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STAT3 restrains RANK- and TLR4-mediated signaling by suppressing expression of the E2 ubiquitin ligase Ubc13

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Abstract

The transcriptional regulator STAT3 curbs pro-inflammatory cytokine production mediated by NF-κB signaling in innate immune cells, yet the mechanism by which this occurs has been unclear. Here we identify STAT3 as a pivotal negative regulator of Ubc13, an E2 ubiquitin-conjugating enzyme that facilitates TRAF6 K63-linked ubiquitination and NF-κB activation. Ubc13 accumulates intracellularly in the absence of STAT3. Depletion of Ubc13 in *Stat3*-deficient macrophages subdues excessive RANKL- or LPS-dependent gene expression, indicating Ubc13 overexpression mediates enhanced transcriptional responses in the absence of STAT3. In RANKL-activated macrophages, STAT3 is stimulated by autocrine IL-6 and inhibits accrual of Ets-1, Set1 methyltransferase and trimethylation of histone H3 lysine 4 (H3K4me3) at the *Ube2n* (Ubc13) promoter. These results delineate a mechanism by which STAT3 operates as a transcriptional repressor on *Ube2n*, thus modulating NF-κB activity by regulation of Ubc13 abundance. Our data suggest this pathway plays important roles in bone homeostasis and restraint of inflammation.

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Introduction

Signal transducer and activator of transcription 3 (STAT3) is a cytokine-responsive transcription factor involved in important immune functions including both pro- and anti-inflammatory mechanisms [1]. A major unresolved question centers on how STAT3 restrains pro-inflammatory cytokine production from innate immune cells such as macrophages or dendritic cells (DCs) upon Toll-like receptor (TLR) stimulation [2,3]. Conditional *Stat3* deletion in murine myeloid or DC lineages, as well as pan-hematopoietic deletion, leads to intestinal inflammation resembling human inflammatory bowel disease [2-4], underscoring the vital nature of the STAT3-mediated anti-inflammatory response. This response may be conserved, as humans with heterozygous inactivating *STAT3* mutations in autosomal dominant Hyper-immunoglobulin E syndrome (AD-HIES) present with immunodeficiency accompanied by disordered inflammation and elevated pro-inflammatory cytokines, a phenotype recapitulated in a mouse model of AD-HIES [5-7]. Strikingly, STAT3-deficiency in humans and mice also associates with bone abnormalities and elevated osteoclast amounts [5,8,9] suggesting effects on the receptor activator of nuclear factor κB (RANK) signaling pathway. By contrast, chronic STAT3 activation in tumor microenvironments induces immune suppression [10,11]. The underlying basis for the immunosuppressive and anti-inflammatory functions of STAT3 has been poorly understood.

Nuclear factor B (NF-κB) signal transduction is necessary for TLR-triggered production of pro-inflammatory cytokines from myeloid cells and DCs. Like STAT3, NF-κB has numerous roles in immunity and development, and is frequently co-opted to regulate tumorigenesis and tumor-promoting inflammation [12]. NF-κB is activated by signaling cascades involving post-translational modifications including ubiquitination. Tumor necrosis factor (TNF) receptor associated factor (TRAF6) is a critical E3 ligase that mediates NF-κB signal transduction from TLRs as well as members of the TNF receptor superfamily such as RANK [13]. TRAF6 is activated upon interaction with the ubiquitin-conjugating E2 enzyme Ubc13, which stimulates formation of the signal-promoting lysine 63 (K63) linked polyubiquitination modification [14]. Ubc13 has been implicated in multiple cellular processes including inflammation [15], although little is known about mechanisms that control its expression.

The multifunctional cytokine interleukin-6 (IL-6) links the NF-κB and STAT3 signaling cascades in inflammatory and immune responses [16]. IL-6 production is triggered by NF-κB upon TLR activation, a response that is often accompanied by concomitant generation of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNFα. IL-6 interacts with its cell surface gp130-containing receptor to elicit intracellular STAT3 and STAT1 signal transduction. Similar to STAT3, IL-6 has both pro- and anti-inflammatory roles [1]. For example, in cancer IL-6 contributes to tumor-promoting inflammation, while endogenous IL-6 restrains osteoclastogenesis as well as the level of pro-inflammatory cytokines elicited during local or systemic acute inflammation [16-18].

Here, we identify a new link between the STAT3 and NF-κB signaling pathways that may explain the anti-inflammatory function of STAT3. We found that IL-6-activated STAT3

functions as a transcriptional repressor for *Ube2n*, the gene encoding Ubc13. Thus, STAT3 inhibits accumulation of Ubc13 and thereby restrains NF-κB-mediated signaling responses.

Results

STAT3 suppresses RANK signaling and Ubc13 expression

Previous studies indicated that hematopoietic *Stat3*-deficient mice and individuals with AD-HIES due to *STAT3* mutation develop osteoporotic phenotypes [9,19]. We found tartrate-resistant acid phosphatase-positive ($TRAP^+$) osteoclasts are present in greater abundance in femurs from hematopoietic *Stat3*-deficient mice compared to *Stat3*-sufficient animals. Moreover, *Stat3*-deficient bone marrow-derived macrophages showed an increased propensity to form osteoclasts in RANK ligand- (RANKL) cultures (Figs. 1a and b). These results suggest *Stat3*-deficiency enables elevated responses to RANKL. *Stat3*-deficiency in hematopoietic cells also renders heightened TLR4 signaling [2]. TLR4 and RANK responses rely on NF-κB activation through the adaptor protein TRAF6 [13], suggesting a common mechanism of STAT3 function that converges upon TRAF6. We therefore examined RANK signal transduction to TRAF6 in bone marrow-derived macrophages. Our results show enhanced total and K63-linked ubiquitination of TRAF6 in *Stat3*-deficient macrophages upon RANKL stimulation, relative to *Stat3*-sufficient controls (Figs. 1c and d). TRAF6 induces activation of NF-κB as well as the mitogen-activated protein kinase (MAPK) cascade downstream of RANK or TLR4 [20]. We detected increased RANKL-stimulated NF-κB DNA binding activity in *Stat3*-deficient macrophages, without effects on RANK or macrophage marker protein expression (Fig. 1e and Supplementary Fig.1). Moreover, c-Jun N-terminal kinase (JNK) and p38 activation by RANKL was increased in the absence of STAT3 (Fig. 1f). These results suggest STAT3 restrains the amplitude and duration of signal transduction pathways activated by RANKL by modulating K63-linked ubiquitination of TRAF6.

