Clathrin Assembly Protein CALM Plays a Critical Role in KIT Signaling by Regulating Its Cellular Transport from Early to Late Endosomes in Hematopoietic Cells

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Abstract

CALM is implicated in the formation of clathrin-coated vesicles, which mediate endocytosis and intracellular trafficking of growth factor receptors and nutrients. We previously found that CALM-deficient mice suffer from severe anemia due to the impaired clathrin-mediated endocytosis of transferrin receptor in immature erythroblast. However, CALM has been supposed to regulate the growth and survival of hematopoietic stem/progenitor cells. So, in this study, we focused on the function of CALM in these cells. We here show that the number of Linage-^Sca-1^KIT^+^ (LSK) cells decreased in the fetal liver of CALM^-/-^ mice. Also, colony forming activity was impaired in CALM^-/-^ LSK cells. In addition, SCF, FLT3, and TPO-dependent growth was severely impaired in CALM^-/-^ LSK cells, while they can normally proliferate in response to IL-3 and IL-6. We also examined the intracellular trafficking of KIT using CALM^-/-^ murine embryonic fibroblasts (MEFs) engineered to express KIT. At first, we confirmed that endocytosis of SCF-bound KIT was not impaired in CALM^-/-^ MEFs by the internalization assay. However, SCF-induced KIT trafficking from early to late endosome was severely impaired in CALM^-/-^ MEFs. Furthermore, SCF-induced phosphorylation of cytosolic KIT was enhanced and prolonged in CALM^-/-^ MEFs compared with that in WT MEFs, leading to the excessive activation of Akt. Similar hyperactivation of Akt was observed in CALM^-/-^ KIT^+^ cells. These results indicate that CALM is essential for the intracellular trafficking of KIT and its normal functions. Also, our data demonstrate that KIT located in the early endosome can activate downstream molecules as a signaling endosome. Because KIT activation is involved in the pathogenesis of some malignancies, the manipulation of CALM function would be an attractive therapeutic strategy.

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Introduction

Clathrin-mediated endocytosis (CME) is an active cellular process for membrane trafficking, which mediates the entry of nutrients and growth factor receptors into the cells [1]. When the target molecule binds to plasma membrane receptor, clathrin and accessory molecules such as AP2, epsin, clathrin assembly lymphoid myeloid leukemia protein (CALM) are recruited from cytoplasm into the membrane, resulting in the formation of clathrin-coated vesicles. Clathrin-coated vesicles transport target molecules from the membrane to early and late endosomes. Also, it regulates the intercellular trafficking among endosomes, trans-Golgi network (TGN), and lysosomes, thereby regulating the degradation of the target molecules [1–3].

Receptor tyrosine kinases (RTKs) such as KIT, epidermal growth factor receptor (EGFR) and MET play crucial roles in the development and maintenance of the cells, on which they are expressed [4–7]. Upon the ligand binding, activated RTKs are internalized and transferred to early endosome. Then, clathrin-coated RTKs are partially sorted back to the plasma membrane via recycling endosome (RE). The remaining RTKs are transported to the intraluminal vesicles (ILV) of late endosomes/multivesicular body (MVB). MVB can be fused to the lysosomes, where RTKs are degraded [8]. Previously, it was believed that several signaling molecules such as MAPKs, JAK/STATs, and PI3K/AKT are activated by RTKs on the plasma membrane. However, several recent reports showed that these signaling molecules can also be activated by clathrin-coated RTKs.
located in the endosome, leading to the establishment of the concept "signaling endosome" [9–11].

**CALM** encodes a 652 aa protein with multiple domains such as AP180 N-terminal homology (ANTH) domain, DPF motif, NPF motif, and type I and II clathrin-binding sequences (CBS I and II), of which expression is ubiquitously observed in various organs [12–18]. Knockdown of CALM by RNA interference leads to the formation of larger and more irregular, clathrin-coated vesicles in HeLa cells, indicating that CALM is required for proper formation of clathrin-coated vesicles [19]. **CALM** was originally isolated as a part of the fusion gene **CALM/AF10**, which results from the chromosomal translocation t(10;11) (p13;q14) [20]. This translocation is found in acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML) and malignant lymphomas [21]. Also, it was shown that overexpression of **CALM/AF10** in primary murine bone marrow (BM) cells resulted in the development of an aggressive form of leukemia in a murine BM transplantation model [22,23]. These results suggest that CALM would play an important role in the growth and differentiation of hematopoietic cells. This hypothesis was subsequently supported by the reports that **fit1** mutants, which contain nonsense point mutations in the **CALM** gene [24,25]. In these mice, the number of early hematopoietic progenitor cells was severely reduced and numerous morphologic and functional defects were observed in the peripheral blood. However, detailed analysis on the hematopoietic defects in **fit1** mutants has not been performed.

