TRIM32 promotes neural differentiation through retinoic acid receptor-mediated transcription

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Summary

Retinoic acid (RA), a metabolite of vitamin A, plays versatile roles in development, differentiation, cell cycles and regulation of apoptosis by regulating gene transcription through nuclear receptor activation. Ubiquitinylation, which is one of the post-translational modifications, appears to be involved in the transcriptional activity of intranuclear receptors including retinoic acid receptor α (RARα). Mutations in the tripartite motif-containing protein 32 gene (*TRIM32*; also known as E3 ubiquitin-protein ligase) have been reported to be responsible for limb-girdle muscular dystrophy type 2H in humans, and its encoded protein has been shown to interact with several other important proteins. In this study, we found that TRIM32 interacts with RARα and enhances its transcriptional activity in the presence of RA. We also found that overexpression of TRIM32 in mouse neuroblastoma cells and embryonal carcinoma cells promoted stability of RARα, resulting in enhancement of neural differentiation. These findings suggest that TRIM32 functions as one of the co-activators for RARα-mediated transcription, and thereby TRIM32 is a potential therapeutic target for developmental disorders and RA-dependent leukemias.

Key words: Neural differentiation, RARα, Retinoic acid, TRIM32, Ubiquitin

Introduction

Nuclear receptors strictly regulate the activity of many genes as ligand-dependent transcriptional factors. It is now apparent that nuclear receptors and their specific ligands play a role in pathogenesis and treatment of various diseases (Maden, 2007). It has been reported that retinoic acid (RA), a vitamin A metabolite, plays crucial roles in cell proliferation, differentiation, tumorigenesis and apoptosis (Altucci et al., 2001; Dokmanovic et al., 2002; Donato et al., 2007; Kitareewan et al., 2002; Rochette-Egly and Chambon, 2001). A recent report has shown that RA is an important signal molecule that participates in induction of neural differentiation and axon outgrowth (Clagett-Dame et al., 2006). RA is also involved in neuronal patterning and maintenance of the differentiated state of neurons. Therefore, dysfunction of the RA signaling system possibly causes Alzheimer's disease and motor neuron disease (Goodman, 2006; Husson et al., 2006). However, in hematological disorders, acute promyelocytic leukemia (APL) often shows the chromosomal translocation *t* (15; 17) (q22; q21), causing the juxtapositioning of the promyelocytic leukemia gene (*PML*) and the retinoic acid receptor α gene (*RARa*), resulting a *PML–RARa* gene fusion. Differentiating agents, including all-trans retinoic acid (ATRA), tamibarotene (Am80) and arsenic trioxide (As_2O_3), are administered to APL patients in order to induce differentiation or apoptosis of leukemic blast cells (Jiang et al., 2008). Nuclear retinoic acid receptors (RARs) upregulate or downregulate target gene expression, depending on co-activators or co-repressors, respectively. There are three subtypes of RARs: α (NR1B1), β (NR1B2) and γ (NR1B3), and they function as ligand-dependent transcriptional factors by heterodimerizing with retinoid X receptor (RXR). Retinoic acid binds to a transcriptional complex composed of RARs and RXR, and the heterodimeric pair binds to a specific DNA sequence called a retinoic acid response element (RARE). Although several co-activators of nuclear receptors have been identified, the functions of those molecules have not been fully elucidated (Maden, 2007; McGrane, 2007; Rochette-Egly and Germain, 2009).

Ubiquitinylation is an important post-translational modification system used by eukaryotic cells (Hershko and Ciechanover, 1998). The ubiquitin-mediated proteolytic pathway plays a crucial role in the elimination of short-lived regulatory proteins including those that contribute to the cell cycle, cell signaling, DNA repair, morphogenesis, protein quality control and transcriptional regulation (Peters, 1998). Ubiquitin conjugation is catalyzed by ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). E3 is a scaffold protein that mediates between E2 and the substrate. The resulting polyubiquitinylated conjugates are immediately recognized and degraded by the 26S proteasome. E3 is thought to be the component of the ubiquitin conjugation system that is most directly responsible for substrate recognition (Hershko et al., 1983; Huibregtse et al., 1995; Scheffner et al., 1995). On the basis of structural similarity, E3 enzymes have been classified into three families: the HECT (homologous to E6-AP C-terminus) family (Hershko and Ciechanover, 1998; Huibregtse 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). It has been reported that some nuclear receptors such as RARs (Gianni et al., 2002; Kopf et al., 2000; Zhu et al., 1999), estrogen receptor (Lonard et al., 2000; Nakajima et al., 2007) and androgen receptor (Kikuchi et al., 2009; Miyajima et al., 2008) are regulated strictly by the ubiquitin–proteasome system, and ubiquitinylation of those transcriptional factors plays a role not only in the degradation signal but also in the activation signal at certain transcriptional stages.

Tripartite motif (TRIM) proteins (also known as E3 ubiquitin–protein ligases) are characterized by the presence of a RING finger, one or two zinc-binding motifs called B-boxes, and an associated coiled-coil region (RBCC) (Meroni and Diez-Roux, 2005; Nisole et al., 2005; Reymond et al., 2001). The TRIM protein family is currently known to comprise ~77 members in humans. TRIM32 is a scaffold protein with E3 ubiquitin ligase activity, and contains six repeats of the NHL (NCL-1, HT2A and LIN-41) motif (Slack and Ruvkun, 1998). Point mutations of the human TRIM32 gene have been reported in two genetic disorders: limb-girdle muscular dystrophy type 2H (LGMD2H) (Borg et al., 2009; Frosk et al., 2002), which is a myopathy with a predominant involvement of the pelvic or shoulder girdle musculature, and Bardet–Biedl syndrome (BBS), which is characterized by retinal degeneration, genitourinary tract malformations, cognitive impairment, obesity and polydactyly (Chiang et al., 2006). Recently, it has been reported that deficiency of TRIM32 in mice produces many of the features observed in LGMD2A or sarcotubular myopathy (STM) in humans (Kudryashova et al., 2009). We previously found that TRIM32 binds to Abl-2 interactor 2 (Abi2), which is known as a tumor suppressor and a cell migration inhibitor, and we showed that TRIM32 mediates the ubiquitinylation of Abi2 (Kano et al., 2008). In addition, it has been reported that TRIM32 functions as an E3 ligase for actin (Kudryashova et al., 2005), Piasy (Liu et al., 2010) and dysbindin (Locke et al., 2009). TRIM32 downregulates endogenous actin in HEK293 cells by means of its E3 ubiquitin ligase activity and interacts with myosin, but not with actin. It has been shown that TRIM32 regulates keratinocyte apoptosis through induction of NF-κB by promoting degradation of Piasy, and it has recently been shown that CC chemokine ligand 20, which is an inflammatory chemokine responsible for recruitment of leukocytes to sites of inflammation, is regulated by TRIM32 and Piasy in keratinocytes. It has also been reported that TRIM32 binds to and ubiquitinylates dysbindin, which is implicated in the genetic pathoetiology of schizophrenia. Moreover, it has been reported that *TRIM32* mRNA is highly expressed in the occipital region of patients with Alzheimer's disease (Yokota et al., 2006). Although it has been reported that TRIM32 is involved in regulation of several proteins, its physiological roles remain to be resolved.

In this study, with the aim of elucidating the function of TRIM32 in neural differentiation, we identified RARα as a novel TRIM32-interacting protein by using a comprehensive dual luciferase reporter assay and a biochemical approach including immunoprecipitation. We showed that TRIM32 stabilizes endogenous RARα and that TRIM32 acts as a co-activator of RARα in neuroblastoma cells and embryonal carcinoma cells, suggesting that TRIM32 is an important molecule for neural cell differentiation.

