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STAT5 Contributes to Interferon Resistance of Melanoma Cells

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Summary

Background: Malignant melanoma is a highly aggressive neoplastic disease whose incidence is increasing rapidly. In recent years, the use of interferon α (IFNα) has become the most established adjuvant immunotherapy for melanoma of advanced stage. IFNα is a potent inhibitor of melanoma cell proliferation, and the signal transducer and activator of transcription STAT1 is crucial for its antiproliferative action. Although advanced melanomas clinically resistant to IFNα are frequently characterized by inefficient STAT1 signaling, the mechanisms underlying advanced-stage interferon resistance are poorly understood.

Results: Here, we demonstrate that IFNα activates STAT5 in melanoma cells and that in IFNα-resistant cells STAT5 is overexpressed. Significantly, the knockdown of STAT5 in interferon-resistant melanoma cells restored the growth-inhibitory response to IFNα. When STAT5 was overexpressed in IFNα-sensitive cells, it counteracted interferon-induced growth inhibition. The overexpressed STAT5 diminished IFNα-triggered STAT1 activation, most evidently through upregulation of the inhibitor of cytokine-signaling CIS.

Conclusions: Our data demonstrate that overexpression and activation of STAT5 enable melanoma cells to overcome cytokine-mediated antiproliferative signaling. Thus, overexpression of STAT5 can counteract IFNα signaling in melanoma cells, and this finally can result in cytokine-resistant and progressively growing tumor cells. These findings have significant implications for the clinical failure of IFNα therapy of advanced melanoma because they demonstrate that IFNα induces the activation of STAT5 in melanoma cells, and in STAT5-overexpressing cells, this contributes to IFNα resistance.

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Introduction

Over the past few decades, the incidence of malignant melanoma has increased at a rate that exceeds that of any other solid tumor. With a limited efficiency of chemotherapy, the use of interferon α (IFNα) has become the most established adjuvant immunotherapy for melanoma of advanced stage. However, the results of multiple clinical trials are controversial and difficult to interpret partly because of a great heterogeneity between the parameters of the trials [1, 2].

IFNα mediates its effects through activation of the janus kinases JAK1 and TYK2, which in turn phosphorylate and activate the signal transducers and activators of transcription: STAT1, STAT2, and STAT3. Activation of these latent transcription factors results in the formation of STAT homo- and heterodimers, their nuclear translocation, and *trans*-activation of the interferon-stimulated genes (ISGs) [3]. ISGs are characterized by the presence of γ activating sequences (GAS) and/or interferon response elements (ISRE) in their promoters. GAS elements are the targets for STAT homo- or heterodimers, whereas ISRE sites are bound by the interferon-stimulated gene factor 3 (ISGF3) in response to IFNα [4]. ISGF3 is a trimeric complex and consists of STAT1, STAT2, and the interferon-stimulated gene factor 3γ (p48/ISGF3γ/IRF-9). Although IFNα activates gene expression through GAS elements, the major part of the IFNα-induced gene expression is brought about by ISGF3's binding to ISRE sites [5]. The essential role of STAT1 in the mediation of an IFNα response (and, in general, interferon signaling) is seen in STAT1 (–/–) mice, which are unresponsive to interferons but remain responsive to other cytokines [6].

In addition to immunomodulatory activity, IFNα has a direct growth-inhibiting effect on melanoma cells [7, 8]. However, cell lines originating from melanoma of advanced stage are frequently unresponsive to the growth-inhibiting effect of IFNα. Such interferon-resistant cells often show defective JAK-STAT signaling, which correlates either with a significant reduction in the level of STAT1 protein or inefficient phosphorylation of STAT1 [7, 9, 10]. However, whereas inefficient STAT1 phosphorylation in response to IFNα and reduced STAT1 levels have also been observed in melanoma patients [9, 11, 12], other studies describe only slight variations in STAT1 expression levels in melanoma biopsies and no loss of function in interferon-resistant cell lines [13, 14]. Thus, the mechanism underlying the cellular IFNα resistance seems to be complex. For the successful application of IFNα in melanoma therapy, it is, however, important to understand the specific function of this interferon in melanoma cells.

It is noteworthy that stimulation of interferon receptors can also lead to the activation of STAT5 [15–17]. In contrast to STAT1, STAT5 is involved in mitogenic as well as in antiapoptotic signaling [6]. STAT5 is constitutively active in a variety of transformed cells, which include *Xiphophorus* melanoma cells [18–20]. Although this suggests an involvement of STAT5 in promoting oncogenesis in pigment cells, nothing is known about its functional role in human melanoma development.

To investigate whether STAT5 plays a role in the development of melanoma, we analyzed its expression in melanoma biopsies and several human melanoma cell lines. STAT5 was found to be overexpressed in IFNα-resistant cell lines and in situ in advanced melanoma lesions. Overexpression of STAT5 in IFNα-sensitive cells revealed that STAT5 can counteract the STAT1-mediated IFNα response by upregulating the cytokine inhibitor CIS. Furthermore, depletion of STAT5 from IFNα-resistant cells by RNA interference (RNAi) resulted in enhanced sensitivity toward IFNα-induced growth inhibition. In summary, our findings strongly suggest a role for STAT5 in the frequently found clinical resistance of advanced melanoma to interferon.

Results

STAT5 Expression in Melanocytic Lesions Correlates with Tumor Progression

In human skin, STAT5 expression was detected specifically in melanocytes located in the basal membrane of the epidermis (STAT5 FITC, Figure 1A), where it colocalized with the melanocyte differentiation antigen MART-1. No significant expression of the STAT protein was seen in surrounding keratinocytes, and this was confirmed in the keratinocyte cell line HaCat (Figure 1B). Both STAT5a and STAT5b were expressed in primary human melanocytes (mel 1043 and mel 775) to a level similar to that found in the human primary melanoma cell line IFB, whereas STAT5 expression was increased in Mel19 metastatic melanoma cells (Figure 1B). STAT1 was expressed in melanoma cells, in melanocytes, and in keratinocytes (Figure 1B), underlining the specificity of STAT5 expression in pigment cells of the epidermis.

Staining of melanocytic skin lesions with anti-STAT5 detected the expression of STAT5 in both benign and neoplastic melanocytic lesions (Figures 1C and 1D). Because STAT5 expression is not restricted to melanocytic cells but can also be seen in leukocytes, double staining for STAT5 and the melanocyte differentiation antigen MART-1 was performed. This confirmed that the majority of STAT5-expressing cells within the tested lesions were of melanocytic origin (Figure 1D). Strikingly, the number of STAT5-expressing cells was significantly elevated in neoplastic lesions compared to benign nevi (Figures 1C and 1D, $p \leq 0.001$). Analysis of the cellular distribution in MART-1-positive cells revealed that although the relative amount of cytoplasmic STAT5 to nuclear-localized STAT5 did not significantly change during tumor progression, the absolute number of cells showing intranuclear localization increased from benign to neoplastic and primary to metastatic lesions (Figure 1D).

