Biochimica et Biophysica Acta 1813 (2011) 1784–1792

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

TRIM8 regulates Nanog via Hsp90β-mediated nuclear translocation of STAT3 in embryonic stem cells

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ARTICLE INFO

Article history:
Received 20 January 2011
Received in revised form 13 April 2011
Accepted 24 May 2011
Available online 2 June 2011

Keywords:
TRIM8
Hsp90β
STAT3
Nanog
Ubiquitin

ABSTRACT

TRIM8 is a member of a protein family defined by the presence of a common domain structure composed of a tripartite motif including a RING-finger, one or two B-box domains and a coiled-coil motif. Here, we show that TRIM8 interacts with Hsp90β, which interacts with STAT3 and selectively downregulates transcription of *Nanog* in embryonic stem cells. Knock-down of TRIM8 increased phosphorylated STAT3 in the nucleus and also enhanced transcription of *Nanog*. These findings suggest that TRIM8 modulates translocation of phosphorylated STAT3 into the nucleus through interaction with Hsp90β and consequently regulates transcription of *Nanog* in embryonic stem cells.

1. Introduction

Ubiquitin-mediated proteolysis plays an important role in the elimination of short-lived proteins [1], 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 [2,3]. This pathway includes two key steps: covalent attachment of multiple ubiquitin molecules to the protein substrate and degradation of the ubiquitinated protein by the 26S proteasome complex. The system responsible for the attachment of ubiquitin to the target protein consists of several components that act in concert [4,5], 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 [5,6]. On the basis of structural similarity, E3 enzymes have been classified into three families: HECT (*h*omologous to *E*6-AP *C*OOH *t*erminus) family [3,7], U-box family [8–10] and RING finger-containing protein family [11–13].

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 (so-called RBCC motif) [14,15]. The conservation of these domains in TRIM proteins from various species indicates that the RBCC motif is the defining characteristic of this superfamily. TRIM proteins are conserved throughout the metazoan kingdom and have expanded rapidly during vertebrate evolution [16]. There are now more than 70 known TRIM proteins in humans and mice. Many TRIM proteins are induced by type I and type II interferons (IFNs), suggesting that TRIM proteins play an important role in anti-viral and anti-microbial systems [17].

It has been reported that TRIM8 is expressed in various human tissues, including the brain, lung, breast, gut, placenta, kidney, muscle and germinal center B cells [18,19]. TRIM8 contains an N-terminal RING finger, followed by two B-boxes and a coiled-coil domain, and thus belongs to the TRIM/RBCC family. It has been reported that TRIM8 localized to specific nuclear bodies and cytosolic speckles in U2OS and HeLa cells [19,20]. TRIM8 has been identified as a suppressor of cytokine signaling (SOCS)-1-interacting protein [21]. It has been reported that *TRIM8* mRNA can be induced by IFN-γ in murine B lymphoid M12 cells, murine fibroblasts and HeLa cells and that amino-terminal 204 amino acids of TRIM8 accelerate the degradation of SOCS-1 and reverse SOCS-1-mediated inhibition of Janus kinase (JAK)-STAT activation by IFN-γ [21]. However, it is not

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doi:10.1016/j.bbamcr.2011.05.013

clear whether full-length TRIM8 truly regulates the JAK-STAT pathway. We have recently reported that TRIM8 interacts with and inhibits the function of the protein inhibitor of activated STAT3 (PIAS3) [20]. It has been reported that PIAS3 inhibits DNA-binding activity of STAT3, followed by the suppression of STAT3-mediated gene activation [22]. 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 JAK family [23–26].

STAT3 has been shown to play an important role in the signaling pathway dependent on leukemia inhibitory factor (LIF) in ES cells to maintain a pluripotent state [27]. Recently, it has been shown that LIF-dependent signaling pathways in mouse ES cells consist of two parallel pathways [28]. In brief, the JAK-STAT3 pathway and the phosphatydylinositol-3-phosphate (PI3) kinase-Akt pathway regulate Klf4 and Tbx3, respectively. Klf4 mainly regulates Sox2, whereas Tbx3 mainly regulates Nanog [28]. Transfection of *Tbx3* or *Klf4* into the CCE-derived MGZ5 ES cell line using an integrated copy of the transgene resulted in the formation of LIF-independent clones [28]. However, no stable transfectants were recovered when MGZ5 ES cells were transfected with *Tbx3* and *Klf4* using an episomal vector system that confers a high expression level of the transgene, indicating a tight dosage effect of these genes [27]. Tbx3, Klf4 and Nanog were heterogeneously expressed in ES cells and were not always coordinated, although 24% of the cells were triple-positive [28]. These findings clearly indicate that ES cells are not simply regulated by LIF in a conventional culture condition but are also modulated by other signals. In contrast, Oct3/4 expression was quite homogeneous [28,29], indicating that the fluctuating expression of upstream transcription factors converged to a constant expression of Oct3/4, maintaining a steady state of self-renewal [28]. However, it has not been clarified how upstream fluctuating signals are controlled as a constant signal to Oct3/4.

In this study, we showed that TRIM8 binds to Hsp90β in mouse ES cells. Hsp90β has been shown to interact with STAT3 and to accelerate nuclear translocation of STAT3 in mouse ES cells [30]. Knock-down of TRIM8 in mouse ES cells resulted in accumulation of tyrosine 705 (Y705)-phosphorylated STAT3 in the nucleus and also resulted in up-regulation of transcription of Nanog, suggesting that TRIM8 has an important role in the regulation of STAT3-mediated signaling in ES cells.

2. Materials and methods

2.1. Plasmid construction

FLAG–TRIM8(WT or ΔRING) and HA-TRIM8(WT or ΔRING) were described previously [20]. TRIM8 cDNA was ligated into *Eco*R I and *Sal* I sites of pET30a (Merck, Whitehouse Station, NJ) or pMAL-c2 (New England BioLab, Ipswich, MA). pSUPER-retro-puro (OligoEngine, Seattle, WA) was utilized for knock-down of TRIM8. pSUPER-retro-puro containing a nonfunctional random sequence or TRIM8 sequences (#2: 5′-GAACACCAAGTCTGTGAAA-3′, and #5: 5′-GATTCTCGTCTGTTC TGTG-3′) was constructed according to the manufacturer′s protocol.

