



Title	Leukemogenic kinase FIP1L1-PDGFR α and a small ubiquitin-like modifier E3 ligase, PIAS1, form a positive cross-talk through their enzymatic activities
Author(s)	Ibata, Makoto; Iwasaki, Junko; Fujioka, Yoichiro; Nakagawa, Koji; Darmanin, Stephanie; Onozawa, Masahiro; Hashimoto, Daigo; Ohba, Yusuke; Hatakeyama, Shigetsugu; Teshima, Takanori; Kondo, Takeshi
Citation	Cancer Science, 108(2), 200-207 https://doi.org/10.1111/cas.13129
Issue Date	2017-02
Doc URL	http://hdl.handle.net/2115/65802
Rights(URL)	http://creativecommons.org/licenses/by-nc/4.0/
Type	article
File Information	Ibata_et_al-2017-Cancer_Science.pdf



[Instructions for use](#)

Leukemogenic kinase FIP1L1-PDGFR α and a small ubiquitin-like modifier E3 ligase, PIAS1, form a positive cross-talk through their enzymatic activities

Makoto Ibata,^{1,6} Junko Iwasaki,^{1,6} Yoichiro Fujioka,² Koji Nakagawa,³ Stephanie Darmanin,⁴ Masahiro Onozawa,¹ Daigo Hashimoto,¹ Yusuke Ohba,² Shigetsugu Hatakeyama,⁵ Takanori Teshima¹ and Takeshi Kondo¹

Departments of ¹Hematology; ²Cell Physiology, Hokkaido University Graduate School of Medicine, Sapporo; ³Laboratory of Pathophysiology and Therapeutics, Hokkaido University Faculty of Pharmaceutical Sciences, Sapporo, Japan; ⁴Center for Hematology and Regenerative Medicine, Department of Medicine, Karolinska University Hospital, Huddinge, Sweden; ⁵Department of Biochemistry, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Key words

FIP1L1-PDGFR α , leukemogenesis, phosphorylation, PIAS1, sumoylation

Correspondence

Takeshi Kondo, Department of Hematology, Hokkaido University Graduate School of Medicine, Kita 15, Nishi 7, Kita-ku, Sapporo, Hokkaido 060-8638, Japan.
Tel: +81-11-706-7214; Fax: +81-11-706-7823;
E-mail: t-kondoh@med.hokudai.ac.jp

[†]These authors contributed equally to this work.

Funding Information

Japan Society for the Promotion of Science (Kakenhi grant nos. 25461404 [to T.K.] and 26890001 [to M.O.]) and by a research fund from the North Japan Hematology Study Group.

Received August 31, 2016; Revised November 21, 2016; Accepted November 30, 2016

Cancer Sci 108 (2017) 200–207

doi: 10.1111/cas.13129

Fusion tyrosine kinases play a crucial role in the development of hematological malignancies. FIP1L1-PDGFR α is a leukemogenic fusion kinase that causes chronic eosinophilic leukemia. As a constitutively active kinase, FIP1L1-PDGFR α stimulates downstream signaling molecules, leading to cellular proliferation and the generation of an anti-apoptotic state. Contribution of the N-terminal FIP1L1 portion is necessary for FIP1L1-PDGFR α to exert its full transforming activity, but the underlying mechanisms have not been fully characterized. We identified PIAS1 as a FIP1L1-PDGFR α association molecule by yeast two-hybrid screening. Our analyses indicate that the FIP1L1 portion of FIP1L1-PDGFR α is required for efficient association with PIAS1. As a consequence of the association, FIP1L1-PDGFR α phosphorylates PIAS1. Moreover, the kinase activity of FIP1L1-PDGFR α stabilizes PIAS1. Therefore, PIAS1 is one of the downstream targets of FIP1L1-PDGFR α . Moreover, we found that PIAS1, as a SUMO E3 ligase, sumoylates and stabilizes FIP1L1-PDGFR α . In addition, suppression of PIAS1 activity by a knockdown experiment resulted in destabilization of FIP1L1-PDGFR α . Therefore, FIP1L1-PDGFR α and PIAS1 form a positive cross-talk through their enzymatic activities. Suppression of sumoylation by ginkgolic acid, a small molecule compound inhibiting a SUMO E1-activating enzyme, also destabilizes FIP1L1-PDGFR α , and while the tyrosine kinase inhibitor imatinib suppresses FIP1L1-PDGFR α -dependent cell growth, ginkgolic acid or siRNA of PIAS1 has a synergistic effect with imatinib. In conclusion, our results suggest that sumoylation by PIAS1 is a potential target in the treatment of FIP1L1-PDGFR α -positive chronic eosinophilic leukemia.

Post-translational modifications are intrinsic for numerous cellular processes. One such post-translational modification is sumoylation, through which the small ubiquitin-like modifier (SUMO) protein is covalently attached to lysine residues in target proteins. Sumoylation regulates the functional roles of target proteins, such as subcellular localization, protein stability, protein–protein interactions, and activities of transcriptional factors. Similar to the ubiquitin system, SUMO attachment to a substrate passes through three enzymatic steps: catalysis by a SUMO E1-activating enzyme, a SUMO E2-conjugating enzyme, and a SUMO E3 ligase. A SUMO E3 ligase mediates an E2 enzyme and specific substrates, and it facilitates SUMO transfer.^(1,2) One of the representative E3 enzymes is protein inhibitor of activated signal transducer and activator of transcription (STAT)1 (PIAS1), which was initially isolated as a molecule that interacts with activated STAT1 and inhibits STAT1-mediated gene activation.⁽³⁾ Although PIAS1 regulates many transcriptional factors associated with cytokine signaling, PIAS1 also controls molecules that play crucial roles in cell proliferation and oncogenesis.⁽⁴⁾

Another post-transcriptional modification is phosphorylation. Many tyrosine kinases are stimulated by growth factors, and the activation of tyrosine kinases leads to cell proliferation. In addition, these kinases are closely associated with cancer development.⁽⁵⁾ The fusion tyrosine kinase FIP1L1-PDGFR α was identified from patients with idiopathic hypereosinophilic syndrome.^(6,7) This fusion gene has been observed in 10–20% of patients with eosinophilia and, therefore, eosinophilia with FIP1L1-PDGFR α is now diagnosed as chronic eosinophilic leukemia (CEL) according to the WHO disease classification.^(8–13) This fusion kinase is constitutively active and its kinase activity is essential for cellular transformation.^(6,7,14–16) As proliferation of CEL cells is dependent on the kinase activity of FIP1L1-PDGFR α , imatinib, which was originally developed for treatment of CML but also inhibits the kinase activity of PDGFR α , is also effective for patients with CEL.^(6,8,9,11,12)

As a leukemogenic fusion kinase, FIP1L1-PDGFR α stimulates downstream effectors. Some effector molecules, including phosphatidylinositol 3-kinase, ERK1/2, JNK, p38 MAPK, JAK2, STAT5, protein kinase B (PKB/c-akt), and

Src-homology 2 domain-containing phosphatase 2, have been identified in the context of leukemic transformation.^(15,17–20) Although the C-terminal kinase portion of FIP1L1-PDGFR is essential for activation of downstream substrates, the N-terminal FIP1L1 portion also plays a crucial role in cellular transformation. The FIP1L1 portion is necessary for the transforming activity of human primary hematopoietic progenitor cells in which the FIP1L1 portion is indispensable for activation of STAT5 and PKB/c-akt.⁽¹⁵⁾ In addition, full-length FIP1L1-PDGFR accumulates in the nucleus and has a higher proliferating activity than that of the C-terminal PDGFR portion of FIP1L1-PDGFR.⁽¹⁶⁾ Based on these reports, it is thought that the FIP1L1 portion directs FIP1L1-PDGFR into the nucleus and plays a crucial role in the development of CEL. However, little is known about the transforming pathway mediated by the FIP1L1 portion.