STAT5 Expression Is Enhanced in Interferon-Resistant Melanoma Cells

Analysis of STAT5 protein levels in various melanoma cell lines showed a significantly enhanced expression in D10, MM96, Mel19, and WM164 cells (Figure 2A). Two of the STAT5-overexpressing cell lines, D10 and MM96, are known to be resistant toward interferon-induced growth inhibition [7, 9], and our analysis revealed a noticeable correlation of STAT5 overexpression with IFNα resistance (Figures 2A and 2B). Notably, STAT1 was expressed in all cell lines regardless of their reactivity toward IFNα, and although slight differences in the protein level of STAT1, as well as those of STAT2 and STAT3 were observed, they did not strictly correlate with interferon resistance (Figures 2A and 2B).

In all interferon-resistant cell lines, IFNα stimulation resulted in phosphorylation of JAK1 and TYK2 (Figure 2C), indicating that the receptors are active. However, whereas STAT1 became strongly phosphorylated in response to IFNα in all sensitive cell lines and in human primary melanocytes, the STAT1 phosphorylation level was reduced or completely abolished in three (MM96, Mel19, and WM164) of four resistant cell lines (Figure 2D).

Because interferon-resistant cell lines appear to overexpress STAT5, we asked both whether STAT5 might interfere with IFNα induced STAT1 activation and whether STAT5 plays a significant role in IFNα signaling in melanoma cells.

Activation of STAT1 Is Required for an Efficient IFNα Response in Melanoma Cells

For our further experiments, we chose A375 cells because they showed the highest IFNα sensitivity in our hands. Stimulation of A375 cells with IFNα resulted in phosphorylation of STAT1 (see Figure 2D) and nuclear translocation (Figure 3A). IFNα also induced binding of the STAT1-containing IGSF3 complex to ISRE sequences (Figure 3B). Already 30 min after addition of IFNα, all three components of IGSF3 were found to bind to the ISRE sequence (Figure 3B). However, after 22 hr IFNα stimulation, the amount of ISRE bound STAT1, STAT2, and p48 was considerably enhanced. This delayed increase correlated with the *trans*-activation of an ISRE-containing promoter, which was weak (1.3-fold) after 3 hr but much stronger (7.3-fold) after 22 hr (Figure 3C) and was also reflected in the induction of $hPNPase^{old\text{-}35}$ (Figure 3D), an interferon-inducible gene whose expression is regulated through an ISRE site in melanoma cells [21].

The importance of STAT1 for IFNα signaling was seen in A375 cells expressing a dominant-negative STAT1, in which tyrosine 701 is mutated to phenylalanine [22] and which therefore shows no significant tyrosine phosphorylation in response to IFNα (Figure 3E, lower panel). The lack of STAT1 activation in STAT1F-expressing cells resulted in a substantial reduction in IFNα-induced growth inhibition when compared to mock transfected or STAT1/WT-expressing cells (Figure 3F), clearly demonstrating the importance of STAT1 for the IFNα response in melanoma cells. Our data also suggest that prolonged STAT1 activation is required for significant induction of ISRE-driven gene expression in melanoma cells.

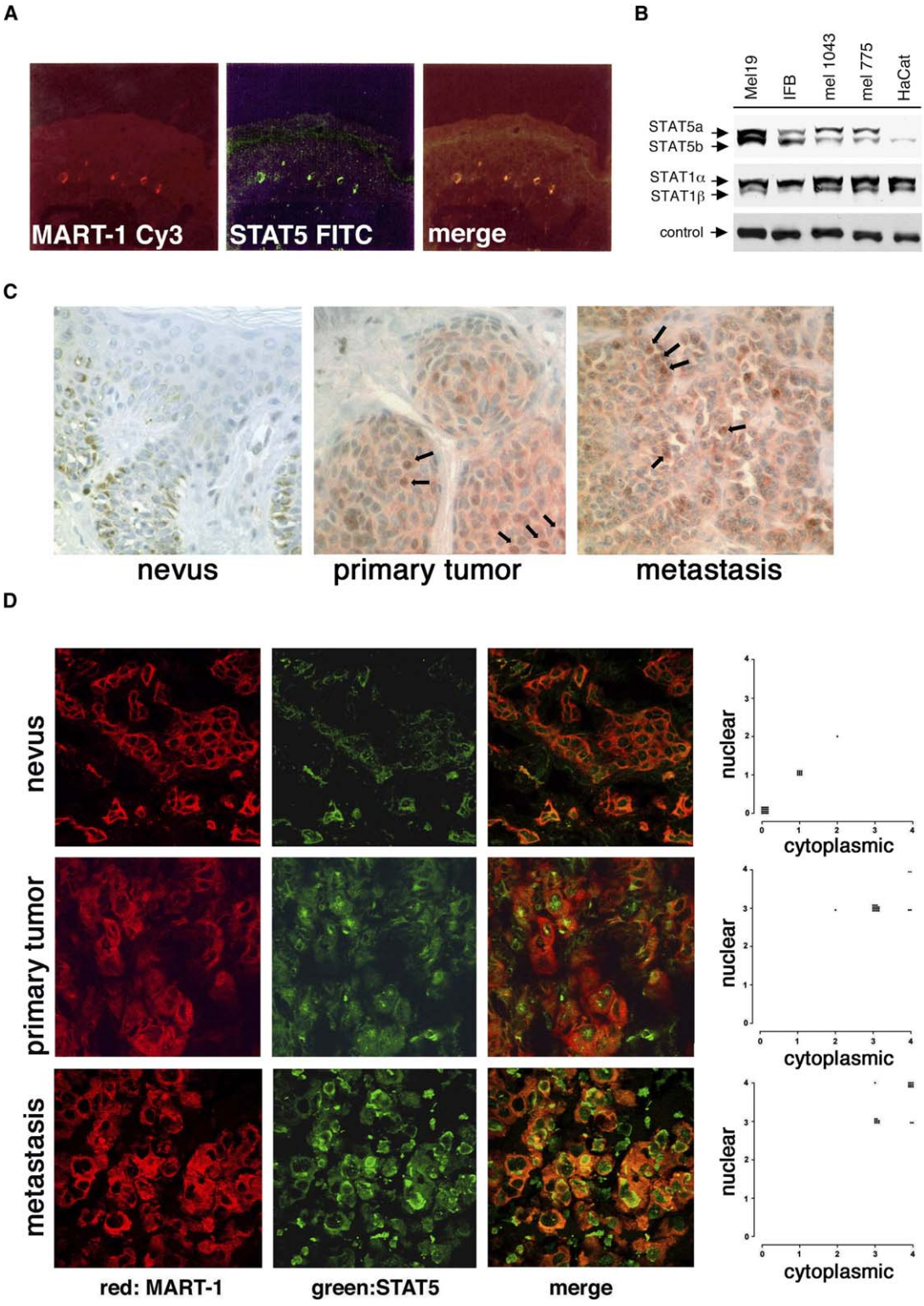

Figure 1. Expression of STAT5 in Normal Human Skin, Nevi, and Melanoma

(A) A section of normal human skin stained with anti-MART-1 (red) and anti-STAT5 (green).

