

# TRIM8 modulates STAT3 activity through negative regulation of PIAS3

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## Summary

TRIM8 is a member of the protein family defined by the presence of a common domain structure composed of a tripartite motif: a RING-finger, one or two B-box domains and a coiled-coil motif. Here, we show that TRIM8 interacts with protein inhibitor of activated STAT3 (PIAS3), which inhibits IL-6-dependent activation of STAT3. Ectopic expression of TRIM8 cancels the negative effect of PIAS3 on STAT3, either by degradation of PIAS3 through the ubiquitin-proteasome pathway or exclusion of PIAS3 from the nucleus. Furthermore, expression of TRIM8 in NIH3T3 cells enhances Src-dependent tumorigenesis. These findings indicate that TRIM8 enhances the STAT3-dependent signal pathway by inhibiting the function of PIAS3.

**Key words:** TRIM8, PIAS3, Ubiquitin, STAT3, IL-6

## Introduction

The ubiquitin-mediated proteolytic pathway has an important role in the elimination of short-lived regulatory proteins (Peters, 1998), including those that contribute to the cell cycle, cellular signaling in response to environmental stress or extracellular ligands, morphogenesis, secretion, DNA repair and organelle biogenesis (Hershko and Ciechanover, 1998). The system responsible for the attachment of ubiquitin to the target protein consists of several components that act in concert (Hershko and Ciechanover, 1992; Scheffner et al., 1995), including a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin-protein isopeptide ligase (E3). E3 is thought to be the component of the ubiquitin conjugation system that is most directly responsible for substrate recognition (Scheffner et al., 1995). On the basis of structural similarity, E3 enzymes have been classified into three families: the HECT (homologous to E6-AP COOH terminus) family (Hershko and Ciechanover, 1998; Huijbrechtse et al., 1995), the RING-finger-containing protein family (Freemont, 2000; Joazeiro and Weissman, 2000; Lorick et al., 1999) and the U-box family (Aravind and Koonin, 2000; Cyr et al., 2002; Hatakeyama et al., 2001).

The superfamily of tripartite-motif-containing (TRIM) proteins is defined by the presence of a tripartite motif composed of a RING domain, one or two B-box motifs and a coiled-coil region (the so-called RBCC motif) (Meroni and Diez-Roux, 2005; Nisole et al., 2005). Many TRIM proteins are induced by type I and type II interferons (IFNs), suggesting that TRIM proteins have an important role in anti-viral and anti-microbial systems (Rajsbaum et al., 2008).

The human *TRIM8* gene is expressed in a variety of tumors, including anaplastic oligodendroglioma, and maps to chromosome 10q24.3, a region that shows frequent deletion or loss of heterozygosity in glioblastomas (Vincent et al., 2000). Therefore, TRIM8 is also designated glioblastoma-expressed RING-finger protein (GERP). It has been reported that TRIM8 localizes to

specific nuclear bodies and cytosolic speckles in U2OS and HeLa cells (Reymond et al., 2001). TRIM8 has also been shown by yeast two-hybrid screening to be a suppressor of cytokine signaling (SOCS)-1 interacting protein (Toniato et al., 2002). *TRIM8* mRNA can be induced by IFN $\gamma$  in murine B lymphoid M12 cells, murine fibroblasts and HeLa cells and the N-terminal 204 amino acids of TRIM8 accelerate the degradation of SOCS-1 and reverse SOCS-1-mediated inhibition of JAK-STAT activation by IFN $\gamma$  (Toniato et al., 2002). However, it is not clear whether full-length TRIM8 truly regulates the JAK-STAT pathway.

Protein inhibitor of activated STAT3 (PIAS3) has been reported to inhibit the DNA-binding activity of signal transducer and activator of transcription 3 (STAT3), followed by the suppression of STAT3-mediated gene activation (Chung et al., 1997). Since many cytokine receptors do not have intrinsic tyrosine-kinase activity, ligand engagement leads to the activation of receptor-associated tyrosine kinases, which are usually members of the Janus kinase (JAK) family (Darnell, 1998; Heinrich et al., 2003; Stark et al., 1998; Yu et al., 2007). STAT3 is also activated by growth-factor receptors, including epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGFR), platelet-derived growth factor receptor (PDGFR) and vascular endothelial growth factor receptor (VEGFR) (Yu et al., 2007). Furthermore, many tumor-produced factors, such as IL-10, IL-6 and VEGF, which are crucial for both tumor growth and immunosuppression, activate STAT3 to create an efficient 'feed-forward' mechanism to ensure increased STAT3 activity both in tumor cells and in tumor-associated immune cells (Yu et al., 2007).

STAT3 is constitutively activated in cells transformed by the oncoprotein Src, which is a non-receptor tyrosine kinase (Yu et al., 1995). Since inhibition of STAT3 signal blocks the transformation of fibroblasts by Src, STAT3 has been shown to be an important molecule for oncogenesis by Src (Bromberg et al., 1998; Turkson et al., 1998). However, a direct role of STAT3 in oncogenesis was shown using a constitutively active STAT3 mutant, which

transforms fibroblasts in culture and allows the transformed cells to form tumors in mice (Bromberg et al., 1999). Under physiological conditions in normal cells, the activation of STAT proteins is rapid and transient because they are negatively regulated by proteins such as SOCS and PIAS (Alexander, 2002; Kubo et al., 2003; Shuai and Liu, 2005).

In this study, we performed yeast two-hybrid screening using TRIM8 as bait and found that PIAS3 is a TRIM8-interacting protein. TRIM8 negatively regulates PIAS3 by degradation through the ubiquitin-proteasome pathway and/or exclusion of PIAS3 from the nucleus. These findings show that expression of TRIM8 might cause prolonged activity of STAT3, and thereby could induce oncogenesis.

