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Persistently Activated Stat3 Maintains Constitutive NF-κB Activity in Tumors

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SUMMARY

NF-κB (RelA) is constitutively active in many cancers, where it upregulates antiapoptotic and other oncogenic genes. While proinflammatory stimulus-induced NF-κB activation involves IKK-dependent nuclear translocation, mechanisms for maintaining constitutive NF-κB activity in tumors have not been elucidated. We show here that maintenance of NF-κB activity in tumors requires Stat3, which is also frequently constitutively activated in cancer. Stat3 prolongs NF-κB nuclear retention through acetyltransferase p300-mediated RelA acetylation, thereby interfering with NF-κB nuclear export. Stat3-mediated maintenance of NF-κB activity occurs in both cancer cells and tumor-associated hematopoietic cells. Both murine and human cancers display highly acetylated RelA, which is associated with Stat3 activity. This Stat3/NF-κB interaction is thus central to both the transformed and nontransformed elements in tumors.

INTRODUCTION

NF-κB is a central transcription factor in both innate and adaptive immunity. There has been increasing interest in NF-κB's role in both cancer initiation (in particular, inflammation-induced carcinogenesis) and maintenance of established cancer, where it is frequently constitutively activated and plays a major role in the transcriptional activation of multiple antiapoptotic and other oncogenic genes. NF-κB consists of five Rel-related proteins, and the prototypical NF-κB complex is a RelA/p50 heterodimer, which is important for NF-κB-mediated antiapoptotic effects (Karin et al., 2002). In the absence of appropriate stimuli, NF-κB is sequestered in the cytoplasm by IκBα protein. IκB kinases (IKKs) are activated upon stimulation of Toll-like receptors and intracellular sensors such as RIG-I, MDA-5, and NOD1/2 by various pathogen-associated molecular patterns (PAMPs) or proinflammatory cytokines such as TNF, leading to serine phosphorylation of IκBα and its subsequent proteasome-mediated degradation, which is critical for NF-κB nuclear translocation (Ghosh et al., 1998). An important role of IKKβ-dependent NF-κB activation involving RelA/p50 has been documented both during pathogen infection and in cancers caused by chronic inflammation and other stimuli (Naugler et al., 2007; Stancovski and Baltimore, 1997). However, with only a few exceptions, such as lymphoid tumors, where activating mutations of upstream IKK activators such as CARD11 have been identified (Lenz et al., 2008; Pomerantz et al., 2002), IKK is not often continuously activated in cultured cancer cells but rather is inducible by proinflammatory stimuli. Several studies have provided evidence that secretion of cytokines and growth factors, many of which are encoded by NF-κB target genes, is critical for constitutive activation of NF-κB in cancer cells (Lu et al., 2004; Lu and Stark, 2004). Since IKK is not often constitutively activated in tumors, the question remains whether one or more additional mechanisms involving signaling pathways or molecules downstream of some of these cytokines and growth factors might directly contribute to constitutive activation of NF-κB.

SIGNIFICANCE

Development of innate and adaptive immunity in response to proinflammatory stimuli requires induction of NF-κB, which involves its nuclear translocation followed by expression of proinflammation/immunity-related genes. In contrast, NF-κB can be constitutively activated without continuous proinflammatory stimuli in cancer cells, where it serves a very different role: upregulating genes necessary for tumor progression. How NF-κB stays constitutively active in cancer remains to be fully defined. The current work reveals a mechanism whereby constitutively activated Stat3 maintains constitutive NF-κB activity in cancers by inhibiting its export from the nucleus. This Stat3/NF-κB interaction observed in cancer provides insights into carcinogenesis and strategies for developing cancer therapeutics.

It has been suggested that NF-κB/IκBα can shuttle in and out of the nucleus in the absence of stimuli, although the rate of nuclear export is greater than the rate of nuclear import (Huang et al., 2000). Recent studies have demonstrated that the amplitude and half-life of nuclear NF-κB can be influenced by acetylation of RelA (Chen and Greene, 2004), which requires prior RelA phosphorylation (Chen et al., 2005). In particular, endogenous RelA is acetylated in a signal-coupled manner following stimulation (Chen et al., 2001, 2002; Chen and Greene, 2004). The p300/CBP cofactors are acetyltransferases that mediate RelA acetylation, which is subject to deacetylation by histone deacetylases (HDACs) (Chen et al., 2001). Reversible acetylation of RelA is essential for the duration of NF-κB activity, due to its role in regulating the assembly of RelA/IκBα complexes necessary for RelA nuclear export and its presence in the cytoplasm. Acetylated RelA interacts only weakly with IκBα, while deacetylation of RelA by HDACs markedly increases the binding of RelA to IκBα (Chen et al., 2001; Chen and Greene, 2004). Although RelA acetylation has been studied in the context of inflammatory stimuli, whether it has a role in constitutive NF-κB activation in cancer is unknown.

Recent research has documented the importance of cytokines and growth factors secreted by tumor cells, which often depend on IKK-mediated NF-κB activation for their production, as a causative factor in constitutive NF-κB activation in cancer cells and tumors (Greten et al., 2004; Lu et al., 2004; Lu and Stark, 2004). Some of these cytokines and growth factors, such as interleukin-6 (IL-6) and fibroblast growth factor (FGF), are activators of signal transducer and activator of transcription 3 (Stat3) (Deo et al., 2002; Zhong et al., 1994). Stat3 is a transcription factor that can promote oncogenesis (Bromberg et al., 1999), and it is commonly activated in cancer (Darnell, 2002; Yu and Jove, 2004) as well as in tumor-associated myeloid cells (Kortylewski et al., 2005; Kujawski et al., 2008). Stat3 and NF-κB stimulate a highly overlapping repertoire of prosurvival, proliferative, and proangiogenic genes (Catlett-Falcone et al., 1999; Darnell, 2002; Lo et al., 2005; Yu and Jove, 2004). A recent study further demonstrated that Stat3 interaction with RelA leads to upregulation of the immunosuppressive IL-23/*p19* gene (Kortylewski et al., 2009). Although Stat3 has been implicated in inhibiting IKK activity in normal immune cells (Welte et al., 2003), whether constitutively activated Stat3 and RelA directly interact in both cancer cells and immune cells within the tumor microenvironment remains unknown. In the current study, we explored the possibility that constitutively activated Stat3 maintains NF-κB activity in tumors.

RESULTS

Stat3 Is Required to Maintain Tumor NF-κB Activity

It has been shown that phospho-IκBα (p-IκBα) levels are increased in *Stat3*$^{-/-}$ dendritic cells (DCs), suggesting that Stat3 signaling inhibits IKK activity in the context of normal immune responses (Welte et al., 2003). We confirmed that Stat3 negatively regulates IKK activity in normal immune cells, by determining the ratio of p-IκBα to IκBα in splenic cells with or without *Stat3*. Generation of mice containing a functional deletion of *Stat3* alleles in the myeloid compartment has been described previously (Lee et al., 2002). Data from these experiments showed that the p-IκBα/IκBα ratio was higher in *Stat3*-deficient splenocytes relative to their wild-type (WT) counterparts (see Figure S1A available online). Using in vitro IKK kinase assays, we further demonstrated that in tumor cells with both constitutive Stat3 and NF-κB activity, such as human A2058 melanoma and DU145 prostate cancer cell lines, blocking Stat3 by a small-molecule Stat3 inhibitor (Turkson et al., 2004) or siRNA also increased IκBα phosphorylation by IKK, which was further upregulated by TNFα (Figure S1B).