(B) Melanoma lines Mel19 and IFB, human primary melanocytes mel1043 and mel755, and HaCat cells were analyzed with anti-STAT5 and anti-STAT1.

(C) Sections of benign nevi, primary cutaneous melanoma, or skin metastases were stained with anti-STAT5 using AEC (red). Slides were counterstained with hematoxylin (blue nuclei). Examples of nuclear STAT5 staining are indicated by arrows.

(D) Sections of benign nevi, primary cutaneous melanoma, or skin metastases were stained with anti-MART-1 (Cy3-red) and anti-STAT5 (FITC-green). The frequency of STAT5 staining in MART-1-positive cells was classified as follows: 0 = none, 1 = <10%, 2 = <30%, 3 = <60%, and 4 = >60%.

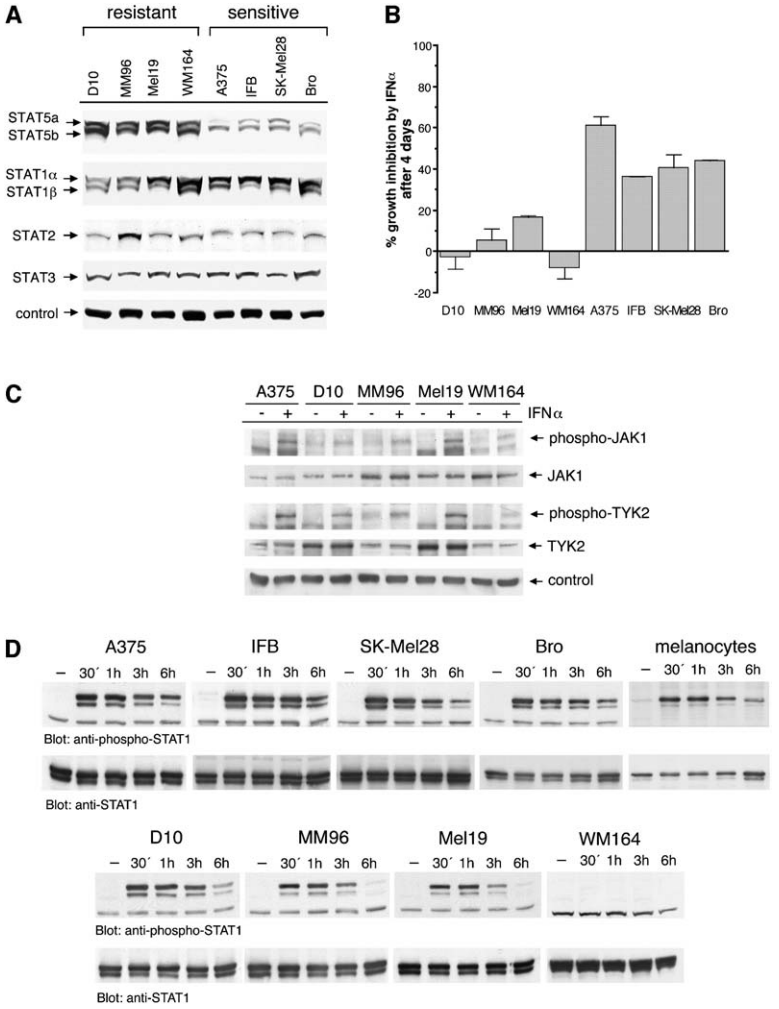

Figure 2. STAT5 Overexpression Correlates with IFNα Resistance

(A) Human melanoma cell lines were analyzed for STAT protein expression with the indicated antibodies. Analysis of ERK2 expression was used as a loading control.

(B) Melanoma cell lines were cultured for 4 days in IFNα (1000 U/ml) containing medium. Control cells were grown without IFNα. After 4 days, cells were counted and the cell number of untreated cells was set 100%. The error bars above the columns indicate standard deviation (SD) from the mean.

(C) Melanoma cells were stimulated with IFNα for 30 min, and the phosphorylation of JAK1 and TYK2 was analyzed with phospho-specific antibodies. Analysis of ERK2 expression was used as a loading control.

(D) The indicated cell lines were stimulated with IFNα for the indicated times, and the phosphorylation of STAT1 was analyzed with anti-phospho(Y701)STAT1. The amount of total STAT protein was determined with anti-STAT1.

Overexpression of STAT5 Reduces the IFNα Response in Melanoma Cells

Stimulation of A375 cells with IFNα also resulted in phosphorylation of STAT5 (Figure 4A), its nuclear accumulation (not shown), and specific DNA binding to GAS sequences (GAS-WT; Figure 4B). Furthermore, a reporter construct containing a STAT5-responsive promoter became activated by IFNα. Activation was seen after 3 hr, whereas after 22 hr, the promoter induction was almost back to basal level (Figure 4C), suggesting that STAT5-induced promoter activation in response to IFNα is an early event.

Strikingly, transient overexpression of STAT5A or STAT5B clearly had a significant inhibitory effect on the IFNα induction of an ISRE-containing promoter (Figure 4D). STAT1, on the other hand, did not suppress IFNα-induced induction of this promoter. To further analyze effects of STAT5 overexpression, we established A375 cells stably expressing STAT51*6 (Figure 4E), a STAT5A version bearing mutations (H299R, S711F) that stabilize the tyrosine-phosphorylated form of the protein [23]. However, basal constitutive tyrosine phosphorylation of STAT51*6 was low in A375-1*6 cells (Figure 4F), reflecting a low basal activity of the respective upstream kinases. Stimulation of A375-1*6 cells with IFNα resulted in phosphorylation of STAT51*6. This phosphorylation was stronger, lasted longer than that of endogenous STAT5 in A375 cells, and resembled the strong phosphorylation, seen in the resistant cell lines D10, MM96, Mel19, and WM164, of STAT5 (Figure 4F and Figure S1 in the Supplemental Data available with this article online).

As seen after transient overexpression of STAT5, luciferase induction from an ISRE-containing promoter was greatly reduced in A375-1*6 cells compared to control A375svneo cells even at high IFNα concentrations (Figure 4G). The suppression of ISRE-mediated transcription was also seen when IFNα-induced *hPNPase*$^{old-35}$ expression was analyzed in A3751*6 cells compared to A375 cells or STAT1-overexpressing A375 cells (Figure 4H). Moreover, in STAT51*6-expressing cells, the IFNα-induced growth-inhibition response was considerably reduced (Figure 4I). Together, these data suggest that overexpression of STAT5 can antagonize IFNα-induced gene expression and growth inhibition in melanoma cells.

