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Cooperation between STAT3 and c-Jun Suppresses *Fas* Transcription

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Summary

Decreased Fas expression during tumor progression often results in a loss of Fas-ligand (FasL)-mediated apoptosis. Human and mouse melanoma exhibit an inverse correlation between the degree of Fas cell surface expression, tumorigenicity, and metastatic capacity. The expression of dominant negative Stat3 or c-Jun in melanoma cells efficiently increased Fas expression and sensitized cells to FasL-induced apoptosis. *Stat3*$^{+/-}$ as well as *c-Jun*$^{-/-}$ cells exhibited increased Fas cell surface expression and higher sensitivity to FasL-mediated apoptosis. Suppression of Fas expression by Stat3 and c-Jun is uncoupled from Stat3-mediated transcriptional activation. Our findings indicate that Stat3 oncogenic activities could also be mediated through its cooperation with c-Jun, resulting in downregulation of Fas surface expression, which is implicated in the tumor's ability to resist therapy and metastasize.

Introduction

Fas (CD95/Apo-1) and Fas-ligand (FasL) are complementary receptor-ligand proteins that play central roles in regulating programmed cell death. Upon interaction with FasL, Fas forms a complex with the Fas-associated death domain protein (FADD), which directly binds and activates caspase-8, resulting in the induction of apoptosis (reviewed in Nagata, 1999). Impaired Fas signaling is frequently observed during tumor progression and has been attributed in most cases to downregulation of Fas expression. Loss of Fas function has been implicated in increased resistance of tumors to apoptosis induced by chemical and physical stimuli, as well as in the acquisition of the metastatic phenotype (Hug, 1997; Owen-Schaub et al., 1998; Shin et al., 1999; Mottolese et al., 2000). Although Fas is constitutively expressed in a variety of cell types (Leithauser et al., 1993), ultraviolet (UV) irradiation, viral infection, p53 expression, and chemotherapeutic agents efficiently increase *Fas* transcription (Tanaka et al., 1994; Owen-Schaub et al., 1995; Leverkus et al., 1997; Muller et al., 1997). Whereas nuclear factor-κB (NF-κB) activates *Fas* transcription (Chan et al., 1999), p38 attenuates NF-κB effects, which result in the suppression of Fas expression (Ivanov and Ronai, 2000). While constitutive and inducible regulatory pathways govern the level of *Fas* transcription, the mechanisms underlying the loss of Fas cell surface expression in human tumors are poorly understood. In this study, we demonstrate that downregulation of Fas expression in human melanoma-derived cell lines is mediated by Stat3 cooperation with c-Jun.

Signal transducers and activators of transcription (STAT) proteins are dual-function molecules that can be activated by diverse extracellular stimuli to transmit signals from the cell surface to the nucleus and directly participate in gene regulation (Ihle, 1995; Darnell, 1997). Activation of Stats results in the expression of genes that control critical cellular functions (reviewed in Bowman et al., 2000; Bromberg and Darnell, 2000). An increasing number of tumor-derived cell lines as well as samples from human cancers contain constitutively activated Stat proteins, which in most cases are Stat3 and Stat5 (Gouilleux-Gruart et al., 1996; Chai et al., 1997; Garcia et al., 1997; Garcia and Jove, 1998).

Stat3 elicits transcriptional signals as a dimer and also via its interaction with c-Jun (Zhang et al., 1999). Similarly, the association of Stat1 with SP1 (Look et al., 1995) and of Stat5 with glucocorticoid receptor (Stocklin et al., 1996) or CCAAT-enhancer binding protein (cEBP) (Delphin and Stavnezer, 1995) contribute to Stat-mediated transcription. Stats have also been implicated in suppression of target genes; interferon-γ suppression of c-myc in wild-type mouse embryo fibroblasts is Stat1 dependent (Ramana et al., 2000).

A constitutively active form of Stat3 is capable of immortalizing fibroblasts and causes cellular transformation (Bromberg et al., 1999). Among Stat target genes that are associated with oncogenesis through regulation of cell cycle progression and/or apoptosis are Bcl-x_L (Catlett-Falcone et al., 1999a), cyclin D1 (Bromberg et al., 1999), $p21^{WAF1/CIP1}$ (Chin et al., 1996; Ouchi et al., 2000), and c-myc (Bromberg et al., 1999).

The notorious resistance of melanoma cells to radiation and chemical-induced apoptosis (reviewed in Chin et al., 1998; Sauter and Herlyn 1998), which inversely correlates with the level of Fas cell surface expression, prompted us to explore further the mechanisms underlying suppression of Fas cell surface expression. Here, we demonstrate that the combined activity of Stat3 and c-Jun mediates suppression of *Fas* transcription.

Results

Fas Cell Surface Expression Inversely Correlates with Melanoma Resistance to UV irradiation

Analysis of cell surface Fas expression in six melanoma-derived cell lines (OM431, FEMX, and HHMSX are highly

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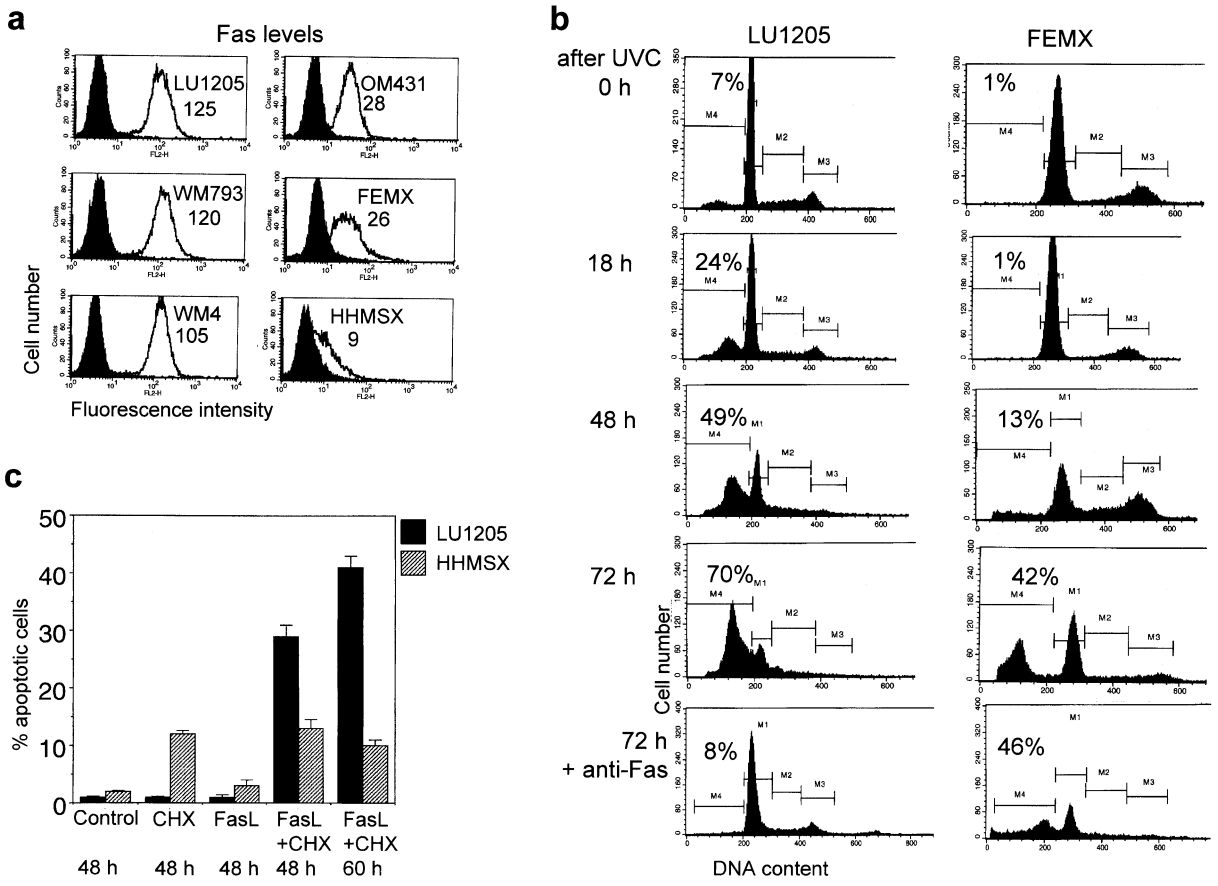

Figure 1. UVC-Induced Apoptosis of Human Melanoma LU1205 Cells Is Fas Dependent

(a) Cell surface Fas expression was determined by staining with PE-conjugated anti-human Fas antibodies and flow cytometry. Filled histogram represents nonspecific staining with mouse Ig-PE. Mean fluorescence intensity (MFI) is indicated.