Results

TRIM32 activates RARα-mediated transcriptional activity

We showed that some members of the TRIM family regulate transcriptional factors: TRIM25 and TRIM68 upregulate estrogen receptor alpha (ERα)-mediated transcription and androgen receptor (AR)-mediated transcription, respectively (Kikuchi et al., 2009; Miyajima et al., 2008; Nakajima et al., 2007). Therefore, we hypothesized that a certain TRIM family protein might regulate RARα-mediated transcription. To screen TRIM proteins that regulate RARα-mediated transcription we performed a luciferase reporter assay using an RAR promoter-driven luciferase construct (RAR–Luc) in P19 cells. Expression vectors encoding TRIM family proteins and RAR–Luc were transfected into P19 cells and then luciferase activity was measured with or without ATRA. We compared the ratios of transcriptional activity with and without ATRA (Fig. 1A–D). TRIM32 significantly enhanced retinoic acid-dependent RARα-mediated transcriptional activity in P19 cells. It has been reported that TRIM2 and TRIM3 are structurally similar to TRIM32, which is classified as subfamily C-VII (Short and Cox, 2006) and that the TRIM19 (*PML*) gene is fused to the *RARa* gene in APL. Therefore, we performed luciferase reporter assays using expression vectors encoding TRIM2, TRIM3, TRIM19 and TRIM32 in P19 cells. Luciferase assays showed that TRIM2, TRIM3 and TRIM19 had no influence on RARα-mediated transcriptional activity, whereas TRIM32 significantly enhanced RARα-mediated transcriptional activity (Fig. 1C). These findings suggest that TRIM32 specifically enhances retinoic-acid-dependent RARα-mediated transcriptional activity in P19 cells. Moreover, the luciferase assays showed that TRIM32 enhanced retinoic-acid-dependent RARα-mediated transcriptional activity in a dose-dependent manner in P19 cells (Fig. 1E). To determine whether TRIM32 specifically drives RARα-mediated transcriptional activity, we examined the effect of TRIM32 on the androgen-dependent AR-reporter system. We performed a luciferase reporter assay using an MMTV–Luc reporter plasmid in CWR22Rv1 cells (Fig. 1F). The luciferase assay showed that TRIM68 facilitates androgen-receptor-mediated transcriptional activity with dihydrotestosterone as reported previously (Miyajima et al., 2008), whereas TRIM32 did not affect androgen receptor-mediated transcriptional activity. These findings suggest that TRIM32 specifically regulates RARα-mediated transcriptional activity.

TRIM32 interacts with RARα

To test whether TRIM32 physically interacts with RARα in vivo, we transfected expression vectors encoding HA-tagged TRIM32 and/or FLAG-tagged RARα into HEK293T cells. Cell lysates were subjected to immunoprecipitation with an antibody to HA or FLAG, and the resulting precipitates were subjected to immunoblot analysis with an antibody to FLAG or HA, respectively. Immunoprecipitation showed that HA–TRIM32 was selectively co-precipitated by anti-FLAG antibody and that FLAG–RARα was also co-precipitated by anti-HA antibody (Fig. 2A and supplementary material Fig. S1). Furthermore, we also examined whether there was interaction between TRIM32 and RXR, using immunoprecipitation, but did not find any, suggesting that TRIM32 interacts weakly with RXR or that TRIM32 interferes with the RARα–RXR complex (Fig. 2B). There was a possibility that a TRIM32–RXR complex was not detected because TRIM32 negatively regulates the expression of RXR. To rule out this

