Schwarz *et al*. Supplemental Information

Supplementary Materials and Methods

Cell lines and Mutagenesis. Cell lines (ATCC) were maintained in IMEM/10% FBS (Gibco) and authenticated by short tandem repeat profiling using Sanger sequencing (March 2011). Long-term estrogen deprived (LTED) cells were generated and maintained in phenol red-free IMEM with 10% dextran/charcoal-treated FBS (DCC-FBS) as described (1). The LYN^{D189Y}, LYN^{E159K}, LYN^{K209N}, LYN^{A370T}, LYN^{G418R} and LYN^{A503D} mutations were created in pDONR223-LYN [Addgene plasmid 23905 (2)] using the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent) according to the manufacturer's protocol. Primers were designed using the QuikChange Primer Design website from Agilent. To generate the LYN^{D189Y} mutation, pDONR223-LYN was generated using the following sequence: g78t:GCACTACAAAATTAGAAGTCTGTATAATGGGGGCTATTACATCTC; g78t antisense: GAGATGTAATAGCCCCCATTATACAGACTTCTAATTTTGTAGTGC. The correct mutation was verified by Sanger Sequencing. The remaining mutations were also generated from pDONR223-LYN by GENEWIZ (South Plainfield, NJ), including E159K LYN ($LYN^{E159K)}$), D189Y LYN (LYN^{D189Y}), K209N LYN (LYN^{K209N}), A370T LYN (LYN^{A370T}), G418R LYN (LYN^{G418R}) and A503D LYN (LYN^{A503D}). All inserts were fully sequenced verified. All pDONR223_LYN constructs, including LYN wild-type (LYN^{WT}) and pENTRY-GFP [Addgene plasmid 15301 (3)] were recombined into the pLX302 Gateway destination vector [Addgene plasmid 25896 (4)] containing a C-terminal V5 tag using Gateway LR Clonase II Enzyme Mix (Invitrogen). For lentiviral transduction, 293T cells were transfected with 2 μg vector, 1 μg PAX2 and 1 μg pSPAX2 using PEI transfection reagent (Polyscience, Inc.). MCF-7, HCC-1428 and ZR 75-1 cells were transduced with viral supernatant in the presence of Polybrene (4 μg/mL). MCF-7,

HCC-1428 and ZR-75-1 cells stably expressing pLX302-GFP, pLX302-LYN^{WT} and pLX302-LYN^{D189Y} were maintained in IMEM/10% FBS plus 2 μg/ml Puromycin. Same maintenance was used for MCF-7 cells stably expressing pLX302-LYN^{E159K}, pLX302-LYN^{K209N}, pLX302-LYN^{A370T} and pLX302-LYN^{G418R}

Deep Kinome Sequence Analysis. Prior to alignment, standard FASTQs created for each specimen [following demultiplexing using fastq-multx (5)] were processed using fastq-mcf (5) [ver. EA-Utils 1.0.5: -p 5 -q 7 custom-adapter-file]. The clipped fastqs were then aligned to hg19 using BWA (6) [ver. 0.5.9: -n 0.02 -l 12] in paired-end fashion [sample] to produce BAM files for each specimen. The resulting BAMs were used to create pileup files, using samtools (7) mpileup [ver 0.1.15:]. These pileup files were used to call variants as well as calculate depth of coverage statistics. To generate the latter, we used a custom script to provide estimates of coverage, depths, uniformity and percent on target. A custom variant-caller, mpileup-variants [-g 1 -d 20 -q 20 -R 7 -p 1] was used to generate variant calls for each specimen. This caller required minimum depth and quality, with a minimum depth for minority allele set to 3 and homopolymer filter of 7 (variants at the end of homopolymer stretches >7 are ignored). While currently not available through EA-Utils, copies of parseGenomePileup and mpileup-variants can be made available upon request. Annotations of these variants was provided by a custom annotation program, chrdex_annotator, which can use standard flat file annotation (such as what is available from UCSC, 1000 Genomes, dbSNP, pGp, etc.) by creating indexes using the Tidx module in perl [Text:Tidx ver 0.92] to match genomic positions of variants with those with each flat file.

