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Jab1/CSN5 Induces the Cytoplasmic Localization and Degradation of RUNX3

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ABSTRACT

Runt-related (RUNX) transcription factors play pivotal roles in neoplastic development and have tissue-specific developmental roles in hematopoiesis (*RUNX1*), osteogenesis (*RUNX2*), as well as neurogenesis and thymopoiesis (*RUNX3*). *RUNX3* is a tumor suppressor in gastric carcinoma, and its expression is frequently inactivated by DNA methylation or its protein mislocalized in many cancer types, including gastric and breast cancer. Jun-activation domain-binding protein 1 (Jab1/CSN5), a component of the COP9 signalosome (CSN), is critical for nuclear export and the degradation of several tumor suppressor proteins, including p53, $p27^{Kip1}$, and Smad4. Here, we find that Jab1 facilitates nuclear export of RUNX3 that is controlled by CSN-associated kinases. RUNX3 sequestered in the cytoplasm is rapidly degraded through a proteasome-mediated pathway. Our results identify a novel mechanism of regulating nuclear export and protein stability of RUNX3 by the CSN complex. J. Cell. Biochem. 107: 557–565, 2009.

KEY WORDS: Jab1; CSN; CK2; RUNX; TUMOR SUPPRESSOR

RUNX proteins comprise a family of conserved transcription factors that are critically involved in normal and neoplastic development. In mammals, there are several genes that encode members of the RUNX family, including *RUNX1/AML1*, *RUNX2/CBFA1*, and *RUNX3/AML2* [van Wijnen et al., 2004]. *RUNX1* and *RUNX2* are required for hematopoiesis and osteogenesis, respectively, and are genetically altered in leukemia and bone diseases [Komori et al., 1997; Lee et al., 1997; Look, 1997; Otto et al., 1997; Speck and Gilliland, 2002; Kim et al., 2006]. *RUNX3* is required for the development of CD8-lineage T cells [Chi et al., 2002; Woolf et al., 2003] and tyrosine kinase receptor C (TrkC)-dependent dorsal root ganglion neurons [Inoue et al., 2002, 2003; Levanon et al., 2002]. RUNX3 also functions as a tumor suppressor. The deregulation of RUNX activity is associated with a spectrum of human diseases, which indicates that RUNX proteins are tightly regulated during normal cell differentiation. Decreased levels of RUNX1 and RUNX2 activity result in leukemia [Osato et al., 1999; Song et al., 1999] and cleidocranial cysplasia [Quack et al., 1999], respectively, while increased levels of *RUNX1* expression are associated with a small subset of childhood B-cell acute lymphoblastic leukemias [Niini et al., 2000].

The tumor suppressor activity of *RUNX3* was originally identified in a gastric carcinoma [Li et al., 2002]. Subsequently, it has been reported that *RUNX3* is frequently inactivated by DNA methylation in nearly all types of cancer, including lung, colon, pancreatic, liver, prostate, bile duct, breast, laryngeal, esophageal, endometrial, uterine cervical, testicular yolk sac, and bladder [Ito, 2004]. In addition to gene silencing, RUNX3 protein mislocalization has been identified as an important mechanism for RUNX3 inactivation in gastric cancer [Ito et al., 2005a] and breast cancer [Lau et al., 2006; Subramaniam et al., 2009]. For example, RUNX3 was found to be inactive in 82% of gastric cancers through either gene silencing (44%) or protein mislocalization (38%). Interestingly, the subcellular location of RUNX3 can be modulated by TGF-β signaling in colon cancer cells [Ito et al., 2005a], while the nuclear/cytoplasmic distribution of the closely related RUNX2 protein can be altered by the anticancer drug Taxol and leptomycin [Pockwinse et al., 2006]. To date, however, the molecular mechanism that control the subcellular localization of RUNX3 remains to be defined.

The COP9 signalosome (CSN) is a highly conserved eight-subunit complex found in all higher eukaryotes. It is structurally similar to the regulatory lid of the 26S proteasome. The eight core subunits of CSN are termed CSN1–8. Jab1/CSN5 was originally described as a transcriptional co-activator of the AP1 proteins (particularly c-Jun and JunD) [Bianchi et al., 2000; Wei and Deng, 2003], and has subsequently been implicated in numerous signaling pathways,

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including those that regulate light signaling in plants, larval development in *Drosophila*, integrin signaling, cell cycle control, and steroid hormone signaling [Tomoda et al., 1999]. Recently, Jab1/CSN5 was shown to play a critical role in the nuclear export and degradation of several tumor suppressors, including $p27^{Kip1}$ [Tomoda et al., 1999], p53 [Bech-Otschir et al., 2001], and Smad4 [Wan et al., 2002]. In agreement with its ability to inactivate tumor suppressors, the overexpression of *Jab1* has also been implicated in cancer initiation and progression [Richardson and Zundel, 2005]. Recently, in addition to the eight core components of the CSN, Casein kinase 2 (CK2), Protein Kinase D (PKD) and inositol 1,3,4-trisphosphate 5/6-kinase (5/6 kinase) were identified as CSN-associated kinases. While CK2 does not directly interact with Jab1, it regulates CSN activity through the phosphorylation of CSN2 and CSN7 [Uhle et al., 2003]. The inhibition of CSN-associated kinases results in the inhibition of CSN-mediated degradation [Bech-Otschir et al., 2001].