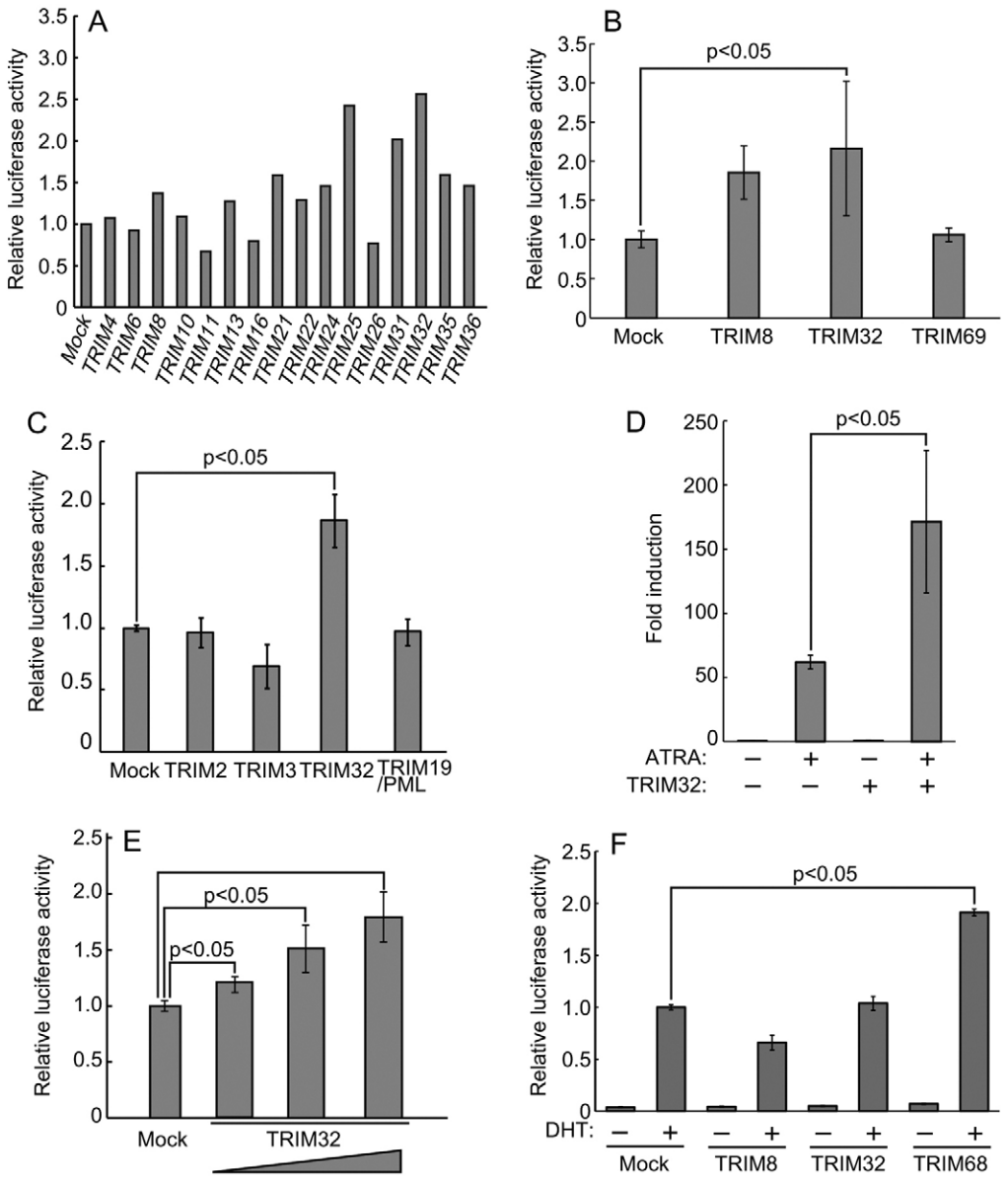

Fig. 1. TRIM32 enhances RARα-mediated transcriptional activation. (**A**) TRIM32 enhances RARα-mediated transcriptional activity in P19 cells. RAR luciferase reporter vector (RAR–Luc) with expression vectors encoding TRIM family proteins and RARα were transfected into P19 cells and then the cells were incubated in culture medium containing 10% fetal bovine serum for 24 hours. Cells were incubated with or without ATRA (20 μM) for 24 hours and then harvested and assayed for luciferase activity. The ratio of the luciferase activity with ATRA to that without ATRA in cells that had been transfected with an empty vector was defined as 1. (**B**) TRIM32 significantly enhances RARα-mediated transcriptional activity in P19 cells. Data are means ± s.d. from three independent experiments. *P*-values for the indicated comparisons were determined by Student's *t*-test. (**C**) RARα-mediated transcriptional activity using subfamily C-VII and TRIM19 (PML). RAR luciferase reporter vector (RAR–Luc) with expression vectors encoding TRIM2, TRIM3, TRIM19, TRIM32 and RARα were transfected into P19 cells. Data are means ± s.d. from three independent experiments. *P*-values for the indicated comparisons were determined by Student's *t*-test. (**D**) TRIM32 enhances RARα-mediated transcriptional activity in P19 cells in the presence or absence of ATRA (20 μM). Data are means ± s.d. from three independent experiments. *P*-values for the indicated comparisons were determined by Student's *t*-test. (**E**) TRIM32 enhances RARα-mediated transcriptional activity in a dose-dependent manner. RAR luciferase reporter vector, RARα expression vector and various amounts of TRIM32 expression vector were transfected into P19 cells. Transfected cells were incubated in culture medium containing 10% fetal bovine serum for 24 hours and then incubated with or without ATRA (20 μM) for 24 hours. Data are means ± s.d. from three independent experiments. *P*-values for the indicated comparisons were determined by Student's *t*-test. (**F**) TRIM32 does not affect AR-mediated transcriptional activity. An MMTV–luciferase reporter vector (MMTV–Luc) and expression vectors encoding several TRIM family proteins were transfected into CWR22Rv1 cells. Transfected cells were incubated in 10% charcoal-treated FBS medium for 24 hours and then treated with or without dihydrotestosterone (DHT; 10 nM) for 24 hours. The cells were then harvested and assayed for luciferase activity. Data are means ± s.d. from three independent experiments. *P*-values for the indicated comparisons were determined using Student's *t*-test.

possibility, we examined the expression levels of endogenous and exogenous RXR by overexpression of TRIM32. Immunoblot analysis showed that overexpression of TRIM32 did not downregulate the expression of RXR (Fig. 2C).

Next, we performed immunofluorescence staining to determine the subcellular localization of TRIM32 and RARα. COS-7 cells were transfected with expression vectors encoding HA-tagged TRIM32 or FLAG-tagged RARα and then stained with antibodies to FLAG or HA and Hoechst 33258 to reveal the subcellular distribution of HA-tagged TRIM32 (green), FLAG-tagged RARα (red) and nuclei (blue). As previously reported (Locke et al., 2009), HA-tagged TRIM32 was predominantly cytosolic, whereas FLAG-tagged RARα was predominantly nuclear. However, we found that TRIM32 was partially colocalized with RARα at the intranuclear or perinuclear region (Fig. 2D). In addition, immunofluorescence staining showed that

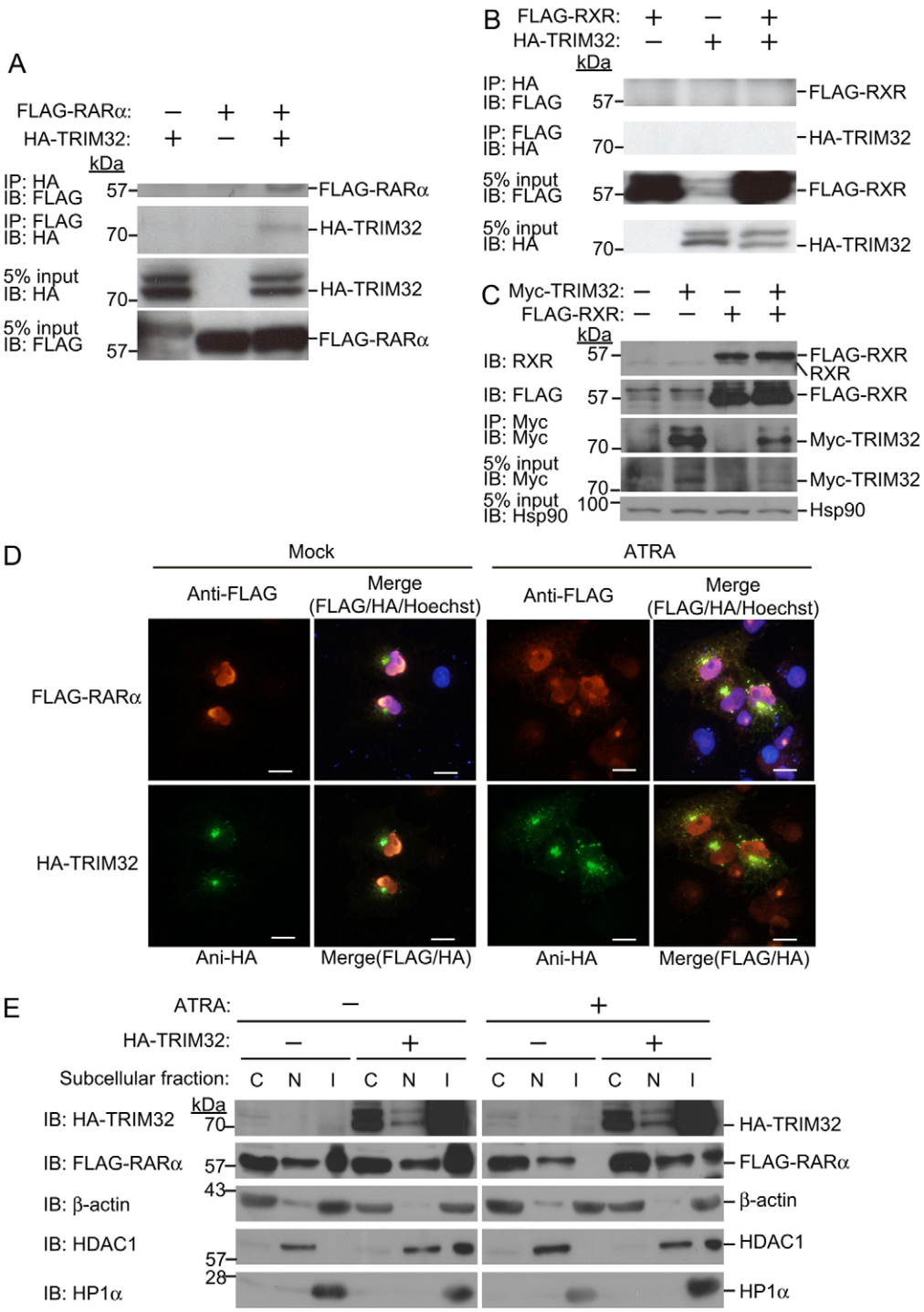

Fig. 2. See next page for legend.

TRIM32 also colocalizes with RARα in the presence of ATRA. However, ATRA caused no significant difference in interaction of TRIM32 and RARα, as determined by immunofluorescence staining. Furthermore, we performed biochemical subcellular fractionation using COS-7 cells to confirm subcellular localization of TRIM32. Immunoblot analysis showed that TRIM32 is localized in the nucleus as well as in the cytosol (Fig. 2E). In accordance with the results obtained by immunofluorescence staining, ATRA caused no significant difference in the expression level of TRIM32 in the nuclear fraction.

TRIM32 mediates ubiquitinylation of RARα

We previously showed that TRIM32 has an E3 ubiquitin ligase activity through its RING finger domain and that deletion of the RING domain of TRIM32 causes a remarkable decrease in E3 ubiquitin ligase activity (Kano et al., 2008). To examine whether TRIM32 ubiquitinylates RARα, we used a deletion mutant lacking a RING finger domain of TRIM32 [TRIM32 (ΔR)] for a ubiquitinylation assay (Fig. 3A). After transfecting expression vectors encoding FLAG-tagged RARα, HA-tagged ubiquitin and Myc-tagged full-length TRIM32 [TRIM32(FL)] or Myc-tagged TRIM32(ΔR) in which the RING domain was lacking, cells were immunoblotted with anti-FLAG antibody and then immunoblot analysis was performed using anti-HA antibody. Overexpression of Myc-tagged TRIM32 caused ubiquitinylation of FLAG-tagged RARα, whereas Myc-tagged TRIM32(ΔR) did not accelerate ubiquitinylation of FLAG-tagged RARα (Fig. 3B). To further confirm ubiquitinylation of RARα by TRIM32 in the denatured condition with urea, we performed an in vivo ubiquitinylation assay. Expression vectors encoding FLAG-tagged RARα, HA-tagged TRIM32 and His_6-tagged-ubiquitin were transfected into HEK293T cells. Proteins conjugated with His_6-tagged ubiquitin were affinity purified with metal affinity resin in a buffer containing urea and immunoblotted with antibodies against FLAG tag. Immunoblot analysis showed that overexpression of TRIM32 facilitates ubiquitinylation of FLAG-tagged RARα (Fig. 3C). These findings suggest that RARα is regulated by TRIM32 by ubiquitinylation through its RING domain.

