Functional involvement of Daxx in gp130-mediated cell growth and survival in BaF3 cells

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Abbreviations: Daxx: Death domain-associated protein, LDH: Lactate dehydrogenase, G-CSFR: G-CSF receptor, Bcl2: B-cell lymphoma 2,

Abstract

Death domain-associated protein (Daxx) is a multifunctional protein that modulates both cell death and transcription. Several recent studies have indicated that Daxx is a mediator of lymphocyte death and/or growth suppression, although the detailed mechanism is unclear. Previously, we reported that Daxx suppresses IL-6 family cytokine-induced gene expression by interacting with STAT3. STAT3 is important for the growth and survival of lymphocytes; therefore, we here examined the role of Daxx in the gp130/STAT3-dependent cell growth/survival signals. We found that Daxx suppresses the gp130/STAT3-dependent cell growth and that Daxx endogenously interacts with STAT3 and inhibits the DNA-binding activity of STAT3. Moreover, small-interfering RNA-mediated knockdown of Daxx enhanced the expression of STAT3-target genes and accelerated the STAT3-mediated cell cycle progression. In addition, knockdown of Daxx attenuated lactate dehydrogenase leakage from cells, indicating that Daxx positively regulates cell death during gp130/STAT3-mediated cell proliferation. Notably, Daxx specifically suppressed the levels of Bcl2 mRNA and protein, even in cytokine-unstimulated cells, indicating that Daxx regulates *Bcl2* expression independently of activated STAT3. These results suggest that Daxx suppresses gp130-mediated cell growth and survival by two independent mechanisms: inhibition of STAT3-induced transcription and down-regulation of *Bcl2* expression.

Introduction

Death domain-associated protein (Daxx) is a multifunctional protein that modulates both cell death and transcription [1]. Daxx is present in most cell types and is mainly located in the nucleus. Because previous studies have demonstrated that Daxx can bear both pro- and anti-cell-death activities depending on the stimulus and the cell type [1, 2], the precise role of Daxx, in particular its ability to promote or hinder cell death, remains controversial. Nevertheless, in lymphocytes, the following observations indicate that Daxx has pro-cell death and/or growth suppressing functions. First, Daxx is induced by type I interferon in pro-B cells and is required for interferon-induced apoptosis [3]. Second, in Con A-stimulated splenocytes, Daxx up-regulation and interaction with PML correlate with the induction of B-cell apoptosis [4]. Third, CD40-induced proliferation is profoundly reduced in transgenic B-cells over-expressing Daxx [5]. Fourth, analysis of T-cell specific transgenic mice expressing a dominant negative form of Daxx (Daxx-DN) has reveled that Daxx-DN protects activated T-cells from Fas-induced cell death and that T-lymphocytes expressing Daxx-DN exhibit increased proliferative T-cell responses [6]. These findings have indicated that Daxx is a mediator of lymphocyte death and/or growth suppression. However, the precise mechanism of how Daxx affects cell survival remained unclear.

Daxx interacts with and regulates the transcriptional activities of several DNA-binding transcription factors, including ETS1 [7], PAX5 [8], Glucocorticoid receptor [9], RelA [10], RelB [11], TCF4 [12], SMAD4 [13], C/EBPβ [14], AIRE [15] and STAT3 [16]. Considering that Daxx binds histone deacetylases [17], DNA methyltransferases and their associated proteins [18-20], and the chromatin-modifying protein α-thalassemia syndrome protein [21, 22], it seems possible that Daxx regulates

cellular processes by regulating transcription of specific genes under different conditions. We have previously shown that Daxx suppresses STAT3-mediated transcriptional activity and has a role in regulating IL-6 family cytokine signaling and gene induction in several cancer cell lines [16].

The IL-6 family of cytokines utilizes the membrane glycoprotein gp130 as a critical signal-transducing receptor component [23]. Dimerization of gp130 activates the JAK family of protein tyrosine kinases, which phosphorylate and activate cytoplasmic STAT3 [24, 25]. Activated STAT3 dimerizes and translocates to the nucleus where it binds to specific DNA response elements and induces expression of STAT3-regulated genes. STAT3 has been known to play critical roles in the regulation of various cellular events, malignancies and autoimmune diseases [26-28]. In lymphocyte proliferation, STAT3 activation is responsible for IL-6-dependent T cell proliferation by preventing apoptosis [29] and STAT3 is also indispensable for IL-27-mediated cell proliferation [30]. Moreover, Chou *et al.* demonstrated that STAT3 is required for maintaining pro-B cell survival and for efficient B lymphopoiesis [31].

In the present study, we examined the role of Daxx in the gp130/STAT3-mediated cell proliferation signal. We found that Daxx represses STAT3 activity through the inhibition of DNA-binding, thereby inhibiting cytokine-dependent growth and survival of BaF3 cells. Moreover, Daxx also regulates *Bcl2* gene expression independently of STAT3 regulation. These results suggest that Daxx exerts growth suppression through the modulation of transcription.

Results

Daxx suppresses gp130/STAT3-dependent cell proliferation

We used an IL-3-dependent mouse pro-B cell line, BaF3-derived BaF-G133, as a model for gp130/STAT3-mediated cell proliferation. It has been reported that, in the absence of IL-3, G-CSF-treatment of BaF-G133 cells induces dimerization of GCSFR/gp130 chimeric receptors causing activation of STAT3 and STAT3-dependent cell growth [32].

To examine the role of Daxx in gp130/STAT3-mediated cell growth, we introduced siRNA against Daxx (siDaxx) into BaF-G133 cells. Daxx protein levels were significantly reduced by siDaxx while siDaxx had no effect on the levels of STAT3 or β-actin (Fig. 1A). We then investigated whether this reduction of Daxx in BaF-G133 cells could affect the rate of cell proliferation. After transfection of siRNA, cells were washed in IL-3-free medium and incubated for 48 h in the absence or presence of G-CSF at various concentrations. Silencing of endogenous Daxx by siRNA significantly enhanced the proliferation of BaF-G133 cells at all G-CSF concentrations tested (Fig. 1B). To further elucidate the role of Daxx in gp130/STAT3-mediated cell growth, we established BaF-G133 transfectants over-expressing Daxx (Fig. 1C; BaF-G133/Daxx#3, BaF-G133/Daxx#5, BaF-G133/Daxx#6) and performed growth assays in the presence or absence of either G-CSF. Over-expression of Daxx caused a reduction in cell number when cells were stimulated with G-CSF (Fig. 1D). These results indicated that Daxx has the ability to suppress the gp130/STAT3-mediated cell growth.

Daxx suppresses cell cycle progression in BaF-G133 cells

To clarify how Daxx regulates cell proliferation, we examined whether

gp130-induced cell cycle progression could be affected by knockdown of Daxx. After transfection of siControl or siDaxx, BaF-G133 cells were synchronized in G0/G1 by withdrawing IL-3 and cells were then treated with G-CSF. Cells were fixed and stained with propidium iodide and then DNA content per cell was determined by flow cytometry. As shown in Fig. 2A, BaF-G133 cells transfected with Daxx siRNA exhibited a significant acceleration in gp130-induced cell cycle progression to S and G2/M phases compared with those transfected with control siRNA.