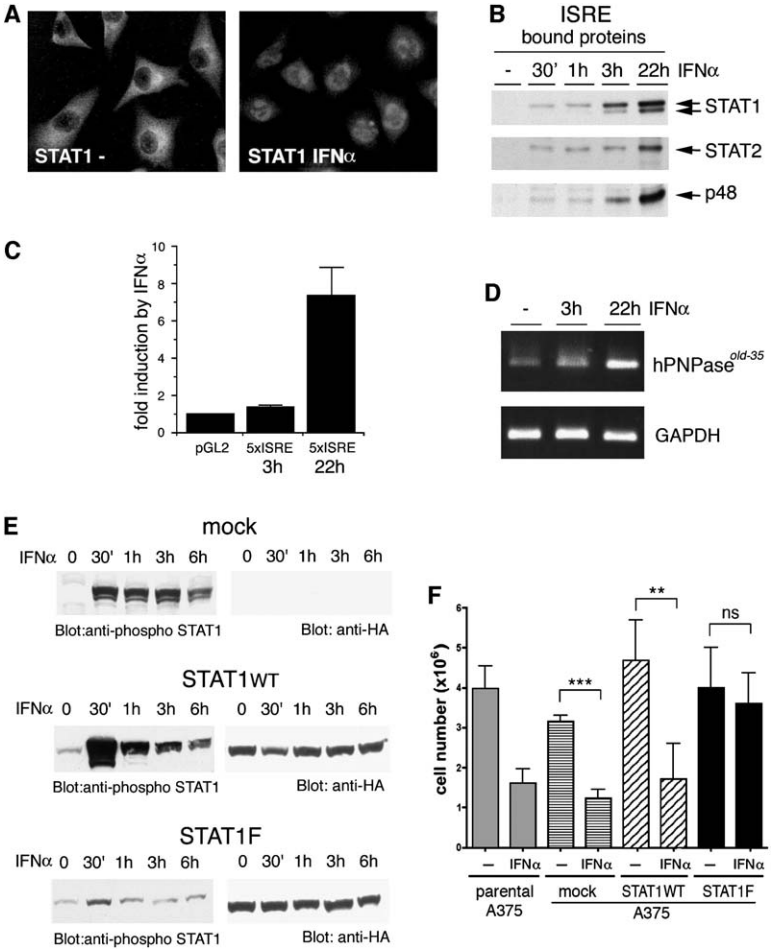

Figure 3. STAT1 Activation Is Required for IFNα Signaling

(A) A375 cells were either stimulated with IFNα for 30 min or left untreated (–). Fixed cells were stained with anti-STAT1 and Cy2-conjugated secondary antibodies.

(B) Cellular extracts of A375 cells either stimulated with IFNα for the indicated times or left untreated (–) were incubated with oligonucleotides carrying ISRE sites. Bound proteins were eluted and analyzed with anti-STAT1, anti-STAT2, and anti-p48 on a western blot.

(C) Luciferase expression from an ISRE-containing promoter or the empty vector pGL2 was measured after 3 hr or 22 hr of IFNα stimulation of A375 cells. Luciferase activities were normalized to β-galactosidase activities, and error bars represent SD from the mean.

(D) A375 cells stimulated with IFNα for the indicated times were analyzed for *hPNPase*$^{old-35}$ expression by RT-PCR. Amplification of GAPDH served as control.

(E) A375 cell lines expressing either HA-tagged STAT1 (STAT1WT) or an HA-tagged dominant-negative mutant STAT1F were stimulated with IFNα for the indicated times and analyzed with anti-phospho(Y701)-STAT1. The amount of STAT1WT and STAT1F protein was determined with anti-HA. Control (mock) cells had been transfected with the empty vector.

(F) A375 cells, mock-transfected cells, and STAT1WT- or STAT1F-expressing cells were cultured for 4 days in IFNα-containing medium before they were counted. Control cells were grown in IFNα-free medium. The error bars above the columns indicate SD from the mean.

STAT5 Counteracts IFNα-Induced STAT1 Activation and Upregulates the Cytokine Inhibitor CIS

Whereas STAT1 became phosphorylated in control transfected A375svneo cells with similar intensity and duration compared to the parental cells (compare with Figure 2D), its phosphorylation was weaker and almost back to basal level after 6 hr of IFNα stimulation in A375-1*6 cells (Figure 5A). Notably, this activation profile strongly resembled the profile observed in the resistant MM96 and Mel19 cells (see Figure 2D).

Analysis of the IFNα-induced expression of suppressors of cytokine signaling (SOCS) revealed no differences in the induction of *SOCS1* and *SOCS2* (not shown), but the induction of *SOCS3* expression by IFNα was significantly reduced in STAT51*6-expressing A375 cells (Figure 5B). Most strikingly, whereas the STAT5 target gene *CIS* [24] was induced very transiently by IFNα in A375 and A375svneo cells (Figure 5B), its expression was induced to a high level and persisted at least 6 hr in STAT5-overexpressing A375-1*6 cells (Figure 5B). This was reflected at the protein level, and in A375-1*6 cells, a strong increase of CIS protein was detectable up to 22 hr (Figure 5C). Basal CIS expression in interferon-resistant and -sensitive cells did not differ significantly although SK-Mel28 cells expressed very low levels of the protein (Figure 5D). However, in contrast to the interferon-sensitive cells A375, IFB, SK-Mel28, and Bro, CIS expression was significantly induced by IFNα in the resistant cell lines (Figure 5D).

When CIS was overexpressed in interferon-sensitive A375 cells, the IFNα-stimulated induction of an ISRE-containing promoter was suppressed (Figure 5E), suggesting that enhanced CIS expression can counteract IFNα-induced signaling.

To fully assess the consequence of enhanced CIS expression for IFNα-mediated growth inhibition, we analyzed the effect of CIS overexpression on the interferon response of A375 cells in a colony-formation assay. To further compare the effect produced by CIS with the capacity of STAT5 to counteract IFNα-mediated growth inhibition, we analyzed overexpression of STAT5 as well as STAT51*6 in the same assay. In agreement with the results of Figure 4I, overexpression of STAT51*6 significantly enhanced the number of colonies formed by A375 cells in the presence of IFNα when compared to cells transfected with an empty vector (Figure 5F). Strikingly, also wild-type STAT5A had a significant effect on the interferon-mediated growth inhibition (Figure 5F), indicating that STAT5 overexpression alone is sufficient to antagonize the IFNα response. Also, over-

