Published in final edited form as:

Cancer Res. 2008 October 1; 68(19): 7736–7741. doi:10.1158/0008-5472.CAN-08-1125.

Activation of Stat3 through a Phosphomimetic Serine727 Promotes Prostate Tumorigenesis Independent of Tyrosine705 phosphorylation

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Abstract

Aberrantly activated Stat3 is implicated in the development of various human cancers. Y705 phosphorylation is conventionally thought to be required for Stat3 signal-dependent activation and appears to play an essential role in some malignancies. Recently, it was shown that Stat3 is activated through novel and non-canonical mechanisms, including phosphorylation at S727. Here, we investigate S727 phosphorylation activation of Stat3 and subsequent effects in prostate cancer development, independent of Y705 phosphorylation, using mutated Stat3 in the human prostate cancer cell line LNCaP. We demonstrate mutation of S727 to the phosphomimetic residue Glu and inactivation of Y705 (Y705F/S727E) resulted in a remarkable growth advantage in low-serum, enhanced anchorage-independent growth in soft agar and increased tumorigenicity in NOD/SCID mice, possibly by direct activation of downstream proto-oncogenes *c-myc*, *mcl-1* and *survivin*. Y705F/S727E mutant cells were more invasive than Y705F/S727A (inactivation of Y705 and S727) mutant cells, and more Y705F/S727E mutant Stat3 was localized in the nuclei relative to Y705F/S727A mutant Stat3 at the steady-state. Furthermore, the Y705F/S727E but not the Y705F/S727A mutant induced anchorage-independent growth of noncancerous prostate epithelial cells (RWPE-1). We further show that Stat3 is phosphorylated at S727 in 65% of malignant prostate tissues (n=20) relative to 25% of normal prostate tissues (n=4). Moreover, there is a positive correlation between phosphoS727-Stat3 expression and Gleason score in these prostate cancer tissues ($P = 0.05$). Our data suggests for the first time that S727 phosphorylation is sufficient to activate Stat3, thereby driving prostate tumorigenesis independent of Y705 phosphorylation.

Keywords

Stat3; Tyr705; Ser727; phosphorylation; prostate cancer

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Introduction

Prostate cancer (PCa) is the most prevalent noncutaneous cancer and the second leading cause of cancer deaths in males in the United States (1). It develops to an advanced stage in ~25% of patients (2). Androgen ablation remains the primary treatment for advanced PCa, but hormone-refractory PCa invariably recurs within 1-2 years and currently no therapy substantially prolongs survival (3). Improved understanding of the molecular mechanisms of PCa development may provide a basis for development of effective treatment strategies.

Signal transducers and activators of transcription 3 (Stat3) is a latent transcription factor that, upon activation, regulates transcription of downstream genes involved in cell proliferation, survival, cell migration and tumorigenesis (4-7). Stat3 is aberrantly activated in various human cancers (6) and its signaling pathway contributes to the development of androgen-independent PCa (4). Conventional dogma for Stat3 signaling is phosphorylation on a single tyrosine residue (Y705) following stimulation resulting in Stat3 homodimerization and translocation into the nucleus, where it binds to promoters transactivating downstream genes. In addition to Y705 phosphorylation, phosphorylation of another conserved Stat3 residue, serine727 (S727), has also been documented to activate Stat3 signaling. S727 phosphorylation enhances the transcriptional activities of Stat3, while mutations of S727 compromise Stat3 transactivational activity and downstream effects (8). Cooperation of both tyrosine and serine phosphorylation is necessary for full activation of Stat3 (4). Nevertheless, many studies on the role of S727 phosphorylation were done under the presumption that Y705 phosphorylation occurs prior to S727 phosphorylation, thus, confounding the role of S727 phosphorylation by itself. Recent studies show that Stat3 can be activated through S727 phosphorylation in the absence of Y705 phosphorylation in noncancerous cells (9,10). To determine the role of S727 phosphorylation independent of Y705 phosphorylation in PCa, we generated double mutants Y705F/S727E that replaces the serine (S) with a glutamic acid (E), resulting in a phosphomimetic at residue 727 (9), and replaces the tyrosine (Y) with a phenylalanine (F), resulting in an unphosphorylatable residue at position 705; and Y705F/S727A that replaces the serine (S) with an alanine (A) at position 727, and the tyrosine (Y) with a phenylalanine (F) at position 705 and is unphosphorylatable at both sites. An Y705F single mutant was also generated and served as a control, where appropriate. We further established stable cell lines expressing each of the mutants in PCa cell line LNCaP. LNCaP was chosen because it does not produce autocrine IL-6 (11), a major stimulator of Stat3, thus greatly minimizing the possibility of endogenous Stat3 phosphorylation. Furthermore, we used late-passage LNCaP cells, which have acquired the capacity to proliferate in an androgen-independent manner. Therefore, the LNCaP mutant cell lines we generated are suitable *in vitro* models to study the role of Stat3-S727 phosphorylation in androgen-independent PCa. To determine the tumorigenic capacity of Stat3-S727 phosphorylation in noncancer prostate cells, stable cell lines expressing the same Stat3 mutants were also generated in RWPE-1, a noncancerous prostate epithelial cell line. More importantly, we determined the expression of Stat3-S727 phosphorylation in PCa patient and normal prostate specimens. Using these systems, we demonstrate that S727 phosphorylation activates Stat3 and promotes PCa tumorigenesis independent of Y705 phosphorylation.

