Supplemental Material

STAT5A-mediated SOCS2 expression regulates Jak2 and STAT3 activity following c-Src inhibition

Supplemental Methods

Cells and Reagents

Dasatinib was purchased from Selleck chemicals (Houston, TX) and the clinical pharmacy. INCB16562 was provided by Incyte Corporation (Wilmington, DE). Both were prepared as 10 mmol/L stock solutions in DMSO. Antibodies used included c-Src, pSFK (Y416), pSTAT3 (Y705), pJak2 (Y221), pJak2 (Y1007/1008), pSTAT5 (Y694) XP, and SOCS2 (Cell Signaling Technology, Danvers, MA); total phosphotyrosine and total STAT5B (Upstate Biotechnology, Billerica, MA); SOCS1 and total Jak2 (BD Biosciences, Franklin Lakes, NJ); total STAT5A (Abcam Inc, Cambridge, MA); and β-Actin (Sigma Chemical Co, St. Louis, MO).

Human HNSCC cell lines were obtained from Dr. Jeffrey Myers and maintained as described previously (Sen *et al.*, 2009). All cell lines were validated by cross-comparing their allelic short tandem repeat profiling (Johns Hopkins Fragment Analysis Core facility, Baltimore, MD) and patterns generated with the PowerPlex 1.2 platform (Promega Corp, Madison, WI) to those from the American Type Culture Collection repository database.

Medium transfer to recipient cells

TU167 cells were transfected with nontargeting scrambled and c-Src specific siRNA as described above. Twenty-four hours prior to medium transfer, transfected cells were

supplemented with fresh medium (donor cells), whereas TU167 cells (recipient cells) were serum starved for 24 h prior to receiving conditioned medium from transfected cells. After serum starvation, recipient cells were supplemented with their respective conditioned media and incubated for indicated times at 37°C with 5% CO_2. Cell lysates were analyzed by Western blot analysis.

Fig S1

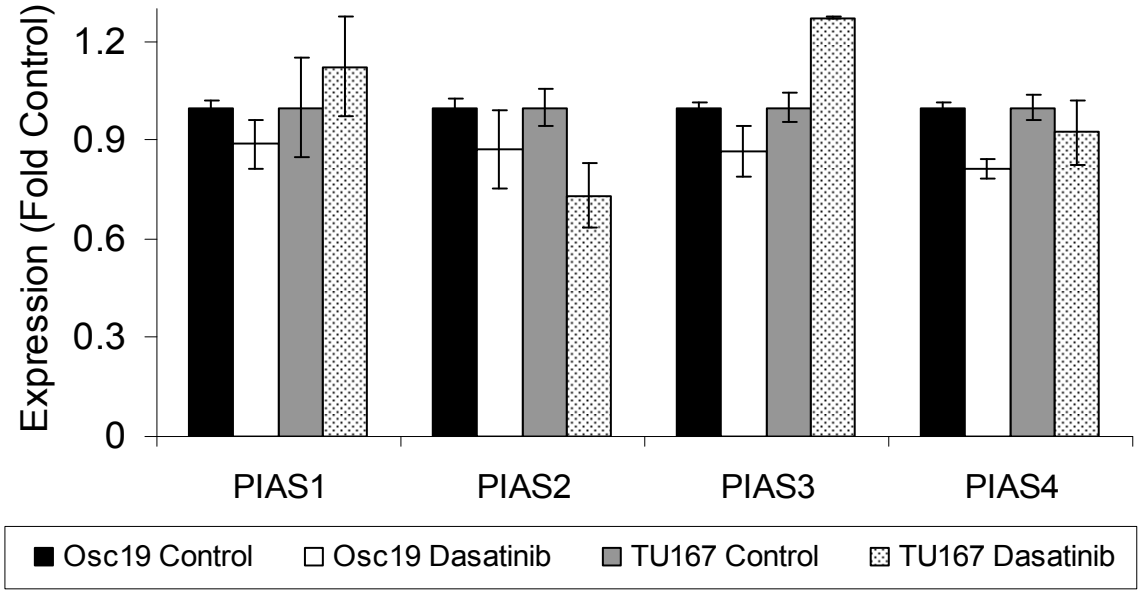

Figure S1

c-Src inhibition does not consistently affect PIAS expression in HNSCC cell lines. Two HNSCC cells were incubated with 100 nM dasatinib for 7 h and mRNA levels of all the known PIAS molecules were measured by qPCR and expressed as fold control (vehicle treatment).