Daxx increases cell death in BaF-G133 cells

Daxx has been reported to positively or negatively regulate cell death [1, 2]. Thus, we tested whether Daxx is involved in the regulation of cell death in BaF-G133 cells. Leakage of LDH into the medium was used as a marker for membrane breakage and cell death. In cells transfected with control siRNA and cultured without IL-3, G-CSF treatment, in a concentration-dependent manner, increased the number of viable cells (as shown in Fig. 1B) with a concomitant reduction of cell death, (Fig. 2B, open circle). Knockdown of endogenous Daxx significantly augmented G-CSF-induced reduction of LDH leakage compared with control cells (Fig. 2B, closed circle).

LDH leakage was increased by about 1.8-fold in Daxx over-expressing transfectants compared with control BaF-G133 cells (Fig. 2C). In addition, BaF-G133/Daxx#3 cells exhibited an increased percentage of Annexin-V-positive cells compared with control BaF-G133 cells (Fig. 2D).

These results indicated that Daxx has the ability to suppress cell cycle progression and to increase cell death, thereby controlling cell proliferation in BaF-G133 cells.

Daxx binds to STAT3 and inhibits its activity

We previously showed that Daxx interacts with STAT3 in transiently transfected 293T cells [16]. STAT3 is essential for gp130-mediated G1 to S phase transition in BaF-G133 cells [33]; therefore, we considered STAT3 to be a potential target of the anti-proliferative function of Daxx. To test whether a Daxx-STAT3 interaction occurs in BaF-G133 cells, we performed co-immunoprecipitation assays (Fig. 3A). An anti-Daxx antibody, but not control rabbit IgG, co-immunoprecipitated endogenous STAT3, regardless of G-CSF stimulation. These data suggest that Daxx endogenously forms a complex with STAT3 in BaF-G133 cells in a constitutive manner.

We next tested whether Daxx affects gp130-mediated phosphorylation of STAT3 and ERK/MAPK. We found that the gp130-mediated upregulation of STAT3 or ERK/MAPK phosphorylation in both BaF-G133/Daxx#3 and /Daxx#6 cells was comparable to that in BaF-G133 cells (data not shown). Therefore, we assumed that Daxx does not participate in the modulation of the activation steps for STAT3 and ERK/MAPK signaling pathways.

We have previously shown that the DNA-binding domain of STAT3 (320–493) interacts with Daxx [16]; therefore, we next examined whether a reduction of Daxx expression affects the DNA-binding activity of STAT3. We introduced siControl or siDaxx into BaF-G133 cells. Cells were washed, starved for 12 h and stimulated with G-CSF for 10 and 30 min. As shown in Fig. 3B, G-CSF-induced STAT3-binding to its consensus oligonucleotide was clearly enhanced by siDaxx treatment, suggesting that Daxx has an inhibitory effect on the DNA-binding activity of STAT3. To investigate the effects of Daxx on the DNA-binding activity of STAT3 in the context of chromatin structure, we performed chromatin immunoprecipitation analysis on the STAT3-binding

site in the 5' region of the *JunB* gene (Fig. 3C). The STAT3-binding site was amplified by PCR in all of the sheared DNA samples (top, indicated as "Input"). Also, this region was amplified by PCR from the anti-STAT3 immunoprecipitates obtained from G-CSF-stimulated cells (middle, lane 2), but not from control Ig immunoprecipitates (bottom), indicating that STAT3 activated by G-CSF bound to this element. Importantly, siDaxx transfection enhanced this binding (middle, lane 4). These findings indicate that Daxx also inhibits DNA-binding activity of STAT3 in chromatin.

To further delineate the functional relevance of Daxx in STAT3 regulation, we examined the status of STAT3-target gene expression in BaF-G133 cells. Either siControl- or siDaxx-transfected cells were treated with G-CSF for 30 min, followed by RNA extraction, reverse transcription and real-time PCR analysis using mouse *Socs3*-specific primers. The *Socs3* mRNA levels were normalized against *Actb*. As shown in Fig. 3D, *Socs3* was induced 6-fold with G-CSF treatment in the control cells and *Socs3* was induced by almost 13-fold in the siDaxx-transfected cells, suggesting that the siDaxx-transfected cells have enhanced expression of STAT3-target genes. Consistent with these observations, knockdown of Daxx in BaF-G133 cells also resulted in enhanced induction of other known STAT3-target genes, *JunB* and *Pim2* (Fig. 3E).

Daxx is involved in the regulation of Bcl2 mRNA and protein levels

Dimerization of G-CSFR-gp130 chimeric receptors in BaF-G133 cells transduces anti-apoptotic signal through the induction of *Bcl2* [32]. Thus, we next examined the effects of Daxx knockdown on the mRNA levels of *Bcl2* family genes. After washing with IL-3-free medium, BaF-G133 cells were stimulated with G-CSF and mRNA levels were analyzed by semi-quantitative RT-PCR, as indicated in Fig. 4A. In control

siRNA-transfected cells, G-CSF treatment induced the upregulation of *Bcl2* mRNA levels, but not of *Bcl2l1* which encodes the Bcl-x protein. The mRNA level of pro-apoptotic *Bax* was unaffected by G-CSF. Notably, knockdown of Daxx increased the basal level of *Bcl2* mRNA, indicating that Daxx regulates transcription factors involved in *Bcl2* expression other than activated STAT3. Reciprocally, BaF-G133 cells over-expressing Daxx exhibited decreased *Bcl2* mRNA levels regardless of G-CSF stimulation (Fig. 4B). In addition, we introduced siDaxx into BaF-G133 to knockdown endogenous Daxx and found an increase in protein levels of Bcl2 in the absence of G-CSF (Fig. 4C). We also prepared cell extracts from BaF-G133 or BaF-G133/Daxx#3 cells and analyzed endogenous protein levels of Bcl2 and Bax by western blotting. As shown in Fig. 4D, reduced expression of Bcl2 was observed in BaF-G133/Daxx#3 cells in G-CSF-treated conditions and also in non-G-CSF-treated conditions. Protein levels of Bax, however, were unaltered by G-CSF stimulation or Daxx expression. Interestingly, we also found that a decrease of Daxx protein preceded the up-regulation of Bcl2 after G-CSF-stimulation. Collectively, these data indicate that Daxx rather specifically regulates *Bcl2* expression at the mRNA level under both basement and G-CSF-stimulated conditions.

