学　位　論　文

A leukemogenic kinase, FIP1L1-PDGFRA, and a SUMO E3 ligase, PIAS1, form a positive crosstalk via their enzymatic activities.

(白血病原因キナーゼである FIP1L1-PDGFRA と SUMO 化 E3 リガーゼ

である PIAS1 は、その酵素活性により正の相互作用を形成する.)

北　海　道　大　学

井端　淳

A leukemogenic kinase, FIP1L1-PDGFRA, and a SUMO E3 ligase, PIAS1, form a positive crosstalk via their enzymatic activities.

Runnning title: Sumoylation of FIP1L1-PDGFRA

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(Abstract)
Fusion tyrosine kinases play a crucial role in the development of hematological malignancies. FIP1L1-PDGFRA is a leukemogenic fusion kinase that causes chronic eosinophilic leukemia. As a constitutively active kinase, FIP1L1-PDGFRA 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-PDGFRA to exert its full transforming activity, but the underlying mechanisms have not been fully characterized. We identified PIAS1 as a FIP1L1-PDGFRA-association molecule by yeast two-hybrid screening. Our analyses indicate that the FIP1L1 portion of FIP1L1-PDGFRA is required for efficient associatiation with PIAS1. As a consequence of the association, FIP1L1-PDGFRA phosphorylates PIAS1. Moreover, the kinase activity of FIP1L1-PDGFRA stabilizes PIAS1. Therefore, PIAS1 is one of the downstream targets of FIP1L1-PDGFRA. Moreover, we found that PIAS1, as a SUMO E3 ligase, sumoylates and stabilizes FIP1L1-PDGFRA. In addition, suppression of PIAS1 activity by a knockdown experiment resulted in destabilization of FIP1L1-PDGFRA. Therefore, FIP1L1-PDGFRA and PIAS1 form a positive crosstalk via their enzymatic activities. Suppression of sumoylation by ginkgolic acid, a small molecule compound inhibiting a SUMO E1-activating enzyme, also destabilizes FIP1L1-PDGFRA, and while the tyrosine kinase inhibitor imatinib suppresses FIP1L1-PDGFRA-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-PDGFRA-positive chronic eosinophilic leukemia.

(Introduction)

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 STAT1 (PIAS1). PIAS1 was initially isolated as a molecule that interacts with activated STAT1 and inhibits STAT1-mediated gene activation [3]. While 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]. FIP1L1-PDGFRA is a fusion tyrosine kinase that was identified from patients with idiopathic hypereosinophilic syndrome [6, 7]. This fusion gene has been observed in 10% to 20% of patients with eosinophilia and, therefore, eosinophilia with FIP1L1-PDGFRA is now diagnosed as chronic eosinophilic leukemia (CEL) according to the WHO disease classification [8,9,10,11,12,13]. This fusion kinase is constitutively active and its kinase activity is essential for cellular transformation [6,7,14,15,16]. Since proliferation of CEL cells is dependent on the kinase activity of FIP1L1-PDGFRA, imatinib, which was originally developed for treatment of chronic myelogenous leukemia (CML) but also inhibits the kinase activity of PDGFRA, is also effective for patients with CEL [6,8,9,11,12].

As a leukemogenic fusion kinase, FIP1L1-PDGFRA stimulates downstream effectors. Some effector molecules, including PI3K, ERK1/2, JNK, p38 MAPK, JAK2, STAT5, PKB/c-akt and SHP2, have been identified in the context of leukemic transformation [15,17,18,19,20]. Although the C-terminal kinase portion of FIP1L1-PDGFRA 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-PDGFRA accumulates in the nucleus and has a higher proliferating activity than that of the C-terminal PDGFRA portion of FIP1L1-PDGFRA [16]. Based on these reports, it is thought that the FIP1L1 portion directs FIP1L1-PDGFRA 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-PDGFRA to elucidate the leukemogenic role of the FIP1L1 portion, and we isolated PIAS1 as a FIP1L1-PDGFRA-association molecule. Our data show that there is a positive crosstalk between FIP1L1-PDGFRA and PIAS1. FIP1L1-PDGFRA phosphorylates and stabilizes PIAS1. On the other hand, PIAS1 sumoylates and stabilizes FIP1L1-PDGFRA. The reciprocally positive interaction between FIP1L1-PDGFRA and PIAS1 via enzymatic activities could be crucial for the transforming activity of FIP1L1-PDGFRA. Moreover, the sumoylation system by PIAS1 could be a potential target in the treatment of FIP1L1-PDGFRA-positive CEL.

(Materials and Methods)

Plasmid construction

Flag-tagged or T7-tagged expression vectors of full-length FIP1L1-PDGFRA (FIP1L1-PDGFRA-FL), a kinase-dead mutant of FIP1L1-PDGFRA (FIP1L1-PDGFRA-KD), and a deletion mutant with only the C-terminal portion of PDGFRA (PDGFRA-C) have been described previously. These vectors are named pFLAG-FIP1L1-PDGFRA-FL, pFLAG-FIP1L1-PDGFRA-KD, pFLAG-PDGFRA-C, pCGT-FIP1L1-PDGFRA-FL, pCGT-FIP1L1-PDGFRA-KD, and pCGT-PDGFRA-C, respectively. For yeast two-hybrid screening, full-length FIP1L1-PDGFRA cDNA was cloned into pBTM116 (Clontech) and named pBTM116-FIP1L1-PDGFRA-FL. Full-length human PIAS1 cDNA was amplified by polymerase chain reaction (PCR) from a HeLa cDNA library. A 6xMyc-tagged expression vector of PIAS1 was generated by inserting human PIAS1 cDNA into a pCI-neo-6xMyc vector that had been generated by inserting α fragment containing six copies of the Myc epitope into pCI-neo (Promega), and the vector was named pCI-6xMyc-PIAS1. A 6xMyc-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-6xMyc-PIAS1-C351S. The 6xMyc-tagged PIAS1 was amplified by PCR and cloned into the pTRE3G-ZsGreen1 (Clontech) vector for a tetracycline-inducible experiment, and it was named pTRE3G-6xMyc-

PIAS1. A T7-tagged expression vector of SUMO-1, pCGT-T7-SUMO-1, was previously described [22]. For constructing retroviral vectors, FLAG-tagged FIP1L1-PDGFRA-FL or FIP1L1-PDGFRA-KD cDNA was amplified by PCR and cloned into pDON-5 Neo (TaKaRa), and these vectors were named pDON-FLAG-FIP1L1-PDGFRA-FL and pDON-FLAG-FIP1L1-PDGFRA-KD, respectively. FIP1L1-PDGFRA-T671I 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-PDGFRA, we transfected yeast strain L40 stably expressing pBTM116-FIP1L1-PDGFRA-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% fetal bovine serum. BAF-B03 cells were obtained from Dr. Masao Seto at Aichi Cancer Center Research Institute and cultured in RPMI 1640 containing 10% fetal bovine serum and 1 ng/ml murine IL-3 (Medical and Biological Laboratories). For transient transfection experiments, the indicated expression vectors were transfected into HEK293 cells in a 6 cm dish by Lipofectamine 2000 (Invitrogen) and then cultured for 36-48 hours 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 co-transfected into HEK293 cells with either pFLAG-FIP1L1-PDGFRA-FL or pFLAG-FIP1L1-PDGFRA-KD. After four hours, 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 of doxycycline. After 24 hours of incubation, the culture media were replaced with fresh media without doxycycline, and this point was set as the starting time. Then the cells were harvested after 24 hours and the cell lysates were subjected to immunoblotting. To establish a HEK293-derived stable cell line expressing FIP1L1-PDGFRA, HEK293 cells were transfected with pFLAG-FIP1L1-PDGFRA-FL. After two days of transfection, the cells were selected with

500 μg/ml G418 (Sigma). The established cell line, HEK293-FIP1L1-PDGFRA-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-PDGFRA and its mutants, we used the retrovirus packaging kit Eco (TAKARA). BAF-B03 cells were infected with pDON-FLAG-FIP1L1-PDGFRA-FL or each mutant of FIP1L1-PDGFRA, and the cells were selected with 500 μg/ml of G418. Ginkgolic acid was purchased from Calbiochem 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-PDGFRA.

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 MBL, anti-T7 tag antibody was from Novagen, anti-phosphotyrosine antibody (PY-20) was from Beckman Coulter, anti-PDGFRA antibody (#3164) was from Cell Signaling, and anti-PIAS1 antibodies (ab32219 and ab77231) were from Abcam. 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, 0.5% sodium deoxycholate) supplemented with 10 mM *N*-ethylmaleimide, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM NaF and 0.5 mM Na_3VO_4. 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), and the band intensity was quantified using ImageQuant TL software (GE Healthcare).

For immunostaining, HEK293 cells were transfected with pCGT-FIP1L1-PDGFRA-FL or pCGT-PDGFRA-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). For DNA staining, fixed cells were stained with 4′,6′-diamidino-2-phenylindole-dihydrochloride (DAPI). Fluorescent images were acquired with an FV-10i confocal microscope

(Olympus, Tokyo, Japan) and analyzed with Metamorph software (Universal Imaging).

Apoptosis assay

BAF-derived cells were treated with imatinib and/or ginkgolic acid at the indicated concentrations for 24 hours. Induction of apoptosis was quantitated using the MEBCYTO Apoptosis Kit (Medical and Biological Laboratories). Briefly, the cells (2×10^5) were collected, washed with PBS, and suspended in 90 μl of binding buffer (containing 10 μl of annexin V-FITC and 1 μl of 100 μg/mL DAPI). Thereafter, the samples were incubated in the dark for 15 min at room temperature and then analyzed by FACSCanto™ II (Becton Dickinson) after addition of 400 μl of the binding buffer.

