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Pim-1 Kinase Phosphorylates and Stabilizes RUNX3 and Alters Its Subcellular Localization

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ABSTRACT

The loci of the *Pim* and *Runx* gene families have been identified as targets for viral insertions in *CD2-myc* mice. Synergistic cooperation between *Pim* and *RUNX* was also found in the *CD2-Runx2* transgenic mouse lymphoma model. *RUNX* genes have come to prominence recently because of their roles as essential regulators of cell fate in development. Paradoxically, they appear to function either as tumor-suppressor genes or dominant oncogenes according to the cellular context. However, the molecular mechanism of the ambiguous roles played by this family of transcription factors in cancer has remained largely uninvestigated. Here we demonstrate that Pim-1 phosphorylates four Ser/Thr residues within the Runt domain and stabilizes RUNX3 protein. In addition, Pim-1 markedly altered the cellular localization of RUNX3 from the nucleus to the cytoplasm. Our results demonstrate that the subcellular localization of RUNX3 is altered by phosphorylation. We propose that *RUNX* family members may behave as oncogenes if mislocalized to a cellular micro-compartment. J. Cell. Biochem. 105: 1048–1058, 2008.

KEY WORDS: RUNX; Pim-1; SUBCELLULAR LOCALIZATION; PHOSPHORYLATION

Members of the *RUNX* family (RUNXs) are transcription factors that play pivotal roles in normal development and neoplasias. RUNX proteins are heterodimeric complexes composed of and subunits [Bae and Ito, 1999]. The subunit contains a conserved region, the Runt domain, which is required for dimerization with the subunit and for recognition of its cognate DNA binding sequence. In mammals, there are three genes encoding subunits, *RUNX1*/*Aml1*, *RUNX2*/*Cbfa1*, and *RUNX3*/*Pebp2αC*/*Aml2* [van Wijnen et al., 2004]. *RUNX1* is required for definitive hematopoiesis and is a frequent target for chromosome translocation in leukemia [Look, 1997]. *RUNX2* is essential for osteogenesis and *RUNX3* is involved in neurogenesis and thymopoiesis and functions as a tumor suppressor [Ito, 2004]. Decreased levels of RUNX activities are associated with a spectrum of human diseases, including leukemia caused by *RUNX1* deficiency, Cleidocranial Dysplasia (CCD) caused by *RUNX2* deficiency and various tumors caused by defective *RUNX3* expression [Li et al., 2002; Ito, 2004; Kim et al., 2005]. On the other hand, increased levels or mislocalization of RUNX proteins appears to be associated with tumorigenesis. For example, *RUNX1* is amplified in a small subset of childhood B-cell acute lymphoblastic leukemias [Niini et al., 2000]. Overexpression of *RUNX3* is associated with human basal cell carcinomas [Salto-Tellez et al., 2006] and mislocalization of the protein has been observed in some gastric tumors [Ito et al., 2005].

Recently, in MoMuLV-infected *CD2-myc* mice, the loci of all members of *RUNX* family were identified as targets for viral insertion [Blyth et al., 2001, 2005, 2006]. Pim-1 kinase was also identified as a target for proviral activation in the MoMuLV-induced T-cell lymphoma [Cuypers et al., 1984] and in pre-B-cell lymphomagenesis in *Eμ-myc* transgenic mice [van Lohuizen et al., 1991; Mikkers et al., 2002]. Synergistic cooperation between *Pim-1* and *Runx2* was also found in the *CD2-Runx2* transgenic mouse lymphoma model [Blyth et al., 2001]. These findings indicate that there is cooperation among *Pim-1*, *Myc* and members of the *RUNX* family and that RUNX family members may function as oncogenes as well as tumor suppressors. However, the molecular mechanism underlying this ambivalency has remained largely uninvestigated [Blyth et al., 2005].

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Recently, RUNX family transcription factors were identified as targets of Pim-1 by yeast two hybrid screening using Pim-1 as a bait [Aho et al., 2006]. By performing the same screening method using the Runt domain of RUNX3 as the bait, we identified Pim-1 as an interaction partner of RUNX3. In this report, we demonstrate that Pim-1 stabilizes RUNX3 protein by phosphorylating Ser/Thr residues within the Runt domain and alters its subcellular localization from the nucleus to the cytoplasm.

RESULTS

IDENTIFICATION OF Pim-1 AS A RUNX3 BINDING PARTNER

To search for novel targets of RUNX3, we performed yeast two-hybrid screening using a LexA AD-fused fetal liver cDNA library and LexA DB-fused RUNX3 (aa 1–187) as bait. We identified 32 positive clones. DNA sequencing revealed that about a quarter of the positive clones were *CBFβ*, a known RUNX3 partner [Ogawa et al., 1993]. Among the remaining positive clones, we found *Pim-1* (Fig. 1A). Since *Pim-1* and *Runx3* [Blyth et al., 2001] were identified as common loci for provirus insertion in *Myc*-transgenic mice, we studied the functional significance of the interaction between Pim-1 and RUNX3.

In vivo interaction analysis showed that full-length RUNX3 co-precipitated with Pim-1 (Fig. 1B). Interestingly, when *RUNX3* was coexpressed with *Pim-1*, the level of RUNX3 protein increased dramatically. RUNX3 protein migrated as a smear during SDS–PAGE gel electrophoresis (Fig. 1C). Consistent with this finding, we observed that overexpression of Pim-1 resulted in the increase of endogenous RUNX3 protein (Fig. 1D). Analysis of the half-life of RUNX3 protein in the presence and absence of Pim-1 revealed that the RUNX3 protein was much more stable when coexpressed with Pim-1 (Fig. 1E). Quantitation of the band intensity by densitometric analysis showed that the half life of RUNX3 was extended from 4 h in the absence of Pim-1 to longer than 12 h in the presence of Pim-1 (Fig. 1F).

MAPPING OF THE MINIMAL INTERACTING REGIONS

To elucidate the minimal region necessary for the interaction, we generated *Pim-1* deletion mutants tagged with the *Flag* epitope (Fig. 2A). Subsequent immunoprecipitation (IP) and immunoblotting (IB) experiments indicated that the region of Pim-1 spanning aa 97–230 was sufficient for interaction with RUNX3 (Fig. 2B). This region of Pim-1 is located within the kinase domain (aa 38–290).

Flag-tagged *RUNX3* mutants covering the N-terminal region, the Runt domain, and the C-terminal region were constructed (Fig. 2C). Each of the *RUNX3* constructs was transfected alone or with *Pim-1*, and the expression of each of the constructs was confirmed by Western blot analysis (Fig. 2D). Immunoprecipitation of RUNX3 with anti-Flag antibody and Western blotting analysis with anti-Myc antibody, which was used to reveal Pim-1, showed that the Runt domain played a major role in the interaction (Fig. 2E). To test for the requirement of the Runt domain, we constructed a plasmid expressing *RUNX3* deleted for the Runt domain (Runt-del). Immuno-coprecipitation analysis revealed that, in marked contrast to full length RUNX3, Runt-del could only interact weakly with Pim-1. Additionally, the migration pattern and stability of Runt-del were unaffected by Pim-1 expression (Fig. 2F). These results demonstrated that the Runt domain is necessary and sufficient for the interaction of RUNX3 with Pim-1.