It has been shown that the cyclic AMP response element (CRE) is a major positive regulatory site in the *Bcl2* promoter in B-cells and that mutation of the CRE abolishes the binding of CREB/ATF and CREB Binding Protein (CBP) transcription factors to the *Bcl2* promoter and greatly diminishes the binding of NF-κB factors [34]. Because Daxx can interact with RelA [10] and RelB [11], two members of the NF-κB family of transcription factors, as well as CBP [35], we next examined the binding of these proteins to the region of the *Bcl2* promoter containing the CRE site. The ChIP assays

were performed with G-CSF-treated BaF-G133 and BaF-G133/Daxx#3 cells, and an anti-rabbit IgG was used as a nonspecific immunoprecipitation control. The results demonstrated that in BaF-G133 cells, RelA and CBP, but not RelB, binds to the CRE site (Fig. 4E). In addition, a substantial decrease in the binding of RelA and CBP was observed in the BaF-G133/Daxx#3 cells (Fig. 4E). These results indicated that Daxx negatively regulates RelA and CBP binding to the Bcl2 promoter in BaF-G133 cells.

Over-expression of Bcl2 represses Daxx-mediated cell death

We then analyzed whether Daxx-mediated cell death of BaF-G133 cells was repressed by over-expression of Bcl2. In G-CSF-treated conditions, over-expression of Bcl2 in BaF-G133/Daxx#3 cells decreased LDH leakage to a level comparable with that observed in control BaF-G133 cells (Fig. 5A, left and 5B). Treatment with HA14-1, a small-molecule Bcl2 inhibitor [36] decreased cell viability in a dose-dependent manner in G-CSF-treated BaF-G133 cells (Fig. 5C), confirming that Bcl2 function contributes to gp130-mediated survival of this cell line. These data indicate that the Daxx-mediated increase in cell death is due, at least in part, to changes in Bcl2 expression in G-CSF-treated BaF-G133 cells.

Discussion

Our present manipulation of Daxx expression has revealed that Daxx negatively regulates gp130-mediated signals. We here proposed and discussed two possible mechanisms concerning to STAT3 and Bcl2. Daxx constitutively interacted with STAT3, resulting in the impaired binding of STAT3 to its consensus DNA sequence in chromatin. In addition, Daxx preferentially down-regulated *Bcl2* expression at the mRNA level. These cellular modifications mediated by Daxx are likely to suppress gp130-mediated cell proliferation and survival.

The involvement of STAT3 in IL-6/gp130-mediated growth responses has been indicated by many investigators. For example, the G1 to S phase progression of cell cycle was shown to be a STAT3-dependent process [33]. In BaF-G133 cells, G-CSF-treatment was reported to induce dimerization of G-CSFR/gp130 chimeric receptors causing activation of STAT3 as well as STAT3-dependent cell growth [32]. In lymphocytes, STAT3 activation is reported to be responsible for IL-6-dpendent T-cell proliferation [29] and for normal early B-cell development [31]. With regard to this gp130-mediated STAT3 function, we here showed constitutive interactions between endogenous Daxx and STAT3. Of importance, our data clearly indicated that these interactions gave rise a decrease of the DNA-binding activity of STAT3 rather than that of the activation steps of STAT3. Our previous report telling that the DNA-binding domain of STAT3 (320–493) was a major domain to interact with Daxx [16] might explain the inhibition of STAT3 transcription by Daxx.

Although Daxx negatively regulated gp130-mediated *Bcl2* mRNA induction, it also reduced basal level of *Bcl2* expression at the mRNA level. This is likely to suggest that Daxx must have further targets for repression, in addition to activated STAT3. In this

regard, we first considered ETS1 to be a candidate, because *Bcl2* is induced by over-expression of ETS1 [7]. However, we could not detect any *Ets1* mRNA expression in BaF-G133 cells (data not shown), indicating that ETS1 is not involved in the Bcl2 expression in those cells. *Bcl2* is also a well known anti-apoptotic target gene of NF-κB. Daxx can interact with RelA [10], RelB [11] and CBP [35], we therefore assessed the effect of Daxx over-expression on Rel and CBP binding to the *Bcl2* promoter. Our ChIP experiments using the primers amplifying the Bcl2 promoter region containing the CRE clearly indicated that Daxx negatively regulates RelA and CBP in BaF-G133 cells. In addition, our experiments using a Bcl2 specific inhibitor indicated that gp130-mediated Bcl2 expression is indeed involved in the maintenance of cell survival and that Daxx specifically inhibits the *Bcl2* expression. During apoptosis of lymphocytes, an inverse correlation of Daxx and Bcl2 expression levels has been suggested to be important. For example, gene expression profiling of interferon-β-treated progenitor B cells revealed enhanced Daxx expression and nuclear accumulation, with subsequent down-regulation of *Bcl2*, followed by apoptosis [3]. Another report said that certain mantle cell lymphomas have markedly decreased levels of *Daxx* expression and exhibit over-expression of *Bcl2* and *Bcl2l1* [37]. It has also been reported that ectopic over-expression of Daxx down-regulates *Bcl2* in malignant lymphocyte Jurkat T-cells and sensitizes cells to the apoptosis-inducing effect of chemotherapeutic agents [38]. These findings and our results support the idea that Daxx participates in repression, especially that of *Bcl2* gene expression and thereby sensitizes cell death.

As described above, Daxx inhibited gp130-mediated cell growth and survival in BaF-G133 cells. In a case of IL-3 signals, which mainly utilize STAT5 [39-41], manipulation of Daxx expression failed to modify their growth and/or survival (data not

shown). This different involvement of Daxx may come from the difference in activated STAT proteins between gp130- and IL-3-mediated signaling. In accordance with this notion, we could not detect the specific interactions between Daxx and STAT5 in our co-immunoprecipitation experiments (data not shown). These observations indicate that Daxx preferentially interacts with and inhibits STAT3 rather than STAT5.

The functions of Daxx are related to its protein level. It has been reported that the peptidyl-prolyl isomerase, Pin1, inhibits Daxx-mediated apoptosis by inducing Daxx ubiquitination and degradation [42], and that the BTB domain-containing speckle type POZ protein/Cul3 ubiquitin ligase complex inhibits the transcriptional repression function of Daxx by mediating the proteasome-dependent degradation of Daxx [43]. Furthermore, Mdm2 and Hausp regulate Daxx functions by controlling Daxx ubiquitination and stability [44]. Interestingly, we found that gp130-mediated up-regulation of Bcl2 is accompanied by a reduction in the level of endogenous Daxx protein. This reduction of Daxx protein may be regulated by the above factors and may be an important process in the 'de-repression' mechanism for the regulation of both STAT3 activity and Bcl2 expression, although the details of this are still unclear.

G-CSF is a common inducer of the release of hematopoietic progenitor cells (HPC) from the bone marrow into the peripheral blood [45]. One of the molecular mechanisms underlying this action of G-CSF has been implicated to involve the phosphorylation of JAK1/STAT3 pathway [46]. Daxx has been reported to be expressed in HPC [47]. Therefore it is possible to speculate that Daxx also could influence G-CSF-mediated mobilization of HPC and the further study will be required to clarify this point.