Reverse-phase protein array analysis (RPPA). Core needle biopsies were obtained before treatment with letrozole and at the time of surgical resection. Lysates from ten post-letrozole

tumors were prepared as described and analyzed by RPPA as described previously (1, 8, 9). FFPE tumor sections from the same biopsies were subjected to IHC with Ki67 antibody (DAKO #M7240). In brief, lysis buffer was used to homogenize the frozen cores. Protein concentrations were determined by BCA assay (Pierce). Lysates were diluted to 1 µg/µL protein and boiled with 1% SDS; the supernatants were manually diluted in six to eight 2-fold serial dilutions with lysis buffer. An Aushon Biosystems (Burlington, MA) 2470 arrayer created 1,056-sample arrays on nitrocellulose-coated FAST slides (Schleicher & Schuell BioScience, Inc.) from the serial dilutions. Slides were then probed with validated primary antibodies against proteins and phospho-proteins (antibodies described at http://app1.bioinformatics.mdanderson.org/tcpa/_design/basic/index.html), including Y416 P-Src. The antibody signal was amplified using a DakoCytomation–catalyzed system. A secondary antibody was used as a starting point for amplification. The slides were scanned, analyzed and quantitated using Microvigene software (VigeneTech Inc.) to generate serial dilution-signal intensity curves for each sample with the logistic fit model: $\ln(y) = a + (b - a)/(1 + \exp\{c*[d - \ln(x)]\})$. A representative natural logarithmic value of each sample curve on the slide (curve average) was then used as a relative quantification of the amount of antigen in each sample. The resulting quantification values were correlated to the post-letrozole Ki67 score in the surgical specimen.

siRNA library screen. MCF-7/LTED cells were transiently transfected with the Dharmacon RTF Protein Kinase siRNA library using methods similar to those described in ref. (10). The siRNA library included ten 96-well plates containing SMARTpool siRNAs targeting 779 protein kinases. MCF-7/LTED cells were reverse-transfected in 96-well plates (10^4 cells/well in 10% DCC-FBS) containing 5 pmol of siRNA/well using Dharmafect 1 (Dharmacon) per the manufacturer's instructions. Each 96-well plate also contained one well with only medium (no cells), which

served as the blank, and ≥3 wells with 5 pmol/well of non-silencing control siRNA (siGENOME Non-Targeting siRNA Pool #2, Dharmacon). Twenty-four h after transfection, cells were trypsinized and divided into three identical replicate black 96-well plates with clear bottoms (Costar) containing 10% DCC-FBS. Cell viability was measured four days later using the Alamar Blue Assay (Invitrogen) according to the manufacturer's instructions. After 4-h incubation with Alamar Blue, fluorescence values were read using a Spectramax M5 spectrophotometer (544 nm excitation, 590 nm emission). The fluorescence reading for each well on a plate was expressed relative to the median fluorescence value of all wells on the plate. Median-centered global normalization was performed to normalize fluorescence signals between plates (CV=0.3305). The fluorescence reading for each well on a plate was standardized within each plate to generate a Z-score (robust version). The mean Z-score of triplicate wells for each siRNA within an experiment was calculated. The experiment was repeated four times; the median Z-score across experiments [median of (Z-scoremean1, Zscoremean2, Z-scoremean3, Z-scoremean4)] was calculated. Median Z-scores were ranked and graphed.

***In vitro* kinase assay.** Five hundred μg of protein extracted from MCF-7/LYN^{WT} and MCF-7/LYN^{D189Y} cells were immunoprecipitated with a V5 tag antibody. The precipitates were washed twice in NP-40 lysis buffer, twice in kinase buffer (20 mM HEPES [pH 7.5], 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM dithiothreitol, 0.1 mM Na_3VO_4) and then aliquoted on ice into two equal portions, each brought up to a final volume of 35 μl with kinase buffer. ATP was added to one of the two aliquots (final concentration 0.2 mM). The kinase reaction was allowed to proceed for 5 min at 30°C and then terminated by adding 5x SDS-PAGE loading buffer and boiling for 3 min before separation by 8% SDS-PAGE followed by immunoblot analysis using V5, P-Tyr and Y507 P-LYN antibodies.