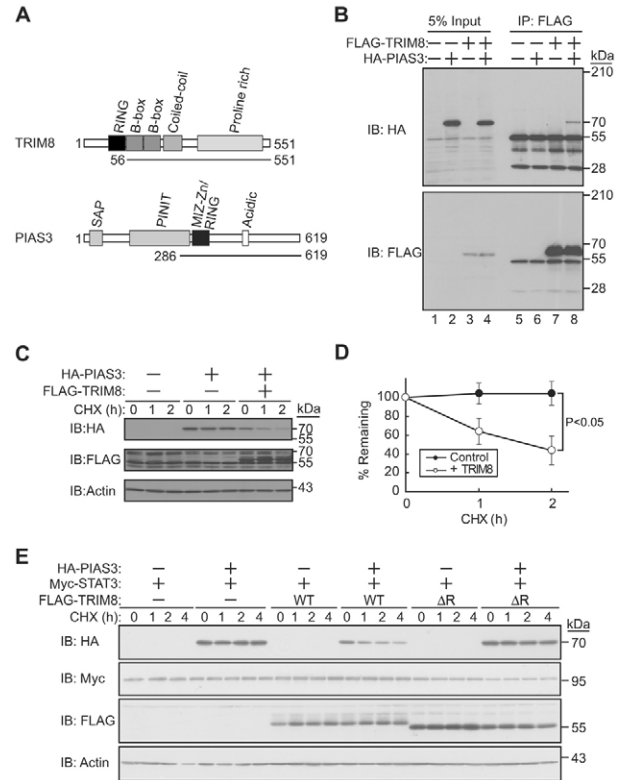
## Results

### TRIM8 interacts with PIAS3

Yeast two-hybrid screening was performed to identify proteins that interact with TRIM8. A mouse T-cell cDNA library was screened using TRIM8 lacking a RING domain ( $\Delta$ RING) as a bait, because immunoblot analysis showed that full-length TRIM8 was only faintly expressed in yeast cells. From  $5 \times 10^5$  transformants that were able to grow on Leu- and Trp-deficient medium, seven positive clones were isolated after two rounds of growth in the absence of His and screening for  $\beta$ -galactosidase activity. The nucleotide sequence of one of these seven clones led to the identification of *PIAS3*. As shown in Fig. 1A, TRIM8 has a RING domain, two B-boxes and a coiled-coil domain. PIAS3 also has a RING domain (MIZ-Zn finger) as well as a SAP box, a PINIT domain, and an acidic domain (Duval et al., 2003). The region that includes the RING domain and acidic domain of PIAS3 was identified as a TRIM8-interacting region by yeast two-hybrid screening. To determine whether TRIM8 also interacts with PIAS3 in mammalian cells, FLAG-tagged full-length TRIM8 and HA-tagged PIAS3 were expressed in HEK293T cells. Lysates of the transfected cells were then subjected to immunoprecipitation with anti-FLAG antibodies, and the resulting precipitates were subjected to immunoblot analysis with anti-HA antibodies (Fig. 1B). HA-PIAS3 was detected in FLAG-TRIM8 immunoprecipitates but not in control precipitates, indicating that PIAS3 specifically interacts with TRIM8 in mammalian cells. We further examined whether TRIM8 selectively interacts with PIAS3 by using PIAS1 as control, and we found that TRIM8 did not interact with PIAS1 (supplementary material Fig. S1).

### TRIM8 destabilizes PIAS3

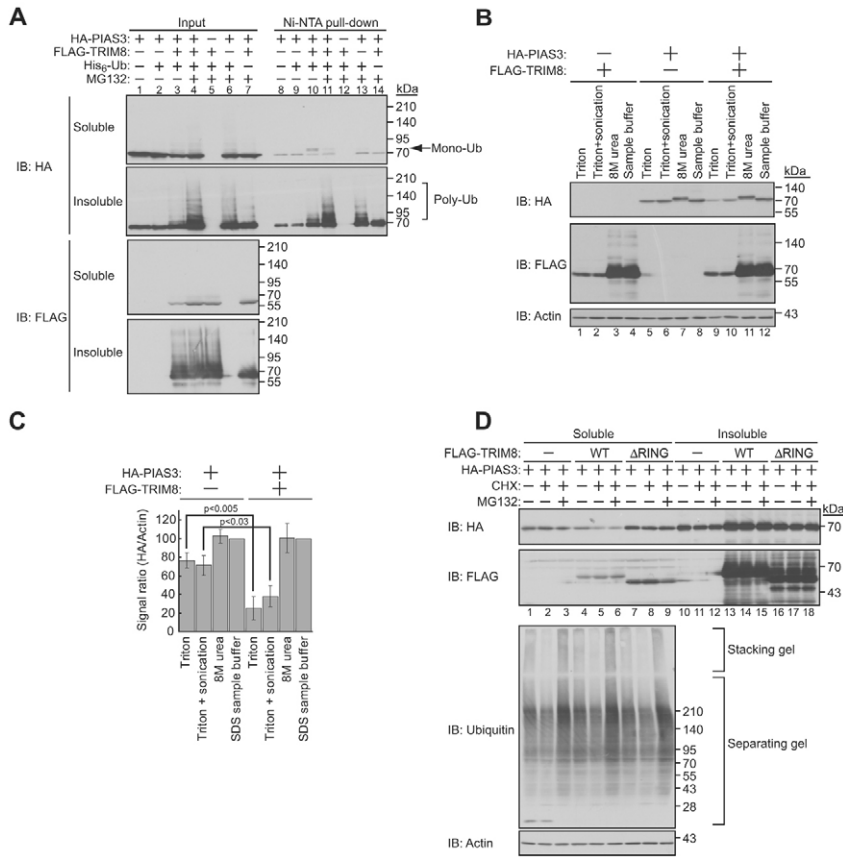
Next, we examined the stability of PIAS3 in the presence or absence of FLAG-TRIM8. PIAS3 was stable without ectopic expression of TRIM8, but expression of TRIM8 markedly promoted the degradation of PIAS3 (Fig. 1C). The half-life of PIAS3 was about 2 hours in the presence of TRIM8 (Fig. 1D). Since the RING domain of ubiquitin ligase is responsible for ligase activity in many cases, we deleted the RING domain of TRIM8 and examined the stability of PIAS3 in the presence of mutant TRIM8( $\Delta$ RING) (Fig. 1E). PIAS3 was stable for up to 4 hours without expression of TRIM8, whereas wild-type TRIM8 destabilized PIAS3. Mutant TRIM8( $\Delta$ RING) did not affect the stability of PIAS3, suggesting that the RING domain of TRIM8 is important for destabilization of PIAS3. Given that PIAS3 is able to interact with STAT3 (Chung et al., 1997), we hypothesize that TRIM8 affects the stability of STAT3. However, we did not detect any effect of TRIM8 on the stability of STAT3 for at least 4 hours (Fig. 1E).



**Fig. 1. Destabilization of PIAS3 by TRIM8.** (A) Schematic representation of TRIM8 and PIAS3. The binding regions of PIAS3 and TRIM8 are underlined. (B) TRIM8 binds to PIAS3. FLAG-tagged TRIM8 was expressed in HEK293T cells with or without HA-tagged PIAS3, followed by immunoprecipitation (IP) with anti-FLAG antibody. Immunoprecipitates were subjected to immunoblot (IB) analysis with anti-HA or FLAG antibody. (C) TRIM8 affects the stability of PIAS3. HEK293T cells were transfected by plasmids encoding FLAG-TRIM8 and/or HA-PIAS3, followed by cycloheximide (CHX) treatment (25  $\mu$ g/ml) for 0, 1 or 2 hours. The cell lysates were subjected to immunoblot (IB) analysis with anti-HA, anti-FLAG or anti- $\beta$ -actin antibodies.  $\beta$ -actin is shown as a loading control. (D) The intensity of the HA-PIAS3 bands was normalized to that of the corresponding  $\beta$ -actin bands shown in C and indicated as a percentage of the normalized value at 0 hour. Data are means  $\pm$  s.d. of values from three independent experiments. Open circle, FLAG-TRIM8; closed circle, Mock. (E) Destabilization of PIAS3 is dependent on the RING domain of TRIM8. HEK293T cells were transfected with plasmids encoding Myc-STAT3, FLAG-TRIM8 (WT or  $\Delta$ RING) and/or HA-PIAS3. The cell lysates were subjected to immunoblot (IB) analysis with anti-HA, Myc, FLAG or  $\beta$ -actin antibodies.  $\beta$ -actin is shown as a loading control.