2.2. Reagents

Cycloheximide, cisplatin, etoposide, retinoic acid, trichloroacetic acid, anti-FLAG-M2 agarose, FLAG peptide and protein G sepharose were purchased from Sigma (St. Louis, MO). Protein A sepharose (GE Healthcare Bioscience, Piscataway, NJ, USA) and DMP (ThermoFisher, Waltham, MA, USA) were purchased from the indicated manufacturers.

2.3. Cell culture and transfection

293T and HeLa cells were cultured as described previously [31]. Mouse embryonic stem cells (E14) were cultured as described previously [32]. Recombinant LIF was produced by CHO cells stably expressing LIF (gift from Dr. Ichiro Taniuchi, RIKEN, Japan). 293T cells were transfected using calcium phosphate precipitation as described previously [31]. For small-scale transfection, Fugene HD reagent (Roche, Mannheim, Germany) was used according to the manufacturer's protocol.

2.4. Immunoprecipitation and immunoblot analyses

Immunoprecipitation and immunoblot analyses were performed as reported previously with some modifications to detect endogenous interaction between Hsp90β and TRIM8 [33]. In brief, ProteinA/G sepharose beads were incubated with anti-TRIM8 antibody overnight and then washed with 0.2 M phosphate (pH 8.0) followed by crosslinking with 20 mM DMP at room temperature for 1 h. The beads were washed with TBS and blocked with 5% skim milk at 4 °C for 7 h. The beads were washed with lysis buffer and incubated with E14 cell lysates at 4 °C overnight. Then the beads were washed again with lysis buffer and boiled in SDS-PAGE sample buffer.

2.5. Antibodies

Antibodies against β-actin (0.2 μg/ml; AC15, Sigma), FLAG (1 μg/ml; M2 and M5, Sigma), HA (1 μg/ml; HA.11, Covance, Berkeley, CA), TRIM8 (1:1000 dilution, B01, Abnova, Taipei City, Taiwan), Nanog (0.1 μg/ml; Abcam, Cambridge, MA), p53 (0.2 μg/ml; Pab240, Santa Cruz Biotechnology, Santa Cruz, CA), Histone H2B (0.2 μg/ml; FL-126, Santa Cruz), PARP (1:1000 dilution, 46D11, Cell Signaling, Danvers, MA), Hsp90β (0.25 μg/ml; SMC-107A/B, StressMarq, Victoria, Canada), PRMT1 (0.1 μg/ml; PRMT1-171, Santa Cruz), HP1α (0.5 μg/ml; BMP001, MBL, Nagoya, Japan), Akt (1:1000 dilution, #9272, Cell Signaling), STAT3 (1:1000 dilution, #9132, Cell Signaling), p-STAT3 (1:1000 dilution, #4113, Cell Signaling), and HDAC1 (1:1000 dilution, #2062, Cell Signaling) were purchased from the indicated manufacturers. Rabbit anti-TRIM8 antibody was generated using recombinant His_6-TRIM8, which was purified from *Escherichia coli* by Ni-NTA agarose (Invitrogen, Carlsbad, CA) in the presence of 8 M urea. Anti-TRIM8 antibody was further purified by maltose binding protein (MBP)-fused TRIM8 according to the protocol of the manufacturer (New England BioLabs).

2.6. Retrovirus infection

Approximately 50% confluent 293T cells in 60-mm dishes were cotransfected with 1 μg pSUPER-retro-puro containing a nonfunctional random sequence or TRIM8 sequences along with 1 μg amphotrophic packaging plasmid pCL10A1 by the calcium phosphate precipitation method. Media containing retrovirus were collected 48 h after transfection, and retroviral supernatant was added to E14 cells in 60-mm gelatinized dishes with 8 μg/ml polybrene (Sigma). Cells were cultured in 5 μg/ml puromycin for 1 week following retroviral transduction and resistant cells were pooled.

2.7. Quantitative PCR analysis

Total RNA was isolated from E14 cells with the use of an ISOGEN (Nippon Gene, Tokyo, Japan), followed by reverse transcription (RT) by ReverTra Ace (Toyobo, Osaka, Japan). 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 mouse *TRIM8* and the promoter region of *Nanog* were as follows: mouse *TRIM8*, 5′-AGAAGAACCT-GAAGCTCACCAAC-3′ and 5′-GCAGGCAGACCTTCTGTG-3′; promoter region #2, 5′-AGAGAGAGAGTGGTGTAAACAGTGGGTCTG-3′ and 5′-

GGCTGGTTGACCTCTGTCCACATCCAGCTG-3′. The primer sequences for promoter region #1 and *Nanog* were reported previously [34]. Primers for mouse *p21*, *Noxa* and *GAPDH* were reported previously [32]. Primers for mouse *Klf4*, *Sox2* and *Oct3/4* were reported previously [28].

2.8. Chromatin immunoprecipitation (ChIP)

ChIP was performed as reported previously [35].

2.9. Subcellular fractionation of ES cell

Cytosolic, nuclear and detergent-insoluble fractions were prepared as reported previously [20].

2.10. Isolation and identification of TRIM8 complex

ES cells and 293T cell lysates were mixed and incubated at 37 °C for 10 min followed by incubation at 4 °C for 1 h. Twenty μl of M2-agarose (50% slurry) was added and the mixture was incubated for a further 1 h. M2-agarose was washed three times according to the manufacturer's protocol, with TBS buffer containing 0.1% NP40. Bound proteins were eluted with 200 μg/ml FLAG peptide in TBS buffer containing 0.1% NP40, followed by TCA precipitation and washing with cold acetone. Precipitates were resolved by SDS-PAGE. Bands were excised from gels, cut into 1 mm pieces, washed in 100 mM ammonium bicarbonate, dried, and digested overnight in sequencing grade modified trypsin (#V5111, Promega). Peptides were extracted using 0.1% trifluoroacetic acid (TFA) in 0, 33 and 70% acetonitrile, combined and dried. Using a 1:1 dilution of CHCA matrix (Applied Biosystems 4700 Proteomics Analyzer Mass