In summary, we suggest that Daxx has two functional roles corresponding to cell death regulation: STAT3 repression and down-regulation of Bcl2. Our results suggest

that the ability of Daxx to repress transcription is relevant to its ability to sensitize cell death. In addition, our finding that STAT3 function can be modulated by Daxx will provide insight into the regulation of various cellular events, malignancies and autoimmune diseases in which STAT3 plays critical roles.

Materials and methods

Cell culture

BaF-G133 cells were a kind gift from Dr. Toshio Hirano (Osaka Univ., Osaka, Japan) and have been described [32]. BaF-G133 cells are derived from an IL-3-dependent mouse pro-B cell line, BaF3, and were maintained in RPMI 1640 medium supplemented with 10% FBS (Trace Biosystems, Sydney, Australia), 10% conditioned medium from WEHI-3B cells as a source of IL-3, 100 U/ml penicillin and 100 μg/ml streptomycin. BaF-G133 cells stably express the chimeric receptor G133, which is composed of the extracellular domain of the G-CSF receptor and the transmembrane and cytoplasmic domains of gp130 (truncation of gp130 occurs 133 amino acids from the transmembrane domain). G133 contains gp130 Tyr767, which is required for STAT3 activation and STAT3 is essential for both gp130-mediated cell survival [32] and gp130-mediated cell-cycle transition [33]. To establish Daxx transfectants, 20 μg of human Daxx expression vector (pCDNA3–FLAG–Daxx) was co-transfected with 2 μg of pMIK–Hyg into BaF-G133 cells by electroporation. Daxx transfectants were selected using 200 μg/ml hygromycin. Independent clones were established using a limiting dilution procedure. Expression levels of Daxx were analyzed by western blotting. Human G-CSF was kindly provided by Chugai Pharmaceutics (Tokyo, Japan).

siRNA Experiments

Mouse Daxx stealth siRNA was purchased from Invitrogen (Carlsbad, CA, USA). The sequence of the mouse Daxx siRNA was 5'-AAGUAGAAGAGACCAUGCCUGCUCC-3'. Stealth siRNA negative control medium GC (Invitrogen) was used as a negative control. BaF-G133 cells were

transfected using a Nucleofector (Amaxa Biosystems, Cologne, Germany). Cells were transfected with 200 pmol siRNA in Nucleofector solution V using program X-001. Immediately following transfection medium was added to the BaF-G133 cells, which were then plated in 6-well tissue culture plates and incubated overnight. Cells were collected the following day and analyzed for protein expression by western blotting.

Proliferation assays

Cell proliferation was determined using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, cells were plated in a 96-well plate at 1×10^4 cells/well and left untreated or were treated with G-CSF at concentrations indicated in figures. Ten microliters of WST-8 mixture was then added to each well, and the plates were incubated at 37°C in 5% CO_2 for 4 h. The absorbance of each well was then measured on a microplate reader at 450 nm. All assays were performed in triplicate and repeated at least three times. To investigate the role of Bcl2 in G-CSF-induced cell growth, a small-molecule Bcl2 inhibitor, HA14-1, (Calbiochem, San Diego, CA, USA) was used.

Immunoprecipitation, STAT3 DNA-binding assay and western blotting

The immunoprecipitation and western blotting assays were performed as described previously [48]. The DNA-binding activity of STAT3 in cell extracts was measured using an immobilized STAT3 consensus oligonucleotide–Sepharose conjugate (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as described previously [49]. The immunoprecipitates or consensus oligo-binding proteins from cell lysates were resolved by SDS–PAGE and transferred to PVDF membranes (PerkinElmer, Boston, MA, USA).

The membranes were then immunoblotted with the different primary antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore, Bedford, MA, USA). Antibodies used in this study were: anti-Daxx (sc-7152), anti-STAT3 (sc-482) and anti-Bcl2 (sc-7382) from Santa Cruz Biotechnology, anti-Bax (#2772) from Cell Signaling technology (Beverly, MA, USA) and anti-Actin (A1978) from Sigma.

Cell cycle analysis

BaF-G133 (2×10^5) cells were IL-3-starved for 12 h and then treated with 30 ng/ml G-CSF. Cells were then washed once with ice-cold PBS, suspended with 100 μl of PBS and then fixed by the addition of 900 μl of ethanol. Cells were incubated at -20°C for 20 min, pelleted, resuspended with 300 μl of staining buffer (1 mg/ml RNase, 20 μg/ml propidium iodide, 0.01% NP-40 in PBS) and incubated at 37°C for 20 min. The DNA content of nuclei was analyzed using a FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA, USA).

Lactate dehydrogenase (LDH) release assay

Cytotoxicity was determined by measuring the release of LDH using an LDH Cytotoxicity Detection Kit (Takara, Otsu, Japan) according to the manufacturer's instructions. The reaction was initiated by mixing 50 μl of cell-free supernatant with a potassium phosphate buffer containing NADH and sodium pyruvate to a final volume of 100 μl in a 96-well plate. The absorbance of the sample was read at 490 nm. Data were normalized to the activity of LDH released from control cells (100%) and are expressed as a percentage of the control value.

Chromatin immunoprecipitation

Cells were treated with 1% formaldehyde for 20 min to crosslink proteins to DNA. Formaldehyde was neutralized for 5 min by the addition of glycine to 125 mM. Cells were washed twice with cold PBS. Cells were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0) and incubated at 4°C for 10 min. The suspension was then sonicated with five 10 sec pulses and then clarified by centrifugation for 10 min in a microcentrifuge. The supernatant was diluted 10-fold with dilution buffer (1% Triton X-100, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl at pH 8.0) to yield the solubilized chromatin solution. The resulting material was used for immunoprecipitation with anti-STAT3 (sc-482), anti-RelA (sc-372), anti-RelB (sc-226), anti-CBP (sc-369) from Santa Cruz Biotechnology or with rabbit IgG as a negative control. After overnight incubation with antibody, DNA-protein complexes were collected by the addition of 10 μL of salmon sperm DNA-protein A agarose (Upstate Biotechnology, Lake Placid, NY, USA) and incubated at 4°C with rotation for 2 h. Following immunoprecipitation, beads were washed in RIPA buffer (1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl at pH 8.0), followed by RIPA/500 mM NaCl, LiCl buffer (250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl at pH 8.0), and finally, TE buffer. The immunoprecipitated complexes were eluted in a buffer containing 10 mM Tris pH 8.0, 300 mM NaCl, 5 mM EDTA, 0.5% SDS at 65°C for 6 h. The samples were then treated with proteinase K for 1 h, and DNA was purified by phenol/chloroform extraction and ethanol precipitation. Immunoprecipitates were analyzed by PCR. PCR primers were designed to amplify -454/-186 of the *JunB* promoter, as described

previously [50] and to amplify -1560/-1461 of the *Bcl2* promoter region containing the CRE as follows: the forward primer was 5'-GCTCAGAGGAGGGCTTTCTT-3' and the reverse primer was 5'-GGCCCGGCCTCTTACTTC-3'. PCR products were electrophoresed on agarose gels and visualized by EtBr staining.