We have therefore tried to characterize a molecule interacting with FIP1L1-PDGFR to elucidate the leukemogenic role of the FIP1L1 portion, and we isolated PIAS1 as a FIP1L1-PDGFR association molecule. Our data show that there is a positive cross-talk between FIP1L1-PDGFR and PIAS1. FIP1L1-PDGFR phosphorylates and stabilizes PIAS1. PIAS1 sumoylates and stabilizes FIP1L1-PDGFR. The reciprocally positive interaction between FIP1L1-PDGFR and PIAS1 through enzymatic activities could be crucial for the transforming activity of FIP1L1-PDGFR. Moreover, the sumoylation system by PIAS1 could be a potential target in the treatment of FIP1L1-PDGFR-positive CEL.

Materials and Methods

Plasmid construction. Flag-tagged or T7-tagged expression vectors of full-length FIP1L1-PDGFR (FIP1L1-PDGFR-FL), a kinase-dead mutant of FIP1L1-PDGFR (FIP1L1-PDGFR-KD), and a deletion mutant with only the C-terminal portion of PDGFR (PDGFR-C) have been described previously. These vectors are named pFLAG-FIP1L1-PDGFR-FL, pFLAG-FIP1L1-PDGFR-KD, pFLAG-PDGFR-C, pCGT-FIP1L1-PDGFR-FL, pCGT-FIP1L1-PDGFR-KD, and pCGT-PDGFR-C, respectively. For yeast two-hybrid screening, full-length FIP1L1-PDGFR cDNA was cloned into pBTM116 (Clontech, Mountain View, CA, USA) and named pBTM116-FIP1L1-PDGFR-FL. Full-length human PIAS1 cDNA was amplified by PCR from a HeLa cDNA library. A 6×Myc-tagged expression vector of PIAS1 was generated by inserting human PIAS1 cDNA into a pCI-neo-6×Myc vector that had been generated by inserting a fragment containing six copies of the Myc epitope into pCI-neo (Promega, Madison, WI, USA), and the vector was named pCI-6×Myc-PIAS1. A 6×Myc-tagged expression vector of a PIAS1 mutant lacking SUMO-E3 ligase activity⁽²¹⁾ was generated by introducing a cysteine-to-serine mutation at amino acid position 351 of PIAS1, by means of site-directed mutagenesis, and the vector was named pCI-6×Myc-PIAS1-C351S. The 6×Myc-tagged PIAS1 was amplified by PCR and cloned into the pTRE3G-ZsGreen1 (Clontech) vector for a tetracycline-inducible experiment, and it was named pTRE3G-6×Myc-PIAS1. A T7-tagged expression vector of SUMO-1, pCGT-T7-SUMO-1, was previously described.⁽²²⁾ For constructing retroviral vectors, FLAG-tagged FIP1L1-PDGFR-FL or FIP1L1-PDGFR-KD cDNA was amplified by PCR and cloned into pDON-5 Neo (TaKaRa, Kusatsu, Shiga, Japan), and these vectors were named pDON-FLAG-FIP1L1-PDGFR-FL and pDON-FLAG-FIP1L1-PDGFR-KD, respectively. FIP1L1-PDGFR-T67II is an imatinib-resistant mutant that was generated by replacing

671-threonine with isoleucine by means of site-directed mutagenesis.⁽⁶⁾

Two-hybrid screening. To screen for molecules that associate with FIP1L1-PDGFR, we transfected yeast strain L40 stably expressing pBTM116-FIP1L1-PDGFR-FL with a murine B cell lymphoma Matchmaker cDNA library in pACT (Clontech) by the lithium acetate method. The cells were cultured on plates of a medium lacking tryptophan, leucine, and histidine, and positive clones were obtained. Then DNA fragments from the positive clones were subjected to DNA sequence analysis.

Cell lines, transfection experiments, retroviral infection, and drug treatment. HEK293 cells were cultured in DMEM supplemented with 10% FBS. BAF-B03 cells were obtained from Dr. Masao Seto (Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya, Japan) and cultured in RPMI-1640 containing 10% FBS and 1 ng/mL murine interleukin-3 (IL-3) (Medical and Biological Laboratories, Nagoya, Japan). For transient transfection experiments, the indicated expression vectors were transfected into HEK293 cells in a 6-cm dish by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and then cultured for 36–48 h and subsequently subjected to analysis. The amount of the transfected vector was determined by adjusting the expression level of the product. A tetracycline-inducible system (Clontech) was used to analyze the stability of PIAS1. pTRE3G-Myc-PIAS1 and pCMV-Tet3G were cotransfected into HEK293 cells with either pFLAG-FIP1L1-PDGFR-FL or pFLAG-FIP1L1-PDGFR-KD. After 4 h, the cells were divided into four culture dishes and cultured with fresh media. Cells in one dish were cultured without doxycycline, and cells in the other three dishes were cultured with 1 µg/mL doxycycline. After 24 h of incubation, the culture media were replaced with fresh media without doxycycline, and this point was set as the starting time. The cells were then harvested after 24 h and the cell lysates were subjected to immunoblotting. To establish an HEK293-derived stable cell line expressing FIP1L1-PDGFR, HEK293 cells were transfected with pFLAG-FIP1L1-PDGFR-FL. After 2 days of transfection, the cells were selected with 500 µg/mL G418 (Sigma, St. Louis, MO, USA). The established cell line, HEK293-FIP1L1-PDGFR-FL, was used for a knockdown experiment. For RNA interference, siRNAs for human PIAS1 (Stealth RNAi VHS41400 and VHS41401) and for murine PIAS1 (Stealth RNAi MSS244277 and MSS285778) and a negative control (#12935-200) were purchased from Invitrogen. To establish BAF-B03-derived stable cell lines expressing FIP1L1-PDGFR and its mutants, we used the retrovirus packaging kit Eco (TaKaRa). BAF-B03 cells were infected with pDON-FLAG-FIP1L1-PDGFR-FL or each mutant of FIP1L1-PDGFR, and the cells were selected with 500 µg/mL G418. Ginkgolic acid was purchased from Calbiochem (San Diego, CA, USA) and used for an experiment to inhibit sumoylation. Imatinib was a kind gift from Novartis and was used to inhibit the kinase activity of FIP1L1-PDGFR.

Immunoprecipitation, immunoblotting, and immunostaining. Anti-FLAG M2 antibody and anti-β-actin antibody (AC-15) were purchased from Sigma, anti-T7 tag antibody (PM022) and anti-Myc antibody (PL14) were from Medical and Biological Laboratories, anti-T7 tag antibody was from Novartis (Basel, Switzerland), anti-phosphotyrosine antibody (PY-20) was from Beckman Coulter (Fullerton, CA, USA), anti-PDGFR antibody (#3164) was from Cell Signaling (Danvers, MA, USA), and anti-PIAS1 antibodies (ab32219 and ab77231) were from Abcam (Cambridge, UK). For immunoblotting, the cells were lysed in RIPA buffer (50 mM

Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate) supplemented with 10 mM *N*-ethylmaleimide, 5 μ g/mL aprotinin, 5 μ g/mL leupeptin, 1 mM NaF, and 0.5 mM Na₃VO₄. Immunoprecipitation and immunoblotting were carried out as previously described.⁽²³⁾

Briefly, whole cell lysates were immunoprecipitated with the indicated antibody, and the immunoprecipitates were washed with RIPA buffer. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblot signals were detected by ECL Prime Western blotting detection reagent and ImageQuant LAS4000 mini system (GE Healthcare, Buckinghamshire, UK), and the band intensity was quantified using ImageQuant TL software (GE Healthcare).