Fig S2

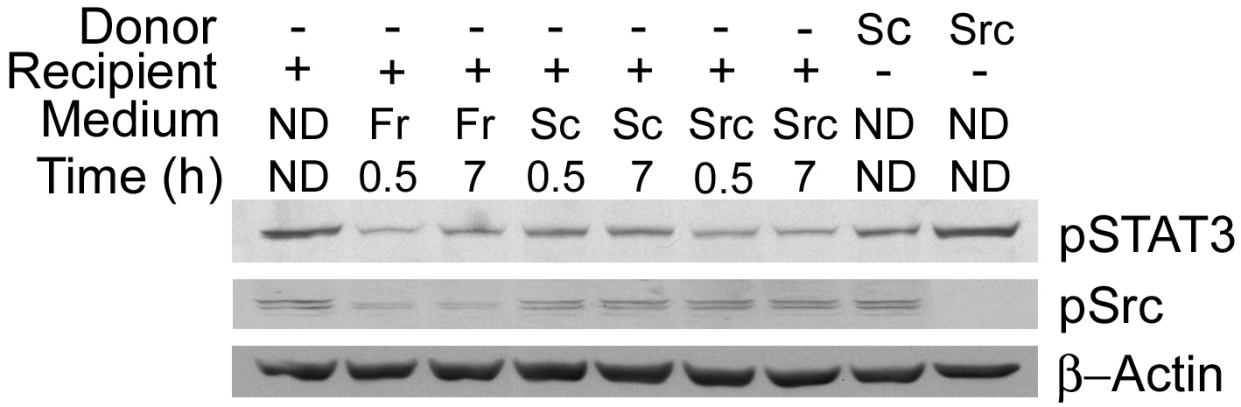

Figure S2

STAT3 reactivation is not regulated by a soluble secreted factor. Conditioned medium from TU167 donor cells incubated with c-Src siRNA (Src) or nontargeting siRNA (Sc) [lanes 8-9] was added to TU167 recipient cells [lanes 4-7]. As a control for the effects of medium change alone, fresh medium (Fr) was added to cells in lanes 2-3. The cells assayed in lane 1 were untreated with no medium changes. As expected, c-Src knockdown led to STAT3 reactivation (lane 9). The medium from cells with c-Src knockdown did not affect STAT3 activation in recipient cells (lanes 6-7).