In the current study, we identified Jab1 as a RUNX-interacting protein, and discovered a novel mechanism for regulating the subcellular localization and stability of RUNX3 through its association with Jab1. Our principal finding is that Jab1 facilitates chromosomal region maintenance 1 (CRM1)-dependent nuclear export and proteasome-mediated degradation of RUNX3 by the CSN complex.

MATERIALS AND METHODS

REAGENTS AND ANTIBODIES

Anti-Jab1 monoclonal (6C3), anti-CK2α monoclonal (1AD9), and anti-Myc polyclonal (A-14), anti-RUNX3 polyclonal (H-50), anti-$p21^{Waf/Cip}$ polyclonal (C-19) antibodies were purchased from Santa Cruz Biotechnology (CA). Anti-RUNX3 monoclonal (5G4) antibody was purchased from Abcam (Cambridge, UK). The anti-Myc (9E10) monoclonal antibody was purchased from Roche Diagnostics. The anti-Flag (M2) and anti-tubulin antibodies were purchased from Sigma (MO). The Alexa Fluor 488 anti-mouse and Alexa 594 anti-rabbit antibodies were purchased from Molecular Probes (Invitrogen, OR). Curcumin, MG132, leptomycin B, 4,6-diamidino-2-phenylindole (DAPI), and cycloheximide were purchased from Sigma. Emodin was purchased from Calbiochem (CA).

PLASMID CONSTRUCTS

The vectors expressing full-length *RUNX3* (*Myc-RUNX3*) and *RUNX* deletion mutants fused to the myc-epitope tag were constructed using the CMV promoter-derived mammalian expression plasmid *pCS4–3Myc*. Human *Jab1* (NM_006837) and full-length human *CK2α* (NM_001895) cDNAs were generated by PCR and then subcloned into pCS4–Flag to generate *Flag-Jab1* and *Flag-CK2α*, respectively. The *Jab1* deletion mutants were generated by PCR and then subcloned into *pCS4–Flag* to generate proteins in which the Flag epitope is fused to Jab1 deletion mutants.

YEAST TWO-HYBRID SCREENING

The yeast strain AH109 was transformed with a bait plasmid encoding the N-terminal region of RUNX3, including the Runt-homology domain (pGBKT-RUNX3-187). Transformants containing pGBKT-RUNX3-187 were mated with pre-transformation yeast that contained a Match Maker cDNA library derived from human thymus (Clontech, CA). Two-hybrid screening was performed according to the manufacturer's protocol. Briefly, yeast strains were mixed and incubated overnight, and then putative interacting clones were selected on Trp/Leu/Ade/His medium. Positive interactions were confirmed by the blue colony phenotype of clones expressing LacZ. To characterize the RUNX3-interacting proteins, plasmids were recovered from yeast strains that exhibited a positive interaction, and sequences were determined by DNA sequencing.

CELL CULTURE AND DNA TRANSFECTION

HEK293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Invitrogen, CA), while SNU5 cells were maintained in RPMI 1640 medium with the same supplements. Transient transfections were performed using Lipofectamine Plus reagent (Invitrogen), according to the manufacturer's instructions.

CO-IMMUNOPRECIPITATIONS

Cells were lysed in 20 mM Tris–HCl (pH 7.4), 5 mM EDTA, 10 mM $Na_2P_2O_7$, 10 mM NaF, 2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, and 1× protease inhibitor mixture (Roche Applied Science, IN). Cell lysates were incubated with the appropriate primary antibody for 2 h at 4°C and then with 50 μl of a 50% slurry of protein A/G-Sepharose for the next hour. Bound proteins were eluted by boiling and then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by immunoblot analysis.

IMMUNOBLOT ANALYSIS

HEK293 cells were transiently transfected with the indicated expression plasmids, and cell extracts were prepared 24 h post-transfection. Extracts were separated by SDS–PAGE, and proteins were transferred electrophoretically to nitrocellulose membranes. Membranes were probed with the appropriate polyclonal or monoclonal primary antibody, followed by horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat immunoglobulin G (IgG). Immunoreactive proteins were visualized by chemiluminescence, according to the manufacturer's instructions (Pierce, IL). For the inhibition of protein degradation, cells were treated with the proteasome inhibitor carbobenzoxy-L-leucyl-L-leucyl-L-leucinal (MG132, 20 μM) after transfection. Images of the immunoblots were acquired using an LAS-3000 mini imaging system (Fujifilm, Japan) and the band intensities were quantitated using Fujifilm Image Gauge Version 3.0 software.

LUCIFERASE ASSAY

HEK293 cells were plated in 24-well plates 1 day before transfection. Cell lysates were prepared 36 h post-transfection in 200 μl of lysis buffer (Promega, Madison). Luciferase activity was determined using luciferase assay reagent (Promega), and β-galactosidase