Materials and Methods

Full Materials and methods are given in the supplemental material.

Cell culture and reagents

LNCaP and RWPE-1 cells were obtained from ATCC and were maintained in RPMI-1640+10% FBS and keratinocyte SFM (Invitrogen Corp., Carlsbad, CA), respectively.

Cell growth assay

LNCaP mutants were seeded in 24-well plates (2×10^4 cells/well) in triplicate. Cell numbers were counted after 24 h and then every other day for 7 days. Dead cells were excluded by trypan blue dye staining.

Anchorage-independent colony formation assay

Colony formation assays were done essentially as described (12), with the exception of plating 5×10^3 of LNCaP cells and 5×10^4 of RWPE-1 cells per 60-cm plate.

Tumorigenicity assay

NCI-Frederick is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals. Animal Care was provided in accordance with the procedures outlined in the 'Guide for Care and Use of Laboratory Animals' (13). Cells at 2×10^4 and 2×10^5 were harvested, mixed with equal volume of Matrigel (BD Biosciences, Franklin Lakes, NJ), and injected subcutaneously (100 μ l) into the flanks of the 7-week-old male NOD/SCID mice (Charles River Laboratories, Wilmington, MA). Animals were monitored daily and tumor sizes measured weekly for 5 weeks. Tumors were removed; protein lysates were extracted and examined for mutant Stat3 by immunoblotting with anti-Flag.

Cell invasion assay

Cell invasion assay was done using 24-well BD BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA) following the manufacturer's instruction. Invaded cells were stained with a three-step staining kit (Richard-Allan Scientific, Kalamazoo, MI) and photographed using a light inverted microscope (Nikon Eclipse TS 100) at 20-fold magnification and counted.

TMA

Prostate tissue arrays (20 malignant and four normal tissue cores in duplicate per array) were used and details are in supplemental materials.

Chromatin immunoprecipitation (ChIP)

ChIP assays were done as previously described (14) with minor modifications and details are in supplemental material.

Real-time RT-PCR

Total RNA was isolated with Trizol (Invitrogen). RNA (2 μ g) was reversed transcribed using SuperScript II (Invitrogen). Real-time PCR was done as described (15). *GAPDH* served as an internal reference control. Primer sequences for all the genes are in Supplemental Table 3.

Statistics

Kolmogorov-Smirnov test was used for invasion assay, Fisher's exact test and Kaplan-Meier test (non-parametric) for *in vivo* tumorigenicity assay, and Pearson's linear correction test for TMA analysis. One-way ANOVA was used for the rest of the data, where appropriate.

Results and Discussion

Much emphasis has been placed on Stat3 Y705 phosphorylation and its signaling in various cancers. Recent findings suggest that S727 phosphorylation may activate Stat3 in the absence of Y705 phosphorylation (9,10), thus warranting further investigation of the role of S727 phosphorylation in cancer. In the present study, we evaluated the role of S727 phosphorylation in PCa progression using the LNCaP cell line stably expressing Stat3 mutants Y705F, Y705F/S727E and Y705F/S727A. Expression of mutant Stat3 was confirmed by immunoblot with anti-Flag antibody (Fig. 1A, left panel). Endogenous S727 and Y705 phosphorylation was very low and undetectable, respectively, in all the cell lines tested but was remarkably stimulated upon IL-6 treatment (Fig. 1A, right panel). We then used this model system to study the role of S727 phosphorylation in prostate tumorigenesis by examining cell survival and growth in low-serum media, colony formation in soft agar, tumorigenicity in mice, and Matrigel invasion.