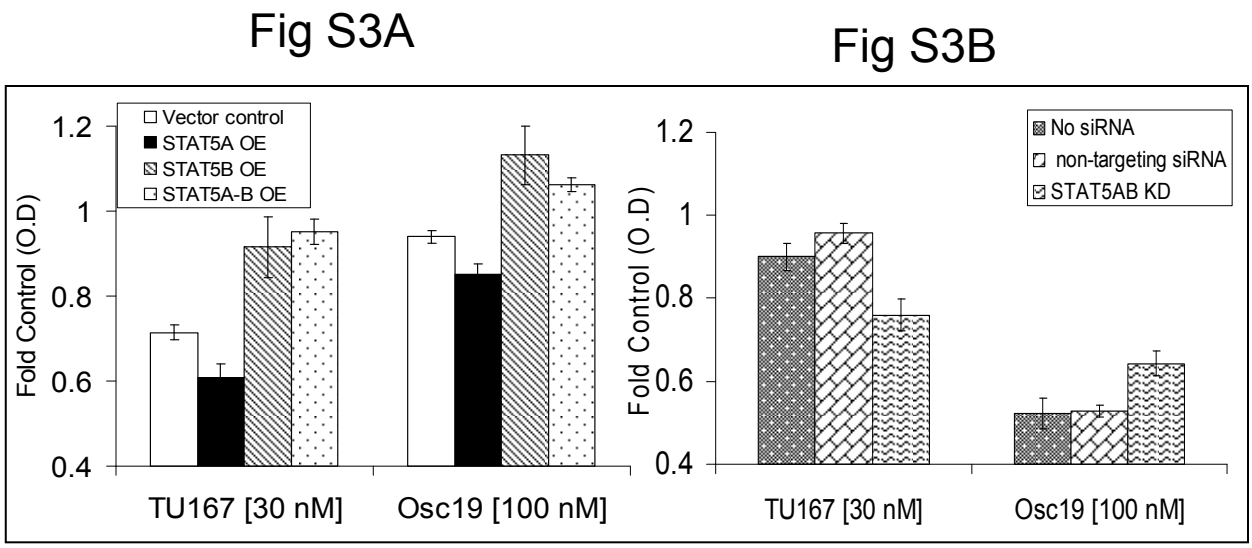

Figure S3

The effect of STAT5A, B, or AB expression on cell viability after c-Src inhibition. TU167 and Osc19 cells were transiently transfected with pUSE-Amp (+) empty vector, or CA-STAT5A or CA-STAT5B or both (A), or non targeting siRNA and STAT5AB-specific siRNA (B). Twenty-four hours after transfection, respective cells were seeded in 96-well plates for MTT assay. Cell viability was assayed in the presence of the indicated concentrations of dasatinib for 72 h.

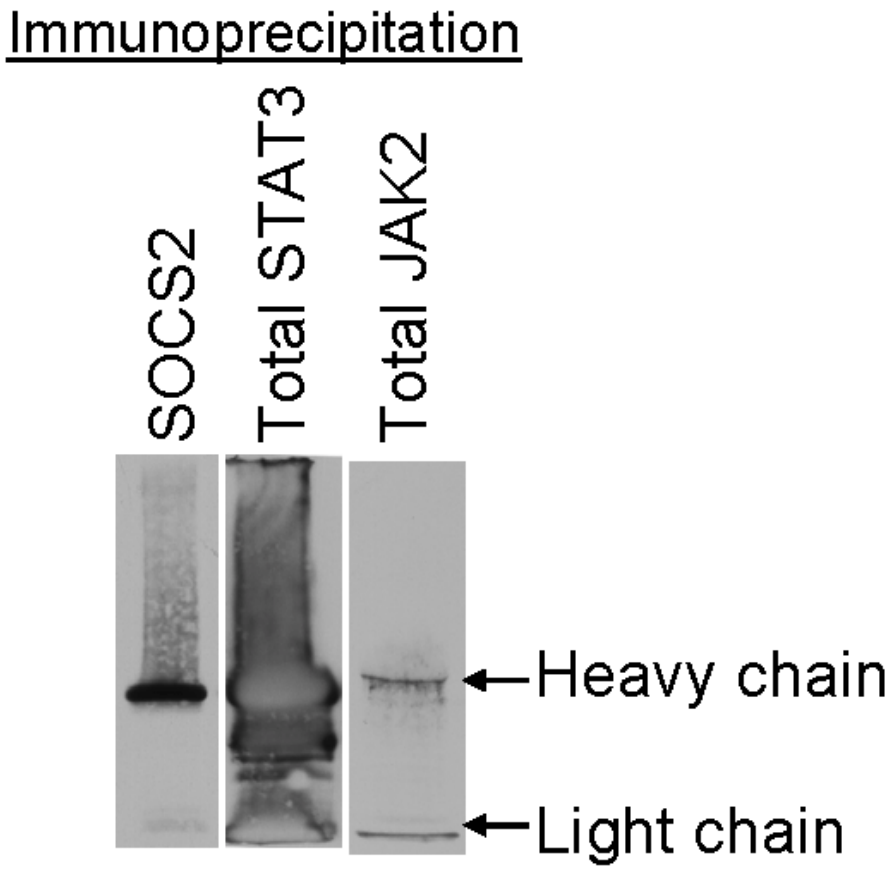

Figure S4: Immunoglobulin control immunoprecipitation for Figure 5A.