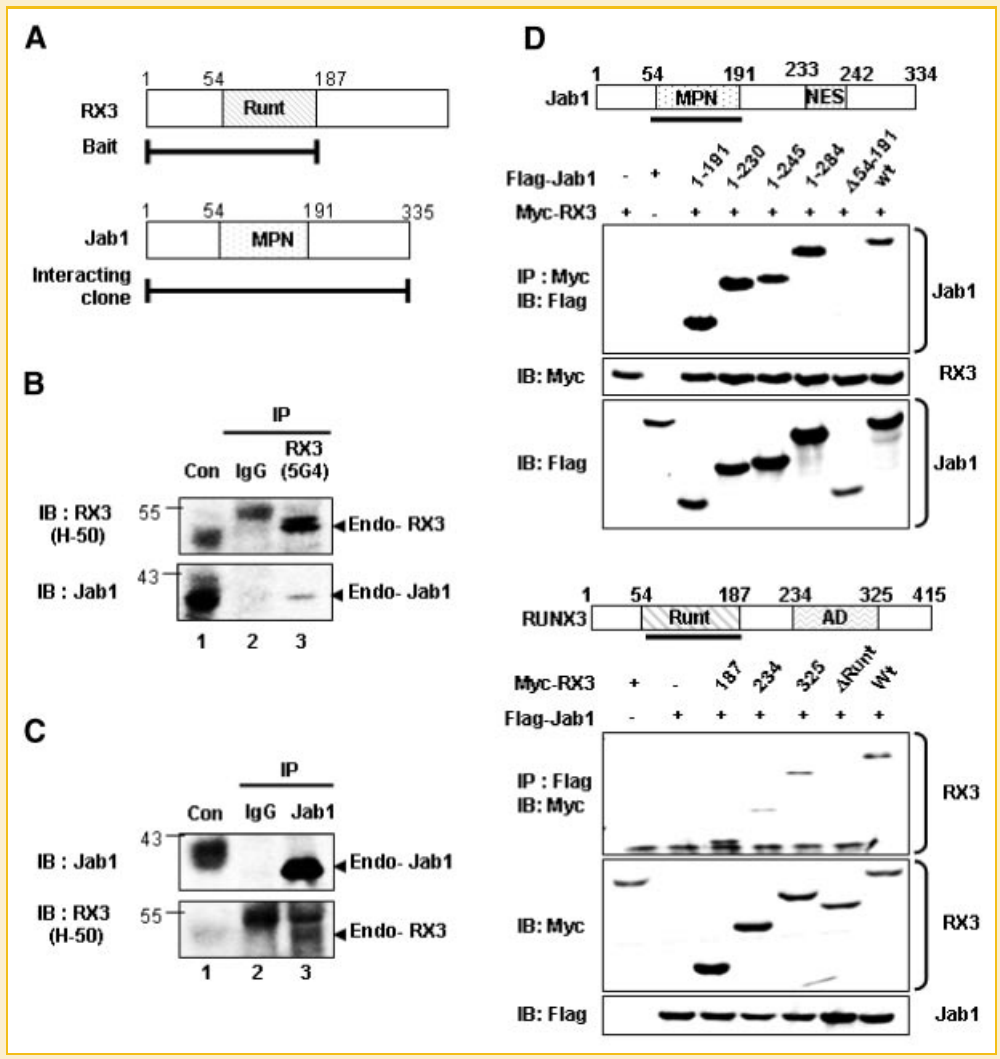

Fig. 1. RUNX3 physically interacts with Jab1. A; Schematic representations of RUNX3 (RX3) and Jab1. The N-terminal region of RUNX3, including the Runt domain, was used as a bait for yeast two hybrid screening. The *Jab1* clone that was isolated is indicated. B: Physical interaction between endogenous RUNX3 and Jab1 in vivo. Cell lysate was obtained from SNU5 cells and immunoprecipitated (IP) with pre-immune antibody (IgG) or anti-RUNX3 monoclonal antibody (5G4) (lane 3). The immunoprecipitate was immunoblotted (IB) with anti-RUNX3 polyclonal antibody (H-50) or anti-Jab1 antibody. C: The same cell lysate was analyzed by IP with anti-Jab1 antibody and IB with anti-Jab1 or anti-RUNX3 antibody. D: HEK293 cells were co-transfected with expression vectors for *Myc-RUNX3* and either full length *Flag-Jab1* or *Flag-Jab1* deletion mutants. Cell lysates were analyzed by IP and IB using the indicated antibodies (upper panels). Similarly, cells were co-transfected with expression vectors for *Flag-Jab1* and *Myc-RUNX3* deletion mutants and the cell lysates were analyzed by IP and IB using the indicated antibodies (lower panels).

activity was determined using a kit from Tropix. All measurements were performed using a Berthold Lumat LB 960 luminometer (BERTHOLD, Wildbad, Germany).

IMMUNOFLUORESCENCE STAINING

HeLa cells were grown on 22-mm cover slips (Fisher) and then transfected with the indicated expression vectors. Cells were washed with phosphate-buffered saline (PBS), and then fixed in a solution of 4% formaldehyde for 15 min at room temperature. Cells were incubated in a solution of 10% fetal bovine serum in PBS containing 0.1% Triton X-100 for 45 min, followed by the indicated primary antibody overnight at 25°C and Alexa Fluor 488 anti-mouse or Alexa 594 anti-rabbit antibody for 1 h at 25°C. The cells were then stained with DAPI for 7 min. Cells were examined with a Leica TCS-NT laser scanning confocal microscope using an oil immersion objective lens and the appropriate emission filters, settings, and controls to exclude bleed-through effects.

SMALL INHIBITORY RNAs

The Jab1-specific small inhibitory RNA (siRNA) (SC-35717) was purchased from Santa Cruz Biotechnology. The following CK2α-specific siRNAs were synthesized (Bioneer Co, Korea): CK2α #1 sense, 5-CAU UUA GUU ACU GGG CAU A-3; CK2α #1 antisense, 5-UAU GCC CAG UAA CUA AAU G-3; CK2α #2 sense, 5-GAU CCA CGU UUC AAU GAU A-3; CK2α #2 antisense, 5-UAU CAU UGA AAC GUG GAU C-3. HEK293 cells were seeded at 50% confluency 1 day before siRNA transfection.