RNA extraction and RT-PCR analysis

Total RNA was extracted from BaF-G133 cells using TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). First-strand cDNA was synthesized from 1 μg of total RNA with Revertra Ace reverse transcriptase (TOYOBO, Osaka, Japan) using random nonamers. The cDNA was the template for PCR using Gene Taq polymerase (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's instructions. PCR products were electrophoresed on agarose gels and visualized by EtBr staining. The primer pairs used in this study were listed in table 1. Quantitative real-time PCR analysis of *Socs3* mRNA transcripts was carried out using the assay-on-demand™ gene-specific fluorescently labeled TaqMan MGB probe in an ABI Prism 7000 sequence detection system.

Statistical analysis

Statistical evaluation of differences between populations was determined using Student's unpaired, two-tailed t test. Results shown are the means and standard deviations.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure legends

Figure 1. Involvement of Daxx protein in the regulation of gp130/STAT3-dependent cell proliferation. (A) BaF-G133 cells were transfected with Daxx-specific and control siRNA and Daxx knockdown was validated by western blot analysis of Daxx, STAT3 and β-actin. Knockdown of Daxx was comfirmed until 3 days after siRNA transfection. Data is representative of three independent experiments. (B) G-CSF-induced cell proliferation of control or Daxx siRNA transfected BaF-G133 cells was determined by the WST8 assay. Data show mean ± SD of triplicate samples and representative of three independent experiments. *$p<0.05$ and **$p<0.01$ (Student's t-test). (C) Lysates from stable transfectants of BaF-G133 cells over-expressing Daxx were subjected to western blot analysis of Daxx and β-actin. Data is representative of three independent experiments. (D) The WST8 assay was performed on BaF-G133 cells and Daxx transfectants. Data show mean ± SD of triplicate samples and representative of three independent experiments. **$p<0.01$ (Student's t-test).

Figure 2. Daxx regulates cell cycle progression and cell death during gp130/STAT3-dependent cell growth. (A) BaF-G133 cells were treated with control siRNA or Daxx siRNA. Cells were IL-3-starved for 12 h and then treated with 30 ng/ml G-CSF for the indicated times. Cells were then fixed and subjected to cell cycle analysis using FACS. The percentages of cells in S plus G2M phases of the cell cycle are indicated (Mean±SD, n = 3). *$p<0.05$ (Student's t-test). (B) Daxx knockdown and control cells were cultured with the indicated concentrations of G-CSF for 48 h and the effect of Daxx knockdown on the G-CSF-induced reduction in cell death of BaF-G133

cells was evaluated by lactate dehydrogenase (LDH) activity in cell culture supernatants. Results are presented as the percentage of the maximum LDH release observed in untreated control cells (Mean±SD, n = 3). **$p<0.01$ (Student's t-test). (C) The LDH assay was performed on BaF-G133 cells and Daxx transfectants, cultured with 30 ng/ml of G-CSF for 48 h. Results are presented as OD at 490 nm (Mean±SD, n = 3). **$p<0.01$ (Student's t-test). (D) BaF-G133 and BaF-G133/Daxx#3 cells were treated with 30 ng/ml G-CSF for the indicated times and the percentages of Annexin-V-positive cells were determined by FACS analysis (Mean±SD, n = 3). *$p<0.05$ (Student's t-test).

Figure 3. Daxx interacts with STAT3 and inhibits its activity. (A) BaF-G133 cells (5×10^7 cells) were stimulated with G-CSF (30 ng/ml) for 30 min. The cells were lysed, immunoprecipitated with control IgG or anti-Daxx antibody and subjected to western blot analysis of STAT3 and Daxx. An aliquot of total cell extract (input) was also analyzed. (B) BaF-G133 cells (2×10^6 cells) were treated with control siRNA or Daxx siRNA, and cells were stimulated with G-CSF (30 ng/ml) for the indicated periods. Cell extracts were prepared and subjected to pull-down experiments using the immobilized STAT3 consensus oligonucleotide-sepharose conjugate to evaluate DNA binding activity of STAT3. The precipitates and an aliquot of total cell extract were subjected to western blot analysis of STAT3. (C) BaF-G133 cells (4×10^6 cells) were treated with control siRNA or Daxx siRNA and cells were stimulated with G-CSF (30 ng/ml) for the indicated periods. Samples for ChIP were prepared as described in Materials and methods. STAT3-DNA binding complexes were immunoprecipitated with the anti-STAT3 antibody or with control IgG. The immunoprecipitated DNA was eluted and subjected to PCR. (D) BaF-G133 cells were treated with control or Daxx

siRNA and cells were stimulated with G-CSF (30 ng/ml) for 30 min. Total RNA samples isolated from these cells were subjected to quantitative real-time PCR analysis using *Socs3* or *Actb* primers. Data represent the levels of *Socs3* mRNA normalized to that of an *Actb* internal control and are expressed relative to the value of control siRNA-treated samples without G-CSF-stimulation. Results are representative of three independent, duplicate experiments. (E) BaF-G133 cells were treated with control or Daxx siRNA and cells were stimulated with G-CSF (30 ng/ml) for the indicated periods. Total RNA samples isolated from these cells were subjected to semi quantitative RT-PCR analysis using *JunB*, *Pim2*, *Daxx* or *Gapdh* primers. Data in (A), (C) and (E) are representative of three independent experiments; (B) is representative of two independent experiments.

Figure 4. Daxx suppresses Bcl2 mRNA and protein levels. (A) cDNA samples, prepared as described in Fig. 3E, were subjected to semi quantitative analysis using *Bcl2*, *Bcl2l1* and *Bax* primers. (B) BaF-G133 or BaF-G133/Daxx#3 cells were treated with G-CSF at 30 ng/ml for 12 h. Total RNA was then extracted and subjected to RT-PCR analysis. (C) BaF-G133 cells were transfected with control siRNA or Daxx siRNA and endogenous Bcl2 protein was detected by western blot analysis. (D) BaF-G133 and BaF-G133/Daxx#3 cells were treated with 30 ng/ml G-CSF for the indicated times. Protein levels of Bcl2, Bax and Daxx in cells were detected by western blot analysis. (E) BaF-G133 or BaF-G133/Daxx#3 cells (4×10^6 cells) were stimulated with G-CSF (30 ng/ml) for 6 h. Samples for ChIP were prepared as described in Materials and methods. Transcription factor-DNA binding complexes were immunoprecipitated with indicated antibodies or with control IgG. The

immunoprecipitated DNA was analyzed by PCR using primers that amplified a 100-bp product that includes the CRE site in the *Bcl2* promoter. Data in this figure are representative of three independent experiments.