For immunostaining, HEK293 cells were transfected with pCGT-FIP1L1-PDGFR α -FL or pCGT-PDGFR α -C. After 2 days, the cells were fixed with 3.7% formaldehyde and incubated with anti-PIAS1 antibody (ab32219) and anti-T7 antibody (Novagen) as primary antibodies and then incubated with Alexa Fluor 488 anti-mouse antibody and Alexa Fluor 594 anti-rabbit antibody (Life Technologies, Palo Alto, CA, USA). For DNA staining, fixed cells were stained with DAPI. Fluorescent images were acquired with an FV-10i confocal microscope (Olympus, Tokyo, Japan) and analyzed with Metamorph software (Universal Imaging, Downingtown, PA, USA).

Apoptosis assay. BAF-derived cells were treated with imatinib and/or ginkgolic acid at the indicated concentrations for 24 h. Induction of apoptosis was quantitated using the MEB-CYTO Apoptosis Kit (Medical and Biological Laboratories). Briefly, the cells (2×10^5) were collected, washed with PBS, and suspended in 90 μ L binding buffer (containing 10 μ L

annexin V-FITC and 1 μ L of 100 μ g/mL DAPI). The samples were incubated in the dark for 15 min at room temperature and then analyzed by FACSCanto II (Beckton Dickinson, Franklin Lakes, NJ, USA) after addition of 400 μ L binding buffer.

Results

FIP1L1-PDGFR α associates with PIAS1. To identify an intracellular protein that interacts with FIP1L1-PDGFR α , yeast two-hybrid screening was initially carried out, and 18 colonies were obtained from 3×10^6 library transformants. One of them was found to encode murine PIAS1. First, we examined whether PIAS1 could associate with FIP1L1-PDGFR α in mammalian cells. We transfected the FLAG-tagged expression vector of FIP1L1-PDGFR α -FL or PDGFR α -C into HEK293 cells. As shown in Figure 1(a), FIP1L1-PDGFR α -FL associated with a limited amount of endogenous PIAS1, with less than 1% of input PIAS1 being co-immunoprecipitated with FIP1L1-PDGFR α -FL. PDGFR α -C also associated with PIAS1, but the amount of PIAS1 associated with PDGFR α -C was much less than that with FIP1L1-PDGFR α -FL. These results suggest that the FIP1L1 portion is required for efficient association between FIP1L1-PDGFR α and PIAS1. Therefore, we examined the intracellular localization of FIP1L1-PDGFR α and PIAS1 by using confocal microscopy, as previous studies showed that PIAS1 is a nuclear protein and that FIP1L1-PDGFR α accumulates in the nucleus.^(16,21) FIP1L1-PDGFR α -FL efficiently colocalized with PIAS1 in the nucleus, whereas PDGFR α -C predominantly localized in the cytoplasm

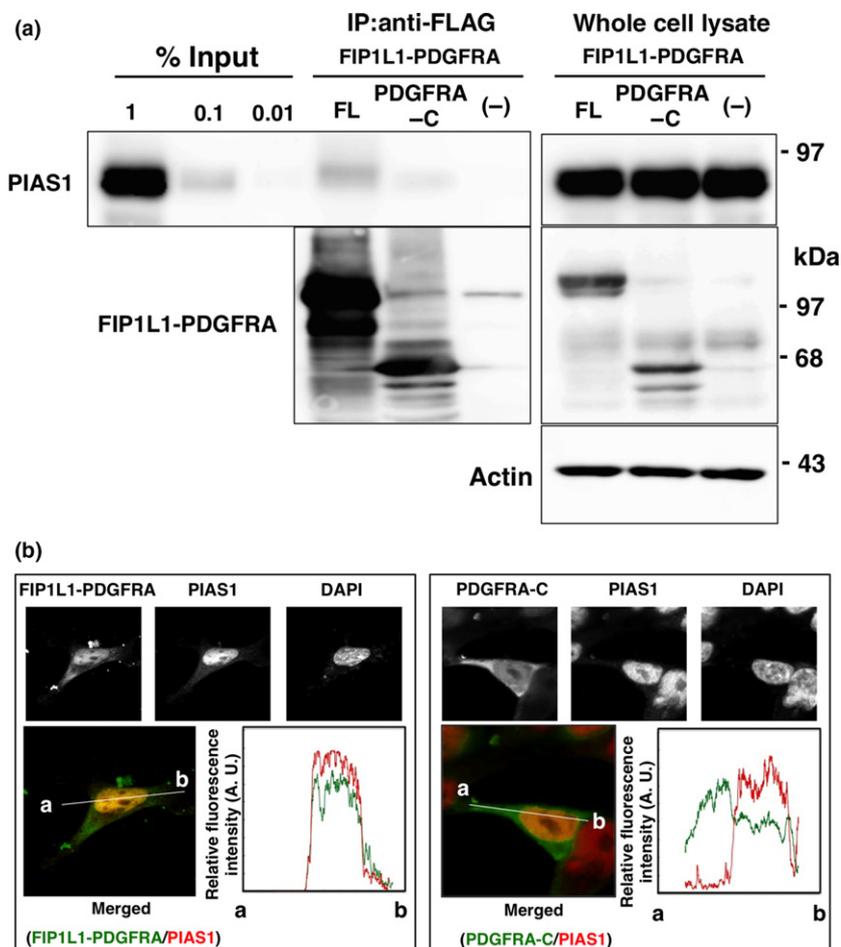


Fig. 1. Leukemogenic kinase FIP1L1-PDGFR α associates with small ubiquitin-like modifier E3 ligase PIAS1 in the nucleus. (a) FIP1L1-PDGFR α associates with PIAS1. HEK293 cells were transfected with a control vector, pFLAG-FIP1L1-PDGFR α -FL or pFLAG-PDGFR α -C. The association between PIAS1 and FLAG-FIP1L1-PDGFR α -FL or FLAG-PDGFR α -C was analyzed by immunoprecipitation (IP) with anti-FLAG M2 antibody and immunoblotting with anti-PIAS1 antibody. Immunoblotting of whole cell lysates with anti-PIAS1 antibody and anti-PDGFR α antibody confirmed the expression. The amounts of transfected vectors were 3 μ g control vector or pFLAG-PDGFR α -C and 1 μ g pFLAG-FIP1L1-PDGFR α -FL. (b) FIP1L1-PDGFR α colocalizes with PIAS1 in the nucleus. HEK293 cells were transfected with 2 μ g pCGT-FIP1L1-PDGFR α -FL (left panel) or pCGT-PDGFR α -C (right panel). The cells were fixed and immunostained with anti-T7 antibody (Alexa Fluor 488, green) and anti-PIAS1 antibody (Alexa Fluor 594, red). The nucleus was simultaneously visualized by DAPI. Fluorescence intensities of Alexa Fluor 488 and Alexa Fluor 594 along the line (a-b) were plotted.