Y705F/S727E showed better survival and growth than Y705F/S727A mutant in low-serum

Numerous findings suggest that activated Stat3 promotes cell survival and proliferation in cancer (4-7), while inhibition of Stat3 suppresses cancer cell growth (6). Since no difference was observed in cell proliferation among LNCaP cell lines carrying the mutant plasmids under normal growth conditions (normal growth media supplemented with 10% FBS; data not shown), we determined the survival and growth difference under stressful conditions (0.5% FBS). Although all mutants grew at a much slower rate in 0.5% FBS compared to 10% FBS (data not shown), Y705F/S727E mutant cells demonstrated a significant survival and growth advantage over the rest of the cells. At day seven, Y705F/S727E cells grew about 2-fold faster than Y705F/S727A cells (Fig. 1B). No apparent growth difference was observed among cells carrying either the empty vector, or Y705F or Y705F/S727A expression vectors. Liu *et al.* demonstrated that S727 phosphorylation activated Stat3 signaling and subsequently promoted macrophage survival in the absence of detectable Y705 phosphorylation (10). This was further corroborated by findings demonstrating that phosphorylation at S727, but not Y705, was critical in enhancing survival effects in neuronal stem cells (9).

Y705F/S727E led to increased anchorage-independent growth *in vitro* and tumorigenicity *in vivo*

To determine the effect of Stat3-S727 phosphorylation on tumorigenesis, we analyzed anchorage-independent growth of our Stat3-modulated LNCaP cells. Y705F/S727E formed 3-fold more colonies in soft agar than Y705F/S727A mutant cells ($P < 0.05$; Fig. 1C). No significant difference was observed among the rest of the cell lines tested. We further determined the transforming capacity of Stat3-S727 phosphorylation in noncancerous prostate epithelial cells, RWPE-1. Expression of mutant Stat3 was confirmed by immunoblot with anti-Flag (Fig 1D, upper panel). Similar to LNCaP mutant cells, RWPE-1 Y705F/S727E mutant formed ~ 3.6-fold more colonies in soft agar than Y705F/S727A mutant (Fig 1D, bottom panel). Representative plates are shown in Supplemental Fig. 1. Furthermore, *in vivo* tumorigenicity showed a dramatic increase in tumor formation in mice subcutaneously injected with LNCaP cells expressing Y705F/S727E relative to Y705F/S727A mutant Stat3. By 5 weeks, nine out of ten mice subcutaneously injected with 2×10^4 Y705F/S727E mutant cells formed tumors as compared to three out of ten injected with Y705F/S727A mutant cells ($P < 0.05$; Fig. 1E, top panel) and 10/10 mice injected with Y705F indicating that S727 in these cells may be phosphorylated at the steady state as seen in Figure 1A, right panel. Mutant Stat3 in tumors was confirmed by immunoblot using anti-Flag antibody (Fig. 1E, bottom panel). It is well established that activated Stat3 can function as an oncogene through activating transcription, inducing transformation, and enhancing

tumorigenicity (5,16). Bromberg *et al.* generated a constitutively active form of Stat3, Stat3-C, that when transfected into 293T cells, was serine phosphorylated but not tyrosine phosphorylated (5). They further showed that overexpressed Stat3-C was capable of driving transcription, inducing transformation and enhancing tumorigenicity in cultured cells, suggesting that it was the serine but not tyrosine phosphorylation that might be indispensable for Stat3-C-mediated oncogenic events, at least in that cell line. In addition, Stat3 may form homodimers in the absence of Y705 phosphorylation (17), and nuclear translocation of Stat3 is independent of Y705 phosphorylation in some tumors and primary cells (18,19). Similarly, we show here that the Y705F/S727E mutant, which is unphosphorylatable at Y705, and is phosphomimetic at S727, results in an activated Stat3 that enhances anchorage-independent growth *in vitro* and tumorigenicity *in vivo*, whereas blocking S727 phosphorylation by the Y705F/S727A mutation impairs the oncogenic capacity of Stat3.