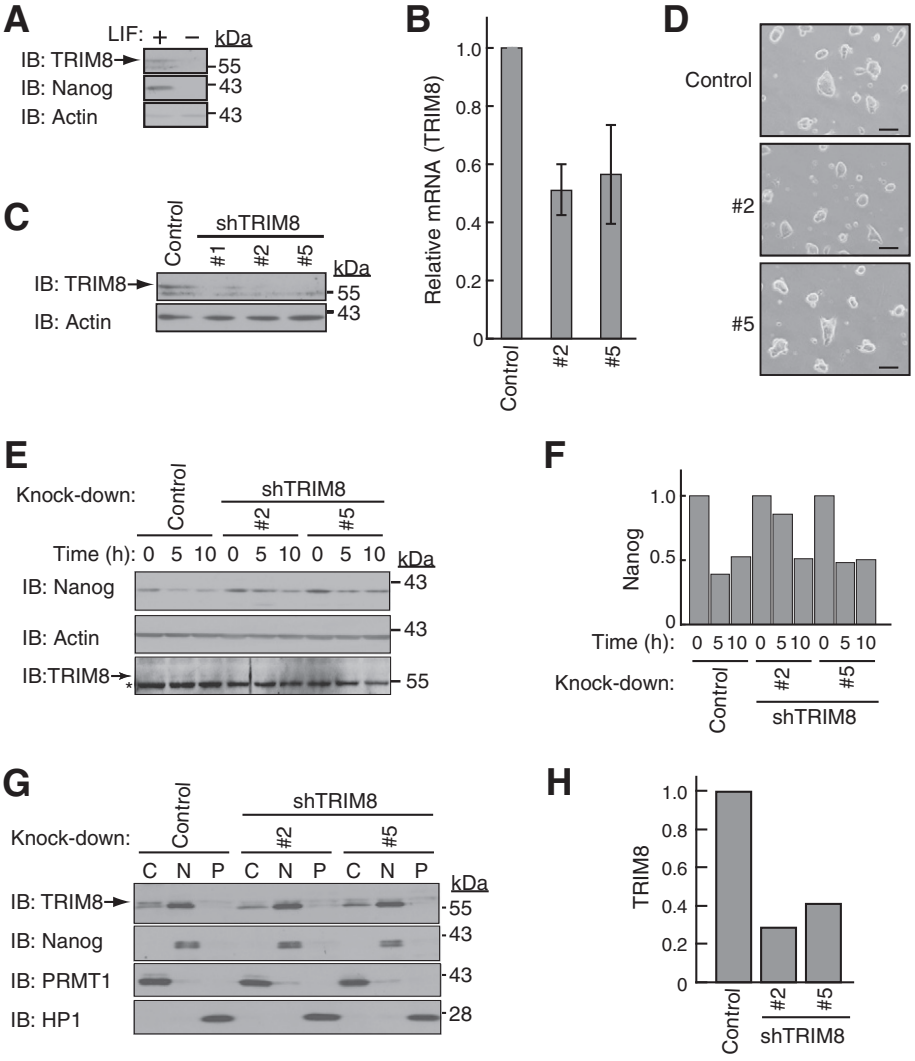

Fig. 1. Expression of TRIM8 in undifferentiated ES cells. (A) Expression of endogenous TRIM8 in ES cells. E14 cells were cultured in the presence or absence of LIF for 6 days and cell lysates were prepared. Nanog and actin are shown as a pluripotent marker and loading control, respectively. (B) Knock-down of TRIM8 in ES cells. E14 cells were infected with either retroviruses containing shRNA targeting *TRIM8* (#2 and 5) or mock, followed by selection with puromycin to establish cell lines stably expressing shRNA. Two different target sequences (#2 and #5) for *TRIM8* were used for the experiments in this study. Total RNA was reverse-transcribed and utilized for quantitative PCR. The amounts of *TRIM8* were normalized by *GAPDH*. Data are means ± SD of values from four independent experiments. (C) Immunoblot analysis of TRIM8 in knocked-down ES cells. ES cell lines were subjected to immunoblotting with anti-TRIM8 (B01) antibody. Actin is shown as a loading control. (D) ES cell lines grown on gelatinized dishes in the presence of LIF. Scale bar, 100 μm. (E) Differentiation of ES cell lines by removal of LIF. Pluripotency was monitored by the expression level of Nanog. Actin is shown as a loading control. Asterisk, non-specific signal. (F) The intensity of Nanog shown in (E) was normalized by that of the corresponding actin. The value of chase period at 0 h was defined as 1. (G) Subcellular localization of TRIM8 in ES cells. Cytosolic, nuclear and detergent-insoluble fractions of E14 cell lines were subjected to immunoblotting with anti-TRIM8 (B01), Nanog, PRMT1 or HP1α antibody. Antibodies against Nanog, PRMT1 and HP1α were used as nuclear, cytoplasmic and detergent-insoluble markers, respectively. Endogenous TRIM8 was detected only in the cytosolic fraction and is indicated by an arrow. (H) Quantification of endogenous TRIM8 in control or TRIM8 knocked-down cell lines. The intensity of TRIM8 shown in (G) was normalized by that of the corresponding cytosolic protein PRMT1.

Standards Kit), samples were spotted to an ABI 4700 Proteomics Analyzer MALDI-TOF target plate (Applied Biosystems). One thousand shots were fired to acquire the initial MS spectrum, and intense peaks were selected for MS/MS analysis. All MS/MS spectra were processed by a MASCOT distiller for generation of peak list files and were subjected to a database search by the MASCOT algorithm (Matrix Science, London, UK) against the non-redundant National Center for Biotechnology Information (nrNCBI) database.

2.11. Electroporation

E14 cells (1×10^7) were suspended in 0.8 ml of PBS including a plasmid (5 μg) in an electroporation cuvette and then electroporated using a Gene-Pulser II (Bio-Rad) under the conditions of 300 V and 500 μF. Cells were selected by treatment with puromycin (2 μg/ml) for 1 week.