### Ubiquitylation of PIAS3 by TRIM8

Since TRIM8 promoted degradation of PIAS3 (Fig. 1), we performed an *in vivo* ubiquitylation assay of PIAS3 in the presence of TRIM8 (Fig. 2A). PIAS3 and TRIM8 were expressed in HEK293T cells with or without His<sub>6</sub>-ubiquitin to detect ubiquitylation of PIAS3. We detected monoubiquitylation of PIAS3, but not polyubiquitylation, in the fraction soluble in 1% Triton X-100 (Fig. 2A). We therefore examined the Triton-insoluble fraction and detected polyubiquitylation of PIAS3, which is partially dependent on expression of TRIM8, suggesting that TRIM8 functions as a putative ubiquitin ligase for PIAS3 (Fig. 2A, lanes 3 and 10). Treatment with the proteasome inhibitor MG132 increased polyubiquitylation of PIAS3 (Fig. 2A, lanes 4 and 11),



**Fig. 2. Polyubiquitylation and translocation of PIAS3 into the detergent-insoluble fraction by TRIM8.**

(A) Polyubiquitylation of TRIM8 in the detergent-insoluble fraction. HEK293T cells were transfected with plasmids encoding FLAG-TRIM8, HA-PIAS3 and/or His<sub>6</sub>-tagged ubiquitin. Proteasome inhibitor MG132 (2 μM for 15 hours) was used to detect polyubiquitylation. The cell lysates were subjected to Ni-NTA pull-down to purify the proteins modified by His<sub>6</sub>-ubiquitin, followed by immunoblot (IB) analysis with anti-HA or FLAG antibody. (B) Solubility of PIAS3 in different lysis-buffer solutions. HEK293T cells were transfected with plasmids encoding FLAG-TRIM8 and HA-PIAS3, followed by lysis with 1% Triton X-100 lysis buffer with or without sonication, 8 M urea in PBS or SDS-PAGE sample buffer. The lysates were subjected to immunoblot (IB) analysis with anti-HA, FLAG, or β-actin antibody. β-actin is shown as a loading control. (C) The intensity of the HA-PIAS3 bands in B was normalized to that of the corresponding β-actin bands and indicated as a percentage of the normalized value of that in SDS sample buffer. Data are means ± s.d. of values from three independent experiments. (D) Reduction of soluble PIAS3 in 1% Triton X-100 lysis buffer by TRIM8. HEK293T cells were transfected with plasmids encoding FLAG-TRIM8 (WT or ΔRING) and/or HA-PIAS3. Transfected cells were treated with cycloheximide (25 μg/ml) in the presence or absence of MG132 (10 μM) for 3 hours. Triton-soluble or -insoluble fractions were subjected to immunoblot (IB) analysis with anti-HA, anti-FLAG, anti-ubiquitin or anti-β-actin antibodies. β-actin is shown as a loading control. Anti-ubiquitin antibodies were used to confirm the effect of the proteasome inhibitor MG132 and CHX.

indicating that TRIM8 ubiquitylated PIAS3 and contributed to the proteasomal degradation. PIAS3 was polyubiquitylated without TRIM8 expression in the presence of MG132 (Fig. 2A, lanes 6 and 13) and we did not detect endogenous TRIM8 in HEK293T cells (data not shown), suggesting that there are other ubiquitin ligases for PIAS3. We also noticed that there was a large amount of TRIM8 in the Triton-insoluble fraction in this condition. Based on these findings, we speculate that TRIM8 removes PIAS3 from the detergent-soluble fraction in a few hours (Fig. 1) and transfers PIAS3 to the detergent-insoluble fraction, followed by the degradation via the proteasome.

To confirm this idea, we examined the solubility of PIAS3 in different lysis buffers and under different conditions (Fig. 2B,C). Without expression of TRIM8, about 80% of PIAS3 was recovered by the Triton lysis buffer, compared with SDS-PAGE sample buffer (Fig. 2B, lanes 5 and 8). However, when TRIM8 was co-expressed, only about 20% of PIAS3 was recovered by Triton lysis buffer, compared with SDS-PAGE sample buffer (Fig. 2B, lanes 9 and 12). These results indicate that not only is TRIM8 a ubiquitin ligase for PIAS3, but it also acts as a carrier protein for PIAS3 to remove PIAS3 from the detergent-soluble fraction, which might act as an inhibitory pathway for PIAS3 (Fig. 2C).

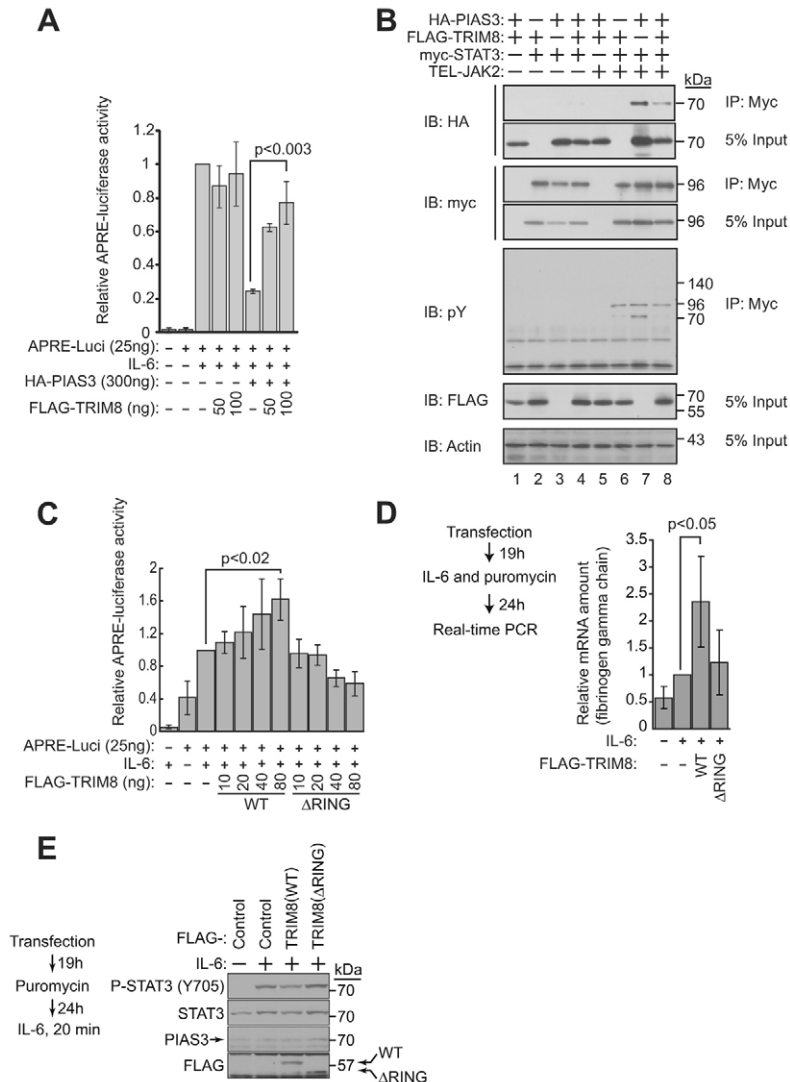
Since polyubiquitylation of PIAS3 by TRIM8 mainly occurred in the Triton-insoluble fraction, we also examined the protein stability of PIAS3 in the insoluble fraction (Fig. 2D). Reduction of detergent-soluble PIAS3 by TRIM8 was not inhibited by the proteasome inhibitor MG132 (Fig. 2D, lanes 4-6). However, overexpression of TRIM8 increased detergent-insoluble PIAS3 (Fig. 2D, lanes 13-15) compared with control samples (Fig. 2D, lanes 10-12) or samples expressing TRIM8(ΔRING) (Fig. 2D,