(b) Apoptosis analysis of human melanoma cells irradiated with UVC (60J/m^2); the percentage of apoptotic cells at 18, 48, and 72 hr after treatment is indicated. The effect of antagonistic anti-*Fas* mAb (which was added to cultures prior to treatment) on the level of apoptosis is shown in the lower panel.

(c) Sensitivity of melanoma cells that exhibit high (LU1205) or very low (HHMSX) levels of Fas cell surface expression to FasL-mediated apoptosis (in absence or presence of 10 μg/ml cycloheximide, as indicated). The degree of apoptosis was monitored at the indicated time points.

metastatic cell lines) revealed an inverse correlation between stage (chemical/radiation resistance and metastatic potential) of the tumor and level of Fas expression (Figure 1a). For example, whereas the LU1205 late-stage melanoma cells exhibited 24% apoptosis 18 hr after UV irradiation, the more aggressive FEMX melanoma-derived cell line, which expressed lower levels of Fas on the cell surface, did not undergo apoptosis at all (Figure 1b). These differences can be attributed to the level of Fas expression, as the addition of antagonistic anti-Fas antibodies prior to UV treatment decreased the degree of UV-induced apoptosis of LU1205 cells (from 70% to 8%), whereas it had no effect on the FEMX cells (Figure 1b). To assess further the role of Fas cell surface expression in the sensitivity of the melanoma cells to undergo apoptosis, we have selected to analyze response of LU1205 and HHMSX cells, which express high (125 MFI) or almost no (9 MFI) Fas, respectively (Figure 1a), to FasL. In the presence of cycloheximide, FasL mediated apoptosis in LU1205 but not in the HHMSX cells (Figure 1c), pointing to the role of Fas cell surface expression in the acquisition of resistance to radiation-induced apoptosis.

Role of STAT3 and c-Jun in the Regulation of Fas Transcription

The presence of three γ interferon activation site (GAS) elements within the *Fas* promoter prompted us to elucidate the possible contribution of Stat to *Fas* transcription. Because each of the GAS elements on *Fas* promoter is adjacent to an AP1 site and Stat3–c-Jun cooperation contributes to Stat3-mediated transactivation (Zhang et al., 1999), we focused our studies on Stat3. To determine the possible role of Stat3 in *Fas* transcription, we used Stat3β, a spliced form of Stat3α that attenuates Stat3α transcriptional activities and thus serves as a dominant negative of Stat3α (Bromberg et al., 1996; Caldenhoven et al., 1996). Forced expression of Stat3β led to a 4-fold increase in *Fas*-promoter-driven luciferase activity, whereas expression of the Stat3α construct resulted in a 2-fold decrease (Figure 2a). The degree of increase in *Fas* transcription elicited by Stat3β

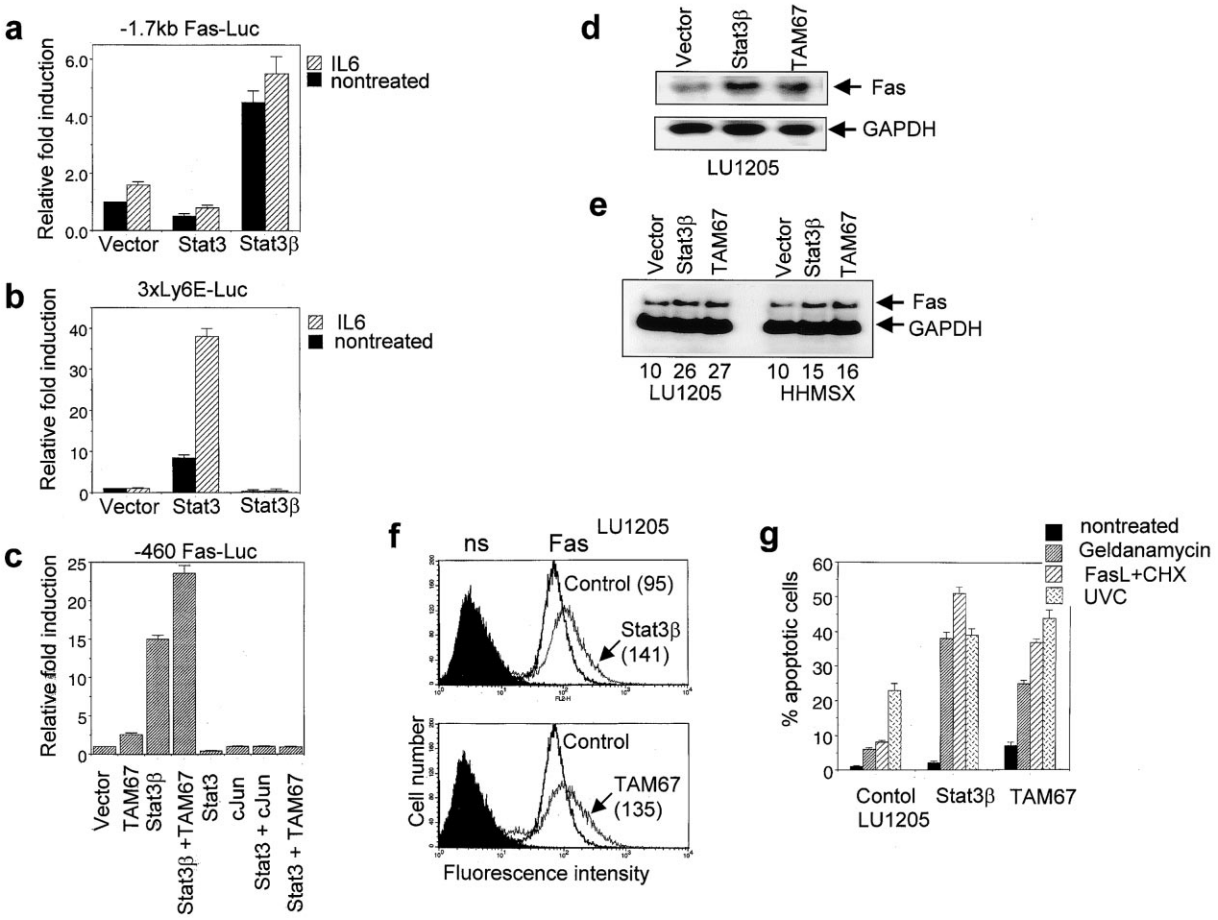

Figure 2. Stat3β and TAM67 Upregulate Fas Expression

(a and b) Effect of Stat3 and Stat3β on activity of −1.7 kb *Fas*-Luc (a) or 3xLy6E-Luc (b) reporter constructs in the presence or absence of IL-6. The normalized ratio of luciferase activity to β-galactosidase is shown.

(c) Effect of c-Jun, TAM67, Stat3, Stat3β, and their combination on activity of −460 bp *Fas*-Luc reporter was carried out as indicated in Experimental Procedures.

(d) Northern blot analysis of *Fas* mRNA levels after transient transfection of Stat3β or TAM67 into LU1205 cells. Control cells were transfected by empty pcDNA3 vector.

(e) RT-PCR analysis of Fas transcripts in two melanoma cell lines that stably express Stat3β or TAM67. Control cells were stably infected with empty vector. Levels of GAPDH transcripts, which were coamplified, are shown. The numbers reflect the relative intensity of the corresponding Fas transcript bands as measured via phosphorimager.

(f) LU1205 cells were infected with empty (control), Stat3β, or TAM67 cDNA cloned into retroviral vector (pBabe). A mixed population of puromycin-resistant cells was assayed for changes in Fas surface expression by staining with anti-Fas-PE mAb followed by flow cytometric analysis. Filled histogram represents nonspecific (ns) staining with mouse Ig-PE. Empty histograms represent control (empty vector) and Stat3β- or TAM67-expressing cells, as indicated. The numbers reflect MFI values.