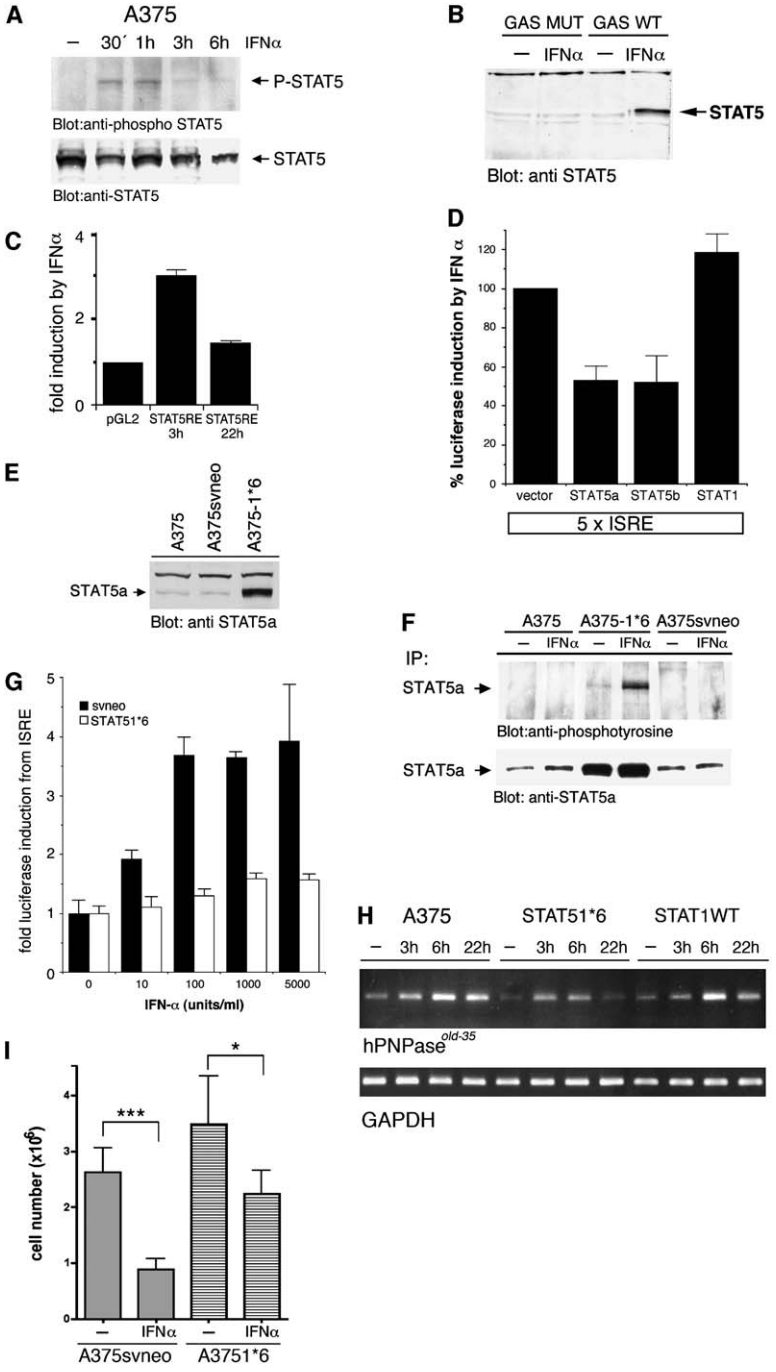

Figure 4. STAT5 Is Activated by IFNα in Melanoma Cells

(A) A375 cells were stimulated with IFNα for the indicated times and analyzed for phospho(Y694)-STAT5 and total STAT5.

(B) A375 cells were either left untreated (–) or stimulated with IFNα for 30 min. Cellular extracts were analyzed for specific DNA binding activity by incubation with oligonucleotides carrying either a GAS (GAS WT) sequence or a mutated binding deficient sequence (GAS MUT). Bound proteins were analyzed with anti-STAT5.

(C) A375 melanoma cells were transfected with either a luciferase construct containing six STAT5 binding sites (STAT5-RE TK luc) or a control vector (pGL2), and luciferase induction by IFNα was measured after 24 hr.

(D) A375 melanoma cells were transfected with an ISRE-containing luciferase construct and with plasmids for STAT5a, STAT5b, STAT1, or an empty vector. Twenty-two hours after the transfection, luciferase induction was measured. Luciferase activities in (C) and (D) were normalized to β-galactosidase activities, and error bars represent SD from the mean.

(E) Analysis of STAT5 expression in parental A375, control A375svneo, and A375-1*6 cells with anti-STAT5a.

(F) Tyrosine phosphorylation of STAT51*6 before and after 10 min of stimulation with IFNα was detected by immunoprecipitation with a STAT5A specific antibody and western-blot analysis with anti-phosphotyrosine. The membrane was reprobed with anti-STAT5A.

(G) A375svneo and A375-1*6 cells were transfected with an ISRE-containing luciferase construct and stimulated with the indicated IFNα concentrations. After 24 hr, luciferase induction was measured. The error bars above the columns indicate SD from the mean.

(H) A375, A375-1*6, and STAT1WT-overexpressing A375 cells stimulated with IFNα for the indicated times were analyzed for *hPNPase*$^{old-35}$ expression by RT-PCR. Amplification of GAPDH served as control.

(I) A375svneo and A375-1*6 cells were cultured for 4 days in IFNα-containing medium before they were counted. The error bars above the columns indicate SD from the mean.

expression of CIS affected the interferon response of A375 cells (Figure 5F), but the effect reached only 50% of the effect produced by STAT5, suggesting that STAT5 antagonizes IFNα-induced growth inhibition through additional factors.

Depletion of STAT5 Enhances the IFNα Response of Melanoma Cells

To establish conclusively its function in interferon signaling in melanoma cells, we depleted STAT5 with a specific small interfering RNA (siRNA) during IFNα stimulation. Treatment of A375 cells with the STAT5-specific siRNA resulted in efficient depletion of the STAT protein, whereas a control siRNA (con) did not affect STAT5 expression (Figure 6A). The STAT5-specific siRNA also produced a significant reduction in the amount of the transcription factor in the STAT5-overexpressing IFNα-resistant MM96 cells (Figure 6A).

Depletion of STAT5 in MM96 cells had a significant effect on STAT1 activation in response to IFNα (Figure 6B). Whereas in untreated cells and cells treated with the control siRNA, STAT1 phosphorylation was almost