Our data and that of others (Welte et al., 2003) suggest that Stat3 signaling has inhibitory effects on stimulus-induced IKK in both immune and tumor cells. However, these findings raise the question of how constitutively activated Stat3 and NF-κB can coexist in cancer cells. When we examined growing B16 melanoma tumors for NF-κB activity, we found that tumor RelA was constitutively bound to its consensus DNA sequence as determined by EMSA (Figure 1A, left); we also observed increased RelA (Ser536) and Stat3 (Tyr705) phosphorylation. B16 tumors growing in mice with *Stat3*$^{-/-}$ myeloid cells displayed reduced Stat3 activity (Figure 1A, left), likely due to the interruption of Stat3-mediated crosstalk between tumor cells and tumor stromal myeloid cells (Kortylewski et al., 2005; Kujawski et al., 2008). To our surprise, there was little constitutive RelA activity in B16 tumors when Stat3 activity was abrogated (Figure 1A, left). These observations suggested a possible requirement of Stat3 for constitutive RelA activity in tumors. The human A2058 melanoma cell line represents a typical example of a tumor with both constitutive Stat3 (Niu et al., 2002) and NF-κB activity (Figure 1A, right). Similar to in B16 tumors, when *Stat3* is silenced in A2058 tumor cells, NF-κB (RelA) activity is greatly diminished in the tumor cells. These results obtained from both murine tumors and human tumor cells are the opposite of what would be predicted if the primary effect of Stat3 on the NF-κB pathway in tumors were inhibition of IKK, and if IKK were mainly responsible for maintaining constitutive NF-κB activity in tumor cells. In contrast to Stat3 siRNA treatment, silencing *IKK*β alone did not affect nuclear NF-κB activity over a 48 hr period (Figure 1A). Similarly, knocking down *IKK*α alone had no effect on NF-κB in tumor cells (data not shown). However, silencing both *Stat3* and *IKK*β with siRNA greatly diminished NF-κB activity in cancer cells (Figure 1A). While these results indicated that maintenance of existing constitutive NF-κB activity in tumors depends more on Stat3 than IKK activity, they did not rule out the need for IKK to initiate NF-κB activation by facilitating its nuclear translocation. They nonetheless reveal an important IKK-independent downstream mechanism for enhanced NF-κB activity in tumors.

Given our data suggesting that Stat3 also inhibits IKK (Figures S1A and S1B), we investigated whether the effect of Stat3 on NF-κB activity depended on NF-κB activity being driven by a proinflammatory stimulus (and was thus dependent on IKK activity) or whether it was constitutively activated, independent of immune stimuli. A2058 tumor cells display relatively high RelA activity, which is difficult to further increase by additional proinflammatory stimuli. However, DU145 prostate cancer cells have lower RelA activity (Figure 1B) and were thus used to confirm our findings that Stat3 facilitates maintenance of constitutive NF-κB activity in tumor cells, and to further test the role of Stat3 in inhibiting inflammatory signal-induced NF-κB activity. As in A2058 tumor cells, siRNA knockdown of *Stat3*, but not siRNA knockdown of *IKK*β, resulted in the reduction of endogenous RelA activity in DU145 tumor cells (Figure 1B). The immunostimulatory

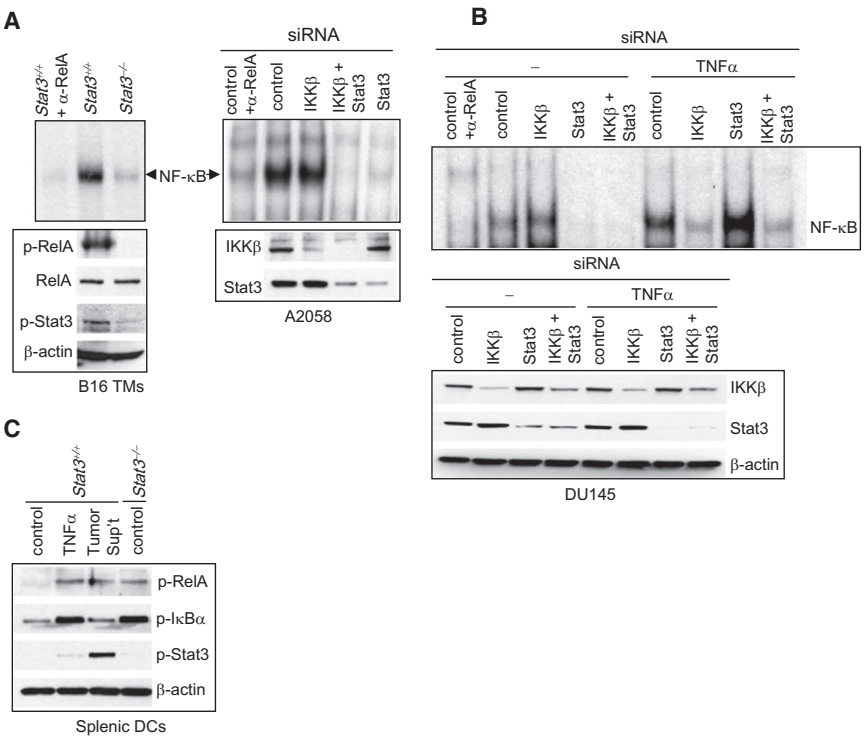

Figure 1. Stat3 Is Required for Maintaining Constitutive NF-κB Activity in Tumors

(A) Stat3, but not IKKβ, is required to maintain constitutive NF-κB (RelA) activity in tumors. Left: DNA binding of NF-κB was determined by EMSA using nuclear extracts prepared from B16 tumors grown in mice with *Stat3*$^{+/+}$ and *Stat3*$^{-/-}$ myeloid cells. Phospho-RelA(S536) (p-RelA) and phospho-Stat3(Y705) (p-Stat3) protein levels in tumors are shown by western blot below. Right: DNA binding activity of endogenous NF-κB in A2058 tumor cells transfected with the indicated siRNAs is shown at top. Supershifting with an anti-RelA antibody (α-RelA) was included in the first lane to verify that the band corresponded to the RelA complex. Effects of siRNA treatments are shown below.

(B) Stat3 is required for maintaining endogenous RelA activity while inhibiting IKK-induced RelA activation in DU145 cancer cells. Cells were transfected with the indicated siRNAs and treated for 15 min with TNFα. NF-κB activity was determined by EMSA. Gene silencing effects by the indicated siRNAs are shown below.

(C) TNFα induces RelA phosphorylation through activating IKK/p-IκBα, whereas RelA phosphorylation induced by tumor factors is associated with Stat3 activation. Freshly isolated splenic dendritic cells (DCs) from mice with *Stat3*$^{+/+}$ and *Stat3*$^{-/-}$ hematopoietic systems were treated with either TNFα or medium conditioned with tumor supernatant and then subjected to western blotting with indicated antibodies.

cytokine TNFα can further stimulate RelA activity in DU145 cells. In contrast to the constitutive, proinflammatory signal-independent RelA activity, TNFα-induced RelA activity was diminished by *IKK*β silencing, while *Stat3* silencing resulted in upregulation of TNFα-induced RelA activity due to abrogation of IKKβ inhibition (Figure 1B). The different effects of Stat3 on RelA activity through two distinct mechanisms were not restricted to tumor cells, as they were also observed in myeloid cells exposed to a proinflammatory stimulus versus tumor-derived factors that activated Stat3 (Figure 1C). Both TNFα and conditioned medium with 10% tumor supernatant prepared from cultured C4 mouse melanoma tumor cells, which have high Stat3 activity and whose secreted factors present in the supernatant have been shown to activate Stat3 effectively (Kujawski et al., 2008), activated RelA in splenic DCs, as indicated by an increase in RelA phosphorylation. However, while TNFα-induced phosphorylation of RelA was associated with an increase in p-IκBα, tumor factor-induced RelA phosphorylation was associated with Stat3 activation but not with an increase in p-IκBα (Figure 1C). Additionally, *Stat3* gene functional deletion resulted in an increase in p-IκBα, consistent with published data (Welte et al., 2003) and results shown in Figure S1A. These data suggested that Stat3 activity, which is elevated in diverse immune cells in the tumor microenvironment, might also contribute to constitutive NF-κB activation in tumor-associated immune cells.

