Supporting Information

Zammarchi et al. 10.1073/pnas.1108482108

SI Materials and Methods

STAT3 Minigene Construction. Human genomic DNA (Promega) was amplified by Platinum Pfx (Invitrogen) as per instructions for 35 cycles with an annealing temperature of 60 °C and a 3-min extension time using STAT3-specific primers (I22F and I24R) with overhanging restriction sites for KpnI and BamHI (Promega), respectively. The amplified region spans the entire exon 22 (43 nt), intron 22 (280 nt), exon 23 (113 nt), intron 23 (988 nt), and 985 nt of exon 24, which include the full-length stop codon at position 56 of the exon. The entire exon 24, including the full UTR, is 2,477-nt long. The resulting PCR product was subcloned into pcDNA3.1+ (Invitrogen). The ligation reaction was used to transform MAX Efficiency DH5a-competent cells, and colony PCR was used to screen for positive colonies that were then grown overnight at 37 °C. Plasmid DNA was prepared (Qiagen) and the resulting vector was confirmed by restriction enzyme digestion and sequencing.

Primer Sequences for STAT3 Minigene Construction. Introns 22F (I22F) 5′-CGACGGTACCTCCAGCTCTGCTTACTGAATGCGA-3′

Introns 24R (I24R) 5′-CGGTGGATCCGCTTAAAGCACCAAGGAGGCTGTT-3′

Minigene Mutant Construction. Mutants were prepared by site-specific mutagenesis using overlap extensions (1). Briefly, complementary PCR primers forward mutant (FM) and reverse mutant (RM) were designed to have either a deletion or a point mutation within the primer, flanked by at least 14 bp on each side. Two PCR reactions were performed using the FM with I24R reverse primer and RM with I22F forward primer. The resulting PCR products were gel-purified, and equal amounts were used as a template for a second PCR, with I22F and I24R primers. All PCR was performed with Platinum Pfx (Invitrogen), with an annealing temperature of 60 °C and an extension time 1 min/kb as per instructions. The resulting products were then subcloned as previously described and verified by sequencing.

Primer Sequences for STAT3 Minigene Deletion Mutants in WT Context.

ΔA-F 5′-CTGCCCCCCATTTCCTACAGAAGCAATACCATTGACCTGCCGATG-3′
ΔA-R 5′-CATCGGCAGGTCAATGGTATTGCTTCTGTAGGAAATGGGGGGCAG-3′
ΔB-F 5′-CCCCATTTCCTACAGAACGACCACCATTGACCTGCCGATGTCC-3′
ΔB-R 5′-GGACATCGGCAGGTCAATGGTGGTCGTTCTGTAGGAAATGGGG-3′
ΔC-F 5′-CATTTCCTACAGAACGACCTGCAGCGACCTGCCGATGTCCCC-3′
ΔC-R 5′-GGGGACATCGGCAGGTCGCTGCAGGTCGTTCTGTAGGAAATG-3′
ΔD-F 5′-CTACAGAACGACCTGCAGCAATACCCCGATGTCCCCCCGC-3′
ΔD-R 5′-GCGGGGGGACATCGGGGTATTGCTGCAGGTCGTTCTGTAG-3′
ΔE-F 5′-ACGACCTGCAGCAATACCATTGACTCCCCCCGCACTTTAGATTCATTGATG-3′
ΔE-R 5′-CATCAATGAATCTAAAGTGCGGGGGGAGTCAATGGTATTGCTGCAGGTCGT-3′
ΔF-F 5′-GCAGCAATACCATTGACCTGCCGCGCACTTTAGATTCATTGATGCAGTTTGGA-3′
ΔF-R 5′-TCCAAACTGCATCAATGAATCTAAAGTGCGCGGCAGGTCAATGGTATTGCTGC-3′
ΔG-F 5′-AATACCATTGACCTGCCGATGTCCTTAGATTCATTGATGCAGTTTGGAAATAATGGTGAA-3′
ΔG-R 5′-TTCACCATTATTTCCAAACTGCATCAATGAATCTAAGGACATCGGCAGGTCAATGGTATT-3′
ΔA1-F 5′-CTGCCCCCCATTTCCTACAGAACCTGCAGCAATACCATTGACCTG-3′
ΔA1-R 5′-CAGGTCAATGGTATTGCTGCAGGTTCTGTAGGAAATGGGGGGCAG-3′
ΔA2-F 5′-TGCCCCCCATTTCCTACAGAACGTGCAGCAATACCATTGACCTGCC-3′
ΔA2-R 5′-GGCAGGTCAATGGTATTGCTGCACGTTCTGTAGGAAATGGGGGGCA-3′
ΔA3-F 5′-GCCCCCCATTTCCTACAGAACGACCAGCAATACCATTGACCTGCCGATG-3′
ΔA3-R 5′-CATCGGCAGGTCAATGGTATTGCTGGTCGTTCTGTAGGAAATGGGGGGC-3′
ΔB2-F 5′-CCCATTTCCTACAGAACGACCTGCAATACCATTGACCTGCCGATGTCCC-3′
ΔB2-R 5′-GGGACATCGGCAGGTCAATGGTATTGCAGGTCGTTCTGTAGGAAATGGG-3′
ΔB3-F 5′-CATTTCCTACAGAACGACCTGCAGCACCATTGACCTGCCGATGTCC-3′
ΔB3-R 5′-GGACATCGGCAGGTCAATGGTGCTGCAGGTCGTTCTGTAGGAAATG-3′
ΔH-F 5′-GCCGATGTCCCCCCGCACTTTAGAGTTTGGAAATAATGGTGAAGGTGCTGAACCC-3′
ΔH-R 5′-GGGTTCAGCACCTTCACCATTATTTCCAAACTCTAAAGTGCGGGGGGACATCGGC-3′
ΔK-F 5′-CCCCCCGCACTTTAGATTCATTGATTAATGGTGAAGGTGCTGAACCCTC-3′
ΔK-R 5′-GAGGGTTCAGCACCTTCACCATTAATCAATGAATCTAAAGTGCGGGG-3′
ΔL-F 5′-CGCACTTTAGATTCATTGATGCAGTTTGGAGGTGCTGAACCCTCAGCAG-3′
ΔL-R 5′-CTGCTGAGGGTTCAGCACCTCCAAACTGCATCAATGAATCTAAAGTGCG-3′
ΔM-F 5′-GCACTTTAGATTCATTGATGCAGTTTGGAAATAATGGACCCTCAGCAGGAGGGC-3′
ΔM-R 5′-GCCCTCCTGCTGAGGGTCCATTATTTCCAAACTGCATCAATGAATCTAAAGTGC-3′
Δ*N*-F 5′-GATGCAGTTTGGAAATAATGGTGAAGGTGCAGGAGGGCAGTTTGGTGAGTATTTG-3′
Δ*N*-R 5′-CAAATACTCACCAAACTGCCCTCCTGCACCTTCACCATTATTTCCAAACTGCATC-3′
ΔO-F 5′-GAAATAATGGTGAAGGTGCTGAACCCTCGTTTGGTGAGTATTTGGTTGACAGACTTTGTC-3′
ΔO-R 5′-GACAAAGTCTGTCAACCAAATACTCACCAAACGAGGGTTCAGCACCTTCACCATTATTTC-3′
ΔH1-F 5′-CCGCACTTTAGAATTGATGCAGTTTGGAA-3′
ΔH1-R 5′-TTCCAAACTGCATCAATTCTAAAGTGCGG-3′
ΔH2-F 5′-CCGCACTTTAGATTCGATGCAGTTTGGA-3′
ΔH2-R 5′-TCCAAACTGCATCGAATCTAAAGTGCGG-3′
ΔH3-F 5′-CCGCACTTTAGATTCATTGCAGTTTGGAA-3′
ΔH3-R 5′-TTCCAAACTGCAATGAATCTAAAGTGCGG-3′
ΔK1-F 5′-CCGCACTTTAGATTCATTGATGTTTGGAAATAATGG-3′
ΔK1-R 5′-CCATTATTTCCAAACATCAATGAATCTAAAGTGCGG-3′
ΔK2-F 5′-CCGCACTTTAGATTCATTGATGCATGGAAATA-3′