(g) Apoptosis analysis of LU1205 cells that constitutively express Stat3β or TAM67 or empty retroviral vector (control) after treatment with UVC (60 J/m^2) or geldanamycin (0.5 μM) or FasL (10 ng/ml) and cycloheximide (10 μg/ml) as indicated. Cells were stained with Annexin-V-FITC 18 hr after treatment and analyzed by flow cytometry.

was similar to that seen after interleukin-6 (IL-6) treatment, whereas expression of Stat3α attenuated the modest effect of interleukin-6 (IL-6) on *Fas*-Luc activity (Figure 2a). These observations imply that Stat3α negatively regulates *Fas* transcription. Under the same conditions, the expression of Stat3α increased GAS-dependent (3xLy6E)-Luc activities and more so after IL-6 treatment, and Stat3β efficiently blocked IL-6-induced GAS-Luc activity (Figure 2b). These findings suggest that Stat3's ability to reduce *Fas*-Luc activity is uncoupled from the conventional Stat3-dependent expression of a multimerized GAS element reporter gene (Figure 2b). The latter observation led us to hypothesize that Stat3 may require cooperation with another transcription factor(s) to elicit suppression of *Fas* promoter.

The proximity of an AP1 site to each of the three GAS elements on the *Fas* promoter led us to determine the possible contribution of c-Jun alone and in combination with Stat3 to the regulation of *Fas* transcription. Forced expression of c-Jun did not affect *Fas* transcription, probably because of the abundant amount of transcriptionally active c-Jun in late-stage melanoma cells (Ivanov and Ronai, 1999). Melanoma cells that express TAM67, a dominant negative form of c-Jun (Brown et

al., 1994), revealed a 3-fold increase in *Fas*-Luc activity, which was attenuated upon coexpression of Stat3 (Figure 2c). These observations suggest that c-Jun may also contribute to the suppression of *Fas* transcription. Forced expression of Stat3β increased Fas-Luc activity, which was further elevated upon coexpression of Stat3β and TAM67 (Figure 2c). These data point to the possible cooperation of c-Jun and Stat3 in the downregulation of *Fas* transcription. These observations were made using a Fas promoter fragment (−460), which contains one GAS, AP1, and NF-κB sites and which represents the activities seen by the full-length 1.7 kb promoter region (data not shown; Chan et al., 1999).

Northern blot analysis of RNA extracted from the cells that were transiently transfected with either TAM67 or Stat3β revealed an increase in *Fas* mRNA levels (Figure 2d). The extent of this effect is probably underestimated because of the limited efficiency of transient transfection. Reverse transcription-polymerase chain reaction (RT-PCR) confirmed an increase of Fas mRNA levels in two melanoma-derived cell lines that express either TAM67 or Stat3β (Figure 2e). These data demonstrate that the changes seen in the *Fas*-Luc assays are reflected at the level of *Fas* transcripts.

To elucidate further Stat3 and c-Jun effects on Fas expression, melanoma cells were subjected to retrovirus-mediated infection with either Stat3β or TAM67, and a mixed population of clones that constitutively express these constructs was further characterized. A clear increase in the level of Fas cell surface expression was observed in both the TAM67- and Stat3β-expressing cells (Figure 2f). Similarly, analysis of LU1205 cells that were transiently transfected with these expression vectors and green fluorescent protein (GFP) demonstrated a noticeable increase of Fas surface levels in Stat3β-expressing cells, which was comparable with the effect of TAM67 (60 to 110 units of MFI; data not shown). These findings confirm that Stat3- and c-Jun-mediated changes at the level of *Fas* transcription result in a coordinated decrease in the cell surface protein expression level.

We next monitored changes in the fraction of Stat3β- or TAM67-expressing melanoma cells that underwent apoptosis upon treatment with either FasL, UV, or geldanamycin. Melanoma cells that express either Stat3β or TAM67 exhibited a 5- to 8-fold increase in the degree of early apoptosis in response to FasL. UV irradiation or geldanamycin treatment also caused greater degree of apoptosis (2- to 6-fold) in cells that expresses higher cell surface Fas, because of the suppression of Stat3 or c-Jun (Figure 2g). These observations suggest that attenuating Stat3 or c-Jun activity efficiently sensitizes melanoma cells to FasL and DNA-damaging, agent-induced apoptosis.

Inhibition of STAT3 or c-Jun Rescues Fas Expression in Fas-Negative Melanoma Cells

The human melanoma cell line HHMSX exhibits almost undetectable levels of Fas cell surface expression (Figure 1a) and is resistant to FasL treatment (Figure 1c). Forced expression of TAM67 in the HHMSX cells led to a 5-fold increase in the level of *Fas* promoter-mediated transcription (Figure 3a). Stat3β caused an over 3-fold increase when transfected alone and slightly augmented the increase mediated by TAM67. The ability to rescue *Fas* transcription by inhibition of Stat3 or c-Jun was also reflected at the level of Fas cell surface expression where transient expression of TAM67 or Stat3β alone caused a noticeable change in the percentage of cells that express low levels of Fas on their surface (data not shown). Similarly, HHMSX cells that were infected with retrovirus carrying either Stat3β or TAM67 exhibit a 5- to 7-fold increase in Fas cell surface expression (Figure 3b). Constitutive expression of TAM67 or Stat3β in WM4 melanoma cells also increased Fas transcription (data not shown) and cell surface expression (Figure 3c). Exposure of WM4 cells that constitutively express either TAM67 or Stat3β to FasL caused an approximately 2-fold increase in the level of apoptosis (Figure 3d). These data demonstrate that increased Fas cell surface expression upon inhibition of either c-Jun or Stat3 efficiently sensitize melanoma cells to FasL-mediated apoptosis.

Stat3/c-Jun-Dependent Fas Expression in Mouse Melanoma-Derived Cells

To assess further the role of c-Jun and Stat3 in Fas expression, we used a mouse melanoma model in which the degree of tumorigenicity and metastatic potential inversely correlates with level of Fas cell surface expression (Owen-Schaub et al., 1998). SW1 cells are among the more metastatic and exhibit a marked loss of Fas cell surface expression when compared with the K1735p or C-19 tumors (Figure 4b) (Owen-Schaub et al., 1998). A decrease in Fas cell surface expression coincides with the increased Stat3 expression (Figure 4a). Infection of SW1 cells with retroviral constructs for either Stat3β or TAM67 led to a marked increase in Fas cell surface expression (Figure 4c) and sensitized them to FasL-mediated apoptosis (Figure 4d). These observations establish the role of Stat3 and c-Jun in downregulation of Fas cell surface expression in mouse melanomas.

Increased Fas Expression in *Stat3*$^{+/-}$ Cells

We have next examined the expression of Fas in embryonic stem (ES) as well as in mouse fibroblast cells lacking one of the *Stat3* alleles (Raz et al., 1999, and unpublished observations). ES-*Stat3*$^{+/-}$ cells express approximately 50% of the Stat3 protein found in ES-*Stat3*$^{+/+}$ cells (Figure 5a). ES-*Stat3*$^{+/-}$ cells exhibited a 70% increase in basal *Fas* promoter activities when compared with the activity seen in ES-*Stat3*$^{+/+}$ cells (Figure 5b). This observation points to an inverse correlation between Stat3 expression and *Fas* transcription. Stat3β elicited a 3-fold increase in *Fas*-Luc activity in ES-*Stat3*$^{+/+}$ cells, as compared with the less than 2-fold increase seen in the ES-*Stat3*$^{+/-}$ cells. *Fas* promoter mutated within the GAS element exhibited a 5-fold increase in transcriptional activity in the *Stat3*$^{+/+}$ cells, as compared with a 2-fold increase in *Stat3*$^{+/-}$ cells (Figure 5b). This finding establishes the relationship between Stat3 expression and suppression of *Fas* promoter through the GAS element. Coexpression of GAS mutant *Fas*-Luc and Stat3β did not cause a further increase in the degree of transcriptional

