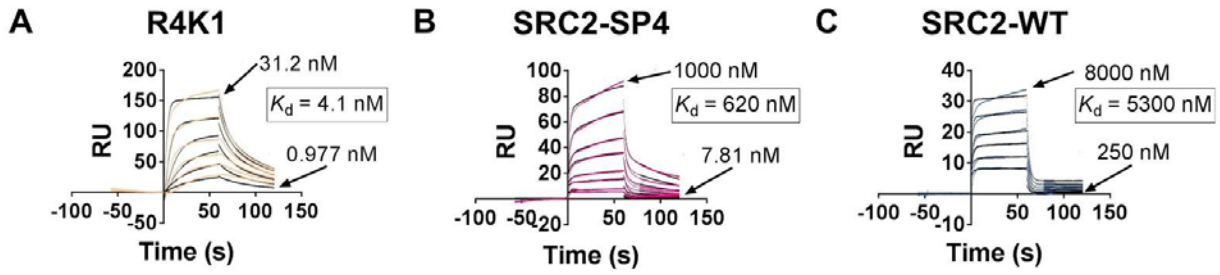

Figure S8. A surface plasmon resonance (SPR) assay using immobilized estrogen receptor α ligand binding domain was used to determine dissociation constants for R4K1 (beige, A), SRC2-SP2 (magenta, B), and SRC2-WT (blue, C). Data were analyzed using a kinetic fit.

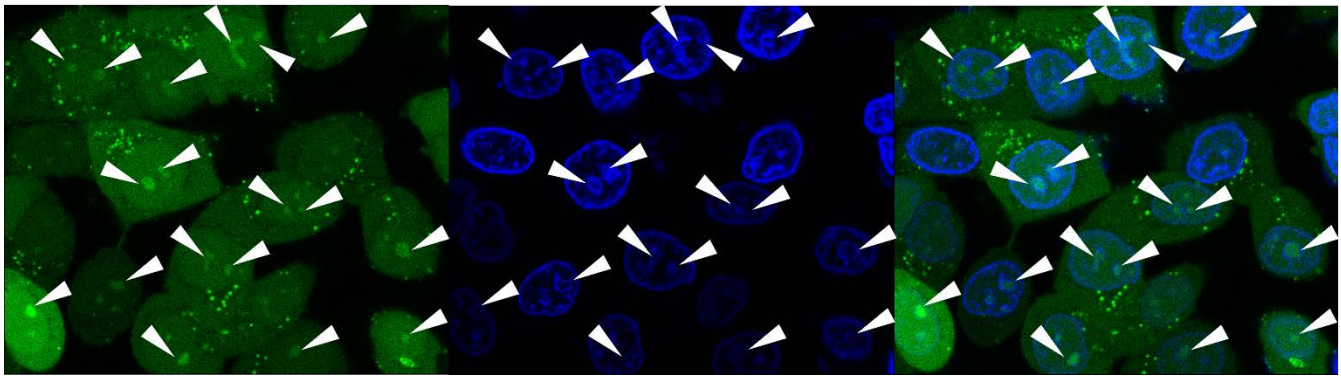

Figure S9 R4K1 localization to nucleoli. MCF-7 cells were treated for 24 hours with 15 μM fluorescein isothiocyanate (FITC)-labeled R4K1. Images from left to right include FITC channel, Hoechst stained nucleus, and FITC/Hoechst overlay at 63× magnification. White arrows indicate peptide localization to nucleoli.

Abbreviations. DIPEA, diisopropylethylamine; DMF, dimethylformamide; TFA, trifluoroacetic acid; Fmoc, 9-fluorenylmethylformamide; MeCN, acetonitrile; MBHA, 4-methyl-benzylhydrylamine; S5, (*S*)-2-(4-pentenyl)Ala; DCE, dichloroethane; PyClock, 6-Chloro-benzotrizole-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole hydrate; HPLC, high performance liquid chromatography; SPPS, solid phase peptide synthesis; MALDI-TOF, matrix assisted laser desorption ionization time of flight; β_{ala}, 3-aminopropanoic acid; FITC, fluorescein isothiocyanate; λ^s, (2*S*,4*S*)-2-amino-2,4-dimethylhept-6-enoic acid.