RESULTS

PHYSICAL INTERACTION BETWEEN RUNX3 AND Jab1

To identify putative RUNX3-interacting proteins, we screened a human thymus cDNA library by the yeast two hybrid system. A truncated form of RUNX3 that contained the amino-terminal and Runt domain, but lacked the C-terminal region of the protein [RUNX3 (1–187)] was used as bait (Fig. 1A). We isolated eight independent clones, one of which contained the sequence of full-length Jab1 (Fig. 1A).

Physical interaction between endogenous RUNX3 and Jab1 protein was confirmed by co-immunoprecipitation assay. Total cellular extract from human gastric cancer SNU5 cells was immunoprecipitated with anti-RUNX3 or with normal mouse IgG. Immunoblotting analysis revealed that endogenous Jab1 was co-immunoprecipitated with endogenous RUNX3 (Fig. 1B, lane 3). In the reciprocal experiments, endogenous RUNX3 was co-immunoprecipitated with endogenous Jab1 (Fig. 1C, lane 3). These results indicate that RUNX3 associates with the Jab1 in vivo.

To identify the region of Jab1 that mediates the binding of RUNX3, we constructed a set of *Jab1* deletion mutants. Wild-type *Jab1* or *Jab1* deletion mutants were co-expressed with full-length *RUNX3*, and cell lysates were subjected to immunoprecipitation and immunoblot analysis. Wild-type Jab1 and the Jab1 deletion mutants Jab1(1–191), (1–230), (1–245), and (1–284) were able to bind to RUNX3, whereas Jab1(Δ54–191) which lacks the MPN (Mpr1, Pad1 N-terminal) domain, was not (Fig. 1D, upper panels). These results suggested that the MPN domain of Jab1 is required for the physical interaction of Jab1 and RUNX3.

To identify the region of RUNX3 that mediate the interaction with Jab1, wild-type *RUNX3* or *RUNX3* deletion mutants were co-expressed with full-length *Jab1*, and cell lysates were subjected to immunoprecipitation and immunoblot analysis. All RUNX3 deletion mutants, with the exception of RUNX3 (ΔRunt), which lacks the runt domain, were co-immunoprecipitated with Jab1 (Fig. 1D, lower panels). These results indicate that the runt domain of RUNX3 is required for the interaction with Jab1. Therefore, the runt domain of RUNX3 and the MPN domain of Jab1 are critical for the physical interaction of RUNX3 and Jab1.

Jab1 MEDIATES THE DEGRADATION OF RUNX3

Jab1 has been found to induce the degradation of multiple proteins that are known regulators of disease progression in diverse cancers [Tomoda et al., 1999; Bech-Otschir et al., 2001; Wan et al., 2002]. Thus, it is possible that Jab1 also induces degradation of the RUNX3 through direct interaction with RUNX3. To further characterize the Jab1-mediated degradation of RUNX3, HEK293 cells were co-transfected with an expression vector for *Myc-RUNX3*, and increasing amounts of *Flag-Jab1*. As shown in Figure 2A, *Jab1* expression decreases the levels of exogenous RUNX3 in a dose dependent manner. Over-expression of *Jab1* also decreases the