lanes 16-18). These results suggest that down-regulation of detergent-soluble PIAS3 by TRIM8 is achieved by translocation to the detergent-insoluble fraction, followed by proteasomal degradation.

### Enhancement of STAT3 activity by TRIM8 through inhibition of PIAS3

To investigate the biological function of TRIM8, we used a previously established luciferase assay with HepG2 cells (Nakajima et al., 1996). In this system, a reporter luciferase gene is driven by the acute-phase response element (APRE) (Wegenka et al., 1993), which is a preferential target for the STAT1 homodimer and STAT1-STAT3 heterodimer (Horvath et al., 1995). As reported previously (Nakajima et al., 1996), we detected IL-6-dependent transcription from APRE (Fig. 3A), which was inhibited by the STAT3-specific inhibitor PIAS3, as reported previously (Chung et al., 1997). Expression of TRIM8 did not affect IL-6-dependent transcription driven by APRE in the absence of HA-PIAS3. Most importantly, expression of TRIM8 rescued IL-6-dependent transcription that was inhibited by HA-PIAS3 in a dose-dependent manner.

Since PIAS3 binds to STAT3 and inhibits STAT3-mediated gene activation (Chung et al., 1997), we further examined the effect of TRIM8 on the interaction between PIAS3 and STAT3 (Fig. 3B). We used TEL-JAK2, which is a fusion protein consisting of human translocated ets leukemia (TEL) (codons 1-162) and mouse JAK2 JH1 domain (codons 839-1127) to activate STAT3 (Kamizono et al., 2001). This fusion gene corresponds to TEL-JAK2 found in B-cell lymphoblastic leukemia patients (Schwaller et al., 1998). TEL-mediated oligomerization of JAK2 results in constitutive activation of tyrosine kinase (Lacronique et al., 1997). We did not detect



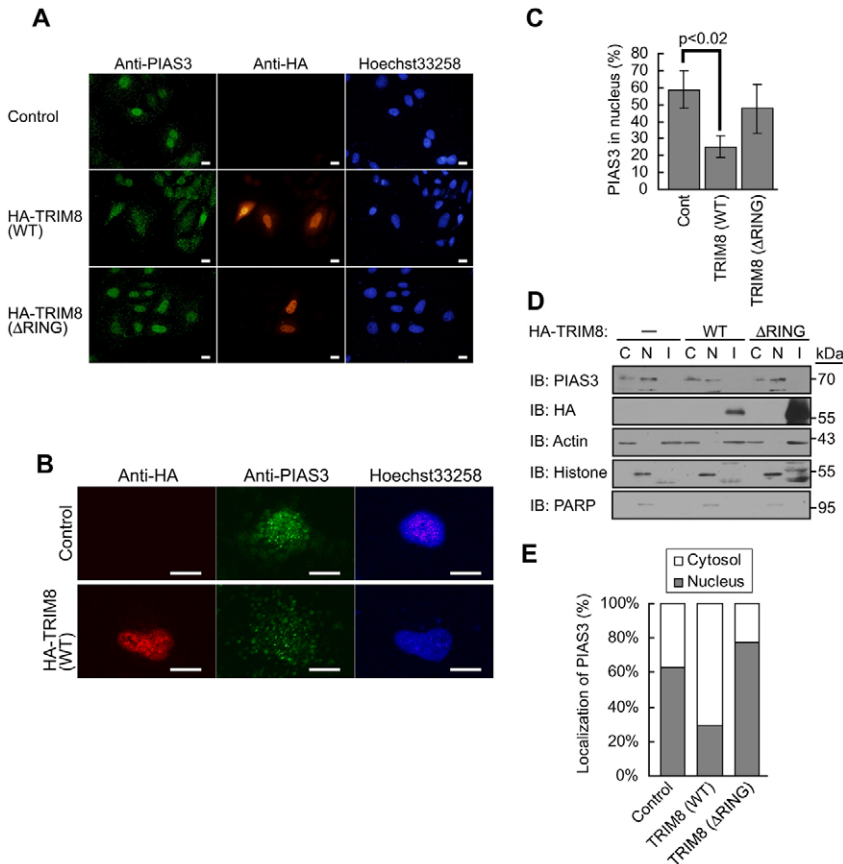
**Fig. 3. PIAS3-mediated transcriptional inhibition is negatively regulated by TRIM8.** (A) HepG2 cells were transfected with plasmids encoding FLAG-TRIM8, HA-PIAS3, and a reporter plasmid containing four copies of APRE in front of the minimal *junB* promoter linked to the firefly luciferase gene. 36 hours after transfection, HepG2 cells were stimulated with IL-6 (25 ng/ml) for 5 hours as indicated, and then luciferase activity was measured. Data are means  $\pm$  s.d. of values from three independent experiments. (B) Interaction between PIAS3 and STAT3 is inhibited by TRIM8. HEK293T cells were transfected with plasmids encoding FLAG-TRIM8, HA-PIAS3, Myc-STAT3 and/or TEL-JAK2, followed by immunoprecipitation (IP) with anti-Myc antibody. Immunoprecipitates were subjected to immunoblot (IB) analysis with anti-HA, anti-Myc, anti-phosphotyrosine (pY), anti-FLAG or anti- $\beta$ -actin antibodies.  $\beta$ -actin is shown as a loading control. (C) HeLa cells were transfected with plasmids encoding FLAG-TRIM8 and a luciferase-encoding reporter plasmid. 36 hours after transfection, HeLa cells were stimulated with IL-6 (25 ng/ml) for 5 hours as indicated, and then luciferase activities were measured. Data are means  $\pm$  s.d. of values from three independent experiments. (D) Upregulation of IL-6-dependent endogenous gene transcription by TRIM8. HeLa cells were transfected with a plasmid encoding both FLAG-TRIM8 (WT or  $\Delta$ RING) and puromycin-resistance gene. 19 hours after transfection, the cells were treated with puromycin (5  $\mu$ g/ml) and IL-6 (25 ng/ml), followed by further incubation for 1 day. mRNA was purified and reverse-transcribed to perform real-time PCR. Data are means  $\pm$  s.d. of values from three independent experiments. (E) Tyrosine phosphorylation status of STAT3 by IL-6 stimulation in the presence or absence of TRIM8 (WT or  $\Delta$ RING). HeLa cells were transfected with plasmid encoding both FLAG-TRIM8 (WT or  $\Delta$ RING) and puromycin-resistance gene. 19 hours after transfection, cells were treated with puromycin (5  $\mu$ g/ml), followed by further incubation for 1 day. Cells were stimulated by IL-6 (25 ng/ml) for 20 minutes and then subjected to immunoblot analysis with anti-STAT3, anti-phospho-STAT3, anti-PIAS3 and anti-FLAG antibodies.