Immunoblot analysis. Cell lysates were subjected to SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblot using primary antibodies against LYN, Src, IGF-IRβ, ERα (F-10), PR, STAT3 (Santa Cruz Biotechnology), P-$Src_{Y416,}$ P-Lyn_{Y507}, P-$FAK_{Y576/577}$, P-FAK_{Y397}, P-FAK_{Y925}, FAK, P-$Paxillin_{Y118}$, Paxillin, P-IGF-$IR\beta_{Y1131}$/P-$InsR\beta_{Y1146}$, P-$EGFR_{Y845}$, P-$EGFR_{Y1173}$, P-$EGFR_{Y1068}$, EGFR, P-$HER3_{Y1197}$, HER3, P-$STAT3_{Y705}$, P-AKT_{S473}, P-AKT_{T308}, AKT, P-$PRAS40_{T246}$, PRAS40, P-$S6_{S240/244}$, S6, P-p44/42 $MAPK_{T202/Y204}$, p44/42 MAPK, IRS, calnexin (Cell Signaling), 4G10 pTyr, P-$IRS\text{-}1_{Y941}$ (Millipore), V5 (Invitrogen) and actin (Sigma-Aldrich), followed by species-specific HRP-conjugated secondary antibodies (Promega). Immunoreactive signals were detected by ECL western blotting substrate or Supersignal ELISA Pico CL substrate (Pierce).

Real-Time qPCR. Cells grown in 10% DCC-FBS were harvested and their RNA extracted using the RNeasy Mini Kit (Qiagen); 1 μg of RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR reactions were conducted in 96-well plates using the iCycler iQ (Bio-Rad) and primers obtained from SABiosciences (Qiagen). Threshold cycle values were normalized for the housekeeping gene 36B4 and each cDNA was assayed in triplicate.

Xenograft studies. Female ovariectomized, athymic, 4- to 5-week old Balb/c mice (Harlan Sprague Dawley) were implanted s.c. with a short-term, 14-day-release 17β-estradiol pellet (0.17 mg; Innovative Research of America) in their dorsum. The next day, 10^7 parental MCF-7 cells or stably transfected LYN^{WT}, LYN^{D189Y} suspended in IMEM and mixed with Matrigel (BD Biosciences) at 1:1 ratio were injected s.c. into the right flank of each mouse. After 4 weeks, mice bearing tumors ≥150 mm^3 were randomized to treatment with vehicle (80 mM sodium citrate buffer pH 3), dasatinib (15 mg/kg/day, p.o.), BKM120 (30 mg/kg/day, p.o.) + fulvestrant (5

mg/wk, s.c.), or BKM120 + fulvestrant + dasatinib. Tumor diameters were measured using calipers twice weekly and volume in mm^3 calculated by the formula volume = $width^2$ x length/2. Mice were weighed daily and those that lost >10% of body weight were given a 1-ml i.p. injection of Dextrose-Free Lactated Ringer's Injection USP (Baxter Healthcare Corp.). Tumors were harvested 4 h after the last dose of dasatinib or BKM120 or 24 h after the last dose of fulvestrant and flash-frozen in liquid nitrogen or fixed in 10% formalin prior to paraffin-embedding. Frozen tumors were homogenized using the TissueLyser II (Qiagen). Five-µm paraffinized sections were used for IHC using Y416 P-Src (Cell Signaling #2101). Sections were scored by two trained pathologist (M.G.K. and M.V.E.) blinded to the type of treatment.

References

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Supplementary Table S1. Pre- and post-treatment Ki67 levels and tumor characteristics for four sequenced ER+/HER2– breast tumors

	Ki67	Ki67			
Tumor	**Pre**	**Post**	**ER**	**PR**	**HER2**
A	78.5	24.5	+	-	-
B	21.9	21.5	+	-	-
C	33.2	14.8	+	+	-
D	80.2	16.8	+	+	-

For **Supplementary Table S2-S3**, see Excel Spreadsheet.

Supplemental Table S4. Pre- and post-treatment Ki67 levels and tumor characteristics of 10 tumors analyzed by RPPA

Tumor	Ki67 Pre	Ki67 Post	ER	PR	pSRC
1	8.4	1.3	ER+	PR-	0.12772
2	4.8	4	ER+	PR-	0.16211
3	21.6	10.3	ER+	PR+	0.15054
4	17.3	3.1	ER+	PR+	0.15293
5	21.9	21.5	ER+	PR-	0.21007
6	80.2	16.8	ER+	PR+	0.20125
7	0	1.3	ER+	PR+	0.12797
8	18.4	5	ER+	PR+	0.1904
9	33.2	14.8	ER+	PR+	0.1671
10	10.1	3.1	ER+	PR+	0.14533