Requirement of Stat3 for Phospho-RelA Nuclear Retention in Cancer Cells

To assess how Stat3 might enhance RelA activity in cancer cells, we utilized immunofluorescence staining and confocal microscopy to detect phospho-Stat3 (p-Stat3) and phospho-RelA (p-RelA) in A2058 cancer cells transfected with control and Stat3 siRNAs. Our results showed that knocking down *Stat3* expression by siRNA decreased p-RelA levels in the nucleus (Figure 2A). To confirm this, nuclear and cytoplasmic extracts were prepared from tumor cells, and levels of phospho- and total Stat3 and RelA proteins were determined by western blot. Consistent with data in Figure 2A, both A2058 and DU145 cells exhibited decreased nuclear p-RelA levels after *Stat3* knockdown (Figure 2B, left), whereas the nuclear protein HDAC1 was not affected. p-Stat3 and p-RelA also physically interacted with each other in A2058 cells as demonstrated by immunoprecipitation with either anti-RelA (Figure 2B, third panel from left, top) or anti-Stat3 antibody (Figure 2B, third panel from left, bottom). Stat3 affected nuclear p-RelA levels, but not total RelA (Figure 2B, right).

Stat3 Facilitates Constitutive Activation of NF-κB via RelA Acetylation

Our data led us to further explore the potential mechanism (or mechanisms) that could facilitate Stat3-mediated NF-κB (p-RelA) nuclear accumulation in cancer cells. It has been shown that RelA acetylation can prolong its nuclear retention (Chen et al., 2001). Importantly, acetylation of RelA requires prior phosphorylation of Ser276 and Ser536 (Chen et al., 2005). We therefore tested the possibility that constitutively activated Stat3 might mediate enhanced RelA acetylation, thereby prolonging retention of p-RelA in cancer cell nuclei. While *Stat3* knockdown in A2058 cancer cells by siRNA did not affect total levels of endogenous RelA protein (Figure 3A), it reduced the relative amount of acetylated RelA (Ac-RelA) (Figure 3A). Given recent findings suggesting

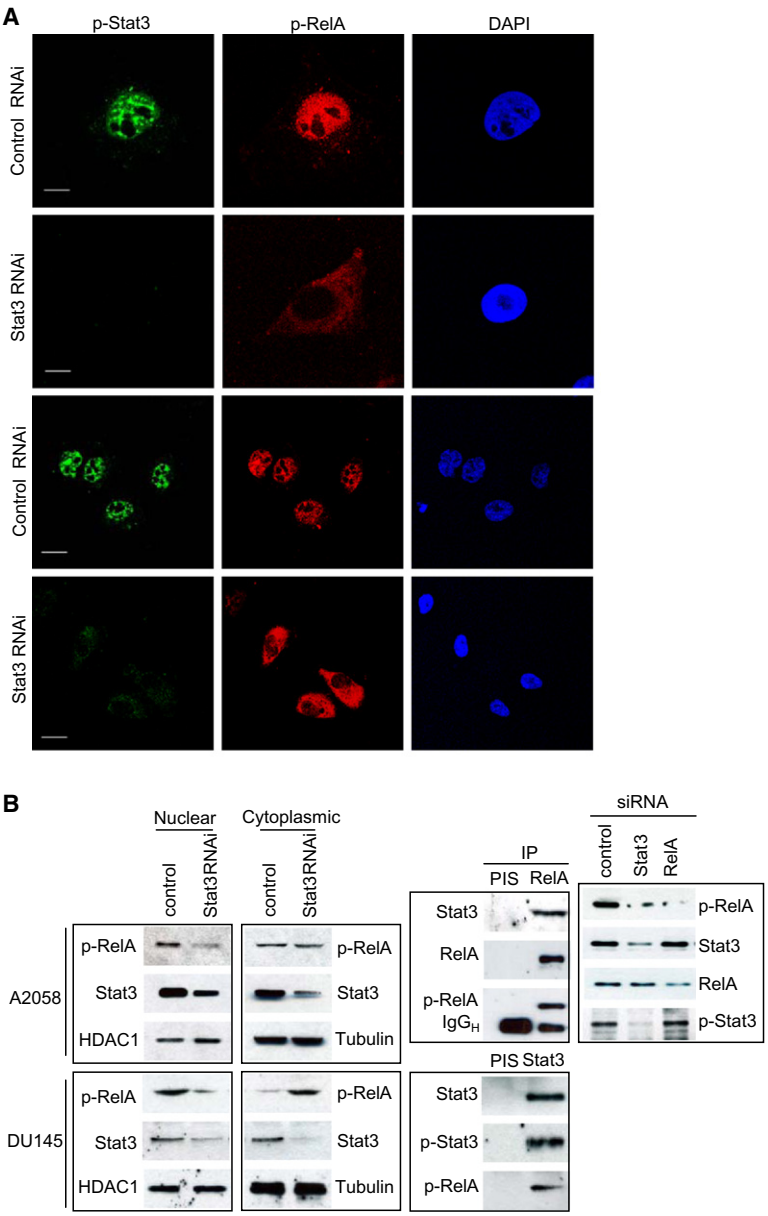

Figure 2. Stat3 Modulates Nuclear NF-κB Retention in Tumor Cells

(A) Cellular localization of p-Stat3 (green) and p-RelA (red) was observed by confocal microscopy after immunofluorescence staining of A2058 cancer cells treated with Stat3 or control siRNAs. DAPI was used to stain nuclei. Images at higher magnification are shown in the top two rows. Scale bars = 10 μm.

(B) Stat3-dependent p-RelA accumulation in tumor cells. First two pairs of panels at left: western blot analyses of nuclear and cytoplasmic p-RelA in the indicated cancer cells transfected with either control or Stat3 siRNAs. HDAC1 and tubulin proteins were used as nuclear and cytoplasmic controls, respectively. Third pair of panels from left: physical interaction of p-RelA with p-Stat3 in tumors. Nuclear extracts from A2058 tumor cells were immunoprecipitated using anti-RelA antibody or anti-Stat3 or preimmune serum (PIS) control, followed by immunoblotting analysis with specified antibodies. Rightmost panel: effects of knocking down *Stat3* and *RelA* on p-RelA versus total RelA in the nucleus.

that NF-κB p50 is an acetylated protein (Chen and Greene, 2003; Deng and Wu, 2003), we tested whether p50 acetylation was also regulated by Stat3. Compared to RelA, the p50 acetylation level was relatively low in A2058 tumor cells and was not affected by *Stat3* knockdown (Figure S2).