Y705F/S727E mutant is more invasive compared to Y705F/S727A mutant

The effect of the Stat3 mutants on LNCaP cell invasion was determined using a Matrigel assay. Y705F/S727E mutant cells were ~3-fold more invasive relative to Y705F and Y705F/S727A mutant cells ($P < 0.01$; Fig. 2A & B). Since epithelial-to-mesenchymal transition (EMT) is often considered a prerequisite for cancer cell invasion, we determined the expression levels of the epithelial differentiation marker, E-Cadherin, and the mesenchymal marker, vimentin. Immunocytochemistry showed similar levels of vimentin expression in both cell lines (data not shown), but a notable decrease in E-Cadherin expression was observed in Y705F/S727E mutant, relative to Y705F and Y705F/S727A mutants, as determined via both immunocytochemistry and immunoblot (Fig. 2C & D). Consistent with our findings, it was shown that oncostatin M-induced Stat3 S727 phosphorylation promoted EMT in the breast cancer cell line MCF-7 (8), whereas inhibition of S727 phosphorylation decreased invasion of the PCa cell line DU145 (4). Taken together, phosphorylation at S727 is sufficient to mediate EMT and tumor cell invasion regardless of the phosphorylation state of Y705.

Increased Stat3 nuclear localization is observed in Y705F/S727E mutant at the steady-state

Stat3 is a cytoplasmic transcription factor that requires nuclear entry to activate transcription. Because it is known that Stat3 constantly shuttles between nuclear and cytoplasmic compartments (20), we examined the nuclear localization of Stat3 at the steady-state in the two double mutants, and found that Stat3 largely localized in the nucleus in Y705F/S727E mutant, but was primarily cytoplasmic in Y705F/S727A mutant (Fig. 3A). This finding was further confirmed by immunoblot (Fig. 3B). Our data suggests that nuclear translocation of Stat3 is independent of Y705 phosphorylation, but is associated with the phosphorylation status of S727. In agreement with our data, nuclear translocation of Stat3 was shown to be independent of tyrosine phosphorylation and its subsequent dimerization (16,17), but is mediated by nuclear import domain of Stat3, importin- $\alpha 3$ (19). It is possible that in the absence of Y705 phosphorylation, Stat3 enters the nucleus through importin- $\alpha 3$, while phosphorylation of S727 is needed to activate Stat3, which can occur either before or after nuclear translocation of Stat3. Once entering the nucleus, Stat3 activation through S727 phosphorylation can further mediate transcription of downstream genes important in tumorigenesis.

Phosphorylation of Stat3 at S727 is more abundant in malignant relative to normal prostate tissues

A tissue microarray containing twenty malignant and four normal prostate tissue cores in duplicate were analyzed with immunohistochemistry to detect the expression of phosphor-S727-Stat3 (pS727) and the staining was evaluated and scored by an experienced pathologist, who was blinded to clinical information. We observed 65% positive staining (>

10% of cells staining positive) in malignant specimens relative to 25% in normal specimens. Furthermore, there is a statistically significant positive correlation between the staining score (staining intensity multiplied by staining extent) and the Gleason score ($P = 0.05$; Supplemental Table 4; Fig. 3C). Representative images are shown in Fig. 3D. Therefore, pS727 Stat3 may play a role in the progression of PCa.

Stat3 binds the promoters of downstream genes *mcl-1*, *survivin* and *c-myc*, subsequently upregulates transcription and translation in Y705F/S727E mutant