Peptide Synthesis. All peptides were manually prepared on 30 µmol scale using standard Fmoc solid phase peptide synthesis and rink amide MBHA resin. Fmoc deprotection was carried out for 2 x 10 minutes using 25% piperidine in DMF with 0.1 M HOBt. Amino acids were coupled using 5 eq of amino acid, 5 eq of PyClock, and 10 eq of DIPEA in 0.75 mL of DMF. Stapling amino acid S5 was coupled for 2 hrs, amino acids following S5 were coupled for 2 x 1.5 hrs, and all other amino acids were coupled for 2 x 20 min. Ring closing metathesis was performed 2 x 120 min at 55 ^{o}C using 1 mL of 4.94 mg/mL Grubb's 1st generation catalyst in DCE. Acetylation or FITC labeling, and peptide cleavage/deprotection were carried out as previously described [1] The crude peptides were purified to >95% homogeneity by semi-preparative HPLC (Solvent System MeCN:H_2O with 0.1% formic acid; 0-4 min, 10% MeCN; 4-24 min 10-50% MeCN; 24-25 min, 50-80% MeCN; 25-30 min, 80% MeCN; 30-31 min 80-10% MeCN. Column: Phenomenex Luna 5 µm C18(2), 100 Å, 250 x 10 mm). Peptide purity was confirmed using analytical HPLC (Solvent System MeCN:H_2O with 0.1% trifluoroacetic acid; 0-2 min, 4% MeCN; 2-12 min 4-70% MeCN; 12-13 min, 70% MeCN; 13-14 min, 70-4% MeCN; 14-17 min 4% MeCN. Column: Phenomenex Kinetex 5 µm C18, 100 Å, 50 x 4.6 mm). Peptide mass was measured using a Bruker Autoflex MALDI-TOF mass spectrometer (Table S1). The matrix used to prepare dried droplet samples was composed of a saturated solution of α-cyano-4-hydroxycinnamic acid in 50:50 water/acetonitrile with 0.1% TFA.

Table S1: MALDI-TOF Analysis of Peptides

Peptide	Sequence	Chemical Formula [M+H$^+$]	Exact Mass	Observed Mass
SRC2-WT	Ac-HKILHRLLQDS-NH_2	$C_{62}H_{106}N_{21}O_{16}$+	1400.813	1400.870
SRC2-SP4	Ac-HKS^5LHRS^5LQDS-NH_2	$C_{64}H_{106}N_{21}O_{16}$+	1424.813	1424.850
R4K1	Ac-RRRRKS^5LHRS^5LQDS-NH_2	$C_{82}H_{147}N_{34}O_{19}$+	1912.158	1912.164
SRC2-SP-FITC	FITC-β_{ala}-HKλ^{s5}LHRS^5LQDS-NH2	$C_{87}H_{122}N_{23}O_{21}S$+	1856.891	1856.896
R4K1-FITC	FITC-β_{ala}-RRRRKS^5LHRS^5LQDS-NH_2	$C_{104}H_{161}N_{36}O_{24}S$+	2330.221	2330.216
SRC2-WT-FITC	FITC-β_{ala}-HKILHRLLQDS-NH_2	$C_{84}H_{120}N_{23}O_{21}S$+	1818.874	1818.954