To clarify the physiological role of **CALM** in vivo, we recently generated **CALM**-deficient mice [26]. Although **CALM**+/− mice didn’t show an apparent phenotype, **CALM**−/− mice exhibited retarded growth in utero and were dwarfed throughout their shortened life-spans. Moreover, **CALM**-deficient mice suffered from severe anemia due to the impaired CME of transferrin in immature erythroblast. Meanwhile, based on the report of **fit1** mutants [24,25], **CALM** has been supposed to regulate the growth and survival of hematopoietic stem/progenitor cells. So, in this study, we focused on the molecular mechanism through which **CALM** regulates their growth and survival. We here show that KIT-mediated growth was impaired in **CALM**-deficient hematopoietic stem/progenitor cells, probably due to the defect in the KIT trafficking from early to late endosomes.

**Materials and Methods**

**Ethics Statement**

This study was approved by the Committee of Animal Experiments, Kinki University Faculty of Medicine (approval ID: 06-13).

**Recombinant Growth Factors and Inhibitors**

Recombinant murine stem cell factor (SCF), Flt-3 ligand (FL), thrombopoietin (TPO), interleukin-3 (IL-3), and IL-6 were purchased from PeproTech. (Rocky Hill, NJ). Imatinib mesylate (STI571) purchased from Selleck (Houston, TX) was used to inhibit KIT activity. Bafilomycin A1 purchased from Sigma Aldrich (St. Louis, MO) was used to inhibit the protein transport from early to late endosome [27].

**Isolation and Immortalization of Murine Embryonic Fibroblasts (MEFs)**

Primary MEFs were isolated from wild-type (WT) and **CALM**-deficient mice on embryonic day 14.5 (E14.5) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 15% fetal calf serum (FCS) and 5% CO2 at 37°C. To immortalize MEFs, we transfected the expression vector for SV40 large T antigen into MEFs by Lipofectamine 2000 Reagent (Invitrogen, Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Stable immortalized clones were obtained by serial dilution.

**Retrovirus Transduction**

Murine full-length Kit cDNA kindly provided from Dr. Mizuki M. (Osaka University, Osaka, Japan) was subcloned into pMSCV-IRESCatGFP bicistronic retrovirus vector. The retrovirus vector was transfected into a packaging cell line 293T containing the expression plasmids for gag and pol, which was cultured in DMEM supplemented with 10% FCS. The supernatant was collected 48 h after transfection. MEFs were plated onto the 5.5 cm dish coated with fibronectin fragment (RetroNectin, Takara Bio, Shiga, Japan) and cultured with 1 ml virus supernatant for 72 h. Retrovirus-infected MEFs were isolated as GFP-positive cells by FACS Aria (BD Biosciences, San Jose, CA).

**Purification of Murine Lineage− Sca-1+ KIT+ (LSK) Cells**

Murine fetal liver cells were harvested from E14.5 embryos and mononuclear cells (MNCs) were separated by density gradient centrifugation. Then, MNCs were incubated with the antibodies (Abs) as follows: anti-lineage Abs (a cocktail of biotinylated Abs against CD3e (145-2C11), CD45R/B220 (RA3-6B2), Gr-1 (RB6–8C5), and TER-119 (TER-119)), fluorescein isothiocyanate (FITC)-conjugated anti-Sca-1 Ab (D7), allophycocyanin (APC)-conjugated anti-c-KIT Ab (2B8), and streptavidin-phycocerythrin (PE)-cy-cyan (cy7 (BD Biosciences). After staining, LSK cells were sorted by FACS Aria. Non-viable cells were eliminated by the staining with 7-amo-actinomycin D (Calbiochem, Merck Millipore, Darmstadt, Germany).