2.12. Statistical analysis

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

3. Results

3.1. Expression of TRIM8 in undifferentiated mouse ES cells

We previously checked around thirty different types of cell lines to detect endogenous TRIM8 [20]. However, we failed to detect endogenous TRIM8 in those cell lines. We then checked ES cells because TRIM8 regulates PIAS3 and STAT3 is one of the regulators to mediate the LIF-dependent signaling pathway [20,27]. Interestingly, endogenous TRIM8 was detected in undifferentiated mouse ES cells (Fig. 1A). Furthermore, differentiated ES cells cultured without LIF did not express endogenous TRIM8, suggesting that the physiological expression of TRIM8 is linked to maintenance of a pluripotent state of ES cells. To study the roles of TRIM8 in ES cells, we knocked down endogenous TRIM8 in ES cells. The expression levels of mRNA and protein of TRIM8 were confirmed by quantitative PCR (Fig. 1B) and immunoblot analysis (Fig. 1C). We used several target sequences in *TRIM8* mRNA for knock-down experiments and we found that #2 and #5 shRNA were most efficient. Knock-down of TRIM8 did not affect colony formation of ES cells and the cells were able to be cultured for

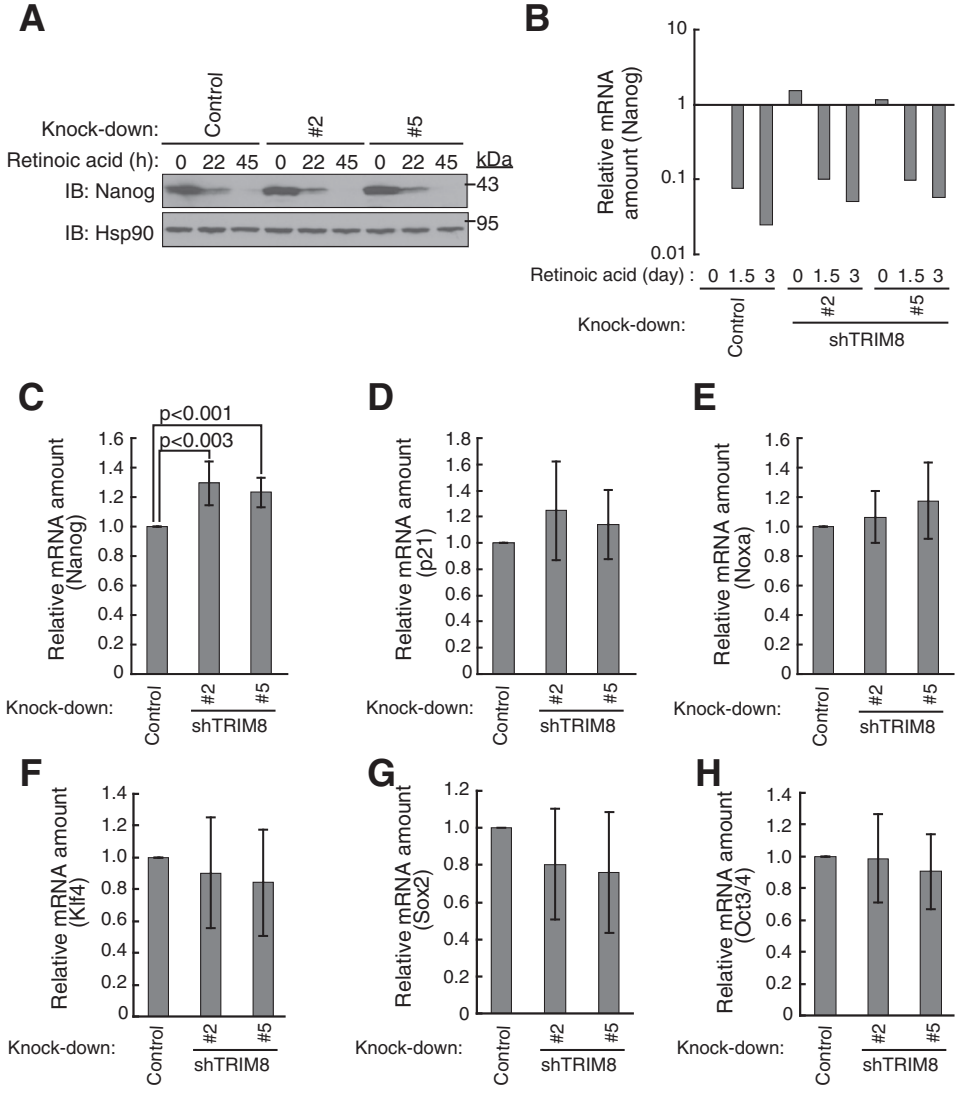

Fig. 2. Selective inhibition of the transcription of Nanog by TRIM8. (A) Differentiation of ES cell lines by retinoic acid was not affected by knock-down of TRIM8. ES cell lines were differentiated by retinoic acid (2.5 μM) for the indicated periods. Cell lysates were subjected to immunoblotting with anti-Nanog or Hsp90. (B) Upregulation of mRNA of *Nanog* by knock-down of TRIM8. ES cell lines were differentiated by retinoic acid (0.5 μM) for the indicated periods. Total RNA was subjected to quantitative RT-PCR. (C) Statistical analysis of upregulation of *Nanog* by knock-down of TRIM8. Each cell line was subjected to quantitative RT-PCR. Data are means ± SD of values from five independent experiments. (D–H) Statistical analysis of (*D*) *p21*, n = 5, (*E*) *Noxa*, n = 3 (*F*) *Klf4*, n = 4 (*G*) *Sox2*, n = 4 and (*H*) *Oct3/4*, n = 4.

at least one month without a differentiated phenotype (Fig. 1D). Qualities of those cell lines were compared by deprivation of LIF and quantify expression level of Nanog (Fig. 1E and F). The protein amounts of Nanog in TRIM8 knocked-down ES cells showed almost the same patterns as those in control cells after removal of LIF. These findings indicated that TRIM8 is expressed in undifferentiated ES cells but does not affect differentiation of ES cells by removal of LIF. We previously reported that overexpressed TRIM8 was localized in the nuclear-detergent insoluble fraction in HeLa cells [20]. We checked whether endogenous TRIM8 also localizes in the nuclear-detergent-insoluble fraction in ES cells. We fractionated ES cells into 1% Triton X-100-soluble cytoplasmic and nuclear fractions as well as insoluble fraction (Fig. 1G). Since only an upper band in the cytoplasmic fraction of control cells was downregulated by knock-down of TRIM8, endogenous TRIM8 is probably an upper band in detergent-soluble cytoplasmic fraction in ES cells, suggesting that the effect of TRIM8 knockdown was approximately 60–70% (Fig. 1H). Interestingly, different from HeLa cells, endogenous TRIM8 was expressed in the detergent-soluble cytoplasmic fraction in ES cells.