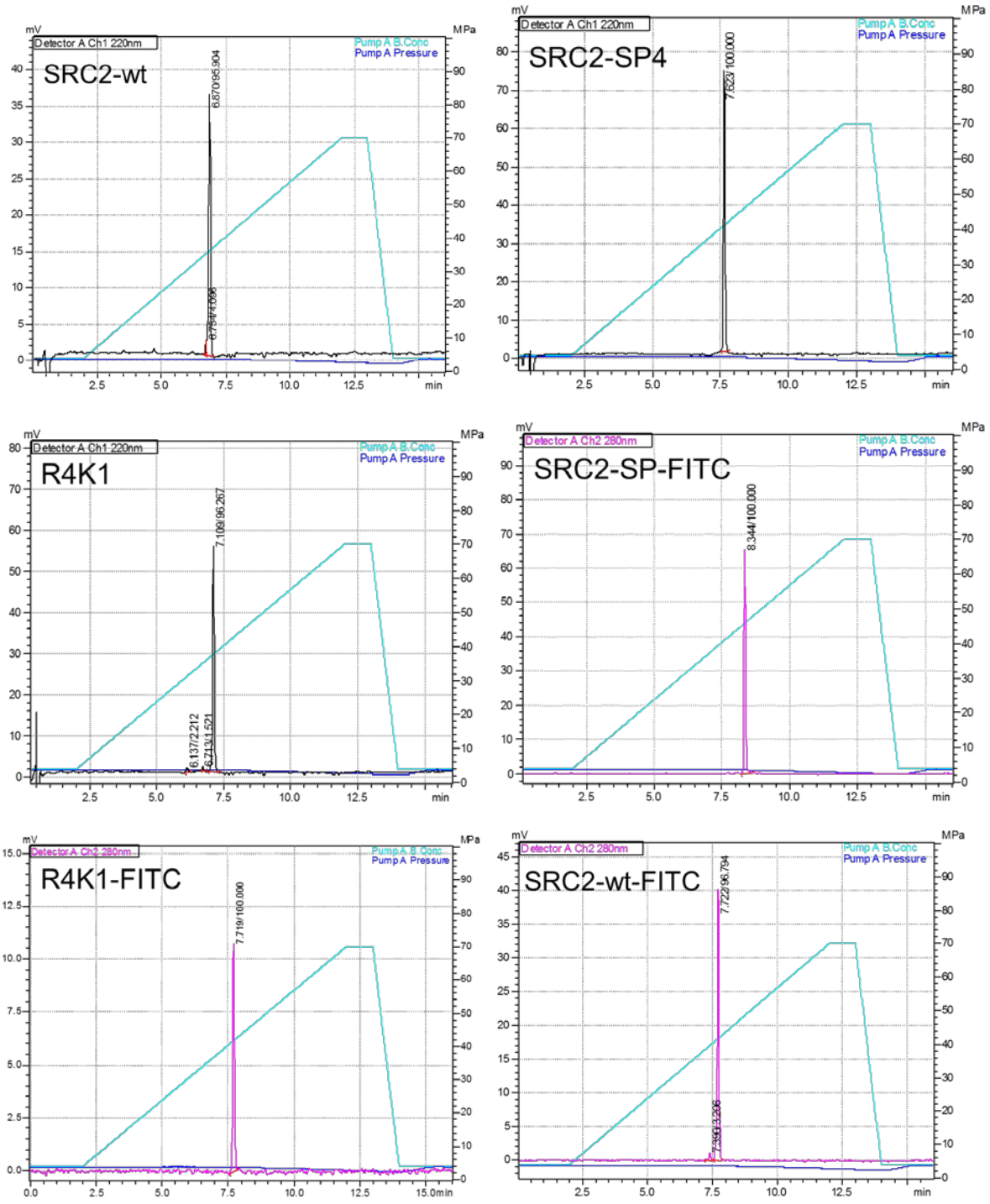

Figure S10 HPLC analysis of purified peptides.

Computational modeling of stapled peptide

The molecular system of SRC2-SP4 was constructed starting from the x-ray crystal of estrogen receptor α complexed with diethylstilbestrol (DES) in the ligand binding pocket and GRIP-1 NR box II peptide bound to the coactivator binding groove as previously described.[2] The phi and psi angles for H687 or R684-R687 and K688 were manually adjusted to prepare three different starting conformations. For SRC2-SP4, conformation 1 aligns with the SRC2 peptide of PDB Code 3ERD and the stapled peptide reported in PDB Code 2YJA, conformation 2 aligns with the structure of SRC2-SP4 bound to estrogen receptor alpha Y537S (PDB 5DXE), and the phi and psi angles for conformation 3 were randomly assigned. The MOE protein builder module was then used to build R4K1 by deleting residue H687 and appending four arginine residues to the N-terminus of the SRC2-SP4 peptide. The phi and psi angles for residues 684 to 688 of R4K1 were randomly adjusted to generate three conformations of R4K1. The six resulting structures were exported from MOE in PDB format.

The PSFGEN plugin within VMD[3] was used to construct molecular systems in CHARMM format (PSF+PDB). A patching protocol was used to perform ring-closing metathesis *in silico* and cap the ends of coactivator peptide with an N-terminal acetyl and C-terminal amide. The N- and C-terminal ends of estrogen receptor were capped using acetyl and N-methylamido patches. The SOLVATE plugin was used to add TIP3P[4] water to 20 Å on each side of the receptor. The AutoIonize plugin was used to neutralize the system and place Na^+ and Cl^- ions > 5 Å from the receptor to yield a final salt concentration of 0.1 M NaCl. The fully solvated systems SRC2-SP4 and R4K1 totaled ~64.4 k or ~65 k atoms respectively.