**Clonogenic Assay**

LSK cells from WT or **CALM**−/− mice were plated onto Complete Medium with murine Cytokines MethoCult GF M3434 (StemCell Technologies, Vancouver, BC, Canada). The numbers of colony forming unit-mixed (CFU-Mix), CFU-granulocyte macrophage (CFU-GM), and burst-forming unit-erythroid (BFU-E) were counted under the inverted microscope 14 days after plating.

**Proliferation Assay**

LSK cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Gibco, Life Technologies, Carlsbad, CA) with 10% FCS containing 100 ng/ml, SCF, 100 ng/ml FL, 100 ng/ml TPO, 100 ng/ml IL-3, 100 ng/ml IL-6 at 37°C. Number of viable LSK cells was measured by the Cell Titer Glo Reagent (Promega, Madison, WI) from the intensity of the luminescence using an Envision plate reader (1420 ARVO MX-2, Wallac, PerkinElmer, Inc., Waltham, MA).

**Flow Cytometric Analysis**

The expression of surface molecules was examined by FACS Aria using the appropriate Abs and these results were analyzed by BD FACS Diva software (BD Biosciences) or Flowjo software (TreeStar, Ashland, OR). To analyze cytoplasmic AKT phosphorylation in KIT+ hematopoietic cells by flow cytometry, MNCs isolated from BM were fixed with 3.7% (w/v) formaldehyde in PBS for 15 min, and permeabilized with 1% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Triton X-100 in PBS for 15 min. These cells were incubated with the Alexa647-conjugated anti-phosphorylated Akt Ab (Cell Signaling Technology, Danvers, MA).
in combination with the Abs to identify KIT+ cells as described above. After staining, these cells were analyzed by FACS Aria.

Internalization Assay
Internalization for KIT was performed as described previously [26]. Briefly, WT and CALM−/− MEFs both engineered to express KIT were cultured with biotinylated SCF (R&D systems, Minneapolis, MN) for 60 min, and further incubated with the APG-conjugated streptavidin (Biolegend, San Diego, CA) for 30 min at 4 °C. Then, these cells were incubated at 37 °C up to 20 min to allow internalization. After stripping unincorporated SCF with acidic buffer (20 mM MES pH 5.130 mM NaCl, 2 mM CaCl2 and 0.1% BSA), relative amount of internalized SCF-KIT complex was evaluated from the fluorescence intensity by FACS at the indicated times compared with the initial amount of membrane KIT.

Immunofluorescence Analysis
KIT-transfected MEFs were transferred onto the coverslips and cultured in DMEM supplemented with 15% FCS at 37°C for 48 h. After the stimulation with 100 ng/ml SCF for the indicated times, the cells were washed with ice-cold PBS and then fixed with 3.7% (w/v) formaldehyde in PBS for 15 min. After incubation in blocking buffer with 1% (v/v) bovine serum albumin (BSA) and 0.1% (v/v) Triton X-100 in PBS for 15 min, the fixed cells were incubated with the primary and then with the secondary Abs suspended in the reagents for each 4 min. The utilized Abs and reagent were as follows: biotinylated anti-KIT (2B8, Biolegend), anti-Rab5 (C6B1), anti-Rab7 (D95F2), anti-Rab11 (D4F5) Abs (Cell Signaling Technology), AlexaFluor 488-conjugated anti-rabbit IgG Ab, AlexaFluor 568 streptavidin conjugates (Invitrogen). After washing with PBS, the coverslips were mounted on glass slides using Prolong Gold antifade reagent (Invitrogen) and observed under the confocal microscopy (LSM-410, Nikon, Tokyo, Japan).

Subcellular Protein Fractionation
Subcellular protein fractionation was performed using OptiPrep density gradient medium (Axis-Shield, Oslo, Norway) following their protocol (S23) with some modification. Briefly, cell lysates were centrifuged at 1000 g for 5 min to pellet nuclei and cell debris. Supernatant was loaded on the Opti-Prep discontinuous gradient (30, 25, 20, 15, 10, 5%) and centrifuged at 90,000 g for 45 min. The supernatant was centrifuged at 10,000 g for 10 min to pellet membranes. The remaining supernatant was further centrifuged at 100,000 g for 30 min to pellet ribosomes. The supernatant was used as a cytoplasmic extract. Then, the remaining membranes were resuspended in membrane extraction buffer at 4 °C for 20 min and centrifuged at 4 °C for 10 min and centrifuged at 500 g for 5 min. This supernatant was used as a membrane extract.