(Results)

FIP1L1-PDGFRA associates with PIAS1.

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

association with PIAS1 because of the nuclear accumulation of FIP1L1-PDGFRA directed by the FIP1L1 portion.

FIP1L1-PDGFRA phosphorylates PIAS1 on tyrosine residues and increases the stability of PIAS1.

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

To examine this possibility, myc-tagged PIAS1 was co-expressed with FIP1L1-PDGFRA 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-PDGFRA-FL but not by FIP1L1-PDGFRA-KD or PDGFRA-C (Figure 2b). Although PDGFRA-C is kinase-active and weakly associated with PIAS1 (Figure 1a), tyrosine phosphorylation of PIAS1 was not detected (Figure 2b, lane 3). This result suggests that the FIP1L1 portion is required not only for efficient association between FIP1L1-PDGFRA and PIAS1 but also for tyrosine phosphorylation of PIAS1 by FIP1L1-PDGFRA.

While examining the association between FIP1L1-PDGFRA and PIAS1, we noticed that the amount of PIAS1 associated with FIP1L1-PDGFRA was greater in cells expressing FIP1L1-PDGFRA-FL than in cells expressing FIP1L1-PDGFRA-KD. Moreover, transient expression experiments, in which expression vectors of FIP1L1-PDGFRA and PIAS1 were transfected, showed that the expression level of PIAS1 tended to be higher in cells co-transfected with FIP1L1-PDGFRA-FL than in cells co-transfected with FIP1L1-PDGFRA-KD. These results indicate the possibility that FIP1L1-PDGFRA stabilizes PIAS1 via 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 (Figure 2c). The expression of PIAS1 was efficiently induced when FIP1L1-PDGFRA-FL was co-expressed (Figure 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 co-expressed with FIP1L1-PDGFRA-KD (Figure 2c, right panel). Since this experiment was performed by transient transfection, we next established cell lines stably expressing FIP1L1-PDGFRA to analyze the functional relation between FIP1L1-PDGFRA and PIAS1. We treated BAF-B03-derived stable cell lines, BAF-FIP1L1-PDGFRA-FL, BAF-FIP1L1-PDGFRA-KD and BAF-FIP1L1-PDGFRA-T674I, with imatinib (Figure 2d). As previously described [14,15,16], parental BAF-B03 cells are IL-3-dependent pro-B cells, which become IL-3-independent upon the introduction of a kinase-active FIP1L1-PDGFRA. Thus, BAF-FIP1L1-PDGFRA-FL cells and BAF-FIP1L1-PDGFRA-T674I cells proliferate in the absence of IL-3. By treatment with imatinib, kinase activity of FIP1L1-PDGFRA-FL was suppressed, resulting in a decrease of PIAS1 expression. On the other hand, the expression level of PIAS1 in BAF-FIP1L1-PDGFRA-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-PDGFRA-T674I cells was also not changed by treatment with imatinib. Collectively, the results suggest that FIP1L1-PDGFRA stabilizes PIAS1 via its kinase activity.

PIAS1 sumoylates and stabilizes FIP1L1-PDGFRA.

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

Since one of the physiological roles of sumoylation is regulation of protein stability, we hypothesized that PIAS1 regulates the stability of FIP1L1-

PDGFRA. To prove this hypothesis, we inhibited the expression of PIAS1 in BAF-FIP1L1-PDGFRA-FL cells by transfecting PIAS1-specific siRNA. As a consequence of the inhibition of PIAS1, the expression level of FIP1L1-PDGFRA was decreased (Figure 3c, left panel, lanes 2 and 3). Based on this result, the down-regulation of FIP1L1-PDGFRA may also affect the expression level of PIAS1 in BAF-FIP1L1-PDGFRA-FL cells. Therefore, we also performed the same experiment in a HEK293-derived stable cell line expressing FIP1L1-PDGFRA, which manifests FIP1L1-PDGFRA-independent growth. As was the case for BAF-FIP1L1-PDGFRA-FL, the expression level of FIP1L1-PDGFRA was decreased by knockdown of PIAS1 (Figure 3c, right panel, lanes 2 and 3). These results support our notion that PIAS1 regulates the expression level of FIP1L1-PDGFRA.
Collectively, the results suggest that PIAS1 sumoylates and stabilizes FIP1L1-PDGFRA.

PIAS1 is a potential therapeutic target for CEL treatment.

Our results suggest that sumoylation regulates the expression level of FIP1L1-PDGFRA, 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], and we thus examined the effect of ginkgolic acid on FIP1L1-PDGFRA expression. To analyze the effect of ginkgolic acid on FIP1L1-PDGFRA-dependent cell growth, we treated BAF-FIP1L1-PDGFRA-FL cells with different concentrations of ginkgolic acid and examined the expression levels of FIP1L1-PDGFRA. Ginkgolic acid decreased the expression level of FIP1L1-PDGFRA in both BAF-FIP1L1-PDGFRA-FL cells and BAF-FIP1L1-PDGFRA-KD cells (Figure 4a). Treatment of BAF-FIP1L1-PDGFRA-FL cells with 20 μM ginkgolic acid alone had a minimal effect in inducing apoptosis, while BAF-FIP1L1-PDGFRA-FL cells underwent apoptosis upon inhibition of FIP1L1-PDGFRA 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-PDGFRA-FL cells. When BAF-FIP1L1-PDGFRA-FL cells were treated with a combination of 20 nM imatinib and 20 μM ginkgolic acid, ginkgolic acid augmented the effect of imatinib (Figures 4b, left panel). This effect seemed to be mediated by suppression of the kinase activity of FIP1L1-PDGFRA, because these compounds had little effect on BAF-FIP1L1-PDGFRA-KD cells that manifest IL-3-dependent growth (Figure 4b, right panel).
Moreover, we examined whether knockdown of PIAS1 augments the effect of imatinib on BAF-FIP1L1-PDGFRA-FL cells. The expression of PIAS1 in BAF-

FIP1L1-PDGFRA-FL cells was inhibited by transfecting PIAS1-specific siRNA as described in the legend of Figure 3c, and subsequently the cells were treated with imatinib. The knockdown of PIAS1 in the transfected cells was confirmed by immunoblotting (data 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 (Figure 4c, left panel). On the other hand, there was no effect of PIAS1-specific siRNAs on induction of apoptosis in BAF-FIP1L1-PDGFRA-FL cells (Figure 4c, right panel). These results indicate that downregulation of PIAS1 sensitizes BAF-FIP1L1-PDGFRA-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-PDGFRA and is a potential target for FIP1L1-PDGFRA-positive CEL treatment.

(Discussion)

To understand the mechanisms by which FIP1L1-PDGFRA exerts its transforming activity via the FIP1L1 portion, we identified PIAS1 as a FIP1L1-PDGFRA-associating molecule and demonstrated a positive crosstalk between FIP1L1-PDGFRA and PIAS1 for phosphorylation and sumoylation.

We found that PIAS1 associates with FIP1L1-PDGFRA and that the FIP1L1 portion is necessary for efficient association. Some molecules have been reported to directly associate with FIP1L1-PDGFRA. The lymphocyte adaptor protein Lnk binds to both PDGFRA and FIP1L1-PDGFRA and acts as a negative regulator of these molecules [25]. c-Cbl is phosphorylated by both PDGFRA and FIP1L1-PDGFRA, but it efficiently ubiquitinates and destabilizes only PDGFRA [26]. The association of Lnk and c-Cbl with FIP1L1-PDGFRA seems to be mediated by the PDGFRA portion, as these molecules associate with the full length of PDGFRA. On the other hand, efficient association between PIAS1 and FIP1L1-PDGFRA required the FIP1L1 portion, because the FIP1L1 portion directs FIP1L1-PDGFRA into the nucleus, where PIAS1 is localized. As a kinase, FIP1L1-PDGFRA phosphorylated PIAS1 on tyrosine residues and this phosphorylation also required the FIP1L1 portion. Moreover, the kinase activity of FIP1L1-PDGFRA 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-PDGFRA is mediated by phosphorylation of PIAS1. Identification of tyrosine residues that are phosphorylated by FIP1L1-PDGFRA is necessary for further characterization of the underlying mechanism for PIAS1 regulation.

The kinase activity of FIP1L1-PDGFRA activates many downstream molecules via FIP1L1-dependent or -independent pathways. It has been reported that the FIP1L1 portion is necessary for activation of PKB/c-akt by FIP1L1-PDGFRA 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-PDGFRA and subsequently activate PKB/c-akt.

Moreover, PIAS1 sumoylated FIP1L1-PDGFRA and regulated its stability as a consequence of the association between FIP1L1-PDGFRA and PIAS1. Although imatinib is highly effective against FIP1L1-PDGFRA-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-PDGFRA. As a consequence, treatment of BAF-FIP1L1-PDGFRA-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-PDGFRA-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 crosstalk between FIP1L1-PDGFRA and PIAS1 may be associated with maintenance of leukemia stem cells in FIP1L1-PDGFRA-positive leukemia.

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Disclosure of Conflicts of Interest

The authors disclose no potential conflicts of interest.

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Figure Legends

Figure 1. FIP1L1-PDGFRA associates with PIAS1 in the nucleus.

a) FIP1L1-PDGFRA associates with PIAS1. HEK293 cells were transfected with a control vector, pFLAG-FIP1L1-PDGFRA-FL or pFLAG-PDGFRA-C. The association between PIAS1 and FLAG-FIP1L1-PDGFRA-FL or FLAG-PDGFRA-C was analyzed by immunoprecipitation (IP) with anti-FLAG M2 antibody and immunoblotting with anti-PIAS antibody. Immunoblotting of whole cell lysates with anti-PIAS1 antibody and anti-PDGFRA antibody confirmed the expression. The amounts of transfected vectors were 3 μg of a control vector or pFLAG-PDGFRA-C and 1 μg of pFLAG-FIP1L1-PDGFRA-FL.

b) FIP1L1-PDGFRA co-localizes with PIAS1 in the nucleus. HEK293 cells were transfected with 2 μg of pCGT-FIP1L1-PDGFRA-FL (left panel) or pCGT-PDGFRA-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.