Total TRAF6 protein amounts did not vary upon RANKL treatment (Figs. 1c and d), suggesting an additional factor mediates enhanced RANKL signaling in *Stat3*-deficient cells. We found Ubc13 expression was higher in *Stat3*-deficient macrophages under basal conditions, and was substantially increased in response to RANKL relative to *Stat3*-sufficient controls (Fig. 1g), correlating with elevated RANKL-dependent signal transduction and osteoclast gene expression (Figs. 1c-f, h). To examine mechanisms that lead to Ubc13 accumulation, we investigated A20, a deubiquitinase that triggers proteasome-dependent degradation of Ubc13 and negatively regulates TRAF6-mediated NF-κB and MAPK activation [21]. We found that A20 was induced by RANKL similarly in the absence or presence of STAT3 (Fig. 1i), indicating A20 is not involved in the STAT3-dependent mechanism that restrains Ubc13 expression and RANKL signaling in macrophages. Together, our results suggest Ubc13 accumulation in *Stat3*-deficient cells is an important response, regulated independently of A20, which results in augmented RANKL and inflammatory signal transduction.

RANK and TLR4 signaling is enhanced by Ubc13 upregulation

To examine involvement of Ubc13, we investigated the effects of manipulating its expression in *Stat3*-deficient cells. We generated mice that contained a tamoxifen-inducible cre transgene and floxed alleles of *Ube2n* (Ubc13) and *Stat3*. Using bone marrow cells from these animals, we deleted *Ube2n* and *Stat3* ex vivo in bone marrow-derived macrophages and investigated signaling responses to RANKL and lipopolysaccharide (LPS). We found that concomitant *Ube2n* and *Stat3* deletion repressed expression of RANKL- or LPS-induced genes to levels at or below those found in *Stat3*-sufficent cells (Figs. 2a and b), indicating a central non-redundant role for Ubc13 in rendering elevated transcriptional responses in the absence of STAT3. Importantly, shRNA-mediated knockdown of Ubc13 in *Stat3*-deficient macrophages, to amounts found in *Stat3*-sufficient cells, decreased RANKL- or LPS-dependent gene expression to levels observed in the presence of STAT3 (Supplementary Figs. 2a-c). Since Ubc13 expression in knockdown conditions approximated levels present in *Stat3*-sufficient cells, the results suggest accumulated Ubc13 as a major factor in the excessive RANKL- or LPS-mediated transcriptional response elicited in the absence of STAT3. By contrast, enforced Ubc13 overexpression in *Stat3*-sufficient macrophages rendered substantially elevated osteoclast-specific mRNA expression in response to RANKL (Supplementary Figs. 2d and e), confirming that increases in Ubc13 amounts result in enhanced gene expression.

STAT3 also stimulates the inhibitor suppressor of cytokine signaling 3 (SOCS3), which facilitates interaction of signaling proteins with the ubiquitin-mediated degradation pathway [1,22]. This raised the possibility that SOCS3 contributes to repression of Ubc13. We ruled out SOCS3 involvement by observing that enforced SOCS3 expression in *Stat3*-deficient cells did not affect Ubc13 protein abundance or LPS-dependent gene expression (Supplementary Figs. 3a and b). In comparison, STAT3 overexpression in *Stat3*-deficient cells reduced LPS-dependent gene expression and Ubc13 protein amounts (Supplementary Figs. 3c and d). Therefore, our data implicate Ubc13 as a key factor in the pathway by which STAT3 dampens RANKL- and LPS-mediated gene expression.

Autocrine IL-6-STAT3 signaling represses Ubc13 expression

We next addressed the nature of the signal that induces STAT3 activity and the mechanism by which STAT3 controls Ubc13 expression. We noted that IL-6 inhibits osteoclastogenesis [17]. This suggested an IL-6-STAT3 autocrine loop might suppress Ubc13 amounts and RANKL-mediated signaling. We found that RANKL induced *Il6* mRNA in macrophages (Fig. 3a). To test whether autocrine IL-6 regulated RANKL signaling, we used IL-6 antibody blockade or $Il6^{-/-}$ cells. We found IL-6 neutralization by antibody blockade enhanced RANKL-responsive osteoclast gene expression and Ubc13 protein amounts in bone marrow-derived macrophages (Figs. 3b and c). Moreover, $Il6^{-/-}$ macrophages showed elevated Ubc13 protein amounts, NF-κB activity and osteoclast gene expression following RANKL treatment (Figs. 3d-f). By contrast, stimulation with recombinant IL-6 suppressed Ubc13 amounts in *Stat3*-sufficient but not *Stat3*-deficient cells (Figs. 3g and Supplementary Fig. 3d). Collectively, our results indicate IL-6 triggers STAT3-dependent repression of Ubc13 expression, inhibiting RANKL- and LPS-induced signaling.

STAT3 utilizes a transcriptional mechanism to restrain *Ube2n*

Previous studies indicated the anti-inflammatory function of STAT3 relies on transcription, eliciting a response that dampens NF-κB signaling [23]. We found that direct stimulation of cells with recombinant IL-6 repressed *Ube2n* (Ubc13) mRNA amounts in macrophages, in addition to its inhibitory effects on Ubc13 protein (Figs. 3g and 4a), suggesting potential to suppress *Ube2n* transcription. Moreover, interleukin-10 (IL-10), the classic anti-inflammatory cytokine utilizing STAT3, similarly dampens *Ube2n* mRNA amounts (Supplementary Fig. 4a). To examine whether STAT3 inhibits *Ube2n* transcription, we inspected the *Ube2n* promoter sequence. We identified a conserved STATx consensus site within 100bp of the predicted *Ube2n* transcriptional start site (TSS) (Supplementary Fig. 4b). Using reporter gene assays to test the function of this conserved STATx element, we found STAT3 inhibited activity of the *Ube2n* promoter while mutation of the STATx binding site abolished STAT3-mediated repression (Fig. 4b). These data suggest STAT3 mediates transcriptional repression of *Ube2n* via the STATx site located near the TSS.