Supplementary Figures

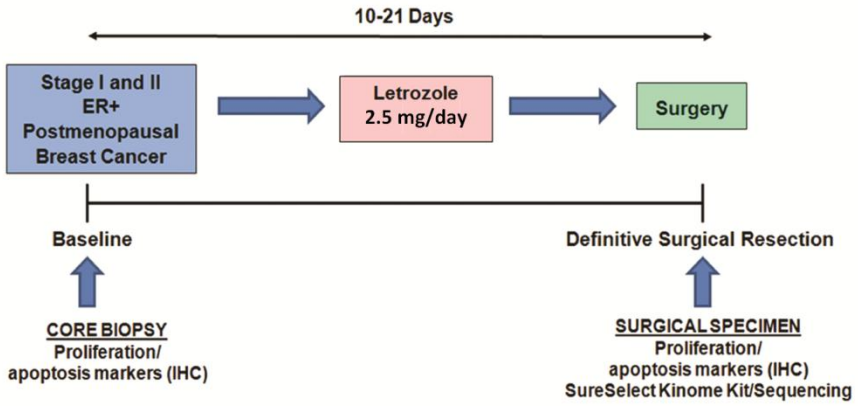

Figure S1A. Schema of clinical trial with presurgical letrozole. Post-menopausal patients with stage I and II operable ER+/HER2-negative breast cancer were treated with letrozole (2.5 mg/day) for 10-21 days prior to surgery (Trial NCT00651976). Tumor cell proliferation was assessed by Ki67 IHC in pre- and post-treatment (surgical) biopsies.

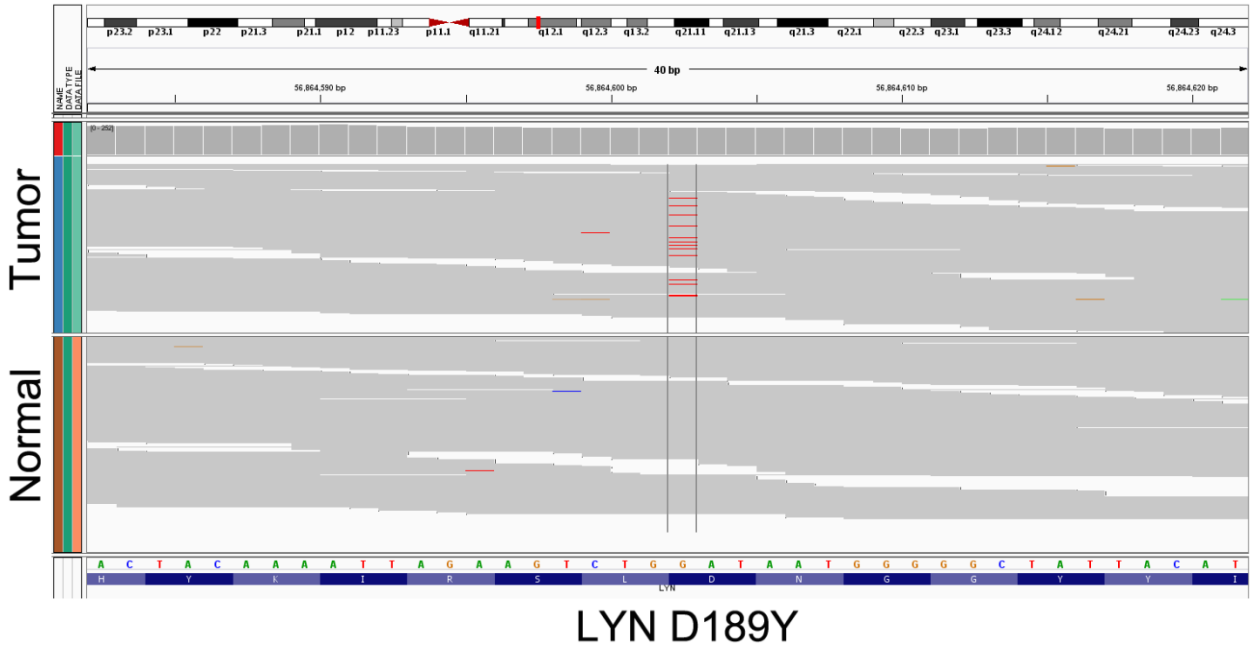

Figure S1B. Integrated Genome Viewer image of tumor (upper) and matched normal (lower) sequencing reads demonstrating somatic status of the LYN D189Y mutation. D189Y was not detected in DNA extracted from normal breast tissue.