3.2. Selective inhibition of transcription of Nanog by TRIM8

Since it has been reported that Nanog is downregulated by retinoic acid-induced differentiation [36], we checked the effect of knock-down of TRIM8 on the down-regulation of Nanog by retinoic acid at the protein level (Fig. 2A) and mRNA level (Fig. 2B). We found that TRIM8 did not play a role in the differentiation of ES cells by retinoic acid. However, we noticed that mRNA of Nanog was modestly but significantly upregulated by knock-down of TRIM8 (Fig. 2B and C). Since it has been reported that ES cells express endogenous p53 and that p53 prevents transcription of Nanog in ES cells [34], we also checked expression levels of other p53-targeted genes, *p21* and *Noxa*. However, these genes were not affected by knock-down of TRIM8 (Fig. 2D and E). Actually, knock-down of TRIM8 did not change sensitivity to DNA damage including damages caused by UV-irradiation, cisplatin or etoposide (Fig. S1).

To test whether TRIM8 selectively regulates the transcription of *Nanog*, we also checked other pluripotent marker genes, *Klf4*, *Sox2* and *Oct3/4* (Fig. 2F–H), and we found that these genes were not affected by knock-down of TRIM8, suggesting that TRIM8 selectively regulates *Nanog* mRNA level.

3.3. Interaction of TRIM8 with Hsp90β

To clarify the role of TRIM8 in ES cells, we purified a TRIM8 containing complex to identify binding proteins of TRIM8. Since we could not establish an ES cell line that stably expresses FLAG–TRIM8, probably because of putative spontaneous differentiation of ES cells by overexpression of TRIM8, we first transiently expressed FLAG–TRIM8 in 293T cells and then incubated the cell lysates from transfected 293T cells with ES cell lysates, followed by purification by anti-FLAG-agarose beads and elution with FLAG peptide (Fig. 3A). As a result, some proteins were identified as candidates to interact with FLAG–TRIM8 (Fig. 3B). Heat shock protein 90β (Hsp90β) was one of the candidates, and it has been reported that Hsp90β interacts with STAT3 in ES cells and regulates LIF signaling [30]. Interaction between endogenous Hsp90β and FLAG–TRIM8 was confirmed (Fig. 3C). Physiological interaction between endogenous TRIM8 and endogenous Hsp90β was also shown (Fig. 3D).

3.4. Regulation of nuclear translocation of STAT3 by TRIM8 and Hsp90β

It has been reported that Hsp90β interacts with STAT3 and regulates nuclear translocation of STAT3 in ES cells [30]. Given that TRIM8 interacts with Hsp90β, TRIM8 may affect nuclear translocation of phosphorylated STAT3(Y705). To study the role of TRIM8 in the Hsp90β–STAT3 signaling pathway, ES cells were fractionated into cytoplasmic, nuclear and detergent-insoluble fractions (Fig. 4A). Knock-down of TRIM8 resulted in accumulation of a large amount of total STAT3 and phosphorylated STAT3(Y705) in the nucleus compared to that in control cells (Fig. 4A). Experiments were repeated three times independently, and statistical analysis showed significant accumulation of phosphorylated STAT3(Y705) in the nucleus

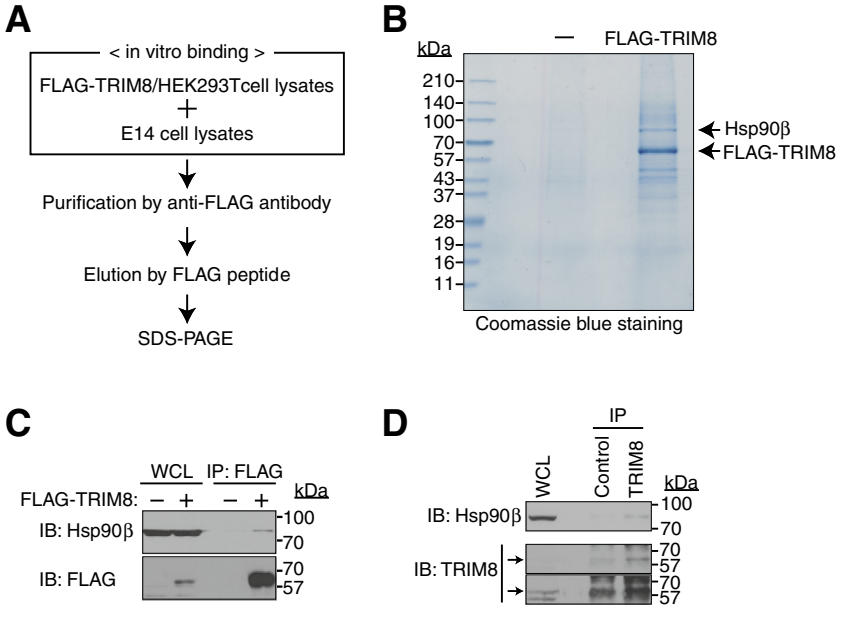

Fig. 3. Interaction of TRIM8 with Hsp90β. (A) Protocol to identify TRIM8-interacting proteins. (B) Coomassie blue staining of TRIM8 complex. TRIM8 complex purified by the protocol as (A) was separated by SDS-PAGE and stained with Coomassie blue. (C) Interaction between Hsp90β and FLAG–TRIM8. FLAG–TRIM8 complex purified as in (A) was subjected to immunoblotting with anti-Hsp90β or FLAG antibody. (D) Endogenous interaction between Hsp90β and TRIM8 in ES cells. ES cell lysates were subjected to immunoprecipitation with anti-TRIM8 antibody, followed by immunoblotting with anti-Hsp90β or TRIM8 antibody. Long exposure for anti-TRIM8 immunoblotting (bottom) was shown to detect endogenous TRIM8 in the input lane.