interaction between PIAS3 and STAT3 without expression of TEL-JAK2 (Fig. 3B, lane 3). The expression of TEL-JAK2 induced tyrosine-phosphorylation of STAT3 as shown by immunoblotting with anti-phosphotyrosine antibodies (Fig. 3B, lanes 6–8). As reported previously (Chung et al., 1997), tyrosine phosphorylation of STAT3 caused interaction between PIAS3 and STAT3 (Fig. 3B, lane 7). TRIM8 expression reduced detergent-soluble PIAS3, resulting in down-regulation of the PIAS3-STAT3 interaction, although the same amount of STAT3 was immunopurified (Fig. 3B, lanes 7 and 8). Since HeLa cells express a larger amount of endogenous PIAS3 than do HepG2 cells (supplementary material Fig. S2), we expressed TRIM8 in HeLa cells to investigate whether expression of TRIM8 inhibits the function of endogenous PIAS3. Wild-type TRIM8 enhanced IL-6-dependent transcription of luciferase in a dose-dependent manner, whereas TRIM8( $\Delta$ RING) did not have any effect (Fig. 3C). We further investigated the effect of TRIM8 on IL-6-dependent endogenous gene expression. Since it has been reported that fibrinogen gamma chain (FGG),  $\alpha$ 2-macroglobulin and  $\alpha$ 1-acid glycoprotein (Orosomucoid) are regulated by APRE (Wegenka et al., 1993), we checked gene expression by real-time PCR and found that the expression of only FGG was clearly upregulated by IL-6 in HeLa cells. Wild-type

FLAG-TRIM8 or FLAG-TRIM8( $\Delta$ RING) expression plasmids that also contain a puromycin-resistance gene were transfected into HeLa cells, and untransfected cells were removed by puromycin treatment. Overexpression of TRIM8(WT) upregulated endogenous FGG expression, whereas that of TRIM8( $\Delta$ RING) did not (Fig. 3D). It has been reported that STAT3-dependent gene expression was regulated by tyrosine phosphorylation of STAT3 (Yu et al., 2007). However, overexpression of TRIM8 did not affect the phosphorylation status of STAT3 by IL-6 stimulation (Fig. 3E). These findings suggest that TRIM8 positively regulates STAT3-dependent gene expression but that the regulation is not dependent on tyrosine phosphorylation (Y705) of STAT3.

#### TRIM8 disrupts nuclear localization of PIAS3

Since it has been reported that PIAS3 is a nuclear protein (Duval et al., 2003), we next examined the localization of endogenous PIAS3 in HeLa cells in the presence or absence of TRIM8. PIAS3 mainly localized in the nucleus, but co-expression of HA-TRIM8 caused disruption of nuclear localization of PIAS3 (Fig. 4A,B and supplementary material Fig. S3). Only strong expression of HA-TRIM8 was detected by immunofluorescence staining, and it was thought that weak expression of HA-TRIM8 that was not obviously



**Fig. 4. TRIM8 translocates PIAS3 from the nucleus to cytosol.**

(A) Localization of PIAS3 with or without TRIM8 (WT or  $\Delta$ RING). HeLa cells were transfected with a plasmid encoding HA-TRIM8 (WT or  $\Delta$ RING) and stained with anti-PIAS3 or anti-HA antibody, followed by incubation with anti-mouse antibodies conjugated with Alexa Fluor 488 or anti-rabbit antibodies conjugated with Alexa Fluor 546. Nuclei were visualized using Hoechst 33258. Scale bars: 10  $\mu$ m. (B) Representative images at high magnification taken by confocal microscopy. Scale bars: 10  $\mu$ m. (C) Statistical analysis of cells in which PIAS3 was localized mainly in the nucleus. Approximately 60 cells from three independent experiments were counted. Data are means  $\pm$  s.d.

(D) Translocation of PIAS3 from the nucleus to cytosol by TRIM8. HeLa cells were transfected with a plasmid encoding HA-TRIM8 (WT or  $\Delta$ RING). Cytosolic, nuclear and insoluble fractions were subjected to immunoblot (IB) analysis with anti-PIAS3, anti-HA, anti- $\beta$ -actin, anti-Histone H1 or anti PARP antibodies.  $\beta$ -actin is shown as cytosolic protein and insoluble protein, and Histone H1 and PARP are shown as nuclear proteins.

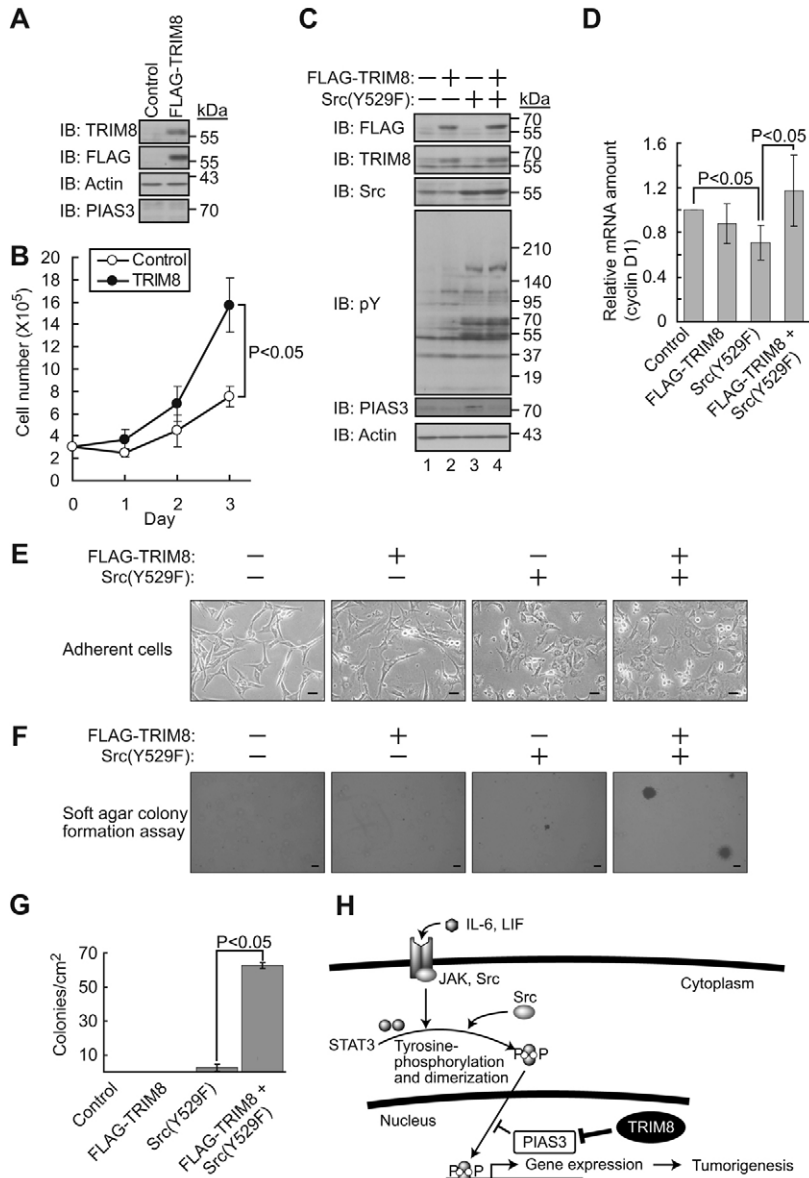
(E) Quantification of PIAS3 in cytosol or nuclear fractions. The intensities of PIAS3 bands in D were quantified using a densitometer.