A kinase dead mutant of Pim-1 (Pim-1-K67R) was efficiently immuno-coprecipitated with RUNX3, indicating that the kinase activity of Pim-1 was dispensable for its interaction with RUNX3 (Fig. 2G). However, the mutant Pim-1 failed to increase the stability of RUNX3. These results suggest that the kinase enzyme activity of Pim-1 is required for RUNX3 stabilization and that the Runt domain might be a target for phosphorylation by Pim-1 kinase.

THE RUNT DOMAIN OF RUNX3 IS PHOSPHORYLATED BY Pim-1

In order to elucidate the nature of the smeared RUNX3 signal seen in the presence of Pim-1 expression, we performed an in vitro kinase assay using -p32-ATP (Fig. 3A). The results clearly indicated that Pim-1 phosphorylates the RUNX3 protein *in vitro*. The weak phosphorylation of the control (RUNX3-alone) may be due to the endogenous activity of other kinases, such as ERK and PKA, which also phosphorylate RUNX3 [Bae and Lee, 2006]. The smeared pattern of RUNX protein almost completely disappeared following treatment with protein phosphatase PP1 and PPase (Fig. 3B), indicating that the smeared migration pattern of RUNX3 was due to phosphorylation by Pim-1.

To narrow down the phosphorylated region, *RUNX3* was serially deleted from its C-terminal end to generate various deletion constructs, RX3-325 (aa 1–325), RX3-284 (aa 1–284), RX3-234 (aa 1–234), RX3-220 (aa 1–220), RX3-206 (aa 1–206), and RX3-187 (aa 1–187). Co-expression of the deletion constructs of *RUNX3* with *Pim-1* followed by Western blot analysis indicated that all of the deletion constructs of *RUNX3* were stabilized and smeared by Pim-1 expression (Fig. 3C), suggesting that the phosphorylation sites are located mainly, if not all within the aa 1–187 region.

To determine the precise phosphorylation sites of Pim-1-mediated phosphorylation, *Myc*-tagged *RUNX3* was co-expressed with *Pim-1* in HEK293 cells and purified by pull down with G-Sepharose-anti-Myc antibody beads. The purified RUNX3 protein was analyzed by MALDI-TOF and ion-trap mass spectrometry which revealed that Ser 149, Thr 151, Thr 153, and Thr 155 of RUNX3 were consistently phosphorylated by Pim-1 (Fig. 3D). Consistent with the notion that the Runt domain might be the target region of Pim-1-mediated phosphorylation, all these four Ser/Thr residues are located within the Runt domain. The four amino acid residues were then substituted by Ala (RUNX3-4A) residues and co-expressed with Pim-1. The results showed that Pim-1 phosphorylation of the RUNX3-4A mutant was dramatically decreased (Fig. 3E), although the interaction of Pim-1 with the mutant was not affected (data not shown). Densitometric analysis of the band intensities in Figure 3E revealed that Pim-1 expression increased the levels of wild type RUNX3 and RUNX-4A by up to 6 times and 1.5 times, respectively. This result demonstrates that Ser 149, Thr 151, Thr 153, and Thr 155 are the major targets of Pim-1 phosphorylation within RUNX3 and that phosphorylation at these sites is associated with the stabilization of RUNX3.