ΔK2-R 5′-TATTTCCATGCATCAATGAATCTAAAGTGC-GG-3′
ΔK3-F 5′-CGCACTTTAGATTCATTGATGCAGTTAAATA-ATGGTG-3′
ΔK3-R 5′-CACCATTATTTAACTGCATCAATGAATCTA-AAGTGCG-3′
Py^-F 5′-ATGTCCCCCCTCCCTTTAGATTCATTG-3′
Py^-R 5′-CAATGAATCTAAAGGGAGGGGGGACAT-3′

Transfection of Minigenes. Minigene transfections into HeLa cells were done with FuGENE 6 (Roche) as per instructions. Twenty-four hours after transfection, cells were collected for RNA extraction.

RNA Extraction and RT-PCR. Total RNA was extracted using either the RNAqueous Kit (Ambion) (for cell lines) or TRIzol reagent (Invitrogen) (for tumor samples) according to the manufacturers' instructions. Contaminating DNA was removed using TURBO DNA-free (Ambion) following product instruction. Total RNA was then reverse-transcribed with SuperScript III RT (Invitrogen) and the resulting cDNA was used as template for PCR analysis. All PCR reactions were performed with Platinum Taq (Invitrogen) as per instructions with an annealing temperature of 60 °C and an extension time of 30 s for a total of 30 cycles.

Primers used to amplify STAT3 and GAPDH were as follows.

STAT3 α/β splicing (minigene).
Forward 5′-GCTAGCGTTTAAACTTAAGCTTGG-3′
Reverse 5′-ACTCCGAGGTCAACTCCATGTCAA-3′

STAT3 α/β splicing (endogenous).
Forward 5′-TGCTACCAATATCCTGGTGTCTCC-3′
Reverse 5′-ACTCCGAGGTCAACTCCATGTCAA-3′

STAT3 exon 6 skipping.
Forward 5′-TGGTGACGGAGAAGCAGCAGAT-3′
Reverse 5′-TGCACGTACTCCATCGCTGACAAA-3′

GAPDH primers.
F 5′-TGCACCACCAACTGCTTAGC-3′
R 5′-GGCATGGACTGTGGTCATGAG-3′

Cell Culture. MDA-MB-435s, MDA-MB-468, MDA-MB-453, HeLa, A549, DU145, K562, 4T1, and B16 cells were purchased from the American Type Culture Collection. Phoenix-Ampho packaging cells were a generous gift of Scott Lowe (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Human breast cancer cell lines were grown in a mixture (1:1, vol/vol) of DMEM and Ham's F-12 medium supplemented with 1% nonessential amino acids (Invitrogen), 1% sodium pyruvate (Invitrogen), 1% penicillin/streptomycin (Invitrogen), and 10% FBS (Sigma). HeLa and Phoenix-Ampho cells were grown in DMEM supplemented with 1% penicillin/streptomycin (Invitrogen) and 10% FBS (Sigma). A549, DU145, K562, 4T1, and B16 were grown in RPMI 1640 media supplemented with 1% penicillin/streptomycin (Invitrogen) and 10% FBS (Sigma). Cells were incubated at 37 °C in a 5% CO_2 atmosphere.

Antibodies. Total-STAT3 (Cell Signaling; 9132), phospho-STAT3 Y705 (Cell Signaling; 9131), total-STAT1 (BD Biosciences; 610115), lens epithelium-derived growth factor (LEDGF) (BD Biosciences; 611714), peroxisomal biogenesis factor 1 (PEX1) (BD Biosciences; 611719), p300/CBP-associated factor (PCAF) (Cell Signaling; 3378), α-tubulin (Sigma; T5168), and actin (Sigma; A4700).

Western Blot. Whole-cell lysates were prepared using M-PER protein extraction buffer (Thermo Scientific) with Complete Protease Inhibitor Mixture (Roche) as per instructions. Protein concentration was determined using the Bradford assay (Bio-Rad). Samples were subjected to an 8% SDS/PAGE, transferred to an Immobilon-FL PVDF membrane (Millipore), and blotted in 5% milk for 2 h at room temperature. Blots were incubated overnight at 4 °C with the respective primary antibodies. Next, the blots were washed, incubated with the corresponding HRP-conjugated secondary antibody, washed again, and detected using SuperSignal West Femto Substrate (Pierce).

Morpholino Treatments. Morpholino oligos (Gene Tools) were mixed at the desired concentration with media plus 4% FBS and added to the cells. Endo-Porter (Gene Tools) was used as delivery reagent at a final concentration of 6 μM.

Vivo-Morpholino oligos (2) were either intratumorally injected at a final concentration of 0.12 mg/30 μl or administered intravenously at 15 mg/kg. Treatments were administered twice a week for either 1 or 3 wk.

Sequences of morpholinos were as follows.

ST1, 5′-CTGCAGGTCGTTCTGTAGGAAAT-3′
ST2, 5′-ATTGCTGCAGGTCGTTCTGTAGG-3′
ST3, 5′-GGTATTGCTGCAGGTCGT-3′
ST6, 5′-CATTTTCTGTTCTAGATCCTGTT-3′
ST7, 5′-TTCACTTGCCTCCTTGACTCTTG-3′
INV, 5′-GGATGTCTTGCTGGACGTCGTTA-3′

Small Interfering RNA. Oligofectamine (Invitrogen) was used to transfect siRNA into MDA-MB-435s cells as per instructions. siRNAs were purchased from the HTSCF (High-Throughput Screening Core facility) (Memorial Sloan-Kettering Cancer Center). As a control, we used an siRNA oligo directed against GFP. siRNAs against PEX1, PCAF, and CyclinC (CCNC) were used at a final concentration of 100 nM each and compared with MDA-MB-435s cells treated with 100 nM GFP siRNA oligo. siRNA against LEDGF was used at a final concentration of 50 nM and compared with MDA-MB-435s cells treated with 50 nM GFP siRNA oligo. Each treatment was done in triplicate. Cells were treated for 72 h and then collected for quantitative (q)PCR/Western blot and trypan blue analysis.