All MD simulations were performed using NAMD 2.12 software package.[5] The protein and ions were described using the refined CHARMM36m force field.[6,7] Parameters required to describe the staple were manually assigned by analogy from the CHARMM36m and CGenFF[8] parameter sets. The parameters required for diethylstilbestrol were assigned using the CGenFF Program webserver.[8] MD simulations were performed for NPT ensembles with a target pressure of 1 atm and temperature of 310 K using a Nosé-Hoover thermostat and a Langevin piston with a period of 100 fs, decay of 50 fs, and damping coefficient of 0.5/ps.[9,10] Periodic boundary conditions were applied, and non-bonded interactions were treated using an exponential switching function starting from 10 Å with a cutoff at 12 Å. Full system periodic electrostatics were computed using the particle mesh Ewald (PME) method[11] with a grid density >1.0/$Å^3$. Nonbonded forces were computed at every timestep (2 fs/step), PME calculations were performed at every other step, and atomic coordinates were recorded every ps.

The solvated and ionized SRC2-SP4 and R4K1 molecular systems were energy minimized for 10,000 steps and equilibrated for 0.1 ns using a harmonic positional restraint (k = 1 kcal/mol·$Å^2$) applied to all protein, coactivator, and ligand heavy atoms except protein residues 460–471 and coactivator residues 684 to 694—residues that were either missing from the x-ray crystal structure or modified to apply the staple or arginine residues. Water and ion atoms were allowed to equilibrate without any external restraints during this simulation. Production simulations were performed under equilibrium conditions for 250 ns per system. In total, the current study comprises 1.5 μs of production simulation time.

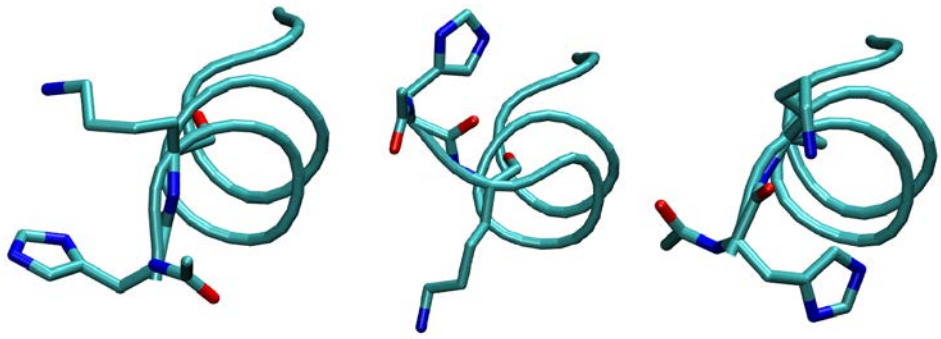

Figure S11. Three different conformations of His687 and Lys688 from SRC2-SP4 at the start of the production simulation.

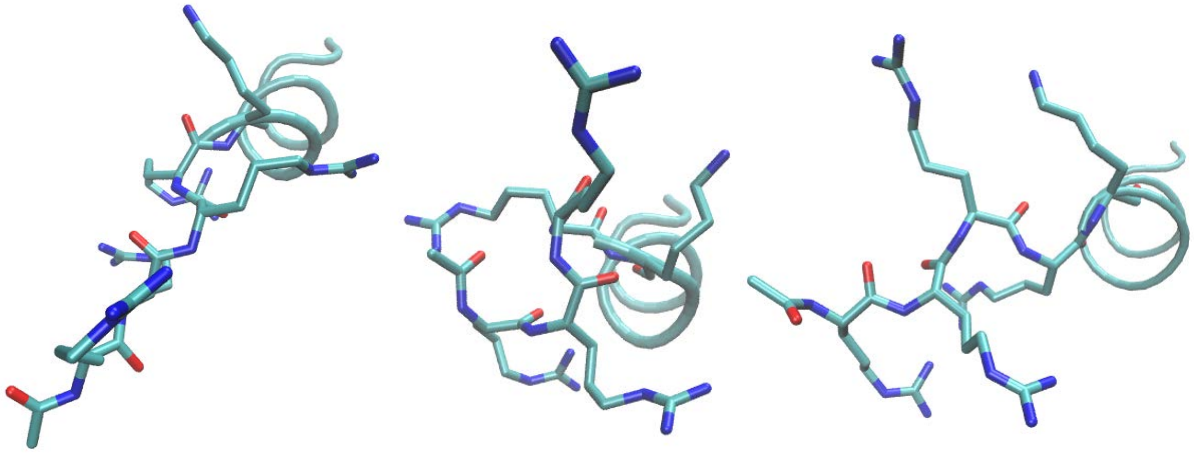

Figure S12. Three different conformations of Arg684, Arg685, Arg686, Arg687 and Lys688 from R4K1 at the start of the production simulation.