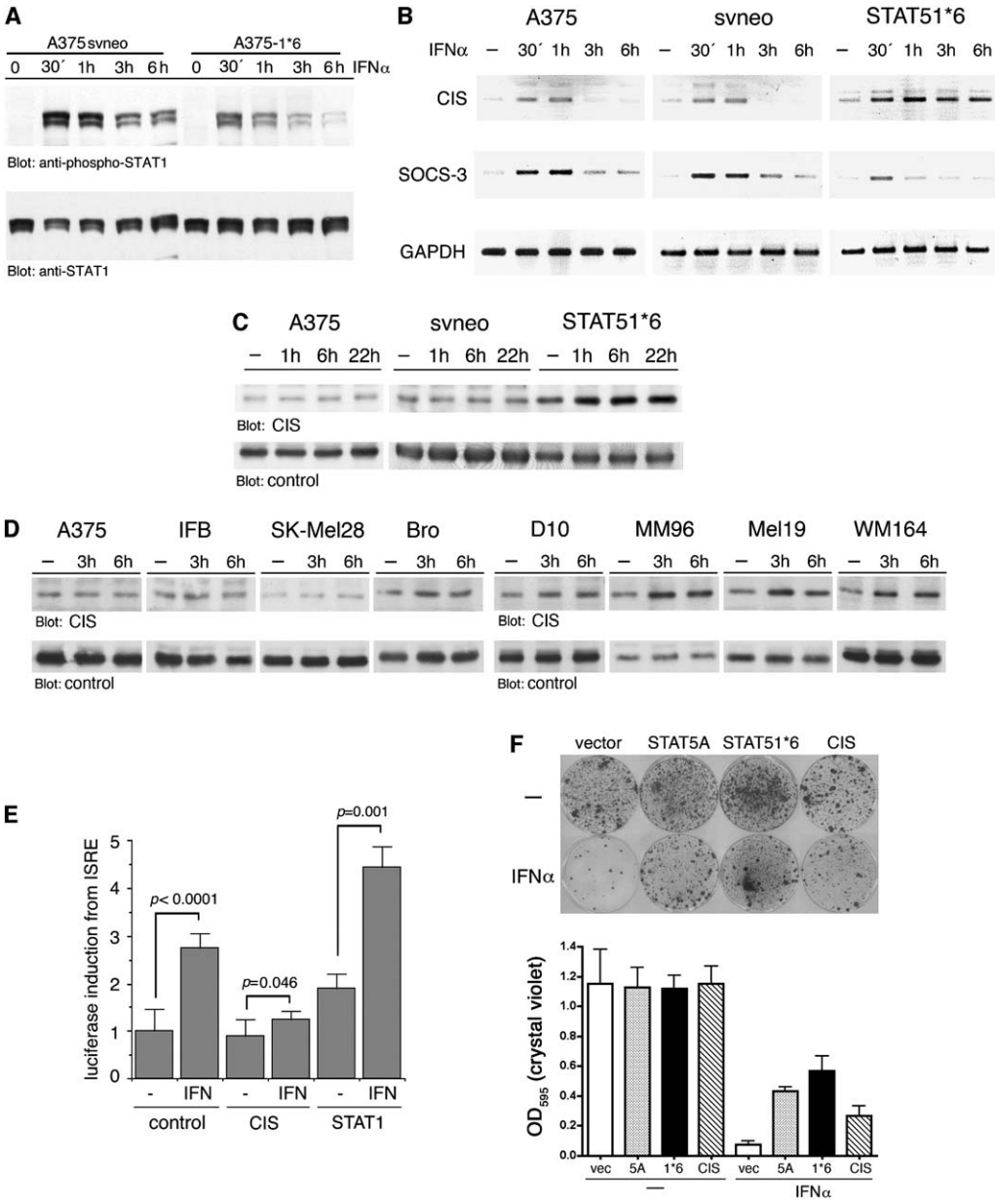

Figure 5. Overexpression of STAT51*6 in Human Melanoma Cells Leads to an Altered Expression of IFNα Target Genes

(A) A375svneo and A375-1*6 cells were stimulated with IFNα for the indicated times and analyzed with anti-phospho(Y701)STAT1. The membrane was reprobed with anti-STAT1.

(B) A375, A375svneo, and A375-1*6 cells were stimulated with IFNα for the indicated times and analyzed for CIS and SOCS3 expression by RT-PCR. Amplification of GAPDH served as control.

(C) Analysis of CIS protein expression in A375, A375svneo, and A375-1*6 cells stimulated with IFNα for the indicated times.

(D) Analysis of CIS protein expression in the indicated cell lines stimulated with INFα for 3 hr or 6 hr.

(E) A375 cells were transfected with a luciferase construct containing five ISRE sites and expression plasmids for CIS, STAT1, or an empty vector (control). Twenty-two hours after the transfection, luciferase induction was measured.

(F) A375 cells were transfected with a Hygromycin resistance plasmid and either an empty vector or a CIS, STAT5A, or STAT51*6 expression vector. Cells were selected for Hygromycin resistance and stained for colonies with crystal violet 19 days after the transfection. Quantification was performed by measuring the absorbance of resolubilized crystal violet. A representative stained cell image is shown on top of the graph.

back to basal level 6 hr after IFNα stimulation, reduction of STAT5 levels in MM96 cells resulted in a more prolonged activation of STAT1 in response to the cytokine (Figure 6B). An even more striking consequence of STAT5 depletion in MM96 cells was apparent when the responsiveness to the antiproliferative potential of IFNα was examined. Whereas MM96 cells treated with the control siRNA (con) were completely resistant to the growth-inhibitory effect of IFNα, the proliferation of cells treated with the STAT5-specific siRNA was considerably inhibited (Figure 6C). Similar effects were seen in the resistant cell line Mel19 (data not shown).

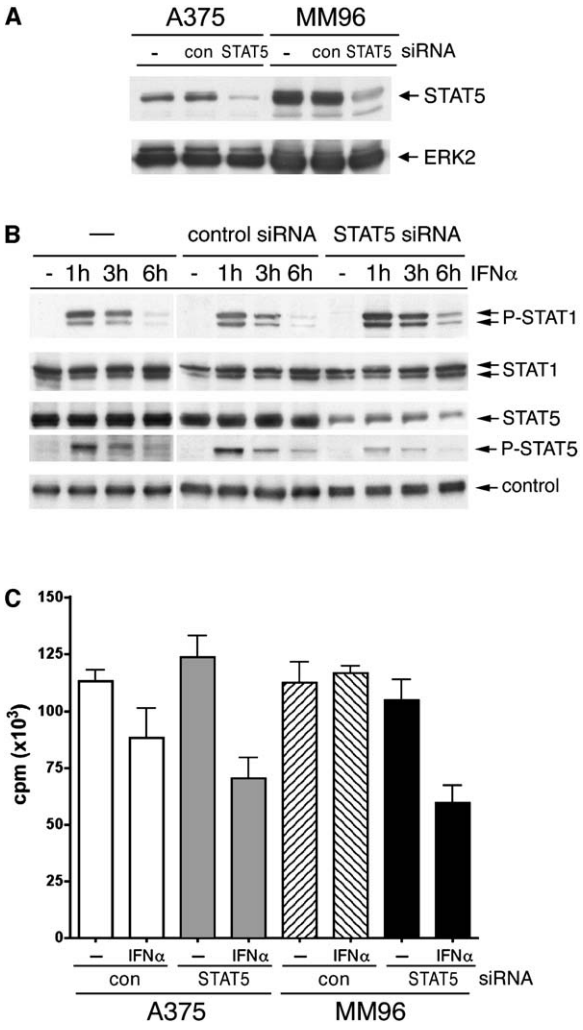

Figure 6. Depletion of STAT5 in Human Melanoma Cells Results in INFα Sensitivity

(A) A375 and MM96 cells were left untreated (–) or were transfected with either a control siRNA (con) or a STAT5-specific siRNA. Seventy-two hours after the transfection, the expression of STAT5 was analyzed by western blotting with anti-STAT5. Analysis of ERK2 expression was used as a loading control.

