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# N822K- or V560G-mutated KIT activation preferentially occurs in lipid rafts of the Golgi apparatus in leukemia cells

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## Abstract

**Background:** KIT tyrosine kinase is expressed in mast cells, interstitial cells of Cajal, and hematopoietic cells. Permanently active *KIT* mutations lead these host cells to tumorigenesis, and to such diseases as mast cell leukemia (MCL), gastrointestinal stromal tumor (GIST), and acute myeloid leukemia (AML). Recently, we reported that in MCL, KIT with mutations (*D816V*, human; *D814Y*, mouse) traffics to endolysosomes (EL), where it can then initiate oncogenic signaling. On the other hand, KIT mutants including *KIT*<sup>D814Y</sup> in GIST accumulate on the Golgi, and from there, activate downstream. *KIT* mutations, such as *N822K*, have been found in 30% of core binding factor-AML (CBF-AML) patients. However, how the mutants are tyrosine-phosphorylated and where they activate downstream molecules remain unknown. Moreover, it is unclear whether a KIT mutant other than *KIT*<sup>D816V</sup> in MCL is able to signal on EL.

**Methods:** We used leukemia cell lines, such as Kasumi-1 (*KIT*<sup>N822K</sup>, AML), SKNO-1 (*KIT*<sup>N822K</sup>, AML), and HMC-1.1 (*KIT*<sup>V560G</sup>, MCL), to explore how KIT transduces signals in these cells and to examine the signal platform for the mutants using immunofluorescence microscopy and inhibition of intracellular trafficking.

**Results:** In AML cell lines, *KIT*<sup>N822K</sup> aberrantly localizes to EL. After biosynthesis, KIT traffics to the cell surface via the Golgi and immediately migrates to EL through endocytosis in a manner dependent on its kinase activity. However, results of phosphorylation imaging show that KIT is preferentially activated on the Golgi. Indeed, blockade of *KIT*<sup>N822K</sup> migration to the Golgi with BFA/M-COPA inhibits the activation of KIT downstream molecules, such as AKT, ERK, and STAT5, indicating that KIT signaling occurs on the Golgi. Moreover, lipid rafts in the Golgi play a role in KIT signaling. Interestingly, *KIT*<sup>V560G</sup> in HMC-1.1 migrates and activates downstream in a similar manner to *KIT*<sup>N822K</sup> in Kasumi-1.

**Conclusions:** In AML, *KIT*<sup>N822K</sup> mislocalizes to EL. Our findings, however, suggest that the mutant transduces phosphorylation signals on lipid rafts of the Golgi in leukemia cells. Unexpectedly, the *KIT*<sup>V560G</sup> signal platform in MCL is similar to that of *KIT*<sup>N822K</sup> in AML. These observations provide new insights into the pathogenic role of KIT mutants as well as that of other mutant molecules.

**Keywords:** Leukemia, KIT tyrosine kinase, Golgi, Lipid rafts, AKT, STAT5, ERK, Endocytosis

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## Background

KIT is a member of the type III receptor tyrosine kinase (RTK) family that includes platelet-derived growth factor receptor A/B (PDGFRA/B), *fms*, and *fms*-like tyrosine kinase 3 (FLT3) [1–3]. It is known to participate in tyrosine phosphorylation signals at the PM, ensuring cell growth and survival in hematopoietic cells, mast cells, interstitial cells of Cajal, germ cells, and melanocytes [4–6].

KIT is composed of *N*-glycosylated immunoglobulin domains in the amino-terminal extracellular portion, transmembrane region, juxta-membrane (JM) region, and the carboxy-terminal cytoplasmic tyrosine kinase domain [6, 7]. Stem cell factor (SCF), a ligand for KIT, stimulates KIT phosphorylation on selective tyrosine residues, such as Y703 and Y721, and these phospho-tyrosines serve as docking sites for downstream molecules [7–9]. SCF-KIT activates the phosphatidylyl 3-kinase-AKT pathway and the RAS-MEK-ERK cascade, which control gene expression, metabolism, and cytoskeletal architecture [6, 9–11]. The JM region plays a role in autoinhibition of the receptor through *intra*-molecular binding [12]. Thus, constitutively active mutations of *KIT* allow host cells to autonomously proliferate, resulting in the development of AML, MCL, GIST, germ cell tumors, and melanoma [6, 13–16]. In particular, *KIT* mutations in the JM region (eg, *V560G*, *deletion* etc.) are found in 70% of GIST patients [17–19]. A tyrosine kinase inhibitor, imatinib mesylate (Gleevec), has been developed for the treatment of GIST, and it has dramatically improved the prognosis of patients [19, 20]. However, *KIT*-bearing mutations in the kinase activation loop (AL), such as *D816V*, cause a loss of sensitivity to imatinib [17, 18, 21, 22]. In comparison to JM mutants (mut), *KIT*<sup>N822K</sup> is also imatinib-resistant but to a lesser degree than *KIT*<sup>D816V</sup> [17, 22, 23].

Previously, we reported that in MCL, *KIT*<sup>D816V</sup> (human) and *KIT*<sup>D814Y</sup> (mouse) activate AKT and the signal transducer and activator of transcription 5 (STAT5) in EL and on the ER, respectively (Table 1) [24, 25]. Furthermore, previous studies showed that in cells other than those of MCL, such as GIST and NIH3T3 cells,

JM-mut or AL-mut accumulates on the Golgi apparatus, where it initiates oncogenic signals (Table 1) [26–29]. These studies raised important questions as to whether mutant *KIT* initiates signaling from intracellular compartments in other cancers such as AML, and whether the mutation status of *KIT* affects the platform for oncogenic signaling.

*KIT* mutations have been found in approximately 30% of CBF-AML patients who have chromosome aberrations [31–33]. Recent studies showed that active *KIT* mutations are correlated with a poor prognosis in AML patients [31, 32]. The major activating *KIT* mutations are found at D816 and N822 (26 cases and 14 cases in 63 *KIT* mutation-positive patients, respectively) [33]. Although spatio-temporal analyses of *KIT*<sup>D816V</sup> signals have been performed [24, 25, 28], it is unclear whether the *N822K* mutation in leukemia affects *KIT* localization and the signal platform.

We then investigated the relationship between *KIT*<sup>N822K</sup> localization and tyrosine phosphorylation signals in Kasumi-1 cells (an AML cell line) that endogenously express *KIT*<sup>N822K</sup>. Furthermore, we examined whether *KIT*<sup>V560G</sup> in HMC-1.1 (MCL) caused signaling on the Golgi, ER, PM, or EL. In Kasumi-1, *KIT* is preferentially found in EL. Newly synthesized *KIT* in the ER traffics to the PM through the Golgi and subsequently relocates to EL through endocytosis in a manner dependent on its kinase activity. Our immunofluorescence assay, however, showed that *KIT* autophosphorylation preferentially occurs on the Golgi. Indeed, *KIT*<sup>N822K</sup> activates AKT, ERK, and STAT5 on the Golgi in Kasumi-1 cells. Moreover, lipid rafts in the Golgi play a role in *KIT* signaling. Interestingly, *KIT*<sup>V560G</sup> in MCL transduces signals in the Golgi in a similar manner to *KIT*<sup>N822K</sup> in AML but not to *KIT*<sup>D816V</sup> in MCL. Our study demonstrates that both *KIT*<sup>N822K</sup> and *KIT*<sup>V560G</sup> are mainly present in EL, but that their signal platform in leukemia cells is the lipid rafts of the Golgi. Furthermore, blockade of mutant *KIT* incorporation into the lipid rafts may provide a new strategy for suppression of growth signals in leukemia cells.