Figure 2. FIP1L1-PDGFRA phosphorylates and stabilizes PIAS1.

a) PIAS1 that associated with kinase-active FIP1L1-PDGFRA slowly migrated by SDS-PAGE. HEK293 cells were transfected with a control vector, pFLAG-FIP1L1-PDGFRA-FL or pFLAG-FIP1L1-PDGFRA-KD. Immunoprecipitation and immunoblotting were performed as described in the legend of Figure 1a. The amounts of the transfected vectors were 3 μg of a control vector or pFLAG-FIP1L1-PDGFRA-KD and 1 μg of pFLAG-FIP1L1-PDGFRA-FL.

b) FIP1L1-PDGFRA phosphorylates PIAS1 on tyrosine residues. pCI-6xMyc-PIAS was transfected into HEK293 cells together with pFLAG-FIP1L1-PDGFRA-FL, pFLAG-FIP1L1-PDGFRA-KD or pFLAG-PDGFRA-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-PDGFRA antibody confirmed the expression of Myc-PIAS1, FLAG-FIP1L1-PDGFRA and its derivatives. The amounts of transfected vectors were as follows: 1 μg of pCI-6xMyc-PIAS1 was co-transfected with 1 μg of pFLAG-FIP1L1-PDGFRA-FL, and 5 μg of pCI-6xMyc-PIAS1 was co-transfected with 5 μg of pFLAG-FIP1L1-PDGFRA-KD or pFLAG-PDGFRA-C.

c) FIP1L1-PDGFRA stabilized PIAS1 via kinase activity.

The effect of FIP1L1-PDGFRA 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-PDGFRA-FL (left panel) or FIP1L1-PDGFRA-KD (lower panel) was co-expressed 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 hr, doxycycline (+)) was arbitrarily assigned to be 1.0 and the results are shown as means ± SE. The expression levels of Myc-tagged PIAS1 were quantitated and statistically compared by the t-test. n.s.: not significant. Analysis was performed in triplicate assays and the results were reproducible.
d) PIAS1 decreased upon imatinib treatment in BAF-PDGFRA-FL cells. BAF-PDGFRA-FL, BAF-PDGFRA-KD and BAF-PDGFRA-T674I cells were treated with 50 nM imatinib for 20 hours, and the expression levels of PIAS1 were examined by immunoblotting.

Figure 3. PIAS1 sumoylates and stabilizes FIP1L1-PDGFRA.
a) FIP1L1-PDFRA is sumoylated by PIAS1. HEK293 cells were transfected with a combination of pCI-6xMyc-PIAS1, pFLAG-FIP1L1-PDGFRA-FL and pCGT-SUMO-1. The total amount of transfected vectors was 6 μg, with 2 μg of each vector being used and an empty vector being used as a mock. FLAG-FIP1L1-PDGFRA was detected by anti-PDGFRA antibody and Myc-PIAS1 was detected by anti-Myc antibody. FIP1L1-PDGFRA was immunoprecipitated with anti-FLAG M2 antibody and subsequently analyzed by immunoblotting. Sumoylation of FIP1L1-PDGFRA was detected by anti-T7 antibody.
b) Knockdown of PIAS1 by siRNA attenuated sumoylation of FIP1L1-PDGFRA. HEK293 cells were transfected with pFLAG-FIP1L1-PDGFRA-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-PDGFRA (lanes 3 and 4).
c) Knockdown of PIAS1 resulted in a decrease of FIP1L1-PDGFRA.
BAF-FIP1L1-PDGFRA-FL cells were transfected with two different murine PIAS1-specific siRNAs or a negative control. HEK293-derived cells expressing FIP1L1-PDGFRA were transfected with two different human PIAS1-specific siRNAs or a negative control. After 2 days, the expression levels of PIAS1 and FIP1L1-PDGFRA were analyzed by immunoblotting with anti-PDGFRA antibody and anti-PIAS1 antibody.

Figure 4. Inhibition of sumoylation targets FIP1L1-PDGFRA.

a) Ginkgolic acid decreased the expression level of FIP1L1-PDGFRA in a dose-dependent manner. BAF-FIP1L1-PDGFRA-FL cells and BAF-FIP1L1-PDGFRA-KD cells were treated with the indicated concentrations of ginkgolic acid (GA) for 24 hours. The expression levels of FIP1L1-PDGFRA were examined by immunoblotting with anti-PDGFRA antibody. The expression levels of FIP1L1-PDGFRA were quantitated and statistically compared by the t-test. For this purpose, the expression level of FIP1L1-PDGFRA treated with mock was arbitrarily assigned to be 1.0 and the results are shown as means ± SE. Analysis was performed in triplicate assays and the results were reproducible.

b) Ginkgolic acid and imatinib synergistically induced apoptosis in BAF-FIP1L1-PDGFRA-FL cells. BAF-FIP1L1-PDGFRA-FL cells (left panel) and BAF-FIP1L1-PDGFRA-KD cells (right panel) were treated with 20 nM imatinib (IM) with or without 20 μM ginkgolic acid (GA) for 24 hours. Annexin V-positive cells were analyzed by flow cytometry and statistically compared by the t-test. n.s.: not significant. Analysis was performed in triplicate assays and the results were reproducible.

c) Knockdown of PIAS1 sensitized BAF-FIP1L1-PDGFRA-FL cells to imatinib. BAF-FIP1L1-PDGFRA-FL cells (left panel) and BAF-FIP1L1-PDGFRA-KD cells (right panel) were transfected with two different murine PIAS1-specific siRNAs or a negative control. After two days, the cells were treated with mock, 10 nM or 20 nM imatinib (IM). Annexin V-positive cells were analyzed by flow cytometry and statistically compared by one-factor ANOVA. n.s.: not significant. Analysis was performed in triplicate assays and the results were reproducible.

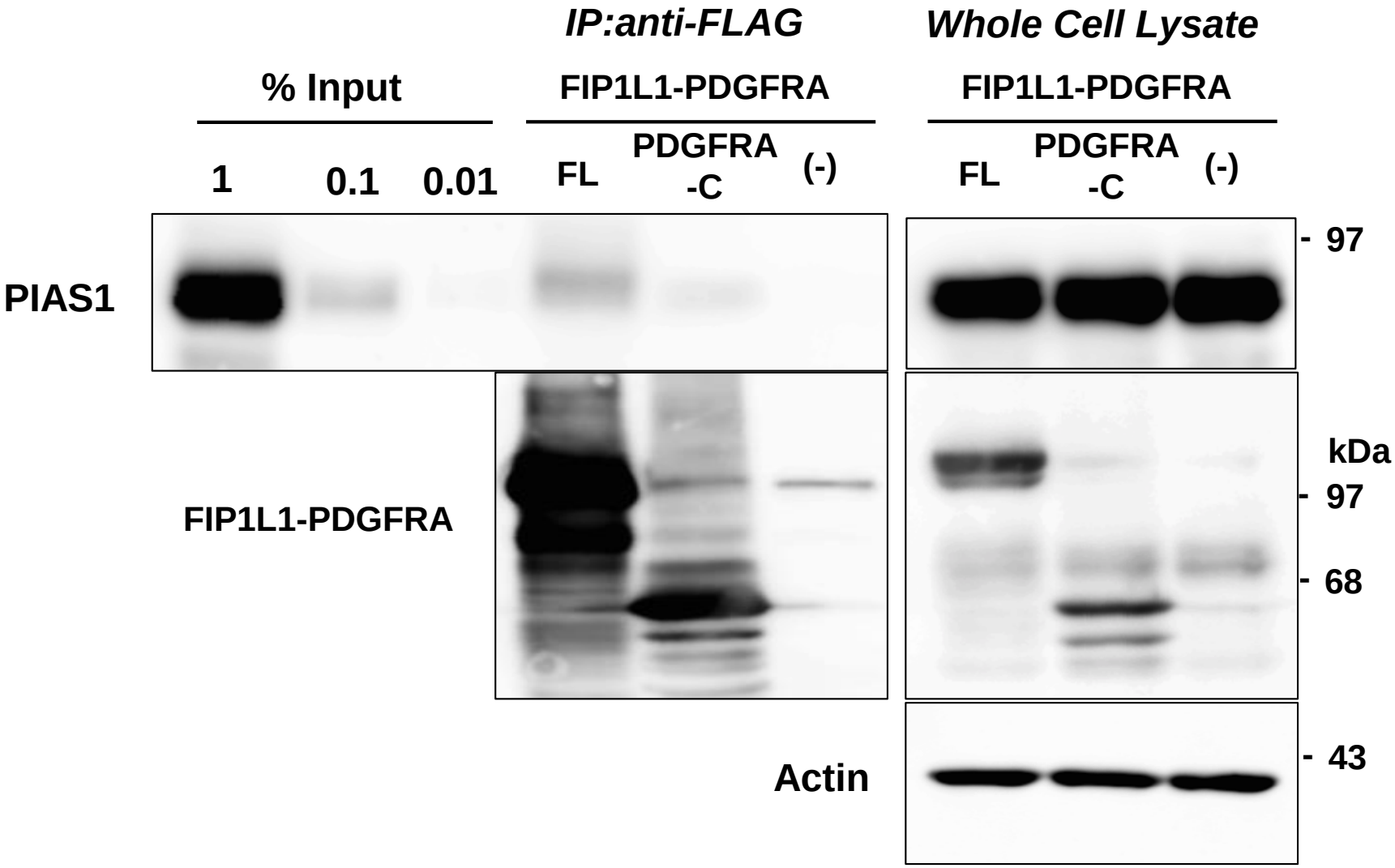
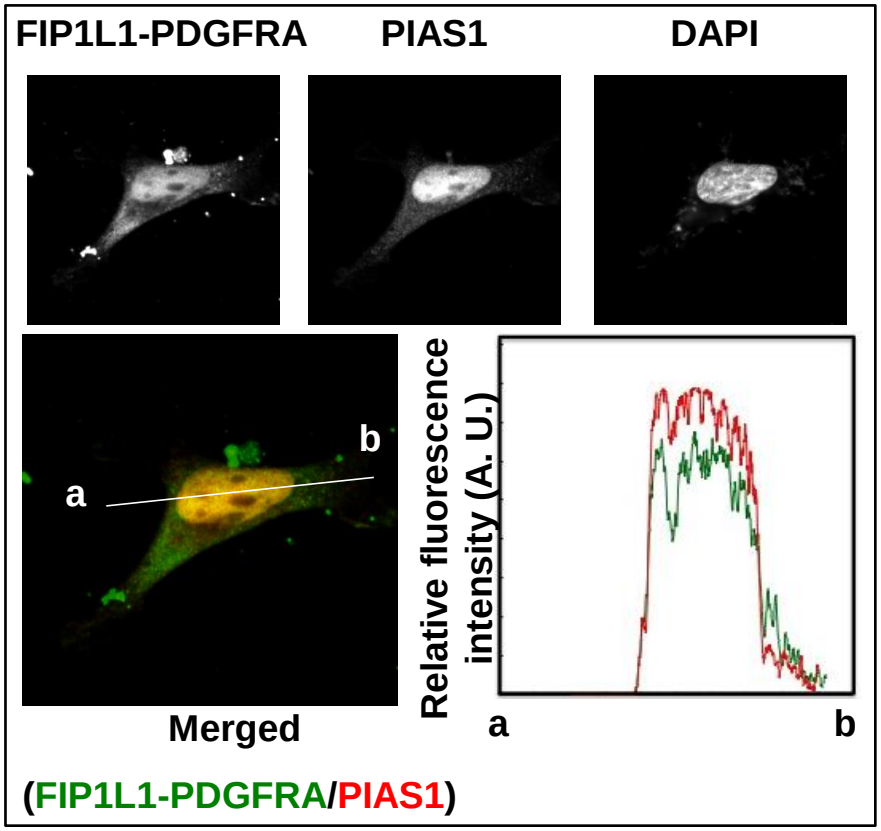