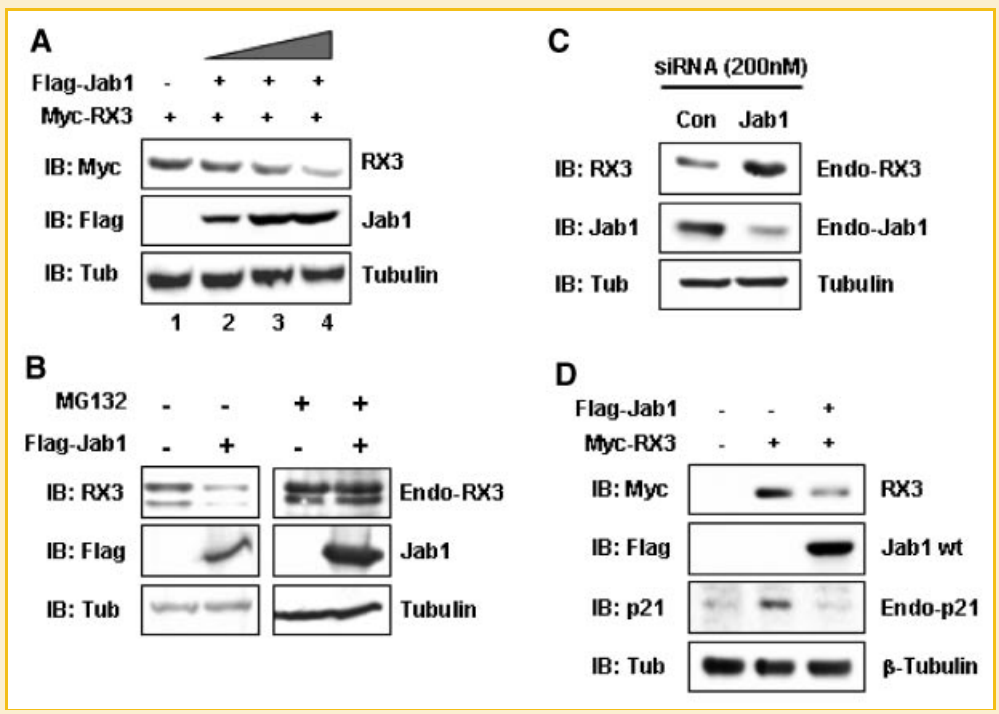

Fig. 2. Jab1 stimulates RUNX3 degradation. A: HEK293 cells were transfected with a fixed amount of expression vector for *Myc-RUNX3* (0.2 μg) and increasing amounts of *Flag-Jab1* (0.2, 0.5, 1.0 μg). Total cell extract was analyzed by IB. B: HEK293 cells were transfected with an expression vector for *Flag-Jab1* (lanes 2 and 4) or empty vector (lanes 1 and 3), and then treated with 20 μM MG132 (lanes 3 and 4) for 4 h before harvesting. Cell lysates were analyzed by IB using anti-RUNX3, anti-Flag, and anti-β-Tubulin antibodies. C: HEK293 cells were transfected with either a control siRNA or *Jab1*-siRNA. Total cell lysate was harvested and the levels of endogenous Jab1, RUNX3, and β-tubulin were analyzed by IB. D: HEK293 cells were transfected with the indicated combinations of plasmids expressing *Myc-RUNX3* or *Flag-Jab1*. The Myc-RUNX3 and Flag-Jab1 levels were monitored using anti-Myc and anti-Flag antibodies. The level of endogenous $p21^{Waf/Cip}$ was detected using anti-p21 antibody.

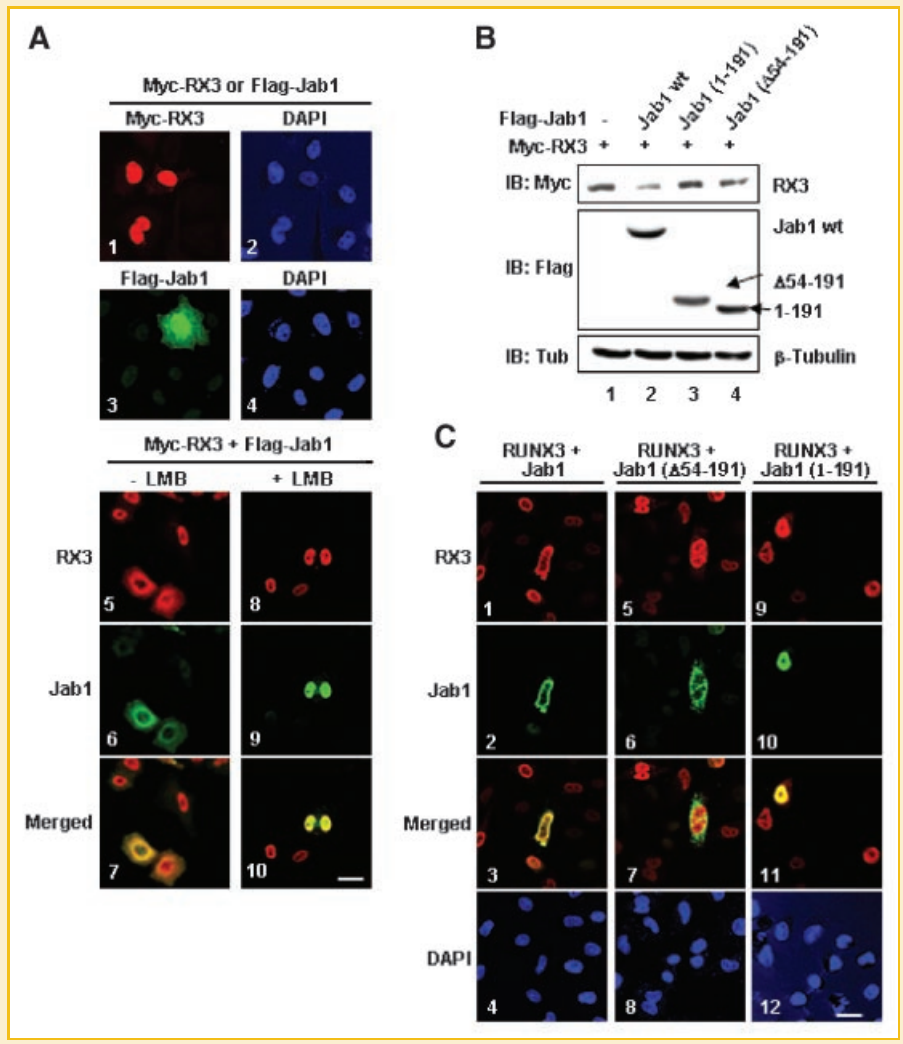

Fig. 3. The MPN domain and the NES region of Jab1 are required for the nuclear export and degradation of RUNX3. A: HeLa cells were transfected with expression vectors for *Myc-RUNX3* (panels 1 and 2), *Flag-Jab1* (panels 3 and 4), or both (panels 5–10), and then cells were immunostained with an anti-Flag monoclonal antibody followed by an Alexa Fluor 488 anti-mouse antibody (green), or polyclonal anti-Myc antibodies followed by an Alexa-594 anti-rabbit antibody (red). Cells were treated with (panels 8–10) or without (panels 1–7) LMB (20 nM) for 4 h, as indicated. The scale bar indicates 25 μm. Nuclei were stained with DAPI. B: HEK293 cells were transfected with expression vectors for *Myc-RUNX3* and either *Flag-Jab1*, *Flag-Jab1(Δ54–191)*, or *Flag-Jab1(1–191)*. The levels of RUNX3 were analyzed by IB using an anti-Myc antibody. C: Myc-RUNX3 was co-expressed with *Flag-Jab1* (panels 1–4), Flag-Jab1(Δ54–191) (panels 5–8) or Flag-Jab1(1–191) (panels 9–12) in HeLa cells and the cells were immunostained as described above. The scale bar indicates 25 μm.