Trypan Blue. Cells were treated with either the morpholino oligos or with the siRNA oligos as described above. At the end of the treatment, cells were trypsinized, counted with a hemocytometer, and scored as dead/total. Data were analyzed by calculating the fold change in dead/total cells compared with the control treatment.

Apoptosis and Cell-Cycle Analysis. Apoptosis was determined by annexin V/propidium iodide (AnxV/PI) staining. Cells (1×10^5) were resuspended in AnxV/PI buffer (Annexin V Binding Buffer; Biolegend). Alexa Fluor 647-conjugated annexin V (Biolegend) was added to the cell suspension and incubated for 15 min at room temperature. Cells were further resuspended in AnxV/PI buffer, and PI (Sigma-Aldrich; 1 μg/mL) was added before flow analysis by Beckman Coulter cyan flow cytometer.

For cell-cycle analysis, harvested cells (5×10^5) were washed twice with PBS + 0.5% BSA and fixed in cold 70% (vol/vol) ethanol for 30 min. Cells were then washed twice and further treated with RNase (100 μg/mL; Sigma-Aldrich) and PI (50 μg/mL). Samples were incubated at 37 °C for 15 min and analyzed by flow cytometry. Data were analyzed using FlowJo software (Tree Star).

ELISA. IL8 levels in MDA-MB-435s supernatants were measured with an IL8 ELISA Kit (Quantikine; R&D Systems) according to the manufacturer's protocol.

Cell Proliferation Assay. Cells were plated in 96-well plates for 24 h and then treated with either 1 μM pan-Jak inhibitor P6 (a tetracyclic pyridone; 2-tert-butyl-9-fluoro-3,6-dihydro-7H-benz[h]-imidaz[4,5-f]isoquinoline-7-one, pyridone 6) (3) or DMSO in association with 6 μM Vivo-Morpholino control oligonucleotide

inverted sequence (INV) or with just 6 μM Vivo-Morpholino oligonucleotides INV, ST2, and ST6 for 48 h. A CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega) was performed according to the manufacturer's instructions. Absorbance was measured at 490 nm using a microplate reader (Bio-Rad).

TUNEL. TUNEL assay was carried out according to the manufacturer's instructions (Roche).

Immunohistochemistry. Immunohistochemical staining was carried out on 5-μm sections from formalin-fixed, paraffin-embedded tumor specimens. Following routine deparaffinization and rehydration, the sections were subjected to staining with phospho-STAT3 (Y705: Cell Signaling, 9131; S727: Epitomics, 1121-1), total-STAT3 (Cell Signaling; 9132), and caspase 3 (Cell Signaling; 9661) antibodies using the automated staining processor Discovery from Ventana Medical Systems.

Quantification of Immunohistochemical Staining. TUNEL and cleaved caspase 3-stained cells were estimated in 10 randomly selected fields of tumor at 40× magnification, and the percentage of positive tumor cells was calculated as number of positive cells/total number of cells × 100.

Affymetrix Oligonucleotide Microarray. MDA-MB-435s cells were treated with 16 μM ST2, ST6, or INV morpholino for 4 d and total RNA was extracted at the end of treatment. The quality of the RNA was ensured before labeling by analyzing 20–50 ng of each sample using the RNA 6000 NanoAssay and a Bioanalyzer 2100 (Agilent). Samples with a 28S:18S ribosomal peak ratio of 1.8–2.0 were considered suitable for labeling. For samples meeting this standard, 2 μg of total RNA was used for cDNA synthesis using an oligo (dT)-T7 primer and the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). Synthesis, linear amplification, and labeling of cRNA were accomplished by transcription in vitro using the MessageAmp aRNA Kit (Ambion) and biotinylated nucleotides (Enzo Diagnostics). Ten micrograms of labeled and fragmented cRNA was then hybridized to the mouse genome U133 plus 2.0 array (Affymetrix), which interrogates ~39,000 transcripts, at 45 °C for 16 h. Automated washing and staining were performed using the Affymetrix Fluidics Station 400 according to the manufacturer's protocols. Finally, chips were scanned with a high-numerical aperture and flying objective lens in the GS3000 scanner (Affymetrix). Raw expression data were analyzed using GCOS 1.4 (Affymetrix). Data were normalized to a target intensity of 500 to account for differences in global chip intensity.

Real-Time Quantitative PCR. Primers for qPCR were designed for use with an annealing temperature of 60 °C and a product size of less than 150 nucleotides through the oligo-design program at http://www.idtdna.com. When possible, primers were designed to span exon–exon junctions to prevent the amplification of contaminating genomic DNA. PCR reactions were performed per SYBR Green instructions (Invitrogen). Primers initially were tested for linearity with a template curve from 200 ng to 2 pg (10-fold dilutions) with retro-transcribed Universal Human RNA (Stratagene) and by checking the PCR product size on an agarose gel stained with ethidium bromide. A single gel band and a single peak on the melting curve were verified initially and used to confirm the PCR product thereafter. The ddC(t) method was used for qPCR determination (4). Hypoxanthine phosphoribosyl transferase (HPRT) and β-2-microglobulin (B2M) were used as housekeeping genes.

Real-Time Primer Sequences.

EGR1. F 5′-AGCCCTACGAGCACCTGAC-3′
R 5′-AGCGGCCAGTATAGGTGATG-3′
FAS. F 5′-ATAAGCCCTGTCCTCCAGGTGAAA-3′
R 5′-ATTCTGGGTCCGGGTGCAGTTTAT-3′
Hsp27. F 5′-AAGGATGGCGTGGTGGAGATCA-3′
R 5′-TGATCTCGTTGGACTGCGTGGCTA-3′
SOCS3. F 5′-GAAGATCCCCCTGGTGTTGA-3′
R 5′-TTCCGACAGAGATGCTGAAGAGT-3′
VEGF. F 5′-TTTCTGCTGTCTTGGGTGCATTGG-3′
R 5′-AAGATGTCCACCAGGGTCTCGATT-3′
IL8. F 5′-AGAAACCACCGGAAGGAACCATCT-3′
R 5′-AATTTCTGTGTTGGCGCAGTGTGG-3′
LEDGF. F 5′-GTGTTTCAAAGGAAGATACCGACC-3′
R 5′-TTGCTGTTGTCACTACTCCTGCCT-3′
CCNC. F 5′-CAAGATCTGTTGAAGGAGCGCCAA-3′
R 5′-CTTGAAATATACCGTAGCAGTGGC-3′
STAT1β. F 5′-AGCCAATGCAACTTGATGGCCCTA-3′
R 5′-TCAACAAGTTCAGCTGTGATGGCG-3′
PCAF. F 5′-GGCAGACTTACAGCGAGTCTTTACCA-3′
R 5′-TTGCTTTAGGCACACTGCTTGGTG-3′
PEX1. F 5′-AGCTGCATGCTGTTTCCCTTGAAC-3′
R 5′-TATTCTCTTTGGCTCGGCGTGTCT-3′
SURVIVIN. F 5′-TTCGTCCGGTTGCGCTTTCC-3′
R 5′-TCGATGGCACGGCGCACTTT-3′
B2M. F 5′-GGCTATCCAGCGTACTCCAAA-3′
R 5′-CGGCAGGCATACTCATCTTTTT-3′
HPRT. F 5′-GACCAGTCAACAGGGGACAT-3′
R 5′-AACACTTCGTGGGGTCCTTTTC-3′