IP:anti-FLAG
Whole Cell Lysate
% Input
FIP1L1-PDGFRA
FIP1L1-PDGFRA
1
0.1
0.01
FL
PDGFRA -C
(-)
FL
PDGFRA -C
(-)
PIAS1
FIP1L1-PDGFRA
Actin
kDa
97
97
68
43

Figure 1a


FIP1L1-PDGFRA
PIAS1
DAPI
a
b
Merged
Relative fluorescence intensity (A. U.)
a
b
(FIP1L1-PDGFRA/PIAS1)

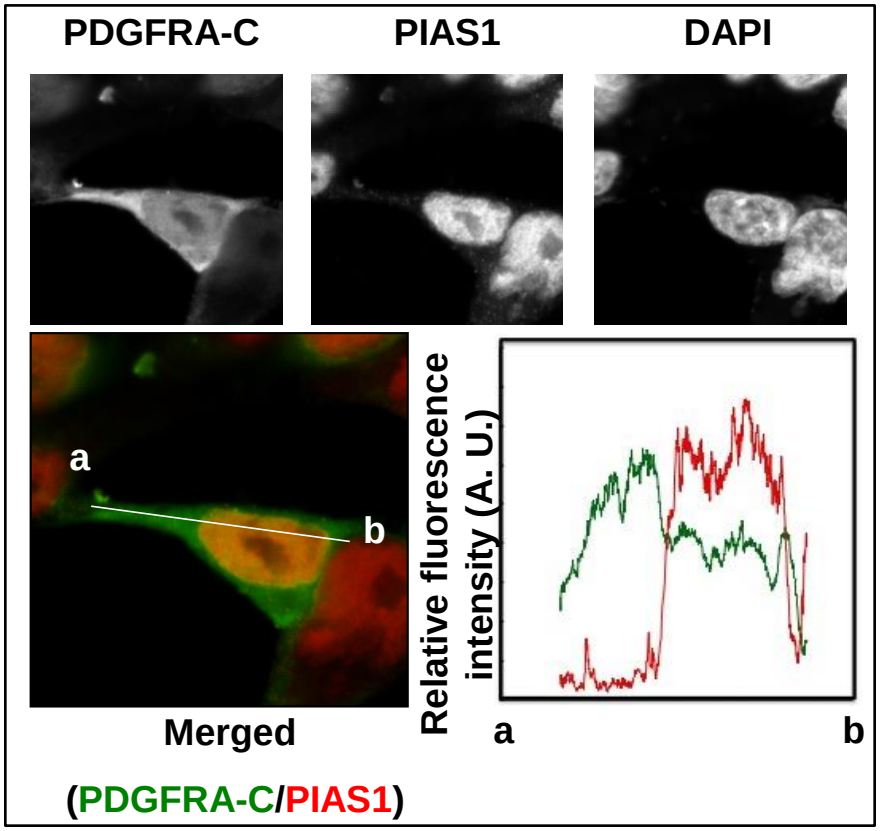

PDGFRA-C
PIAS1
DAPI
a
b
Merged
Relative fluorescence intensity (A. U.)
a
b
(PDGFRA-C/PIAS1)

Figure 1b

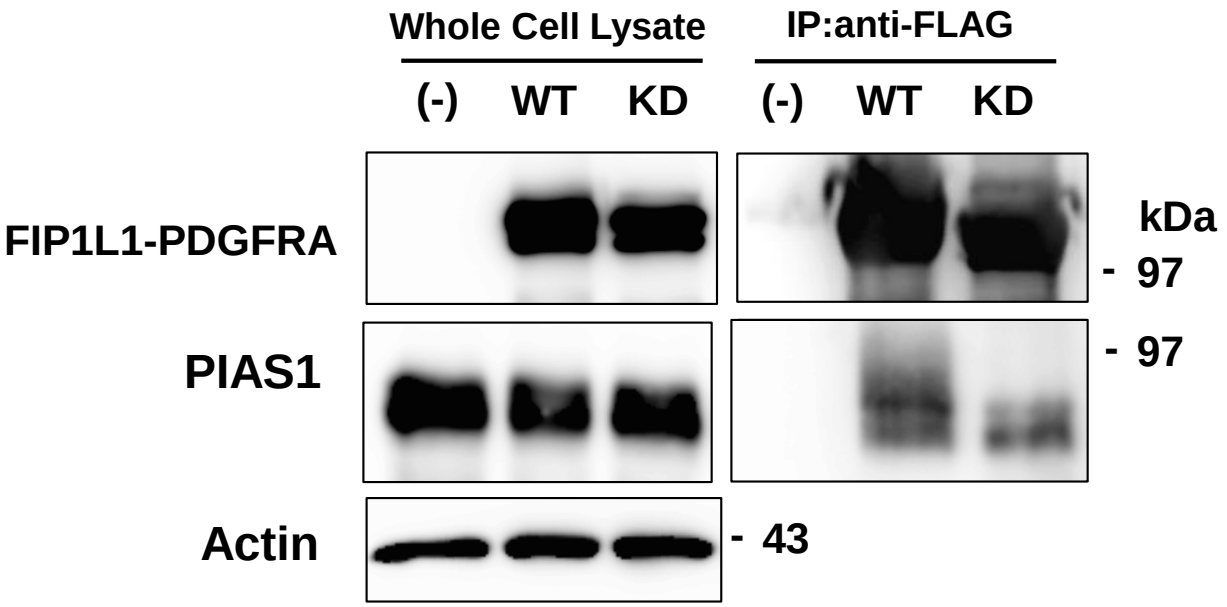

Whole Cell Lysate
IP:anti-FLAG
(-)
WT
KD
(-)
WT
KD
FIP1L1-PDGFRA
PIAS1
Actin
kDa
- 97
- 97
- 43