These findings prompted us to further investigate a potential role of Stat3 in regulating RelA acetylation. Trichostatin A (TSA), a selective inhibitor of multiple HDACs, has been shown to increase acetylation of RelA and inhibit its interaction with IκBα (Chen et al., 2001). Treatment of A2058 melanoma cells with TSA increased the proportion of acetylated RelA (Figure 3B). Moreover, the presence of Stat3C, a constitutively activated Stat3 mutant (Bromberg et al., 1999), led to further enhancement of RelA acetylation accompanied by the loss of RelA/IκBα complexes, as shown by the absence of IκBα protein in RelA complexes assessed by immunoprecipitation (Figure 3B). This finding suggested that Stat3 activity inhibits RelA affinity for IκBα. To test this, nuclear RelA complexes were isolated from A2058 tumor cells transfected with either a control siRNA or Stat3 siRNA, followed by incubation with recombinant IκBα. We found that nuclear RelA from *Stat3* knockdown tumor cells showed increased affinity for IκBα (Figure 3C).

Cytokines and growth factors elevated in the tumor environment are known to activate Stat3 in normal cells, including DCs, macrophages, and myeloid-derived suppressor cells in the tumor stroma, promoting tumor immunosuppression and angiogenesis (Kortylewski et al., 2005; Kujawski et al., 2008; Yu et al., 2007). NF-κB is also constitutively activated in immune cells in the tumor stroma (Greten et al., 2004). To determine whether Stat3-mediated RelA acetylation and nuclear retention observed in cancer cells were also operative in immune cells exposed to the tumor milieu, we tested whether Stat3 activity promoted RelA acetylation in DCs stimulated by the Stat3 activator IL-10, which is frequently elevated in cancer. Normal splenic DCs were isolated from mice with $Stat3^{+/+}$ and $Stat3^{-/-}$ myeloid compartments. We found that IL-10 activation of Stat3 in DCs from mouse spleens was coupled with an increase in RelA acetylation that was greater than that observed with TNFα stimulation (Figure 3D). Lack of Stat3 in $Stat3^{-/-}$ splenic DCs effectively blocked IL-10-induced RelA acetylation (Figure 3D), indicating a critical requirement of Stat3 for RelA acetylation in immune cells exposed to the tumor milieu.

RelA Acetylation by p300 Acetyltransferase Requires Stat3 Phosphorylation and DNA Binding

To further test Stat3's role in mediating RelA acetylation, we investigated whether Stat3 facilitates RelA acetylation through p300, as previous studies have shown that RelA acetylation is mediated by p300 (Chen et al., 2001), which also can serve as a Stat3 coactivator (Darnell, 2002). Cotransfection of p300 with the constitutively activated Stat3 mutant Stat3C led to greater acetylation of RelA

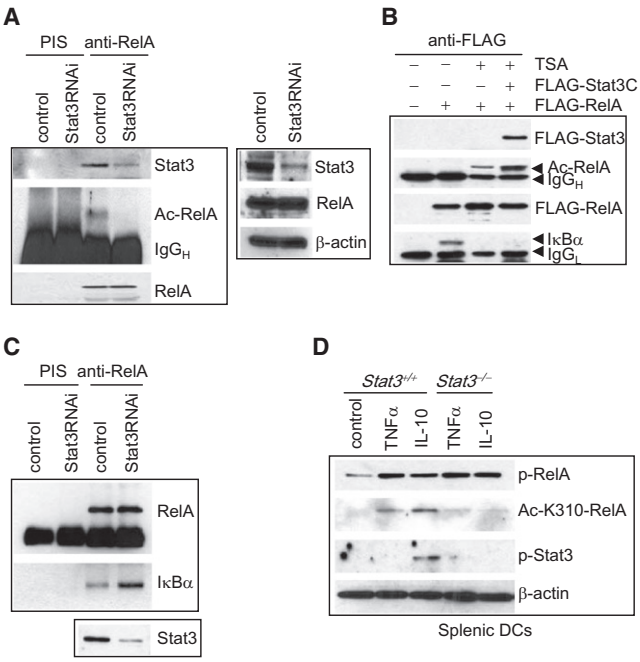

Figure 3. RelA Acetylation Is Mediated by Stat3

(A) Inhibition of Stat3 decreases RelA acetylation in A2058 cells. siRNA-transfected cells were immunoprecipitated with anti-RelA antibody; endogenously acetylated RelA protein (Ac-RelA) was then detected by anti-Ac lysine antibody (left). Stat3 inhibition by siRNA is shown at right.

(B) Constitutively active Stat3C enhances trichostatin A (TSA)-mediated RelA acetylation while inhibiting interaction between RelA and IκBα. BALB/c 3T3 cells were transfected with the indicated expression vectors and treated for 18 hr with TSA. Western blot analyses with specified antibodies are shown.

(C) Stat3 activity inhibits tumor nuclear RelA's affinity to IκBα. Nuclear RelA from A2058 tumor cells treated with control or Stat3 siRNA was allowed to interact with recombinant IκBα, followed by western blot analysis. The effects of Stat3 siRNA are shown below.

(D) Stat3 activators frequently elevated in tumors, such as IL-10, induce acetylation of RelA in primary immune cells in a Stat3-dependent manner. Splenic DCs isolated from *Stat3*$^{+/+}$ or *Stat3*$^{-/-}$ animals were treated with either TNFα or IL-10, followed by western blot analysis.

than transfection of either one alone (Figure 4A, left). Coexpression of Stat3C and p300 in 3T3 fibroblasts also resulted in increased nuclear RelA levels (data not shown). Furthermore, levels of p300 and acetylated RelA in the complex were Stat3 dependent in both B16 melanoma (Figure 4A, right) and A2058 cancer cells (data not shown). To demonstrate that RelA activity in tumors was dependent upon the interaction with Stat3 and p300, we performed NF-κB DNA binding assays using an NF-κB DNA-binding oligonucleotide and nuclear extracts prepared from growing B16 melanoma tumors. Constitutive NF-κB activity in tumors was blocked by preincubation with either Stat3 or p300 antibody (Figure 4B), but not by anti-c-Rel antibody, suggesting that constitutive activation of the NF-κB complex in tumors involves both Stat3 and p300 (Figure 4B, upper left). To further confirm that the NF-κB DNA-binding complex contained Stat3 and p300, an oligo binding assay using biotin-labeled NF-κB DNA-binding sequences was performed. After incubation with nuclear extract, NF-κB complexes were pulled down by streptavidin-conjugated magnetic beads, followed by western blot analysis. Our results suggested Stat3, p300, and NF-κB were in the same DNA-binding complex in tumors (Figure 4B, upper right). Moreover, the interaction between DNA-bound RelA and p300 was reduced in growing tumors from *Stat3*$^{-/-}$ mice (Figure 4B, upper right). Similar to the observations in murine melanoma tumors, p300, Stat3, and RelA were in the same DNA-binding complex in A2058 melanoma cells (Figure 4B, lower left), and the RelA/p50 heterodimer was detected in the DNA-binding complex (Figure 4B, lower left). In contrast, NF-κB DNA complexes induced by TNFα in DC 2.4 cells, which have low Stat3 activation levels (data not shown), did not contain Stat3 and p300 (Figure 4B, lower right).