Stable and Transient Overexpression. STAT1β, PCAF, IL8, and CCNC full-length cDNAs were isolated by RT-PCR from MDA-MB-435s cells and subcloned into the retroviral vector pLPC (5). The pLPC vector is a retroviral expression vector with a puromycin resistance marker (a gift from S. Lowe, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). LEDGF cDNA was a generous gift of Marja Jäättelä Danish Cancer Society, Copenhagen, Denmark, and it was also subcloned into the retroviral vector pLPC. The plasmids were verified by sequence analysis to confirm the absence of mutations. To generate stable cell pools for STAT1β, PCAF, IL8, CCNC, and the empty vector (pLPC), MDA-MB-435s cells were infected using the supernatant from transiently transfected Phoenix-Ampho packaging cells. Transfection of the Phoenix-Ampho packaging cells was done using FuGENE 6 (Roche) for a period of 48 h. After this time the media were filtered, supplemented with polybrene (4 μg/mL), and added to MDA-MB-435s cells. Cells were subjected to two rounds of infection every 12 h. After 24 h from the last infection, cells were trypsinized and selected with puromycin (2.5 μg/mL) for 72 h. After selection, cells were analyzed for protein expression by Western blot.

To overexpress LEDGF, MDA-MB-435s cells were transiently transfected with either empty vector (pLPC) or LEDGF using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. Cells were analyzed for protein expression by Western blot.

Xenograft Mouse Model. Athymic female mice 8–10 wk old were s.c. inoculated with 10×10^6 MDA-MB-435s cells. When tumors reached an average of 100 mm^3 in size, mice were randomly assigned to treatment groups and injections of Vivo-Morpholino were administered as described in *SI Morpholino Treatments*. Tumor-bearing mice were assessed for weight loss and tumor volume twice weekly. Tumor volumes were determined by caliper measurement using the following equation: length × $(\text{width})^2 \times \pi/6$. Four days after the last treatment, mice were killed and tumors were fixed in formalin. All animal studies were conducted in accordance with guidelines approved by the Memorial Sloan-Kettering Cancer Center Institutional Animal Care and Use Committee and the Research Animal Resource Center.

Statistical Analysis. The statistical significance of differences for both the morpholino and the siRNA in vitro treatments was calculated using the stratified χ^2 test (6), and bars represent SEs. For both the ELISA and MTT assays a paired *t* test was used to establish statistically significant differences and bars represent SD. Differences were considered statistically significant at a *P* value of <0.05.

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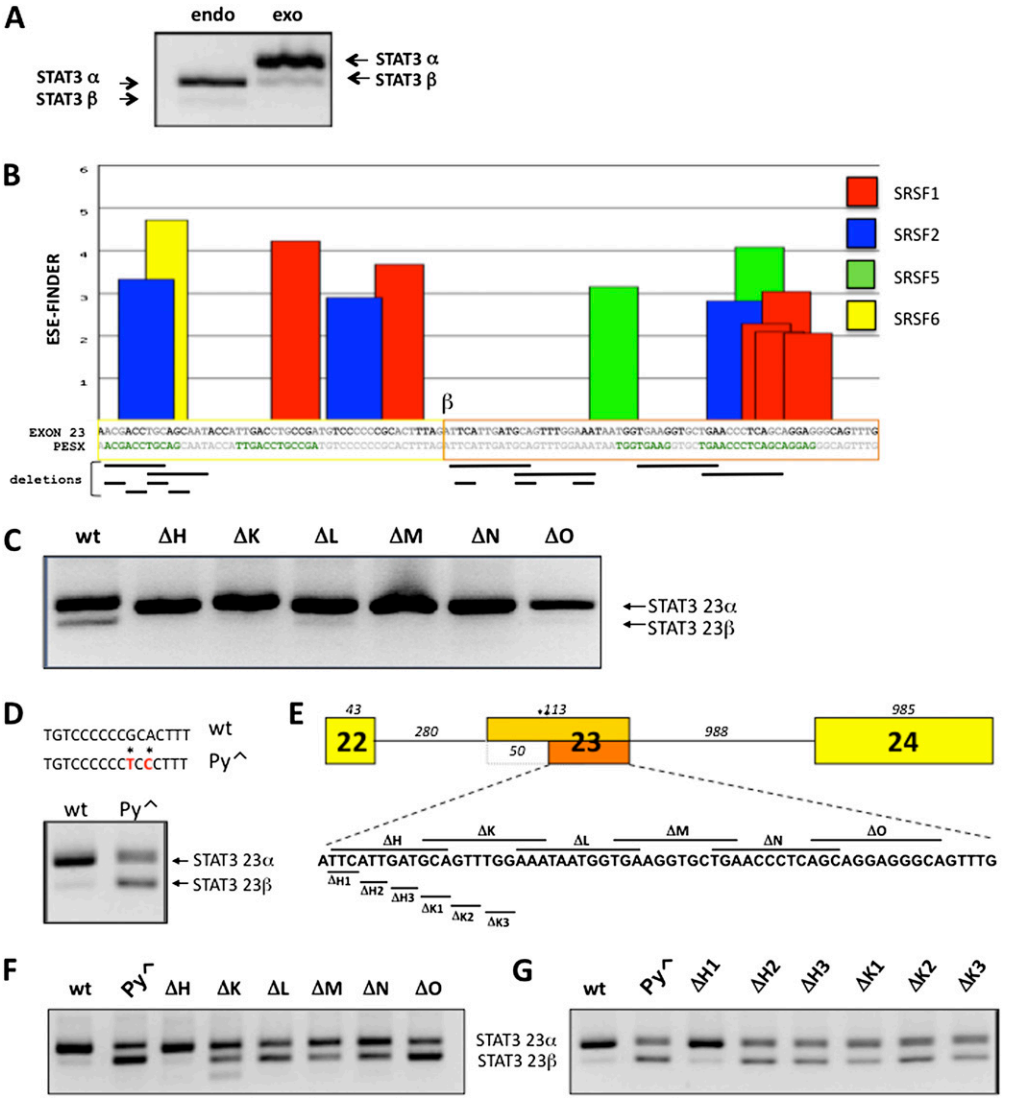