Figure 2a

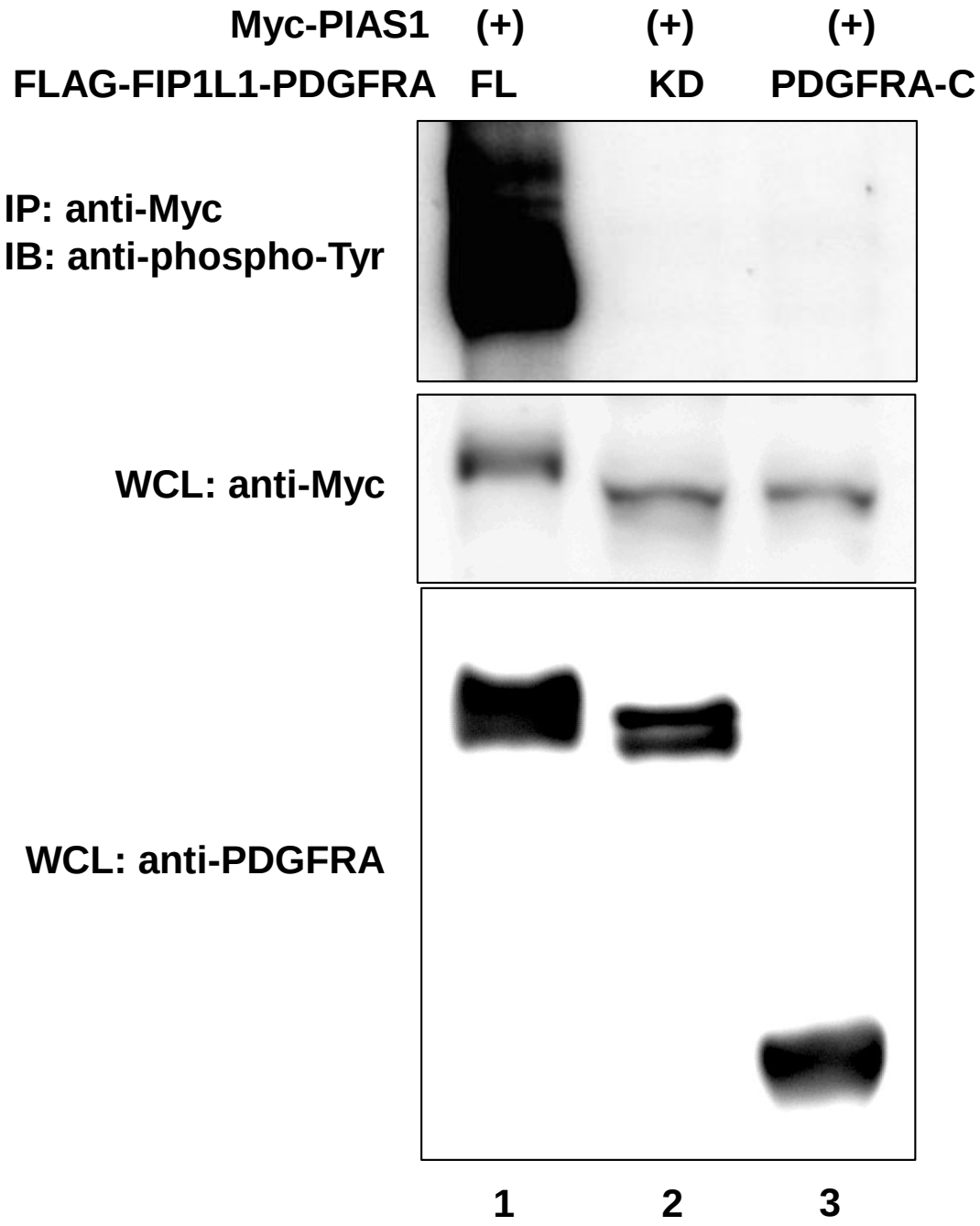
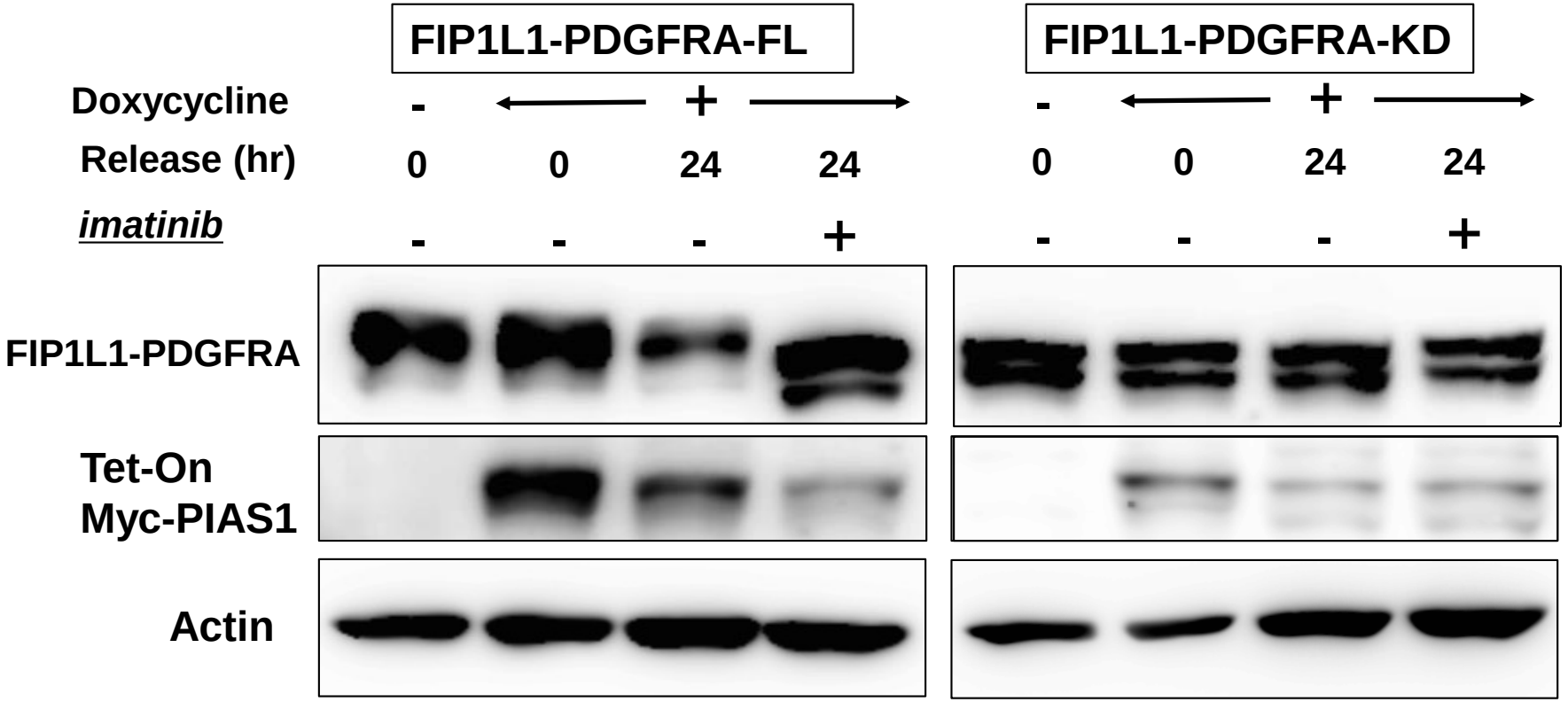

Myc-PIAS1 (+) (+) (+)
FLAG-FIP1L1-PDGFRA FL KD PDGFRA-C
IP: anti-Myc
IB: anti-phospho-Tyr
WCL: anti-Myc
WCL: anti-PDGFRA
1
2
3

Figure 2b


FIP1L1-PDGFRA-FL
FIP1L1-PDGFRA-KD
Doxycycline
- ← + →
- ← + →
Release (hr)
0 0 24 24
0 0 24 24
imatinib
- - - +
- - - +
FIP1L1-PDGFRA
Tet-On
Myc-PIAS1
Actin

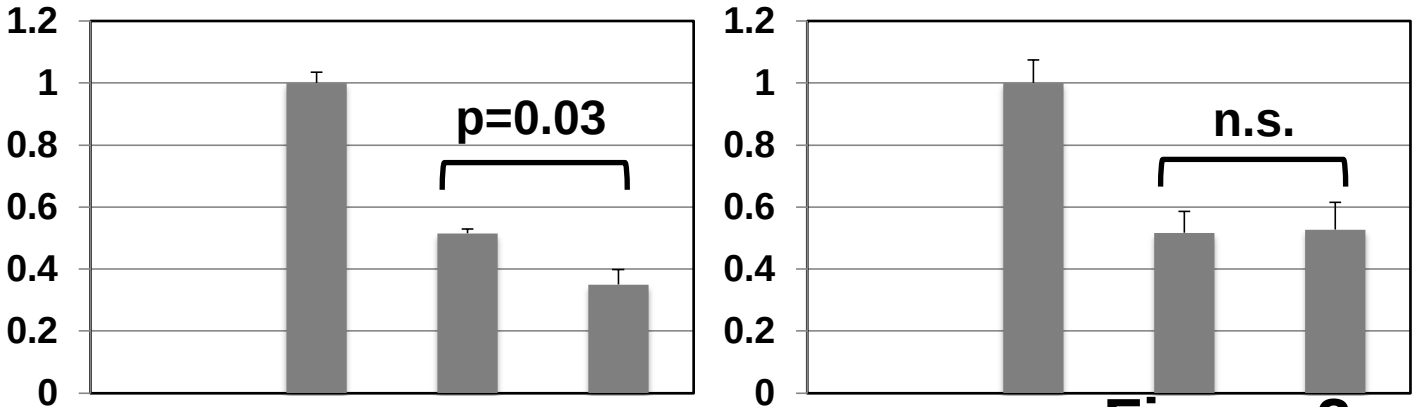

1.2
1
0.8
0.6
0.4
0.2
0
p=0.03
1.2
1
0.8
0.6
0.4
0.2
0
n.s.