levels of endogenous RUNX3 (Fig. 2B, left panel) and the Jab1-induced down-regulation of RUNX3 is inhibited by the proteasome inhibitor MG132 (Fig. 2B, right panel). We also examined the protein levels of endogenous RUNX3 in cells that were transfected with *Jab1*-siRNA. The results demonstrate that knock-down of endogenous *Jab1* increases the levels of endogenous RUNX3 (Fig. 2C). Consistent with these observations, exogenous Jab1 reduces the level of endogenous *p21*, target protein of RUNX3 [Chi et al., 2005] (Fig. 2D).

THE MPN DOMAIN AND THE NES REGION OF Jab1 ARE REQUIRED FOR THE NUCLEAR EXPORT AND DEGRADATION OF RUNX3

Since Jab1 contains the nuclear export signal (NES) and is involved in the translocation of protein substrates from the nucleus to the cytoplasm [Tomoda et al., 1999, 2002], we examined whether Jab1 alters the subcellular localization of RUNX3 by immunofluorescence staining and confocal microscopy. When either *Jab1* or *RUNX3* was expressed, RUNX3 localized exclusively to the nucleus, whereas Jab1 localized in both the nucleus and the cytoplasm (Fig. 3A, panels 1–4). However, when *RUNX3* and *Jab1* was co-expressed, RUNX3 localization was altered from the nucleus to the cytoplasm (Fig. 3A, panels 5–7).

It has been demonstrated that Jab1-mediated nuclear export requires CRM1, a member of the importin-related nuclear transport receptor family [Chi et al., 2002]. To determine whether the Jab1-mediated nuclear export of RUNX3 was CRM1-dependent, HeLa cells were transiently transfected with expression vectors for *RUNX3* and *Jab1*, and then treated with an inhibitor of CRM1, leptomycin B

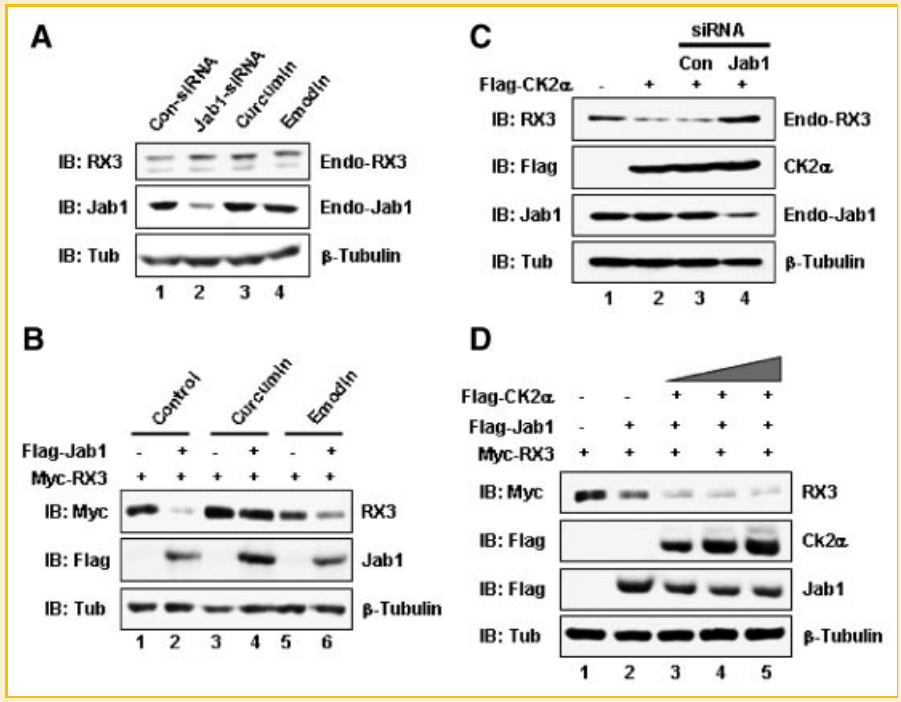

Fig. 4. Inhibition of CSN-specific phosphorylation in HEK293 cell stabilizes RUNX3. A: HEK293 cells were treated with control siRNA (Con-siRNA, lane 1), Jab1-siRNA (lane 2), 10 μM curcumin (lane 3) or 20 μM emodin (lane 4), and then the levels of endogenous RUNX3, Jab1 and β-tubulin were analyzed by IB using the indicated antibodies. Treatment with curcumin and emodin decreased the levels of endogenous RUNX3, but had no effect on the level of Jab1. B: HEK293 cells were transfected with expression vectors for *Myc-RUNX3* and/or *Flag-Jab1* and then treated with vehicle (lanes 1 and 2), 10 μM curcumin (lanes 3 and 4) or 20 μM emodin (lanes 5 and 6). The protein levels of RUNX3, Jab1 and β-tubulin were analyzed by IB. C: HEK293 cells were pretreated with Con-siRNA (lane 3) or *Jab1*-siRNA (lane 4), and then transfected with an expression vector for *Flag-CK2α* (lanes 2–4). Cell lysates were analyzed by IB using anti-RUNX3, anti-Flag, anti-Jab1 and anti-β-tubulin antibodies 48 h after transfection. D: HEK293 cells were co-transfected with expression vectors for *RUNX3* and either *Flag-Jab1* alone, or *Flag-Jab1* together with increasing amounts of *Flag-CK2α*. The effect of CK2α on Jab1-mediated degradation of RUNX3 was analyzed by IB using anti-Myc, anti-Flag and β-tubulin antibodies.