Immunoblot and Immunoprecipitation
Immunoblot analyses were performed as described previously [28]. Briefly, the cultured cells were lysed in lysis buffer containing 1% Nonidet P-40 (NP-40) and protease inhibitors, and insoluble materials were removed by centrifugation. The whole cell lysates (15 μg per each lane) or immunoprecipitated proteins were subjected to SDS-PAGE and electrophoretically transferred onto a polyvinylidene difluoride membrane (Immobilon, Millipore, Bedford, MA). After blocking the residual binding sites on the membrane, immunoblotting was performed with an appropriate Ab. For immunoprecipitation, protein extract was incubated with the appropriate Abs and protein A-Sepharose beads at 4°C for 6 h. After SDS-PAGE, protein was transferred to a nitrocellulose membrane. The membranes were incubated in TBST blocking buffer (4% nonfat dry milk in Tris-buffered saline-Tween 20, 0.15 M NaCl, 0.01 M Tris-HCl pH 7.4, 0.05% Tween 20) followed by the incubation with the primary Ab at room temperature for 1 h. The primary Abs utilized in this study were as follows: anti-CALM (G-18), and anti-actin Ab (Santa Cruz Biotech., Santa Cruz, CA), anti-phospho-tyrosine (4G10), anti-KIT (D13A2), anti-phosphorylated Akt (Ser473) (587F11), anti-Akt (C67E7), anti-phosphorylated p-44/42MAPK (T202/Y204) (20G11), anti-p44/42MAPK (ERK1/2) (137F5), anti-pan-Cadherin (28E12), anti-HSP90 (E289), anti-EEA1 (C8B1), and anti-LAMP1 (C54H11) Abs (Cell Signaling Technology). Then, the membranes were incubated with the appropriate secondary Abs diluted in blocking buffer, and immunoreactive proteins were visualized by enhanced chemiluminescence (LAS4010, GE Healthcare, Cleveland, OH).

Statistical Analysis
Statistical analysis was performed with the Student t test. Error bars indicate the standard deviation (SD) of the mean. P-values less than 0.05 were considered statistically significant.

Results
Number of LSK Cells Decreases in Fetal Liver of CALM−/− Mice
To analyze the role of CALM in hematopoietic stem/progenitor cells, we isolated fetal liver from WT, CALM+/−, and CALM−/− mice on E14.5. Fetal liver from CALM−/− mice was macroscopically small compared with that from WT and CALM+/− mice. In accord with this finding, total number of hematopoietic MNCs significantly decreased in the fetal liver of CALM−/− mice compared with that in WT and CALM+/− mice (Fig. 1A, left). However, the proportions of LSK cells in MNCs were almost same regardless of their genotypes (Fig. 1A, middle). Thus, total number of LSK cells in the fetal liver of CALM−/− mice was about 50% compared with that in WT and CALM+/− mice (Fig. 1A, right).

Colony Forming Activity and Cytokine-dependent Growth Are Impaired in CALM−/− LSK Cells
We also performed colony assays by plating 1,000 LSK cells into the semisolid medium each containing the cytokine cocktail appropriate for the development of CFU-Mix, CFU-GM, and BFU-E. As shown in Fig. 1B, no apparent difference was observed in clonogenic activity between WT and CALM−/− LSK cells. In contrast, CALM−/− LSK cells yielded significantly less numbers of CFU-GM, BFU-E, and GFF-Mix than WT and CALM+/− LSK cells, indicating that colony forming activities were impaired in CALM−/− LSK cells. In addition, we found that the size of CFU (especially of CFU-Mix) formed from CALM−/− LSK cells was apparently smaller than that from WT and CALM+/− cells (Fig. S1). These findings indicate that CALM−/− LSK cells would have less activity to proliferate in response to cytokines.

To confirm this hypothesis, we isolated LSK cells from fetal liver of WT, CALM+/+, and CALM−/− mice and cultured them
with SCF, FL, and TPO. As shown in Fig. 2A, the growth of CALM<sup>-/-</sup> LSK cells was apparently impaired compared with that of WT and CALM<sup>+/+</sup> LSKs. However, when IL-3 and IL-6 were added into this culture medium, the growth of CALM<sup>-/-</sup> LSK cells was not completely but partially recovered compared with that of WT and CALM<sup>+/+</sup> LSK cells (Fig. 2B). On the other hand, WT, CALM<sup>+/+</sup> and CALM<sup>-/-</sup> LSK cells show similar growth responses to IL-3 and IL-6 (Fig. 2C). These results indicate that CALM plays a crucial role in the transmission of growth signal from SCF, FL, and/or TPO but not from IL-3 or IL-6 whereas its haploinsufficiency doesn’t cause a clear defect.