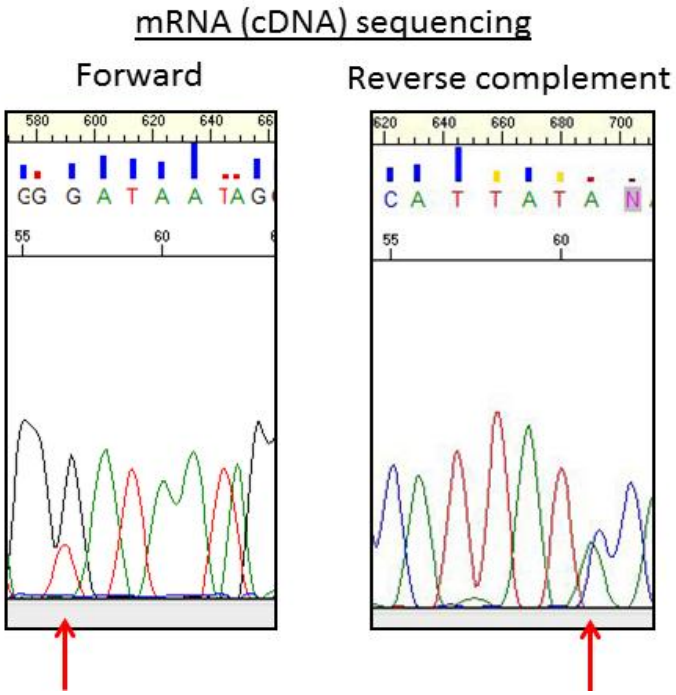

Fig. S1C. Formalin-fixed, paraffin-embedded sections with approximately 20% tumor cellularity were macro-dissected to enrich for tumor nuclei and total RNA was purified using the Qiagen FFPE RNeasy kit. cDNA was synthesized with iScript (BioRad) and LYN mRNA was amplified across exon/exon boundaries by PCR using the following primers: Forward: GGAAGCTTCTCTCTGTCTGTCA; Reverse: AAGCCATCTGCCTGCTTTTG. PCR product was digested with Hpy188III (New England Biolabs), which cuts the wild type but not the GAT>TAT (D189Y) mutant. Undigested product was gel purified and re-amplified prior to Sanger sequencing in the forward and reverse direction.

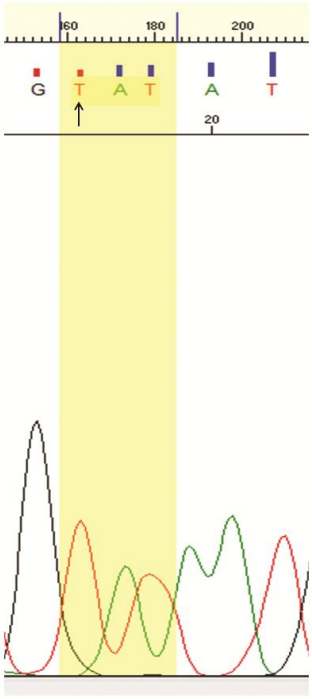

Figure S2. Sanger sequencing of mutant LYN. The D189Y LYN (565G>T) mutation was created in pDONR223-LYN (Addgene plasmid 23905) using the QuikChange Lightning Site-Directed Mutagenesis kit as detailed in Supplemental Materials and Methods. The arrow designates the G residue mutated to T.

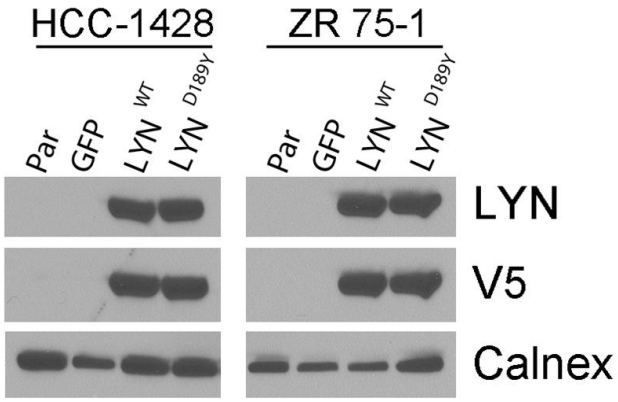

Figure S3. HCC-1428 and ZR75-1 cells stably transduced with GFP, LYN^{WT}, or LYN^{D189Y} lentiviral vectors were plated in 10% FBS; cell lysates were harvested and analyzed by immunoblot using the indicated antibodies.