Fig. S1. Additional analysis of STAT3 exon 23 *cis*-elements. (*A*) RT-PCR analysis to assess the splicing pattern of endogenous STAT3 and transfected minigenes in HeLa cells using specific primers. Endo, endogenous; exo, exogenous. (*B*) Bioinformatic analysis of STAT3 exon 23 sequence using ESEfinder (1) (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) and PESX (2) (http://cubweb.biology.columbia.edu/pesx) algorithms. The upper graph displays the predicted scores for functional binding of four common serine-arginine (SR) proteins (SRSF1, SRSF2, SRSF5, SRSF6), according to the ESEfinder algorithm, whereas in the PESX analysis, green sequences represent putative exonic splicing enhancers. Black bars at the bottom summarize the position of all deletion mutants (from this work) that had some effect on exon 23 splicing, thus identifying putative splicing regulatory elements in exon 23. Sequences boxed in yellow correspond to the region of exon 23 specific to the α isoform, whereas sequences boxed in orange correspond to the region of exon 23 common to both α and β variants. Gray and black in the exon 23 sequence indicate codons in the α reading frame. (*C*) RT-PCR analysis of STAT3 α/β levels in HeLa cells transfected with the 12-nt (ΔH–ΔO) exon 23 deletion mutants in STAT3 minigenes. wt, wild-type. (*D*) (*Upper*) Partial sequence of the wt and pyrimidine-enhanced (Py^) minigenes. Asterisks indicate the exact position of the mutations. (*Lower*) RT-PCR analysis of STAT3 α/β levels in both transfected minigenes in HeLa cells. (*E*) (*Upper*) Diagram of STAT3 minigene. Exon and intron sizes are indicated (nt). (*Lower*) Lines above and below the exon 23 sequence indicate the exact position of the 12-nt (ΔH–ΔO) and 3-nt (ΔH1–ΔK3) deletions generated. (*F* and *G*) RT-PCR analysis of STAT3 α/β levels in HeLa cells transfected with the 12-nt (ΔH–ΔO) and 3-nt (ΔH1–ΔK3) exon 23 deletion mutants, respectively, in the pyrimidine-enhanced minigenes. Py^, pyrimidine-enhanced.

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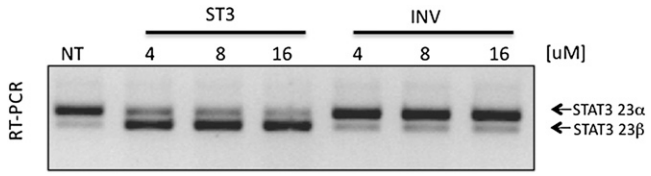

Fig. S2. Specificity of morpholino treatment. RT-PCR analysis of STAT3α/β levels in human chronic myelogenous leukemia cell line K562, treated with increasing amount of ST3 and control INV compounds for 4 d at the indicated concentrations. NT, untreated.

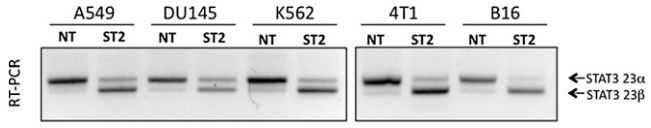

Fig. S3. Morpholino treatment of multiple cell lines. RT-PCR analysis of STAT3α/β levels in human lung cancer cell line A549, human prostate cancer cell line DU145, human chronic myelogenous leukemia cell line K562, mouse breast cancer cell line 4T1, and mouse melanoma cell line B16 treated with 16 μM ST2 for 4 d.

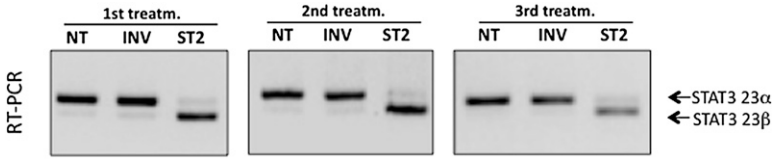

Fig. S4. Sustainable splicing switch by ST2 treatment. RT-PCR analysis of STAT3 α/β levels in MDA-MB-435s cells treated with either INV or ST2 morpholino oligonucleotide for three consecutive treatments of 4 d each.

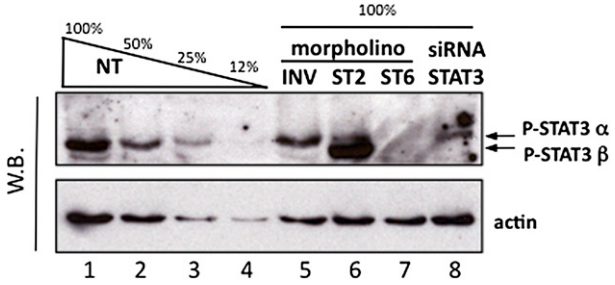

Fig. S5. Side-by-side comparison of forced splicing-dependent nonsense-mediated decay and siRNA effectiveness to knock down STAT3. Western blot (W.B.) of phospho-STAT3 Y705 levels in lysates from treated MDA-MB-435s cells. Lanes 1–4 are a serial dilution of untreated cells (NT) from 100 to 12.5% of input. Lanes 5–7 are 100% of input from cells treated with 16 μM INV/ST2/ST6 oligos. Lane 8 is 100% of input from cells treated for 48 h with 100 nM siRNA oligo against STAT3. Actin was used as loading control.

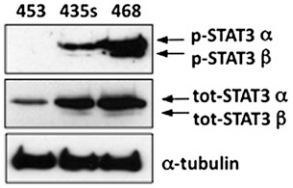

Fig. S6. STAT3 Y705 phosphorylation status in different cell lines. Western blot analysis of phospho-STAT3 Y705 and total levels in lysates from MDA-MB-453, MDA-MB-435s, and MDA-MB-468 cells. α-Tubulin was used as loading control.