## Methods

### Cell culture

Kasumi-1, SKNO-1 (JCRB Cell Bank, Osaka, Japan), HMC-1.1 (Merck Millipore, Darmstadt, Germany), HMC-1.2 and pt18 cells were cultured at 37 °C in RPMI1640 medium supplemented with 10% FCS, penicillin, streptomycin, glutamine (Pen/Strep/Gln), and reducing agents (0.5 mM monothioglycerol or 50 μM 2-mercaptoethanol). For expansion of SKNO-1, 10 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF, Peprotech, Rocky Hill, NJ) was used. GIST-T1 cells (Cosmo Bio, Tokyo, Japan) were cultured at 37 °C in

**Table 1** Summary of *KIT* localization and signaling in AML, MCL, and GIST

Cell line, <i>KIT</i> mutation	<i>KIT</i> localization	Downstream activation	Reference
Kasumi-1 (AML), <i>N822K</i>	EL	Golgi	This study
HMC-1.1 (MCL), <i>V560G</i>	EL	Golgi	
HMC-1.2/RCM (MCL), <i>D816V/D814Y</i>	EL	ER, EL	[24, 25]
GIST cell lines ( <i>ex11/17</i> etc...), NIH3T3 (transfected <i>KIT</i> <sup>D816V</sup> etc...)	Golgi	Golgi	[26–29, 30]

AML acute myeloid leukemia, MCL mast cell leukemia, GIST gastrointestinal stromal tumor, Ex exon, EL endo-lysosomes, ER endoplasmic reticulum

DMEM supplemented with 10% FCS and Pen/Strep/Gln. All human cell lines were authenticated by Short Tandem Repeat analysis at JCRB Cell Bank (Osaka, Japan) and tested for *Mycoplasma* contamination with a MycoAlert Mycoplasma Detection Kit (Lonza, Basel, Switzerland).

### Chemicals

Imatinib (Cayman Chemical, Ann Arbor, MI) and PKC412 (Selleck, Houston, TX) were dissolved in DMSO. Bafilomycin A1, brefeldin A (Sigma, St. Louis, MO), monensin (Biomol, Exeter, UK), and cer-C6 (Cayman Chemical) were dissolved in ethanol. M-COPA (also known as AMF-26) was synthesized as previously described [34, 35] and dissolved in DMSO.

### Antibodies

The sources of purchased antibodies were as follows: KIT ( $M^{-14}$ ), cathepsin D ( $H^{-75}$ ), STAT5 (C-17), ERK2 (K-23), ARF1 (ARFS 3F1), GBF1 (25), PTP1B (D-4), SHP-1 (D-11), and SHP-2 (B-1) from Santa Cruz Biotechnology (Dallas, TX); KIT [pY703] (D12E12), KIT (D13A2), LAMP1 (lysosome-associated membrane protein 1, D4O1S), AKT (40D4), AKT [pT308] (C31E5E), STAT5 (D2O6Y), STAT5[pY694] (D47E7), ERK1/2 (137F5) and ERK [pT202/pY204] (E10) from Cell Signaling Technology (Danvers, MA); PDI (RL90), TFR (transferin receptor, ab84036), giantin (ab24586), and GM130 (EP892Y) from Abcam (Cambridge, UK); TFR (H68.4) from Thermo Fisher Scientific (Rockford, IL); calnexin (ADI-SPA-860) from Enzo (Farmingdale, NY); GM130 (clone 35) from BD Transduction Laboratories (Franklin Lakes, NJ); LAMP1 (L1418) from Sigma (St. Louis, MO) and KIT (104D2) from Biolegend (San Diego, CA). HRP-labeled secondary antibodies were purchased from the Jackson Laboratory (Bar Harbor, MA). Alexa Fluor-conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR).

### Immunofluorescence confocal microscopy

Cells in suspension culture were fixed with methanol for 10 min at  $-20^{\circ}\text{C}$  or with 4% paraformaldehyde for 20 min at room temperature, then cyto-centrifuged onto coverslips. GIST-T1 cells were cultured on poly-L-lysine-coated coverslips and fixed as above. Fixed cells were permeabilized and blocked for 30 min in PBS supplemented with 0.1% saponin and 3% BSA, and then incubated with a primary and a secondary antibody for 1 h each. After washing with PBS, cells were mounted with Fluoromount (DiagnosticBioSystems, Pleasanton, CA). Confocal images were obtained with an Fluoview FV10i (Olympus, Tokyo, Japan) or a TCS SP5 II/SP8 (Leica, Wetzlar, Germany) laser scanning microscope. Composite figures were prepared with Photoshop Elements 10

and Illustrator CS6 software (Adobe, San Jose, CA). Pearson's R were calculated with NIH ImageJ 1.48v software.

### Western blotting

Lysates prepared in SDS-PAGE sample buffer were subjected to SDS-PAGE and electro-transferred onto PVDF membranes. Immunodetection was performed by ECL (PerkinElmer, Waltham, MA). Sequential re-probing of membranes was performed after the complete removal of primary and secondary antibodies in stripping buffer (Thermo Fisher Scientific), or inactivation of peroxidase by 0.1% sodium azide. Results were analyzed with an LAS-3000 with Science Lab software (Fujifilm, Tokyo, Japan) or a ChemiDoc XRC+ with Image Lab software (BIORAD, Hercules, CA).

### Flow cytometry

Leukemia cells were directly collected from a cell suspension by centrifugation. GIST-T1 cells were scraped from culture dishes and then centrifuged. Cells were stained with anti-KIT (104D2) and Alexa Fluor 488-conjugated anti-mouse IgG in PBS supplemented with 0.5% calf serum and 0.1%  $\text{NaN}_3$  at  $4^{\circ}\text{C}$  for 1 h each. Stained cells were fixed with 4% paraformaldehyde for 20 min at room temperature. Flow cytometric data were obtained by FACSCalibur (BD Biosciences, Franklin Lakes, NJ), and results were analyzed with FlowJo software (Tomy Digital Biology, Tokyo, Japan).

### Gene silencing with small interfering RNA (siRNA)

For silencing *ARF1*, *GBF1*, *PTP1B*, *SHP-1*, or *SHP-2* genes, ON-TARGETplus SMARTpool siRNAs were purchased from Dharmacon (Lafayette, CO). ON-TARGETplus Non-targeting Pool (Dharmacon) was used as a source of control siRNAs. Electroporation was performed using a Neon Transfection System (Thermo Fisher Scientific), according to the manufacturer's instructions.

### Cell proliferation assay

To measure Kasumi-1 and HMC-1.1 proliferation, cells were cultured with Gleevec or Midostaurin (referred to henceforth as imatinib and PKC412, respectively) for 18 h, and then treated with [ $^3\text{H}$ ] thymidine deoxyribonucleotide (TdR) for 12 h. Cell proliferation was evaluated by the incorporation of [ $^3\text{H}$ ] TdR.

### Analysis of protein glycosylation

Following the manufacturer's instructions (New England Biolabs, Ipswich, MA), NP-40 cell lysates were treated with endoglycosidases for 1 h at  $37^{\circ}\text{C}$ . The reactions were stopped with SDS-PAGE sample buffer, and products were resolved by SDS-PAGE and immunoblotted.

### Statistical analyses

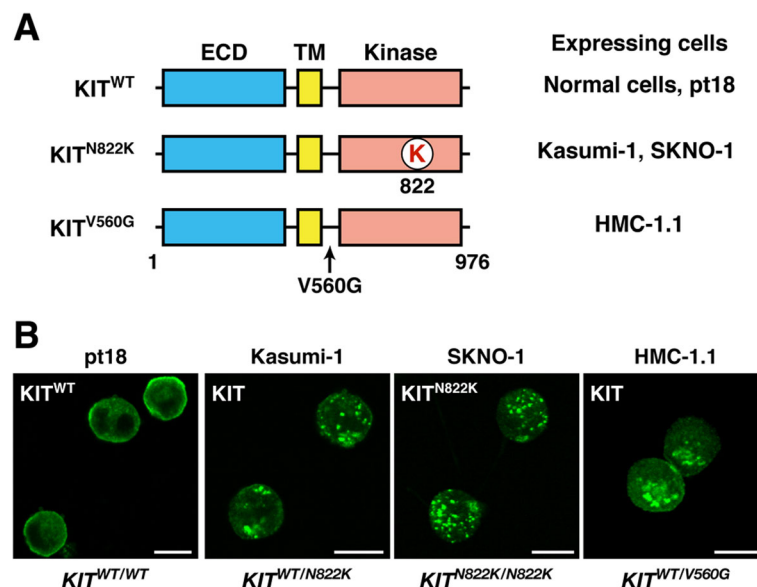
For statistical analysis, experiments were repeated as three biological replicates. Differences between two or more groups were analyzed by the two-tailed Student's *t*-test or one-way analysis of variance followed by Dunnett's multiple comparison *post-hoc* test, respectively. All significant differences stated indicate a 5% level of probability.