(Fig. 1b). These results suggest that FIP1L1-PDGFR α associated with PIAS1 through the PDGFR α portion but that the FIP1L1 portion is necessary for efficient association with PIAS1 because of the nuclear accumulation of FIP1L1-PDGFR α directed by the FIP1L1 portion.

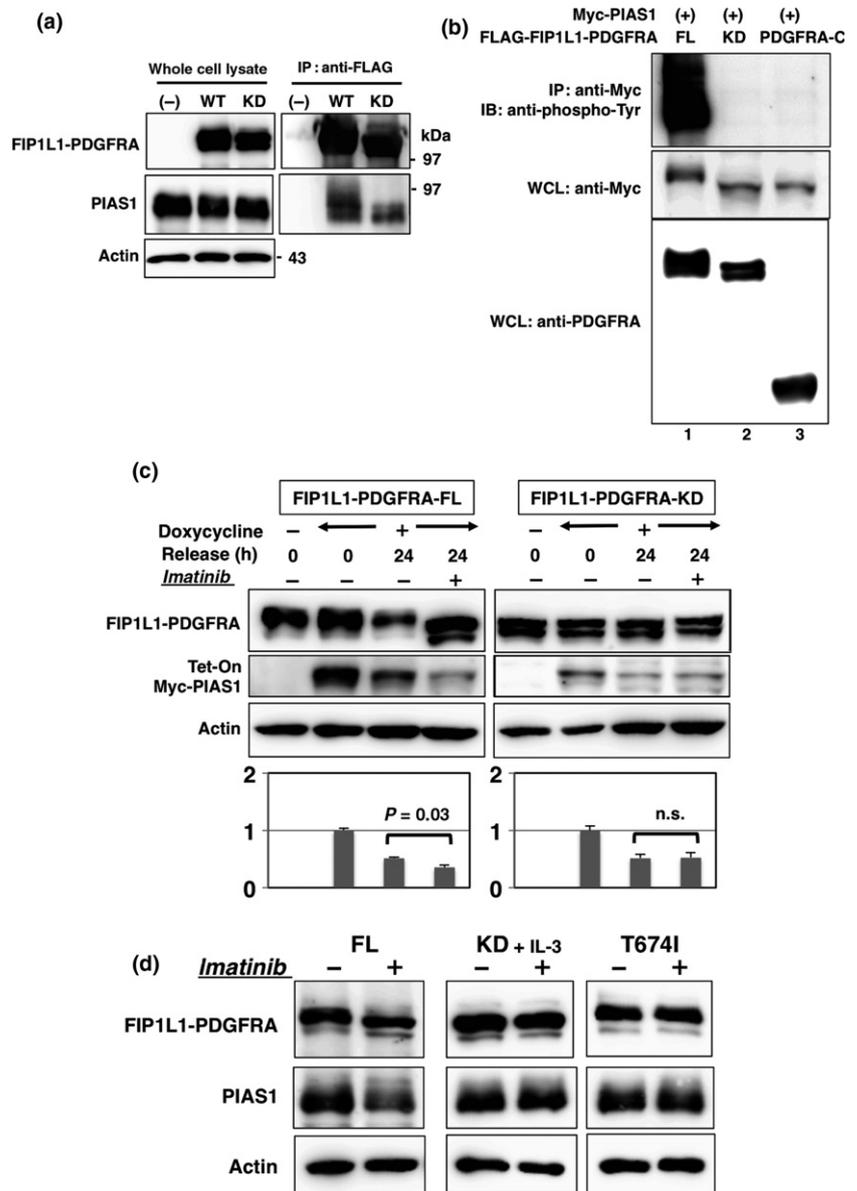
FIP1L1-PDGFR α phosphorylates PIAS1 on tyrosine residues and increases the stability of PIAS1. Immunoblotting of PIAS1 associated with FIP1L1-PDGFR α -FL resulted in slow migration of PIAS1 (Fig. 1a). Therefore, we next examined whether kinase activity of FIP1L1-PDGFR α is required for association between FIP1L1-PDGFR α and PIAS1 and whether FIP1L1-PDGFR α phosphorylates PIAS1. As shown in Figure 2(a), both FIP1L1-PDGFR α -FL and FIP1L1-PDGFR α -KD associated with PIAS1, and PIAS1 that associated with FIP1L1-PDGFR α -FL migrated more slowly than PIAS1 that associated with FIP1L1-PDGFR α -KD. These results raise the possibility that FIP1L1-PDGFR α phosphorylates PIAS1 on tyrosine residues.

To examine this possibility, Myc-tagged PIAS1 was coexpressed with FIP1L1-PDGFR α or its mutants in HEK293 cells,

and phosphorylation of PIAS1 on tyrosine residues was analyzed using an anti-phosphotyrosine antibody. As a result, PIAS1 was phosphorylated on tyrosine residues by FIP1L1-PDGFR α -FL but not by FIP1L1-PDGFR α -KD or PDGFR α -C (Fig. 2b). Although PDGFR α -C is kinase-active and weakly associated with PIAS1 (Fig. 1a), tyrosine phosphorylation of PIAS1 was not detected (Fig. 2b, lane 3). This result suggests that the FIP1L1 portion is required not only for efficient association between FIP1L1-PDGFR α and PIAS1 but also for tyrosine phosphorylation of PIAS1 by FIP1L1-PDGFR α .

While examining the association between FIP1L1-PDGFR α and PIAS1, we noticed that the amount of PIAS1 associated with FIP1L1-PDGFR α was greater in cells expressing FIP1L1-PDGFR α -FL than in cells expressing FIP1L1-PDGFR α -KD. Moreover, transient expression experiments, in which expression vectors of FIP1L1-PDGFR α and PIAS1 were transfected, showed that the expression level of PIAS1 tended to be higher in cells cotransfected with FIP1L1-PDGFR α -FL than in cells cotransfected with FIP1L1-PDGFR α -KD. These results indicate the possibility that

Fig. 2. Leukemogenic kinase FIP1L1-PDGFR α phosphorylates and stabilizes small ubiquitin-like modifier E3 ligase PIAS1. (a) PIAS1 that associated with kinase-active FIP1L1-PDGFR α slowly migrated by SDS-PAGE. HEK293 cells were transfected with a control vector, pFLAG-FIP1L1-PDGFR α -FL, or pFLAG-FIP1L1-PDGFR α -KD, followed by immunoprecipitation and immunoblotting. The amounts of the transfected vectors were 3 μ g control vector or pFLAG-FIP1L1-PDGFR α -KD and 1 μ g pFLAG-FIP1L1-PDGFR α -FL. (b) FIP1L1-PDGFR α phosphorylates PIAS1 on tyrosine residues. pCI-6xMyc-PIAS1 was transfected into HEK293 cells together with pFLAG-FIP1L1-PDGFR α -FL, pFLAG-FIP1L1-PDGFR α -KD, or pFLAG-PDGFR α -C. The tyrosine phosphorylation in immunoprecipitated PIAS1 was examined using an anti-phosphotyrosine antibody. Immunoblotting of whole cell lysates (WCL) with anti-Myc antibody and anti-PDGFR α antibody confirmed the expression of Myc-PIAS1, FLAG-FIP1L1-PDGFR α , and its derivatives. The amounts of transfected vectors were as follows: 1 μ g pCI-6xMyc-PIAS1 was cotransfected with 1 μ g pFLAG-FIP1L1-PDGFR α -FL; and 5 μ g pCI-6xMyc-PIAS1 was cotransfected with 5 μ g pFLAG-FIP1L1-PDGFR α -KD or pFLAG-PDGFR α -C. (c) FIP1L1-PDGFR α stabilized PIAS1 through kinase activity. The effect of FIP1L1-PDGFR α on the stability of PIAS1 was analyzed using a tetracycline-inducible system. After induction of Myc-tagged PIAS1 by doxycycline, exposure of the cells to doxycycline was stopped. FIP1L1-PDGFR α -FL (left panel) or FIP1L1-PDGFR α -KD (lower panel) was coexpressed and the stability of induced PIAS1 was examined in the presence or absence of 100 nM imatinib. For this purpose, the expression level of Myc-tagged PIAS1 just after induction (time 0 h, doxycycline [+]) was arbitrarily assigned to be 1.0 and the results are shown as means \pm SE. The expression levels of Myc-tagged PIAS1 were quantitated and statistically compared by the t-test. Analysis was carried out in triplicate assays and the results were reproducible. n.s., not significant. (d) PIAS1 decreased after imatinib treatment in BAF-PDGFR α -FL cells. BAF-PDGFR α -FL, BAF-PDGFR α -KD, and BAF-PDGFR α -T674I cells were treated with 50 nM imatinib for 20 h, and the expression levels of PIAS1 were examined by immunoblotting.