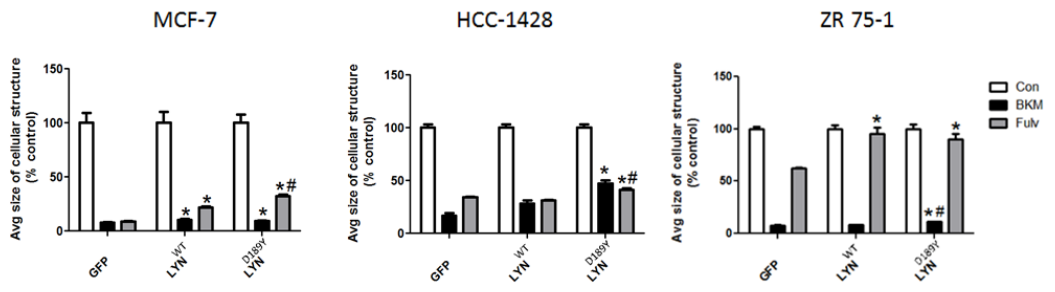

Figure S4. MCF-7, HCC-1428 and ZR75-1 cells stably transduced with GFP, LYN^{WT}, or LYN^{D189Y} were cultured in Matrigel ± 1 μM BKM120 or 1 μM fulvestrant for 15 days. Media and inhibitors were replenished every 3 days. Data are presented as percent of the respective control for each cell line (n=3; *p<0.05 vs. GFP treated with BKM120 or fulvestrant, #p<0.05 vs. LYN^{WT} treated with fulvestrant).

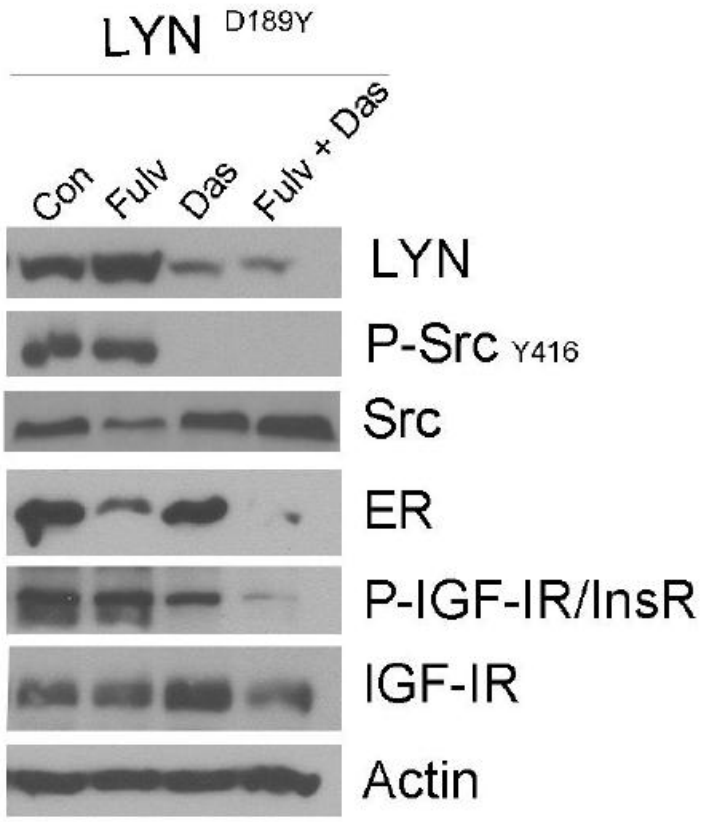

Figure S5. SFK inhibition with dasatinib restores the ability of fulvestrant to reduce ER and P-IGF-IR/InsR levels. MCF-7 cells stably transduced with LYN^{D189Y} were treated with 10% DCC-FBS ± 1 μM fulvestrant (Fulv) or 1 μM dasatinib (Das) for 24 h. Cells were harvested and protein lysates prepared followed by immunoblot analysis with the indicated antibodies.