Fig. 2. TRIM32 interacts with RARα. (**A**) In vivo assay for binding between TRIM32 and RARα. Expression vectors encoding HA-tagged TRIM32 and FLAG-tagged RARα were transfected into HEK293T cells. Whole cell lysates were immunoprecipitated with anti-HA or anti-FLAG antibody and immunoblotted with anti-FLAG and anti-HA antibodies. (**B**) In vivo assay for binding between TRIM32 and RXR. Expression vectors encoding HA-tagged TRIM32 and FLAG-tagged RXR were transfected into HEK293T cells. Whole cell lysates were immunoprecipitated with anti-HA or anti-FLAG antibody and immunoblotted with anti-FLAG and anti-HA antibodies. (**C**) Overexpression of TRIM32 does not affect the expression of RXR. HEK293T cells were transfected with expression vectors encoding Myc-tagged TRIM32 and FLAG-tagged RXRα. Whole cell lysates were immunoblotted with anti-RXR, anti-FLAG and anti-Myc antibodies. Hsp90 was used as an internal control. (**D**) Colocalization of TRIM32 with RARα. COS-7 cells were transfected with expression plasmids encoding FLAG-tagged RARα and HA-tagged TRIM32. 24 hours after transfection, the cells were incubated with or without ATRA (20 μM) for 24 hours and were stained with anti-FLAG and anti-HA antibodies, followed by incubation with Alexa-Fluor-546-labelled anti-mouse IgG antibody and Alexa-Fluor-488-labelled anti-rabbit IgG antibody, respectively. Nuclei were visualized using Hoechst 33258. Scale bars: 20 μm. (**E**) Subcellular fractionation of TRIM32 from COS-7 cells. COS-7 cells were transfected with expression plasmids encoding FLAG-tagged RARα and HA-tagged TRIM32. 24 hours after transfection, the cells were incubated with or without ATRA (20 μM) for 24 hours. After ATRA stimulation, biochemically fractionated cytosolic and nuclear extracts were subjected to immunoblot analysis with anti-HA, anti-FLAG, anti-HDAC1, anti-β-actin and anti-HP1α antibodies. β-actin, HDAC1 and HP1 were used as cytosolic, nuclear and insoluble protein markers, respectively. C, cytoplasmic fraction; N, nuclear fraction; I, insoluble fraction.

TRIM32 stabilizes expression level of RARα

To examine whether TRIM32 affects the expression level of RARα, immunoblot analysis was performed using Neuro2A cells transfected with an expression vector encoding TRIM32. Immunoblot analysis showed that overexpression of TRIM32 causes a high expression of endogenous RARα in Neuro2A cells (Fig. 4A). To confirm that TRIM32 stabilizes RARα in vivo, we performed protein stability analysis for RARα with cycloheximide. HEK293T cells were transfected with vectors for FLAG-tagged RARα and HA-tagged TRIM32, and then the cells were cultured with cycloheximide for the indicated times (Fig. 4B) and analyzed by immunoblotting with anti-FLAG antibody. Pulse-chase analysis showed that expression of TRIM32 promoted stabilization of RARα (Fig. 4B). Next, we performed real-time PCR to compare mRNA levels of tissue transglutaminase (*tTG*, also known as *TGM2*) as a target gene of RARα with and without overexpression of TRIM32 (Citron et al., 2005; Donato et al., 2007; Watashi et al., 2003). Real-time PCR analysis showed that *tTG* mRNA is highly induced by expression of TRIM32 even without ATRA, suggesting that TRIM32 lowers the threshold for RARα-mediated transcriptional activation (Fig. 4C,D).

TRIM32 facilitates neural differentiation of P19 cells

Because the mouse embryonic carcinoma cell line P19 is used as an in vitro neuronal differentiation model, and P19 cells are differentiated with ATRA (Jones-Villeneuve et al., 1982), we hypothesized that TRIM32 also affects neural differentiation of P19 cells, as well as ATRA. We established three P19 cell clones in which FLAG-tagged TRIM32 was stably expressed and a cell line that was transfected with an empty vector (mock-transfected cells) using the lipofection method followed by limiting dilution and drug-resistance selection, and the expression was confirmed by immunoblot analysis (Fig. 5A). Immunoblot analysis showed that endogenous RARα was more highly expressed in cells in which TRIM32 had been stably expressed than in the mock-transfected cells (Fig. 5B). To evaluate neural differentiation of P19 cells with or without overexpression of TRIM32, we induced neural differentiation of these established cell clones with tamibarotene (1 μM), which is approximately ten times more potent than ATRA in inducing cell differentiation, and the cell shape and neurite-like outgrowth was observed for 3 days. Overexpression of TRIM32 facilitated neural cell differentiation with tamibarotene compared with the mock-transfected cells (Fig. 5C). To confirm that TRIM32 induces neural differentiation, we counted neurite-bearing cells after induction with tamibarotene. The cells that had neurites longer than the diameter of their cell body were then counted, and the percentage of cells with neurites was calculated. Overexpression of TRIM32 accelerated neurite-like changes compared with the mock-transfected cells (Fig. 5D). These findings suggest that TRIM32 facilitates neural differentiation of mouse embryonal carcinoma cells in collaboration with RARα.

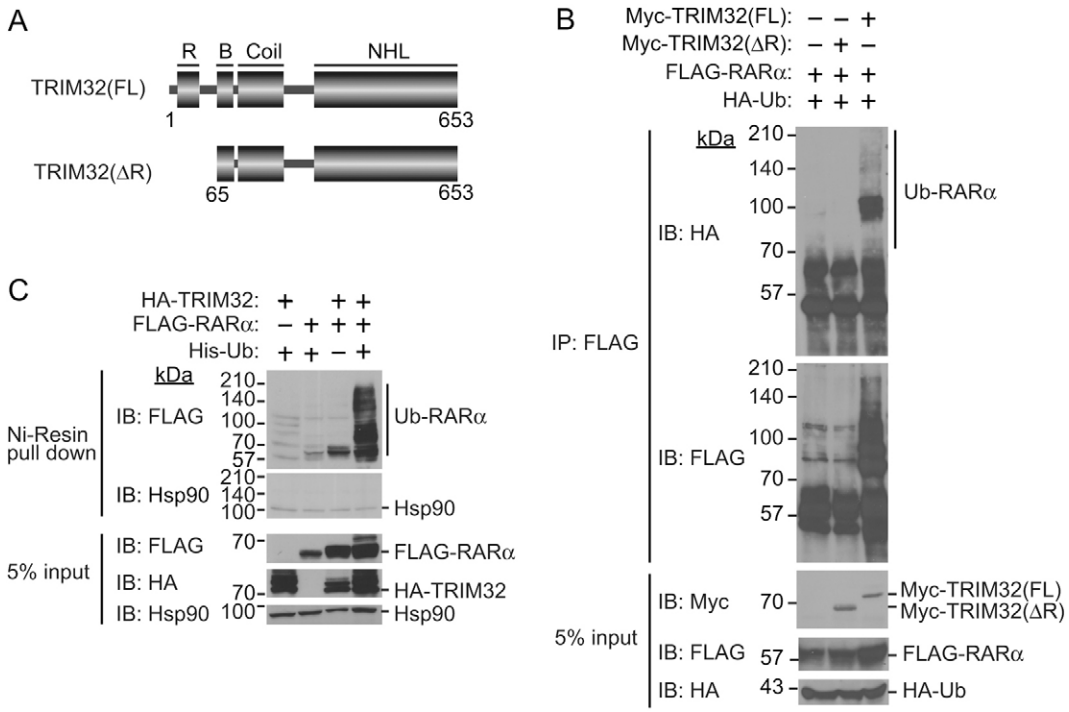

Fig. 3. TRIM32 promotes ubiquitinylation of RARα. (**A**) Schematic representation of full-length TRIM32 [TRIM32(FL)] and TRIM32 lacking the RING domain [TRIM32(ΔR)]. TRIM32 has a RING finger (R), two B-boxes (B), a coiled-coil region (Coil) and an NHL domain (NHL). (**B**) In vivo assay for ubiquitinylation of RARα by TRIM32. Expression vectors encoding FLAG-tagged RARα, HA-tagged ubiquitin, Myc-tagged TRIM32(FL) or TRIM32(ΔR) were transfected into HEK293T cells. Cell lysates were immunoprecipitated with anti-FLAG antibody and then anti-HA immunoblot analysis was performed to detect ubiquitinylation of RARα. (**C**) In vivo assay for ubiquitinylation of RARα by TRIM32 under denaturing conditions. Expression vectors for FLAG-tagged RARα, HA-tagged TRIM32 and His_6-tagged ubiquitin were transfected into HEK293T cells. His_6-tagged ubiquitin was pulled down with metal affinity resin and immunoblotted with antibodies against the FLAG tag and HA tag to detect ubiquitinylation of RARα. Anti-Hsp90 antibody was used as an internal control.