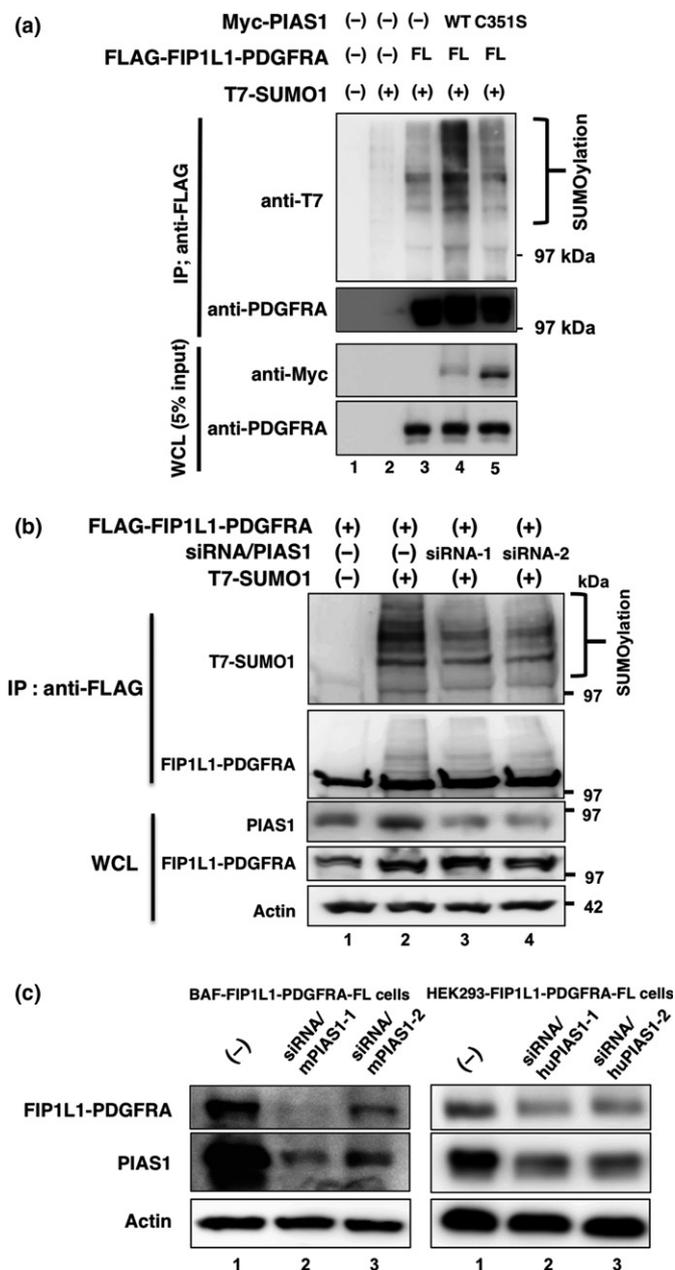


Fig. 3. Small ubiquitin-like modifier E3 ligase PIAS1 sumoylates and stabilizes leukemogenic kinase FIP1L1-PDGFR. (a) FIP1L1-PDGFR is sumoylated by PIAS1. HEK293 cells were transfected with a combination of pCI-6xMyc-PIAS1, pFLAG-FIP1L1-PDGFR-FL, and pCGT-SUMO1. The total amount of transfected vectors was 6 μ g, with 2 μ g each vector used and empty vector used as a mock. FLAG-FIP1L1-PDGFR was detected by anti-PDGFR antibody and Myc-PIAS1 was detected by anti-FLAG M2 antibody. FIP1L1-PDGFR was immunoprecipitated with anti-FLAG M2 antibody and subsequently analyzed by immunoblotting. Sumoylation of FIP1L1-PDGFR was detected by anti-T7 antibody. (b) Knockdown of PIAS1 by siRNA attenuated sumoylation of FIP1L1-PDGFR. HEK293 cells were transfected with pFLAG-FIP1L1-PDGFR-FL and/or pCGT-SUMO1 and/or human PIAS1-specific siRNA. Decreased expression of endogenous PIAS1 by siRNA was confirmed by anti-PIAS1 antibody. Decreased expression of PIAS1 was accompanied by attenuation of sumoylation of FIP1L1-PDGFR (lanes 3 and 4). (c) Knockdown of PIAS1 resulted in a decrease of FIP1L1-PDGFR. BAF-FIP1L1-PDGFR-FL cells were transfected with two different murine PIAS1-specific siRNAs or a negative control. HEK293-derived cells expressing FIP1L1-PDGFR were transfected with two different human PIAS1-specific siRNAs or a negative control. After 2 days, the expression levels of PIAS1 and FIP1L1-PDGFR were analyzed by immunoblotting with anti-PDGFR antibody and anti-PIAS1 antibody.

FIP1L1-PDGFR stabilizes PIAS1 through its kinase activity. To analyze the stability of PIAS1, we used a tetracycline-inducible expression system. After induction of PIAS1 by doxycycline, the culture medium was changed to a fresh medium without doxycycline in the presence or absence of imatinib, a tyrosine kinase inhibitor (Fig. 2c). The expression of PIAS1 was efficiently induced when FIP1L1-PDGFR-FL was coexpressed (Fig. 2c, left panel); however, the kinase activity was suppressed and the expression level of PIAS1 was rapidly decreased by the addition of imatinib. In addition, the expression level of PIAS1 was not affected by imatinib when PIAS1 was coexpressed with FIP1L1-PDGFR-KD (Fig. 2c, right panel). As this experiment was carried out by transient transfection, we next established cell lines stably expressing FIP1L1-PDGFR to analyze the functional relation between FIP1L1-PDGFR and PIAS1. We treated BAF-B03-derived stable cell lines, BAF-FIP1L1-PDGFR-FL, BAF-FIP1L1-PDGFR-KD, and BAF-FIP1L1-PDGFR-T674I, with imatinib (Fig. 2d). As previously described,^(14–16) parental BAF-B03 cells are IL-3-dependent pro-B cells, which become IL-3-independent following the introduction of a kinase-active FIP1L1-PDGFR. Thus, BAF-FIP1L1-PDGFR-FL cells and BAF-FIP1L1-PDGFR-T674I cells proliferate in the absence of IL-3. By treatment with imatinib, kinase activity of FIP1L1-PDGFR-FL was suppressed, resulting in a decrease of PIAS1 expression. In contrast, the expression level of PIAS1 in BAF-FIP1L1-PDGFR-KD cells, which were cultured in the presence of IL-3, was not affected by treatment with imatinib. Moreover, the expression level of PIAS1 in imatinib-resistant BAF-FIP1L1-PDGFR-T674I cells was also not changed by treatment with imatinib. Collectively, the results suggest that FIP1L1-PDGFR stabilizes PIAS1 through its kinase activity.