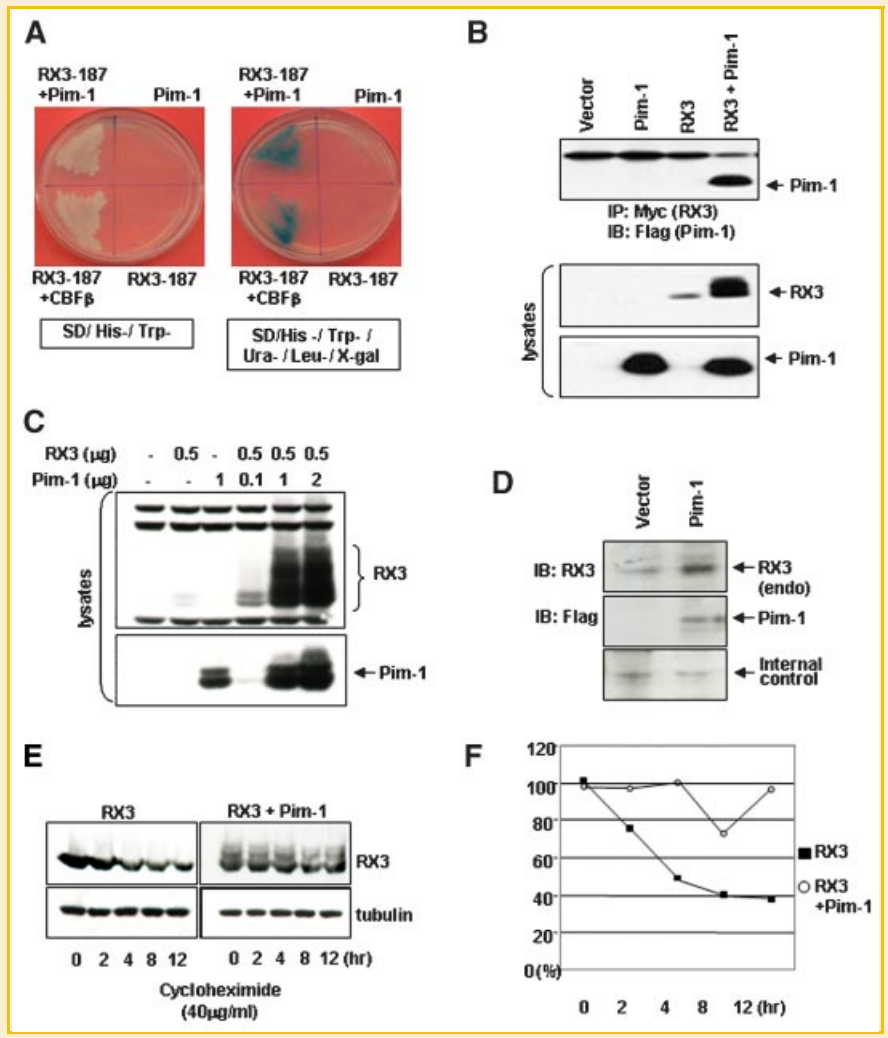


Fig. 1. Protein interaction studies in yeast and HEK 293 cells. A: EGY48 yeast strain was transformed with pEG202-RUNX3-187 or pFG4-5-Pim-1. The *RUNX3-187* transformants were additionally transformed with pJG4-5-Pim-1 or pJG4-5-CBF, plated on SD-Trp$^-$His$^-$ or SD-Trp$^-$His$^-$Ura$^-$Leu$^-$/X-gal for 3 days at 30°C. B: Cotransfection of 293 cells with *Flag-Pim-1* and *Myc-RUNX3* (*RX3*) and analysis by IP with anti-Myc antibody and IB with anti-Flag antibody (top panel). Expression of each gene was checked with the indicated antibodies. C: 293 cells co-transfected with *HA-RUNX3* and increasing concentrations of *Flag-Pim-1* as indicated. HA-RUNX3. *HA-RUNX3* was detected by IB with anti-HA antibody and Pim-1 was detected by anti-Myc antibody. Increases in the amount of the *Pim-1* expression plasmid resulted in an increased amount of RUNX3 protein. D: To examine the effect of Pim-1 on the endogenous RUNX3 protein level, 293 cells were transfected with Flag-Pim-1, followed by immunoblotting with anti-RUNX3 antibody. Endogenous RUNX3 protein level was increased by Pim-1 up to 2.5 times. E: Flag-RUNX3 was co-expressed with Pim-1 in 293 cells as indicated and cultured for the indicated periods in the presence of cycloheximide (40 g/ml). RUNX3 expression was detected by immunoblotting with anti-Flag antibody. F: The band intensities of Figure e were measured by a densitometer. Protein levels are shown as a line graph. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Pim-1 CHANGES THE LOCALIZATION OF RUNX3 FROM THE NUCLEUS TO THE CYTOPLASM

RUNX family proteins are known to be primarily localized to the nucleus, where they interact with many co-factors to modulate the expression of various genes. Pim-1 is reported to shuttle between cytoplasmic and nuclear compartments, depending on cell type and cell physiology [Wang et al., 2002; Ionov et al., 2003]. The confocal data with embryonic kidney cells (HEK 293) demonstrated that Pim-1 is primarily localized to the cytoplasm (Fig. 4A, top panel). When coexpressed with *Pim-1*, RUNX3 moved from the nucleus to the cytoplasm, where it co-localized with Pim-1 (Fig. 4A, second panel). Interestingly, nuclear localization of RUNX3 was not affected by Pim-1-K67R, rather Pim-1-K67R colocalized with RUNX3 in the nucleus (Fig. 4A, third panel). When RUNX3-4A and Pim-1 was cotransfected, RUNX3-4A was localized in the nucleus and Pim-1 was shown to be diffused in both compartment (mainly in cytoplasm and weakly in nucleus) (Fig. 4A, fourth panel). When the ratio of nuclear to cytoplasmic RUNX3 in the presence of Pim-1 was scored, only 10% of the transfected cells showed nuclear localization of RUNX3; the remainder exhibited RUNX3 localization to either the cytosolic or both cellular compartments (Fig. 4B). These results unequivocally demonstrate that Pim-1 sequesters RUNX3 to the cytoplasm and that the kinase activity of Pim-1 is required for this sequestration.