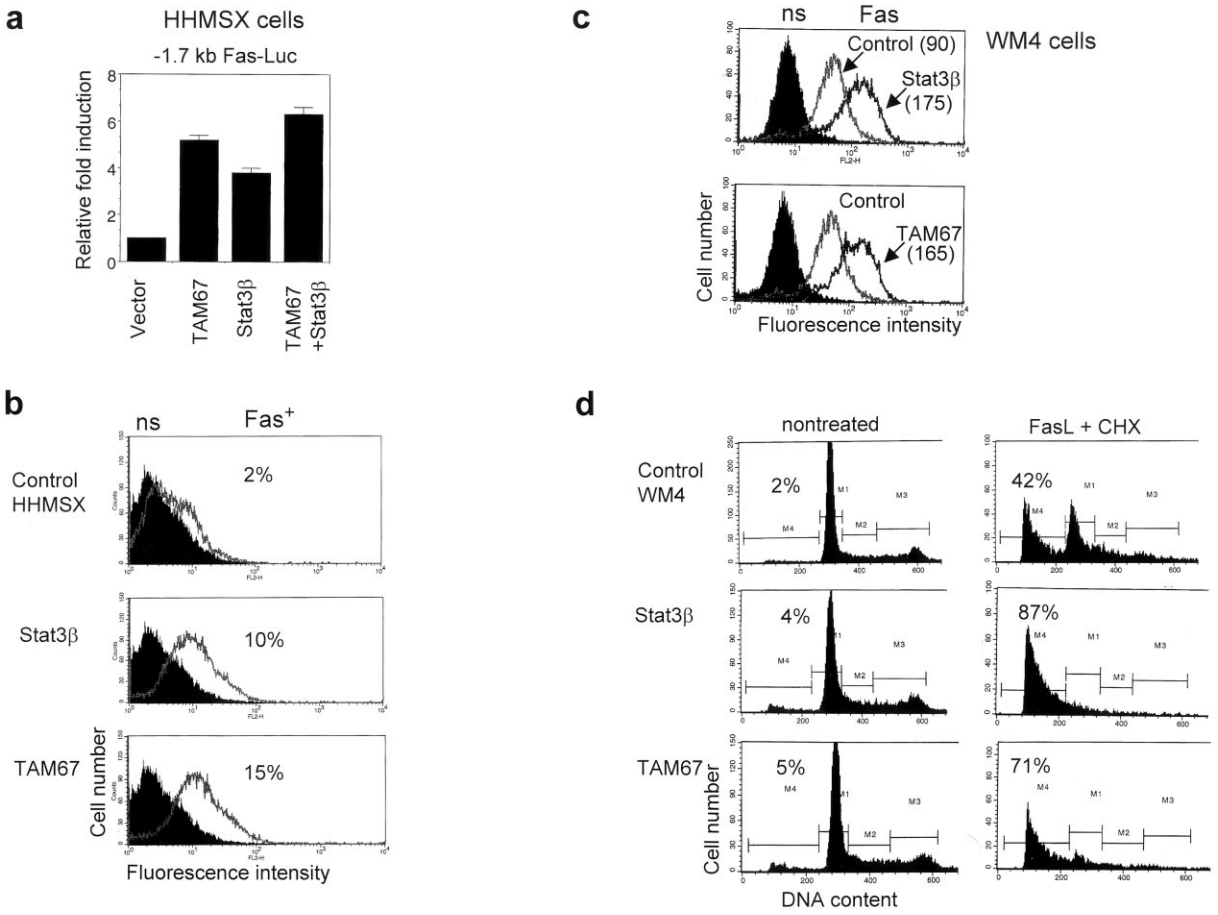

Figure 3. Stat3β and TAM67 Rescue Fas Expression in HHMSX and Upregulate Fas Levels in WM4 Melanoma Cells

(a) Effect of TAM67 and Stat3β on activity of −1.7 kb *Fas*-Luc reporter in HHMSX cells.

(b) Flow cytometric analysis of HHMSX cells infected with retrovirus carrying Stat3β or TAM67 or empty (control) expression vectors. The filled histogram represents nonspecific (Ig-PE) staining, whereas the open histogram represents the level of Fas. The percentage values represent the percentage of Fas-positive cells.

(c) MFI of Fas cell surface expression in WM4 cells, which stably expresses Stat3β or TAM67, or empty pcDNA3 vector (control) is shown.

(d) Apoptosis of WM4 cells that constitutively express TAM67 or Stat3β or empty vector (control) 36 hr after treatment with FasL (50 ng/ml) and cycloheximide (10 μg/ml).

activities seen by GAS mutant *Fas*-Luc alone, further supporting the finding that Stat3 suppression of Fas is mediated through the GAS element.

Differences in the degree of endogenous Fas expression were also reflected at the level of *Fas* cell surface expression. Seventy percent of ES- *Stat3*$^{+/-}$ cells were positive for cell surface Fas expression compared with 28% of the *Stat3*$^{+/+}$ cells (Figure 5c).

Similar to the finding in the ES *Stat3*$^{+/-}$ cells, mouse embryo fibroblasts (MEFs) of *Stat3*$^{+/-}$ mice exhibit a marked increase in Fas cell surface expression when compared with the parent MEF (Figure 5d). Forced expression of Stat3 efficiently reduced the level of Fas, almost to the level seen in the wild-type MEF cells. Sensitivity of *Stat3*$^{+/-}$ fibroblasts to FasL and cycloheximide-mediated apoptosis coincided with the degree of Fas cell surface expression (Figure 5e). The inverse correlation between Stat3 expression and *Fas* transcription as found in *Stat3*$^{+/-}$ cells provides genetic evidence for the role of Stat3 in downregulation of *Fas* transcription.

Increased Fas Cell Surface Expression in *c-Jun*$^{-/-}$ Cells

Given the role of c-Jun in the suppression of Fas cell surface expression, we have next elucidated the level of Fas cell surface expression in *c-Jun*$^{-/-}$ fibroblasts (Wisdom et al., 1999). *c-Jun*$^{-/-}$ fibroblasts exhibit a marked increase (180 vs. 100 MFI) in Fas cell surface expression (Figure 6a). This finding points to an inverse correlation between c-Jun and Fas expression. Differences in Fas expression coincided with sensitivity to FasL-mediated apoptosis; 41% of *c-Jun*$^{+/+}$ cells underwent apoptosis as compared with 83% of the *c-Jun*$^{-/-}$ cells (Figure 6b). Forced expression of c-Jun in the *c-Jun*$^{-/-}$ fibroblasts efficiently reduced the level of Fas cell surface expression (from 92 to 59 MFI where 54 is the basal level seen in *c-Jun*$^{+/+}$ cells; Figure 6c). These data provide additional support for the role of c-Jun in suppression of Fas transcription. Interestingly, forced expression of Stat3β led to a further increase in Fas surface expression, suggesting that in the absence of

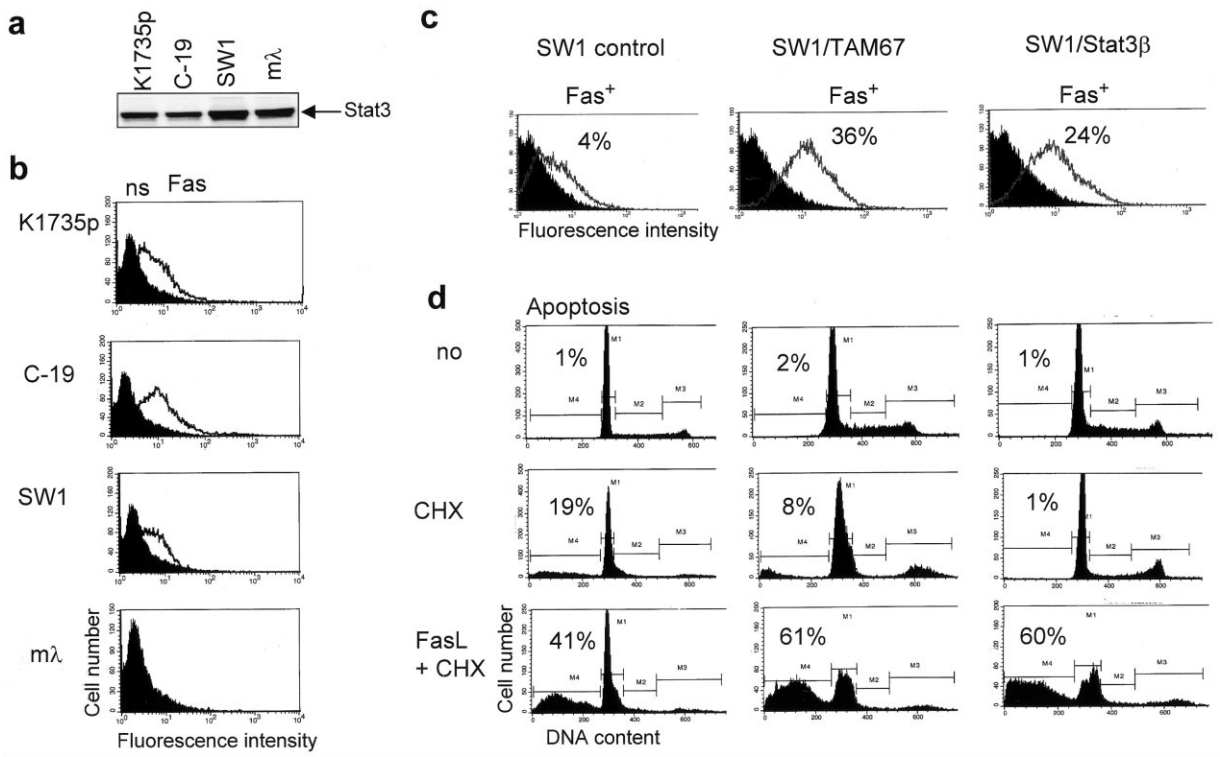

Figure 4. Stat3- and c-Jun-Dependent Fas Surface Expression in Mouse Melanoma Tumors Inversely Correlates with Sensitivity to FasL-Mediated Apoptosis

(a) Western blot analysis of Stat3 expression in mouse melanoma-derived cells of which K1735p is nonmetastatic and exhibit the lower degree of tumorigenicity. C-19 has intermediate tumorigenicity but is nonmetastatic, and both SW1 and mλ1 are highly tumorigenic and metastatic (Owen-Schaub et al., 1998).