Given the observations that Y705F/S727E mutant promotes survival and growth, tumorigenicity, and invasion, we hypothesized that Stat3-Y705F/S727E might exert these effects through direct binding and further upregulating transcription of genes involved in these processes. We performed both database (TFSEARCH, <http://www.cbrc.jp/research/db/TFSEARCH>) and literature searches to identify cancer-related genes that contain a GAS site (TTN_{4.5}AA), the known binding site for Stat3, and compiled a list of target genes, including: genes that regulate cell proliferation and survival (*c-myc*, *cyclin D1*, *mcl-1*, *survivin*, *bcl-2*, *bcl-xl*), genes that regulate tumor cell invasion (*matrix metalloproteinase-2*, *integrin β 6*), and genes that are pleiotropic regulators in tumorigenesis (*c-fos*, *inos*). We performed ChIP assays in the two double mutants to access the recruitment of Stat3 to the GAS site located in the promoters of the above-listed genes. Normal IgG antibody-precipitated samples served as negative controls. Markedly higher occupancy of Stat3 at the promoter regions of *c-myc*, *mcl-1* and *survivin* was observed in Y705F/S727E than in Y705F/S727A mutant cells (Fig. 4A). We did not observe an apparent difference in Stat3 recruitment to the promoters of the rest of the listed genes (data not shown). To determine whether this binding induced transcription, we performed quantitative PCR to measure mRNA levels. We found that mRNA levels of *mcl-1*, *survivin* and *c-myc* were approximately 2.3-, 2.5- and 2.8-fold higher in Y705F/S727E mutant cells than in Y705F/S727A mutant cells, respectively (Fig. 4B). Correspondingly, the protein levels were also notably higher in Y705F/S727E mutant relative to Y705F/S727A mutant (Fig. 4C). To confirm that S727 phosphorylation upregulates these three proteins, the Stat3 Y705F single mutant was employed and treated with IL-6 plus its soluble receptor. We found that S727 was highly phosphorylated after IL-6 treatment and this further led to increased expression of *c-myc*, *mcl-1* and *survivin* (Fig. 4D).

C-Myc is an essential activator of cell growth and proliferation. Mcl-1 is a potent member of the Bcl-2 prosurvival family, and survivin is a member of the inhibitor of apoptosis protein (IAP) family. Overexpression of c-Myc, Mcl-1 and Survivin is frequently observed in many human cancers. It is possible that the direct transcriptional activation of the three proto-oncogenes by a S727-phosphorylation-activated Stat3 triggers cell growth and survival signals that lead to a striking growth advantage in LNCaP cells in low-serum condition, enhanced anchorage independent growth and increased tumorigenicity, while blocking S727 phosphorylation using Y705F/S727A significantly impaired the oncogenic capacity of Stat3. The mechanisms through which S727 phosphorylation promotes LNCaP cell invasion is unresolved and requires further investigation, though it is possible that S727 phosphorylation may mediate invasion through post-transcriptional mechanisms (4,8).

Taken together, to our knowledge, this is the first report demonstrating that Stat3 phosphorylation at S727 is capable of activating Stat3 signaling through direct activation of downstream target gene transcription, further driving prostate tumorigenesis in the absence of Y705 phosphorylation. More importantly, our findings are clinically relevant given that pS727 is more abundant in PCa patients compared to normal tissues and correlates with more aggressive disease, lending support to the notion that Stat3-S727 phosphorylation alone plays an important role in PCa development and progression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Matthew J. Fivash Jr (Senior Statistician, Data Management Services, Inc., National Cancer Institute) for help with the statistical analyses, Donna Butcher (B.S., Pathology/Histology Laboratory, National Cancer Institute) for help with TMA staining, and Miriam R. Anver (D.V.M., PhD, Senior Staff Pathologist, Pathology/Histology Laboratory, National Cancer Institute) for help with histological grading. This project has been funded in whole or in part with federal funds from the National Cancer Institute, National Institutes of Health, under contract N01-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US Government. This research was supported [in part] by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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Figure 1.