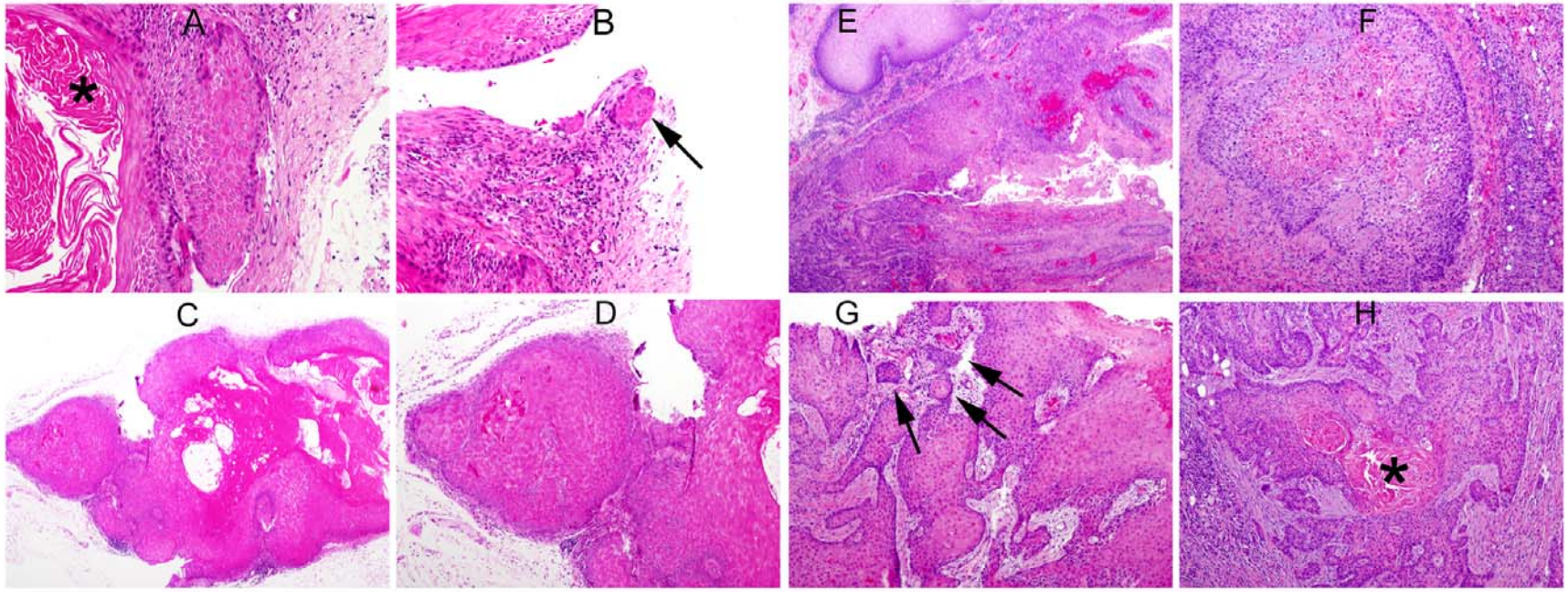

Figure S5. Human HNSCC implanted into mice retains the histologic features of the original tumor from which it was derived. Heterotransplants (A-D) and the patient tumor (E-H) from which it was derived were stained with H&E and examined micrscopically. The resected heterotranplant tumors consist almost entirely of squamous carcinoma (best visualized in C). Both the heterotransplants and the patient tumors demonstrate invasive squamous carcinoma (arrow) with typical keratin production (*).