To further evaluate the mechanism by which STAT3 suppresses *Ube2n* transcription, we used STAT3 isoforms with mutations in regions that mediate transcriptional function. Overexpression of a STAT3 mutant with defective DNA binding activity (STAT3 DN) attenuated the repressive effects of STAT3 signaling on *Ube2n* reporter activity as well as Ubc13 protein amounts (Figs. 4c and d). In addition, we found the C-terminal transactivation domain (TAD) was required for the inhibitory effect of STAT3 on *Ube2n* promoter activity (Supplementary Fig. 5). Blockade of STAT3 SH2 domain function with PM-73G, a small molecule phosphopeptide-based STAT3 inhibitor [24], led to suppressed STAT3 tyrosine phosphorylation, accumulation of Ubc13 and enhanced RANKL-dependent gene expression (Figs. 4e, f and Supplementary Fig. 6a). Moreover, STAT3 V637M, an SH2 domain mutant associated with AD-HIES [6], mediated enhanced LPS-dependent inflammatory gene expression compared to wild type STAT3 (Supplementary Fig. 6b). Taken together, our data suggest STAT3 acts as a transcriptional repressor via its classic pathway (i.e., SH2-dependent dimerization, DNA binding and *trans*-mediated events) to inhibit *Ube2n*/Ubc13 expression and modulate RANKL and inflammatory signaling responses.

Ube2n repression coincides with Ets-1 and Set1 displacement

To evaluate whether STAT3 acts directly as a transcriptional repressor on the *Ube2n* promoter, we examined whether STAT3 interacts with the promoter STATx site in vivo. By performing STAT3 chromatin immunoprecipitations (ChIPs) and quantitative PCR (qPCR), we found IL-6 induced STAT3 binding to the *Ube2n* promoter in proximity to the conserved STATx site (Figs. 5a and b). Similarly, IL-10 stimulation resulted in STAT3 interaction with the *Ube2n* promoter within the region of the STATx element (Figs. 5a and c). These results indicate cytokine-induced STAT3 accumulation at the *Ube2n* promoter, which correlates with reduced mRNA expression, suggesting a direct mechanism of *Ube2n* transcriptional repression.

We considered potential scenarios for STAT3-mediated transcriptional repression, including whether cytokine-inducible STAT3 binding might affect constitutive association of

transcriptional regulators at the *Ube2n* promoter. Further inspection of the *Ube2n* promoter revealed an Ets consensus sequence that overlapped with the STATx site (Fig. 5a and Supplementary Fig. 4b). Using ChIPs, we found that the transcription factor Ets-1 was localized to the *Ube2n* promoter in the absence of cytokine stimulation (Fig. 5d). Ets-1 binding was reduced upon IL-6 stimulation in *Stat3*-sufficient but not *Stat3*-deficient cells (Fig. 5d), suggesting STAT3 mediates the decrease in Ets-1 association at the *Ube2n* promoter in response to cytokine treatment.

A recent study showed genome-wide Ets-1 binding in proximity to the chromatin modification histone H3K4me3, which is linked to transcriptional activation [25]. We analyzed effects of IL-6-STAT3 signaling on H3K4me3 at the *Ube2n* promoter by measuring H3K4me3 abundance across a 2 kb region centered on the *Ube2n* TSS. We found that IL-6 reduced H3K4me3 amounts at the *Ube2n* TSS, relative to unstimulated cells (Figs. 5a and e). Furthermore, we found IL-6-dependent H3K4me3 repression at the *Ube2n* TSS required STAT3, as indicated by the lack of suppression in *Stat3*-deficient macrophages (Fig. 5e). Significantly, the IL-6-STAT3-dependent reduction in H3K4me3 occurred in the region that STAT3 and Ets-1 occupy (Figs. 5a, b and d).

The H3K4me3 modification is deposited by Set domain-containing methyltransferases [26]. Accordingly, we assayed Set1 binding to the *Ube2n* promoter region by ChIPs. We found Set1 associated with the *Ube2n* promoter in the absence of IL-6 stimulation, while IL-6 signaling decreased Set1 accumulation in *Stat3*-sufficient but not *Stat3*-deficient cells (Fig. 5f), implying STAT3 is involved in IL-6-dependent reduction in Set1. Collectively, our results suggest STAT3 acts as a cytokine-inducible transcriptional repressor at the *Ube2n* promoter, leading to displacement of constitutively associated Ets-1, reduction in Set1 abundance and decreased amounts of the transcriptionally active H3K4me3 modification.

Discussion

Here, we demonstrate STAT3 represses Ubc13 expression by direct transcriptional inhibition, causing restraint of TRAF6 K63-linked ubiquitination, NF-κB signaling and RANKL- and LPS-responsive gene expression. To our knowledge, these results provide the first mechanistic description of how STAT3 operates as a transcriptional repressor. Furthermore, they reveal an important molecular pathway by which STAT3 exerts anti-inflammatory activity and suppresses osteoclastogenesis [1]. Our data may explain why hematopoietic-*Stat3* deficient mice and humans with heterozygous *STAT3* inactivating mutations and AD-HIES have increased inflammatory cytokine expression and skeletal abnormalities such as recurrent bone fractures [5,6,8]. By contrast, STAT3-mediated repression of Ubc13 may contribute to autocrine or paracrine effects of persistent IL-6-STAT3 signaling in disease, including cancer-promoting immune suppression [10,11]. Manipulating the STAT3-Ubc13 axis might be an approach to influence inflammatory responses for therapeutic benefit.

An anti-inflammatory role for STAT3 has been observed for decades, without the molecular basis of this function understood. Previous studies showed the anti-inflammatory activity is generated from a wide variety of STAT3-activating receptors, including natural as well as

engineered receptors [27], indicating a general signaling role for STAT3. Moreover, compelling evidence suggests STAT3 acts as a transcription factor to regulate the anti-inflammatory response [23]. Accordingly, STAT3 inhibits NF-κB family members from stimulating transcription at specific gene promoters such as *Il12p40* and *Bcl3* [28,29]; however, these data do not fully explain the wide-ranging anti-inflammatory activity of STAT3. By identifying *Ube2n* as a STAT3-respressed gene, we reveal a mechanism that has a broad effect on Ubc13/TRAF6-mediated signaling to NF-κB, which may explain the molecular basis of the general STAT3 anti-inflammatory response.