Figure 2c

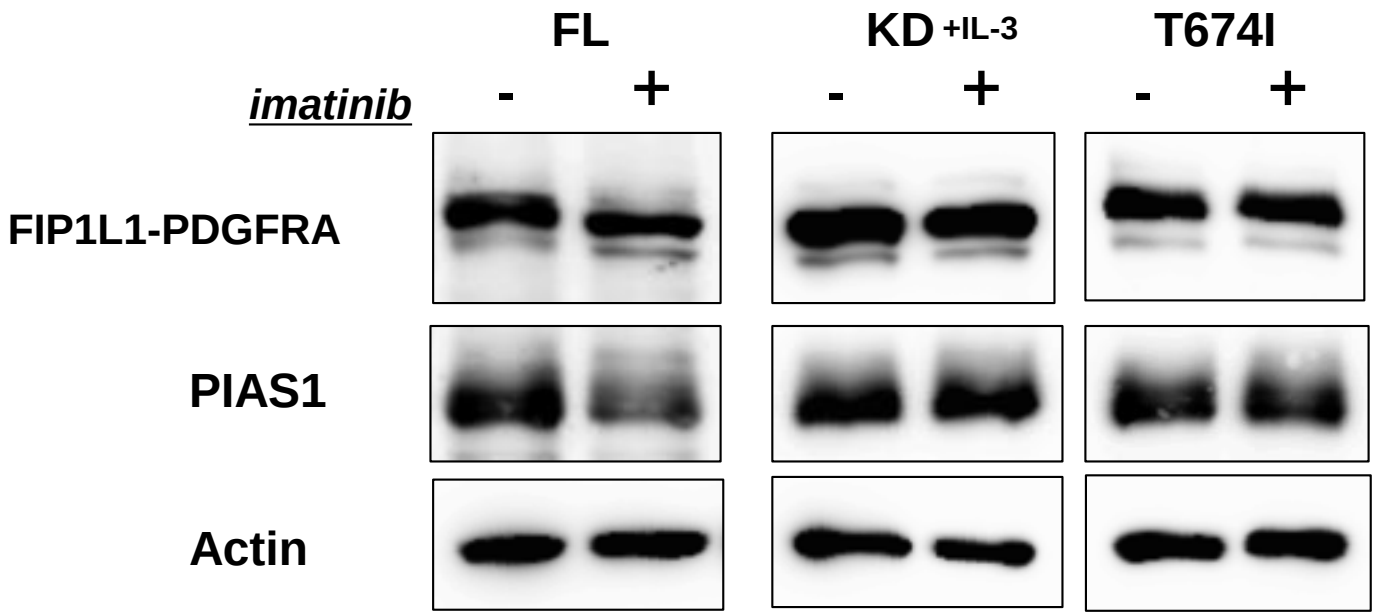


Figure 2d

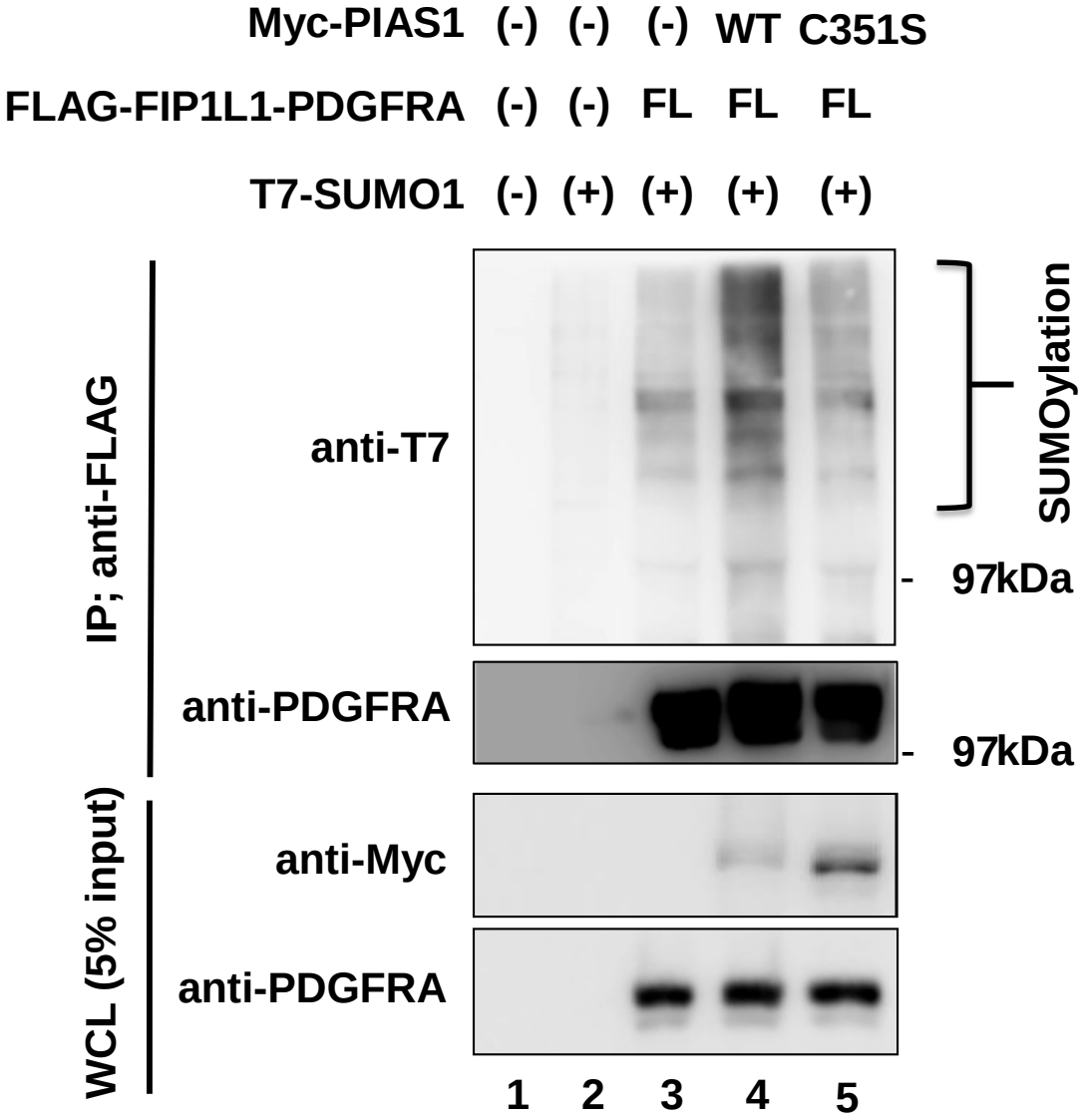

Myc-PIAS1 (-) (-) (-) WT C351S
FLAG-FIP1L1-PDGFRA (-) (-) FL FL FL
T7-SUMO1 (-) (+) (+) (+) (+)
IP; anti-FLAG
anti-T7
SUMOylation
97kDa
anti-PDGFRA
97kDa
WCL (5% input)
anti-Myc
anti-PDGFRA
1 2 3 4 5

Figure 3a

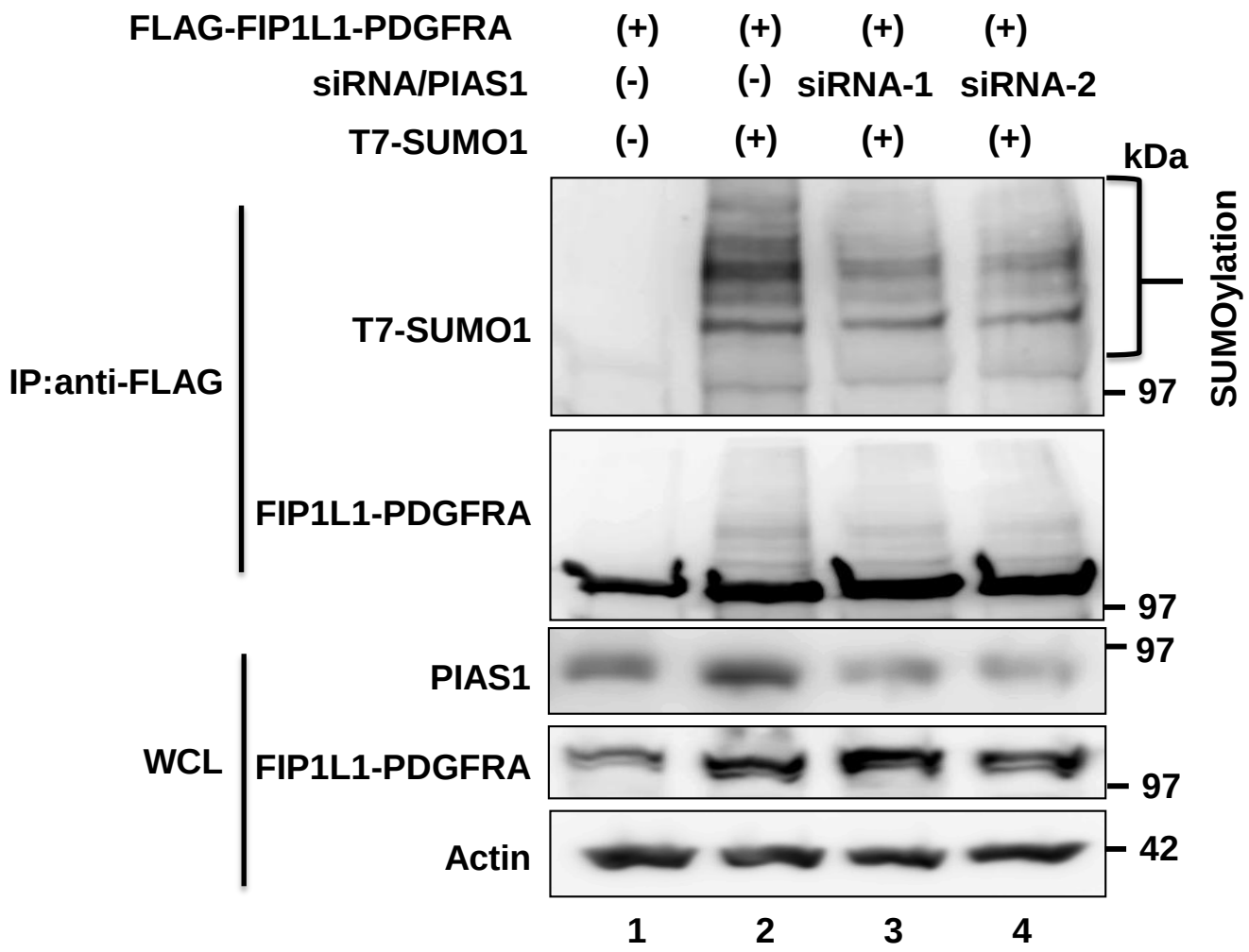

FLAG-FIP1L1-PDGFRA (+) (+) (+) (+)
siRNA/PIAS1 (-) (-) siRNA-1 siRNA-2
T7-SUMO1 (-) (+) (+) (+)
kDa
IP:anti-FLAG
T7-SUMO1
SUMOylation
97
FIP1L1-PDGFRA
97
WCL
PIAS1
97
FIP1L1-PDGFRA
97
Actin
42
1 2 3 4