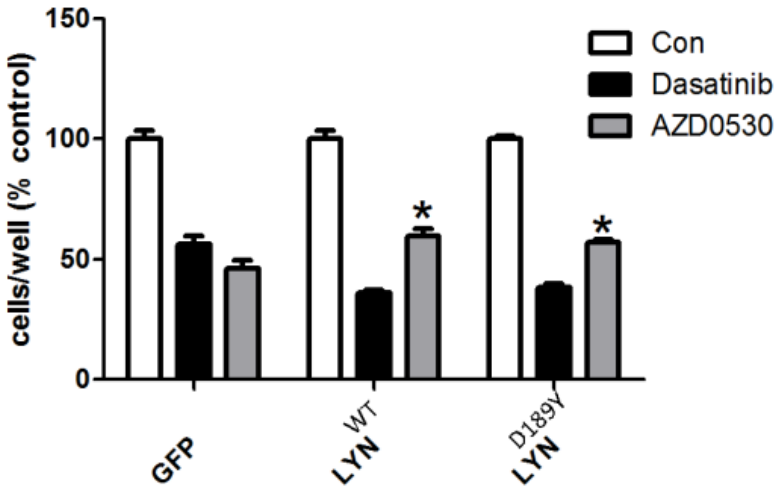

Figure S6. MCF-7 cells stably transduced with GFP, LYN^{WT}, or LYN^{D189Y} were incubated in 10% DCC-FBS ± 1 μM dasatinib or 1 μM AZD0530. After 5 days, monolayers were trypsinized and cells numbers measured in a Coulter counter (n=3; *p<0.05 *vs.* respective GFP).

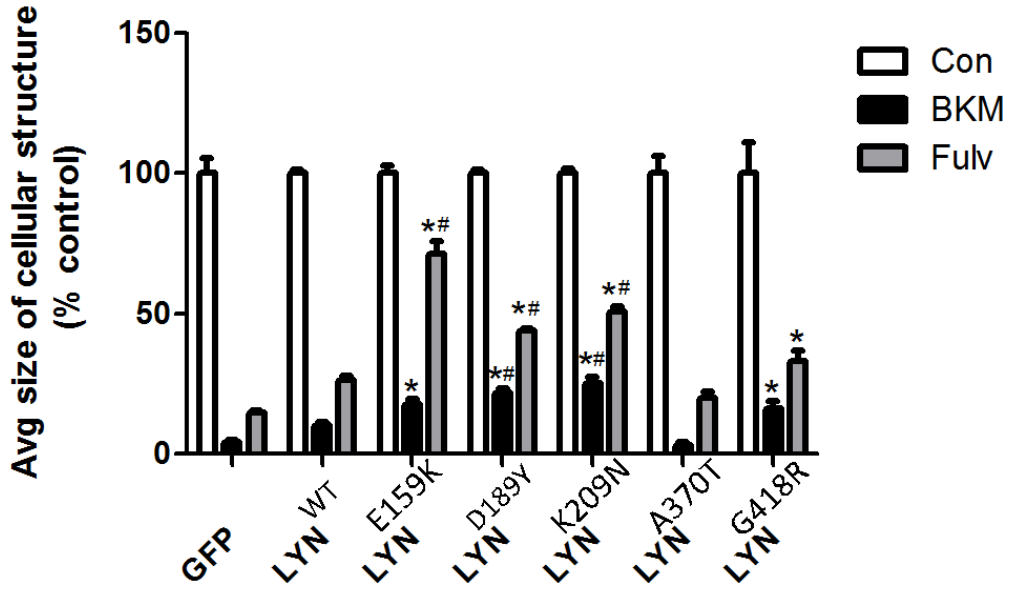

Figure S7. MCF-7 cells stably transduced with GFP, LYN^{WT}, LYN^{E159K}, LYN^{D189Y} , LYN^{K209N} , LYN^{A370T} and LYN^{G418R} were cultured in Matrigel ± 1 μM BKM120 or 1 μM fulvestrant. Media and inhibitors were replenished every 3 days. On day 15, adherent colonies were stained with MTT and photographed with Gelcount reader. Data are presented as percent of the respective control for each cell line (n=3; *p<0.01 vs. respective treatment of GFP controls, #p<0.01 vs. respective treatment of LYN^{WT} cells).