Our studies highlight an important role for autocrine IL-6-activated STAT3 in restraining RANKL- and LPS-induced gene expression. While this is consistent with the known function of IL-6 in suppressing in vitro osteoclastogenesis, IL-6 has complex effects on bone formation and resorption including promotion of RANKL production from osteoblasts and induction of myeloid progenitors that can serve as osteoclast precursors (e.g., colony-forming unit granulocyte-macrophage; CFU-GM). Furthermore, the *Il6*-deficient strain we utilized for in vitro studies may contribute to the effects observed on RANKL signaling, and thus additional work is required to confirm results in vivo [5,8,9,17,30]. Moreover, IL-6 is frequently described as a pro-versus anti-inflammatory cytokine. This characterization appears to be context specific as IL-6 elicits both pro- and anti-inflammatory activities in vivo [16-18]. In fact, recent work underscores the importance of the IL-6 anti-inflammatory function in mediating macrophage alternative activation [31]. By contrast, IL-10 is the archetypal anti-inflammatory cytokine, activity that is attributed to exclusive activation of STAT3 through the IL-10 receptor [23,32]. Importantly, we found IL-10 also recruits STAT3 to the *Ube2n* promoter and suppresses *Ube2n* mRNA, consistent with Ubc13 repression as a key component of STAT3 anti-inflammatory signaling.

Our results, however, failed to support a major function for SOCS3 in curbing LPS-dependent gene responses. SOCS3 has been proposed to mediate the anti-inflammatory function of STAT3, yet *Socs3* gene ablation in mice is protective against LPS-induced sepsis shock [33]. Moreover, mutation of the SOCS3 binding site in gp130, the signaling subunit of the IL-6 receptor, enhanced the anti-inflammatory activity of IL-6 [27,33]. Together, these data indicate SOCS3 restrains anti-inflammatory signaling, consistent with its role as a general inhibitor of cytokine signal transduction, blocking downstream Janus kinase (Jak) and STAT activity. Thus, the collective data are consistent with a potent anti-inflammatory response elicited by STAT3-dependent repression of Ubc13, which may be enhanced by SOCS3 removal.

STATs were first identified as transcriptional activators, although it is increasingly clear that certain STATs also function to repress expression of specific genes [34,35]. Here, to our knowledge, we report the first detailed description of a mechanism by which STAT3 acts as a transcriptional repressor. Our data suggest STAT3 operates via its classical signaling pathway, including SH2-domain-mediated dimerization and DNA binding to suppress *Ube2n* transcription. STAT3 association at the *Ube2n* promoter is accompanied by inhibition of constitutive Ets-1 binding, reduced amounts of the transcriptionally active chromatin mark H3K4me3 and Set1 association. This suggests at least 2 potential mechanisms for *Ube2n* inhibition: direct competition between STAT3 and Ets-1 for *Ube2n*

promoter binding, or indirect displacement of Ets-1 by an as yet unknown STAT3-recruited factor(s). Either scenario would be consistent with requirements for STAT3 SH2, DNA binding and transactivation domains, and will require further investigation to resolve. Moreover, while our results suggest Ets-1 mediates *Ube2n* transcriptional activation and, potentially, Set1 association along with H3K4me3 accumulation, these mechanisms of *Ube2n* regulation remain to be tested directly.

Significantly, the co-localization of STATx and Ets-1 binding sites at the *Ube2n*/*UBE2N* promoter is conserved between mice and humans, suggesting murine and human STAT3 may function to repress *Ube2n*/*UBE2N* similarly. In accordance, AD-HIES patients contain mutations in STAT3 domains involved in transcriptional activity, show bone abnormalities consistent with enhanced osteoclastogenesis and exhibit features of deregulated inflammation [5,8]. Thus, while our data suggest the RANKL-inhibiting and anti-inflammatory role for murine STAT3 is mediated by Ets-1 displacement on the *Ube2n* promoter, it is important to determine whether this mechanism operates on human *UBE2N*, if it occurs at other STAT3-repressed genes, and if it represents a genome-wide response to STAT3 signaling that coordinates other critical biological activities.

IL-6 is widely recognized as an important cancer-promoting cytokine. This activity is attributed to its ability to stimulate proliferation of malignant epithelial cells and induce a pro-tumorigenic microenvironment by enhancing angiogenesis and expansion of myeloid-derived suppressor cells, tumor associated macrophages and $CD4^+$ interleukin-17 (IL-17)-secreting cells [36]. Thus, therapeutic inhibition of the IL-6-STAT3 signaling axis, with both IL-6 and IL-6Rα blocking antibodies, has become a major goal, particularly for cancers induced or promoted by chronic inflammation [36]. As a STAT3-repressed gene, *Ube2n* expression levels would be expected to increase in the presence of IL-6 or STAT3 inhibitors. Accordingly, we observed Ubc13 upregulation in bone-marrow derived macrophages treated with a peptide-based STAT3 inhibitor, as well as *Ube2n* induction lung myeloid cell infiltrates upon delivery of IL-6 blocking antibodies in a mutant K-ras lung cancer model in vivo, a treatment that has a positive correlation with tumor suppression (unpublished work). These results suggest the potential for increases in Ubc13 in myeloid cells to be protective and elicit anti-tumor responses in the tumor environment. In addition, while our studies focused on myeloid responses, the fact that Ubc13 is restrained by STAT3 in murine embryonic fibroblasts (MEFs) suggest the STAT3-Ubc13 pathway may operate in non-hematopoietic cell types. Since Ubc13 has a broad role in regulating NF-κB signaling, it is crucial to understand how Ubc13 expression is affected in tumor cells as well as tumor-infiltrating and niche-associated populations, and whether STAT3-mediated effects on Ubc13 expression in specific cell types associate with pro- or anti-tumor responses. This information may help maximize therapeutic targeting in cancer.