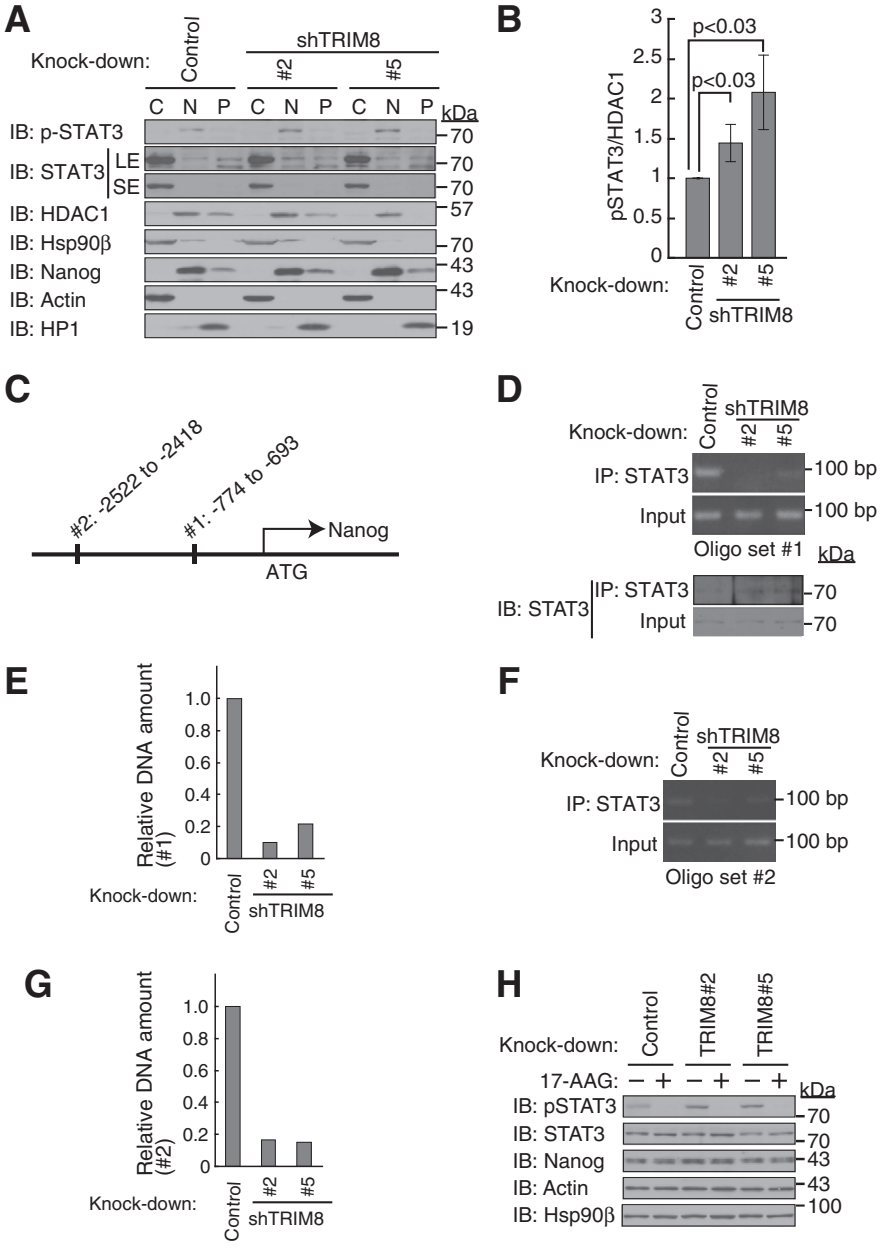

Fig. 4. Regulation of nuclear translocation of STAT3 by TRIM8 and Hsp90β (A) Upregulation of phosphorylated STAT3 in the nucleus by knock-down of TRIM8. ES cell lines were fractionated into cytosolic, nuclear and detergent-insoluble fractions, followed by immunoblotting with anti-phospho STAT3 (p-STAT3), STAT3, HDAC1, Hsp90β, Nanog, Actin or HP1. LE, long exposure; SE, short exposure. (B) Statistical analysis of upregulation of phosphorylated STAT3 (p-STAT3) in the nucleus by knock-down of TRIM8 in ES cells. The intensity of nuclear p-STAT3 shown in (A) was normalized by that of the corresponding HDAC1. The value of control cell line was defined as 1. Data are means ± SD of values from three independent experiments. (C) Schematic representation of the promoter region of *Nanog* and locations of primer sets used to detect the promoter region. (D) Chromatin immunoprecipitation (ChIP) assay with anti-STAT3 antibody. ES cell lines were lysed and utilized for ChIP assay. Immunoprecipitates were purified by phenol/chloroform extraction and utilized for PCR to detect promoter region #1 of *Nanog*. Immunoprecipitates were also subjected to immunoblotting with anti-STAT3 antibody to show that the same amounts of STAT3 were utilized for the CHIP assay. (E) Quantification of the intensity of promoter region #1 in (D) co-purified by STAT3. (F) ChIP assay was performed using other primers. Immunoprecipitates were purified by phenol/chloroform extraction and utilized for PCR to detect promoter region #2 of *Nanog*. (G) Quantification of the intensity of promoter region #2 in (F) co-purified by STAT3. (H) Downregulation of phosphorylation of STAT3 by an Hsp90-specific inhibitor. TRIM8 knocked-down ES cell lines as well as a control cell line were treated with the Hsp90-specific inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG, 1 μM) for 8 h in the presence of LIF, followed by immunoblotting with anti-phospho STAT3 (p-STAT3), STAT3, Nanog, Hsp90β or actin. Actin was used as a loading control.