(LMB) [Yoshida and Horinouchi, 1999]. Treatment with LMB efficiently blocked the Jab1-mediated nuclear export of RUNX3 (Fig. 3A, panels 8–10). These results strongly suggested that that Jab1 facilitates the nuclear export of RUNX3, and that this process is CRM1-dependent.

We then analyzed the regions of Jab1 which is required for the RUNX3 degradation. Co-expression of wild-type *Jab1*, *Jab1(Δ54–191)* (lacking MPN domain) or *Jab1(1–191)* (lacking NES) with *RUNX3* followed by immunoblotting with anti-RUNX3 antibody revealed that both Jab1(Δ54–191) and Jab1(1–191) failed to decrease RUNX3 level, while wild-type Jab1 could (Fig. 3B). We also compared the effect of wild-type Jab1, Jab1(Δ54–191) and Jab1(1–191) on RUNX3 subcellular localization by immunofluorescence staining. RUNX3 showed the cytoplasmic distribution in approximately all the cells expressing wild-type *Jab1*, whereas the cells expressed with *Jab1(Δ54–191)* or *Jab1(1–191)* show nuclear localization of RUNX3 (Fig. 3C). These results suggest that both MPN domain and NES are required not only for the nuclear export of RUNX3 but also for the degradation of RUNX3.

INHIBITION OF CSN-ASSOCIATED KINASES STABILIZES ENDOGENOUS RUNX3

Previously, it was shown that Jab1 mediates the degradation of target proteins in a free form/small Jab1 complex, or as a component of the large COP9 signalosome complex (CSN) [Bech-Otschir et al., 2001; Uhle et al., 2003]. The function of the CSN complex is modulated by CSN-associated kinases [Bech-Otschir et al., 2001; Uhle et al., 2003; Huang et al., 2007]. To determine whether the CSN complex is involved in Jab1-mediated RUNX3 degradation, we treated cells with the CSN inhibitors curcumin and emodin [Osato et al., 1999; Quack et al., 1999], and then analyzed the protein levels of endogenous RUNX3. Similar to the effect of Jab1 knock-down using Jab1-siRNA (Fig. 4A, lanes 1 and 2), treatment of cells with the CSN-associated kinase inhibitors curcumin and emodin increased the protein levels of endogenous RUNX3 (Fig. 4A, lanes 3 and 4). The effect of the CSN inhibitors was independent of the levels of Jab1 in these cells, since curcumin and emodin has no effect on the protein levels of Jab1. We also examined the effect of curcumin and emodin on exogenously expressed RUNX3. The over-expression of *Jab1* accelerates RUNX3 degradation (Fig. 4B, lanes 1 and 2), and treatment with CSN inhibitors prevents the Jab1-mediated degradation of RUNX3 (Fig. 4B, lanes 4 and 6).

To examine the role of CSN associated kinases on RUNX3 degradation more closely, HEK293 cells were transfected with an expression vector for *CK2α*, and then the levels of endogenous RUNX3 were analyzed by immunoblot. Over-expression of *CK2α* decreases endogenous RUNX3 protein levels, and this effect is