Methods

Animals

Hematopoietic *Stat3*-deficient (*Tg(Tek-cre)12Flv Stat3*$^{f/\delta}$) mice were generated by crossing *Tg(Tek-cre)12Flv* and *Stat3*$^{f/f}$ strains [37]. Age and gender-matched wild type controls (*Stat3*$^{w/f}$) were used as indicated [37]. *Il6*$^{-/-}$ (*Il6*^{tm1Kopf}) mice were obtained from Jackson

Labs; *Il6*$^{+/+}$ mice were used as controls. *Ube2n*$^{f/f}$ mice were kindly provided by Dr. Shizuo Akira [38]. Cre-ER *Stat3*$^{f/f}$, Cre-ER *Ube2n*$^{f/f}$ and Cre-ER *Ube2n*$^{f/f}$ *Stat3*$^{f/f}$ mice were obtained by crossing Rosa26^{CreER} mice (kindly provided by Dr. Yong-Jun Liu) with *Stat3*$^{f/f}$, *Ube2n*$^{f/f}$ or *Ube2n*$^{f/f}$ *Stat3*$^{f/f}$ animals, respectively. All strains were on the C57BL/6J background; male and female mice aged 5-12 weeks were used. Randomization, blind allocation or exclusion criteria were not utilized. Animals were housed in a specific pathogen-free barrier facility. Experimental procedures were approved by the Institutional Animal Care and Use Committee at UT MD Anderson Cancer Center.

Plasmids

A 536bp fragment of the murine *Ube2n* promoter, encompassing the consensus STATx site, was amplified from mouse genomic DNA and cloned into the pGL3-Basic vector (Promega) to generate the *Ube2n* reporter (pGL3-*Ube2n*). Mutation of the STATx site in the *Ube2n* reporter was performed with the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) to generate pGL3-*Ube2n*-mut. Sequences encoding murine STAT3C (a persistently activated isoform, kindly provided by Dr. Jim Darnell) or STAT3-TAD (a transactivation domain deletion mutant) were cloned in the pMX-IRES-GFP vector and used as indicated in gene reporter assays. All plasmid inserts were verified by DNA sequencing.

Antibodies and cytokines

The following antibodies were used at the indicated dilutions for immunoblotting: pJNK (#9251, 1:1000), p-p38 (#9211, 1:1000), pERK1/2 (#9101, 1:1000) and pSTAT3 antibodies (#9131, 1:1000), obtained from Cell Signaling; JNK (C-17, 1:1000), p38 (H-147, 1:1000), ERK1/2 (K-23, 1:1000), TRAF6 (H-274, 1:1000), anti-ubiquitin (P4D1, 1:1000), RAN (C-20, 1:1000), STAT3 (C-20, 1:1000), c-Myc (9E10, 1:5,000) and tubulin antibodies (10D8, 1:2000), obtained from Santa Cruz Biotech; and anti-K63 ubiquitin (HWA4C4, 1:750) and anti-Ubc13 antibodies (#371100, 1:1000), obtained from Enzo and Invitrogen, respectively. TRAF6 antibody (H-274) was also used for immunoprecipitation (1 g/sample). For ChIPs, the following antibodies were used at 2 g/sample: STAT3 (C-20) and c-Ets-1 antibodies (C-20), obtained from Santa Cruz Biotech; H3K4me3 (CS200580) and total H3 antibodies (04-928), obtained from Millipore; and Set1 antibody (A300-289A) from Bethyl Laboratories. For flow cytometry, fluorescently labeled antibodies to CD11b (M1/70, FITC-labeled), F4/80 (BM8, PerCP Cy5.5-labeled), M-CSFR (AFS98, APC-labeled) and RANK (R12-31, PE-labeled) from eBioscience were used at a 1:100 dilution. Recombinant murine RANKL, IL-6, IL-10 and IL-6 blocking antibody were purchased from R&D Systems.

Cell lines

HEK293T cells and *Stat3*$^{-/-}$ MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). L929 cells were cultured in DMEM containing 10% FBS for 7 d to generate macrophage colony stimulating factor (M-CSF)-containing conditioned medium [39]. HEK293T and L929 were obtained from ATCC. *Stat3*$^{-/-}$ MEFs were kindly provided by Dr. David Levy.

Bone marrow cultures and in vitro osteoclastogenesis

Bone marrow cells were flushed from femurs and tibia of age-matched mice (5 to 12 weeks). Bone marrow-derived macrophages were prepared by culturing bone marrow cells in DMEM supplemented with 20% FBS and 30% M-CSF-containing conditioned medium for 4-6 d [39]. In vitro osteoclast differentiation was induced by culturing bone marrow-derived macrophages in M-CSF-conditioned media for 4 d, then adding RANKL (25 to 100ng mL^{-1}) for additional 3 to 4 d [40].

Stat3 deletion in vitro

To induce *Stat3* deletion, 4-hydroxytamoxifen (1μM, Sigma-Aldrich) was added to macrophage cultures from Cre-ER $Stat3^{f/f}$, Cre-ER $Ube2n^{f/f}$ or Cre-ER $Stat3^{f/f}$ $Ube2n^{f/f}$ mice every 48h throughout the culture period. As controls, $Stat3^{f/f}$ macrophages were cultured similarly with 4-hydroxytamoxifen.

Flow cytometry

Bone marrow derived macrophages were stained with fluorescently labeled antibodies on ice for 30 min. Cells were analyzed by flow cytometry on an LSRII machine (BD Bioscience). Data was further analyzed by FlowJo.

RNA isolation and qPCR

Total RNA was extracted by Trizol and reverse-transcribed with iScript (Bio Rad); qPCR was performed with SYBR Green PCR Mix (Bio Rad) and a sequence detector (Bio Rad 5000). The expression of individual genes was calculated and normalized to 18s RNA [37]. Data are presented as fold change between the test groups and controls, as indicated in the Figure legends. Gene-specific primers are listed in Supplementary Table I.

Immunoprecipitations and immunoblotting

For TRAF6 immunoprecipitations, cells were lysed under denaturing conditions and immunoprecipitations were performed with TRAF6-specific antibody in buffer containing 50mM Tris pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate and 1% NP-40, supplemented with 1 mM N-ethylmaleimide. Ubiquitinated TRAF6 was detected by immunoblotting with pan-ubiquitin antibodies or antibodies specific for the K63 ubiquitin linkage; total TRAF6 was detected on immunoblots by TRAF6 antibody. For all other immunoblots, whole cell lysates were generated in RIPA buffer supplemented with a phosphatase inhibitor cocktail (Roche) and subjected to immunoblotting [40]. The relative density of protein signals on immunoblots was measured by NIH Image J software. Full images of immunoblots are shown in Supplementary Figures 7-9.