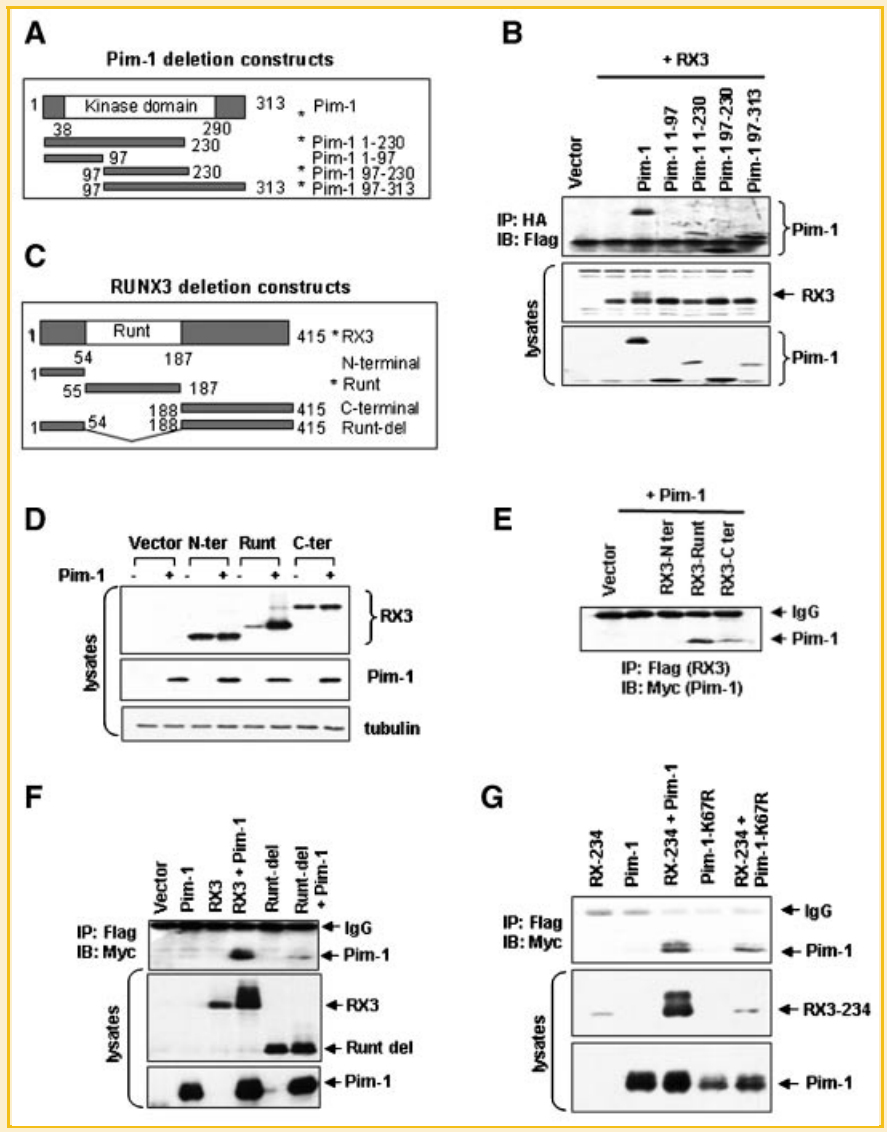


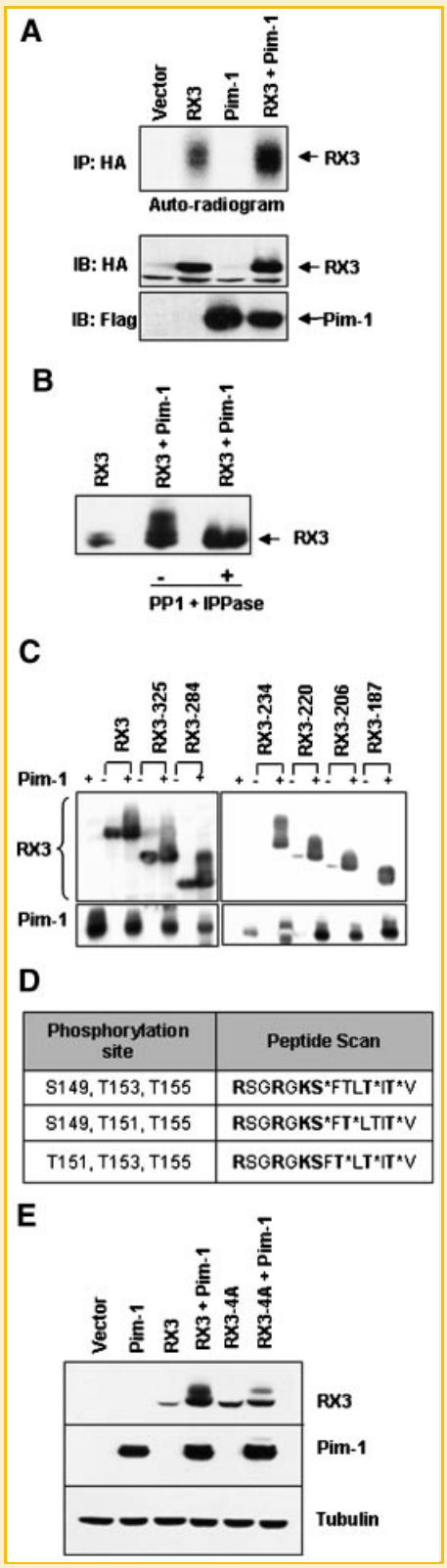
Fig. 2. Characterization of Pim-1 and RUNX3 interaction domains. A: Schematic representation of wild type Pim-1 and deletion constructs. B: *Flag-Pim-1* mutants and *HA-RUNX3* expressed in 293 cells were detected by IP and IB with the indicated antibodies. C: Schematic representation of wild type *RUNX3* and deletion constructs. D: The N-terminal, Runt, or C-terminal region of *Flag-RUNX3* mutants were expressed with or without *Myc-Pim-1* and detected by IB with the indicated antibodies. E: Physical interaction between RUNX3 and Pim-1 was detected by IP with anti-Flag (RUNX3) antibody and IB using anti-Myc (Pim-1) antibody. F: *Flag-RUNX3* and *FLAG-Runt-del* were expressed with or without *Myc-Pim-1*. Expression of the transfected genes was detected with anti-Myc or anti-Flag antibody. Protein interaction was detected by IP and IB using the indicated antibodies. G: *Myc-Pim-1* or *Myc-Pim-1-K67R* was cotransfected with *Flag-RX3-234*. Expression and protein-protein interaction was detected as described in (F).

To understand whether the Pim-1 mediated Runx3 phosphorylation results in inefficient nuclear import or an active nuclear export, the cells were treated with Leptomycin B (LMB) and analyzed the subcellular localization of Runx3. The result revealed that nuclear localization of RUNX3 was not affected by Pim-1 in the presence of LMB, suggesting that Pim-1 actively export Runx3 to cytoplasm (Fig. 4C).

However, it has been reported that EYFP-Runx1 co-localizes with ECFP-Pim-1 to the nucleus of COS-7 cells [Aho et al., 2006], which is contrary to our observations. To explain this discrepancy, we performed similar experiments to those of Aho et al. In contrast to the 293 HEK cells, we found that the subcellular localizations of EYFP-Runx1 and EYFP-RUNX3 were not affected by the coexpression of ECFP-Pim-1 in COS-7 cells (Fig. 4D). Pim-1 was localized primarily to the nucleus in COS-7 cells, which possibly contributed to RUNX nuclear localization. This result suggests that the changes in the subcellular RUNX3 localization induced by Pim-1 could be cell type dependent.

Pim-1 INHIBITS THE TRANSACTIVATION ACTIVITY OF RUNX3

Cytoplasmic sequestration of RUNX3 by Pim-1 suggested that the transactivation activity of RUNX3 might be inhibited by Pim-1. To



Phosphorylation site	Peptide Scan
S149, T153, T155	RSGRGKS*FTLT*IT*V
S149, T151, T155	RSGRGKS*FT*LTIT*V
T151, T153, T155	RSGRGKSFT*LT*IT*V

investigate this possibility, we measured the reporter activity of $p21^{waf/cip}$-promoter-luciferase which is known to be stimulated by RUNX3 [Chi et al., 2005]. As shown in Figure 5A, wild type Pim-1 substantially inhibited RUNX3 stimulation of $p21^{waf/cip}$-promoter activity. To show that this was not a particularity of the $p21^{waf/cip}$ promoter, we also used the pGL3-TRE and pGL3-*M-CSF-R* reporter plasmids which contain a RUNX binding sites and known to be activated by RUNX proteins [Chi et al., 2005]. As with the $p21^{waf/cip}$-promoter-luciferase construct, Pim-1 efficiently blocked RUNX3 transactivation activity of the *M-CSF-R* and TRE promoter, regardless of the presence or absence of CBF, a heterodimeric partner of RUNX3 (Fig. 5B,C). However, the kinase dead mutant (Pim-1-K67R) failed to inhibit RUNX3 mediated transactivation of the TRE promoter (Fig. 5D). Similarly, the RUNX3-4A mutant exhibited resistance to Pim-1 mediated inhibition (Fig. 5E). These results suggest that RUNX3 phosphorylation by Pim-1 is associated with the inhibition of RUNX3 activity.

Pim-1 INHIBITS RUNX3-MEDIATED GROWTH ARREST IN K562 STABLE CELL LINES

To determine if our findings were physiologically relevant, we established K562 human leukemic cell lines stably expressing *RUNX3* (K562-RUNX3), *Pim-1* (K562-Pim-1), or both genes together (K562-RUNX3-Pim-1). K-562 cells can differentiate into progenitors of the erythrocytic, granulocytic, and monocytic lineages [Alitalo, 1990]. After confirming the expression of *RUNX3* and/or *Pim-1* in individual clones (Fig. 6A), the cells were treated with PMA to arrest further growth and differentiation. Cell cycle analysis of PI stained cells using FACS showed that the untreated cell lines (PMA (–)) were essentially identical in their cell cycle profiles. When the cells were treated with PMA, the stably transfected cell lines showed distinct cell cycle profiles. K562-RUNX3 and K562-Pim-1cell populations were mainly arrested in G1 phase and G2/M phase, respectively (Fig. 6B,C). Remarkably, coexpression of *Pim-1* (K562-RUNX3-Pim-1) with *RUNX3* released

Fig. 3. The Runt domain of RUNX3 is phosphorylated by Pim-1. A: 293 cells were transfected with *HA-RUNX3* and *Flag-Pim-1* as indicated. Protein expression was verified by IB with the indicted antibody. RUNX3 was immnunoprecipitated with anti-HA antibody and a radioactive kinase assay was performed using [-^{32}P] ATP. Half volumes of each sample were loaded onto denaturing gel and the radio activity was detected by autoradiography. B: *Flag-RUNX3* was expressed with or without *Myc-Pim-1* in 293 cells. Cell lysates were incubated with PP1 and PPase for 30 min at 30°C and RUNX3 was detected by immnuoblotting with anti-Flag antibody. C: *Flag-RUNX3* mutants were expressed with or without *Myc-Pim-1* in 293 cells and the RUNX3 and Pim-1 proteins were detected by IB with anti Flag and anti-Myc antibody, respectively. D: LC/LC Mass spectra analysis of RUNX3 phosphorylation sites. Two hundred ninety-three cells were transfected with *Flag-RUNX3* and/or *Pim-1* and RUNX3 was immnunoprecipitated with anti-Flag antibody. The immunoprecipitates were analyzed for the phosphorylation sites by LC/LC MS technique. The phosphorylated sites are marked by a * on the peptide sequence. E: Two hundred ninety-three cells were transfected with *Flag-RUNX3* or *Flag-RUNX3-4A* (S149A, T151A, T153A, T155A) or *Myc-Pim-1* or in combination. Effect of Pim-1 on the protein levels of RUNX3 and RUNX3-4A were measured by IB.