detected was probably sufficient to translocate endogenous PIAS3. However, mutant TRIM8 lacking a RING domain did not affect localization of endogenous PIAS3 (Fig. 4A,C). These findings suggest that TRIM8 is localized in the nucleus and affects the localization of PIAS3, and that the RING domain of TRIM8 is important for regulation of the subcellular localization of PIAS3. The effect of TRIM8 on localization of endogenous PIAS3 was further confirmed by biochemical subcellular fractionation of HeLa cells. PIAS3 in cytosolic, nuclear and insoluble fractions was detected by immunoblotting with anti-PIAS3 antibodies (Fig. 4D). Endogenous PIAS3 was mainly localized in the nucleus of control HeLa cells, but was found in the cytosol of TRIM8-expressing cells. The TRIM8 mutant lacking a RING domain did not affect the localization of PIAS3 (Fig. 4D,E). These results suggest that TRIM8 exists mainly in the nuclear detergent-insoluble fraction and that it regulates localization of PIAS3 in HeLa cells.

#### TRIM8 enhances anchorage-independent cell growth by constitutive active Src

STAT3 has a broad range of biological functions, including cell activation, cell proliferation and apoptosis. Activated STAT3 has been shown to protect tumor cells from apoptosis and promote cell proliferation by regulating genes encoding antiapoptotic and proliferation-associated proteins, such as Bcl- $x_L$ , Mcl-1, Bcl-2, Fas, cyclin D1, survivin and Myc (Huang, 2007). To examine the effect of TRIM8 on cell growth, we established a NIH3T3 cell line that stably expresses FLAG-TRIM8 by retrovirus-mediated transduction (Fig. 5A). Expression of TRIM8 markedly increased cell growth rate compared with that of the control cell line (Fig. 5B). Mutant cellular Src(Y529F) has been shown to be a

constitutively active form of cellular Src (Kmieciak and Shalloway, 1987), and viral Src has been shown to contribute to transformation through activation of STAT3 (Bromberg et al., 1998; Turkson et al., 1998). We therefore used Src(Y529F) to study the effect of TRIM8 on the transformation of NIH3T3 cells by activated STAT3. First, we established NIH3T3 cell lines that expressed FLAG-TRIM8 and/or Src(Y529F) (Fig. 5C). Expression of FLAG-TRIM8 did not affect the Src protein level (Fig. 5C, lanes 3 and 4). As expected, expression of Src(Y529F) enhanced tyrosine phosphorylation of some proteins as shown by anti-phosphotyrosine immunoblotting (Fig. 5C). More importantly, constitutive expression of Src(Y529F) upregulated endogenous PIAS3, which was downregulated by co-expression of FLAG-TRIM8 (Fig. 5C). Consistent with this observation, one of the STAT3 target genes, cyclin D1 (*CCND1*), was negatively regulated by stable expression of Src(Y529F), suggesting that induced PIAS3 inhibited STAT3-dependent transcription of *CCND1* (Fig. 5D). Additional expression of FLAG-TRIM8 neutralized the upregulation of PIAS3, which resulted in a modest but significant induction of cyclin D1 compared with that in NIH3T3 cells expressing Src(Y529F). This suggests that TRIM8 regulates the Src-STAT3 signal pathway through downregulation of PIAS3. Interestingly, expression of FLAG-TRIM8 led to a rough cell surface compared with mock-treated cells, which had a smooth surface (Fig. 5E). As observed previously, expression of active Src flattened NIH3T3 cells and induced formation of filopodia (Herrmann et al., 2007), and coexpression of FLAG-TRIM8 and active Src further enlarged and flattened cells. We also noticed that co-expression of FLAG-TRIM8 and active Src produced more rounded cells compared with control cells (Fig. 5E). Next, we examined anchorage-independent cell



**Fig. 5. TRIM8 enhances anchorage-independent cell growth in collaboration with constitutively active Src.**

(A) Establishment of a stable NIH3T3 cell line expressing FLAG-TRIM8. NIH3T3 cells were infected with a control retrovirus or retrovirus encoding FLAG-TRIM8, followed by selection with puromycin. Immunoblot (IB) analysis was performed with anti-TRIM8, anti-FLAG, anti- $\beta$ -actin or anti-PIAS3 antibodies.  $\beta$ -actin is shown as loading control. (B) TRIM8 accelerates cell growth.  $3 \times 10^5$  cells of each NIH3T3 cell line were seeded and the cell number was counted every day. Data are means  $\pm$  s.d. of values from three independent experiments. (C) Establishment of a stable NIH3T3 cell line expressing FLAG-TRIM8 and Src(Y529F). A stably expressing FLAG-TRIM8 NIH3T3 cell line was further infected with a control retrovirus or retrovirus encoding Src(Y529F), followed by selection with hygromycin, and then immunoblot (IB) analysis was performed with anti-FLAG, anti-TRIM8, anti-Src, anti-PIAS3, anti-phosphotyrosine (pY) or anti- $\beta$ -actin antibodies.  $\beta$ -actin is shown as a loading control. (D) Regulation of endogenous *CCND1* gene transcription by Src(Y529F) and TRIM8. mRNAs of four cell lines in C were purified and reverse transcribed to perform real-time PCR. Data are presented as mean  $\pm$  s.d. from two independent experiments, both of which were duplicated ( $n=4$ ). (E) Cell shapes of stable cell lines. Scale bars: 30  $\mu$ m. (F) Anchorage-independent cell growth by expression of a combination of Src(Y529F) and TRIM8. NIH3T3 cell lines were plated into soft-agar medium at a density of  $1 \times 10^5$  cells. Colonies were photographed at day 10 after seeding. Scale bars: 0.2 mm. (G) The numbers of colonies visible in F with a diameter of  $>0.1$  mm in six randomized areas (1 cm<sup>2</sup>). Data are means  $\pm$  s.d. of values from two independent experiments (three areas from one experiment). (H) A putative model of the inhibitory effect of TRIM8 on JAK-STAT signaling. STAT3 is activated by IL-6, LIF or other cytokines through phosphorylation of the tyrosine residue of STAT3 by JAK or Src. Phosphorylation of the tyrosine residue of STAT3 results in homodimerization of STAT3 and translocation into the nucleus to function as a transcription factor. PIAS3 inhibits DNA-binding activity of STAT3, resulting in downregulation of STAT3-dependent transcription. TRIM8 activates STAT3-dependent transcription through inhibition of PIAS3 by proteasomal degradation and/or translocation to the cytosolic fraction.