Fig. S6A

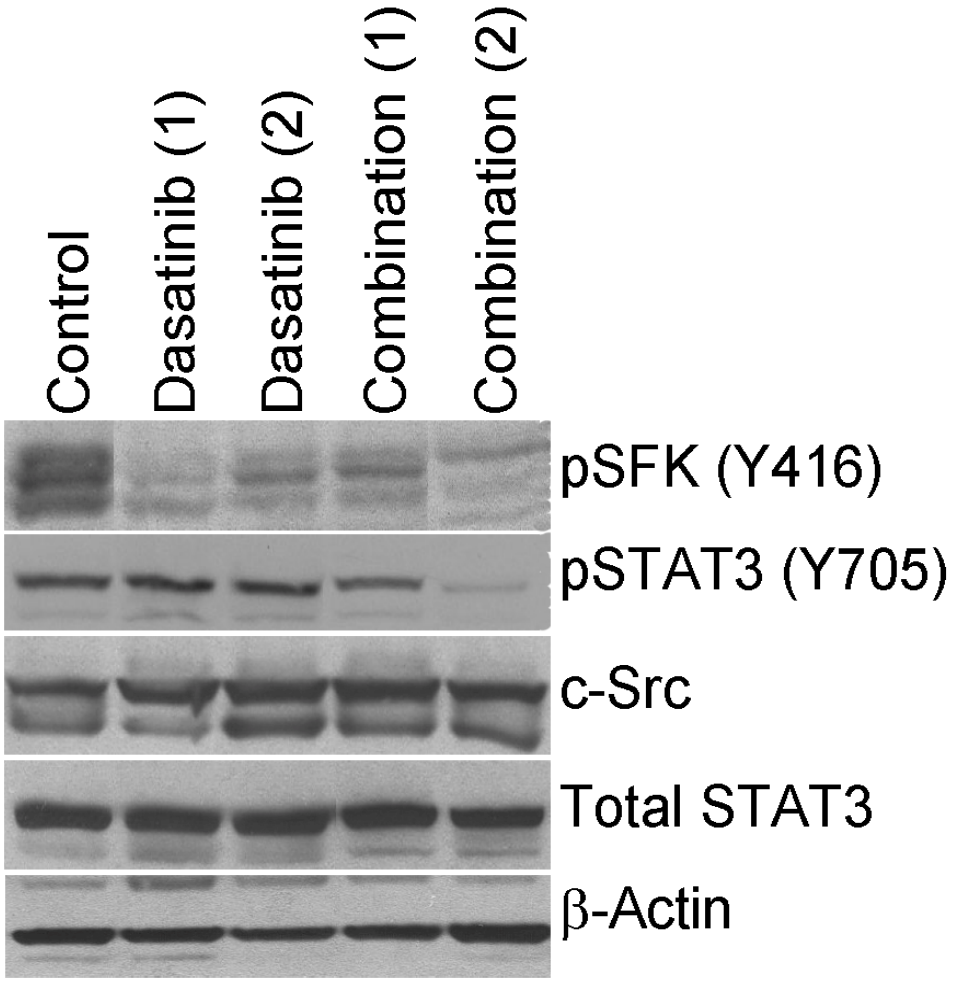
Control
Dasatinib (1)
Dasatinib (2)
Combination (1)
Combination (2)
pSFK (Y416)
pSTAT3 (Y705)
c-Src
Total STAT3
β-Actin