Electrophoretic mobility shift assays (EMSAs)

Nuclear extracts were prepared from bone marrow-derived macrophages, and 10 g of extract was incubated with double-stranded ^{32}P-radiolabelled probes specific for NF-κB, NF-Y or Oct-1 in 12 μl of EMSA assay reaction buffer containing 2 μg of poly (dI-dC), 20 mM HEPES (pH 7.9), 1 mM $MgCl_2$, 50 mM KCl, 0.05 mM EDTA, 0.5 mM dithiothreitol (DTT), 0.05% Igepal-CA630 and 5% glycerol [41]. Protein-DNA complexes were separated

on non-denaturing 5% polyacrylamide gels containing 0.5× Tris-Borate-EDTA buffer (45 mM Tris-borate and 1 mM EDTA). Probes sequences are listed in Supplementary Table I.

Chromatin immunoprecipitations (ChIPs)

Bone marrow-derived macrophages were stimulated with IL-6 or IL-10, as indicated in the Figure legends. ChIPs were performed with antibodies to STAT3 (Santa Cruz), H3K4me3 (Millipore), total H3 (Millipore), Set1 (Bethyl Laboratories), c-Ets-1 (Santa Cruz) or rabbit IgG control (Santa Cruz) using a ChIP assay kit, per the manufacturer's instructions (Millipore). For STAT3, Set1 or c-Ets-1, ChIP results were normalized first to the relevant input sample, and then to the untreated (NT) sample from *Stat3*-sufficient cells to determine fold change. For H3K4me3 ChIPs, results were normalized to data from ChIPs with total H3 antibody. Normalized data was plotted versus the corresponding predicted ChIP amplification product from the *Ube2n* proximal promoter region, as indicated by the schematic diagram in Figure 5.

Reporter gene assays

HEK293T cells were transfected with the *Ube2n* reporter construct and the indicated plasmids (see Figure legend), using Lipofectamine LTX (Invitrogen) per the manufacturer's instructions. Cells were cultured in DMEM for 24 to 48 h. Luciferase activity was measured with a Dual Luciferase assay kit obtained from Promega using a Sirius luminometer (Berthold Detection Systems) [37].

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). The statistical significance between two groups was calculated by a two-tailed *t* test. For multiple groups, significance was evaluated by two-way Annova with Bonferroni multiple comparisons. Graphpad Prism 5 software was used for each type of analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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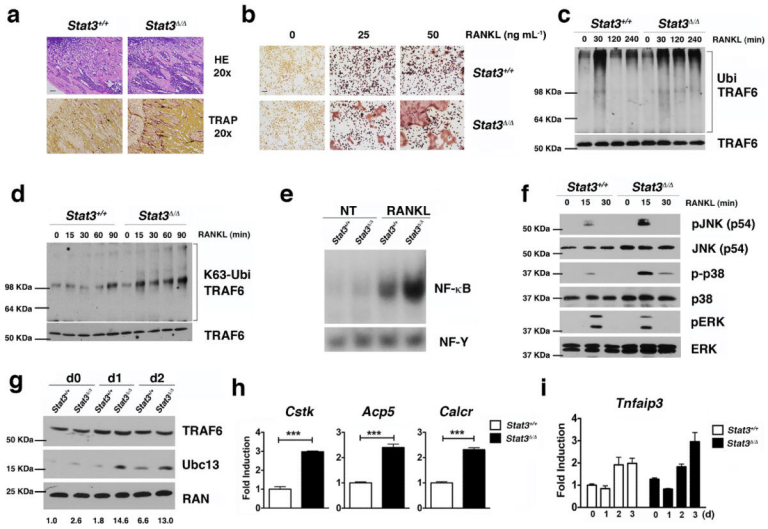

Figure 1. STAT3 suppresses osteoclastogenesis and RANK signal transduction

(a) Femoral sections from *Stat3*-deficient (*Stat3*$^{\delta/\delta}$) and -sufficient (*Stat3*$^{+/+}$) mice, 8 weeks. (**Upper**) Hematoxylin and eosin (HE); (**Lower**) TRAP staining (arrows). Scale bar, 100 M. (**b** to **i**) Bone marrow cells from *Stat3*$^{+/+}$ and *Stat3*$^{\delta/\delta}$ mice were cultured in M-CSF-containing medium for 4 d to generate bone marrow-derived macrophages. (**b**) 2×10^5 cells were cultured in M-CSF and RANKL (0, 25 or 50 ng mL^{-1}) for an additional 4 d. Osteoclasts detected by TRAP staining (pink). Scale bar, 100 M (**c to f**) Macrophages were starved of exogenous growth factor overnight and stimulated with RANKL (100 ng mL^{-1}) or left untreated, as indicated. (**c and d**) TRAF6 ubiquitination was evaluated −/+ RANKL stimulation by immunoprecipitation and immunoblotting for total ubiquitin (**c**) or K63-specific linkage (**d**). Lower panels: total TRAF6. (**e**) Nuclear NF-κB DNA binding activity was measured −/+ RANKL stimulation (15 min) by EMSA. Loading control: NF-Y EMSA. (**f**) MAPK activation was measured −/+ RANKL stimulation by immunoblotting whole cell lysates with antibodies specific for phosphorylated protein (pJNK, p-p38 or pERK). Total protein amounts served as loading controls (JNK, p38 or ERK). (**g**) Macrophages were cultured in M-CSF and RANKL (50 ng mL^{-1}). TRAF6 and Ubc13 amounts were determined by immunoblotting. Loading control: RAN. The relative level of Ubc13 is expressed as a ratio of Ubc13:RAN at each timepoint (indicated below bottom panel); amounts in *Stat3*$^{+/+}$ cells on day 0 = 1.0. (**h**) Macrophages were cultured in M-CSF and RANKL (50 ng mL^{-1}) for 3 d. Expression of osteoclast genes *Cstk, Acp5* and *Calcr* was determined by qPCR. Relative mRNA amounts for each gene expressed as fold induction versus expression in *Stat3*-sufficient cells. (**i**) A20 (*Tnfaip3*) expression was determined after culturing macrophages in M-CSF and RANKL (50 ng mL^{-1}) for 0, 1, 2 or 3 d, using qPCR. (**a**) Results represent n=5 per genotype. (**b to g**) Results represent 3 independent experiments. (**h** and **i**) Data represent mean values of 3 independent experiments. Error bars indicate SEM. (**h**) *t* test used; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ for indicated comparisons. (**i**) two-way Anova with Bonferroni multiple comparison used; not significant.