growth of these cell lines. It has been reported that STAT3 is an important molecule for oncogenesis by Src (Bromberg et al., 1998; Turkson et al., 1998) and we found that stable expression of Src(Y529F) promoted anchorage-independent cell growth (Fig. 5F,G). Most importantly, coexpression of TRIM8 with Src(Y529F) enhanced the anchorage-independent cell-growth rate as judged by the number of colonies larger than 0.1 mm (Fig. 5F,G). The colonies triggered by TRIM8 and Src(Y529F) in the soft agar were increased about 15-fold compared with those promoted by Src(Y529F) alone (Fig. 5G). TRIM8 expression itself did not promote anchorage-independent cell growth, whereas the combination of TRIM8 and Src(Y529F) enhanced anchorage-independent cell growth, suggesting that TRIM8 enhances the STAT3 signal activated by Src(Y529F).

## Discussion

We showed that PIAS3 is a TRIM8-interacting protein. Because PIAS is involved in regulation of the JAK-STAT signal, we further examined the relationship between TRIM8 and PIAS3 and found

that TRIM8 functions as a negative regulator of PIAS3. Negative regulation of PIAS3 enhances the STAT3 signal because PIAS3 is a specific inhibitor for STAT3 (Chung et al., 1997). It has been reported that STAT3 contributes to cell proliferation and oncogenesis (Bromberg et al., 1998; Bromberg et al., 1999; Turkson et al., 1998; Yu et al., 2007). Thus, enhancement of STAT3 signal by TRIM8 probably results in oncogenic transformation. In fact, we also showed that TRIM8 further enhances anchorage-independent cell growth induced by active Src, which supports the possibility that TRIM8 enhances the STAT3 signal by inhibiting PIAS3.

Recombinant TRIM8 protein expressed in the cells by transfection was quite insoluble in 1% Triton X-100 and consequently we failed to prepare a recombinant protein from *E. coli* or insect cell line SF9 as a soluble protein to perform an in vitro ubiquitylation assay. Although we were not able to perform an in vitro ubiquitylation assay, we detected increased polyubiquitylation of PIAS3 by TRIM8 in the Triton-insoluble fraction in an in vivo ubiquitylation assay (Fig. 2A). Ectopically

expressed FLAG-TRIM8 mainly exists in the Triton-insoluble fraction in HEK293T cells and also in HeLa cells. However, interestingly, FLAG-TRIM8 was soluble in NIH3T3 cells (data not shown). Thus, the solubility of TRIM8 in detergent-containing buffer might be dependent on the expression level or cell type.

It has been reported that TRIM8 functions as a SOCS-1-interacting protein and that truncated TRIM8 consisting of the N-terminal 204 amino acids accelerated the degradation of SOCS-1 (Toniato et al., 2002). These findings suggest that TRIM8 is a ubiquitin ligase for SOCS-1 and that TRIM8 thereby regulates the JAK-STAT signal. However, it has not yet been determined whether endogenous TRIM8 is able to accelerate the degradation of SOCS-1. It has also been shown that transcription of *TRIM8* is induced by stimulation by IFN $\gamma$  (Toniato et al., 2002). However, we could not detect induction of endogenous TRIM8 protein by IFN $\gamma$  (data not shown). It is crucial to determine how the TRIM8 expression level is regulated. We tried IFN $\alpha$  and/or IFN $\beta$  (for HeLa cell line) and IL-6 (for HepG2 cell line) but failed to induce endogenous TRIM8 (data not shown). However, we detected mRNA encoding *TRIM8* in HeLa cells by RT-PCR (data not shown), suggesting that TRIM8 expression is inhibited in HeLa cells at the translational level. Given that expression of TRIM8 results in enhancement of the STAT3 signal through inhibition of PIAS3, expression of TRIM8 may be continuously inhibited by undetermined mechanisms. Since sustained expression of TRIM8 induces tumorigenesis, which depends on activation of the STAT3 pathway, it might be important to inhibit the expression of TRIM8. These findings suggest that inhibitors of TRIM8 or activators of PIAS3 could potentially act as carcinostatic drugs.

PIAS3 has been shown to inhibit microphthalmia transcription factor (MITF) (Levy et al., 2002). Recently, it has been reported that a 23-residue peptide derived from PIAS3, which inhibits the transcriptional activity of both *MITF* and *STAT3*, induced apoptosis in a rat basophilic leukemia cell line, RBL-2H3, and in a mouse melanoma cell line, B16F10.9 (Yagil et al., 2009). Hence, it would be interesting to determine whether these cell lines express endogenous TRIM8 in order to clarify the regulation of MITF and STAT3 activity.

It has been reported that PIAS3 also appears to regulate NF- $\kappa$ B (Jang et al., 2004) and estrogen receptor  $\alpha$  (ER $\alpha$ ) (Sentis et al., 2005), suggesting that TRIM8 also modulates several signal pathways. Results of future studies aimed at elucidation of the relation between TRIM8 and binding proteins should be helpful for advances of cancer biology, developmental biology and obstetrics.

## Materials and Methods

### Yeast two-hybrid screening

Yeast two-hybrid screening was performed as described previously (Okumura et al., 2004). In brief, yeast strain L40 (Invitrogen, Carlsbad, CA) was transformed with both with pBTM116 encoding the FLAG-tagged human TRIM8 (GenBank<sup>TM</sup> accession number NM\_030912) and with a mouse T-cell cDNA library in pACT (Clontech Laboratories, Mountain View, CA). The cells were then streaked on plates of medium lacking histidine to detect interaction-dependent activation of HIS3 according to the protocol of the manufacturer (Clontech).