Figure 5. Over-expression of Bcl2 repressed Daxx-mediated cell death. (A) BaF-G133 or BaF-G133/Daxx#3 cells were transfected with empty plasmid or Bcl2 expression plasmid. After treatment with G-CSF (30 ng/ml, left) for 48 h, LDH activities in culture supernatants were measured. Results are presented as OD at 490 nm (Mean±SD, n = 3; **$p<0.01$ and n.s., not significant). (B) Transient over-expression of Bcl2 in BaF-G133 or BaF-G133/Daxx#3 cells was validated by western blot analysis of Daxx, Bcl2 and β-actin. Data is representative of three independent experiments. (C) BaF-133 cells were plated in a 96-well plate in the presence of G-CSF (30 ng/ml) without or with the indicated concentrations of the small-molecule Bcl2 inhibitor, HA14-1. Twenty-four hours after treatment, cell number was determined by the WST8 assay. The value obtained for DMSO-treated (0 μM) cells was set as 100% survival. Data show mean + SD (n=3). **$p<0.01$ (Student's t-test).

Table 1. RT-PCR primers used in this study

target	Forward primer (5' - 3')	Reverse primer (5' - 3')
Bax	TGCAGAGGATGATTGCTGAC	GATCAGCTCGGGCACTTTAG
Bcl2	GTCGCTACCGTCGTCACTTC	ACAGCCAGGAGAAATCAAAC
Bcl2l1	TAGGACTGAGGCCCCAGAAG	CAGTCATGCCCGTCAGGAAC
Daxx	CCCATGGCCACCGATGACAGCAT	AGGGTTAGGGCCCGACGCCTCACT
Gapdh	CAGTAGAGGCAGGGATGATGTTC	GAAATCCCATCACCATCTTCCAGG
JunB	CAGGCAGCTACTTTTCGGGTC	AAGGGTGGTGCATGTGGGAGG
Pim2	AGCACCTCCTCCATGTTGAC	ATGGCCACCTGACGTCTATC

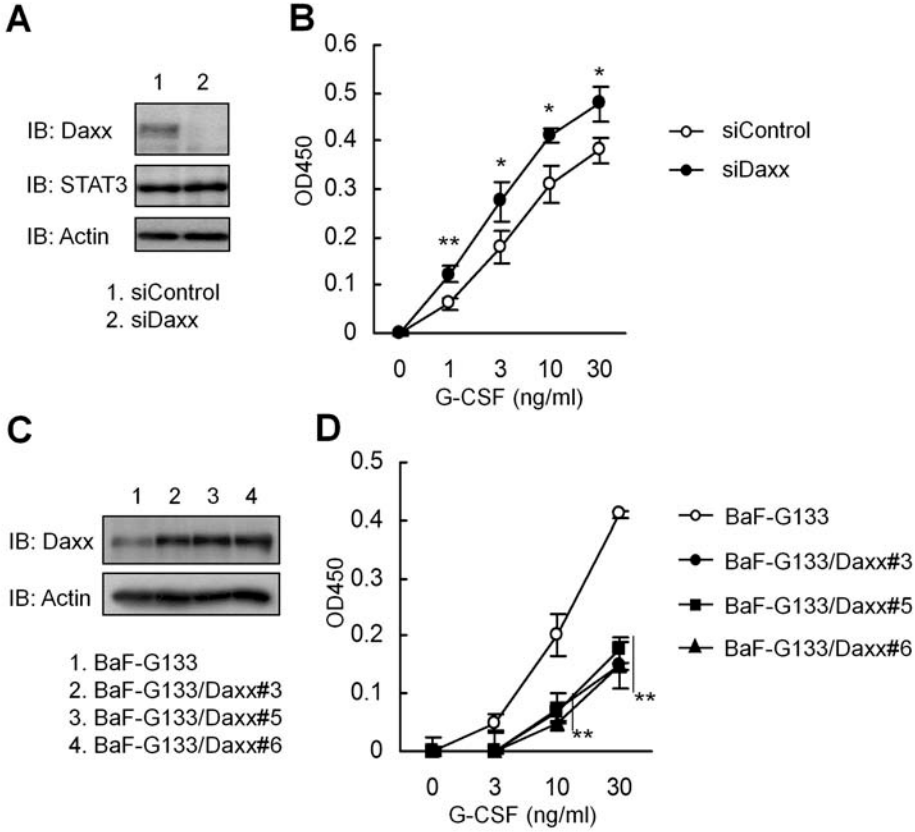

A
1 2
IB: Daxx
IB: STAT3
IB: Actin
1. siControl
2. siDaxx
B
0.6
0.5
0.4
0.3
0.2
0.1
0
OD450
**
*
*
*
0 1 3 10 30
G-CSF (ng/ml)
siControl
siDaxx
C
1 2 3 4
IB: Daxx
IB: Actin
1. BaF-G133
2. BaF-G133/Daxx#3
3. BaF-G133/Daxx#5
4. BaF-G133/Daxx#6
D
0.5
0.4
0.3
0.2
0.1
0
OD450
**
**
0 3 10 30
G-CSF (ng/ml)
BaF-G133
BaF-G133/Daxx#3
BaF-G133/Daxx#5
BaF-G133/Daxx#6