(B) MM96 cells were left untreated (–) or were transfected with either a control siRNA or a STAT5-specific siRNA. Sixty-four hours after transfection, cells were stimulated with IFNα for the indicated times and analyzed for STAT1 phopshorylation with anti-phospho (Y701) STAT1. The amount of total STAT1 protein was determined with an anti-STAT1 antibody, and STAT5 expression in control cells and STAT5-siRNA-treated cells was analyzed with anti-STAT5. Analysis of ERK2 expression was used as a loading control.

(C) A375 and MM96 cells were transfected with either a control siRNA (con) or a STAT5-specific siRNA. Thirty-six hours after transfection, the cells were stimulated with IFNα for 24 hr. For the last 4 hr, 3H-thymidine was added, and incorporated 3H-thymidine was quantified by liquid scintillation counting. The error bars above the columns indicate SD from the mean.

Discussion

Malignant melanoma is a highly aggressive and increasingly common disease. Treatment of metastatic melanoma is notoriously difficult and usually ineffective, frequently because of the acquisition by melanoma cells of resistance to growth-inhibitory cytokines [7, 8, 25]. Because STAT1 is essential for the mediation of antiproliferative effects brought about by interferons, it is not surprising that deficient STAT1 activation is correlated with interferon resistance in melanoma [11, 12].

We present here several lines of evidence for a role of STAT5 in melanoma development and its involvement in interferon resistance. We have shown that STAT5 is overexpressed in cell lines derived from advanced melanoma and that nuclear-localized STAT5 increased with tumor progression in melanoma biopsies. We also found that STAT5 was localized in the cytoplasm in melanocytes and melanoma cells derived from a primary melanoma (IFB and A375 cells), whereas predominantly nuclear localization was seen in all other melanoma cell lines (Figure S2 and data not shown). Because nuclear translocation of STATs is implicated in their cellular activation, this suggests constitutive activation of STAT5 in malignant melanoma. However, future studies involving analysis of STAT5 phosphorylation are required to fully establish the state of STAT5 activation in melanoma.

We found that STAT5 overexpression correlated with IFNα resistance in melanoma cell lines. Significantly, ectopic overexpression of STAT51*6 as well as wild-type STAT5 severely reduced the ability of IFNα to inhibit proliferation in an IFNα-sensitive cell line. Moreover, depletion of STAT5 in IFNα-resistant cells dramatically enhanced the responsiveness to IFNα. This strongly supports the notion that deregulation of STAT5 activity is likely to make a significant contribution to the acquisition of IFNα resistance.

STAT1 activation is crucial for IFNα-induced growth inhibition in melanoma cells [7, 9] but was found to be inhibited by the overexpression of STAT51*6. Because STAT1 is an essential component of ISGF3, the transcription factor that binds to the ISRE in interferon-induced genes [4], ISRE-dependent transcription was strongly suppressed in STAT51*6-expressing cells. We found that in melanoma cells, ISGF3-mediated transcription is a late event that would require sustained STAT1 activation. However, activation of STAT5 resulted in abrogation of sustained STAT1 activation. This was sufficient to affect ISGF3-driven transcription as seen in the suppression of *hPNPase*$^{old-35}$ expression. Notably, enhanced *hPNPase*$^{old-35}$ expression strongly inhibits melanoma cell growth [26], and the suppression of *hPNPase*$^{old-35}$ levels will counteract this antiproliferative effect.

Although STAT51*6 became activated by IFNα immediately after receptor stimulation, its inhibitory effect on STAT1 became apparent only in a later phase of activation, suggesting the involvement of the suppressor of cytokine signaling (SOCS/CIS) factors. A mutual regulation of STATs on the level of SOCS/CIS factors has been found in a variety of cell systems where the suppression of STAT1 tyrosine phosphorylation blocks interferon-induced cellular responses [27, 28]. In STAT51*6-overexpressing cells, impaired STAT1 activation was correlated with a reduced SOCS3 (CIS3) expression and a strongly enhanced CIS (CIS1) expression. We demonstrate here that overexpression of CIS contributes to the effect of STAT5 and leads to a significant suppression of IFNα-induced transcription. Furthermore, overexpression of CIS in IFNα-senstitive cells reduced the interferon response of the cells. This suggests a new role for CIS in the acquisition of interferon resistance in

melanoma. However, the effect brought about by CIS accounted only for 50% of the effect caused by STAT5, which suggests that the STAT5-induced IFNα resistance involves the activation of additional factors.

Although both CIS and SOCS3 inhibit cytokine-induced signaling, they differ in the way they act. SOCS3 affects the activity of JAK kinases, and CIS inhibits the binding of STATs to the activated tyrosine-phosphorylated receptor [29]. Interestingly, we and others have demonstrated that STAT5, but neither STAT1 nor STAT3, can become activated independently from receptor phosphorylation [30, 31]. On the other hand, activation of STAT1 by the IFN receptor is strictly dependent on receptor phosphorylation [32]. Thus, STAT1 activation might be inhibited by overexpression of CIS, whereas STAT5 could still become activated by the receptor.

Another possibility by which overexpression of STAT5 could affect the activation of STAT1 could be competition of STAT5 with STAT1 for activation by the receptor. Accordingly, in cells that overexpress STAT51*6, STAT5 phosphorylation is much stronger (see Figures S1) and STAT1 activation is decreased. Also, when STAT5 expression was lowered by RNAi, its activation was reduced and STAT1 activation was increased (see Figure 6). However, the most striking differences in STAT1 activation between interferon-resistant and -sensitive cells were observed at later time points (3–6 hr), suggesting that reduced STAT1 activity is rather due to enhanced STAT5 downstream signaling than to overexpressed STAT5 preventing STAT1 activation by the receptor through competition. Furthermore, we found that overexpression of either wild-type or dominant-negative STAT1 had no significant effect on the activation of STAT5 (not shown). Similarly, no increase in interferon-induced STAT5 activation was found in T cells from STAT1 (–/–) mice when compared to normal T cells [33], and this points to no influence of STAT1 expression levels on STAT5 activation by the interferon receptor. Together, these data suggest that the effects we observed are not due to competition at the receptor level, but further studies are required to fully address this issue.

Conclusions

The data presented here show that in melanoma cells, STAT1 and STAT5 exhibit a functional antagonism; whereas STAT5 promotes cell growth and protects the cells from the inhibitory effect of IFNα, STAT1 contributes to growth inhibition. Thus, a tight regulation of STAT activation and signaling seems to be a critical step in the cytokine-mediated growth control of melanoma cells. Our findings suggests that loss of STAT1 activity but gain of STAT5 activity can lead to an imbalance in this regulation and enables melanoma cells to overcome cytokine-mediated antiproliferative signaling, thus contributing to melanoma development and progression.