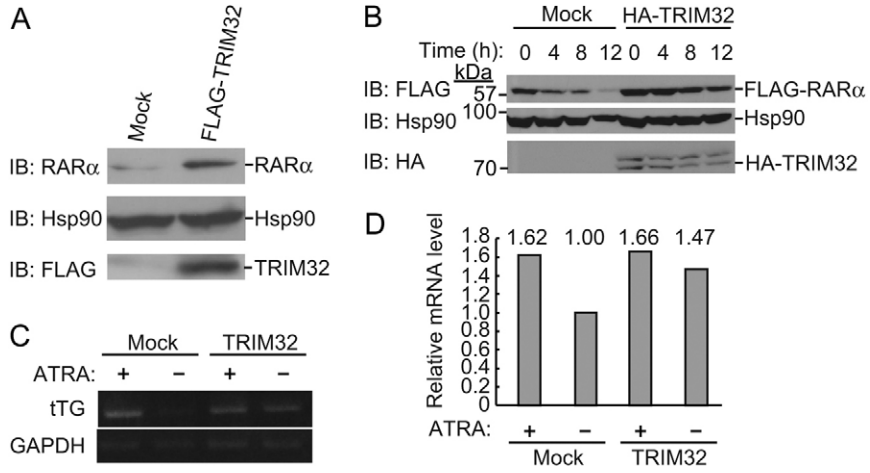

Fig. 4. TRIM32 stabilizes RARα and activates transcription of its target gene. (**A**) Immunoblot analysis of endogenous RARα in Neuro2a cells in which FLAG-tagged TRIM32was expressed. The cell lysates were checked by immunoblot analysis using anti-RARα antibody and anti-FLAG antibody. Anti-Hsp90 antibody was used as an internal control. (**B**) Protein stability analysis of RARα with TRIM32. HEK293T cells were transfected with expression vectors encoding FLAG-tagged RARα and HA-tagged TRIM32 or an empty vector (Mock). After 48 hours, the cells were cultured in the presence of cycloheximide (50 μg/ml) for the indicated times. Cell lysates were then subjected to immunoblot analysis with anti-FLAG, anti-hsp90 or anti-HA antibody. (**C**) Upregulation of RARα-dependent endogenous gene transcription by TRIM32. Neuro2a cells lines stably expressing FLAG-tagged TRIM32 and cell transfected with empty vector (Mock) were established using a retroviral expression system, and used for real-time quantitative PCR of tissue transglutaminase (t*TG*) mRNA, which is one of the RARα target downstream genes. Each cell line was incubated with or without ATRA (20 μM) for 1 day, and then total mRNA was purified and reverse-transcribed to perform real-time PCR. PCR product samples were subjected to agarose gel electrophoresis. (**D**) Quantification of t*TG* mRNA by real-time PCR. The average threshold cycle (Ct) to that of *GAPDH* was determined and the value of the mock-transfected cells without ATRA was defined as 1. Two different sets of primers for *tTG* were used to confirm the results of independent experiments.

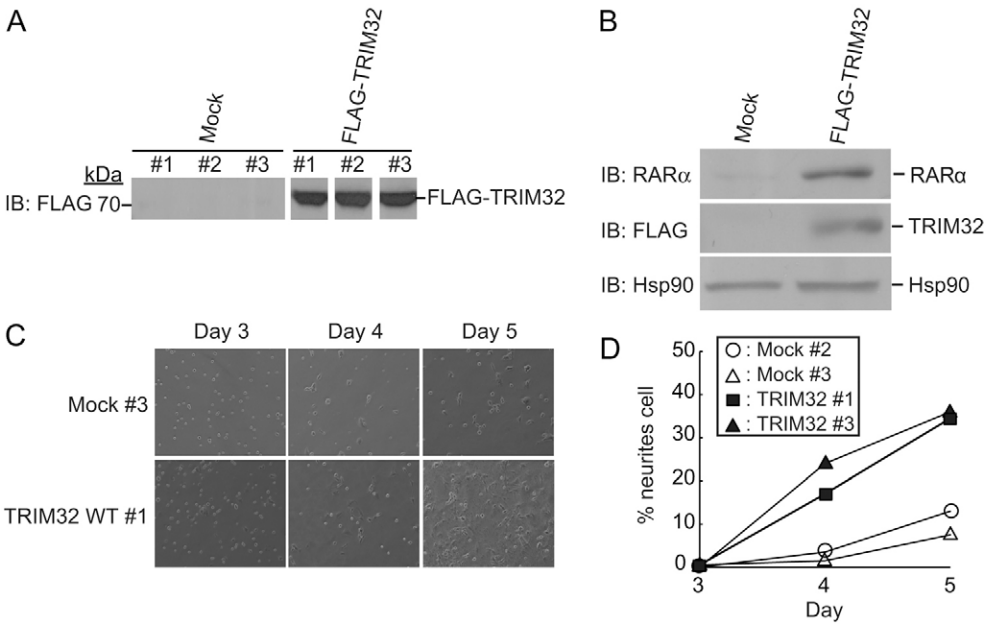

Fig. 5. TRIM32 promotes neural differentiation of P19 cells. (**A**) Establishment of three P19 cell clones stably expressing FLAG-tagged TRIM32 using lipofection followed by limiting dilution and puromycin selection. Three mock-transfected cell clones were also generated with the corresponding empty vector. The cell lines were checked by immunoblot analysis using anti-FLAG antibody. (**B**) Immunoblot analysis of endogenous RARα in P19 cells in which FLAG-tagged TRIM32 was stably expressed. These cell clones were checked by immunoblot analysis using anti-RARα antibody and anti-FLAG antibody. Anti-Hsp90 antibody was used as an internal control. (**C**) TRIM32 affects neural differentiation of P19 cells. P19 cell clones stably expressing FLAG–TRIM32 or empty vector (mock) were seeded at 2×10^4 cells in six-well plates with tamibarotene (1 μM) to induce neurite elongation, and cell shape was observed. Day 0 was the day on which tamibarotene was added to the cell culture medium. (**D**) Quantification of TRIM32-induced neural differentiation of P19 cells. Cells that have neurites longer than the diameter of their cell body were counted and the percentages of cell with neurites were calculated. Data are means from two independent experiments.