Figure 1

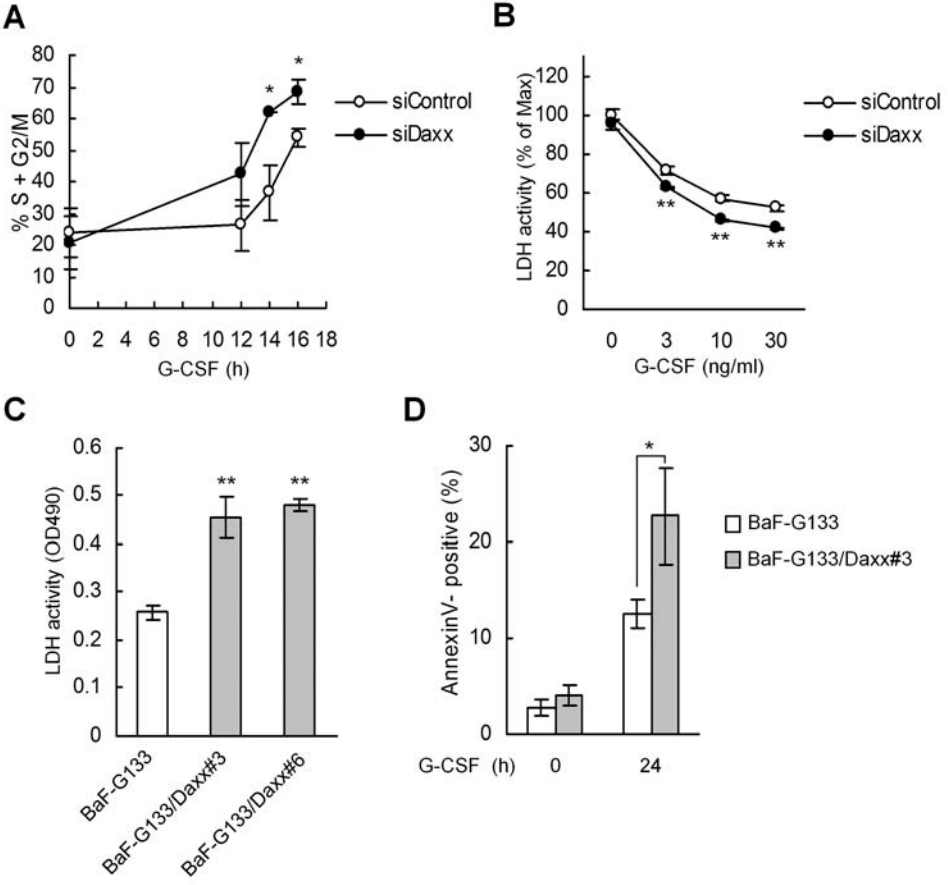

A
% S + G2/M
G-CSF (h)
siControl
siDaxx
B
LDH activity (% of Max)
G-CSF (ng/ml)
siControl
siDaxx
C
LDH activity (OD490)
BaF-G133
BaF-G133/Daxx#3
BaF-G133/Daxx#6
D
AnnexinV- positive (%)
G-CSF (h)
BaF-G133
BaF-G133/Daxx#3

Figure 2

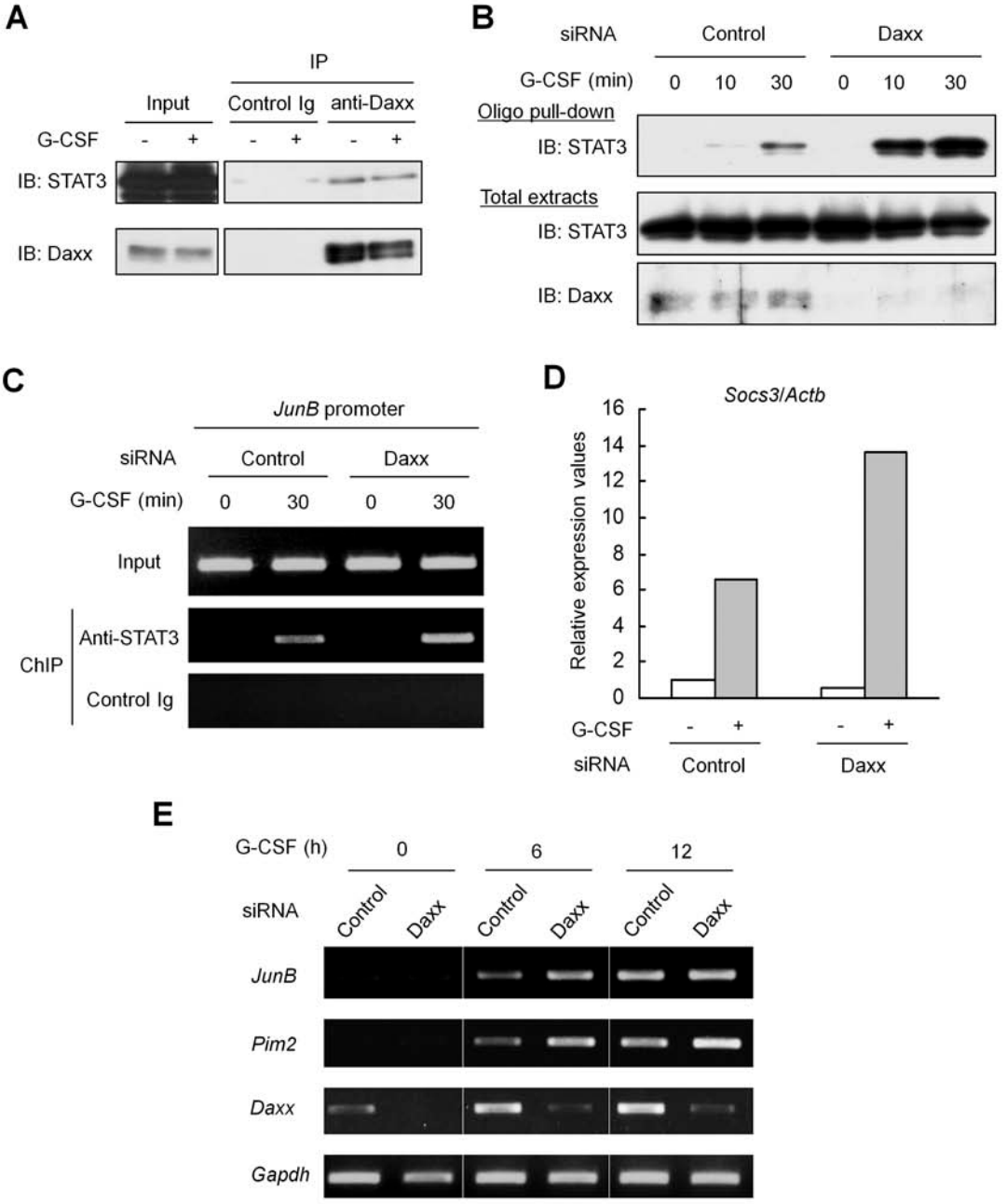
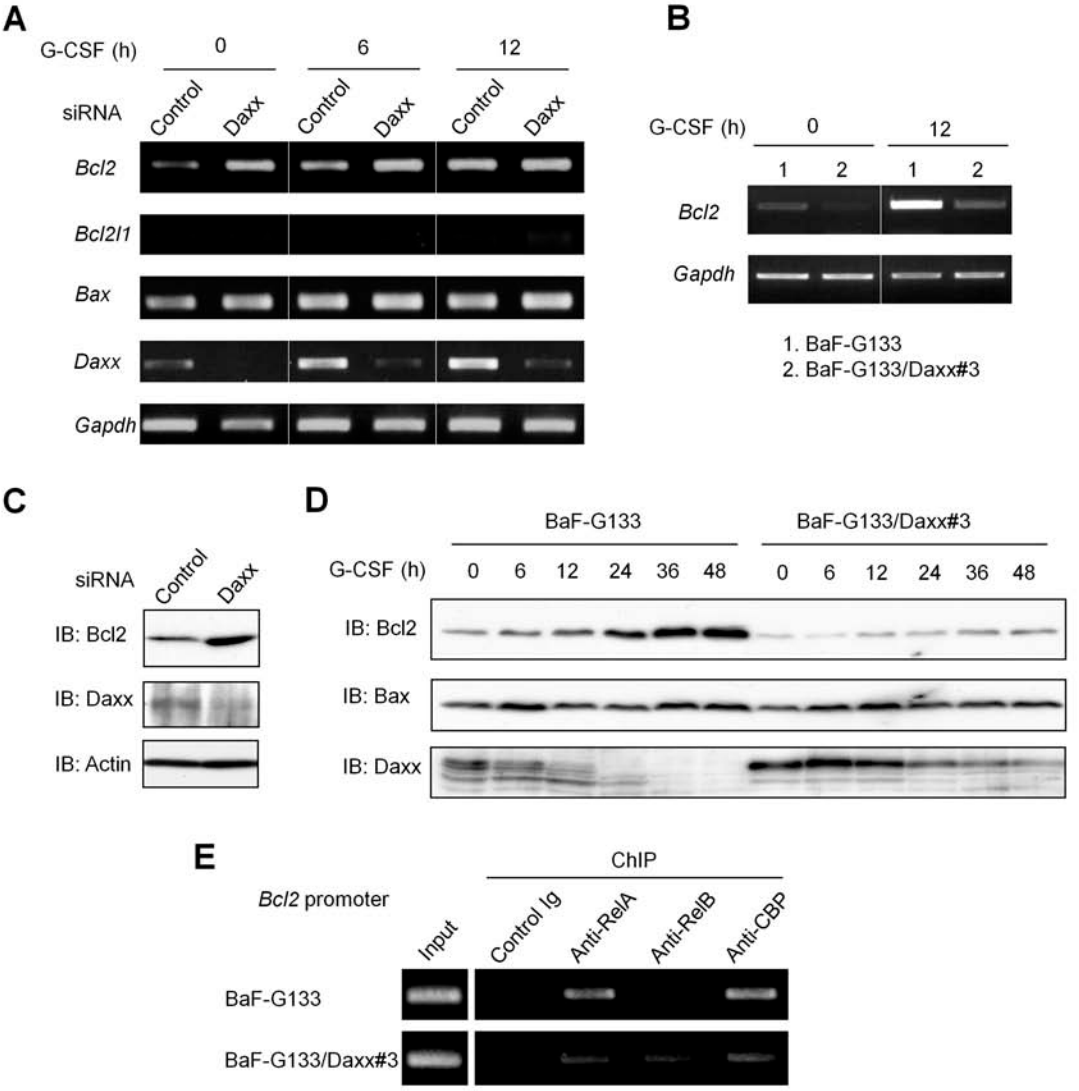