### Results

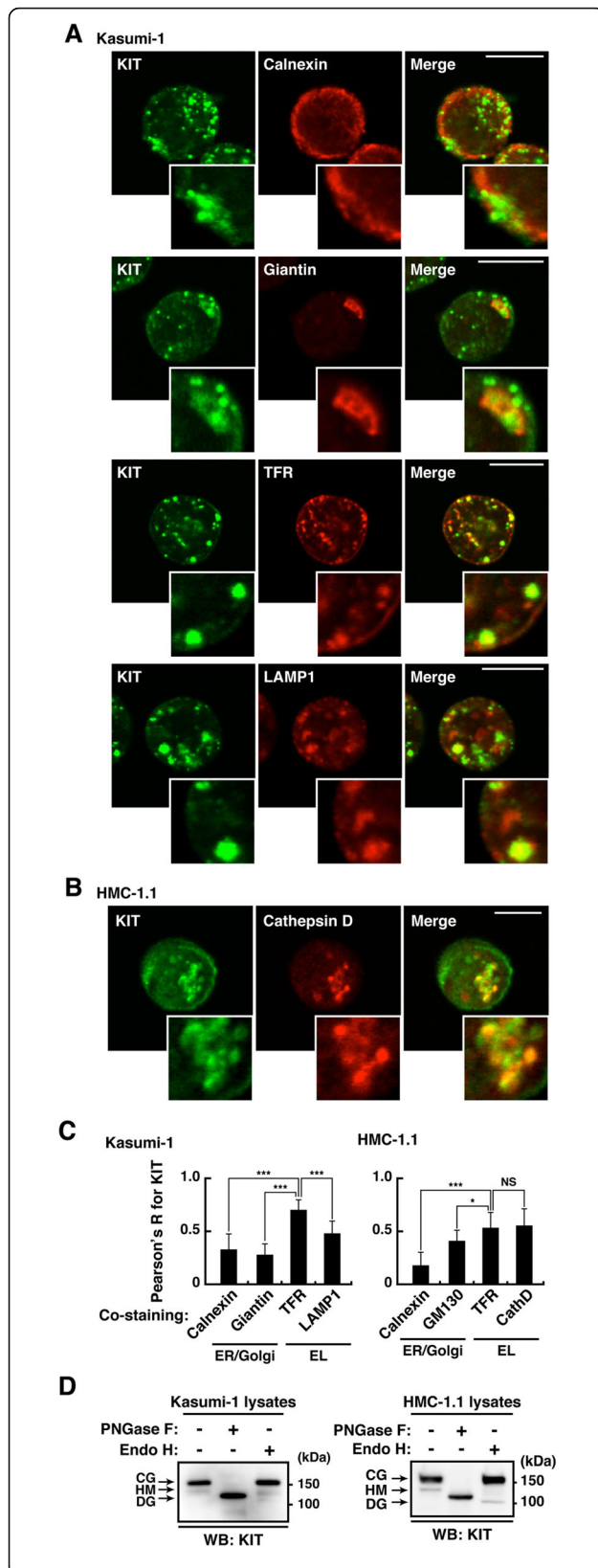
#### $KIT^{N822K}$ and $KIT^{V560G}$ mislocalize to EL in leukemia cells

To investigate the sub-cellular localization of endogenous KIT, we performed confocal immunofluorescence microscopic analyses in pt18 (mouse mast cell line, *KIT* wild-type (*WT*)), Kasumi-1 (human AML, *KIT*<sup>WT/N822K</sup>), SKNO-1 (human AML, *KIT*<sup>N822K/N822K</sup>), and HMC-1.1 (human MCL, *KIT*<sup>WT/V560G</sup>) (Fig. 1a). As previously described [24], most *KIT*<sup>WT</sup> localized to the PM in pt18 (Fig. 1b, left). In contrast, KIT accumulated in vesicular structures in Kasumi-1, SKNO-1, and HMC-1.1 (Fig. 1b). We then performed co-staining assays to identify these structures. In Kasumi-1, KIT was co-localized with TFR (transferrin receptor, endosome marker) and LAMP1 (lysosome marker) rather than with calnexin (ER marker) or giantin (Golgi marker) (Fig. 2a). Similarly, in HMC-1.1, KIT in vesicular structures was colocalized with TFR and cathepsin D (lysosome marker) (Fig. 2b; Additional file 1: Figure S1A). By calculating Pearson's R correlation coefficients (Pearson's R) for

KIT versus organelle markers, the intensity from KIT-TFR was significantly greater than that from KIT-calnexin, -giantin, and -LAMP1 in Kasumi-1 (Fig. 2c, left graph), suggesting that KIT mainly localizes to endosomes. The quantification showed that in HMC-1.1, KIT was colocalized with TFR to a similar extent as with cathepsin D (Fig. 2c, right graph). In both types of cells, KIT was colocalized with EL markers rather than with ER/Golgi markers. We previously showed that in MCL and GISTs, KIT mutants are normally complex-glycosylated in the Golgi [24, 26]. To test for the KIT glycosylation state in Kasumi-1 and HMC-1.1, we treated KIT with endoglycosidase H, which digests immature high-mannose forms of KIT but not mature complex-glycosylated forms. Figure 2d shows that most KIT in these leukemia cells was in a complex-glycosylated form, similar to normal KIT [24, 28, 29]. KIT shifted to a non-glycosylated form following the complete digestion of N-linked glycans by peptide-N-glycosidase F. In SKNO-1, as with Kasumi-1 and HMC-1.1, KIT was complex-glycosylated (Additional file 1: Figure S1B). Pearson's R quantification from immunofluorescence images showed that in SKNO-1, KIT localized to endosomes to a similar extent as to lysosomes and it was found in EL rather than on ER/Golgi (Additional file 1: Figure S1C & D). These results suggest that complex-glycosylated KIT accumulates in EL in leukemia cells but not in the early secretory compartments.



**Fig. 1** N822K- or V560G-mutated KIT mis-localizes to vesicular structures in leukemia cells. **a** Schematic representations of wild-type KIT (*KIT*<sup>WT</sup>) and constitutively active KIT mutants (*KIT*<sup>N822K</sup> and *KIT*<sup>V560G</sup>) showing the extracellular domain (ECD), the transmembrane domain (TM), the kinase domain, the lysine mutation at 822 (K in red), and the glycine mutation at 560 (V560G). **b** Kasumi-1, SKNO-1, HMC-1.1, or pt18 cells were immunostained with anti-KIT. Bars, 10  $\mu$ m. Note that *KIT*<sup>WT</sup> localized to the PM, whereas KIT mutants accumulated on vesicular compartments



**Fig. 2** KIT mutants localize to EL but not to the PM in leukemia cells. **a & b** Kasumi-1 (**a**) or HMC-1.1 cells (**b**) were double-stained with anti-KIT (green) plus the indicated antibody (red). Insets show magnified images. Bars, 10  $\mu$ m. **c** Pearson's R correlation coefficients were calculated by analyzing the intensity of KIT vs. organelle markers. Results are means  $\pm$  s.d. ( $n = 12\sim 22$ ). \* $P < 0.05$ , \*\*\* $P < 0.001$ . NS, not significant. Calnexin (ER marker); giantin (Golgi marker); GM130 (Golgi marker); TFR (endosome marker); LAMP1 (lysosome marker); cathepsin D (cathD, lysosome marker). **d** Lysates from Kasumi-1 (left) or HMC-1.1 cells (right) were treated with peptide N-glycosidase F (PNGase F) or endoglycosidase H (endo H) then immunoblotted with anti-KIT. CG, complex-glycosylated form; HM, high mannose form; DG, deglycosylated form. Note that most KIT was present in a complex-glycosylated form in these leukemia cells

**KIT mutants autonomously migrate from the PM to EL through endocytosis in a manner dependent on their kinase activity**

Next, we examined the role of KIT kinase activity on cell proliferation, growth signals, and KIT localization. As previously reported [36–39], Kasumi-1 and HMC-1.1 proliferate autonomously, and KIT tyrosine kinase inhibitors (TKIs), such as imatinib and PKC412, suppressed cell proliferation in a dose-dependent manner (Fig. 3a). Immunoblotting showed that phosphorylation of KIT, AKT, ERK, and STAT5 occurred in the absence of TKIs, and PKC412 and imatinib reduced these phosphorylations (Fig. 3b), confirming that KIT activates AKT, ERK, and STAT5 in Kasumi-1 and HMC-1.1. Next, we investigated the localization of KIT in TKI-treated cells by immunofluorescence and flow cytometry. In Kasumi-1 cells treated with TKIs, KIT localized more in PM (outside ER staining, Fig. 3c & d) and less in endosomes (Fig. 3e & f). Similar results were seen with HMC-1.1 and SKNO-1 (Additional file 1: Figure S1E). Collectively, in these leukemia cells, newly synthesized KIT in the ER moves to the PM along the secretory pathway and subsequently traffics to EL through kinase activity-dependent endocytosis. In addition to our previous findings that KIT<sup>D816V</sup> and KIT<sup>Δ560–578</sup> in the PM are increased by TKI treatment [24, 26, 40], these results suggest that retention in the PM by TKIs is a ubiquitous feature of KIT mutants.