To explore this interaction in more detail, we tested which part of the Stat3 protein was critical for p300-mediated RelA acetylation. Cotransfection of RelA, Stat3, and p300 expression vectors into *Stat3*-deficient murine embryonic fibroblasts (MEFs), followed by oncostatin M (OSM) treatment to stimulate Stat3, led to an increase in RelA acetylation mediated by p300 and Stat3 (Figure 5A, left panel). Because Stat3 phosphorylation at Ser727 is known to be important for interaction with p300 (Schuringa et al., 2001), we tested whether a Ser→Ala (S/A) mutation in Stat3 at the 727 residue would interfere with p300-mediated RelA acetylation. Cotransfecting the T7-tagged RelA with GFP-Stat3(S/A) reduced p300 incorporation into the RelA protein complex as well as levels of acetylated RelA (Figure 5A). Similarly, when we overexpressed a Stat3 Tyr705 mutant (Y/F) that does not efficiently translocate into the nucleus but is found in both the cytoplasm and nucleus (Pranada et al., 2004), RelA acetylation was inhibited upon OSM stimulation relative to the WT Stat3. Furthermore, when a Stat3 DNA-binding mutant (3D) was expressed in *Stat3*-deficient MEFs, RelA acetylation was compromised relative to the constitutively activated Stat3 mutant, Stat3C (Figure 5A, right panel). A critical role of the DNA-binding domain in mediating Stat3 interaction with NF-κB has been described previously (Yu and Kone, 2004).

These results led us to test whether RelA acetylation was required for Stat3-mediated RelA nuclear retention. We cotransfected Stat3C into *Stat3*$^{-/-}$ MEFs with vectors encoding WT RelA, RelA-K221R (221), RelA-K310R (310), or RelA-K218/221/310R (K/R) mutants, all of which have been reported to have markedly reduced acetylation (Chen et al., 2002). While Stat3C induced nuclear accumulation of WT RelA, it failed to mediate stable nuclear retention of RelA K/R mutants (Figure 5B, left panel). Additional experiments using OSM to stimulate Stat3 further demonstrated that Stat3-mediated RelA nuclear retention required RelA acetylation (Figure 5B, right panel).

The results shown in Figure 5 could have resulted from a Stat3-mediated increase in p300 histone acetyltransferase (HAT) activity. We assessed this possibility by transfecting either WT Stat3 or Stat3 S727A expression vectors into B16 tumor cells with relatively low endogenous Stat3 activity, followed by in vitro acetylation assays. The results of these experiments suggested that Stat3 facilitated p300 recruitment to RelA but did not directly increase p300 HAT enzymatic activity (Figure S3).

RelA Is Highly Acetylated in Mouse and Human Tumors: A Critical Role for Stat3

A correlation between Stat3 and NF-κB activity and cancer progression has been well documented. To validate the

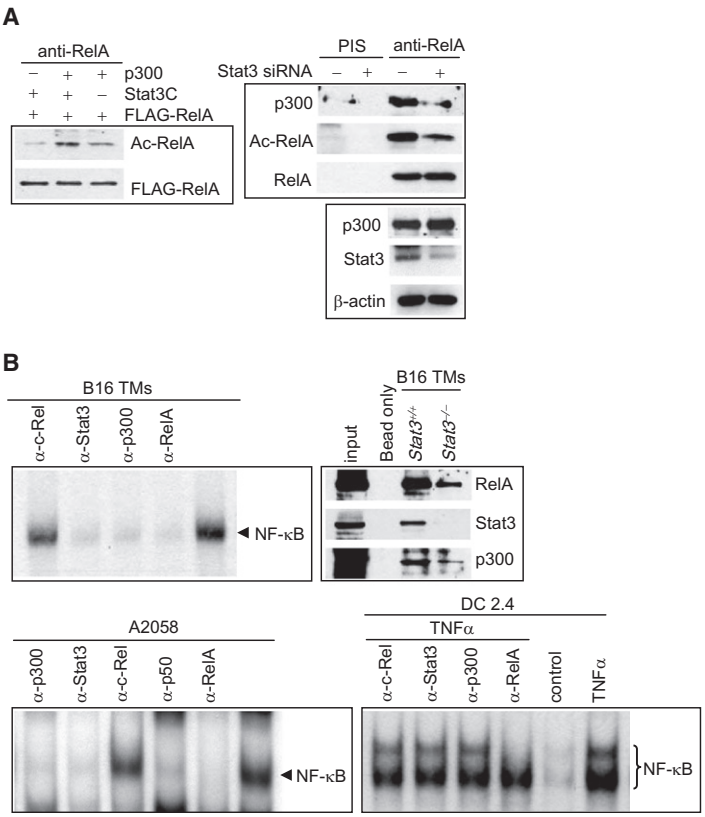

Figure 4. NF-κB Interaction with p300 Acetyltransferase Requires Stat3 Activity

(A) Stat3-associated RelA acetylation is mediated by p300. Left: activated Stat3 increases p300-mediated RelA acetylation. 3T3 cells were transiently transfected with plasmids expressing p300 and/or Stat3C together with RelA, followed by western blotting. Right: p300 protein interacts with acetylated RelA in a Stat3-dependent manner. B16 melanoma cells were transfected with either control or Stat3 siRNA. Nuclear RelA proteins were immunoprecipitated with anti-RelA antibody, followed by western blot analysis with the indicated antibody (top). Total p300 levels were determined in whole cell extracts (bottom).

(B) The tumor NF-κB/DNA complex contains both p300 and Stat3, whereas the TNFα-induced NF-κB/DNA complex in DCs does not. Top left: nuclear extracts prepared from B16 tumors were subjected to EMSA to detect NF-κB DNA binding activity. Specific antibodies as indicated were used to identify Stat3, RelA, and p300 in the binding complex. Top right: nuclear extracts from B16 tumors were incubated with biotin-labeled oligonucleotides containing NF-κB sites, and the DNA-bound RelA complex was pulled down by streptavidin beads, followed by western blotting. Bottom left: NF-κB RelA/p50 complexes with p300 and Stat3 in A2058 cancer cells. Bottom right: NF-κB/DNA complexes in DC 2.4 cells after TNFα treatment were distinct from that found in tumor cells with constitutively activated Stat3.

importance of Stat3-mediated RelA acetylation in cancer, we utilized tumor-associated myeloid cells to examine whether Stat3 was required for RelA acetylation in vivo. While CD11b$^+$ myeloid cells isolated from B16 tumors contained both phosphorylated and acetylated RelA, in vivo targeted functional deletion of *Stat3* in the myeloid compartment diminished levels of both phospho-RelA and acetylated RelA (Figure 6A). Furthermore, immunohistochemical analyses confirmed the heavy presence of acetylated RelA in B16 tumors, mainly in the nuclear compartment (Figure 6B, left panels). In contrast, the level of acetylated RelA was greatly diminished in whole B16 tumors with minimal Stat3 activity (Figure 6B, right panels), due to *Stat3* ablation in the tumor-infiltrating myeloid cells (Figure 1A, left panel). Of the eight slides examined, only one section of B16 tumor tissue from a mouse with a *Stat3*$^{-/-}$ myeloid compartment exhibited detectable (but low) levels of acetylated RelA (data not shown). Other sections did not exhibit detectable acetylated RelA (Figure 6B, right panels). These results emphasize that the Stat3-dependent NF-κB acetylation/nuclear retention described here is not totally cell-autonomous, but rather that crosstalk between tumor cells and nontransformed hematopoietic cells in the tumor microenvironment is particularly important to amplify this interaction in multiple cellular components within the tumor in vivo.

In order to determine whether our findings regarding Stat3-mediated RelA acetylation are important for human cancers, we next analyzed human tumors and normal tissues for phospho-RelA, acetylated RelA, and phospho-Stat3. Malignant human tissues of different origins were subject to immunohistochemical staining and confocal microscopic analyses. We used sections from the same tumor tissues to costain with anti-phospho-Stat3 and anti-Ac-RelA or with anti-phospho-Stat3 and anti-phospho-RelA. Results from these analyses indicated that phospho-Stat3, acetylated RelA, and phospho-RelA were highly elevated in human tumor cells and that they colocalized well in the tumor cell nuclei (Figure 7; Figure S4A). Nuclear colocalization of constitutively activated Stat3 and RelA is also observed in many other types of human tumors (Figure S4B). Furthermore, there was little detectable phospho-Stat3, phospho-RelA, or acetylated RelA in nontransformed human tissues (Figure S4C; spleen and lung tissue slides are shown as examples).