Stat3 Y705F/S727E (YF/SE) mutant induced LNCaP cell survival and growth under low-serum conditions, enhanced colony formation in soft agar and tumorigenicity in NOD/SCID mice. **A**, Left, expression of overexpressed mutant Stat3 and endogenous Stat3 was determined by immunoblot using anti-Flag and anti-Stat3 antibodies, respectively. β -actin served as a loading control. Right, cells were starved overnight before IL-6 treatment for 1h. Expression of Stat3 phospho-S727 and phospho-Y705 was determined by immunoblot. **B**, Growth of LNCaP carrying empty vector, Stat3 Y705F (YF), Y705F/S727E (YF/SE) and Y705F/S727A (YF/SA) mutants in RPMI-1640 supplemented with 0.5% FBS. * $P < 0.05$, as determined by one-way ANOVA. **C**, *Top*, representative plates for anchorage-independent growth assay in soft agar in LNCaP carrying either the empty vector or Stat3 mutants. *Bottom*, quantification of colonies per plate. Columns, mean of triplicate samples; bars, SD. *, $P < 0.05$, as determined by one-way ANOVA. **D**, *Top*, expression of ectopic and endogenous Stat3 in RWPE-1 cells was determined by immunoblot using anti-Flag and anti-Stat3 antibodies, respectively. *Bottom*, YF/SE mutant RWPE-1 cells formed ~3.6-fold more colonies than YF/SA mutants ($P < 0.05$). **E**, Evaluation of tumor growth in male NOD/SCID mice subcutaneously injected with LNCaP carrying either the empty vector, or YF, YF/SE and YF/SA mutant cells. *, $P < 0.05$, as determined by Fisher's exact test for YF/SE as compared to YF/SA cells. *Bottom*, protein was extracted from tumors and Stat3 expression was determined by immunoblot with anti-Flag antibody.

Figure 2.

Stat3 YF/SE mutant enhanced LNCaP cell invasion and EMT. **A.** LNCaP mutants (5×10^4 cells/well) in serum-free media were plated in triplicate onto the Falcon cell culture inserts containing 8-micron pore size PET membrane with a thin layer of Matrigel basement membrane matrix as well as the control inserts (without Matrigels). Ten percent FBS was used as the chemoattractant. After 22 h of incubation, the invaded cells were stained, photographed, and counted. **B.** Quantification of invasion assay. The data are expressed as the percent invasion through the matrigel matrix relative to the migration through the control membrane, and mean $\pm$ standard error was calculated from three independent experiments. *, $P < 0.05$, as determined by Kolmogorov-Smirnov test. **C.** Immunofluorescence staining for E-Cadherin in LNCaP mutant cells. More than 95% of the cells show reduced/lost expression of E-cadherin in Y705F/S727E mutant. Images were visualized with immunofluorescence microscopy. **D.** Immunoblot detection for E-Cadherin protein expression.

Figure 3.

Phosphomimetic-S727-Stat3 is localized in the nucleus and PCa patient tissues show higher levels of phosphoS727 Stat3 than normal prostate tissues. A. Immunofluorescence staining with anti-flag antibody for mutant Stat3 subcellular localization. Images were visualized with immunofluorescence microscopy. Images represent > 95% of the cell population. B. Immunoblot detection of mutant Stat3 nuclear expression in the two mutants using anti-Flag antibody. Histone-H2B and β -actin served as loading controls for nuclear and cytoplasmic protein expression, respectively. C. A scatter plot showing staining scores of pS727 Stat3 correlated with Gleason scores in PCa patients. D. Representative examples of tissue microarray staining showing phospho-S727-Stat3 staining in malignant (Gleason score

indicated) and normal prostate tissue. An isotype control for both malignant and normal tissues is also shown. Total Stat3 levels were comparable between normal and malignant tissues (data not shown).

Figure 4. Stat3-YF/SE bound promoters of downstream genes and further activated transcription. A. ChIP assays were done to detect the recruitment of mutant Stat3 to *mcl-1*, *survivin* and *c-myc* loci. Antisera used in the chromatin immunoprecipitation are indicated. DNA samples from ChIP preparation were analyzed by semi-quantitative PCR analyses. Input values were obtained from samples treated identically as the experimental ones except that no immunoprecipitation steps were done. Data shown are representative of three independent experiments that yielded similar results. B. Relative mRNA expression of *mcl-1*, *survivin*, and *c-myc* was determined by real-time RT-PCR analysis. GAPDH served as an internal control, and mRNA expression in Y705F/S727A mutant was set as 1. The assay was performed in triplicate. *, $P < 0.05$, as determined by one-way ANOVA. C. Mcl-1, survivin, and c-Myc protein expression in the two mutants. Relative density was quantified using ImageQuant TL image analysis software (v2003, Amersham Biosciences, Piscataway, NJ) and indicated above the blots. D. A Stat3 single mutant Y705F (YF) was treated with IL-6 plus its soluble receptor (sIL-6R) at 10 ng/ml for 6 h followed by protein extraction and immunoblot. Relative density was indicated above the blots. NE, nuclear extracts.