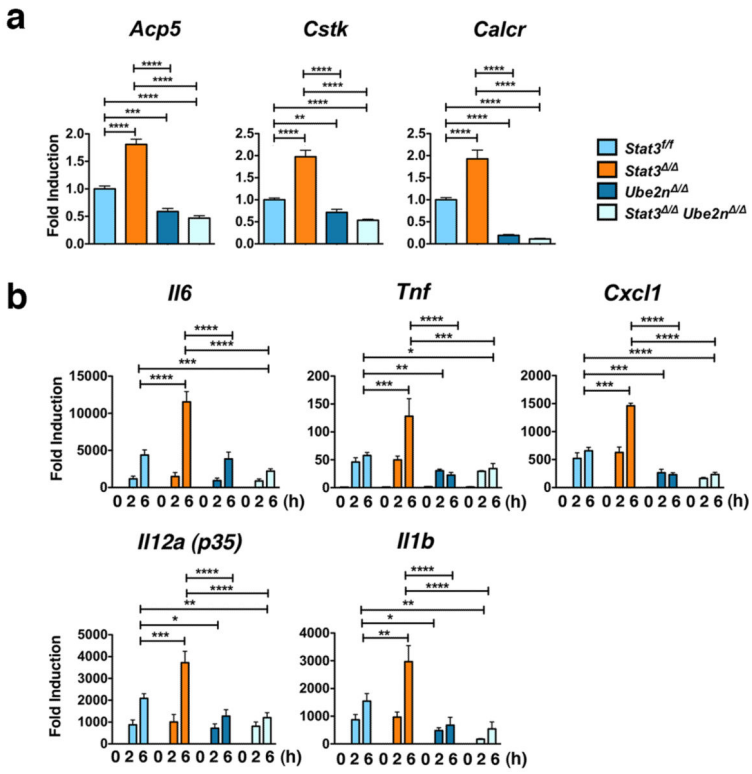

Figure 2. Ubc13 mediates enhanced RANKL- and LPS-responsive gene expression induced by *Stat3*-deficiency

Bone marrow cells from *Stat3*$^{f/f}$, Cre-ER *Stat3*$^{f/f}$, Cre-ER *Ube2n*$^{f/f}$ or Cre-ER *Stat3*$^{f/f}$ *Ube2n*$^{f/f}$ mice were cultured in M-CSF-containing media plus 4-hydroxytamoxifen for 4 d to generate *Stat3*-sufficient (*Stat3*$^{f/f}$), *Stat3*-deficient (*Stat3*$^{\delta/\delta}$), *Ube2n*-deficient (*Ube2n*$^{\delta/\delta}$) or *Stat3; Ube2n* deficient (*Stat3*$^{\delta/\delta}$*Ube2n*$^{\delta/\delta}$) macrophages, respectively. Cells were cultured in M-CSF- and RANKL-containing medium for 3 d (**a**) or stimulated with LPS (**b**), as indicated. Gene expression was analyzed by qPCR. Results represent mean values of 3 independent experiments. Error bars indicate SEM. Two-way Anova with Bonferroni multiple comparison used. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ for indicated comparisons.

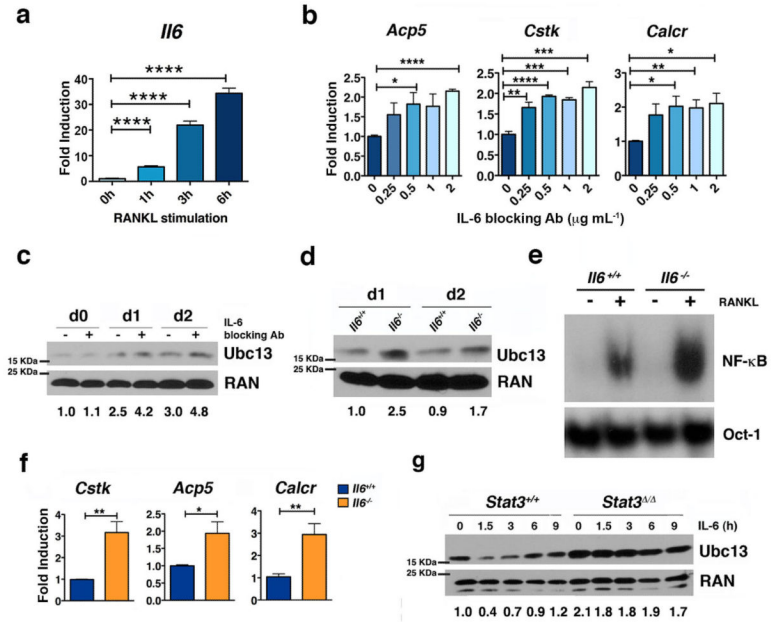

Figure 3. Autocrine IL-6 is required to repress Ubc13 expression

a) *Il6* mRNA expression was measured in bone marrow-derived macrophages by qPCR following RANKL (50 ng mL^{-1}) stimulation, as indicated. (**b and c**) Osteoclast gene expression **(b)** and Ubc13 amounts **(c)** were determined in bone marrow-derived macrophages following culture in M-CSF- and RANKL-containing medium without (−) or with (+) IL-6 blocking antibody by qPCR or immunoblotting, respectively. **(d to f)** Ubc13 amounts, nuclear NF-κB DNA binding activity and osteoclast gene expression were determined in *Il6*$^{+/+}$ and *Il6*$^{-/-}$ bone marrow-derived macrophages following culture in M-CSF- and RANKL-containing medium for 1 or 2 d (**d**), 1 d (**e**), or 3 d (**f**) by immunoblotting, EMSA or qPCR, respectively. **(g)** Ubc13 expression was evaluated in bone marrow-derived macrophages from *Stat3*-sufficient (*Stat3*$^{+/+}$) or hematopoietic *Stat3*-deficient (*Stat3*$^{\Delta/\Delta}$) mice by immunoblotting following IL-6 stimulation, as indicated. (**c**, **d** and **g**) Relative Ubc13 expression vs. untreated (**c**), IL-6-(**d**) or STAT3-sufficient control (**g**) was calculated as indicated in Fig. 1g and displayed below bottom panels. (**a** and **f**) Results represent mean values of 3 independent experiments. Error bars indicate SEM. (**a** and **b**) Two-way Anova with Bonferroni multiple comparison used; (**f**) *t* test used. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ for indicated comparisons. (**c, d, e** and **g**) Results represent 3 independent experiments.