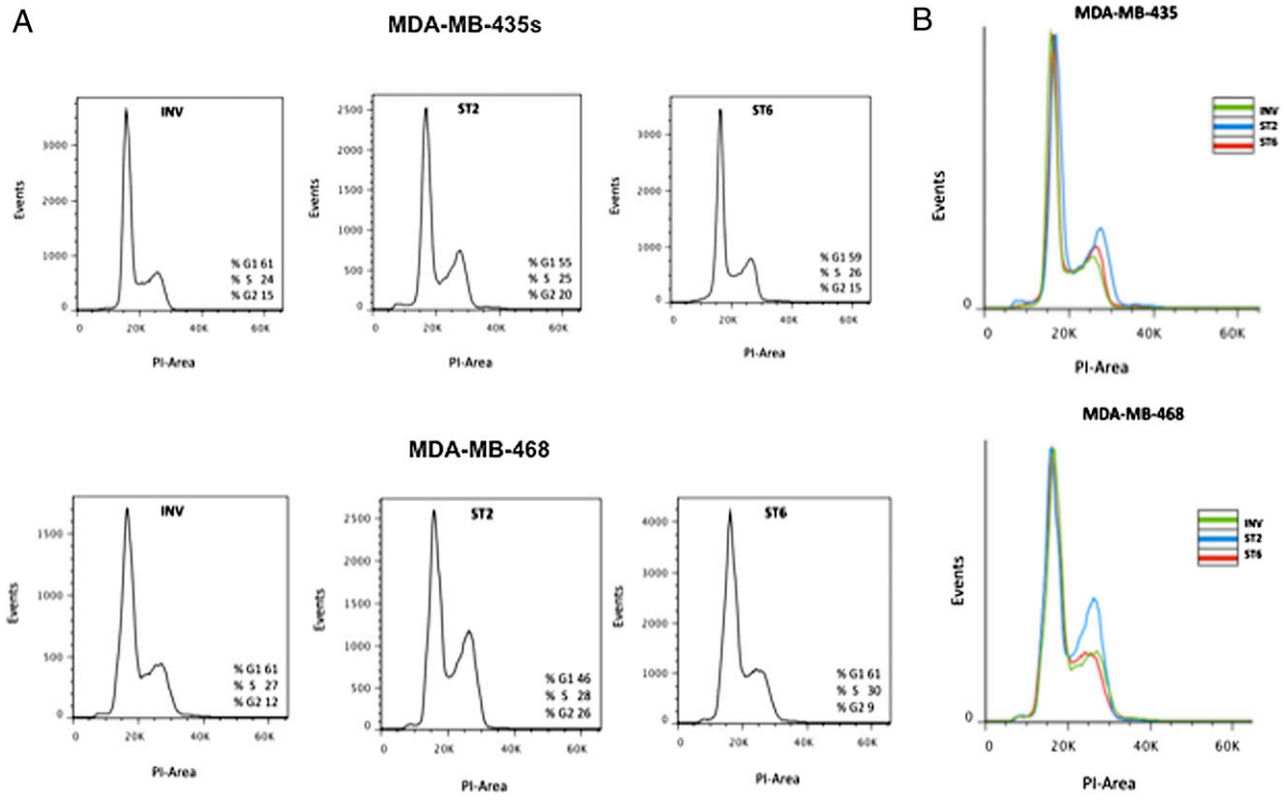

Fig. S7. ST2 but not ST6 treatment inhibits cell-cycle progression in MDA-MB-435s and MDA-MB-468 cells. The effect of ST2 or ST6 vivo-morpholino oligonucleotides on the cell cycle was evaluated by flow cytometric analysis. After 60 h of treatment, MDA-MB-435s cells treated with ST2 displayed an increase in the G2/M population (20.8% ± 0.28 SE) compared with the INV- (14% ± 1.4 SE) and ST6-treated cells (12.5% ± 3.5 SE). Similarly, in MDA-MB-468 cells, the G2/M population increased to 25% ± 1.4 SE following treatment with ST2 (INV: 9% ± 4.24 SE; ST6: 12% ± 0.7 SE). The observed increase of the cell-cycle population at the G2/M phase was accompanied by a decrease of cell population in the G1 phase of the cell cycle. All treatments were done at 4 μM, and the treatment was only for 60 h to ensure the cells were in exponential growth. (*A*) Representative profiles of the analysis of cell-cycle progression. MDA-MB-435s and MDA-MB-468 cells were treated with 4 μM INV, ST2, or ST6 for 60 h. The distribution and percentage of cells in the G1, S, and G2/M phase of the cell cycle are indicated. (*B*) Overlay of the cell-cycle profiles with ST2 treatment, associated with the increase in the G2/M population, showed in blue. ST6 is in red and control (INV) is in green.

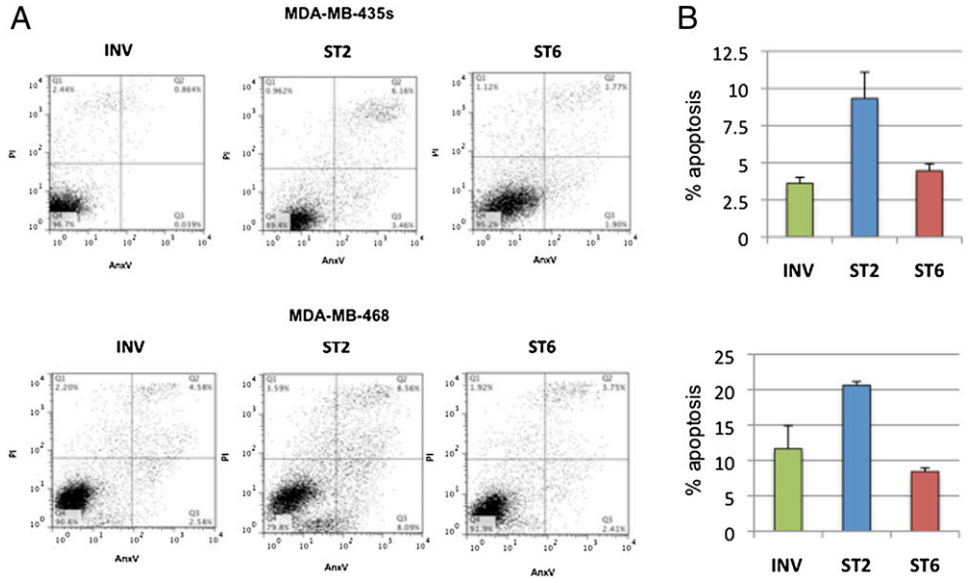

Fig. S8. ST2 but not ST6 treatment induces apoptosis in MDA-MB-435s and MDA-MB-468 cells. The effect of ST2 and ST6 treatment on apoptosis was evaluated with annexin V staining. After 60 h of treatment at 4 μM, an increase in the levels of apoptosis was observed in the MDA-MB-435s (9.33% apoptotic; ±1.76 SE) and MDA-MB-468 cells (20.6% apoptotic; ±0.5 SE), following ST2 treatment. (*A*) Representative scatter plot of flow analysis of MDA-MB-435s and MDA-MB-468 cells following treatment with INV, ST2, or ST6 at 4 μM for 60 h. Cells (1×10^5) (including supernatant) were harvested and analyzed by AnxV/PI as detailed in *SI Materials and Methods* and data were analyzed with FlowJo software. The percentage of apoptotic cells is indicated. (*B*) Assessment of apoptotic population, as determined by AnxV/PI staining, in MDA-MB-435s and MDA-MB-468 cells treated with INV, ST2, or ST6 vivo-morpholinos (error bars indicate SE).