DISCUSSION

Crosstalk between Stat3 and NF-κB has been demonstrated at multiple levels, including activation of Stat3 by NF-κB-regulated factors such as IL-6 (Naugler et al., 2007; Zhong et al., 1994) and Cox-2 (Dalwadi et al., 2005), possible inhibition of IKK activity in normal immune cells by Stat3 (Welte et al., 2003), and nuclear translocation of unphosphorylated NF-κB by unphosphorylated Stat3 (Yang et al., 2007). However, how the two transcriptional factors in their phosphorylated forms interact in the nucleus in cancer has not been elucidated. A number of recent studies have focused on the importance of NF-κB in both epithelial and myeloid cells in epithelial carcinogenesis (Karin and Greten, 2005). TNFα has been found to be frequently elevated in cancer and is critical for inflammation-induced cancer through activating NF-κB (Greten et al., 2004; Pikarsky et al., 2004). Our results do not contradict these findings but rather indicate that NF-κB activity regulating multiple critical oncogenic processes is determined in part by its interaction with activated Stat3. Our results therefore functionally link these two transcription factors that are frequently activated in cancer. Our data further suggest that the shift in equilibrium between acetylation and deacetylation of RelA toward hyperacetylation, which is driven by constitutively

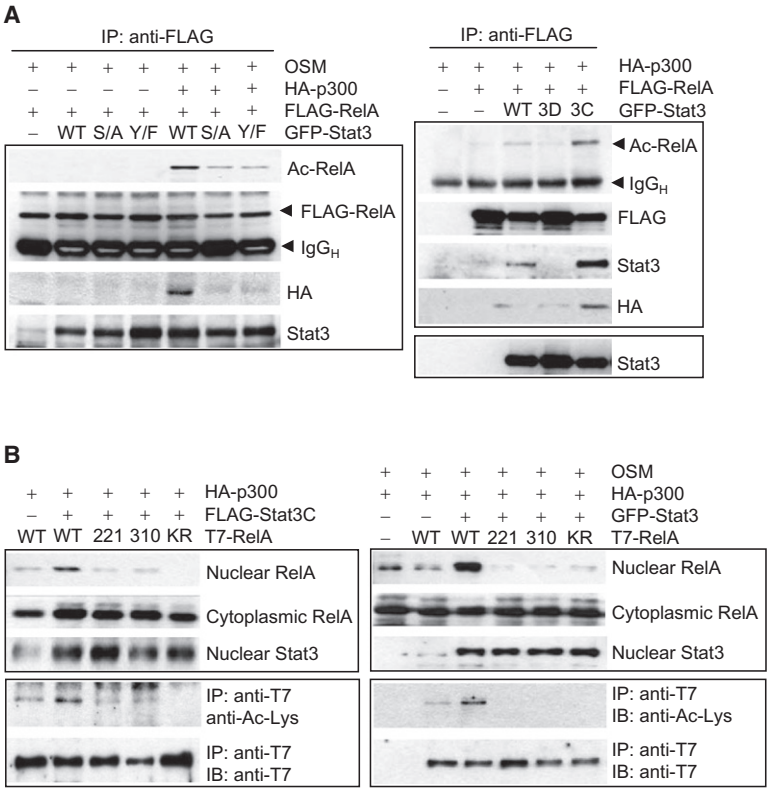

Figure 5. Stat3-Mediated p300 Interaction Regulates RelA Acetylation that Modulates Its Nuclear Retention

(A) Stat3 Ser727, Stat3 Tyr705, and Stat3 DNA binding are all important for RelA acetylation. *Stat3*-deficient MEFs were transfected with wild-type (WT) Stat3, Stat3 S727A (S/A) or Y705F (Y/F), Stat3D (dominant-negative DNA-binding mutant), or Stat3C (constitutively activated mutant). Samples shown in the left panels were treated with oncostatin M (OSM) to stimulate Stat3 activation.

(B) RelA acetylation is critical for Stat3-mediated RelA nuclear retention. Left: Stat3C and p300 were cotransfected with WT RelA, RelA K221R, RelA K310R, or RelA K218/221/310R (KR). RelA levels in nuclear and cytoplasmic extracts were determined by western blot. Coimmunoprecipitation to determine RelA acetylation was performed using the nuclear extract. Right: OSM was used to stimulate Stat3 in *Stat3*$^{-/-}$ MEFs transfected with the indicated vectors. Nuclear and acetylation levels of RelA were detected as described above.

activated Stat3, contributes to NF-κB activation in both tumor cells and the tumor microenvironment. Our studies also reconcile the roles of NF-κB and Stat3 in mediating the complex interactions between the tumor and its immune microenvironment.

The data generated by expressing various Stat3 mutants in MEFs lacking intact *Stat3* alleles suggest that Stat3-mediated RelA acetylation requires serine (727) and tyrosine (705) phosphorylation, as well as the DNA-binding domain of Stat3 protein. The reason that serine phosphorylation is important for RelA acetylation is likely that it is the critical site for Stat3 interaction with p300 (Schuringa et al., 2001). It has been documented that both interaction of RelA with p300 and acetylation of RelA by p300 require phosphorylation of RelA (Chen et al., 2005). Because unphosphorylated Stat3 (Y705F) preferentially interacts with unphosphorylated RelA (Yang et al., 2007), it is plausible that only phosphorylated Stat3 is able to interact with p300/phosphorylated RelA efficiently, leading to RelA acetylation. As for why mutation of the DNA-binding domain of Stat3 inhibits its ability to activate NF-κB, it has been reported that the Stat3 DNA-binding domain is critical for mediating interaction with RelA (Yu and Kone, 2004). Our data are consistent with these reports in that S/A and Y/F Stat3 proteins were able to interact with RelA but Stat3D was not (Figure 5A). At the same time, these Stat3 mutants, which do not efficiently interact with p300, RelA, or phosphorylated RelA, were not able to facilitate RelA acetylation. Although our results suggest an important role of p300 in facilitating acetylation of phosphorylated RelA, it is possible that other acetyltransferases, such as Tip60 or NcoA/SRC1, which interact with Stat3 (Giraud et al., 2002; Xiao et al., 2003), could also contribute to RelA acetylation.