PIAS1 sumoylates and stabilizes FIP1L1-PDGFR. As PIAS1 is a SUMO E3 ligase, we next examined whether PIAS1 sumoylates FIP1L1-PDGFR. When PIAS1, FIP1L1-PDGFR, and SUMO1 expression vectors were cotransfected into HEK293 cells, FIP1L1-PDGFR was efficiently sumoylated (Fig. 3a). Enforced expression of PIAS1 enhanced sumoylation of FIP1L1-PDGFR (Fig. 3a, lane 4). This effect was not observed when ligase-mutant PIAS1-C351S was expressed instead of wild-type PIAS1 (Fig. 3a, lane 5). Sumoylation of FIP1L1-PDGFR was observed in transfected cells that did not express exogenous PIAS1 or expressed PIAS1-C351S (Fig. 3a, lanes 3 and 5). To examine the effect of endogenous PIAS1, we undertook a knockdown experiment. When the expression of PIAS1 was suppressed by PIAS1-specific siRNA, sumoylation of FIP1L1-PDGFR-FL decreased (Fig. 3b), indicating that PIAS1 acts as a SUMO E3 ligase of FIP1L1-PDGFR.

As one of the physiological roles of sumoylation is regulation of protein stability, we hypothesized that PIAS1 regulates the stability of FIP1L1-PDGFR. To prove this hypothesis, we inhibited the expression of PIAS1 in BAF-FIP1L1-PDGFR-FL cells by transfecting PIAS1-specific siRNA. As a consequence of the inhibition of PIAS1, the expression level of FIP1L1-PDGFR was decreased (Fig. 3c, left panel, lanes 2 and 3). Based on this result, the downregulation of FIP1L1-PDGFR may also affect the expression level of PIAS1 in BAF-FIP1L1-PDGFR-FL cells. Therefore, we also undertook the same experiment in an HEK293-derived stable cell line expressing FIP1L1-PDGFR, which manifests FIP1L1-PDGFR-independent growth. As was the case for BAF-FIP1L1-PDGFR-FL, the expression level of FIP1L1-

PDGFRA was decreased by knockdown of PIAS1 (Fig. 3c, right panel, lanes 2 and 3). These results support our notion that PIAS1 regulates the expression level of FIP1L1-PDGFR.

Collectively, the results suggest that PIAS1 sumoylates and stabilizes FIP1L1-PDGFR.

PIAS1 is a potential therapeutic target for CEL treatment. Our results suggest that sumoylation regulates the expression level of FIP1L1-PDGFR, and we therefore assumed that inhibition of sumoylation or PIAS1 activity is a potential target in the treatment of CEL. Recently, it has been reported that ginkgolic acid acts as an inhibitor of a SUMO E1-activating enzyme,⁽²⁴⁾ so we examined the effect of ginkgolic acid on FIP1L1-PDGFR expression. To analyze the effect of ginkgolic acid on FIP1L1-PDGFR-dependent cell growth, we treated BAF-FIP1L1-PDGFR-FL cells with different concentrations of ginkgolic acid and examined the expression levels of FIP1L1-PDGFR. Ginkgolic acid decreased the expression level of FIP1L1-PDGFR in both BAF-FIP1L1-PDGFR-FL cells and BAF-FIP1L1-PDGFR-KD cells (Fig. 4a). Treatment of BAF-FIP1L1-PDGFR-FL cells with 20 μ M ginkgolic acid alone

had a minimal effect in inducing apoptosis, whereas BAF-FIP1L1-PDGFR-FL cells underwent apoptosis following inhibition of FIP1L1-PDGFR kinase activity by imatinib. We then examined whether the combination of ginkgolic acid and imatinib had a synergistic effect to induce apoptosis in BAF-FIP1L1-PDGFR-FL cells. When BAF-FIP1L1-PDGFR-FL cells were treated with a combination of 20 nM imatinib and 20 μ M ginkgolic acid, ginkgolic acid augmented the effect of imatinib (Fig. 4b, left panel). This effect seemed to be mediated by suppression of the kinase activity of FIP1L1-PDGFR, because these compounds had little effect on BAF-FIP1L1-PDGFR-KD cells that manifest IL-3-dependent growth (Fig. 4b, right panel).

Moreover, we examined whether knockdown of PIAS1 augments the effect of imatinib on BAF-FIP1L1-PDGFR-FL cells. The expression of PIAS1 in BAF-FIP1L1-PDGFR-FL cells was inhibited by transfecting PIAS1-specific siRNA as described in the legend of Figure 3(c), and subsequently the cells were treated with imatinib. The knockdown of PIAS1 in the transfected cells was confirmed by immunoblotting (data