A
IP
Input
Control Ig
anti-Daxx
G-CSF
- + - + - +
IB: STAT3
IB: Daxx
B
siRNA
Control
Daxx
G-CSF (min)
0 10 30 0 10 30
Oligo pull-down
IB: STAT3
Total extracts
IB: STAT3
IB: Daxx
C
JunB promoter
siRNA
Control
Daxx
G-CSF (min)
0 30 0 30
Input
ChIP
Anti-STAT3
Control Ig
D
Socs3/Actb
Relative expression values
16
14
12
10
8
6
4
2
0
G-CSF
- +
- +
siRNA
Control
Daxx
E
G-CSF (h)
0
6
12
siRNA
Control
Daxx
Control
Daxx
Control
Daxx
JunB
Pim2
Daxx
Gapdh

Figure 3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G-CSF (h)
0
6
12
siRNA
Control
Daxx
Control
Daxx
Control
Daxx
Bcl2
Bcl2l1
Bax
Daxx
Gapdh
B
G-CSF (h)
0
12
1
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1
2
Bcl2
Gapdh
1. BaF-G133
2. BaF-G133/Daxx#3
C
siRNA
Control
Daxx
IB: Bcl2
IB: Daxx
IB: Actin
D
BaF-G133
BaF-G133/Daxx#3
G-CSF (h)
0 6 12 24 36 48
0 6 12 24 36 48
IB: Bcl2
IB: Bax
IB: Daxx
E
ChIP
Bcl2 promoter
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Control Ig
Anti-RelA
Anti-RelB
Anti-CBP
BaF-G133
BaF-G133/Daxx#3

Figure 4

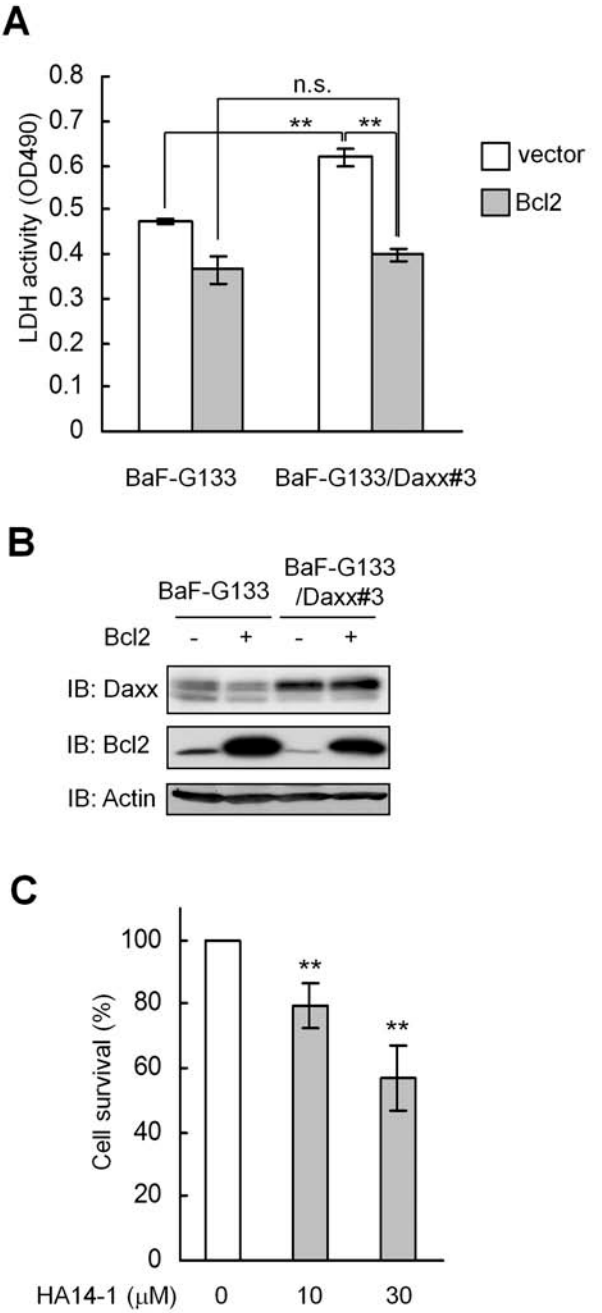
A
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
LDH activity (OD490)
n.s.
**
**
vector
Bcl2
BaF-G133
BaF-G133/Daxx#3
B
BaF-G133
BaF-G133
/Daxx#3
Bcl2
-
+
-
+
IB: Daxx
IB: Bcl2
IB: Actin
C
100
80
60
40
20
0
Cell survival (%)
**
**
HA14-1 (μM)
0
10
30

Figure 5