While IKK gene silencing did not abrogate NF-κB (RelA) activity already present in the tumor cells tested, our results do not challenge the importance of IKK activity in the carcinogenic process. In fact, Stat3-mediated NF-κB nuclear retention during cancer development likely depends on IKK activation. Constitutive activation of NF-κB has been shown to be induced by secreted cytokines and growth factors in cancer cells (Lu et al., 2004; Lu and Stark, 2004). Many of these cytokines and growth factors are encoded by NF-κB target genes that require IKK activation. In several mouse carcinogen or tissue damage-induced inflammation cancer models, IKK is critical for tumor initiation (Karin and Greten, 2005). NF-κB activity in turn upregulates IL-6, which in turn activates Stat3, leading to cancer development (Naugler et al., 2007). Interestingly, in the carcinogen/inflammatory murine cancer model, in which IKK is required for IL-6 production and tumor development, IKK activation is not constitutive, whereas Stat3 is, by carcinogen treatment (Naugler et al., 2007). Recent publications have further demonstrated a critical requirement of Stat3 for tumor growth in IL-6-mediated murine inflammatory cancer models (Bollrath et al., 2009; Grivennikov et al., 2009). Although IKK activity is not constitutive, it is likely that periodic activation of IKK is required for NF-κB activity by facilitating its nuclear entry, while IL-6 production, which activates Stat3, is necessary to maintain oncogenic progression in such models and in certain human cancers. A2058 melanoma cells, like many other tumor cells, sustain mutations, including in c-Src, that activate Stat3 (Niu et al., 2002). In these cultured tumor cells, IKK activation may not be as critical for maintaining NF-κB activity. Nevertheless, a critical role of IKK and many of its activators, including TNFα, in tumor initiation by tissue damage/inflammation and in chronic inflammation-associated cancer has been well documented (Hu et al., 2004; Karin and Greten, 2005; Naugler et al., 2007). At the same time, IKK-mediated cancer-promoting effects could be due to its interactions with molecules and pathways other than NF-κB (Hu et al., 2004; Lee et al., 2007). The interactions between Stat3 and RelA in both cancer cells and immune cells within the tumor microenvironment shown by our results are

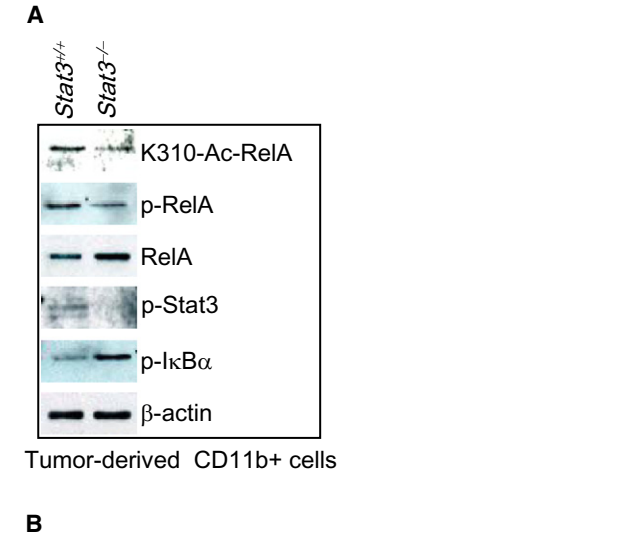

Figure 6. Acetylation of RelA in Growing Tumors Requires Stat3

(A) $CD11b^+$ myeloid cells were purified from B16 tumors harvested from mice with $Stat3^{+/+}$ and $Stat3^{-/-}$ myeloid compartments, followed by western blot analysis utilizing an antibody specific for Ac-RelA(K310).

(B) High levels of acetylated RelA in B16 tumor cell nuclei from mice with $Stat3^{+/+}$ myeloid cells, but not in tumors grown in mice with $Stat3^{-/-}$ myeloid cells. Frozen sections from O.C.T.-embedded tumor tissues were stained with an antibody against acetylated RelA (green) and mounted in medium containing DAPI to show nuclei (blue). The middle panels show magnifications of the left panels. Left and right panel scale bars = 200 μm; middle panel scale bar = 50 μm.

distinct, having opposite consequences for overall NF-κB activity in tumor versus normal immune cells responding to immunostimulatory signals. These findings define a cooperativity between Stat3 and NF-κB in cancer and help explain why both transcription factors appear to stimulate a highly overlapping repertoire of prosurvival, proliferative, and proangiogenic genes (Catlett-Falcone et al., 1999; Darnell, 2002; Lo et al., 2005; Yu and Jove, 2004). Our finding that this Stat3/NF-κB interaction extends to tumor-associated hematopoietic cells emphasizes its importance in the tumor microenvironment. Our study also provides evidence that in both murine tumors and human cancers, RelA is acetylated, which correlates with and is in part regulated by Stat3 activity. These findings may have implications for developing cancer therapeutics.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents

A2058 human melanoma and DU145 human prostate cancer cells were obtained from the American Type Culture Collection. The DC 2.4 mouse dendritic cell line was obtained from K.L. Rock (University of Massachusetts Medical School). The C4 mouse melanoma cell line was kindly provided by I. Fidler (University of Texas M.D. Anderson Cancer Center). Tumor supernatant was prepared with C4 mouse melanoma cells; confluent cells received reduced amounts of fresh medium, which was then collected 24 hr later, filtered to remove cell debris, and added to fresh culture medium at 10% final concentration to stimulate Stat3 in DCs overnight prior to cell harvesting. Polyclonal antibodies recognizing Stat3 (C-20 and C-20x) and RelA (C-20); siRNAs targeting Stat3, RelA, and p50; and control siRNA were from Santa Cruz Biotechnology. siRNA against p300 was from Dharmacon. Antibodies against acetyl-lysine, IκB-α, phospho-IκBα, and phospho-RelA (S536) were from Cell Signaling Technology. Antibody against anti-p300 was from Upstate Biotechnology. T7-tagged RelA WT and RelA K221R, K310, and K218/221/310R mutants as well as anti-acetyl K310 RelA antibody were kindly provided by W. Greene (University of California, San Francisco) and Abcam, respectively.

Transfection

Cells were seeded (5×10^5) in 100 mm plates 24 hr before transfection with siRNA using Lipofectamine 2000 (Invitrogen). For some experiments, cells were treated for 15 min at 48 hr posttransfection with 20 ng/ml TNFα (Endogen). To activate Stat3, cells received 10 ng/ml oncostatin M (Sigma) or 20 ng/ml IL-10 (PeproTech) for 20 min.

Coimmunoprecipitation and Immunoblotting Analysis

Stat3/NF-κB interaction was tested by coimmunoprecipitation analysis. Nuclear extracts (50 μg) from A2058 cells were diluted ten times with modified RIPA buffer containing 50 mM Tris (pH 7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, and protease inhibitor cocktail (Roche) and then incubated overnight with 2 μg of either anti-RelA or anti-Stat3 antibody. Preimmune serum was used as the antibody control in each experiment. Immune complexes were pulled down by addition of protein A agarose (30 μl) followed by further incubation (1 hr). After extensive wash with modified RIPA, immunoprecipitates were separated by SDS-PAGE and subjected to immunoblotting. To detect the level of acetylated RelA protein, cells were transfected with either T7- or FLAG-tagged RelA plasmid DNA for 24 hr and treated overnight with TSA (400 ng/ml) before harvesting. For cell fractionation analysis, cells were transfected with siRNA for 48 hr. Nuclear and cytoplasmic extracts were prepared as described previously (Catlett-Falcone et al., 1999).

Electrophoretic Mobility Shift Assay

DNA binding assays were carried out as described previously (Catlett-Falcone et al., 1999). Briefly, nuclear extracts (4 μg) were incubated for 20 min at room temperature with 50,000 cpm of ^{32}P-labeled probe. The oligonucleotide probe sequence to detect NF-κB binding was 5′-GATCCATTAGGGGATGCCCCT CAT-3′. For antibody supershift, antibody (1 μg) was preincubated with protein for 15 min prior to the addition of radiolabeled probe.