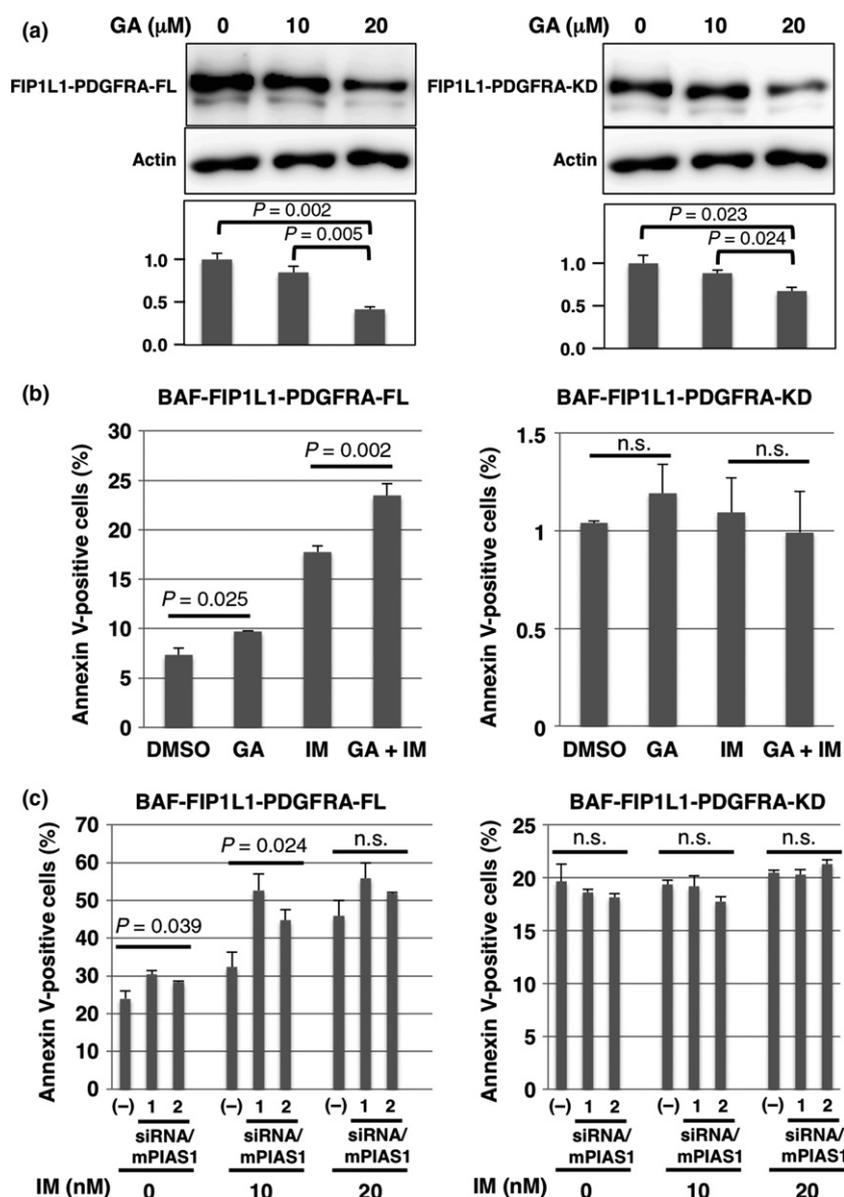


Fig. 4. Inhibition of sumoylation targets FIP1L1-PDGFR. (a) Ginkgolic acid (GA) decreased the expression level of FIP1L1-PDGFR in a dose-dependent manner. BAF-FIP1L1-PDGFR-FL cells and BAF-FIP1L1-PDGFR-KD cells were treated with the indicated concentrations of GA for 24 h. The expression levels of FIP1L1-PDGFR were examined by immunoblotting with anti-PDGFR antibody. The expression levels of FIP1L1-PDGFR were quantitated and statistically compared by the *t*-test. For this purpose, the expression level of FIP1L1-PDGFR treated with mock was arbitrarily assigned to be 1.0 and the results are shown as mean \pm SE. Analysis was carried out in triplicate assays and the results were reproducible. (b) GA and imatinib (IM) synergistically induced apoptosis in BAF-FIP1L1-PDGFR-FL cells. BAF-FIP1L1-PDGFR-FL cells (left panel) and BAF-FIP1L1-PDGFR-KD cells (right panel) were treated with 20 nM IM with or without 20 μ M GA for 24 h. Annexin V-positive cells were analyzed by flow cytometry and statistically compared by the *t*-test. Analysis was undertaken in triplicate assays and the results were reproducible. (c) Knockdown of PIAS1 sensitized BAF-FIP1L1-PDGFR-FL cells to imatinib. BAF-FIP1L1-PDGFR-FL cells (left panel) and BAF-FIP1L1-PDGFR-KD cells (right panel) were transfected with two different murine PIAS1-specific siRNAs or a negative control. After 2 days, the cells were treated with mock, 10 nM IM, or 20 nM IM. Annexin V-positive cells were analyzed by flow cytometry and statistically compared by one-factor ANOVA. Analysis was carried out in triplicate assays and the results were reproducible. n.s., not significant.

not shown). In the treatment with 20 nM imatinib, apoptosis was similarly induced in cells transfected with a negative control and cells transfected with PIAS1-specific siRNAs. However, in the treatment with 10 nM imatinib, induction of apoptosis was significantly greater in the cells transfected with PIAS1-specific siRNAs than in cells transfected with a negative control (Fig. 4c, left panel). There was no effect of PIAS1-specific siRNAs on induction of apoptosis in BAF-FIP1L1-PDGFR-FL cells (Fig. 4c, right panel). These results indicate that downregulation of PIAS1 sensitizes BAF-FIP1L1-PDGFR-FL cells to a low concentration of imatinib.

Taken together, the results indicate that the sumoylation system by PIAS1 regulates the expression level of FIP1L1-PDGFR and is a potential target for FIP1L1-PDGFR-positive CEL treatment.

Discussion

To understand the mechanisms by which FIP1L1-PDGFR exerts its transforming activity through the FIP1L1 portion, we identified PIAS1 as a FIP1L1-PDGFR associating molecule and showed a positive cross-talk between FIP1L1-PDGFR and PIAS1 for phosphorylation and sumoylation.

We found that PIAS1 associates with FIP1L1-PDGFR and that the FIP1L1 portion is necessary for efficient association. Some molecules have been reported to directly associate with FIP1L1-PDGFR. The lymphocyte adaptor protein Lnk binds to both PDGFR and FIP1L1-PDGFR and acts as a negative regulator of these molecules.⁽²⁵⁾ c-Cbl is phosphorylated by both PDGFR and FIP1L1-PDGFR, but it efficiently ubiquitinates and destabilizes only PDGFR.⁽²⁶⁾ The association of Lnk and c-Cbl with FIP1L1-PDGFR seems to be mediated by the PDGFR portion, as these molecules associate with the full length of PDGFR. However, efficient association between PIAS1 and FIP1L1-PDGFR required the FIP1L1 portion, because the FIP1L1 portion directs FIP1L1-PDGFR into the nucleus, where PIAS1 is localized. As a kinase, FIP1L1-PDGFR phosphorylated PIAS1 on tyrosine residues and this phosphorylation also required the FIP1L1 portion. Moreover, the kinase activity of FIP1L1-PDGFR stabilized PIAS1. It has been reported that the function of PIAS1 is regulated by the phosphorylation of serine residues.^(27,28) Our results suggest a novel mechanism of PIAS1 also being regulated by tyrosine phosphorylation. It has not yet been

determined whether stabilization of PIAS1 by FIP1L1-PDGFR is mediated by phosphorylation of PIAS1. Identification of tyrosine residues that are phosphorylated by FIP1L1-PDGFR is necessary for further characterization of the underlying mechanism for PIAS1 regulation.

The kinase activity of FIP1L1-PDGFR activates many downstream molecules by way of FIP1L1-dependent or -independent pathways. It has been reported that the FIP1L1 portion is necessary for activation of PKB/c-akt by FIP1L1-PDGFR and that PIAS1 sumoylates and activates PKB/c-akt.^(15,29) Our results suggest the presence of a potential signaling pathway by which PIAS1 can be upregulated by FIP1L1-PDGFR and subsequently activate PKB/c-akt.

Moreover, PIAS1 sumoylated FIP1L1-PDGFR and regulated its stability as a consequence of the association between FIP1L1-PDGFR and PIAS1. Although imatinib is highly effective against FIP1L1-PDGFR-positive CEL, drug resistance occasionally develops and relapse often occurs after discontinuation of imatinib treatment.^(6,12,30,31) Inhibition of sumoylation by siRNA of PIAS1 or treatment with ginkgolic acid destabilized FIP1L1-PDGFR. As a consequence, treatment of BAF-FIP1L1-PDGFR-FL cells with ginkgolic acid and siRNA of PIAS1 augmented the effect of imatinib. These results suggest that PIAS1-targeted therapy may be effective in treating FIP1L1-PDGFR-positive leukemia. Very recently, it has been reported that PIAS1 plays a crucial role in the maintenance of hematopoietic stem cells.⁽³²⁾ Based on our results, the positive cross-talk between FIP1L1-PDGFR and PIAS1 may be associated with maintenance of leukemia stem cells in FIP1L1-PDGFR-positive leukemia.

Acknowledgments

The authors thank Dr. M. Seto for providing BAF-B03 cells. The authors also acknowledge Ms. M. Yamane, Ms. M. Mayanagi, Ms. I. Sato, and Ms. R. Sekiguchi for technical assistance. This work was supported by Japan Society for the Promotion of Science (Kakenhi grant nos. 25461404 [to T.K.] and 26890001 [to M.O.]) and by a research fund from the North Japan Hematology Study Group.

Disclosure Statement

The authors have no conflict of interest.