TRIM32 promotes differentiation of the mouse neuroblastoma cell line Neuro2A

The mouse neuroblastoma cell line Neuro2a, which is also known as a neuronal differentiation model in vitro, undergoes ATRA-dependent cell differentiation. Because it has been reported that overexpression of TRIM32 induces neuronal differentiation, whereas inhibition of TRIM32 causes daughter cells to retain progenitor cell fate (Schwamborn et al., 2009), it was important to examine whether TRIM32 affects neural cell differentiation using Neuro2A cells. Furthermore, we previously showed that TRIM32 has an E3 ubiquitin ligase activity through its RING finger domain. To examine whether the RING domain of TRIM32 is required for neural differentiation, we established a Neuro2A cell line in which FLAG-tagged TRIM32(FL) or TRIM32(ΔR), which lacks a RING domain, is stably expressed by using a retroviral expression system, and we confirmed the expression by immunoblot analysis (Fig. 6A). To evaluate differentiation of Neuro2A cells with or without overexpression of TRIM32, we induced neural differentiation of these established cell lines with ATRA (20 μM). Overexpression of TRIM32(FL) affected cell differentiation compared with the mock-transfected or TRIM32(ΔR)-expressing cells (Fig. 6B). We counted the number of neurite-bearing cells in the three cell lines with or without ATRA and calculated the percentages of neurite-bearing cells. In the presence of ATRA (20 μM), TRIM32(FL) overexpression promoted more rapid changes in neurite cell shape than occurred in the mock-transfected and TRIM32(ΔR)-expressing cells; no significant difference was observed in the absence of ATRA (Fig. 6C).

Furthermore, to confirm whether knockdown of RARα abolished the effects of TRIM32 on neural differentiation, we tried to generate mock-transfected and FLAG–TRIM32-expressing Neuro2A cell lines in which RARα was knocked down by using short hairpin RNA (shRNA) as previously reported (Chen and Napoli, 2008). Silencing of RARα at the protein level in TRIM32-expressing or mock-transfected Neuro2A cell lines was confirmed by immunoblot analysis with anti-RARα antibody (Fig. 7A). The amount of endogenous RARα in mock-transfected Neuro2A cells was decreased by silencing of RARα, whereas we could not establish stable TRIM32-expressing Neuro2A cell lines in which RARα was knocked down by the same silencing system for RARα. Overexpression of TRIM32 might cause inhibition of RARα knockdown by an unidentified mechanism. Therefore, we transiently infected a retrovirus encoding sh*RARα* into Neuro2A cells in which TRIM32 was stably overexpressed. Then, we determined the percentage of neurite-bearing cells. In the presence of ATRA, knockdown of RARα reduced the number of cells with neurites in which TRIM32 was overexpressed (Fig. 7B,C). These findings suggest that TRIM32 facilitates differentiation of mouse neuroblastoma cells in the presence of ATRA.

Discussion

RAR signaling has an important role in the development, regeneration and differentiation of the nervous system. Neuronal induction by retinoic acid is now commonly used in

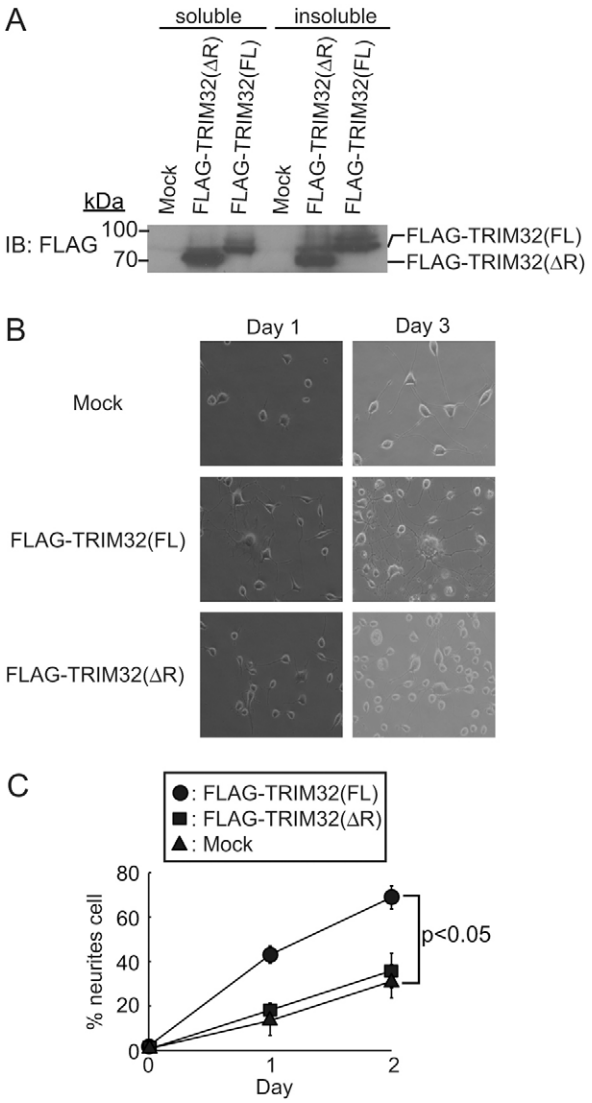

Fig. 6. Overexpression of TRIM32 promotes differentiation of Neuro2a cells. (**A**) Establishment of Neuro2A cell lines stably expressing FLAG-tagged TRIM32(FL) and FLAG-tagged TRIM32(ΔR) using a retroviral expression system. The soluble and insoluble fractions from the cell lines were checked by immunoblot analysis using anti-FLAG antibody. (**B**) TRIM32 affects neural differentiation of Neuro2A cells. Neuro2A cell lines stably expressing FLAG–TRIM32(FL), FLAG–TRIM32(ΔR) or empty vector (Mock) were seeded at 2×10^4 cells in six-well plates with ATRA (20 μM) to induce neurite elongation, and cell shape was observed. Day 0 was the day on which ATRA was added to the cell culture medium. (**C**) Quantification of TRIM32-induced neural differentiation of Neuro2A cells. Cells that had neurites longer than the diameter of their cell body were counted and the percentages of cells with neurites were calculated. Data are means ± s.d. from three independent experiments. *P*-values for the indicated comparisons were determined using Student's *t*-test.

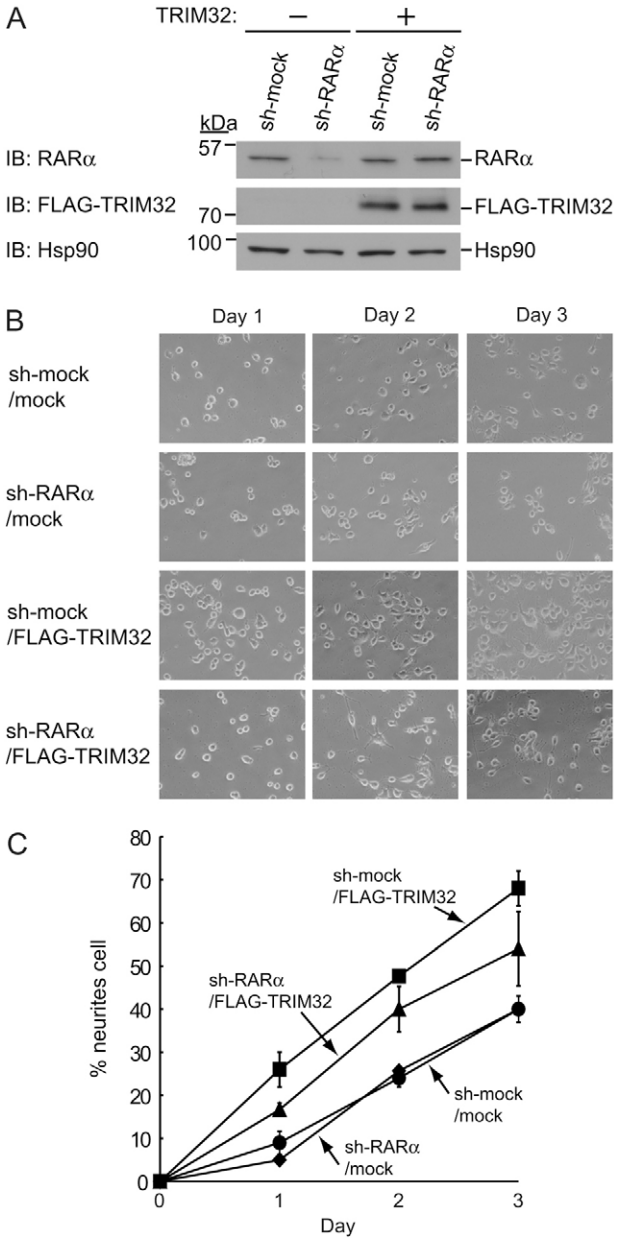

Fig. 7. TRIM32 promotes neural differentiation through RARα. (**A**) Establishment of Neuro2A cell lines stably expressing FLAG-tagged TRIM32 or empty vector (mock) in which RARα was knocked down using a retroviral expression system. The cell lysates were checked by immunoblot analysis using anti-RARα antibody. (**B**) Neuro2A cells in which RARα was knocked down and/or TRIM32 was stably overexpressed were seeded at 2×10^4 cells in six-well plates with ATRA (10 μM), and the cell shape was observed. Day 0 was the day on which ATRA was added to the cell culture medium. (**C**) Quantification of neural differentiation of Neuro2A cells in which RARα was knocked down and/or TRIM32 was overexpressed. Cells that have neurites longer than the diameter of their cell body were counted, and the percentage of cells with neurites was calculated. Data are means ± s.d. from three independent experiments.