(Fig. 4B). These findings suggest that knock-down of TRIM8 enhances the increase in nuclear localization of phosphorylated STAT3. To clarify the effect of phosphorylated STAT3(Y705) accumulated in the nucleus, we performed a chromatin immunoprecipitation (ChIP) assay (Fig. 4C–G). Mock or TRIM8 knocked-down cell lines were fixed with formaldehyde and lysed for immunoprecipitation with an anti-STAT3 antibody. Genomic DNA was purified by chloroform/phenol extraction and utilized for polymerase chain reaction (PCR), which targeted the upstream region of the start codon of *Nanog* (Fig. 4C). Since it has been shown that p53 binds to the promoter region of *Nanog* and inhibits transcription [34], we used the same primers (Fig. 4C, #1) and found that STAT3 was able to interact with the promoter region of *Nanog* (Fig. 4D). Although phosphorylated STAT3 (Y705) accumulated in TRIM8 knocked-down cell lines, we found that binding of STAT3 to the promoter region of *Nanog* was downregulated by knock-down of TRIM8 (Fig. 4D and E). Furthermore, we utilized independent cell lysates prepared on different days and different sets of primers to eliminate technical errors, and we obtained the same

results, suggesting that TRIM8 modulates the interaction between STAT3 and the promoter region of *Nanog* (Fig. 4F and G). Hsp90β is a molecular chaperone that functions as a major regulator of protein conformation and plays a central role in protein homeostasis in the cell. To clarify whether TRIM8 regulates phosphorylation of STAT3 through chaperone activity of Hsp90β, we treated TRIM8 knocked-down ES cell lines as well as a control cell line with the Hsp90-specific inhibitor 17-allylamino-17-demethoxygeldanamycin (17-AAG), which inhibits binding of ATP to Hsp90 (Fig. 4H). Phosphorylation of STAT3 was reduced by 17-AAG treatment in all cell lines, indicating that chaperone activity of Hsp90β was required for at least maintenance of phosphorylation of STAT3. However, the expression levels of Nanog, STAT3 and Hsp90β were not affected by 17-AAG treatment. These findings suggest that TRIM8 modulates the STAT3 signaling pathway in collaboration with Hsp90β to regulate the transcription of *Nanog* in ES cells.

3.5. Differentiation of ES cells by overexpression of TRIM8

Since knock-down of TRIM8 upregulated transcription of *Nanog* (Fig. 2C), we also analyzed ES cells in which FLAG–TRIM8(WT or ΔRING) is constitutively overexpressed (Fig. 5A and B). Overexpression of FLAG–TRIM8(WT) or FLAG–TRIM8(ΔRING) caused disappearance of Nanog (Fig. 5A). Furthermore, p-STAT3, STAT3 and Hsp90β were downregulated in these cell lines (Fig. 5A). However, since TRIM8(ΔRING) also caused decrease in the expression of these proteins, this downregulation would not be regulated by ubiquitin–proteasomal degradation involved in ubiquitin ligase activity of