Oligonucleotide Pull-down Assay

Two complementary oligonucleotides containing the NF-κB site (sequence shown above) were labeled with biotin according to the manufacturer's instructions (Pierce). After labeling, oligonucleotides were annealed and 1 μg of double-stranded oligonucleotides was incubated with 300 μg of nuclear extract from A2058 cells in 500 μl of binding buffer containing 12% glycerol, 12 mM HEPES (pH 7.9), 4 mM Tris (pH 7.9), 150 mM KCl, 1 mM EDTA, 1 mM DTT, and 10 μg poly(dI:dC). Protein complexes bound to oligonucleotides were then pulled down by incubation with 50 μl of streptavidin beads (Promega)

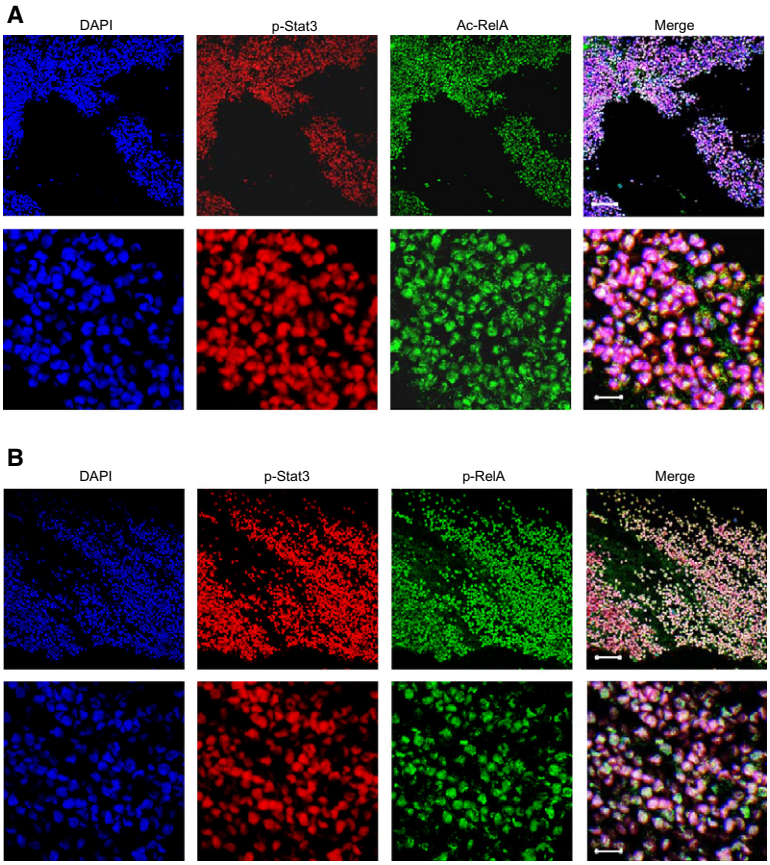

Figure 7. Acetylated RelA Is Prominently Present in Human Tumors and Colocalizes with Activated Stat3 in the Nuclei

RelA is constitutively acetylated (A) and phosphorylated (B) in human tumors and colocalizes with p-Stat3. Sections of human lung cancer specimens were stained with the indicated antibodies, followed by confocal microscopic analysis. In each of (A) and (B), the bottom row of images shows magnifications of the top row of images. Top panel scale bars = 40 μm; bottom panel scale bars = 10 μm.

preadsorbed with 1 mg/ml BSA, 50 μg poly(dI-dC), and 50 μg sheared salmon sperm DNA. After extensive washing with binding buffer, protein complexes were separated by SDS-PAGE, blotted, and probed with antibodies. For in vitro IκBα pull-down assay, tumor RelA complexes were isolated from the nuclei of A2058 cells by immunoprecipitation with anti-RelA antibody and then incubated for 2 hr at room temperature with recombinant IκBα proteins (BioSource). Complexes were then detected by immunoblotting.

Immunofluorescence Staining and Confocal Microscopy

A2058 cells were seeded on coverslips in six-well culture plates and transiently transfected with either control or Stat3 siRNA. Cells were fixed for 20 min with 2% paraformaldehyde, permeabilized for 5 min with PBS containing 0.1% Triton X-100 (PBS-T), quenched with 50 mM NH_4Cl in PBS-T, and blocked with 1% BSA in PBS-T. Immunostaining was performed using antibodies as indicated in Figure 1B, and images were acquired using a Zeiss LSM 510 META NLO confocal microscope with a plan apo 63×/1.4 NA lens.

To prepare frozen sections, B16 tumors harvested from *Stat3*$^{flox/flox}$ or *Cre/Stat3*$^{flox/flox}$ mice were embedded in O.C.T. (Tissue-Tek) and frozen in liquid nitrogen. Sections were air dried, fixed in 2% formaldehyde, and permeabilized in cold methanol before immunofluorescence staining with antibodies. The expression level of each protein in tumor tissues was visualized by a Nikon Eclipse TE2000-U microscope and imaged using SPOT software.

Human tissue array slides (including both normal and malignant tissues), obtained from the archives of the Pathology Laboratory at City of Hope National Medical Center, were used to detect acetylated versus phosphorylated RelA and phosphorylated Stat3 proteins. Preparation of tissue arrays was approved by the Institutional Review Board at City of Hope, and use of these tissue array slides was exempt as anonymous, archived specimens. For antibody staining, tissue slides were deparaffinized, rehydrated through an alcohol series, and then boiled in Antigen Unmasking Solution (Vector). After incubation with a blocking solution containing 10% goat serum (Sigma) in PBS, the sections were stained overnight at 4°C with a 1:50 dilution of a primary antibody. After incubation with a secondary antibody (Alexa 488 for green signal and Alexa 546 for red; Invitrogen), sections were mounted in Vectashield mounting medium containing 4′6′-diamidino-2-phenylindole (DAPI) (Vector). Images obtained by confocal microscopy were prepared by Zeiss LSM Image Browser software.

In Vivo Experiments

Mouse care and experimental procedures were performed under pathogen-free conditions in accordance with established institutional guidance and approved protocols from the Institutional Animal Care and Use Committee of Beckman Research Institute at City of Hope National Medical Center. *Mx1-Cre* mice were obtained from The Jackson Laboratory, and *Stat3*$^{flox/flox}$ mice were generously provided by S. Akira and K. Takeda of Osaka University. Generation of mice with *Stat3*$^{-/-}$ hematopoietic cells by the inducible *Mx1-Cre* recombinase system has been described previously (Kortylewski et al., 2005; Lee et al., 2002; Wang et al., 2004). Tumor challenge was performed in *Stat3*$^{flox/flox}$ or *Cre/Stat3*$^{flox/flox}$ mice 5 days after poly(dI:dC) treatment, which induces *Stat3* ablation mainly in the hematopoietic system. Mice were sacrificed 2–3 weeks after tumor challenge, and spleens and tumor specimens were harvested. Purification of specific immune subsets has been described previously (Kortylewski et al., 2005). Proteins and RNA were prepared from isolated immune cells, whole spleens, and whole tumors for various analyses as indicated.

In Vitro IP Kinase Assay

IKKβ kinase complexes were immunoprecipitated from cells transfected with siRNA in the presence or absence of TNFα. Kinase activity was measured in 20 μl reaction buffer containing 1 mM ATP, 10 μCi [γ-^{32}P]ATP, and 1 μg recombinant IκBα. ELISA-based kinase assay was performed according to the manufacturer's instructions (SuperArray).

HAT Assay

The enzymatic activity of p300 was assayed by incubating p300 immunoprecipitates for 30 min at 30°C with either 2 μg of histone H4 peptides (Upstate) or 0.5 μg of recombinant RelA (BioSource) as substrate. Reaction mixtures were spotted on P81 filters and washed extensively. Incorporation rate of [^{3}H]acetyl-CoA was measured by liquid scintillation counting.

SUPPLEMENTAL DATA

The Supplemental Data include four figures and can be found with this article online at http://www.cancercell.org/supplemental/S1535-6108(09)00071-3.

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