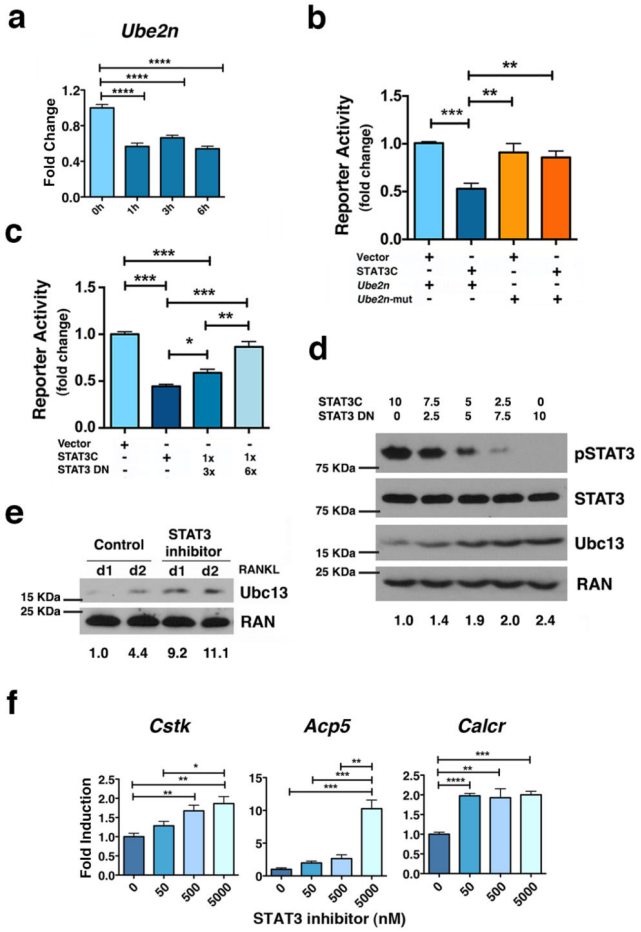

Figure 4. STAT3 transcriptional activity is required to repress *Ube2n* expression

(**a**) *Ube2n* mRNA was measured in bone marrow-derived macrophages from *Stat3*-sufficient mice following treatment with IL-6 as indicated, using qPCR. **(b** and **c)** *Ube2n* promoter activity was measured by reporter assays in HEK293T cells transfected with pGL3-*Ube2n* (*Ube2n*), pGL3-*Ube2n*-mut (*Ube2n*-mut; containing a mutated STATx site), pMX vector (vector), pMX-STAT3C (STAT3C), pMX-STAT3 DN (STAT3 DN) and pTK-Renilla, as indicated. pMX-STAT3 DN and pMX-STAT3C were included in some samples in a 3:1 or 6:1 ratio, as shown. **(d)** *Stat3*$^{-/-}$ MEFs were transfected with pMX-STAT3C, pMX-STAT3 DN or both, at the ratios indicated. The expression level of pSTAT3, total STAT3 and Ubc13 was detected by immunoblotting. Ran served as loading control**. (e** and **f)** Bone marrow-derived macrophages from *Stat3*-sufficient mice were cultured in M-CSF- and RANKL-containing medium in the absence or presence of the STAT3 inhibitor PM-73G, as indicated. Ubc13 amounts were detected by immunoblotting (**e**); osteoclast gene expression was measured by qPCR (**f**). (**d** and **e**) Relative Ubc13 expression vs. STAT3C transfection alone (**d**) or untreated cells on 1 d (**e**) was calculated as indicated in Fig. 1g and displayed below bottom panels. **(a, b, c** and **f)** Data represent mean values of 3 independent experiments. Error bars indicate SEM. Two-way Anova with Bonferroni multiple comparison used. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ for the indicated comparisons. **(d** and **e)** Results represent 3 independent experiments.

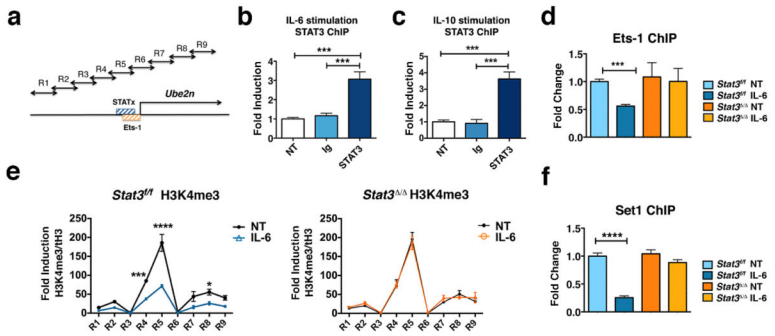

Figure 5. STAT3 represses *Ube2n* transcription by regulating Ets-1 association and H3K4me3 abundance at the proximal promoter

(**a**) Schematic diagram showing the approximate location of the putative STATx (blue) and Ets-1 consensus sites (orange) and primers used for H3K4me3 ChIPs (arrows, sequences are listed in Supplementary Table I). (**b** and **c**) STAT3 association with the *Ube2n* promoter was measured by ChIPs from *Stat3*-sufficient bone marrow-derived macrophages in the absence (NT) or presence of IL-6 (**b**) or IL-10 (**c**) stimulation for 1 h, as indicated. IgG ChIPs were performed as control. (**d** to **f**) Bone marrow cells from *Stat3*$^{f/f}$ or Cre-ER *Stat3*$^{f/f}$ mice were cultured in M-CSF-containing media and 4-hydroxytamoxifen for 5 d to generate *Stat3*-sufficient (*Stat3*$^{f/f}$) or *Stat3*-deficient (*Stat3*$^{\delta/\delta}$) macrophages, respectively. Association of Ets-1 (**d**), H3K4me3 (**e**) or Set1 (**f**) with the *Ube2n* promoter in the vicinity of the STAT3 binding site (**d** and **f**) or the *Ube2n* proximal promoter (−/+ 1kb of TSS) (**e**) was measured by ChIPs, with (IL-6) or without (NT) IL-6 stimulation for 1 h, as indicated. ChIP primers for R5 were used for **d** and **f**. **(b** to **f)** Data represent mean values of 3 independent experiments. Error bars indicate SEM. Two-way Anova with Bonferroni multiple comparison used. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$ for the indicated comparisons.