(b) Level of Fas cell surface expression on each of the mouse melanoma tumor-derived cells.

(c) Retroviral-mediated infection of TAM67 or Stat3β or empty retroviral vector led to marked increase in Fas cell surface expression (open histograms). The percentage of Fas-positive cells is indicated.

(d) Apoptosis of SW1 cells 36 hr after treatment with FasL (50 ng/ml) and cycloheximide (1 μg/ml).

c-Jun, Stat3 may cooperate with other transcription factors that bind to the AP1 site (that is, ATF2) to elicit a certain degree of Fas suppression, which could be alleviated upon Stat3β expression. Transient reconstitution of c-Jun expression in the *c-Jun*$^{-/-}$ cells was capable of reducing the degree of FasL-mediated apoptosis (from 50 to 31%; Figure 6d). Expression of c-Jun in Jun$^{+/+}$ cells did not alter degree of FasL-mediated apoptosis (27%) (data not shown). These results provide genetic support for the role of c-Jun in the suppression of Fas expression.

Stat3 and c-Jun Affect Fas Promoter Activities via GAS and AP1 Elements

To characterize inhibition of *Fas* transcription by Stat3, we mutated the GAS elements within the *Fas* promoter sequences linked to the luciferase reporter gene. Both Stat3β and TAM67 increased luciferase activity mediated from the *Fas* promoter region, although Stat3β was three times as effective (Figure 7a). A mutation within the single GAS element caused a modest increase in *Fas*-Luc activity. The same mutant exhibited a 5-fold increase in *Fas*-Luc activity in the ES-Stat3$^{+/+}$ cells (Figure 5b), implying that in these melanoma cells, Stat3 requires cooperation with another transcription factor(s). Upon Stat3β expression, *Fas* reporter mutated on the GAS element no longer exhibited an increase in transcriptional activities, suggesting that the GAS element is required to relieve *Fas* suppression by endogenous Stat3. Expression of TAM67 led to an over 9-fold increase in luciferase activity mediated by *Fas* promoter that had been mutated within the GAS element (Figure 7a). The marked increase of *Fas*-Luc activities under conditions in which neither Stat3 (GAS mutated site) nor Jun (TAM67 effect) is available suggests that c-Jun cooperation with Stat3 is required to mediate suppression of *Fas* transcription.

Mutation within the AP1 site on the *Fas* promoter caused a modest increase in basal *Fas* transcription and abrogated the effect of TAM67. These findings confirm that c-Jun effects on *Fas* promoter are mediated through the AP1 site and suggest that c-Jun alone may not be sufficient to mediate suppression of *Fas* transcription. Stat3β was capable of eliciting an over 10-fold increase in AP1-mutated *Fas* promoter activities (Figure 7a). Deletion of both AP1 and GAS elements from the *Fas* promoter region revealed a higher increase in *Fas* transcription than observed upon mutating either the

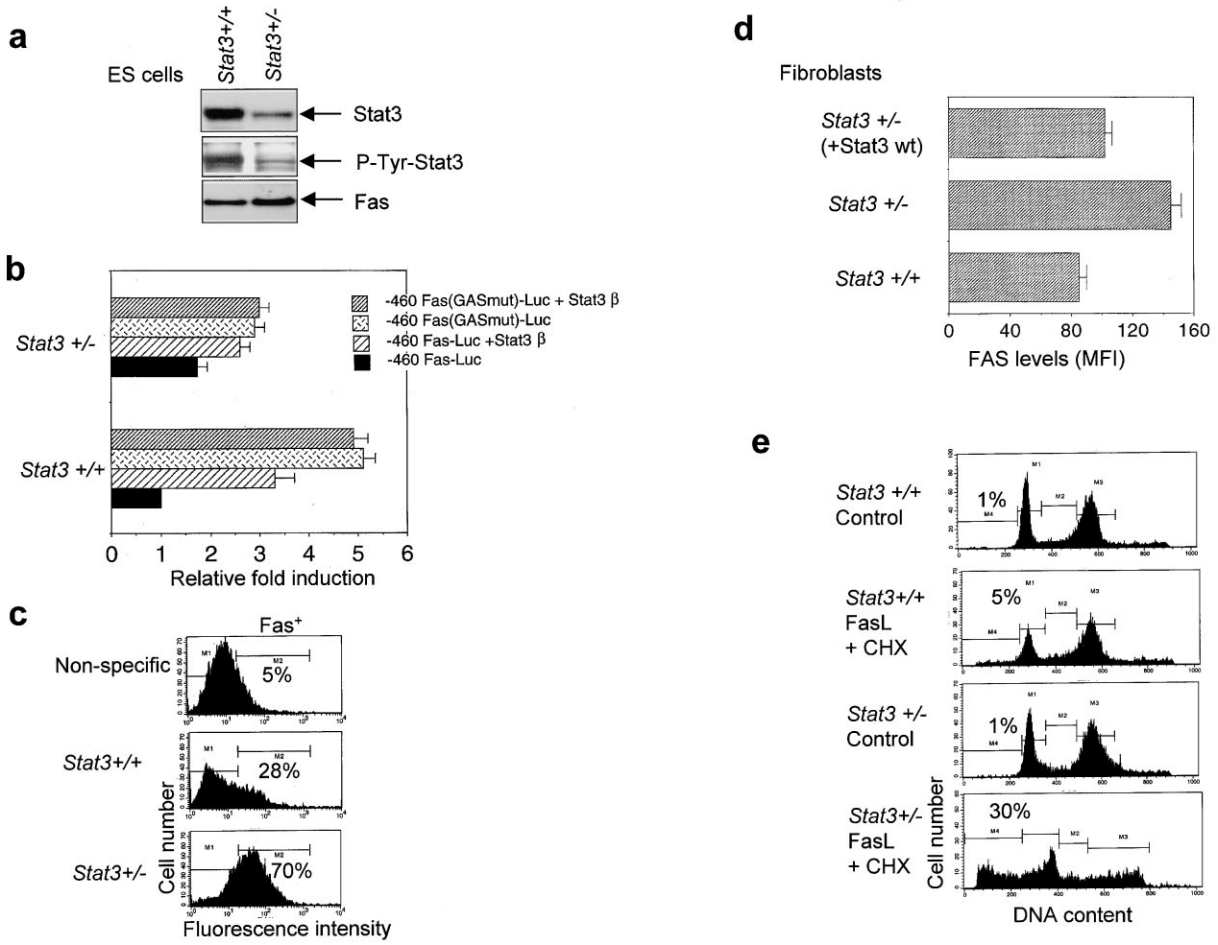

Figure 5. Stat3 Expression Inversely Correlates with Levels of Fas in *Stat3*$^{+/-}$ ES and Mouse Embryo Fibroblast Cells

(a) Western blotting of total cell extracts (70 μg) from ES *Stat3*$^{+/+}$ and *Stat3*$^{+/-}$ cells using anti-Stat3, phospho-Tyr705, or anti-Fas Abs.

(b) *Stat3*$^{+/+}$ or *Stat3*$^{+/-}$ ES cells were transiently transfected with the −460 *Fas* promoter-Luc (0.5 μg) or with a mutated variant of this construct (GASmut) in the presence of 0.25 μg of pCMV-β-gal. The normalized ratio of luciferase activity to β-galactosidase is shown.

(c) Cell surface expression of Fas in ES was determined by anti-Fas-PE mAb and analyzed using flow cytometry. The percentage of Fas-positive cells is indicated.

(d) Level of Fas cell surface expression in *Stat3*$^{+/-}$ mouse embryo fibroblasts 36 hr after cotransfection of pGFP and mock or pGFP + Stat3.