Table S2:TR-FRET Data Analysis

	R4K1	SRC2-SP4	SRC2-WT
Sigmoidal, 4PL, X is log(concentration)			
Best-fit values			
Top	0.524	0.4956	0.5053
Bottom	0.02911	0.01778	0.01084
LogIC50	-8.289	-6.411	-5.953
HillSlope	-1.14	-0.8722	-0.8931
IC50	5.14E-09	3.88E-07	1.11E-06
Span	0.4949	0.4779	0.4945
Std. Error			
Top	0.009898	0.007867	0.009079
Bottom	0.01243	0.01569	0.02845
LogIC50	0.05964	0.06509	0.09057
HillSlope	0.1533	0.09551	0.1349
Span	0.01677	0.01923	0.03212
95% CI (profile likelihood)			
Top	0.5041 to 0.5448	0.4797 to 0.513	0.4864 to 0.5263
Bottom	0.003243 to 0.05325	-0.01813 to 0.0463	-0.06737 to 0.05821
LogIC50	-8.41 to -8.168	-6.538 to -6.277	-6.112 to -5.736
HillSlope	-1.518 to -0.8717	-1.1 to -0.6902	-1.269 to -0.6306
IC50	3.89e-009 to 6.786e-009	2.9e-007 to 5.283e-007	7.727e-007 to 1.838e-006
Goodness of Fit			
Degrees of Freedom	38	31	32
R square	0.9719	0.9824	0.9675
Absolute Sum of Squares	0.05685	0.02177	0.0369
Sy.x	0.03868	0.0265	0.03396
Number of points			
# of X values	48	36	36
# Y values analyzed	42	35	36

Reagents. 17β-estradiol (E2) and 4-hydroxy-tamoxifen (4OHT) were purchased from Sigma, item #E8875 and item #H7904, respectively. Fmoc-S5-OH and Grubb's 1st generation catalyst were purchased from Sigma-Aldrich. Fmoc-protected amino acids and all other reagents are commercially available and were purchased from Chem-Impex, Oakwood, Novabiochem, or Sigma-Aldrich and used as supplied. All primers were purchased from Integrated DNA Technologies (IDT). Cell culture reagents were purchased from Gibco/Life Technologies and cell culture ware from BD Falcon, unless otherwise stated.

Cell Culture. Human MCF-7 and T47D cells were obtained from Dr. Debra Tonetti (University of Illinois at Chicago) and were maintained in Roswell Park Memorial Institute (RPMI) media supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 2 mM L-glutamine, 1% antibiotics penicillin-streptomycin, and 6 ng/mL human recombinant insulin at 37 ˚C in 5% CO_2. Before treatment with ligands, inhibitors, or stapled peptides, cells were cultured in phenol red-free RPMI 1640 media supplemented with 5% charcoal–dextran–stripped fetal bovine serum (i.e. treatment media) for at least 48 hrs. Cell line authentication was previously performed for both MCF-7 and T47D cell lines using short tandem repeats (STR) by the facility (DNAS), Research Resources Center (RRC), UIC.

Table S3. RNA Primers

EGR3 forward: TTCTCGTACAGGGTGGCTCC

EGR3 reverse: GGCAGAGAGCAACCTTCCC

PTGES forward: CTTCCTTTTCCTGGGCTTCG

PTGES reverse: GAAGACCAGGAAGTGCATCCA

PS2 forward: GTGTGCAAATAAGGGCTGCTG

PS2 reverse: TGGAGGGACGTCGATGGTA

PR forward: GTCGCCTTAGAAAGTGCTGTCAG

PR reverse: GCTTGGCTTTCATTTGGAACGCC

ER forward: TGCCCTACTACCTGGAGAAC

ER reverse: CCATAGCCATACTTCCCTTGTC

ICAM1 forward: TGACGAAGCCAGAGGTCTCAG

ICAM1 reverse: AGCGTCACCTTGGCTCTAGG

36B4 forward: GTGTTCGACAATGGCAGCAT

36B4 reverse: GACACCCTCCAGGAAGCGA

GAPDH forward: GTCTCCTCTGACTTCAACAGCG

GAPDH reverse:ACCACCCTGTTGCTGTAGCCAA

RelB forward: TGTGGTGAGGATCTGCTTCCAG

RelB reverse: TCGGCAAATCCGCAGCTCTGAT

TNFa forward: AAGGGTGACCGACTCAGCG

TNFa reverse: ATCCCAAAGTAGACCTGCCCA

IGFBP4 forward: AGCTTCAGCCCCTGTAGCG

IGFBP4 reverse: TCATCTTGCCCCCACTGGT

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