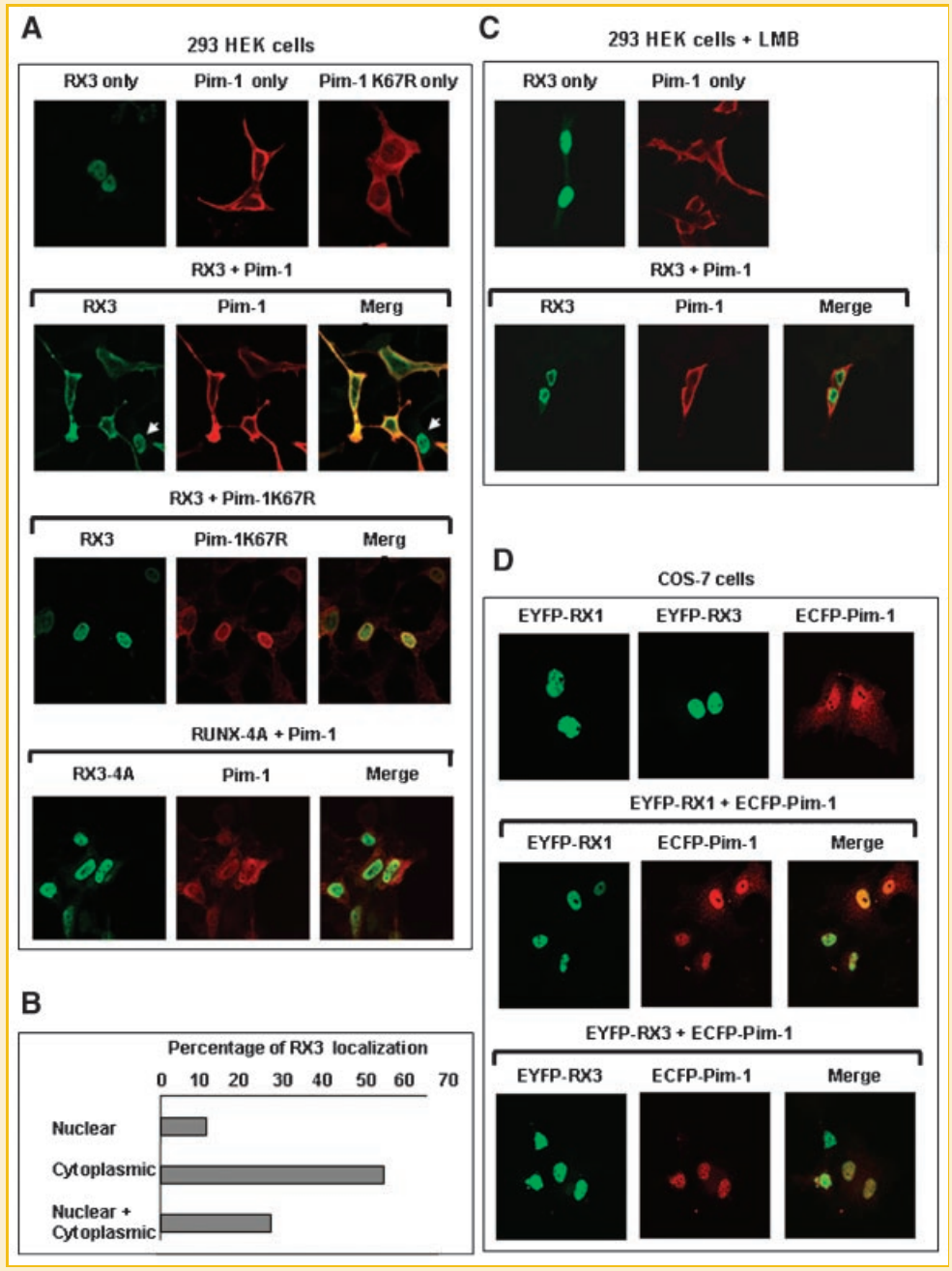


Fig. 4. Co-localization of Pim-1 and RUNX3 in cytoplasm. A: *HA-RUNX3*, *Flag-Pim-1* and *Flag-Pim-1-K67R* were expressed in 293 HEK cells as indicated and the subcellular localization of each protein was visualized by immuno-staining. Pim-1 was probed with anti-Flag antibody emitting red fluorescence and RUNX3 was probed with anti-HA antibody emitting green fluorescence. RUNX3 alone was localized to the nucleus exclusively and Pim-1 and Pim-1-K67R were localized mainly to the cytoplasm (top panel). RUNX3 was sequestered in the cytoplasm only in cells cotransfected with *Pim-1*. Arrows indicate the nuclear localization of RUNX3 in the Pim-1 non-transfected cells (second panel). Pim-1-K67R colocalized with RUNX3 to the nucleus (third panel). RUNX3-4A localized to the nucleus even in the presence of Pim-1 (fourth panel). A merged image is presented on the right. B: One hundred cells co-transfected with *HA-RUNX3* and *Flag-Pim-1* were counted to determine the localizations of the RUNX3 protein (nuclear, cytosol or both). The result is shown as a bar diagram. C: *HA-RUNX3* and *Flag-Pim-1* were expressed in 293 HEK cells as indicated and treated with Leptomycin B (LMB). The subcellular localization of each protein was visualized by immuno-staining. D: *EYFP-RUNX1*, *EYFP-RUNX3* and *ECFP-Pim-1* were expressed in COS-7 cells as indicated and the subcellular localizations of each protein were visualized by capturing the excited fluorescence with a confocal microscope. RUNX3 was localized to the nucleus exclusively. Pim-1 was also localized mainly to the nucleus (top panel). Pim-1 did not alter the subcellular localization of RUNX1 (second panel) or RUNX3 (third panel).

the cells from the block in G1 phase imposed by *RUNX3*, whereas coexpression of the Pim-1 kinase defective mutant Pim-1-K67R had no affect on the RUNX3 cell cycle profile (Fig. 6B,C). These results suggest that *Pim-1* expression suppresses the inhibitory activity of *RUNX3* in the G1-phase of the cell cycle and that the kinase activity of Pim-1 is required for this phenomenon.