Figure 3b

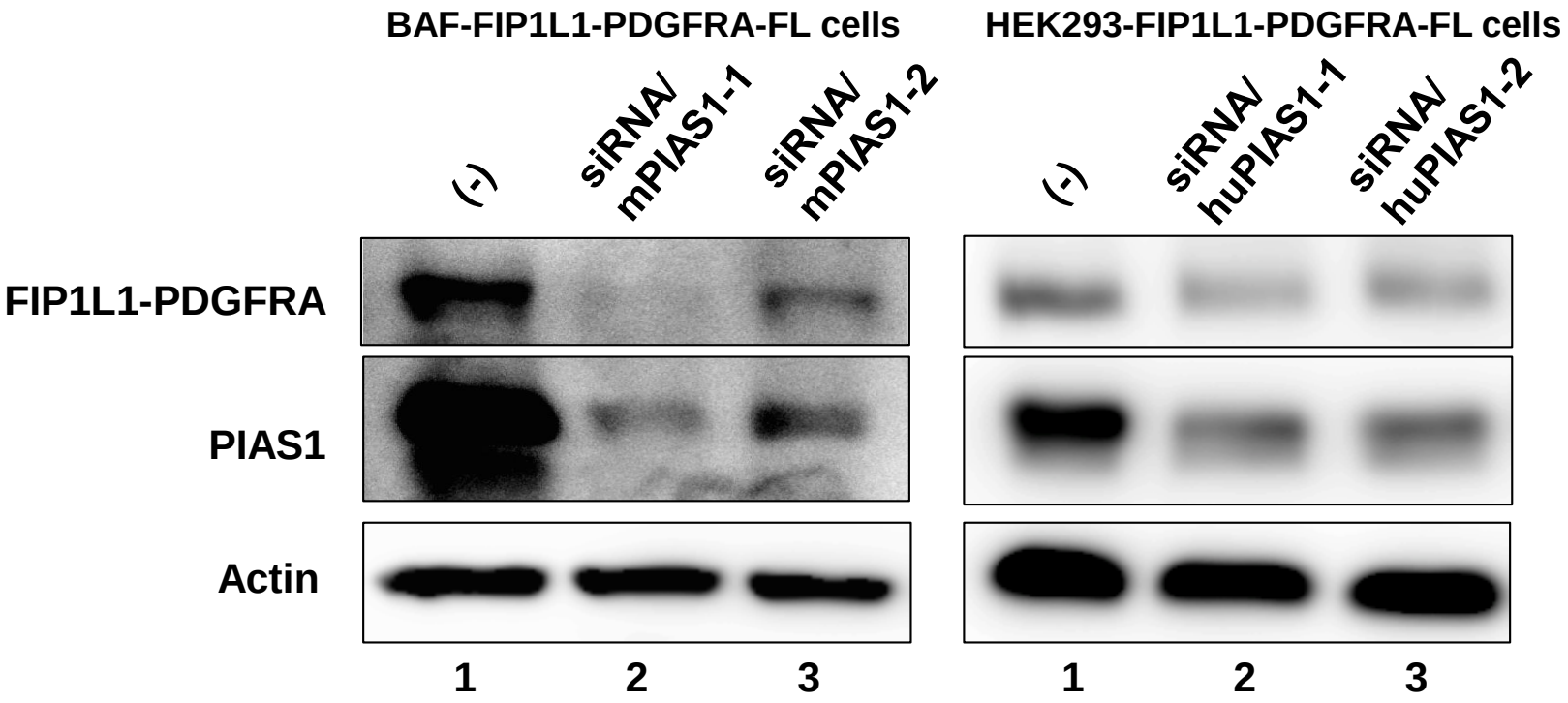
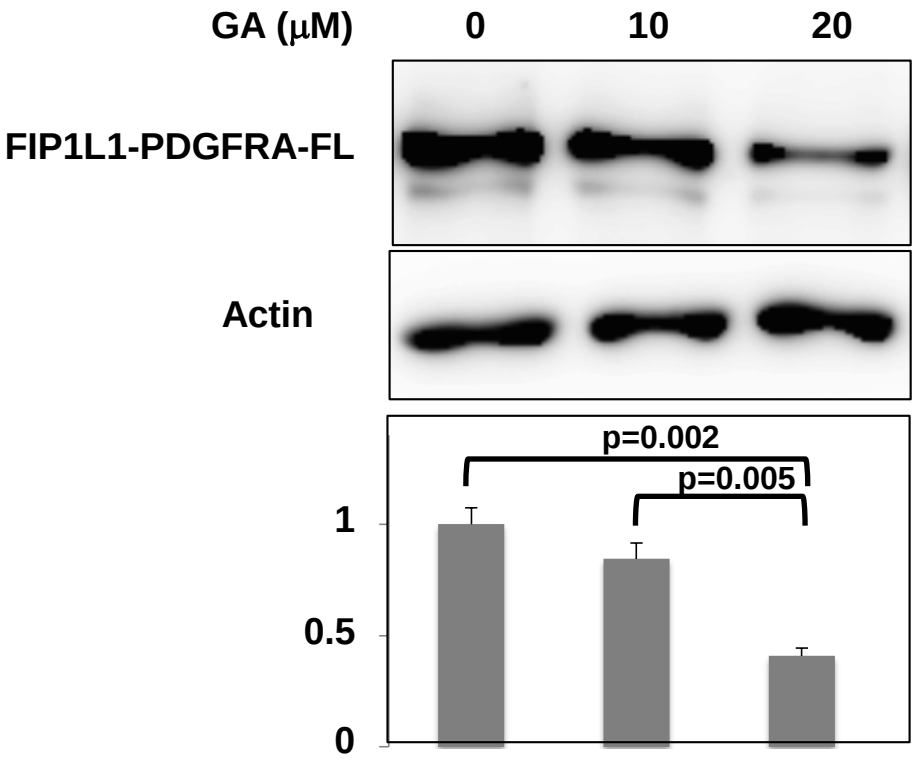

BAF-FIP1L1-PDGFRA-FL cells
HEK293-FIP1L1-PDGFRA-FL cells
(-)
siRNA/ mPIAS1-1
siRNA/ mPIAS1-2
(-)
siRNA/ huPIAS1-1
siRNA/ huPIAS1-2
FIP1L1-PDGFRA
PIAS1
Actin
1
2
3
1
2
3

Figure 3c


GA (μM)
0
10
20
FIP1L1-PDGFRA-FL
Actin
p=0.002
p=0.005
1
0.5
0

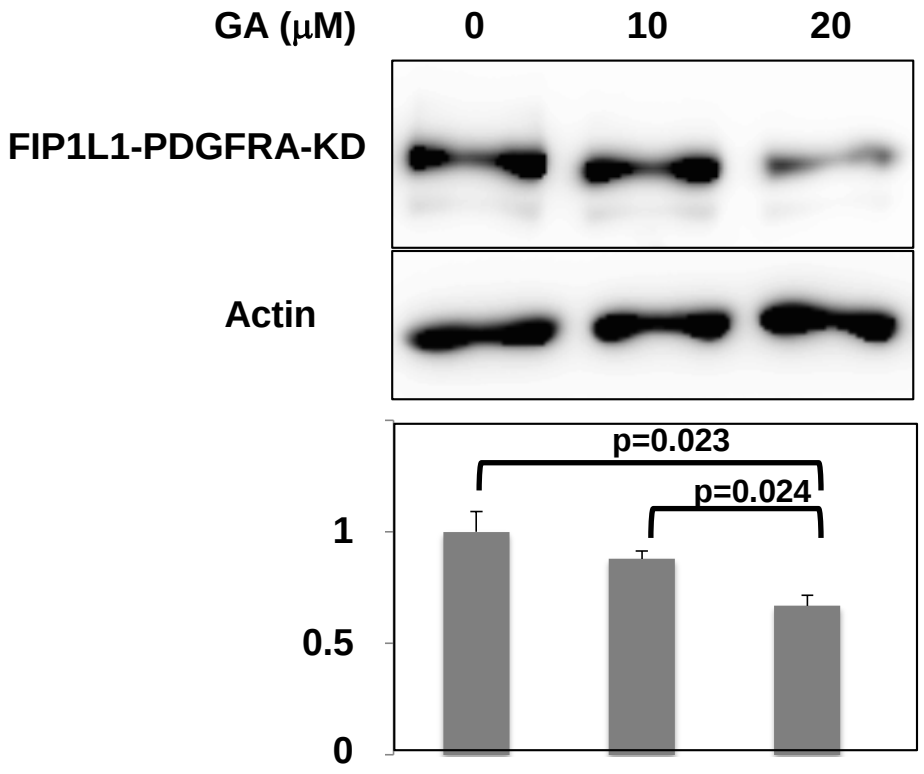
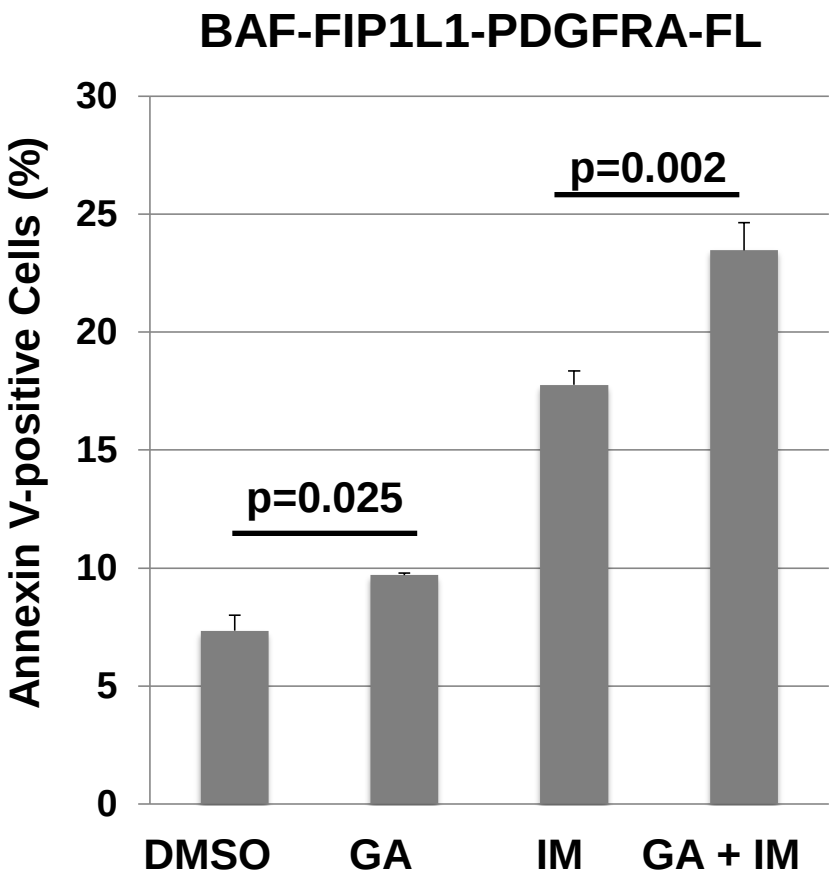