Fig. S6B

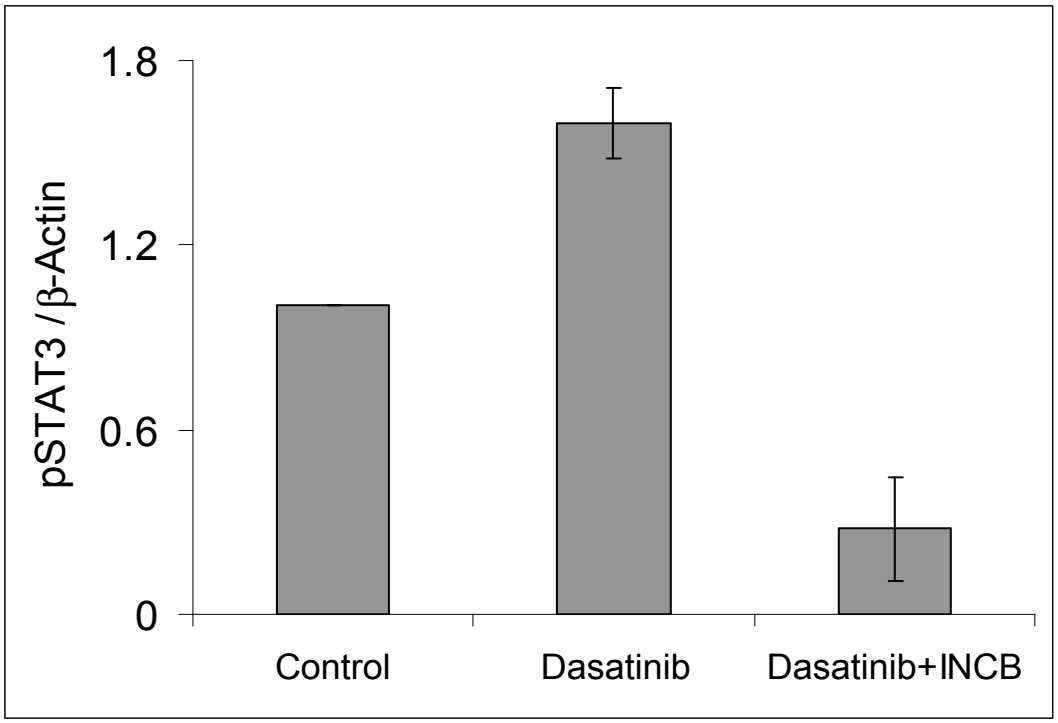

Figure S6

Jak inhibition abrogates dasatinib-induced STAT3 reactivation *in vivo*. Mice bearing orthotopic Osc19 xenografts were treated daily with dasatinib (20 mg/kg/d) alone or in combination with INCB016562 (INCB, 60 mg/kg/d), or with vehicle (control) for 7 d. Tumors lysates were analyzed by Western blot anlysis with the indicated antibodies (A). Western blots for activated STAT3 (pSTAT3, Y705) were analyzed using densitometry and normalized to β-actin (B).