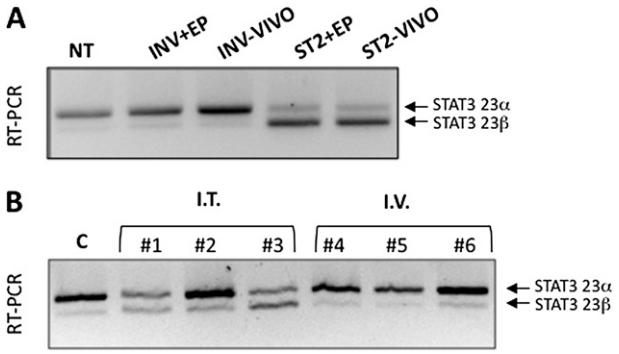

Fig. S9. Effectiveness of Vivo-Morpholino oligonucleotide treatment. (*A*) Side-by-side comparison of naked morpholinos and Vivo-Morpholino efficacy. RT-PCR analysis of STAT3α/β levels in MDA-MB-435s cells treated with either INV or ST2 morpholino plus Endo-Porter or with either INV or ST2 Vivo-Morpholino. All oligos were used at a final concentration of 16 μM for 4 d. EP, Endo-Porter. (*B*) Delivery of Vivo-Morpholino in a xenograft tumor model. RT-PCR analysis of STAT3α/β levels in intratumorally (IT) or intravenously (IV) treated xenograft tumors. MDA-MB-435s cells (10×10^6) were s.c. inoculated in the flanks of female athymic mice. When tumors reached ~100 mm^3 in volume size, mice ($n = 3$ per group) were randomly assigned to one of four treatment groups (Control-IV, ST2-IV, Control-IT, and ST2-IT). Animals were treated twice in 1 wk and, 4 d after the second treatment, tumors were collected and further analyzed. C, control. When similar experiments were attempted with MDA-MB-468 cells under comparable conditions (multiple times), the growth of the resulting tumors was unpredictable, with a high fraction of injections that did not take, whereas the ones that grew did so at very irregular rates, and in some cases the tumor grew but was highly necrotic (regardless of treatment).

A

Group	Size (cm)	Necrosis (%)	Mitotic Count (highest/ 20hpf)
Control			
mean	0.5	12	43
median	0.4	15	53
range	0.4-0.7	0-20	22-55
INV			
mean	0.4	20	137
median	0.45	20	146
range	0.4-0.45	n/a	101-163
ST2			
mean	0.175	2.5	8
median	0.175	2.5	8
range	0.1-0.25	0-5	1-15
ST6			
mean	0.48	23	68
median	0.45	20	75
range	0.43-0.55	5-45	19-109

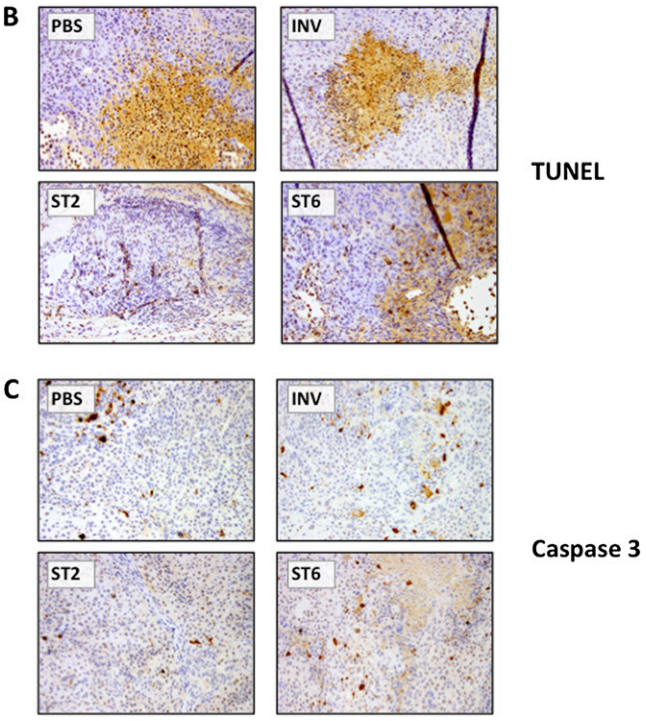

Fig. S10. Histopathological analysis of Vivo-Morpholino-treated tumors. (*A*) Table summarizing the histopathological examination of PBS-, INV-, ST2-, and ST6-treated tumors such as their size (cm), estimated area of necrosis (%), and highest mitotic count per 20 high-power fields (hpf). (*B*) Representative results of the TUNEL staining of tumor sections from each of the four treated groups (40× magnification). (*C*) Representative results of the caspase 3 immunohistochemical staining of tumor sections from each of the four treated groups (40× magnification).

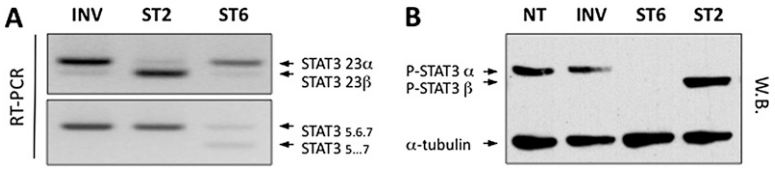

Fig. S11. Validation of treatment efficacy of the samples used for qPCR/microarray analysis. (*A*) (*Upper*) RT-PCR analysis of STAT3 α/β levels in MDA-MB-435s cells treated with 16 μM INV, ST2, or ST6 oligos for 4 d. (*Lower*) RT-PCR analysis of STAT3 exon 6 skipping in MDA-MB-435s cells treated with 16 μM INV, ST2, or ST6 oligos for 4 d. (*B*) Western blot analysis of phospho-STAT3 Y705 α/β levels in MDA-MB-435s cells treated with 16 μM INV, ST6, or ST2 for 4 d. α-Tubulin was used as loading control.

A. ST2 compared to INV

Gene Name	Fold Change	Genbank	Gene Symbol	Description
218972_at	3,667	NM_018259	TTC17	tetratricopeptide repeat domain 17
204565_at	2,614	NM_018473	THEM2	thioesterase superfamily member 2
218104_at	2,579	NM_017746	TEX10	testis expressed sequence 10
201157_s_at	2,547	AF020500	NMT1	N-myristoyltransferase 1
201158_at	2,484	AI570834	NMT1	N-myristoyltransferase 1
220264_s_at	2,326	NM_020960	GPR107	G protein-coupled receptor 107
204809_at	2,309	NM_006660	CLPX	ClpX caseinolytic peptidase X homolog (E. coli)
214358_at	2,288	AW188201	ACACA	acetyl-Coenzyme A carboxylase alpha
209422_at	2,166	AY027523	PHF20	PHD finger protein 20
211506_s_at	0.343055556	AF043337	IL8	interleukin 8
201278_at	0.335416667	N21202	DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)
222118_at	0.315972222	AK023669	BM039	uncharacterized bone marrow protein BM039
203845_at	0.30625	AV727449	PCAF	p300/CBP-associated factor
222156_x_at	0.286111111	AK022459	CCPG1	cell cycle progression 1
203167_at	0.25625	NM_003255	TIMP2	TIMP metallopeptidase inhibitor 2
209337_at	0.23125	AF063020	PSIP1	PC4 and SFRS1 interacting protein 1
201955_at	0.217361111	AL137784	CCNC	cyclin C
205961_s_at	0.193055556	NM_004682	PSIP1	PC4 and SFRS1 interacting protein 1
209969_s_at	0.184027778	BC002704	STAT1	signal transducer and activator of transcription 1, 91kDa
204873_at	0.132638889	NM_000466	PEX1	peroxisome biogenesis factor 1