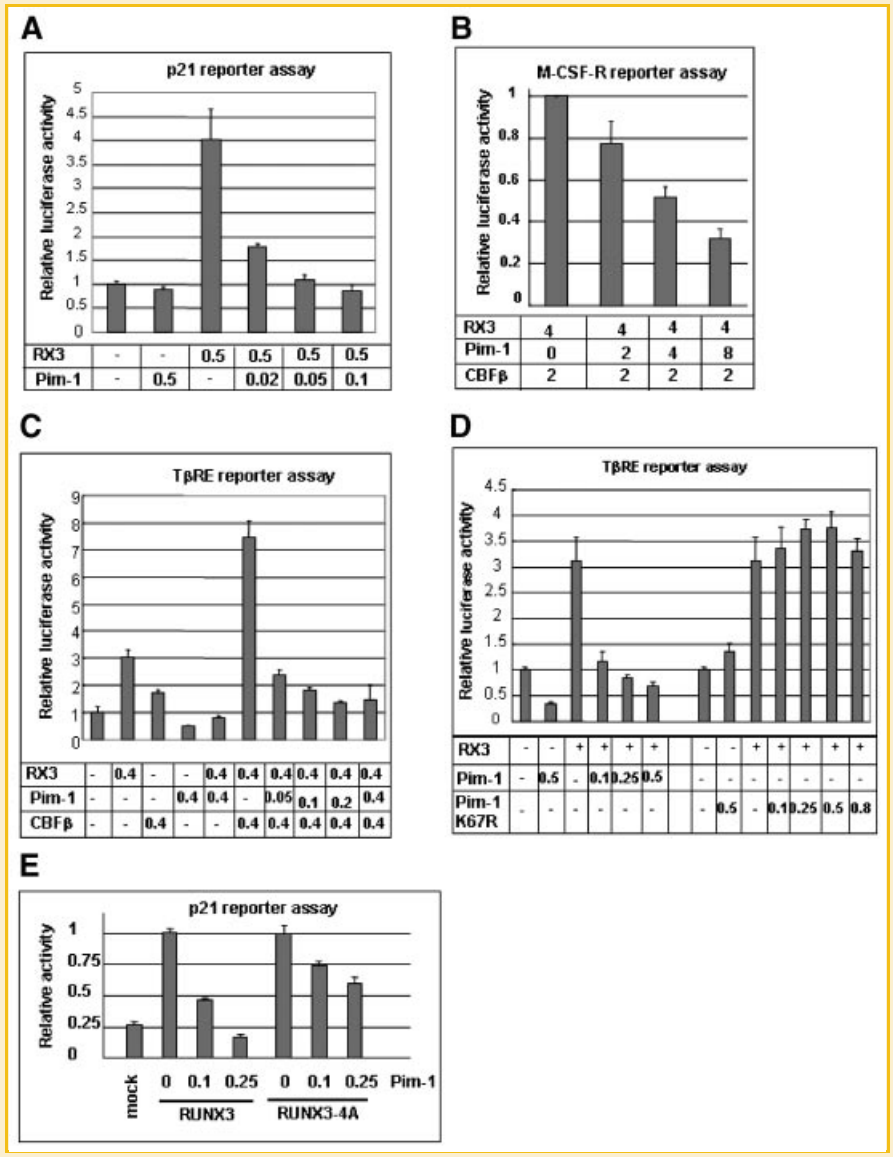


Fig. 5. Pim-1 inhibits RUNX3 transactivation activity on the *p21* promoter. A: Two hundred ninety-three cells were transfected with a fixed amount of pGL3-p21 reporter plasmid (0.05 g) and *RUNX3* expression plasmid (0.5 g) and various amounts of *Pim-1* expression plasmid (0.02, 0.05, 0.1 g). B: Jurkat TAg cells were transfected with the pGL3-MCSF-R reporter plasmid, and *Pim-1* and *CBF* expression plasmids as indicated. C: Two hundred ninety-three cells were cotransfected with the indicated amounts of pGL3-TRE reporter plasmid, pCS4-3Myc-RUNX3, pCS4-3Myc-CBF-, and pCS4-3Myc-Pim-1. D: To measure the effect of the Pim-1-K67R mutant, 293 cells were transfected with *RUNX3* and either the *Pim-1* or *Pim-1-K67R* expression plasmid as indicated. E: To compare the susceptibility of RUNX3 and RUNX3-4A transactiviation activity to *Pim-1* expression, 293 cells were transfected with *RUNX3* or *RUNX3-4A* expression plasmid with or without the *Pim-1* expression plasmid as indicated. Reporter activities were measured with a luminometer and the activities were normalized to that of pCMV--galactosidase (0.05 g), which served as an internal control.

DISCUSSION

Pim-1 plays a crucial role not only in relaying signals from cytokine receptors but also in the malignant transformation of various cell types. In MoMuLV-infected *myc* transgenic mice, the loci of all members of *RUNX* family and Pim family were identified as targets for viral insertion. Recently, synergistic cooperation between *Pim-1* and *RUNX2* was observed in the *CD2-RUNX2* transgenic mouse lymphoma model [Blyth et al., 2001]. This result raised an important question as to why ectopic expression of both *Pim-1* and *RUNX* family genes is more oncogenic than that of *Pim-1* alone. Interestingly, Aho et al. found a physical interaction between Pim-1 and RUNX3 by yeast two hybrid screening using Pim-1 as bait, and showed that Pim-1 phosphorylates RUNXs [Aho et al., 2006]. However, the molecular mechanism for the synergistic effect of *RUNXs* and *Pim-1* on lymphomagenesis was not addressed.

In this study, we confirmed the interaction between these two proteins using the same approach but with the Runt domain as bait. In addition, we found that Pim-1 phosphorylates four Ser/Thr residues within the Runt domain ($\underline{S}^{149}$, $\underline{T}^{151}$, $\underline{T}^{153}$, $\underline{T}^{155}$) of RUNX3 and increases the stability of RUNX3. Phosphorylation appeared to be essential for RUNX3 stabilization, as a mutant Pim-1 that lacks