**Endocytosis of SCF-bound KIT Is Not Impaired in CALM<sup>-/-</sup> MEFs**

We next focused on the KIT signaling, because CALM has been reported to be involved in the internalization and/or intracellular transport of RTKs [29]. As cytoplasmic area of immature hematopoietic cells was rather small and not suitable for immunofluorescence analysis, we utilized MEFs from WT and CALM<sup>-/-</sup> mice, both of which were engineered to express KIT by the retrovirus infection.

At first, we confirmed that KIT was expressed at a similar level on the cell surface of WT and CALM<sup>-/-</sup> MEFs by measuring EGFP, which was expressed together with KIT from a single RNA by the bicistronic promoter of the retrovirus vector (data not shown).

Next, we examined the internalization of KIT in WT and CALM<sup>-/-</sup> MEFs by incubating them with APC-labeled SCF at 37°C up to 60 min. After stripping unincorporated SCF, we quantified the amount of the internalized SCF from the intensity of fluorescence by FACS. As shown in Figure 3, after treatment with APC-conjugated SCF, the amount of SCF-KIT complex in the cytoplasm peaked at 20 min in both WT and CALM<sup>-/-</sup> MEFs, indicating that the internalization of KIT wouldn’t be impaired by CALM deficiency. Then, the level of SCF-KIT complex decreased to the near basal level at 30 min and remained low up to 60 min in WT MEFs, suggesting that the internalized KIT was degraded and/or recycled in WT MEFs. On the other hand, it remained high up to 60 min in CALM<sup>-/-</sup> MEFs, implying that the intracellular trafficking of internalized KIT in CALM<sup>-/-</sup> MEFs would be rather different from that in WT MEFs.

**SCF-Induced KIT Trafficking from Early to Late Endosomes Is Impaired in CALM<sup>-/-</sup> MEFs**

Next, we analyzed intracellular KIT transport in WT and CALM<sup>-/-</sup> MEFs by immunofluorescence analysis. KIT was detected on cell surface prior to SCF stimulation and internalized into cytoplasm 5 min after SCF stimulation in both WT and
CALM regulates intracellular trafficking of KIT

CALM−/− MEFs (Fig. 4, upper and lower panels, time 0 and 5). Then, the internalized KIT adhered to the intracellular compartments surrounding nucleus, and subsequently formed a numerous punctate pattern in the cytoplasm in WT MEFs. Finally, the majority (about 80%) of KIT disappeared at 30 min in WT MEFs (Fig. 4, upper panel). In contrast, KIT was still easily detectable with about 80% of the basal level in CALM−/− MEFs at 30 min (Fig. 4, lower panel), indicating that the clearance of the internalized KIT from cytoplasm was impaired in CALM−/− MEFs.

To analyze cytoplasmic localization of KIT in more detail, we utilized Rab5, Rab7, and Rab11 as a marker of early, late, and recycling endosome, respectively. At first, we confirmed that a significant proportion of CALM was colocalized with Rab5 at 5 min and with Rab7 at 30 min after SCF stimulation (Fig. S2). In accord with the localization of CALM, a substantial proportion of KIT was colocalized with Rab5 in both WT and CALM−/− MEFs 5 min after SCF stimulation (Fig. 5A), indicating that the transport of KIT to the early endosome isn’t disrupted in CALM−/− MEFs. Although only a faint signal of KIT was detectable in WT MEFs after 30-min SCF stimulation (about 20% of the basal level as shown in Fig. 4), the remaining KIT was colocalized with Rab7 in WT MEFs (Fig. 5B, upper panel). In contrast, KIT was scarcely colocalized with Rab7 in CALM−/− MEFs (Fig. 5B, lower panel). As for this reason, we found that KIT still remained in the early endosome in CALM−/− MEFs at this point (Fig. 5C, lower panel). On the other hand, CALM was scarcely colocalized with Rab11 in WT MEFs after SCF-treatment up to 30 min (the lowest panel in Fig. S2). Similarly, KIT wasn’t colocalized with Rab11 neither in WT nor in CALM−/− MEFs.