### Plasmid construction

Human *TRIM8* and *PIAS1* (GenBank<sup>TM</sup> accession number NM\_016166) cDNA were amplified by polymerase chain reaction (PCR) from a HeLa cell cDNA library with PCR primers 5'-gccatgcccggagaattggaag-3' and 5'-gcgttagctcgtcagctagtg-3' for *TRIM8*, 5'-aagatgcccggacagtcggg-3' and 5'-gaatcagccaatgaataatg-3' for *PIAS3*. A deletion mutant of the RING domain of TRIM8 was made with primers 5'-aaccagcgcctcaaccagag-3' and 5'-gcgttagctcgtcagctagtg-3'. The amplified fragment was subcloned into the *EcoRV* site of pBluescriptII (Stratagene, La Jolla, CA), and the sequence was verified. Full-length or RING-domain deletion mutant of *TRIM8*

cDNA was subcloned into *HindIII* and *EcoRI* sites of pFLAG-CMV2 (Sigma). *PIAS1* was subcloned into *SpeI* and *Sall* sites of pCGN-HA. A FLAG-tagged *TRIM8* fragment was amplified by PCR with primers 5'-atggactacaagacgatg-3' and 5'-gcgttagctcgtcagctagtg-3'. The amplified fragment was subcloned into pCAG-puro, pGBK-LexA and pMSCV-puro (Boyapati et al., 2007; Peterson et al., 2007). pCGN-HA-human *PIAS3* was kindly provided by Hidehisa Takahashi (Kyushu University, Fukuoka, Japan), and pcDNA3-Myc-*STAT3* and TEL-JAK were provided by Akihiko Yoshimura (Keio University, Tokyo, Japan). His $_6$ -ubiquitin was described previously (Okumura et al., 2004). APRE-Luc was kindly provided by Toshio Hirano (Nakajima et al., 1996).

### Cell culture and transfection

HEK293T, HepG2 and HeLa cells (ATCC, Manassas, VA) were cultured as described previously (Okumura et al., 2007). NIH3T3 cells (ATCC) were cultured as described previously (Kano et al., 2008). 293T cells were transfected using calcium phosphate precipitation as described previously (Okumura et al., 2007). For small-scale transfection, Fugene HD reagent (Roche, Branchburg, NJ) was used according to the manufacturer's protocol.

### Immunoprecipitation, Ni-NTA pull-down and western blot

Immunoprecipitation, Ni-NTA pull-down and western blot analysis were performed as reported previously (Okumura et al., 2004).

### Antibodies

Antibodies against  $\beta$ -actin (0.2  $\mu$ g/ml; AC15, Sigma), FLAG (1  $\mu$ g/ml; M2 and M5 Sigma), HA (1  $\mu$ g/ml; HA.11, Covance, Berkeley, CA), HA (0.2  $\mu$ g/ml; Y-11, Santa Cruz Biotechnology, Santa Cruz, CA), His $_6$  (0.2  $\mu$ g/ml; H-15, Santa Cruz), Myc (1  $\mu$ g/ml; 9E10, Covance), phosphotyrosine (1  $\mu$ g/ml; 4G10, Upstate, Charlottesville, VA), PIAS3 (0.2  $\mu$ g/ml; C-12, Santa Cruz), poly (ADP-ribose) polymerase (PARP) (1:1000 dilution, 46D11, Cell Signaling, Danvers, MA), Src (1:1000 dilution, GD11, Upstate Biotechnology), STAT3 (1:1000 dilution, Cell Signaling), phospho-STAT3 (Tyr705) (1:1000 dilution, M9C6, Cell Signaling), TRIM8 (1:1000 dilution, B01, Abnova, Taiwan) and ubiquitin (1  $\mu$ g/ml; FK2, Biomol International, Plymouth Meeting, PA) were purchased from the respective manufacturers.

### Retroviral expression system

Approximately 50% confluent HEK293T cells in 100 mm dishes were cotransfected with 10  $\mu$ g pMSCV-puro-FLAG-TRIM8 or an empty plasmid along with 10  $\mu$ g amphotropic packaging plasmid pCL10A1 by the calcium phosphate precipitation. Medium containing retrovirus was collected 48 hours after transfection, and retroviral supernatant was added to NIH3T3 cells in 60 mm dishes with 8  $\mu$ g/ml polybrene (Sigma). Cells were cultured in 10  $\mu$ g/ml puromycin for 1 week following retroviral transduction and resistant cells were pooled. These cells were further infected with retrovirus expressing constitutive active Src or control retrovirus made by pMX-hyg-Src(Y529F) or pMX-puro empty plasmid, as described previously (Kano et al., 2008).

### Immunofluorescence staining

HeLa cells were fixed by phosphate buffer saline (PBS) containing 4% formaldehyde and 0.1% Triton X-100 for 15 minutes at room temperature, followed by blocking with PBS containing 2% BSA and 1% sheep serum for 30 minutes at room temperature. Then the cells were incubated overnight at 4°C with anti-PIAS3 antibodies (1  $\mu$ g/ml) and anti-HA antibodies (Y-11, 0.2  $\mu$ g/ml) in blocking solution containing 0.1% Tween 20. Cells were washed three times with TBST (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20), followed by incubation with Alexa Fluor 546 goat anti-rabbit antibodies and Alexa Fluor 488 goat anti-mouse antibodies (Invitrogen) (both at 1:3000 dilution) in blocking solution for 1 hour at room temperature in the dark. The cells were further incubated with Hoechst 33258 (0.3  $\mu$ g/ml) in PBS for 30 seconds followed by extensive washing with TBST and then photographed with a CCD camera (DP71, Olympus, Japan) or confocal microscope (Eclipse A1, Nikon, Japan).

### Colony formation assay

For the colony formation assay,  $1 \times 10^5$  cells were plated in 60 mm dishes containing 0.4% soft agar and cultured for 10 days. The number of colonies with a diameter of >0.1 mm in six randomized areas (1 cm<sup>2</sup>) was counted.

### Dual-luciferase assay

HepG2 cells were seeded in 24-well plates at  $1 \times 10^5$  cells per well and incubated overnight. Acute-phase response element (APRE)-luciferase reporter plasmid (Nakajima et al., 1996) and pRL-TK *Renilla* luciferase plasmid (Promega, Madison, WI) were transfected in various combinations with FLAG-TRIM8 and HA-PIAS3 expression plasmid into HepG2 cells using the Fugene HD reagent (Roche). 36 hours after transfection, HepG2 cells were stimulated with IL-6 (25 ng/ml) (PeproTech, Rocky Hill, NJ) for 5 hours, as described previously (Nakajima et al., 1996). Then the cells were harvested and assayed for luciferase activity by the Dual-Luciferase Reporter Assay System (Promega). Luminescence was quantified with a luminometer (Promega).

**Real-time PCR analysis**

Total RNA was isolated from HeLa or NIH3T3 cells with the use of an ISOGEN (Nippon Gene, Tokyo, Japan), followed by reverse transcription (RT) by RevaTra Ace (Toyobo). The resulting cDNA was subjected to real-time PCR with a StepOne machine and Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The average threshold cycle (Ct) was determined from three independent experiments and the level of gene expression relative to *GAPDH* was determined. The primer sequences for fibrinogen gamma chain (*FGG*) (GenBank™ accession number NM\_000509.4), *GAPDH* (GenBank™ accession number NM\_002046) and *CCND1* (GenBank™ accession number NM\_007631) were as follows: *FGG*, 5'-ggacatctgtctctactgacacac-3' and 5'-tcttaatgcatatggatggcagactg-3'; *GAPDH*, 5'-gcaattccatggcaccgt-3' and 5'-tcgcccactgatttgg-3'; and *CCND1*, 5'-cctctcctgctaccgcacac-3' and 5'-gcccaggctgactcagaag-3'.

**Statistical analysis**

The Student's *t*-test was used to determine the statistical significance of experimental data.

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Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/123/13/2238/DC1>

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