B. ST6 compared to INV

Gene Name	Fold Change	Genbank	Gene Symbol	Description
218972_at	4,348	NM_018259	TTC17	tetratricopeptide repeat domain 17
218104_at	2,573	NM_017746	TEX10	testis expressed sequence 10
204809_at	2:05	NM_006660	CLPX	ClpX caseinolytic peptidase X homolog (E. coli)
201157_s_at	2,391	AF020500	NMT1	N-myristoyltransferase 1
201158_at	2,388	AI570834	NMT1	N-myristoyltransferase 1
209422_at	2,221	AY027523	PHF20	PHD finger protein 20
204565_at	2,164	NM_018473	THEM2	thioesterase superfamily member 2
203167_at	0.308333333	NM_003255	TIMP2	TIMP metallopeptidase inhibitor 2
203973_s_at	0.296527778	M83667	CEBPD	CCAAT/enhancer binding protein (C/EBP), delta
202238_s_at	0.277083333	NM_006169	NNMT	nicotinamide N-methyltransferase
202237_at	0.275	NM_006169	NNMT	nicotinamide N-methyltransferase
208992_s_at	0:15	BC000627	STAT3	signal transducer and activator of transcription 3
208991_at	0.071527778	BC000627	STAT3	signal transducer and activator of transcription 3

Fig. S12. Microarray data from treated MDA-MB-435s cells. (*A*) Table representing significantly differentially expressed genes (fold change ≥2) filtered from the microarray dataset obtained by comparing ST2-treated versus INV-treated MDA-MB-435s cells. In red are up-regulated genes; in blue are down-regulated genes. (*B*) Table representing significantly differentially expressed genes (fold change ≥2) filtered from the microarray dataset obtained by comparing ST6-treated versus INV-treated MDA-MB-435s cells. In red are up-regulated genes; in blue are down-regulated genes.

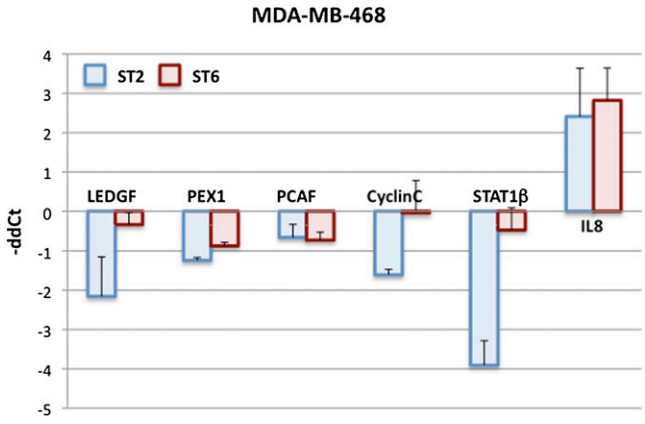

Fig. S13. Validation of STAT3β's transcriptional signature in the MDA-MB-468 cell line. cDNAs from MDA-MB-468 cells treated with 16 μM ST2, ST6, or INV morpholinos for 4 d were analyzed by real-time PCR to validate STAT3β's transcriptional signature previously identified in MDA-MB-435s cells. Results are represented as a comparison between ST2 and INV treatments (blue) or between ST6 and INV treatments (red) and are expressed as −ddC(t) values after normalization to HPRT expression levels. Data represent the average of at least three independent experiments. Bars represent SD.

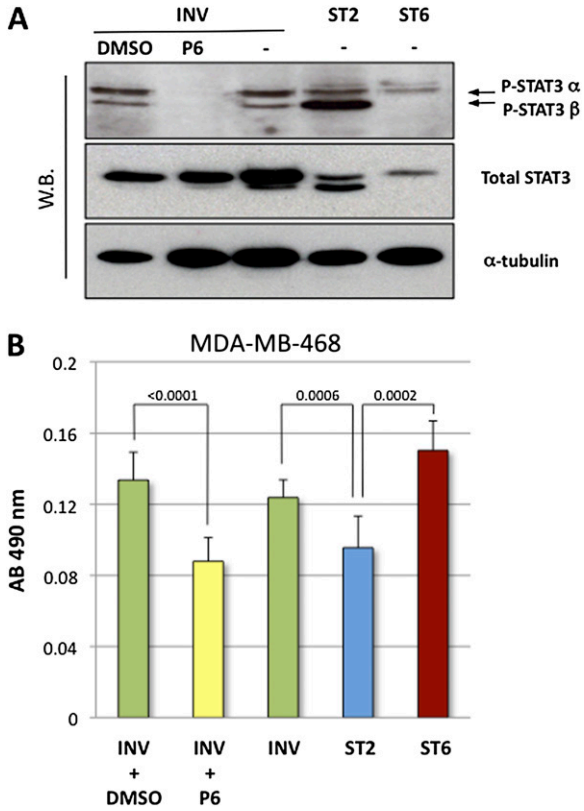

Fig. S14. Comparison of (P6) pan-JAK inhibitor with ST6 down-regulation of STAT3 in the MDA-MB-468 cell line. (*A*) MDA-MB-468 cells were treated for 48 h with 1 μM pan-JAK inhibitor tetracyclic pyridone 6 (P6) or DMSO in combination with morpholino control treatment (INV), total STAT3 knockdown morpholino treatment (ST6), or STAT3 α/β splicing switch treatment (ST2) as indicated. All vivo-morpholinos were used at 6 μM. P6 was used in combination with INV to control for the baseline toxicity associated with morpholino treatment in MDA-MB-468 cells. However, the relative increase in cell death due to P6 versus DMSO is the same whether INV is present or not. Activation status of STAT3 and the effectiveness of treatment were monitored using an antibody against Y705 (*Top*), and total STAT3 levels were also determined by Western blot (*Middle*). Tubulin was used as loading control. (*B*) The effect of the various treatments on cell growth was measured using the MTT assay. Data represent the average of at least five independent experiments. Bars represent SD. Indicated *P* values were calculated by two-tailed Student's *t* test. The results indicate that lack of phospho-STAT3 is not sufficient, per se, to inhibit growth even in cells described as highly sensitive to its depletion such as MDA-MB-468. Rather, another event must occur, such as the induction of STAT3β or the presumable inactivation of additional JAK substrates by pan-Jak inhibitor P6.