**Autophosphorylation of KIT<sup>N822K</sup> and KIT<sup>V560G</sup> predominantly occurs on the Golgi in leukemia cells**

We next examined the site of KIT activation in leukemia cells. To determine the signal platform for KIT, we immuno-stained for phospho-tyrosine residues in the kinase domain which would indicate KIT activation [7–9, 26, 27]. In Kasumi-1 cells, phospho-KIT Y703 (pKIT [Y703]) was clearly detected (Fig. 4a), although pKIT [Y721], [Y730], and [Y936] were undetectable by our immunofluorescence staining (data not shown). Interestingly, compared with KIT localization to EL, pKIT

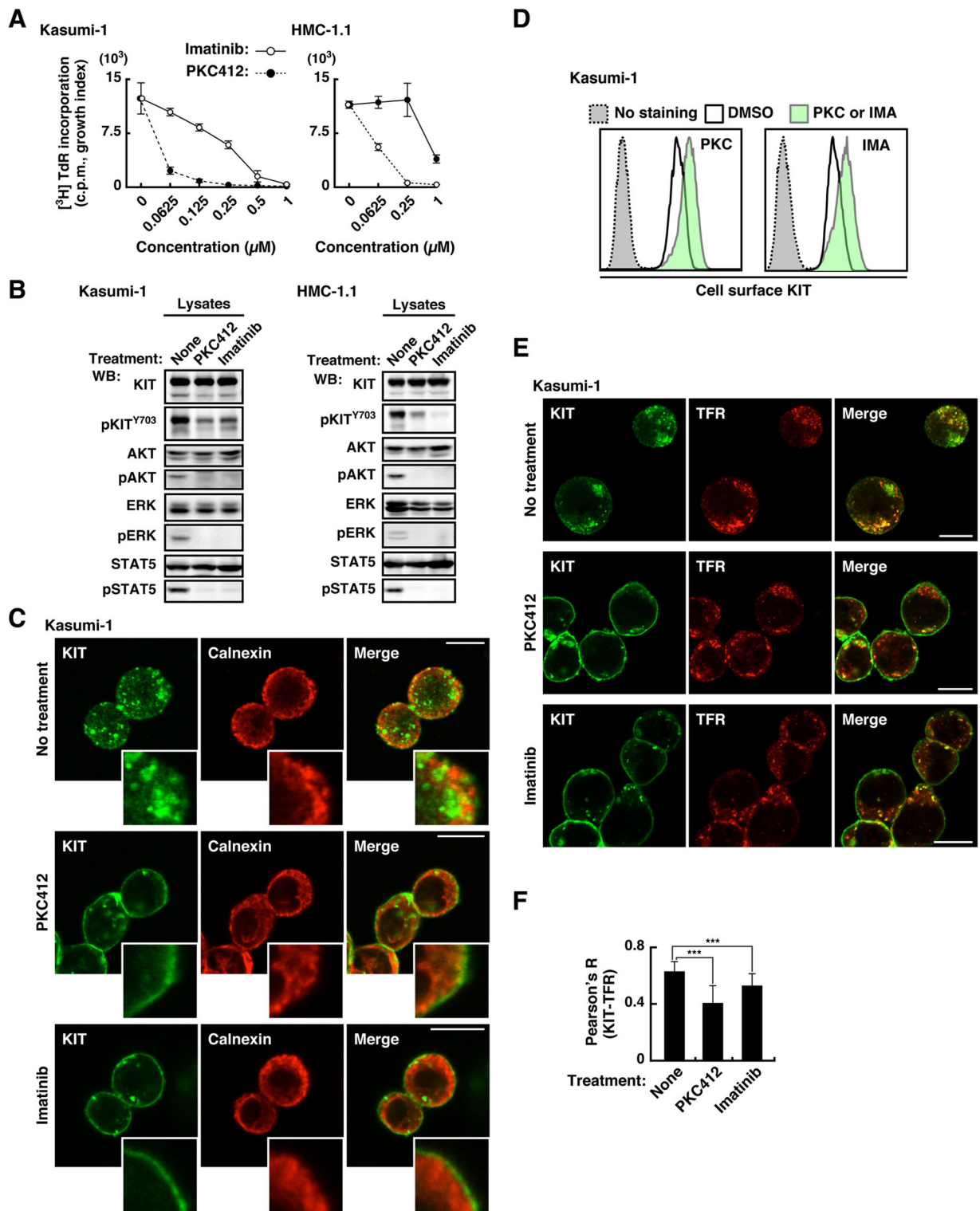


Fig. 3 (See legend on next page.)

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**Fig. 3** KIT migrates to EL through endocytosis in a manner dependent on their kinase activity. **a** Kasumi-1 or HMC-1.1 cells were cultured for 24 h in the presence of KIT kinase inhibitors (imatinib, open circles; PKC412, closed circles). The graphs show the levels of [<sup>3</sup>H] thymidine deoxyribonucleotide (TdR) incorporation into cells (counts per minute, c.p.m., growth index) at the indicated inhibitor concentrations. Results are means ± s.d. (*n* = 3). **b** Kasumi-1 or HMC-1.1 cells were treated for 4 h with 1 μM PKC412 or 1 μM imatinib. Lysates were immunoblotted for KIT, phospho-KIT Y703 (pKIT<sup>Y703</sup>), AKT, pAKT, STAT5, pSTAT5, ERK, and pERK. **c-f** Kasumi-1 cells were treated for 12 h with 1 μM PKC412 (PKC) or 1 μM imatinib (IMA). **c** Cells were immunostained with anti-KIT (green) and anti-calnexin (ER marker, red). Confocal immunofluorescence images are shown. Insets show magnified images of the PM region. Bars, 10 μm. **d** Cell surface KIT levels determined by flow cytometry are shown. Non-permeabilized cells were stained with anti-KIT extracellular domain antibody. Green histogram, with KIT inhibitor treatment; white histogram, no KIT inhibitor; gray histogram, no anti-KIT antibody control. **e** Cells were immunostained with anti-KIT (green) and anti-TFR (endosome marker, red). Confocal immunofluorescence images are shown. Bars, 10 μm. **f** Pearson's R correlation coefficients were calculated by analyzing the intensity of KIT vs. TFR. Results are means ± s.d. (*n* = 22–29). \*\*\**P* < 0.001. Note that these inhibitors lowered KIT in vesicular structures and increased KIT in the PM

[Y703] was restricted to the perinuclear region in Kasumi-1 (Fig. 4a, top panels, arrowheads). Similar to KIT in Kasumi-1, KIT in HMC-1.1 was found in the perinuclear compartment (Fig. 4b). Perinuclear KIT autophosphorylation was colocalized with GM130 (Golgi) rather than with PDI (ER), TFR (endosomes), or LAMP1 (lysosomes) (Fig. 4c; Additional file 1: Figure S2A). Similar results were obtained with SKNO-1 (Additional file 1: Figure S2B & C). These results suggest that in leukemia cells, activation of KIT<sup>N822K</sup> and KIT<sup>V560G</sup> occurs predominantly on the Golgi although KIT itself is found mainly in EL.