Experimental Procedures

Cell Culture, Generation of Cell Lines, and Colony-Formation Assay

A375 (ATCC#CRL1619), SK-Mel 28 (ATCC#HTB72), D10, Mel19, IFB, Bro, and STAT1- and STAT1F-expressing A375 cells [34] were cultured in RPMI/10% FCS; WM164 and MM96 were grown in DMEM/10% FCS. Human primary melanocytes were cultured in DMEM/10% FCS containing 200 nM TPA, 1.2 nM cholera toxin, and 0.3 nM bFGF. For all experiments, if not indicated otherwise, the medium was replaced by DMEM/1% FCS for 24 hr before the cells were stimulated with IFNα at a concentration of 1000 U/ml. For the generation of A375 cells expressing STAT51*6 (a kind gift from T. Kitamura [23]), the cells were transfected with prk5STAT51*6 and selected for G418 (1 mg/ml) resistance. Stable clones were analyzed by western-blot analysis with anti-STAT5a. For the colony-formation assay, cells were transfected in triplicates with 1 μg expression plasmid plus 0.1 μg pCDNA3.1/Hygro and selected in Hygromycin (0.5 mg/ml) for 5 days. After this selection, cells were either treated with IFNα or left untreated for another 14 days before colonies were stained with crystal violet.

Cell Lysis, Immunoprecipitation, Western Blotting, and Antibodies

Cells were rinsed twice with cold PBS and lysed in Triton lysis buffer, and immunoprecipitation and western blotting were performed as described [19]. The following antibodies were used: anti-STAT5b (C-17; crossreacts with STAT5A), anti-STAT5a (L-20; STAT5A specific), anti-STAT1 (E-23), anti-STAT3 (C-20), anti-p48/ISGF3γ (C-20), anti-CIS (C-20), and anti-ERK2 (C-14) were from Santa Cruz Biotechnology. Anti-phospho (Y701) STAT1 and anti-Tyk2 were from UBI; anti-STAT2, anti-JAK1, and anti-phosphotyrosine (PY20) were from BD Transduction Laboratories. Anti-phospho(Y1022/1023)-JAK1, anti-phospho(Y1054/1055)-TYK2, and anti-phopsho(Y694)-STAT5 were from Cell Signaling.

Immunofluorescence and Immunohistological Analysis of Melanoma Biopsies

Methanol/acetone fixed cells were blocked with 1% BSA/PBS and incubated with either anti-STAT5 (C-17) or anti-STAT1 (E-23) and with either Cy2- or Cy3-conjugated anti-rabbit IgG (Dianova). Cells were embedded in mounting medium containing DAPI. For tissue samples, frozen sections were dried overnight and fixed in acetone. All incubation steps including anti-MART-1 (1:150; clone 361M, Biosite), Cy3-conjugated anti-mouse IgG (Dianova), and finally FITC-conjugated anti-STAT5 (1:250; C-17, Santa Cruz Biotechnology) were performed at room temperature (RT). Slides were mounted in vectashield and analyzed with a Leica confocal microscope. Chi-square tests were used to compare the proportions of STAT5-positive cells in benign and malignant lesions. For immunohistology, frozen sections were fixed in acetone followed by quenching of endogenous peroxidase with 0.03% H_2O_2 and blocking of collagenous elements with 10% BSA. Serial sections were incubated for 30 min with biotinylated antibodies at predetermined dilutions (usually 20 μg/ml). Subsequently, the streptavidin-peroxidase complex (DAKO) was applied for 30 min, followed by 15 min incubation with the chromogen AEC (DAKO). Slides were counterstained with hematoxylin (DAKO) and mounted in aquatex (Merck Eurolab).

Preparation of Cell Extracts and STAT5 DNA Binding

Cell extracts were prepared by four freeze-thaw cycles in low salt lysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM NaCl, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). The cleared supernatant was incubated with M280-Streptavidin Dynabeads (Dynal) carrying either multiple ISRE (5′-GATCCAGGCTCCCGGGAAAGCGAA ACCTAAACAGTGGCGC-3′) or GAS elements (5′-GATCAGATTTT AGGAAATCAATCC-3′) or a mutated GAS sequence (5′-GATCA GATTTATTTTAATTCAATCC-3′). Bound proteins were eluted and analyzed by western blotting.

Transfections and Luciferase Reporter Assays

A375 cells were transfected with 1 μg luciferase construct, 0.4 μg expression vector, and 0.4 μg pSV-β-Galactosidase (Promega) with Lipofectamine (Invitrogen). After 24 hr, cells were lysed in RLB buffer (Promega) and analyzed for luciferase activity. The constructs were pGL2, 5xISRE-TK-LUC (a gift from T. Spencer [35]), STAT5RE-TKluc, pXM-STAT5a and STAT5b (kindly provided by R. Moriggl [36]), pcDNAmyc-CIS1 (a kind gift from A. Yoshimura [37]), and pCAGGSNeoSTAT1 [22]. Each experiment was performed at

least three times, and luciferase expression was corrected with β-galactosidase values as a covariate.

RT-PCR Analysis

RNA was isolated with TRIZOL reagent (Invitrogen). First-strand cDNA synthesis was done with 1 μg total RNA and random hexanucleotides. The respective genes were amplified with a number of cycles, where amplification was still linear and analyzed on an agarose gel. The primers were the following: for *cis*, 5′-CTGCTGTG CATAGCCAAGACGTTC-3′ and 5′-CAGAGTTGGAAGGGGTACTGT CGG-3′; for *socs*3, 5′-GGTCACCCACAGCAAGTTTCC-3′ and 5′-GTCCAGGAACTCCCGAATGGG-3′; for *hPNPase*$^{old-35}$, 5′-AAGA GAACTTGGGCATGGTG-3′ and 5′-TTTGCCACTGAAGCTTGTTG-3′; and for *gapdh* 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′.

RNA Interference and Thymidine Incorporation

Melanoma were seeded at 2.2 × 10^3 cells per well of a 6-well plate the day before transfection. Cells were transfected with 120 nM STAT5-specific siRNA (5′-AACGCCACCAUCACGGACAUU-3′) or 120 nM control siRNA (5′-AAGUCCAUGGUGACAGGAGAC-3′) in 1 ml OPTIMEM with 4 μl Lipofectamine. After 5 hr, 1 ml of DMEM containing 10% FCS was added, and the cells were cultured o/n. Then, the medium was replaced by DMEM/1% FCS for 24 hr before the cells were stimulated with IFNα for 24 hr. For the last 4 hr, ^{3}H-thymidine (0.4 μCi/ml) was added, and incorporated ^{3}H-thymidine was quantified by liquid scintillation counting.

Supplemental Data

Supplemental Data include two Supplemental Figures and are available with this article online at: http://www.current-biology.com/cgi/content/full/15/18/1629/DC1/.

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