(e) Apoptosis of *Stat3*$^{+/-}$ or *Stat3*$^{+/+}$ MEF 36 hr after treatment with FasL (50ng/ml) and cycloheximide (1 μg/ml).

GAS or the AP1 sites (Figure 7a). Neither TAM67 nor Stat3β was able to augment the level of *Fas* transcription in the absence of AP1 and GAS elements, further supporting the role of c-Jun and Stat3 in suppression of *Fas* transcription via the corresponding binding sites.

Stat3 Suppression of Fas Transcription Is Uncoupled from Its Transactivation of Multimerized GAS Elements

To analyze Stat3-mediated suppression of *Fas* transcription, a mutant form of Stat3 that is no longer capable of associating with c-Jun was used. Forced expression of Stat3 mutated within its c-Jun binding site (Stat3 QC-1) (Zhang et al., 1999) abolished its ability to mediate *Fas* suppression and was able to increase *Fas* transcriptional activities (Figure 7b). This observation further supports the need for Stat3 cooperation with c-Jun to mediate suppression of *Fas* transcription. Interestingly, expression of Stat3 that has been mutated to enable its constitutive dimerization (Stat3-C) (Bromberg et al., 1999) increased GAS-Luc (Figure 7c), as well as *Fas* promoter activities (Figure 7b). The forms of Stat3 that were able to increase *Fas*-Luc transcription—Stat3β, Stat3-C, and QC1—differ from wild-type Stat3 in their abilities to form certain protein interactions. Stat3-C is locked into a homodimeric configuration, and QC1 cannot bind c-Jun. These findings suggest that Stat3 heteromerization is one of the parameters that confer Stat3's ability to mediate suppression of *Fas* promoter activities. These observations also imply that under conditions in which such association is abolished, Stat3 can elicit a positive signal for *Fas* transcription. Although both Stat3β and TAM67 effectively elevate *Fas*-Luc activities, neither is able to elicit a similar increase in GAS-Luc activities (Figure 7c). These findings further demonstrate that the suppressive effects of Stat3 and c-Jun on *Fas*

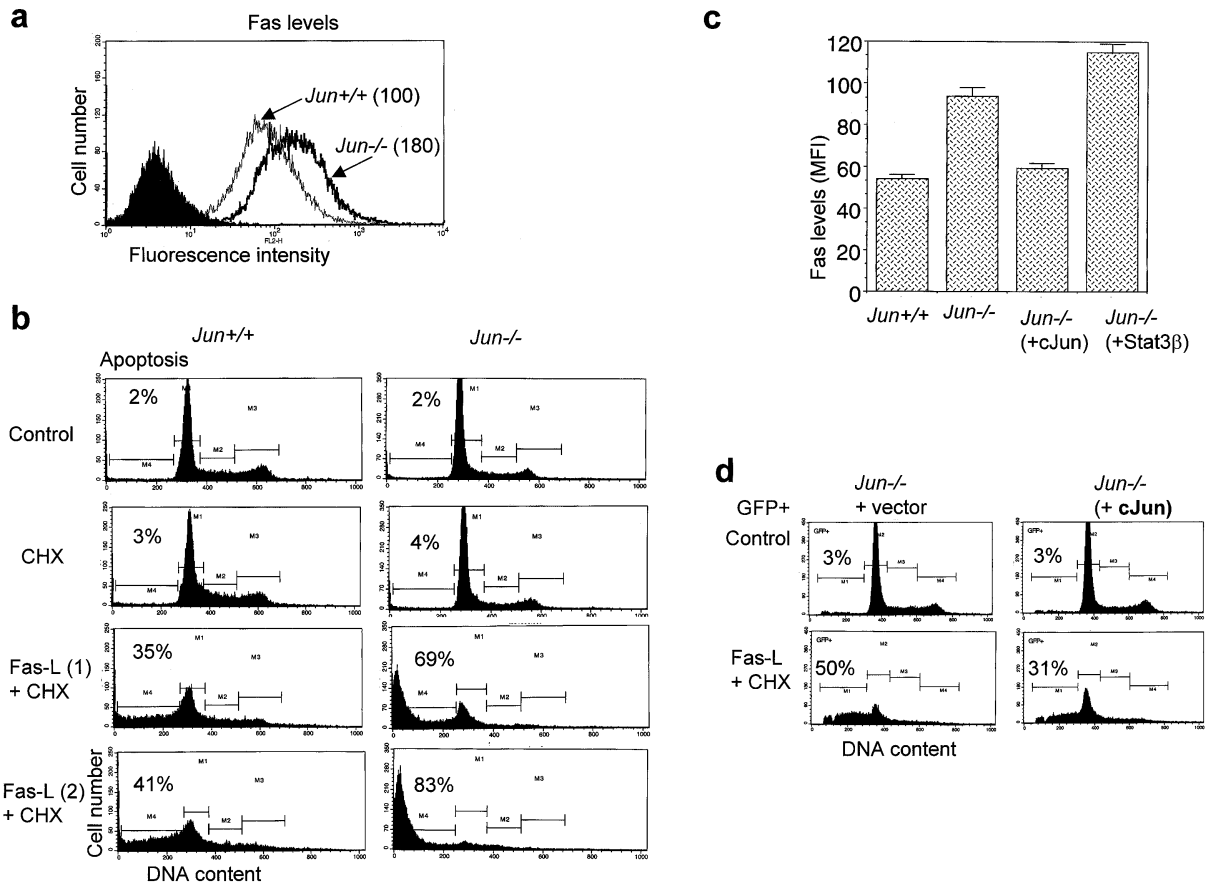

Figure 6. *Jun*$^{-/-}$ Fibroblasts Exhibit Higher Fas Cell Surface Expression and Sensitivity to FasL-Mediated Apoptosis

(a) Analysis of Fas cell surface expression in *Jun*$^{+/+}$ and *Jun*$^{-/-}$ mouse embryo fibroblasts. The numbers reflect MFI.

(b) Apoptosis of *Jun*$^{-/-}$ vs. *Jun*$^{+/+}$ fibroblasts 36 hr after treatment with cycloheximide (1 μg/ml) and FasL at concentrations of 25 ng/ml (1) or 50 ng/ml (2).

(c) Level of Fas cell surface expression in Jun$^{-/-}$ cells that were cotransfected with c-Jun or Stat3β and pGFP.

(d) Level of apoptosis 36 hr after FasL (50 ng/ml) and cycloheximide (1 μg/ml) treatment of *Jun*$^{-/-}$ fibroblasts that were transfected with empty vector and pGFP or c-Jun and pGFP.

promoter are uncoupled from the regulation of the isolated GAS elements.

STAT3-Jun Complex Is Bound to Fas Promoter Sequences

We next monitored the possible association of Stat3 with c-Jun in human melanoma cell lines. Immunoprecipitation of nuclear extracts with antibodies to Stat3 followed by immunoblot analysis using antibodies to c-Jun identified Stat3–c-Jun complexes in both melanoma cell lines (Figure 7d). Incubation of nuclear extracts prepared from nontreated melanoma cells with biotinylated oligonucleotides bearing the GAS-AP1 sequences as present on the *Fas* promoter detected both Stat3 and c-Jun as *Fas* promoter-bound proteins (Figure 7e, panel I). A mutation on both GAS and AP1 elements efficiently abolished the binding of both Stat3 and c-Jun (Figure 7e, panel II; compare wild type with DM lanes). Binding of Stat3 or c-Jun to *Fas* promoter sequences, in which either GAS or AP1 sites were mutated, revealed a substantial decrease in the binding of the respective transcription factor (Figure 7e). These findings provide direct evidence for the presence of the Stat3-Jun complex and for its specific association with the GAS-AP1 target sequences present on the *Fas* promoter.