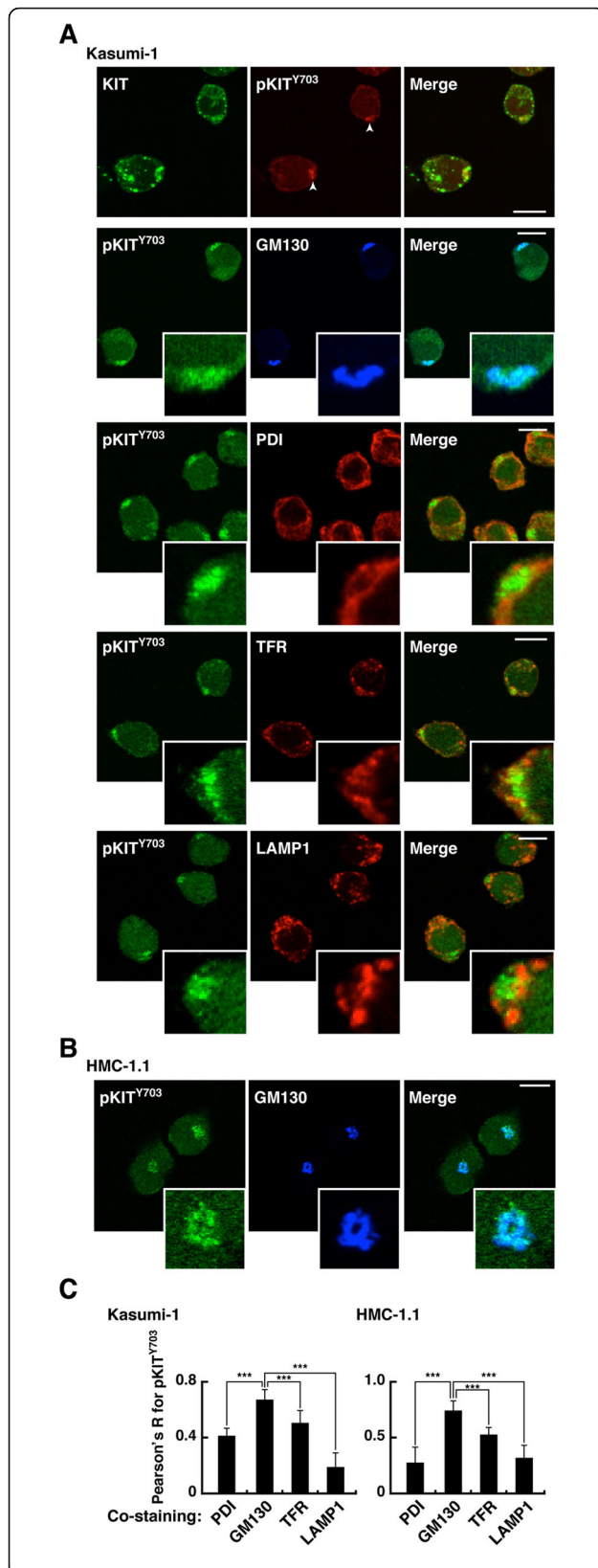
#### KIT<sup>N822K</sup> and KIT<sup>V560G</sup> mainly activate downstream molecules on the Golgi in leukemia cells

We then examined whether KIT activated downstream molecules on the Golgi in leukemia cells. To resolve this question, we used inhibitors of intracellular trafficking, such as brefeldin A (BFA), 2-methylcoprophilnamide (M-COPA) (inhibitors of ER export to the Golgi) [27, 34, 35, 41], monensin (an inhibitor of *intra*-Golgi trafficking) [26, 42], and bafilomycin A1 (BafA1, an inhibitor of endosome-to-lysosome trafficking) [24, 43]. In Kasumi-1 and HMC-1.1, treatment with BFA or M-COPA significantly increased colocalization of KIT with an ER marker, calnexin (Fig. 5a & Additional file 1: Figure S3A), confirming that the treatment inhibited ER export of KIT. Immunoblotting showed that KIT shifted to a lower molecular weight in BFA- or M-COPA-treated cells because of a defect in full glycosylation on the Golgi apparatus (Fig. 5b & c, top panels). KIT on the ER was dephosphorylated and unable to activate downstream molecules (Fig. 5b & c). Previous studies showed that a major target of BFA/M-COPA is Golgi-specific BFA-resistance guanine nucleotide exchange factor 1 (GBF1) that plays a role in the secretory pathway through activation of ADP ribosylation factor 1 (ARF1) [34, 44, 45]. Interestingly, knockdown (KD) of *ARF1* and *GBF1* with siRNAs did not cause a defect in full glycosylation of KIT or inhibition of signaling (Additional file 1: Figure S3B). Since BFA and M-COPA bind not only to the ARF1-GBF1 complex but also to other complexes

[44, 45], the blockers affect KIT trafficking in a manner independent of ARF1-GBF1 inhibition in the leukemia cells used in this study. Further study will be required for understanding how the inhibitors block KIT trafficking from the ER.

Fig. 5d shows that inhibition of the Golgi export of KIT through blocking *intra*-Golgi trafficking did not suppress KIT signaling, suggesting that Golgi-localized KIT is sufficient for oncogenic signaling in Kasumi-1 and HMC-1.1. As shown in Additional file 1: Figure S3C, KIT signals remained in BafA1-treated cells, indicating that endosome-to-lysosome trafficking is unnecessary for downstream activation. Taken together, these results suggest that the Golgi apparatus serves as the platform for KIT activation in leukemia cells. To support this conclusion, we stained for phospho-AKT (pAKT), pERK, and pSTAT5. As shown in Fig. 5e, these phosphorylations were found at the Golgi region in Kasumi-1 cells. Compared with pAKT, total AKT was barely seen in the Golgi (Additional file 1: Figure S3D, upper panels). In Kasumi-1, only part of AKT may be activated by KIT. Furthermore, total ERK and STAT5 were distributed in the Golgi region (Additional file 1: Figure S3D). These results support our hypothesis that KIT activates these downstream molecules on the Golgi in leukemia cells. In HMC-1.1, AKT, ERK, STAT5, and their phospho-forms showed a diffuse distribution compared with those in Kasumi-1 (Additional file 1: Figure S3E). Since pAKT, pERK, pSTAT5, though small, were found in the Golgi region, they could be activated on the Golgi and subsequently move elsewhere.

Recently, we showed that in GISTs, KIT on the ER is dephosphorylated by protein tyrosine phosphatases (PTPs) [27]. We then considered the role of PTPs in KIT inactivation in the ER in leukemia cells. In M-COPA-pretreated Kasumi-1, a 3-h treatment with a PTP inhibitor (sodium orthovanadate, Na<sub>3</sub>VO<sub>4</sub>) [46] restored pKIT [Y703], resulting in downstream reactivation (Fig. 5f, left), indicating that in Kasumi-1, PTPs play a role in KIT inactivation in the ER. In M-COPA-treated HMC-1.1, pKIT [Y703] and pSTAT5 were recovered by Na<sub>3</sub>VO<sub>4</sub> treatment, but AKT and ERK did not become



**Fig. 4** Autophosphorylation of KIT<sup>N822K</sup> and KIT<sup>Y560G</sup> occurs preferentially on the Golgi in leukemia cells. **a** & **b** Kasumi-1 (**a**) or HMC-1.1 cells (**b**) were immunostained for KIT (green), phospho-KIT Y703 (pKIT<sup>Y703</sup>, red or green) together with GM130 (Golgi marker, blue), PDI (protein disulfide isomerase, ER marker, red), TFR (endosome marker, red), or LAMP1 (lysosome marker, red). Insets show magnified images. Bars, 10 μm. **c** Pearson's R correlation coefficients were calculated by analyzing the intensity of pKIT<sup>Y703</sup> vs. organelle markers. Results are means ± s.d. (n = 12~21). \*\*\*P < 0.001. Note that pKIT<sup>Y703</sup> was colocalized with GM130 rather than with PDI, TFR, or LAMP1 both in Kasumi-1 and HMC-1.1 cells

active on PTP inhibition (Fig. 5f, right). Negative regulation of AKT and ERK may differ among cell types. Taken together, these results suggest that ER-localized KIT is inactivated by PTPs. PTP1B, Src homology 2 containing PTP-1 (SHP-1), and SHP-2 have been reported as PTPs for KIT and FLT3 RTKs [47–50]. Thus, we knocked down these PTPs and treated cells with M-COPA to investigate the key PTPs for KIT in the ER. Additional file 1: Figure S4 shows that in M-COPA-treated cells, pKIT [Y703], pAKT, and pERK were not restored by PTP1B or SHP1/2 KD, suggesting that these PTPs are not responsible for this dephosphorylation in the ER. Interestingly, PTP1B but not SHP1/2 KD partially rescued pSTAT5 in M-COPA-treated cells (Additional file 1: Figure S4A, arrows). Although we were unable to identify KIT phospho-tyrosine sites that are dephosphorylated by PTP1B in this study, PTP1B in the ER may play a role in inactivation of the KIT-STAT5 axis.