Figure 3. Endocytosis of SCF-bounded KIT isn’t affected by CALM deficiency. (A) WT and CALM−/− murine embryonic fibroblasts (MEFs) engineered to express KIT were incubated with biotinylated SCF for 60 min, and then with the APC-conjugated streptavidin for 30 min at 4 °C. After stripping unincorporated SCF, the amount of the internalized SCF-KIT complex was quantified from the fluorescence intensity at the indicated times. (B) Uptake of SCF in WT or CALM−/− MEFs. The vertical axis indicates the ratio of mean fluorescence intensity, MFI (internalized SCF-KIT complex/initial surface KIT) (Data represent means ± SD, n = 3, n.s.: not significant (p = 0.079)).

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Figure 4. Localization of KIT after SCF stimulation is altered in CALM−/− MEFs. Localization of KIT was analyzed before and after SCF stimulation under confocal microscopy using WT and CALM−/− MEFs engineered to express KIT. KIT was visualized by the biotinylated anti-KIT antibody (Ab) and AlexaFluor 568 streptavidin conjugates.

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To quantitatively demonstrate the difference in the intracellular localization of KIT between WT and CALM−/− MEFs after SCF treatment, we performed cell fractionation with OptiPrep density gradient. In this experiment, EEA1 was used as a marker of early endosome and LAMP1 as a marker of late endosome and lysosome, because these markers are more sensitive to discriminate each organella than Rab5 and Rab7 in this method. Treatment with SCF for 15 min led to the formation of a peak of KIT within late endosome to lysosome fractions (fractions 13–23) that are enriched in LAMP1 protein (Fig. 6). In contrast, although a small amount of KIT was detected in LAMP1-positive fractions (fractions 15–21), the majority of KIT was detected in EEA1-positive fractions (fractions 5–15) in CALM−/− MEFs. These results again indicate that SCF-induced KIT trafficking from early to late endosome was impaired in CALM−/− MEFs.

Next, we analyzed whether KIT signaling was also altered in CALM−/− MEFs because of the impaired trafficking from early to late endosome. For this purpose, we isolated membrane fraction (plasma, mitochondria and ER-Golgi membranes) and cytosolic fraction (containing endosomes) separately, of which separation was confirmed by the blotting with the Abs against pan-cadherin (reactive to only membrane fraction) and HSP90 (reactive to only cytosolic fraction). These lysates were subjected to immunoprecipitation and immunoblot analyses using the Abs indicated in Fig. 7. As shown in Fig. 7, 4th panel, both a mature (fully glycosylated, plasma membrane-bound, and functional) form and an immature (not fully glycosylated, membrane-unbound, and nonfunctional) form of KIT were detected in the membrane fraction with molecular weight 145 kDa and 120 kDa, respectively, of which levels were almost the same between WT and CALM−/− MEFs before SCF treatment. After 30-min SCF stimulation, the amount of mature KIT similarly decreased in both WT MEFS and CALM−/− MEFS (% decrease 17% and 13% by densitometric analysis, p = 0.67 (n = 5) (data not shown)). In addition, membrane KIT revealed similar phosphorylation pattern after SCF stimulation in both WT and CALM−/− MEFS (3rd panel). In contrast, although cytosolic KIT almost disappeared after 5-min SCF stimulation in WT MEFS, it was detected in CALM−/− MEFS (8th panel). This result seems to be inconsistent with our previous result obtained by immunofluorescence analysis indicating that KIT was still detectable in cytoplasm 5 min after SCF stimulation in WT MEFS (shown in Fig. 4, upper panel). As for this reason, we speculate that a substantial proportion of cytosolic KIT might be extracted as membrane protein because this fraction includes ER-Golgi membranes. Nonetheless, SCF-induced phosphorylation of cytosolic KIT (which is mainly located in early endosomes, Fig. 5A, 5B) was enhanced 5 min after SCF stimulation and prolonged up to 30 min in CALM−/− MEFS compared with that in WT MEFS (7th panel). In accord with this finding, SCF-induced phosphorylation of Akt was enhanced and prolonged in CALM−/− MEFS compared with that in WT MEFS (9th panel). In contrast, an apparent difference was not observed in ERK1/2 phosphorylation between these cells (11th panel). To confirm that the enhanced and prolonged phosphorylation of Akt observed in CALM−/− MEFS was dependent on KIT activity, we analyzed the effect of a KIT inhibitor, imatinib, on the activity of Akt after SCF stimulation in CALM−/− MEFS. As a result, we found that imatinib pretreatment inhibited both SCF-induced phosphorylations of KIT and Akt, implying that SCF-induced Akt phosphorylation was dependent on KIT activity (Fig. S4A). Furthermore, we confirmed that Bafilomycin A1, which inhibits protein transport from early to late endosome, enhanced and prolonged phosphorylation of KIT and Akt in SCF-stimulated WT MEFS as seen in CALM−/− MEFS (Fig. S4B). Taken together, these results indicate that KIT located in the early endosome can activate one of downstream molecules, Akt.