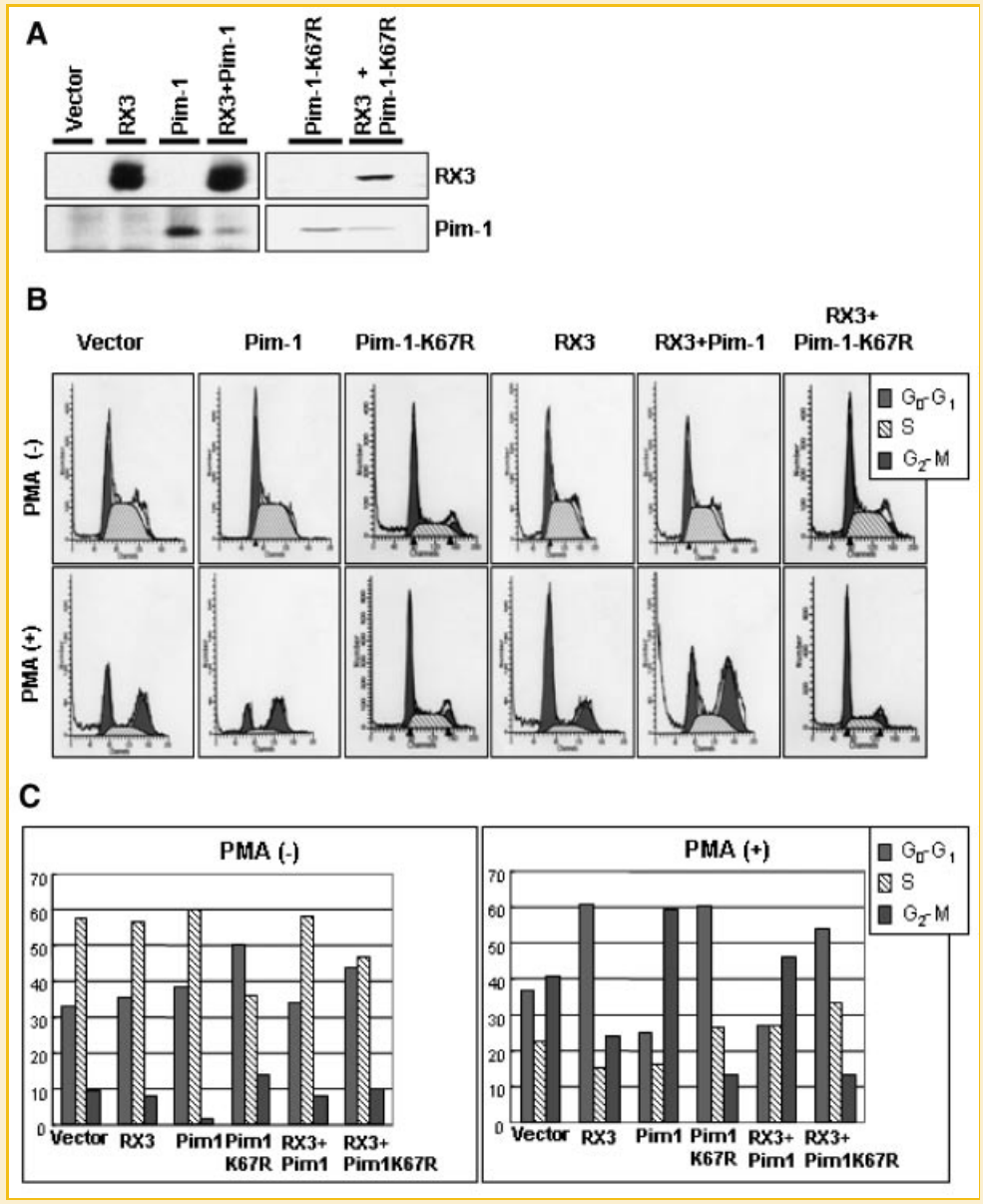


Fig. 6. Cell cycle analysis of stable cell lines expressing *RUNX3* and *Pim-1* by Fluorescence activated cell sorting (FACS). A: Expression of *RUNX3*, *Pim-1*, *Pim-1-K67R* in each K562 stable cell line was checked by immnuoblotting with appropriate antibodies. B: The stable cell lines were incubated for 48 hrs with or without Phorbol 12-myristate 13-acetate (PMA), followed by staining with Propidium Iodide, and FACS analysis. C: The percentage of cells of each cell line in each cell cycle phase, with or without PMA, is presented as a bar diagram.

kinase activity failed to stabilize RUNX3, although it could interact with RUNX3 (Fig. 2F). The phosphorylation sites of RUNX3 (R-S-G-R-G-K-S^{149}-F-T^{151}-L-T^{153}-I-T^{155}) are proceeded by basic amino acid residues. Although it has been reported that Pim-1 can phosphorylate Ser/Thr residues irrespective of the nature of the preceding amino acid residues [Maita et al., 2000; Winn et al., 2003], other studies have reported a preference for basic amino acid residues (R/K-X-R-X-L-S/T) in Pim-1 substrates [Bachmann and Moroy, 2005].

We also found that RUNX3 was sequestered to the cytoplasm when coexpressed with Pim-1. It is worth mentioning that RUNX3 is known to interact with important tumor suppressor proteins, including p300 [Kitabayashi et al., 1998] and Smads [Hanai et al., 1999] as well as with the repair protein Ku70 [Tanaka et al., 2007]. Pim-1-mediated mislocalization of RUNX3 may also result in mislocalization of RUNX3 binding partners. If this is the case, stabilization and sequestration of RUNX3 by Pim-1 would be an effective mechanism for the depletion of components of various tumor suppressor pathways. In support of this interpretation, the ectopic localization of RUNX3 from the nucleus to the cytoplasm has been suggested to be associated with tumorigenesis [Ito et al., 2005]. Aho et al. [2006] reported that the nuclear localization of RUNX1 and RUNX3 is not affected by Pim-1 in COS-7 cells. We also obtained similar results in the same

experimental setting. Thus, it appears that the changes in the subcellular localization of RUNX3 induced by Pim-1 are cell type dependent and that the discrepancy between our results and those of Aho et al. could be due to differences in the cell lines used in the two studies.

We showed that Pim-1 interacts primarily with the Runt domain of RUNX3 and phosphorylates four Ser/Thr residues within the Runt domain. However, we cannot exclude the possibility that Pim-1 also phosphorylates the C-terminal Ser/Thr rich region, since mutation of the four Ser/Thr residues within the Runt domain did not completely abolish the smeared migration pattern of RUNX3 (Fig. 3E). RUNX3 might also be phosphorylated by other protein kinases that are closely associated with the Pim-1/RUNX3 complex. Pim-1 interacts with and affects the activities of two protein kinases, Etk1 and C-TAK1 [Bachmann et al., 2004; Xie et al., 2006]. Conversely, RUNX3 interacts with two kinases, ERK and PKA, which might further phosphorylate RUNX3 downstream of Pim-1.

It has been reported that Pim-1 enhances RUNX1 activity on the *M-CSFR*-promoter [Aho et al., 2006]. However, we found that Pim-1 inhibited the transactivation activity of RUNX3. This could mean that the transactivation activities of RUNX1 and RUNX3 are fundamentally different. In fact, RUNX family members are known to function as transcriptional activators or repressors depending on the promoter context [Yarmus et al., 2006].

Pim-1 was also found to affect RUNX3 activity in the K562 cell line. In the presence of PMA, both K562-RUNX3 and K562-Pim-1 cell lines exhibited distinctly higher G1 phase and G2 phase peaks, respectively. However, the K562-RUNX3-Pim-1 cell line behaved like the K562-Pim-1 cell line, indicating that *Pim-1* can overcome the growth-arrest imposed by *RUNX3* expression.

In conclusion, our findings show that the Runt domain of RUNX3 is phosphorylated by the oncogene Pim-1 and that this is associated with its stabilization and localization from the nucleus to the cytoplasm. Based on these observations and the observation that Pim-1 effectively suppressed the negative effect of RUNX3 on promoter transactivation and cell cycle progression, we propose the intriguing possibility that the function of *RUNX*s as an essential regulator of cell fate or as a tumor suppressor may depend on where it is localized. The increased stability of RUNX3 and localization of RUNX3 to the cytoplasm imposed by Pim-1 could result in the depletion of the nuclear factors associated with RUNX3 and turn *RUNX3*, as well as perhaps other members of the RUNX3 family, into dominant oncogenes.