SKNO-1 cells were similar to Kasumi-1 in phospho-regulation of KIT in intracellular compartments (Additional file 1: Figure S5A & B). However, AKT, ERK, and STAT5 were not activated by KIT<sup>N822K</sup> (Additional file 1: Figure S5C). SKNO-1 requires GM-CSF for proliferation, but the cytokine did not affect the activation of KIT, AKT, ERK, or STAT5 (Additional file 1: Figure S5C, right panels). AKT and ERK were not found in specific compartments in SKNO-1 in the presence or absence of GM-CSF (Additional file 1: Figure S5D). At present, we are unable to find downstream molecules that are activated by KIT<sup>N822K</sup> in SKNO-1 cells. We will investigate the role of KIT<sup>N822K</sup> in SKNO-1 growth in the near future.

**Lipid rafts play a key role in KIT signaling, which occurs on the Golgi apparatus**

Recent studies showed that sphingomyelin-enriched membrane microdomains (lipid rafts) in the Golgi are needed for activation of an innate immunity molecule, STING [51, 52]. Formation of normal lipid microdomains is inhibited by N-hexanoyl-D-erythro-sphingosine (cer-C6) through producing short chain sphingomyelin that disrupts the lipid order [51, 53, 54]. Figure 6a shows



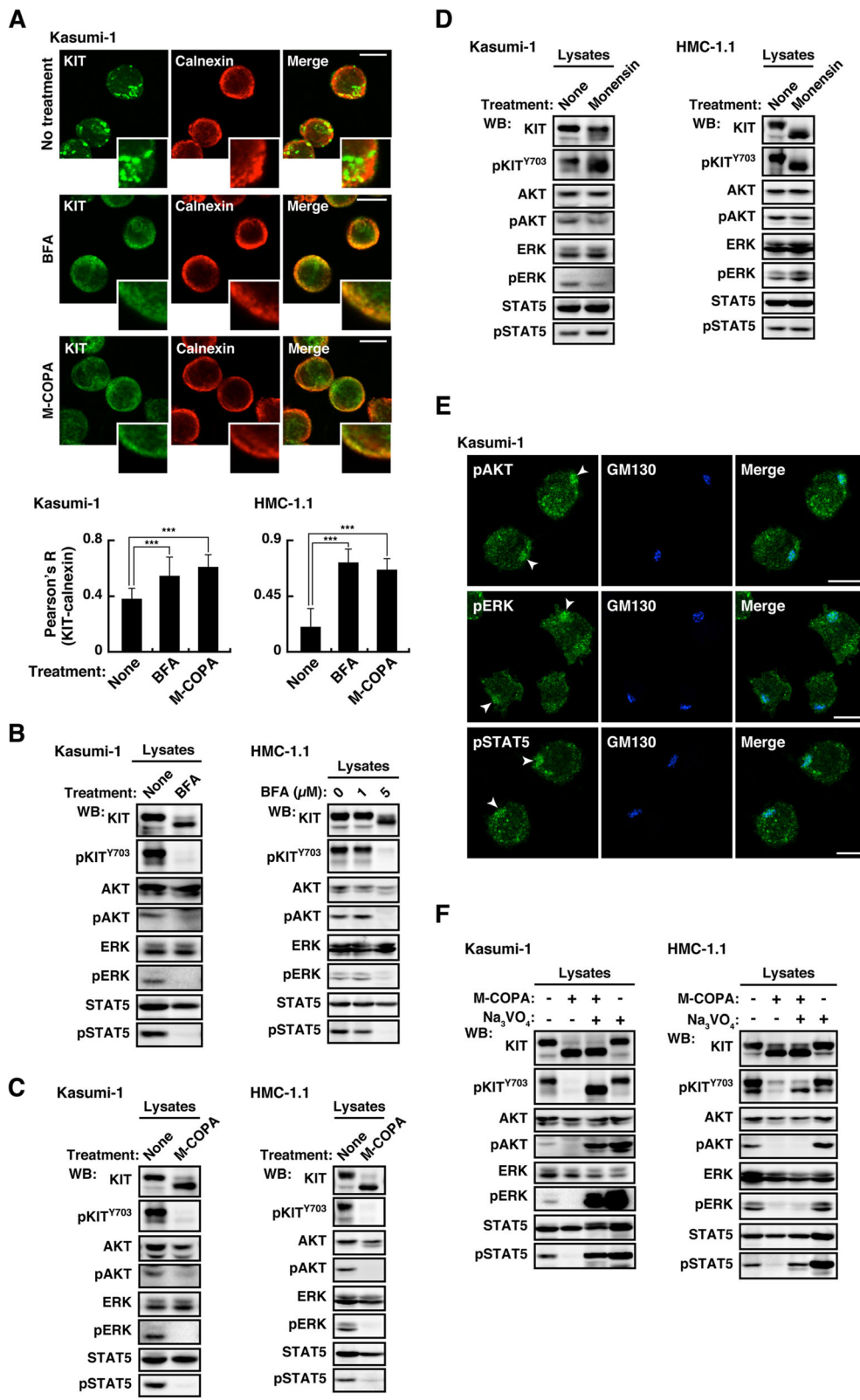


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**Fig. 5** In Kasumi-1 and HMC-1.1, KIT activates downstream pathways on the Golgi apparatus. **a** Kasumi-1 cells were cultured for 12 h in the presence of 1  $\mu$ M BFA or 1  $\mu$ M M-COPA (inhibitors of ER export to the Golgi) and immunostained for KIT and calnexin (ER marker, red). Bars, 10  $\mu$ m. Pearson's R correlation coefficients were calculated by analyzing the intensity of KIT vs. calnexin. The right graph shows Pearson's R (KIT-calnexin) for HMC-1.1 cells treated with 5  $\mu$ M BFA or 1  $\mu$ M M-COPA for 16 h. Results are means  $\pm$  s.d. ( $n = 14\sim 20$ ).  $***P < 0.001$ . **b-d** Kasumi-1 cells were treated for 12 h with 1  $\mu$ M BFA, 1  $\mu$ M M-COPA, or 250 nM monensin (an inhibitor of Golgi export to the PM). HMC-1.1 cells were treated with 1–5  $\mu$ M BFA, 1  $\mu$ M M-COPA for 16 h, or 250 nM monensin for 24 h. Lysates were immunoblotted. **e** Kasumi-1 cells were immunostained for phospho-AKT (pAKT, green), pERK (green), pSTAT5 (green), and GM130 (Golgi marker, blue). Bars, 10  $\mu$ m. Arrowheads indicate the Golgi region. **f** Cells were treated with 1  $\mu$ M M-COPA for 12 h (Kasumi-1) or 16 h (HMC-1.1), including 3 mM  $\text{Na}_2\text{VO}_4$  (a PTP inhibitor) during the last 3 h, then immunoblotted

that in Kasumi-1, cer-C6 treatment lowered the protein levels of KIT and inhibited KIT autophosphorylation and the activation of AKT, ERK, and STAT5 in a dose-dependent manner. The treatment did not decrease but rather increased KIT on the Golgi (Fig. 6b & c). These results suggest that KIT<sup>N822K</sup> and KIT<sup>V560G</sup> require lipid rafts for their stability and activation in the Golgi in these leukemia cells.

Finally, we asked whether lipid rafts play a role in oncogenic signaling by all KIT mutants. GIST-T1 cells (KIT <sup>$\Delta$ 560–578</sup>) grow in a manner dependent on KIT signaling on the Golgi, whereas HMC-1.2 (mast cell leukemia, KIT<sup>V560G/D816V</sup>) requires pAKT on EL and pSTAT5 on the ER [24–27] (Additional file 1: Figure S6A & Table 1). In both cell types, TKIs increased PM distribution of KIT mutants (Additional file 1: Figure S6B), supporting our data obtained with Kasumi-1 that mutant KIT localizes to intracellular compartments in a manner dependent on its kinase activity. In GIST-T1, cer-C6 inhibited the phosphorylation of KIT and downstream molecules (Fig. 6d). Unlike KIT in leukemia cells, that in cer-C6-treated GIST-T1 assumed an immature glycosylated form (Fig. 6e), indicating that KIT is complex-glycosylated in GIST after reaching lipid rafts. Similar to the results using Kasumi-1, the treatment did not decrease but rather increased KIT on the Golgi (Additional file 1: Figure S6C). On the other hand, in HMC-1.2, cer-C6 did not have an inhibitory effect on Golgi export of KIT<sup>D816V</sup> and growth signals (Fig. 6f; Additional file 1: Figure S6D). Therefore, lipid rafts play a critical role in KIT signaling that occurs on the Golgi.