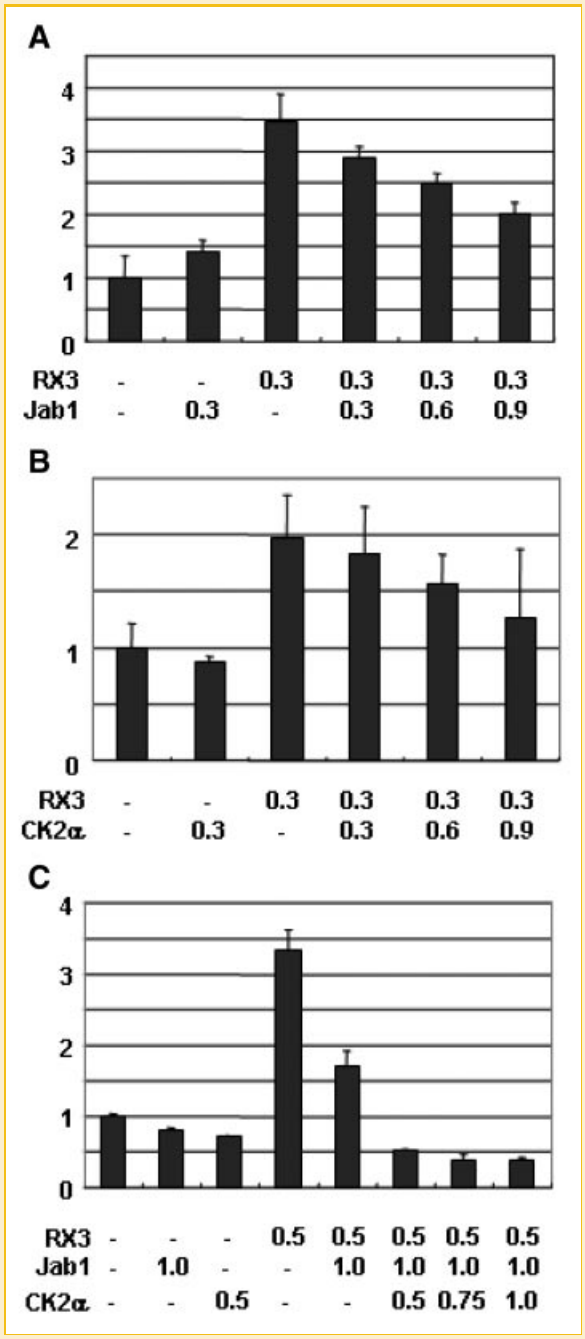

Fig. 5. Jab1 inhibits RUNX3-mediated transcription. A–C: HEK293 cells were co-transfected with a luciferase reporter gene under the control of the $p21^{Waf/Cip}$ promoter (p21-luc) and expression vectors for *RUNX3*, *Jab1* and *CK2α*, as indicated. *pSV-βGal* was also transfected as an internal control. Luciferase reporter activity was measured and normalized to β-galactosidase activity. Data represent the means and standard deviation of at least three independent experiments performed in duplicate.

abrogated by siRNA mediated depletion of *Jab1* (Fig. 4C). Furthermore, CK2α enhances Jab1-mediated RUNX3 degradation in dose-dependent manner (Fig. 4D). These results strongly suggested that the CSN-associated kinase CK2α stimulates Jab1-mediated degradation of RUNX3.

Jab1 inhibits runx3-mediated transcription

To determine whether the Jab1-induced degradation of RUNX3 correlates with the down-regulation of RUNX3-mediated transcriptional activity, we analyzed the transactivation activity of RUNX3 using a p21-promoter reporter assay [Chi et al., 2005]. Exogenous expression of either *Jab1* or *CK2α* reduces the transactivation activity of RUNX3 (Fig. 5A,B), and the co-expression of *Jab1* and *CK2α* has a synergistic effect (Fig. 5C). These results suggest that RUNX3 is inactivated by CSN complex and support our observation that CSN complex regulates nuclear export and degradation of RUNX3.

DISCUSSION

The RUNX family of transcription factors plays a pivotal role in normal development. Changes in RUNX activity are associated with leukemia and the development of solid tumors, autoimmunity, and cleidocranial dysplasia. Haploinsufficiency of *RUNX1* due to heterozygous loss-of-function mutations is associated with familial platelet disorder with a predisposition to acute myeloid leukemia (FPD-AML) [Song et al., 1999], and sporadic heterozygous mutations of *RUNX1* are leukemogenic [Osato et al., 2001]. In addition, the overexpression of *RUNX1* has been implicated in leukemogenesis [Osato and Ito, 2005]. Haploinsufficiency of *RUNX2* can result in cleidocranial dysplasia, an autosomal dominant bone disorder [Lee et al., 1997; Mundlos et al., 1997], and increased levels of *RUNX2* expression are associated with craniosynostosis, another bone disorder [Eswarakumar et al., 2004]. Thus, changes in RUNX protein levels by 50% (either an increase or decrease) result in human disease, which suggests that the levels of RUNX activity are tightly regulated during normal cell differentiation.

To date, several mechanisms of regulation of the stability and activity of RUNX proteins have been reported, including phosphorylation, acetylation, and ubiquitination [Bae and Lee, 2006]. In the current study, we demonstrated that Jab1 facilitates CRM1-dependent nuclear export of RUNX3, and that the sequestered cytoplasmic RUNX3 is subsequently degraded via a proteasome-dependent pathway. These results point to a novel mechanism of regulation of RUNX3.

Jab1 has been reported to facilitate the nuclear export of p27 and p53 by functioning as a molecular link between CRM1 and p27 and p53 [Xirodimas et al., 2004]. Jab1 not only induces the nuclear export of p27 and p53, but also induces their subsequent cytoplasmic degradation. It has been suggested that the direct interaction of Jab1 with p27 provides p27 with a NES, which is required for binding to CRM1 and facilitates nuclear export. Although it is still unclear how Jab1 facilitates CRM1-dependent RUNX3 nuclear export, our results suggest that the mechanism might be similar to that of p27, since deletion of the NES-containing region of Jab1 abrogated the Jab1-mediated nuclear export of RUNX3 (Fig. 3C).

Recent studies have indicated that decreased levels of RUNX3 activity are associated with the initiation and progression of cancer [Li et al., 2002; Kim et al., 2005]. Interestingly, the overexpression of *Jab1* also has been implicated in the initiation and progression of

different types of cancer, including cancer of the lung, pancreas, mouth, thyroid, and breast [Kouvaraki et al., 2003; Shintani et al., 2003; Goto et al., 2004; Ito et al., 2005b; Fukumoto et al., 2006]. These results suggest that the oncogenic activity of Jab1 or CSN activity is associated with the down-regulation of RUNX3. We also found that all RUNX family members interact with similar affinity with Jab1 (our unpublished observation), suggesting that other members of the RUNX family could be down-regulated by a similar manner.

In summary, our results demonstrate that there is a dynamic equilibrium between the biosynthesis and the CSN-mediated nuclear export and degradation of RUNX3, and that this mechanism plays a role in the regulation of endogenous RUNX3.

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