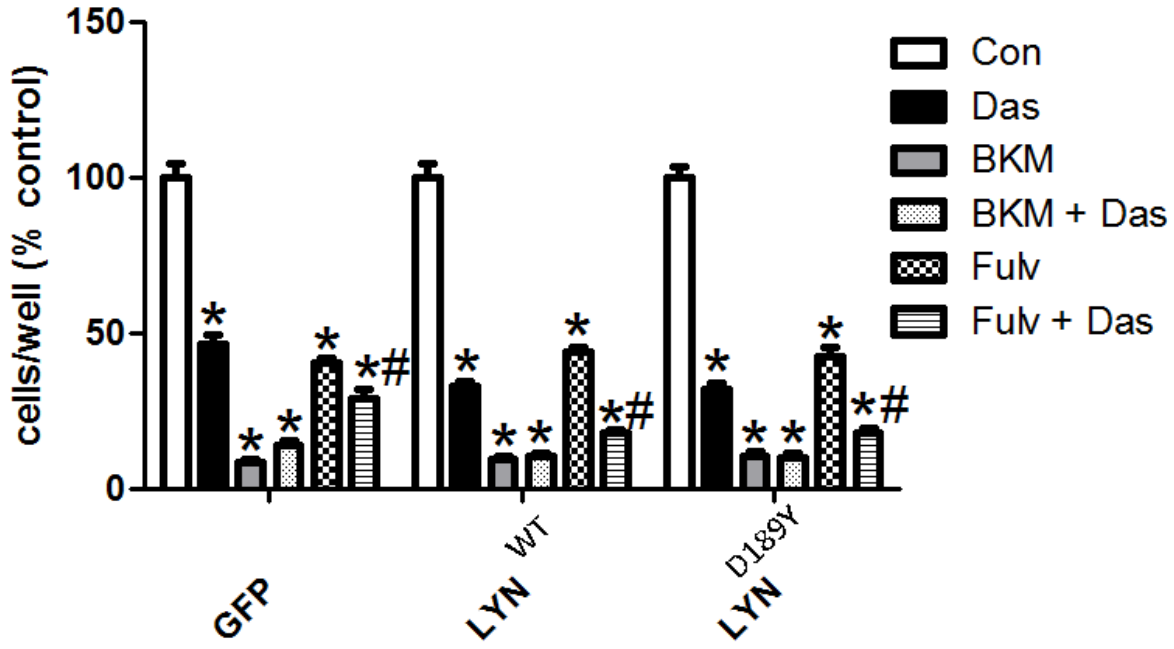

Figure S8. A) MCF-7 cells stably transduced with GFP, LYN^{WT}, or LYN^{D189Y} were treated with 10% DCC-FBS ± 1 µM dasatinib, 1 µM BKM120, or 1 µM fulvestrant. Media and drugs were replenished every 3 days. Cells were counted after 5 days. Data are presented as percent of control (n=3; **p*<0.0001 *vs.* respective Con, #*p*<0.05 *vs*. Fulv).

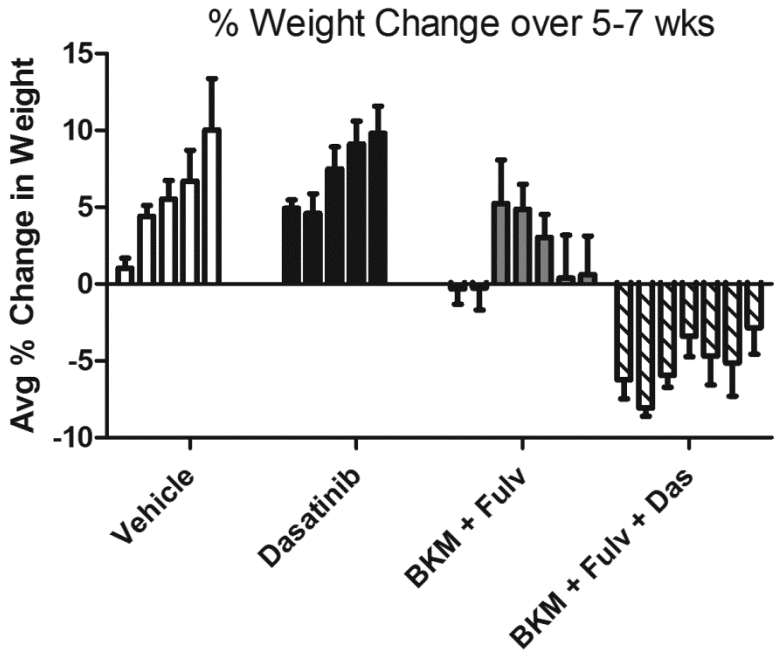

Figure S9. Weight loss of mice treated with dasatinib, BKM120 and/or fulvestrant. MCF-7 cells were injected s.c. into athymic mice supplemented with 14-day release 17β-estradiol pellets. Mice bearing MCF-7 xenografts were treated with vehicle, dasatinib or the indicated combinations as described in Figs.8-9 for seven weeks. Data are presented as the average percent change in weight on any week during treatment.