## Discussion

In this study, we demonstrate that in leukemia cells, localization of KIT<sup>N822K</sup> and KIT<sup>V560G</sup> is clearly different from that of KIT<sup>WT</sup> in normal cells. We provide evidence that after secretory trafficking to the PM, these mutants localize to EL through kinase activity-dependent endocytosis. However, they are autophosphorylated predominantly on the Golgi apparatus, where they activate downstream molecules, such as AKT, ERK, and STAT5. Lipid rafts play a key role in KIT signaling on the Golgi. Moreover, ER-localized KIT is dephosphorylated by PTPs (Fig. 7). This phospho-regulation of KIT is similar

to that in GISTs. Our observations show that in some cases, receptor mutants can initiate signals from the Golgi even if they are mainly present in EL (Table 1).

Recently, we reported that in MCL, AL-mut, such as KIT<sup>D816V</sup> (human) and KIT<sup>D814Y</sup> (mouse), accumulates in EL (Table 1) [24, 25]. Unlike JM-mut, AL-mut in MCL activates AKT and STAT5 on EL and the ER, respectively. Previous studies showed that transfected AL-mut in cell lines other than MCL, such as NIH3T3 and GISTs, localizes to the Golgi, where it initiates oncogenic signals on the Golgi apparatus (Table 1) [26, 28]. The host cell environment rather than the KIT mutation status may determine the mutant's subcellular localization. Considering that JM-mut in MCL activates a downstream pathway on the Golgi, these studies suggest that AL-mut activates downstream molecules on EL and the ER only when it is expressed in an MCL environment. Moreover, there is great interest in further investigation as to whether KIT<sup>D816V</sup> in AML causes growth signaling on the ER, Golgi, or EL. Further studies will be required for understanding the mechanism responsible for the difference in the signal platforms of KIT.

In addition to previous reports on MCL and GISTs [24, 26, 30], this study suggests that KIT<sup>N822K</sup> also mis-localizes to intracellular organelles in AML. Permanently active KIT mutations are found in about 25% of mucosal melanomas and seminomas [22, 55–57]. Thus, we will examine the relationship between KIT localization and oncogenic signaling in these cancers in the near future. Furthermore, recent studies reported that cancer-causing receptor mutants and splice variants also accumulate on intracellular compartments in an aberrant manner [58]. FLT3-internal tandem duplication (FLT3-ITD, AML), FGFR3<sup>K650E</sup> (multiple myeloma), PDGFRA<sup>Y288C</sup> or <sup>V561D</sup> (carcinomas/GIST), and a splice variant of TRKA (TRKAIL, neuroblastoma) are found in early secretory compartments [59–65], and EGFR<sup>L858R/T790M</sup>, GP130 <sup>$\Delta$ YY</sup>, and CSF3R<sup>T618I</sup> mislocalize to endosomes [66–68]. These reports and the findings of our study raised the intriguing possibility that mislocalization in cancer cells is a ubiquitous feature of aberrant receptors.

In innate immune cells, STING binds to exogenous DNA fragments in the ER, then moves to lipid rafts of