ES cell differentiation and is useful for generating homogenous populations of neurons from ES cells. Actually, RA-treated cells give rise to a defined and developmentally restricted neuronal lineage upon differentiation (Glaser and Brustle, 2005). The role of RARα in neuronal development is important but is not clear at the molecular level. In this study, we showed that TRIM32 functions as a novel positive regulator for RARα-mediated transcription. Thus, it is important to clarify TRIM32-mediated RARα activity in neural differentiation. The transcriptional activity of RAR is modulated by cofactors, such as p300/CBP-associated factor (PCAF), and facilitates or represses RAR-mediated gene expression (Weston et al., 2003). Recent advances

have indicated that RAR-mediated transactivation is regulated by post-translational modification, including phosphorylation, acetylation and ubiquitinylation. It has been reported that several TRIM family E3 ubiquitin ligases, including TRIM24, TRIM25 and TRIM68, interact with other nuclear steroid receptors and regulate their transcriptional activities (Kikuchi et al., 2009; Miyajima et al., 2008; Nakajima et al., 2007). However, these E3 ligases probably participate in the regulation of transcriptional activity by a function other than proteolysis. These E3 ligases might ubiquitinylate transcriptional factors and recruit several regulators that recognize the ubiquitinylation on transcriptional factors.

Because several TRIM family proteins regulate activation of nuclear receptors, we tried to identify a regulator for RARα by using an RAR–Luc reporter assay. The present study provided evidence by using a comprehensive dual luciferase reporter assay with TRIM family proteins that TRIM32 is a novel regulator of the activation of RARα in P19 cells. It has been shown that TRIM32 plays an important role in various cellular functions, including neural proliferation and differentiation. Recently, it has been reported that TRIM32 activates microRNAs, by which it ubiquitinylates and degrades the transcription factor Myc but also binds argonaute-1 and thereby increases the activity of specific micro RNAs, which prevents self renewal in mouse neural progenitors. TRIM32 overexpression also induces neuronal differentiation, whereas inhibition of TRIM32 causes daughter cells to retain progenitor cell fate (Schwamborn et al., 2009). Moreover, it has been reported that TRIM32 is responsible for limb-girdle muscular dystrophy type 2H (LGMD2H) and sarcotubular myopathy in humans and that a *TRIM32* knockout model showed a myopathy with a neurogenic component (Kudryashova et al., 2009). In addition, we showed that TRIM32 physically interacts with RARα and enhances RA-dependent RARα-mediated transcriptional activity in a dose-dependent fashion. These findings suggest that TRIM32 controls a novel mechanism of neural differentiation by RARα-mediated regulation in neural progenitor cells. We demonstrated by pulse-chase analysis that overexpression of TRIM32 promotes stabilization of RARα and causes a high expression level of endogenous RARα in P19 cells in which TRIM32 was stably expressed. Because RAR is a ligand-dependent nuclear receptor for transcription, TRIM32 might promote modification of chromatin and formation of a bridge between the DNA-bound RAR intranuclear receptor and the transcriptional mechanism recruiting the chromatin-remodeling proteins to the nuclear receptor target gene. Actually, we showed that TRIM32 physically binds to RARα and that the RING domain is required for neural differentiation in neuroblastoma Neuro2A cells. Previously, we have shown that overexpression of TRIM32 promoted cell growth and transforming activity, whereas TRIM32(ΔR) has a dominant negative effect (Kano et al., 2008). These findings suggest that the RING domain of TRIM32 plays an important role not only in cell growth and carcinogenesis but also in neural cell differentiation.

It has been reported that HECT domain and ankyrin-repeat containing, E3 ubiquitin–protein ligase (HACE1) represses the transcriptional activity of RARα1 (Zhao et al., 2009) and that another E3 ligase (Rnf41) also regulates differentiation of hematopoietic progenitors by modulating the steady state of RARα (Jing et al., 2008). Furthermore, it has been reported that modification of a small ubiquitin-like modifier-2 (SUMO-2) as a post-translational regulatory mechanism controls ATRA-dependent RARα transcription (Zhu et al., 2009). We also tried to prepare recombinant RARα protein from *Escherichia coli* to perform an in vitro ubiquitinylation assay, but we consistently failed to prepare recombinant RARα protein probably because of its unstable characteristics. Therefore, it remains unclear whether TRIM32 acts as a direct E3 ligase for RARα or ubiquitinylates co-activators or repressors interacting with RARα. However, we showed that TRIM32 increases polyubiquitinylation of RARα in an in vivo ubiquitinylation assay under denaturing conditions. Moreover, we demonstrated that TRIM32 probably regulates the level of RARα expression by ubiquitinylation and that the RING domain of TRIM32 contributes to the ubiquitinylation of RARα in cells. However, it is likely that ubiquitinylation of RARα by TRIM32 does not contribute to proteolysis but that TRIM32 stabilizes RARα. It has been reported that the E3 ubiquitin ligase ZFP91 binds to NF-κB-inducing kinase (NIK) and mediates its ubiquitinylation but that ZFP91 stabilizes NIK through K63-linked polyubiquitinylation, suggesting that ZFP91 is an activator for NIK and that stimulation of the MEK/ERK pathway strongly promotes K63-linked ubiquitinylation of RACO-1, and stabilizes RACO-1 by inhibiting the K48-linked ubiquitinylation for degradation (Davies et al., 2010; Jing et al., 2010). Hence, further studies are needed to elucidate whether TRIM32 directly ubiquitinylates RARα and what kind of ubiquitinylation of RARα is induced by TRIM32 (Burgdorf et al., 2004; Metivier et al., 2003).

Retinoids are important drugs for cancer therapy because they have potential effects on cell differentiation and apoptosis. For example, ATRA is a well-known inducer of differentiation, which is used for acute promyelocytic leukemia (APL). The initial treatment for APL is established using ATRA (Jiang et al., 2008). Moreover, neuroblastoma, which is the most common solid tumor of early childhood, is a severe tumor characterized by poor prognosis, and retinoids can induce differentiation of neuroblastoma cells. A 13-cis retinoic acid is administered for maintenance therapy of neuroblastoma, and the agent has dramatically improved the prognosis of this disease (Matthay et al., 1999). Recently, it has been reported that overexpressed TRIM16 reduces neuroblastoma cell growth, enhances retinoid-induced differentiation and reduces tumorigenicity in vivo (Marshall et al., 2010). We also showed that TRIM32 is a positive modulator of transcriptional activity of RARα, suggesting that TRIM32 regulates the function of RARα or cofactors involved in differentiation of neuroblastoma cells. It is crucial to identify the substrates of TRIM32 and pharmacological enhancers of RAR-associated ubiquitin ligases, including TRIM32, in order to be able to develop therapeutic agents for malignancies in which differentiation is regulated by retinoic-acid-dependent RARα-mediated transcription.