Recruitment of c-Jun to Fas Promoter Is Modified after UV Irradiation

Further confirmation for c-Jun and Stat3 binding to the Fas promoter was obtained in vivo via ChIP assays on chromatin samples from untreated and UV-irradiated melanoma cells. Both Stat3 and c-Jun were found in association with Fas promoter in nontreated cells, consistent with the notion that both transcription factors are bound to Fas promoter under conditions in which Fas expression is suppressed. Decreased binding of Stat3 to Fas promoter was seen as early as 2 hr and more so 6 hr after UV treatment. Within 6 hr after UV irradiation, binding of c-Jun was no longer apparent (Figure 7f). Decreased c-Jun binding to the *Fas* promoter sequences coincided with transient upregulation of Fas expression (Figure 7f, panel III). The change in c-Jun and Stat3 binding to *Fas* promoter sequences after UV treatment, which mediates apoptosis through elevated

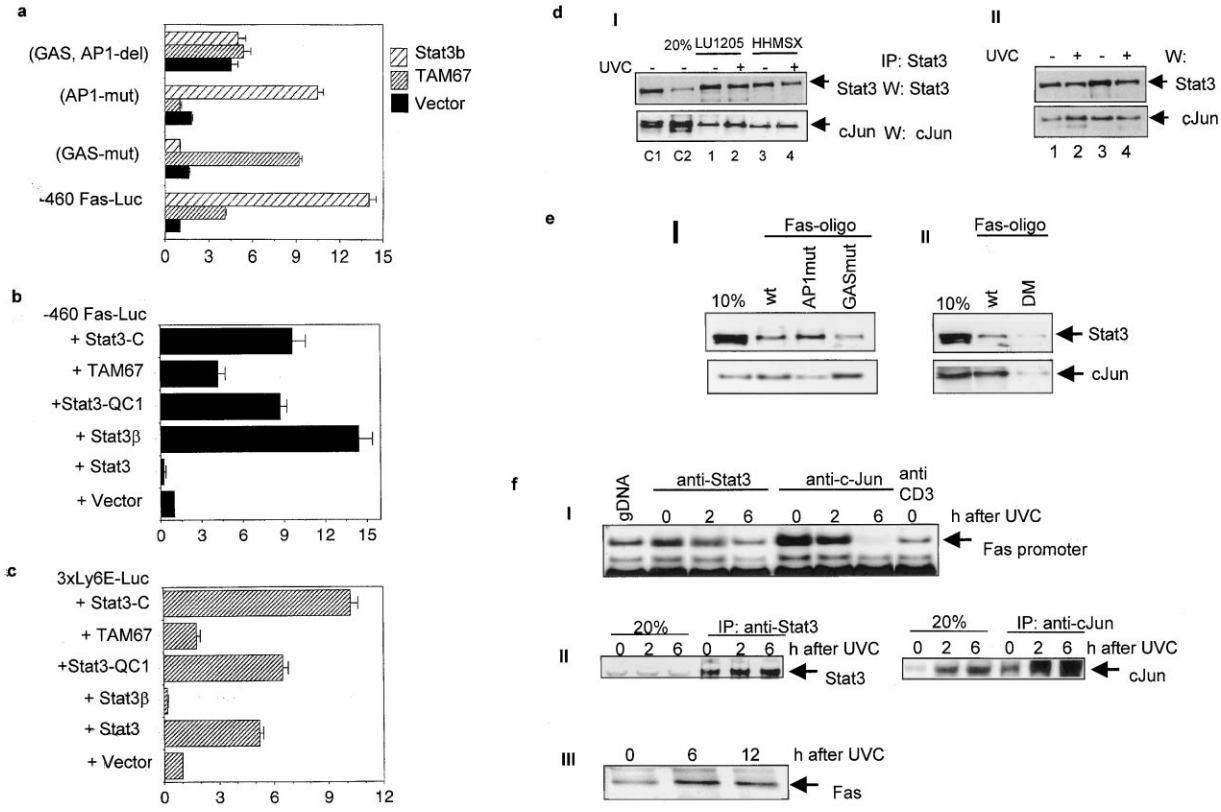

Figure 7. Both AP1 and GAS Elements Are Required for Efficient Suppression of Fas Transcription

(a) LU1205 cells were transiently transfected with indicated constructs. After 14 hr, cells were analyzed for luciferase and β-galactosidase activity. The normalized ratio of luciferase activity to β-galactosidase is shown.

(b) The −460 *Fas*-Luc construct was used with the empty vector TAM67 or with different mutated forms of Stat3, including Stat3β and Stat3QC-1, which lost the ability to interact with c-Jun and Stat3-C, which represented a permanently active form of Stat3.

(c) GAS reporter construct (3xLy6E-Luc) was used for transfection under conditions described in (b).

(d) Stat3 interacts with c-Jun and binds to GAS/AP1 sequences in human melanomas (I) Nuclear extracts (400 μg) from melanoma LU1205 and HHMSX cells prepared prior (−) or at the indicated time points after UVC (+) were immunoprecipitated with anti-Stat3 Ab. Immunoprecipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by Western blotting with indicated Abs. C1 and C2 represent whole cell extract and 20% input of nuclear extracts of LU1205 cells, respectively. (II) Western blotting of nuclear extracts (70 μg) of melanoma LU1205 and HHMSX cells (UVC+ or UVC−) using anti-Stat3 or anti-c-Jun Abs.

(e) Biotinylated oligonucleotides containing GAS-AP1-element sites as present on the *Fas* promoter (Fas-oligo) in wild-type or mutant (on AP1, GAS, or double mutants [DM], as indicated) were incubated with nuclear proteins of LU1205 cells. Biotinylated oligonucleotide-bound proteins were captured on avidin agarose beads and were washed, and GAS-AP1-bound proteins were eluted and analyzed on Western blotting with the indicated Abs.

(f) Panel I, ChIP assays were carried out on chromatin that was immunoprecipitated with antibodies to Stat3 or c-Jun from control or UV-treated cells at the indicated time points. Shown is a *Fas* promoter fragment amplified by PCR from each of the ChIP samples. Control IP was performed with IgG (data not shown) or nonrelevant antibodies to CD3. Amplification of genomic DNA is shown (gDNA). Panel II shows the Western blot of input as well as immunoprecipitated Stat3 and c-Jun from nuclear extracts. Panel III depicts Western blot analysis of Fas expression.

Fas expression (Figure 1b), coincides with increased Fas expression, which is required for the sensitization of these melanoma cells to UV-induced cell death (Ivanov and Ronai, 1999), and points to the reversibility of Stat3-mediated suppression of Fas transcription.

Discussion

The present study demonstrates that suppression of *Fas* transcription and subsequent cell surface expression is mediated via cooperation between two oncogenes, Stat3 and c-Jun. The role of Stat3 and c-Jun in the suppression of Fas transcription, cell surface expression, and sensitivity to FasL-elicited apoptosis is demonstrated in several human and mouse melanoma cell lines. Important confirmation to our findings comes from the use of *Stat3*$^{+/-}$ and *c-Jun*$^{-/-}$ cells that exhibit elevated Fas expression, which could be reduced upon re-expression of Stat3 or c-Jun, respectively.

Because low Fas expression has been associated with resistance to therapy, metastatic capacity, and poor prognosis, the finding that Stat3 contributes to downregulation of *Fas* transcription highlights a novel mechanism for Stat3 oncogenic activities. The suppression of *Fas* transcription by Stat3-Jun is uncoupled from Stat3 ability to elicit transactivation of traditional GAS elements bearing promoters. We provide direct evidence that Stat3 cooperation with c-Jun is required to

elicit downregulation of *Fas* transcription. The requirement for such cooperation is demonstrated via the use of the Stat3-QC mutant, which is mutated on its c-Jun docking site (Zhang et al., 1999) and has lost its ability to inhibit *Fas* transcription. Furthermore, mutation of GAS element on *Fas* promoter together with inhibition of c-Jun (or vice versa) was the most efficient means among those tested in derepressing *Fas*-reporter activities. Downregulation of *Fas* transcription may be augmented by additional cellular components, which can affect Stat3 and/or c-Jun transcriptional activities.

Our ChIP data demonstrate that following UV treatment, there is a decrease in Stat3 and a loss of c-Jun bound to Fas promoter in vivo, which coincides with increased *Fas* transcription and expression and concomitant Fas-dependent apoptosis. This finding provides important support for the cooperation of c-Jun and Stat3 and for the existence of a dynamic regulation of Stat3/c-Jun recruitment to *Fas* promoter, which is required to mediate suppression of *Fas* transcription under relevant physiological conditions. Stat3 cooperation with c-Jun could take place through heterodimerization of the two proteins or through association of c-Jun with a dimerized form of Stat3. A dimer form of Stat3 has been implicated in the association with c-Jun to elicit activation of α_2-macroglobulin promoter bearing adjacent AP1 and GAS elements (Zhang et al., 1999). Stat3β was also reported to cooperate with c-Jun to enhance α_2-macroglobulin promoter activities (Schaefer et al., 1995). The nature of Stat3-Jun-mediated *Fas* suppression, as opposed to transactivation of α_2-macroglobulin, could depend on the proximity of GAS and AP1 elements, as well as the context of adjacent promoter sequences. Alternatively, such differences could stem from the composition of the overall transcriptional complex on the DNA, which was shown to affect strongly DNA bending and transcriptional potency in the case of AP1 (Rajaram and Kerppola, 1997; Chytil et al., 1998; Falvo et al., 2000). Equally relevant is the possibility that the response to c-Jun and Stat3 activities is cell/tissue dependent. Whereas Stat3's ability to elicit transcriptional suppression (pending cooperation with c-Jun) is shown in this study, the ability of c-Jun to elicit transcriptional suppression was documented for Smad3 (Dennler et al., 2000), p53 (Schreiber et al., 1999), and major histocompatibility complex class I (Howcroft et al., 1993).