GA (μM)
0
10
20
FIP1L1-PDGFRA-KD
Actin
p=0.023
p=0.024
1
0.5
0

Figure 4a


BAF-FIP1L1-PDGFRA-FL
Annexin V-positive Cells (%)
30
25
20
15
10
5
0
p=0.002
p=0.025
DMSO
GA
IM
GA + IM

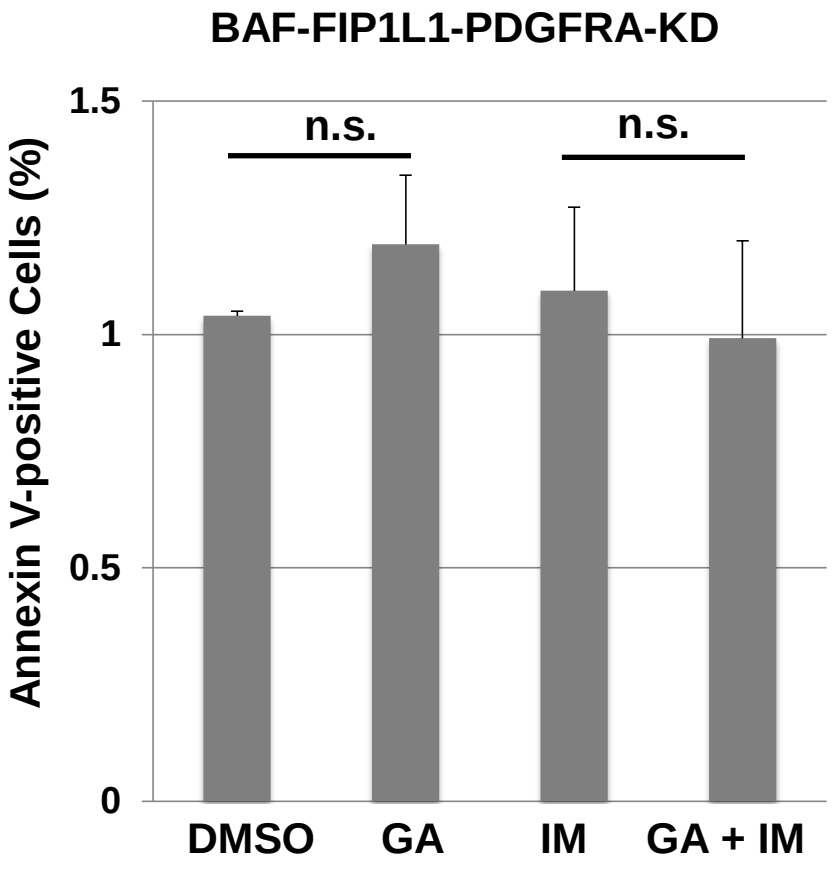

BAF-FIP1L1-PDGFRA-KD
Annexin V-positive Cells (%)
1.5
1
0.5
0
n.s.
n.s.
DMSO
GA
IM
GA + IM

Figure 4b

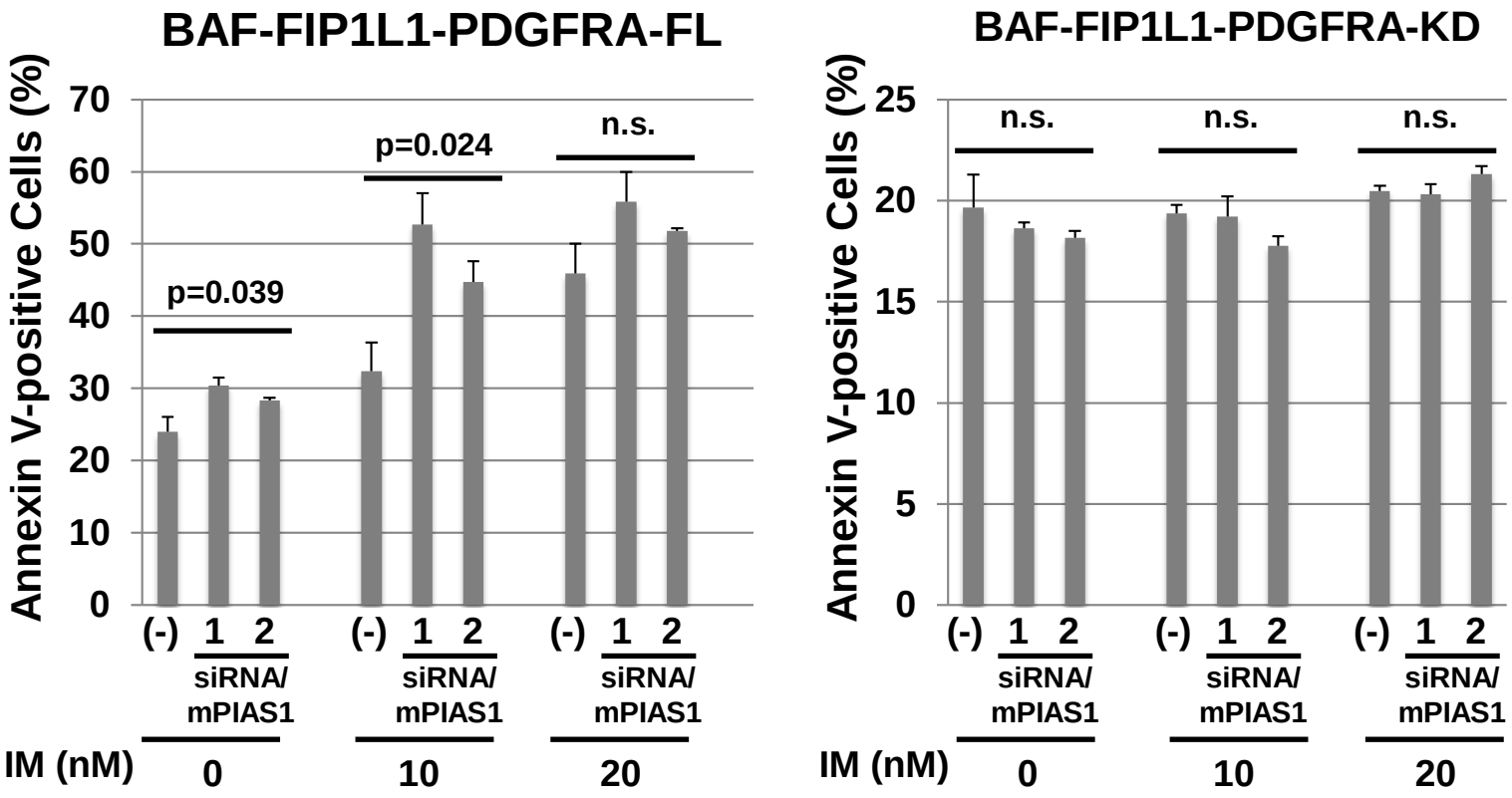

BAF-FIP1L1-PDGFRA-FL
Annexin V-positive Cells (%)
70
60
50
40
30
20
10
0
p=0.039
p=0.024
n.s.
(-) 1 2
(-) 1 2
(-) 1 2
siRNA/
mPIAS1
IM (nM)
0
10
20
BAF-FIP1L1-PDGFRA-KD
Annexin V-positive Cells (%)
25
20
15
10
5
0
n.s.
n.s.
n.s.
(-) 1 2
(-) 1 2
(-) 1 2
siRNA/
mPIAS1
IM (nM)
0
10
20

Figure 4c