Next, we analyzed whether KIT signaling was also altered in CALM−/− hematopoietic stem/progenitor cells. For this purpose, we isolated fetal liver cells from C57BL/6 WT and CALM−/− mice. As shown in Fig. 8A, KIT+ fraction increased in the total fetal liver cells from CALM−/− mice compared with that from WT mice (38.1% vs. 23.8%). We stimulated each fetal liver cells with SCF for the indicated times. Because cell components were different between WT and CALM−/− KIT+ fraction, that is, the erythroid progenitor fraction was larger in CALM−/− KIT+ fraction due to anemia than in WT KIT+ fraction (36.1% vs. 17.6%). So, we...
analyzed Akt phosphorylation in KIT$^{\text{high}}$CD71$^{\text{high}}$Ter119$^{\text{low}}$ erythroid progenitor (Region I: Ery-P) fraction and KIT$^{\text{dim}}$CD71$^{\text{dim}}$Ter119$^{\text{low}}$ non-erythroid progenitor (Region II: non-Ery-P) fraction separately by gating with these markers. As shown in Fig. 8A and 8B, SCF induced excessive Akt phosphorylation to the similar extent in both erythroid and non-erythroid progenitors.

This result indicates that the enhanced and prolonged Akt phosphorylation observed in CALM$^{-/-}$ KIT$^{+}$ fraction was not due to different cell components. Together, these results indicate that KIT signaling was altered in CALM$^{-/-}$ primary hematopoietic cells as well as in MEFs.

Figure 6. SCF-induced KIT trafficking from early to late endosome is impaired in CALM$^{-/-}$ MEFs. Whole cell lysates were prepared from WT and CALM$^{-/-}$ MEFs after 15-min SCF stimulation. These lysates were fractionated with OptiPrep density gradient as described in Materials and Methods. Subcellular localization of KIT was analyzed by immunoblot analysis with the anti-KIT Ab. EEA1 was utilized as an early endosome marker, and LAMP1 as a late endosome to lysosome marker.

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Figure 7. Diminution of cytosolic KIT after SCF stimulation is impaired in CALM$^{-/-}$ MEFs, leading to the enhanced and prolonged activation of KIT and Akt. WT and CALM$^{-/-}$ MEFs were stimulated with SCF and cellular lysates were isolated at the indicated times. Then, cytosolic and membrane fractions were separated, of which separation was confirmed by blotting with the Abs against pan-cadherin (reactive to only membrane fraction) and HSP90 (reactive to only cytosolic fraction). To examine the amounts and phosphorylation status of KIT, immunoprecipitated KIT proteins were subjected to immunoblot analyses with the indicated Abs. Phosphorylations of AKT and ERK1/2 were analyzed by immunoblotting using whole cell lysates.

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Roles of CALM in Endocytosis and Intracellular Transport of KIT

Although cell surface interaction between SCF and KIT has been extensively studied [31–33], the precise mechanisms of their endocytosis and intracellular transport have not been clarified. Meanwhile, CME has been reported to be involved in the endocytosis and/or intracellular transport of some receptors such as epidermal growth factors (EGFs) [29,34], vascular endothelial growth factor (VEGF) [35,36] and Notch [37,38]. So, in this study, we analyzed the roles of CALM in the endocytosis and transport of KIT. As a result, we found that CALM plays a critical role for the signaling from KIT and FLT3, and 8% of AML cases, respectively [40–42], have been considered to cause AML. Also, AML cases harboring these mutations are known to have poor prognosis [43–46]. Based on our data that CALM plays a critical role for the signaling from KIT and FLT3, it was expected that the manipulation of the CALM function would be an attractive new strategy to treat AML cases with such mutations.