Materials and Methods

Cell culture and P19 cell differentiation

HEK293T, Neuro2a, COS-7 and HeLa cells (ATCC, Manassas, VA) are cultured under an atmosphere of 5% CO_2 at 37°C in DMEM (Sigma-Aldrich Corp., St Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). NIH3T3 cells (ATCC) were cultured under the same conditions in DMEM supplemented with 10% calf serum (Camblex, Charles City, IA). P19 cells (ATCC) were cultured under the same conditions in α-MEM (Sigma) supplemented with 10% fetal bovine serum. CWR22Rv1 cells (ATCC) were cultured by the same method in RPMI1640 (Sigma) with 10% fetal bovine serum. P19 cells were differentiated as described previously (Jones-Villeneuve et al., 1982). Briefly, cells were aggregated on bacterial-grade Petri dishes at a seeding

density of 1×10^5 cells/ml in the presence of tamibarotene (1 μM) in α-MEM with 10% fetal bovine serum. After 3 days of aggregation, cells were dissociated into single cells using trypsin and were cultured in cell-culture-grade plates without retinoic acid for a further 3 or 4 days. For neurite elongation, Neuro2a cells were cultured with ATRA (20 μM; Sigma) for 72 hours. Tamibarotene was kindly provided by the Ituu Laboratory (Tokyo, Japan).

Cloning of cDNA and plasmid construction

Human *TRIM32* cDNAs were amplified from HeLa cDNA by PCR with BlendTaq (Takara, Tokyo, Japan) using the following primers: 5′-GCAATGGCTGCAG-CAGCAGCTTCT-3′ (*TRIM32* sense) and 5′-CCCCTATGGGGTGGAATAT-CTTCT-3′ (*TRIM32* antisense). The amplified fragments were subcloned into pBluescript II SK^+ (Stratagene, La Jolla, CA). FLAG-tagged, HA-tagged or Myc-tagged *TRIM32* cDNAs and were then subcloned into pCR, pCGN, pCAG-puro or pcDNA3 (Invitrogen) for expression in eukaryotic cells. Deletion mutants of *TRIM32* cDNA containing 65–653 amino acids, used as a TRIM32 deletion RING finger domain (ΔR), were amplified by PCR and subcloned (Kano et al., 2008). FLAG-tagged *RARa* and *RXR* cDNAs were subcloned into pCR vector for expression in eukaryotic cells.

Transfection, immunoprecipitation and immunoblot analysis

HEK293T cells were transfected using the calcium phosphate method and lysed in a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, leupeptin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 400 μM Na_3VO_4, 400 μM EDTA, 10 mM NaF and 10 mM sodium pyrophosphate. The cell lysates were centrifuged at 16,000 ***g*** for 15 minutes at 4℃, and the resulting supernatant was incubated with antibodies for 2 hours at 4℃. Protein-A–Sepharose (GE Healthcare Bioscience, Piscataway, NJ) that had equilibrated with the same solution was added to the mixture, which was then tumbled for 1 hour at 4℃. The resin was separated by centrifugation, washed five times with ice-cold lysis buffer, and then boiled in SDS sample buffer. Immunoblot analysis was performed with the following primary antibodies: anti-FLAG (1 μg/ml; M2 or M5; Sigma), anti-HA (1 μg/ml, HA.11; Covance Research Products, Berkeley, CA), anti-HA (1 μg/ml; Y11; Santa Cruz Biotechnology, Santa Cruz, CA), anti-TRIM32 (mouse polyclonal; Abnova, Taiwan), anti-HDAC1 (1:1000 dilution; no. 2062, Cell Signaling, Danvers, MA), anti-β-actin (1 μg/ml; AC15, Sigma), anti-HP190 (1 μg/ml; MBL, Nagoya, Japan), anti-Hsp90 (1 μg/ml; 68, Transduction Laboratories, San Jose, CA), anti-c-Myc (1 μg/ml; 9E10, Covance), anti-RXRα (rabbit polyclonal; Cell Signaling) and anti-RARα to mouse or rabbit IgG (1:10,000 dilution; Promega, Madison, WI) and an enhanced chemiluminescence system (GE Healthcare). To fractionate cytosolic or nuclear extracts, cell lysates were treated with a ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany).

Dual-luciferase assay

Cells were seeded in 24-well plates at 1×10^5 cells per well (Neuro2a, CWR22Rv1 and HeLa) or 5×10^4 cells per well (P19) and then incubated at 37℃ with 5% CO_2 for 48 hours. The retinoic acid reporter–luciferase (RAR–Luc) plasmid and mouse mammary tumor virus–luciferase (MMTV–Luc) reporter plasmid were transfected with TRIM32 and/or RARα and RXR expression vectors into Neuro2a, CWR22Rv1, HeLa and P19 cells using Fugene HD reagent (Roche, Branchburg, NJ). Transfected cells were incubated in DMEM (Invitrogen) supplemented with 10% charcoal-treated fetal bovine serum (Equitech-Bio, Kerrville, TX) for 24 hours and then incubated with ATRA (20 μM), tamibarotene (1 μM) or dihydrotestosterone (10 nM) for 24 hours, harvested, and assayed for luciferase activity with a dual-luciferase reporter assay system (Promega). The luminescence was quantified with a luminometer (Promega).

Protein stability analysis with cycloheximide

Transiently transfected HEK293T cells were cultured with cycloheximide (Sigma) at the concentration of 50 μg/ml and then incubated for various times. Cell lysates were then subjected to SDS-PAGE and immunoblot analysis with antibodies to HA, Hsp90 and FLAG.

Retroviral infection

Retroviral expression vectors for FLAG-tagged TRIM32 were constructed with pMX-puro, which was obtained from Toshio Kitamura (University of Tokyo) (Morita et al., 2000). For retrovirus-mediated gene expression, Neuro2a cells were infected with retroviruses produced by Plat-E packaging cells and then cultured in the presence of puromycin (5 μg/ml; Sigma).

RNA interference

pSUPER-retro-neo vector was purchased from OligoEngine (OligoEngine, Seattle, WA). A short hairpin RNA (shRNA) for *RARa* mRNA was designed according to a previous report (Chen and Napoli, 2008) and chemically synthesized (Invitrogen). pSUPER-retro-neo containing an shRNA for *RARa* sequences (sh*RARa*-#1, 5′-GCCTTGCTTTGTTTGTCAA-3′) was constructed according to the manufacturer's protocol. A scrambled shRNA was also used as a negative control with no significant homology to any known gene sequences in human and mouse genomes. Approximately 50% confluent Plat-E cells in 100-mm dishes were transfected with 10 μg pSUPER-retro-neo-sh*RARa* or scrambled shRNA vector using Fugene HD reagent (Roche). Culture supernatant containing the retrovirus was collected 48 hours after transfection, and retroviral supernatant was added to Neuro2A cell lines stably expressing FLAG-tagged TRIM32 or to mock-transfected cells in 60-mm dishes with polybrene (8 μg/ml; Sigma). Cells were cultured with neomycin (1 mg/ml) for 1 week.

Immunofluorescence staining

COS-7 cells expressing FLAG-tagged RARα and HA-tagged TRIM3 were grown on a glass coverslips and fixed for 10 minutes at room temperature with 2% formaldehyde in PBS. They were then incubated for 1 hour at room temperature with a primary antibody to FLAG or HA in PBS containing 0.1% bovine serum albumin and 0.1% saponin. Next they were incubated with Alexa-Fluor-488-labeled goat polyclonal antibody to mouse immunoglobulin or Alexa-Fluor-546-labeled goat polyclonal antibody to rabbit immunoglobulin (Invitrogen) at a dilution of 1:1000. The cells were covered with a drop of Vectashield (Vector Laboratories, Burlingame, CA) and then photographed with a CCD camera (DP71, Olympus, Japan) attached to an Olympus BX51 microscope.

Real-time PCR

Total RNA was isolated from Neuro2a cells using 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 independent experiments and the level of gene expression relative to *GAPDH* was determined. Two different sets of primers for tissue transglutaminase (*tTG*) were used to confirm the results of independent experiments. The primer sequences for *tTG* were 5′-CTGCTCTACAATGCCTGGTG-3′ and 5′-GAGCATCAGGCAGGTATCCA-3′ (*tTG*-1), 5′-AGGCCAACCACCTGAAC-AAA-3′ and 5′-CATACAGGGGATCGGAAAGT-3′ (*t-TG*-2), and those for *GAPDH* were 5′-GCAAATTCCATGGCACCGT-3′ and 5′-TCGCCCCACT-TGATTTTGG-3′.

Statistical analysis

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

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