MATERIALS AND METHODS

CELL LINES AND CELL CULTURE

All culture media and antibiotics were purchased from Invitrogen (CA). HEK 293 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 100 g/ml streptomycin, and K562 cells were maintained in RPMI 1640 medium with the same supplements. The cells were grown at 37°C in a humidified atmosphere of 5% CO_2. Stable K562 cell lines were established by electroporation with pBabe-3HA-Pim-1 or pBabe-3HA-RUNX3 plasmids and by puromycin selection (4 g/ml). To establish cell lines expressing both *RUNX3* and *Pim-1 or RUNX3 and Pim-1-K67R*, K562-Pim-1 or Pim-1-K67R cells were electrophorated with the pcDNA3.1-3Myc-RUNX3 plasmid and selected with hygromycin (200 g/ml). Exogenous expression of *RUNX3* and *Pim-1* was confirmed by Western blotting.

PLASMIDS AND ANTIBODIES

The *RUNX3* full-length type I isoform with the MRIPV N-terminal sequence (Q13761-1) and its deletion mutants were tagged with Myc, FLAG or HA, in the CMV promoter-derived mammalian expression vector (pCS4-3Myc, pCS4-3FLAG and pCS4-3HA, respectively). Deletion constructs of *RUNX3* and *Pim-1* were prepared by PCR before cloning into the pCS4 plasmids. The pGL3-p21 luciferase reporter plasmid was described by Chi et al. [2005]. The pGL3-TRE reporter plasmid containing the TRE was described by Lin and Stavnezer [1992] and Lee et al. [2000]. M-CSF-R-Luc reporter plasmid was kindly provided by Dr. Dong-ER Zhang (Scripps Research Institute, USA). pBabe (puromycin) plasmid was used to clone *RUNX3* or *Pim-1*. pCDNA3.1/hygro (Invitrogen) was used to clone *RUNX3* (3Myc tags). Antibodies against Myc (9E10; Santa Cruz Biotechnology, CA), HA (12CA5; Roche Applied Science, Mannheim, Germany), and FLAG (M2; Sigma, MO) were used.

YEAST TWO-HYBRID SCREEN

Yeast two-hybrid screening was performed using the DupLEX-A™ Yeast Two-Hybrid System, according to the method in the Yeast Protocols Handbook (OriGene, Rockville, MD). The pEG202-RUNX3-187 bait plasmid was prepared by PCR amplification of *RUNX3* region, corresponding to amino acids 1–187 of the type I isoform, followed by subcloning of the amplified fragment into the pEG202 plasmid. Yeast strain EGY48 cells were transformed with pEG202-RUNX3-187 and pSH18-34 (reporter gene plasmid) by the lithium acetate method. The transformants were selected by growth on SD/-His/-Ura media and the expression of RUNX3-187 was detected by Western blotting with an anti-LEX antibody. EGY48 yeast cells expressing RUNX3-187 were transformed with a human fetal liver cDNA library. The transformants were selected by growth on SD/-His/-Ura/-Trp media, and then colonies were isolated and tested on X-gal plates for protein-protein interactions.

DNA TRANSFECTION AND REPORTER ASSAY

Transient transfection was performed using the calcium phosphate method [Chen and Okayama, 1988]. For luciferase assays, 293 cells were plated into 96-well plates 1 day before transfection. At 36 h posttransfection, the cell lysates were analyzed for luciferase activity with a luciferase reporter assay kit (Promega, WI). The pCMV-galactosidase plasmid was included as an internal control for transfection efficiency.

IMMUNOFLUORESCENCE STUDIES

Cells grown on 12-mm cover slips (Fisher Scientific, PA) were transfected and grown as described in above, washed with phosphate-buffered saline, fixed with 4% formaldehyde for 30 min at room temperature, and then blocked with 10% fetal bovine serum. Cells were stained with an anti-HA monoclonal antibody diluted 1:200 to detect HA-RUNX3, and with an anti-FLAG polyclonal antibody diluted 1:2,000 to detect FLAG-tagged Pim-1

and Pim-1-K67R. Cells were incubated with primary antibody for 1 h, washed with phosphate-buffered saline, and incubated for 30 min with a rhodamine-conjugated secondary antibody. After mounting, cells were analyzed with a laser-scanning confocal microscope (Leica).

FLUORESCENCE ACTIVATED CELL SORTING (FACS)

The K562-RUNX3, K562-Pim-1, K562-RUNX3-Pim-1 and K562-RUNX3-Pim-1-K67R stable cell lines were cultured with or without 10 nM 4-Phorbol 12-Myristate 13-Acetate (PMA) for 48 h, stained with propidium iodide (PI), and analyzed by FACS.

IN VITRO KINASE ASSAY

Two hundred ninety-three cells were transfected as indicated and lysed with ice-cold lysis buffer. The resulting supernatants were immunoprecipitated with an anti-HA (RUNX3) antibody, and then incubated with 10 Ci of [-^{32}P]ATP in 30 l of kinase buffer (25 mM HEPES [pH 7.5], 150 mM NaCl, 10 M $MgCl_2$, 2 mM $MnCl_2$) for 30 min at 22°C. Half of each reaction was loaded onto an SDS–PAGE gel (8% polyacrylamide) and evaluated by autoradiography.

PHOSPHATASE TREATMENT OF CELL EXTRACTS

Dephosphorylation of RUNX3 was performed by incubating whole cell extracts with 1 unit of protein phosphatase 1 (PP1) and 400 units of protein phosphatase (PPase) (NEB, MA, USA) at 30°C for 1 h. The reactions were stopped by boiling in SDS sample buffer, followed by SDS–PAGE and Western blotting with an anti-FLAG (RUNX3) antibody.

IDENTIFICATION OF PHOSPHORYLATION SITES BY LC–MS/MS

HEK293 cells transfected with expression plasmids for *Flag-RUNX3* and *Pim-1* by using Lipofectamine Plus reagent (Invitrogen) were harvested after 36 hrs. Flag-tagged RUNX3 proteins were purified by gel filtration (Superdex-200 FPLC) chromatography followed by pull-down with G-Sepharose-anti-Flag antibody beads. The Sephrose beads were washed extensively with MLB buffer and the bounded proteins were separated by 10% SDS–PAGE, and the corresponding RUNX3 band was divided into slices of 1 mm width. In gel, tryptic digests from the entire lane were analyzed by tandem mass spectrometry (MS/MS) using an LTQ linear ion trap mass spectrometer (Thermo Electron). Assignment of MS/MS data was performed using SEQUEST software (Thermo Electron). Resultant matches were entered and compiled into a MySQL database, and proteomics computational analyses were performed using a Hypertext Preprocessor (PHP)-based program. First, peptide identifications were made based on the following criteria: cross-correlation score >1.5, 2.0, and 2.5 for charge states $+1$, $+2$, and $+3$, respectively; delta correlation >0.1; primary score >200; ranking of the primary score <3; and percent fragment ions $>30\%$. Second, protein identifications were assigned when the following criteria were met: the unique peptide match number was greater than or equal to 2, the peptides contributing to protein matches were derived from a single gel slice or from adjacent slices, and the protein was identified in at least two vitreous samples.

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