Activation of Downstream Molecules by KIT-containing Early Endosomes

Several previous papers demonstrated that PI3K/Akt signaling is confined to the plasma membrane and quickly lost once RTKs are internalized. In contrast, recent studies showed that some cytokine receptors and adaptor proteins located in endosomes can transduce their signals to downstream molecules, proposing the concept of “signaling endosome” [9–11]. In addition, it was also reported that oncogenic forms of Met and EGFR are mislocalized in cytoplasm, where they transmit aberrant signals to the downstream molecules. As for KIT, its recruitment to lipid rafts was shown to be necessary to activate PI3K/Akt [47]. However, it was also shown that WT and mutant KIT activate different sets of signaling pathways including Akt and MAPK due to their distinct intracellular localization. In addition, Xiang Z et al. reported that Golgi-localized oncogenic KIT can activate downstream molecules such as Akt, ERK, and STAT3 [40]. In this study, we found that SCF-induced phosphorylation of KIT in early endosomes was enhanced and prolonged in CALM−/− MEFs compared with that in WT MEFs, leading to the excessive activation of Akt. Similar results were also observed in CALM−/− KIT−/− hematopoietic cells. These results indicate that KIT-containing early endosomes can indeed transit KIT signals to the downstream molecules as a signaling endosome. A very recent study showed that Bafilomycin A1, which inhibits protein transport from early to late endosome, enhanced FGF (fibroblast growth factor)-induced activation of ERK1/2 in HEK293 cells [27]. However, in contrast to Akt, we didn’t observe the enhanced phosphorylation of ERK1/2 (Fig. 7). So, it was speculated that the signaling pattern from early endosome would be somewhat different among RTKs.

Significance of Enhanced and Prolonged Activation of Akt in CALM−/− LSK Cells

Many of the previous studies provided evidence that PI3K/Akt signaling is essential or important for the growth and survival in various cell types including hematopoietic cells [49–51].
CALM inhibitor didn’t restore the growth but induced apoptosis in AML, GM, CFU-GM; E, BFU-E. (TIFF)

Figure S1 Picture of CFU formed from WT, CALM−/−, and CALM−/− fetal liver LSKs. LSK cells isolated from fetal liver of WT, CALM−/−, and CALM−/− mice on E14.5 were subjected to clonogenic assays. The size of colonies was observed under the fluorescence microscopy (BZ-X700, Keyence, Osaka, Japan). The representative results were shown. Mix, CFU-Mix; GM, CFU-GM; E, BFU-E (+/+), (+/-), and (-/-) represent the origin of LSK cells: WT, CALM−/−, and CALM−/− mice. (TIFF)

Supporting Information

Figure S2 Distribution of CALM after SCF stimulation in WT MEFs. Distribution of CALM was followed at the indicated times after SCF stimulation by immunofluorescence analyses using the anti-CALM Ab. Rab5, Rab7, Rab11 were used as markers of early, late, and recycling endosomes, respectively. Arrows indicate colocalization (Inset shows region of higher magnification). (TIFF)

Figure S3 Colocalization of KIT and Rab11, a marker of the recycling endosome. KIT and Rab11 were costained with anti-KIT and anti-Rab11 Abs and analyzed by confocal microscopy. (TIFF)

Figure S4 Activation of downstream molecules by KIT localized at early endosomes. (A) CALM−/− MEFs were incubated with 5 μM imatinib or vehicle before the treatment with SCF for 6 h. After SCF-stimulation, the amounts and phosphorylation status of KIT were analyzed at the indicated time points using immunoprecipitated cell lysates. Also, whole cell lysates were subjected to immunoblot analyses using anti-Akt and anti-phospho-Akt Abs. (B) WT MEFs were incubated with 1 μM Bafilomycin A1 or vehicle during the treatment with SCF and whole cell lysates were subjected to the same experiment as Fig. 7A. (TIFF)

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Author Contributions

Conceived and designed the experiments: SR HT TW YK IM. Performed the experiments: SR HT YT MS HO AT KM KT. Analyzed the data: SR HT MS TW IM. Contributed reagents/materials/analysis tools: YM TS TY KO YK. Contributed to the writing of the manuscript: SR HT TW IM.

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