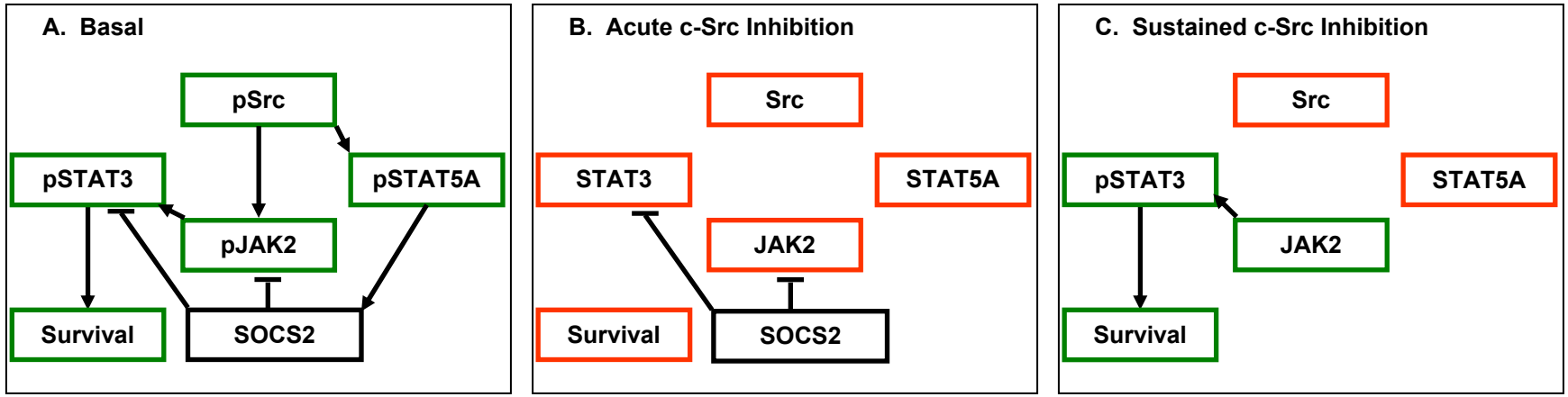

Figure S7

Model for STAT3 reactivation following c-Src inhibition. (A) Basal c-Src activation in cancer cells can overcome the inhibitory effects of basal SOCS2 expression and results in activation of Jak2, STAT3, and STAT5A. (**B**) Acute c-Src inhibition in the presence of basal SOCS2 results in Jak2, STAT3, and STAT5A inhibition. (**C**) Chronic c-Src inhibition results in durable STAT5A inhibition, decreased SOCS2 transcription, and the subsequent loss of SOCS2 protein by depletion. SOCS2 loss allows for Jak2 kinase reactivation, Jak2-STAT3 binding, and STAT3 reactivation. The persistent STAT3 activation allows HNSCC survival.

Table S1. Oligonucleotide sequences of qPCR primers for the SOCS family members.

CIS-1F: 5'-GTT TTA GAC TGC TGC GCT CC-3'
CIS-1R: 5'-GTG TCC ATG GCT GTG TCA TC-3'
CIS-2F: 5'-GCC AGA AGG CAC GTT CTT AG-3'
CIS-2R: 5'-CTC AAT GCG TAC ATT GGT GG-3'
SOCS1-F: 5'-CCCCTGGTTGTTGTAGCAGC-3'
SOCS1-R: 5'-GAGTTCAGGTCCTGGCTCCA-3'
SOCS2-F: 5'-CCCGCAGGGACTCGTTTT-3'
SOCS2-R: 5'-TTCGCGTCCTTCCTTGAAGT-3'
SOCS3-F: 5'-CTTCTTCACGCTCAGCGTCA-3'
SOCS3-R: 5'-ATGCGCAGGTTCTTGGTCC-3'
SOCS4-F: 5'-GCGCAATCTCGGTTCACTG-3'
SOCS4-R: 5'-GAGAATTGCTGGAACCTGGAAG-3'
SOCS5-F: 5'-GAGCTTGGAAAGTCCACACACA-3'
SOCS5-R: 5'-CAGGCACGAGGCAGTGTATG-3'
SOCS6-F: 5'-GAGCTCAGAGGCCGATAAGGT-3'
SOCS6-R: 5'-GACTGTAGTGATGGCTGCGGA-3'
SOCS7-F: 5'-AGTGGGACGCTGCCTACATC-3'
SOCS7-R: 5'-CAAGGAAGACCCCATCGGA-3'
PIAS1-F: 5'-ACCTTGCAGCCTTCCAGGTT-3'
PIAS1-R: 5'-GGTTCCACGCCATTTTTTGT-3'
PIAS2-F: 5'-CTTCAGCTGTGCCAAACCAA-3'
PIAS2-R: 5'-TCCCAATTTCTGATGCCC AA-3'
PIAS3-F: 5'-CATGGTGATGAGTTTCCGGG-3'
PIAS3-R: 5'-GCAAAGCCAAGAAGCACCTG-3'
PIAS4-F: 5'-GATCCGGAACTCCAGGGAAC-3'
PIAS4-R: 5'-ACGACCTGCACGGCTTTAAC-3'
L32-F: 5'-CCTTGTGAAGCCCAAGATCG-3'
L32-R: 5'-TGCCGGATGAACTTCTTGGT-3'

Table S2. *P* values for PCNA staining shown in Figure 6C.

	PCNA Staining		
Treatment group	High	Medium	Low
Control vs. Dasatinib	0.009	0.152	0.031
Control vs. INCB16562	<0.001	0.022	0.002
Control vs. Combination	<0.001	0.005	<0.001
INCB16562 vs. Combination	0.031	0.994	0.358
Dasatinib vs. INCB16562	0.206	0.050	0.020
Dasatinib vs. combination	0.103	0.050	0.015