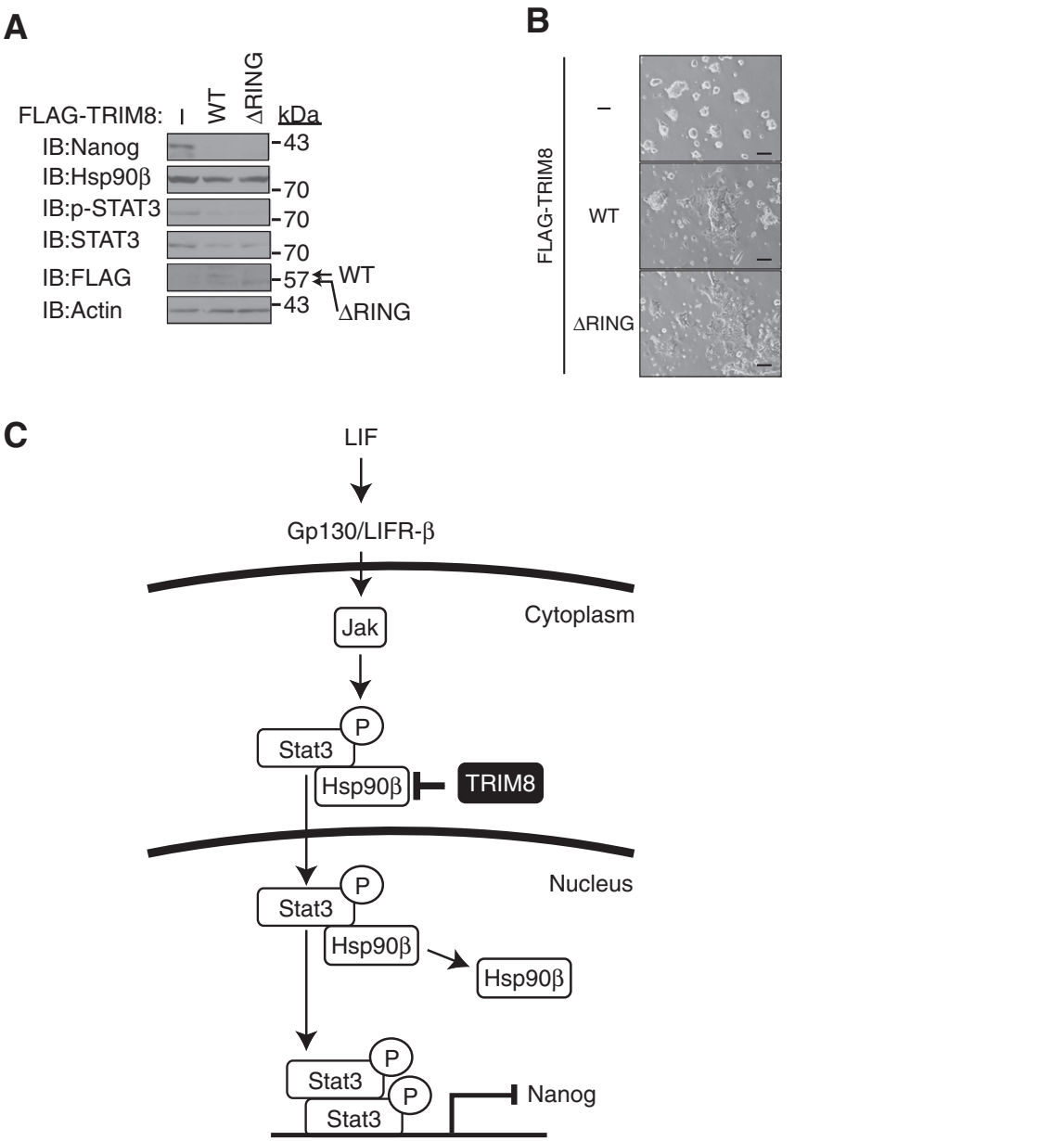

Fig. 5. Fine tuning of pluripotent ES cells by TRIM8. (A) Differentiation of ES cells by overexpression of TRIM8. ES cells were electroporated with a plasmid encoding FLAG–TRIM8(WT or ΔRING) or a control plasmid and were then selected by treatment with puromycin (2 μg/ml) for 1 week. Immunoblot analysis was performed with antibodies to Nanog, Hsp90β, p-STAT3, STAT3, FLAG and actin. Actin was used as a loading control. (B) ES cell lines grown on gelatinized dishes in the presence of LIF for 2 days. Scale bar, 100 μm. (C) Model for biological roles of TRIM8 in ES cells. TRIM8 prevents nuclear translocation of STAT3/Hsp90β heterodimer, resulting in prevention of excessive signal transduction by STAT3. STAT3 at a physiological concentration binds to the promoter region of *Nanog* and regulates transcription. A high concentration of phosphorylated STAT3 may prevent binding to the promoter region of *Nanog* by an unidentified mechanism.

TRIM8. Next, to examine whether TRIM8 maintains an undifferentiated state of ES cells, we evaluated the morphological phenotype of ES cells in which TRIM8 is overexpressed. We seeded each cell line on dishes and cultured the cells with LIF for maintenance of pluripotency. Colonies from control ES cells grew normally and were enlarged in the presence of LIF for 2 days, whereas TRIM8-overexpressing ES cells differentiated into endoderm-like cells even in the presence of LIF (Fig. 5B). These findings suggest that protein expression of TRIM8 is tightly regulated at an appropriate level or that abnormal ES cells spontaneously differentiate to be excluded from the pluripotent population.

4. Discussion

In this present study, we showed that TRIM8 is expressed in undifferentiated ES cells, suggesting that TRIM8 plays an important role for maintaining pluripotency of ES cells. Although we knocked down TRIM8 and established stable cell lines, these cell lines could maintain pluripotency for at least several months. These findings suggest that TRIM8 is one of the regulators in ES cells but not a crucial regulator for maintaining pluripotency. Since ES cells lose pluripotency in an inappropriate condition, they likely differentiate spontaneously and are excluded from the expanding pool as a safety system to maintain the quality of ES cells. Therefore, knock-down of TRIM8 may cause the exclusion of differentiated or apoptotic ES cells from experimental samples and, consequently, we may have not observed an obvious biological effect of TRIM8 on ES cells.

Knock-down of TRIM8 modestly but significantly upregulated transcription of *Nanog* (Fig. 2), suggesting that TRIM8 controls the expression of *Nanog* to maintain the expression at a constant level. Other important transcription factors, including *Klf4*, *Sox2* and *Oct3/4*, were not affected by knock-down of TRIM8, suggesting that TRIM8 selectively affects the expression of *Nanog*. Although transcription of *Nanog* was upregulated, we did not detect upregulation of the protein amount of Nanog. It has been reported that a high expression level of Oct3/4, which is a master regulator of pluripotency and is required for maintenance of an undifferentiated state, induces spontaneous differentiation [37]. Hence, when protein expression of Nanog is upregulated by knock-down of TRIM8, ES cells may differentiate and be excluded from the culture pool.

To identify proteins that bind specifically to TRIM8 in ES cells, we performed an in vitro binding assay combined with mass-spectrometrical analysis by using cell lysates from TRIM8-expressing 293T cells and ES cells. We found that Hsp90β is one of the endogenous binding partners to TRIM8. It has been reported that Hsp90β upregulates nuclear translocation of STAT3 [30]. Therefore, we hypothesized that TRIM8 affects nuclear translocation of Hsp90β/STAT3 complex (Fig. 5C). A relatively large amount of phosphorylated STAT3 was present in the nucleus with knock-down of TRIM8, but knock-down of TRIM8 caused dissociation of STAT3 from the promoter region of *Nanog* gene. It is important to clarify the molecular mechanisms for regulation of the dissociation of STAT3 from the promoter region of *Nanog* in the nucleus by TRIM8 and/or Hsp90β.

TRIM8 is a putative ubiquitin ligase because TRIM8 has a RING domain, which interacts with E2 enzymes for the ubiquitination cascade. Since Hsp90β seems to be a stable protein, TRIM8 may have substrates other than Hsp90β. It is important to identify substrates in ES cells to be ubiquitinated by TRIM8 followed by degradation by proteasomes. Since TRIM8 expression is found only in undifferentiated ES cells, the identification of substrates of TRIM8 from ES cells is critical for understanding the molecular mechanisms by which pluripotency of ES cells is maintained.

Conventional human ES cells share multiple defining features with mouse epiblast stem cells (EpiSCs) rather than mouse ES cells [38]. Conventional human ES cells correspond, at least partially, to the primed pluripotent state rather than to the naïve state of mouse ESCs, and LIF signaling does not seem to be important for maintenance. However, conventional human ES cells and iPS cells were recently converted to a more immature naïve pluripotent state by propagating the cells in the presence of LIF and with ectopic induction of Oct4, Klf4, and Klf2 combined with inhibitors of glycogen synthase kinase 3β (GSK3β) and mitogen-activated protein kinase (ERK1/2) pathway [39]. It is important to determine whether TRIM8 is also involved in the maintenance of naïve human ES cells to make them useful for regenerative medicine.

Acknowledgements

We would like to thank Dr. I. Taniuchi for the CHO cell line expressing LIF, Dr. M. Nishiyama for valuable discussion, and Drs. H. Sabe and Y. Onodera for the confocal microscope.

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology and Mitsubishi Pharma Sources of Funding (to S. Hatakeyama and F. Okumura), Ono Cancer Research Fund and the Research Foundation Ituu Laboratory (to S. Hatakeyama).

Appendix A. Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.bbamcr.2011.05.013.

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