Inhibition of Stat3 and c-Jun efficiently increased *Fas* transcription and cell surface expression, even in melanoma cells that were near null for Fas expression. In all cases, such increases led to a concomitant sensitization of melanoma cells to FasL-induced apoptosis. Reduced Fas expression has also been implicated in the metastatic capacity of human tumors and was further documented in the mouse melanoma model (Owen-Schaub et al., 1998). Forced expression of Stat3β or TAM67 efficiently restored Fas expression, sensitized the more aggressive SW1 tumor cells to FasL-mediated apoptosis and reduced SW1 tumor outgrowth at the orthotopic site in mice (data not shown). The latter are in line with the observation that elevated Fas expression in PC3 and LNCAP prostate cell lines reduced tumor outgrowth (Takeuchi et al., 1996).

In pointing to the role of Stat3-Jun cooperation in the constitutive repression of Fas expression in human and mouse melanoma tumor-derived cell lines, our study identifies the mechanism underlying the notorious resistance of melanoma and possibly other tumor types to therapy.

Experimental Procedures

Cell Lines

Human melanoma cells were maintained in culture as previously described (Ivanov and Ronai, 2000; Ivanov et al., 2000). ES cells E14 (Stat3$^{+/+}$) (Hooper et al., 1987) and E14 clone 3-2 (Stat3$^{+/-}$) were propagated by standard methods on monolayers of mitomycin C-treated mouse fibroblasts (Raz et al., 1999). *Stat3*$^{+/-}$ immortalized mouse embryo fibroblasts were prepared by standard procedures (Aaronson and Todaro, 1968) from mouse embryos heterozygous for *Stat3*, derived from gene-targeted ES cells (Raz et al., 1999). *Stat3*$^{+/-}$ fibroblasts, *c-Jun*$^{-/-}$ fibroblasts, and mouse melanoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Plasmid Constructs

Reporter construct −1.7 kb *Fas*-Luc and −460 *Fas*-Luc were previously described (Chan et al., 1999). Mutations within the GAS or AP1 site were generated using the Quick Change kit (Stratagene, La Jolla, CA). Primers used to generate mutations within the GAS site were GAC AGG AAT GCC CAT TTG TGC **TT**C GAA CCC TGA CTC and GAG TCA GGG TTG G**AA** GCA CAA ATG GGG, within the AP1 site were CCC ATT TGT GCA ACG AAC CC**A AA**C TCC TTC CTC ACC and GGT GAG GAA GGA GTT TGG G**TT** CGT TGC ACA AAT GGG. Deletion of the 24bp sequence containing both AP1 and GAS elements was carried out using primers CCC CGG GAC AGG AAT GCC CAC CTC ACC CTG ACT TCT CCC CCTC and GAG GGG GAG AAG TCA GGG TGA GGT GGG CAT TCC TGT CCG GGGG. Primers used to mutate the distal −677/−669 GAS elements were GGT TAA CTG TCC ATT CCA GG**T T**CG TCT GTG AGC CTC TC and GAG AGG CTC ACA GAC G**AA** CCT GGA ATG GAC AGT TAA CC. Expression vectors pRcCMV containing Stat3, Stat3 QC1 (L148A), or Stat3-C were described previously (Bromberg et al., 1999; Zhang et al., 1999). The expression plasmid pIRES-Stat3β, which encodes the human Stat3β (Catlett-Falcone et al., 1999b), was kindly provided by Dr. R. Jove (Moffit Cancer Center, University of South Floria, Tampa, FL). Retroviral vectors for Stat3β or TAM67 were generated by PCR amplification of the corresponding cDNAs, which were subsequently subcloned into the XhoI (Stat3β) or BamHI/EcoRI (TAM67) sites of the pBabe vector. In all cases, the sequence integrity of modified/cloned constructs was confirmed via sequencing.

Transfection and Luciferase Assay

The luciferase reporter gene containing three GAS elements from the Ly6E gene was previously described (Wen et al., 1995). Transient transfection of different reporter constructs (0.5 μg) together with expression vectors and pCMV-βgal (0.25 μg) into 5×10^5 melanoma cells was performed using Lipofectamine (Life Technologies-BRL, Grand Island, NY). Proteins were prepared for βGal and luciferase analysis 14 hr after transfection. Luciferase activity was determined using the luciferase assay system (Promega, Madison, WI) and normalized based on β-galactosidase levels. Retroviral packaging and infection carried out as previously described (Morgenstern and Land, 1990). Infected cultures were subjected to selection in puromycin (1.5 μg/ml), and a mixed population of resistant cells was analyzed.

Treatment and Apoptosis Studies

Cells were exposed to UV at a wavelength of 254 nM (C) (UVC) at 60J/m^2 as previously described (Ivanov and Ronai, 1999). Antagonistic monospecific antibodies against Fas (clone G254–274; Pharmingen) were added (1–5 μg/ml) 1 hr before UVC treatment. FasL (25–50 ng/ml) was used in combination with cycloheximide. Apoptosis was assessed by quantifying the percentage of hypodiploid nuclei undergoing DNA fragmentation (Nicoletti et al., 1991). Surface expression of Fas was determined using anti-Fas-PE Ab (Pharmingen). Flow cytometric analysis was performed on a FACS Calibur flow cyto-

meter (Becton Dickinson, Mountain View, CA) using the CellQuest program.

Transient Transfection and GFP Assay

Melanoma cells (5×10^5) were transiently cotransfected with expression vectors together with marker plasmid encoding GFP (pGFP; 1 μg and 0.25 μg, respectively) using Lipofectamine (Life Technologies-BRL). Twenty-four hours after transfection, surface Fas expression in GFP-positive cells was determined by staining with PE-anti-Fas Ab and flow cytometry. For apoptosis studies, cells were irradiated with UVC (60J/m^2) 24 hr after transfection and 18 hr later were stained with propidium iodide (PI) and analyzed by flow cytometry.

ChIP assays

The ChIP assays were based on protocol described by Falvo et al. (2000). Human melanoma cells (2.5×10^6 cells per 10 cm diameter plate) were exposed to UV irradiation (60J/m^2), and cells were fixed by formaldehyde at indicated time points. Fifty micrograms of purified chromatin samples were immunoprecipitated with 1 μg of anti-Stat3, anti-c-Jun, or anti-CD3 antibodies. DNA isolated from immunoprecipitated material following reversal of formaldehyde cross-linking was amplified by PCR (17 cycles of 1 min at 94°C, 53°C, and 72°C in the presence of α^{32}P dCTP) using primers (CTCGAGGTCCTCA CCTGAAGTGAGCATGCC and GAAGCCTCGCTGGGGAACGCCCG GGTT) that amplify 188bp fragment of Fas promoter flanking the GAS and AP1 elements.

Western Blot Analysis and Immunoprecipitation

Cell lysates (50–100 μg protein) were resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and processed according to the standard protocols. The Abs used were polyclonal anti-Stat3, anti-c-Jun (NEB), or monoclonal anti-Fas (1:1000 to 1:3000; Pharmingen). The secondary Abs (anti-rabbit or anti-mouse) conjugated to horseradish peroxidase (dilution 1:5000). Signals were detected using the ECL system (Amersham, Arlington Heights, IL). Immunoprecipitation was carried out by standard methods (Ivanov and Ronai, 1999).

Northern Blot and RT-PCR Analysis

Total RNA (10 μg) was fractionated in a 1% agarose formaldehyde gel before transferred to a nylon membrane (Hybond-N; Amersham) followed by prehybridization and subsequently hybridization with ^{32}P-labeled Fas cDNA probe. RT-PCR was carried out on cDNA prepared from the RNA using primers designed to amplify 499bp of Fas promoter and 250bp of the GAPDH cDNAs. Amplifications were carried out for 17 cycles in the presence of α^{32}P dCTP (1 min at 94°C, 55°C, 72°C) followed by separation on polyacrylamide gels and autoradiography.

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