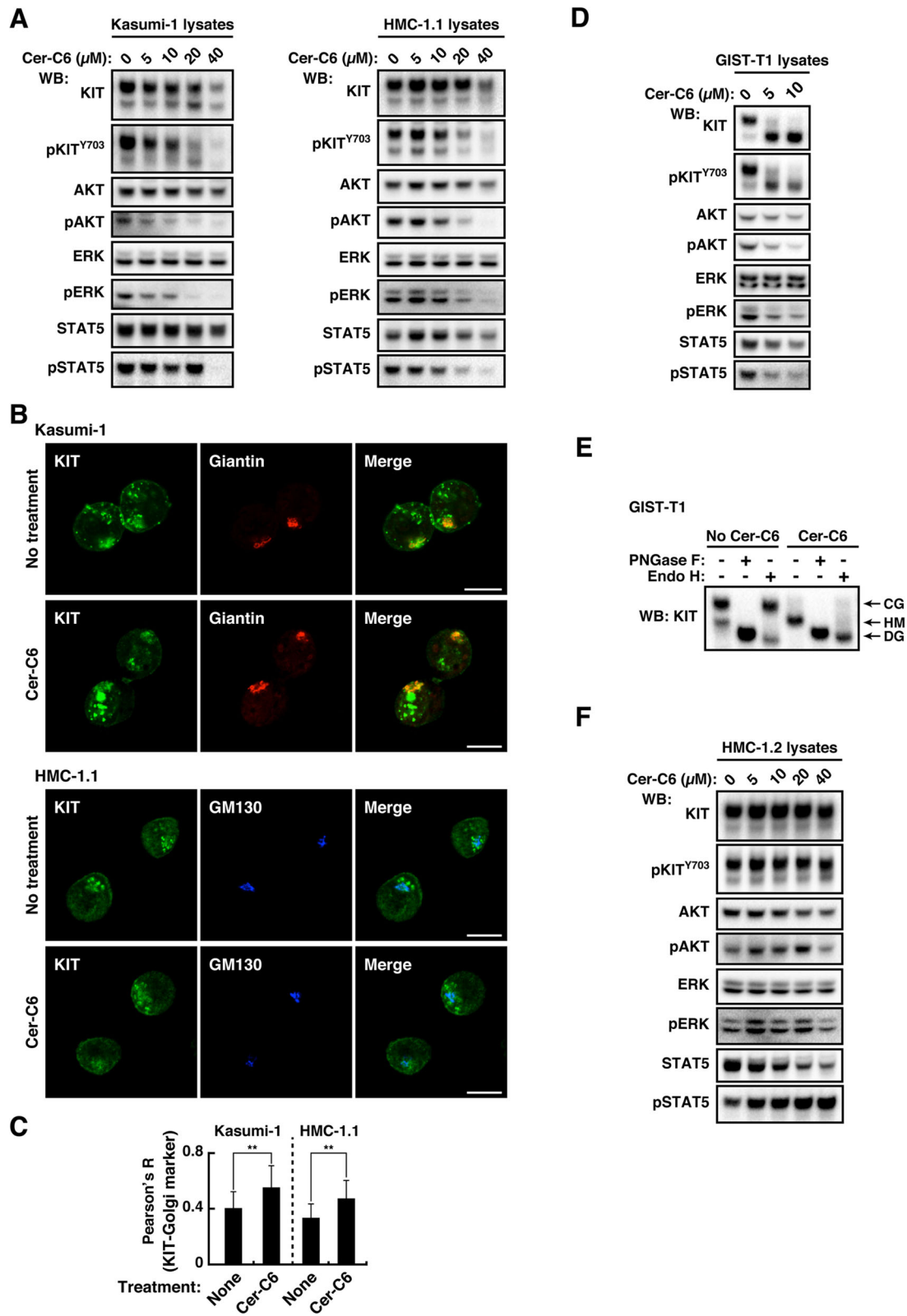


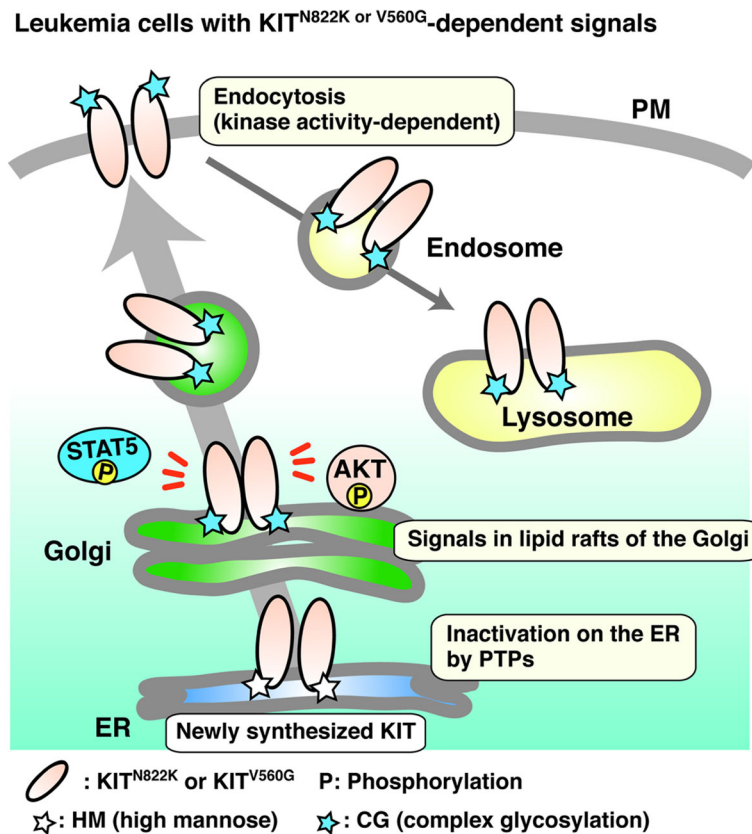
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**Fig. 6** Lipid rafts have a role in KIT signaling at the Golgi apparatus. **a-c** Kasumi-1 or HMC-1.1 cells were treated with 0~40 μM cer-C6 for 8 h (for inhibition of normal lipid raft formation). **a** Lysates were immunoblotted. **b** Cells treated with 40 μM cer-C6 for 8 h were immunostained for KIT (green), giantin (Golgi marker, red), or GM130 (Golgi marker, blue). Bars, 10 μm. **c** Pearson's R correlation coefficients were calculated by analyzing the intensity of KIT vs. giantin (Kasumi-1) or GM130 (HMC-1.1). Results are means ± s.d. (n = 16~22). \*\*P < 0.01. **d & e** GIST-T1 cells were treated with 0~10 μM cer-C6 for 10 h. **d** Lysates were immunoblotted. **e** Lysates were treated with PNGase F or endoglycosidase H then immunoblotted with anti-KIT. CG, complex-glycosylated form; HM, high mannose form; DG, deglycosylated form. **f** HMC-1.2 cells were treated with 0~40 μM cer-C6 for 8 h, then immunoblotted

the Golgi [51, 52]. This requires palmitoylation of its cysteine residues for migration to the lipid rafts, where it can activate the TBK1-IRF3 pathway [51]. MET tyrosine kinase is activated and palmitoylated on its extracellular domain at the Golgi [69, 70], and lipid rafts contribute to receptor distribution and stability [70, 71]. These studies raise the interesting possibility that incorporation of KIT into the lipid rafts of the Golgi is involved in protein acylation. Investigation as to whether palmitoylation is necessary for KIT signaling on the Golgi is now under way.

PTPs play a role in inactivation of KIT in the ER. Our loss of function study showed that PTP1B, SHP-1, and SHP-2 are not major PTPs for KIT dephosphorylation in the ER, suggesting the role of other PTPs in KIT inactivation. In addition, negative regulators of KIT and downstream molecules could be abundant in intracellular compartments other than the Golgi. Further analyses of the localization and functions of these negative regulators should explain how KIT signaling is inactivated in the ER, PM, and EL both in leukemia and GISTs. In



**Fig. 7** Model of mutant KIT trafficking and signals on the Golgi in leukemia cells. Newly synthesized mutant KIT (KIT<sup>N822K</sup> or KIT<sup>V560G</sup>) in the ER traffics to the PM through the Golgi apparatus. They are normally complex-glycosylated in the Golgi. After reaching the PM, mutant KIT immediately undergoes endocytosis in a manner dependent on its kinase activity, then accumulates in EL. However, its full autophosphorylation mainly occurs on the Golgi, where it causes downstream activation. Lipid rafts play a role in KIT signaling. ER-localized KIT is inactivated by PTPs

other words, the study will reveal the mechanism of de-regulation of RTK on signal platforms.

In this study, KIT mutants were retained in the PM in TKI-treated cells, since TKIs inhibit endocytosis of KIT, which depends on the kinase activity. Furthermore, TKIs increase PM localization of EGFR in lung cancers and PDGFRA/KIT in GISTs [26, 63, 72]. A previous report showed that TKI treatment increases the FLT3-ITD PM level in AML, which enhances the effect of FLT3-directed immunotherapy in mice [73]. Moreover, anti-KIT antibody is efficacious for suppression of the autonomous growth of GIST cells [74, 75]. From a clinical point of view, combined therapy with anti-RTK antibodies and TKIs seems attractive.

Small molecule TKIs and antibodies against RTKs have been developed for suppression of proliferative signals in cancers. In this study, blockade of the ER export of KIT with BFA/M-COPA decreased KIT's autophosphorylation in leukemia cells. Together with the results previous reports [25, 27, 76, 77], our findings may offer a trafficking blockade of receptor mutants as a third strategy for inhibition of oncogenic signaling.

## Conclusions

In conclusion, we show that in leukemia cells, N822K- and V560G-mutated KIT can initiate growth signals in lipid rafts of the Golgi apparatus. These observations provide new insights into the pathogenic role of KIT mutants as well as into that of other mutant signaling molecules. Moreover, from a clinical point of view, our findings offer a new strategy for leukemia treatment through that blocks the incorporation of KIT mutants into the lipid rafts of the Golgi.

## Additional file

**Additional file 1: Figure S1.** Mutant KIT localizes preferentially to EL in HMC-1.1 and SKNO-1 cells. **Figure S2.** In HMC-1.1 and SKNO-1, the major site for KIT autophosphorylation is colocalized with the Golgi region. **Figure S3.** Distribution of signal molecules in Kasumi-1 and HMC-1.1 cells. **Figure S4.** Effect of knockdown of *PTP1B*, *SHP-1*, and *SHP-2* on KIT signals. **Figure S5.** KIT<sup>N822K</sup> does not activate AKT, ERK, and STAT5 in SKNO-1 cells. **Figure S6.** Effect of inhibition of normal lipid raft formation on KIT distribution. (PDF 14400 kb)

## Abbreviations

AML: Acute myeloid leukemia; BafA1: Bafilomycin A1; BFA: Brefeldin A; CerC6: N-hexanoyl-D-erythro-sphingosine; EL: Endosome-lysosomal compartments; ER: Endoplasmic reticulum; ERK: Extracellular signal-regulated kinase; FLT3: Fms-like tyrosine kinase 3; GIST: Gastrointestinal stromal tumor; MCL: Mast cell leukemia; M-COPA: 2-methylcoprophilinamide; mut: Mutant; PDGFR: Platelet-derived growth factor receptor; pKIT: Phospho-KIT; PM: Plasma membrane; PTP: Protein tyrosine phosphatase; RTK: Receptor tyrosine kinase; SCF: Stem cell factor; STAT: Signal transducer and activator of transcription; TKI: Tyrosine kinase inhibitor; wt: Wild-type

## Acknowledgments

The authors thank Dr. Hirohisa Saito and Dr. Kenji Matsumoto (National Center for Child Health and Development) for providing HMC-1.2 cells and Dr. Ryo Goitsuka (Tokyo University of Science) for pt18.

## Grant support

The Japan Society for the Promotion of Science (18 K07208 to YO, 19H03722 to TN, and 15 K06843)  
Japan Agency for Medical Research and Development (to IS, KO, YO, and TN)  
Takeda Science Foundation (to YO)  
Friends of Leukemia Research Fund (to YO)  
Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics (to YO)

## Authors' contributions

YO conceived, designed, performed and analyzed the data from all experiments, and wrote the manuscript. YH performed flow cytometric analysis and Western blotting. IS supervised the total synthesis of M-COPA and edited the manuscript. TM carried out the synthesis of M-COPA and helped to draft the manuscript. YT participated in the first half of the synthesis of M-COPA. KS participated in the second half of the synthesis of M-COPA. KI synthesized the key intermediate of M-COPA. ST performed immunofluorescence confocal microscopic analysis. KY carried out Western blotting. TT provided advice on the design of the in vitro experiments. KO analyzed the data and edited the manuscript. TN provided advice on the design of the in vitro experiments and edited the manuscript. RA conceived and supervised the project, analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

## Funding

This work was supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science (18K07208 to YO, 19H03722 to TN, and 15 K06843 to RA), by the Japan Agency for Medical Research and Development (to IS, KO, YO, and TN), and by research grants from the Takeda Science Foundation, the Friends of Leukemia Research Fund, and the Kawano Masanori Memorial Public Interest Incorporated Foundation for Promotion of Pediatrics (to YO).

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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Received: 14 March 2019 Accepted: 22 August 2019

Published online: 04 September 2019

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