References

- 1 Sarge KD, Park-Sarge OK. SUMO and its role in human diseases. *Int Rev Cell Mol Biol* 2011; **288**: 167–83.
- 2 Wilkinson KA, Henley JM. Mechanisms, regulation and consequences of protein SUMOylation. *Biochem J* 2010; **428**: 133–45.
- 3 Liu B, Liao J, Rao X *et al.* Inhibition of Stat1-mediated gene activation by PIAS1. *Proc Natl Acad Sci USA* 1998; **95**: 10626–31.
- 4 Shuai K, Liu B. Regulation of gene-activation pathways by PIAS proteins in the immune system. *Nat Rev Immunol* 2005; **5**: 593–605.
- 5 Drake JM, Lee JK, Witte ON. Clinical targeting of mutated and wild-type protein tyrosine kinases in cancer. *Mol Cell Biol* 2014; **34**: 1722–32.
- 6 Cools J, DeAngelo DJ, Gotlib J *et al.* A tyrosine kinase created by fusion of the PDGFR and FIP1L1 gene as a therapeutic target of imatinib in idiopathic hypereosinophilic syndrome. *N Engl J Med* 2003; **348**: 1201–14.
- 7 Griffin JH, Leung J, Bruner RJ, Caligiuri MA, Briesewitz R. Discovery of a fusion kinase in EOL-1 cells and idiopathic hypereosinophilic syndrome. *Proc Natl Acad Sci USA* 2003; **100**: 7830–5.
- 8 Pardanani A, Brockman SR, Paternoster SF *et al.* FIP1L1-PDGFR fusion: prevalence and clinicopathologic correlates in 89 consecutive patients with moderate to severe eosinophilia. *Blood* 2004; **104**: 3038–45.
- 9 Roche-Lestienne C, Lepers S, Soenen-Cornu V *et al.* Molecular characterization of the idiopathic hypereosinophilic syndrome (HES) in 35 French patients with normal conventional cytogenetics. *Leukemia* 2005; **19**: 792–8.
- 10 Sada A, Katayama Y, Yamamoto K *et al.* A multicenter analysis of the FIP1L1-alphaPDGFR fusion gene in Japanese idiopathic hypereosinophilic syndrome: an aberrant splicing skipping the alphaPDGFR exon 12. *Ann Hematol* 2007; **86**: 855–63.
- 11 Jovanovic JV, Score J, Waghorn K *et al.* Low-dose imatinib mesylate leads to rapid induction of major molecular responses and achievement of complete molecular remission in FIP1L1-PDGFR-positive chronic eosinophilic leukemia. *Blood* 2007; **109**: 4635–40.
- 12 Baccarani M, Cilloni D, Rondoni M *et al.* The efficacy of imatinib mesylate in patients with FIP1L1-PDGFRalpha-positive hypereosinophilic syndrome. Results of a multicenter prospective study. *Haematologica* 2007; **92**: 1173–9.
- 13 Swerdlow SH, Campo E, Harris NL *et al.*, eds. *World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissue*, 4th edn. Lyon, France: IARC Press, 2008.
- 14 Stover EH, Chen J, Folens C *et al.* Activation of FIP1L1-PDGFRalpha requires disruption of the juxtamembrane domain of PDGFRalpha and is FIP1L1-independent. *Proc Natl Acad Sci USA* 2006; **103**: 8078–83.

- 15 Buitenhuis M, Verhagen LP, Cools J, Coffey PJ. Molecular mechanisms underlying FIP1L1-PDGFR α -mediated myeloproliferation. *Cancer Res* 2007; **67**: 3759–66.
- 16 Iwasaki J, Kondo T, Darmanin S et al. FIP1L1 presence in FIP1L1-RARA or FIP1L1-PDGFR α differentially contributes to the pathogenesis of distinct types of leukemia. *Ann Hematol* 2014; **93**: 1473–81.
- 17 Ishihara K, Kitamura H, Hiraizumi K et al. Mechanisms for the proliferation of eosinophilic leukemia cells by FIP1L1-PDGFR α . *Biochem Biophys Res Commun* 2008; **366**: 1007–11.
- 18 Fukushima K, Matsumura I, Ezoe S et al. FIP1L1-PDGFR α imposes eosinophil lineage commitment on hematopoietic stem/progenitor cells. *J Biol Chem* 2009; **284**: 7719–32.
- 19 Li B, Zhang G, Li C et al. Identification of JAK2 as a mediator of FIP1L1-PDGFR α -induced eosinophil growth and function in CEL. *PLoS ONE* 2012; **7**: e34912.
- 20 Noël LA, Arts FA, Montano-Almendras CP et al. The tyrosine phosphatase SHP2 is required for cell transformation by the receptor tyrosine kinase mutants FIP1L1-PDGFR α and PDGFR α D842V. *Mol Oncol* 2014; **8**: 728–40.
- 21 Lee JM, Kang HJ, Lee HR, Choi CY, Jang WJ, Ahn JH. PIAS1 enhances SUMO-1 modification and the transactivation activity of the major immediate-early IE2 protein of human cytomegalovirus. *FEBS Lett* 2003; **555**: 322–8.
- 22 Nakagawa K, Yokosawa H. PIAS3 induces SUMO-1 modification and transcriptional repression of IRF-1. *FEBS Lett* 2002; **530**: 204–8.
- 23 Kondo T, Kobayashi M, Tanaka J et al. Rapid degradation of Cdt1 upon UV-induced DNA damage is mediated by SCF^{Skp2} complex. *J Biol Chem* 2004; **279**: 27315–9.
- 24 Fukuda I, Ito A, Hirai G et al. Ginkgolic acid inhibits protein SUMOylation by blocking formation of the E1-SUMO intermediate. *Chem Biol* 2009; **16**: 133–40.
- 25 Gueller S, Hehn S, Nowak V et al. Adaptor protein Lnk binds to PDGF receptor and inhibits PDGF-dependent signaling. *Exp Hematol* 2011; **39**: 591–600.
- 26 Toffalini F, Kallin A, Vandenberghe P et al. The fusion proteins TEL-PDGFR β and FIP1L1-PDGFR α escape ubiquitination and degradation. *Haematologica* 2009; **94**: 1085–93.
- 27 Liu B, Yang Y, Chernishof V et al. Proinflammatory stimuli induce IKK α -mediated phosphorylation of PIAS1 to restrict inflammation and immunity. *Cell* 2007; **129**: 903–14.
- 28 Stehmeier P, Muller S. Phospho-regulated SUMO interaction modules connect the SUMO system to CK2 signaling. *Mol Cell* 2009; **33**: 400–9.
- 29 Li R, Wei J, Jiang C et al. Akt SUMOylation regulates cell proliferation and tumorigenesis. *Cancer Res* 2013; **73**: 5742–53.
- 30 Score J, Walz C, Jovanovic JV et al. Detection and molecular monitoring of FIP1L1-PDGFR α -positive disease by analysis of patient-specific genomic DNA fusion junctions. *Leukemia* 2009; **23**: 332–9.
- 31 Klion AD, Robyn J, Maric I et al. Relapse following discontinuation of imatinib mesylate therapy for FIP1L1/PDGFR α -positive chronic eosinophilic leukemia: implications for optimal dosing. *Blood* 2007; **110**: 3552–6.
- 32 Liu B, Yee KM, Tahk S, Mackie R, Hsu C, Shuai K. PIAS1 SUMO ligase regulates the self-renewal and differentiation of hematopoietic stem cells. *EMBO J* 2014; **33**: 101–13.