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# Regulation of CD44 expression and focal adhesion by Golgi phosphatidylinositol 4-phosphate in breast cancer

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## Key words

Breast cancer, CD44, focal adhesion, phosphatidylinositol-4-monophosphate, SAC1

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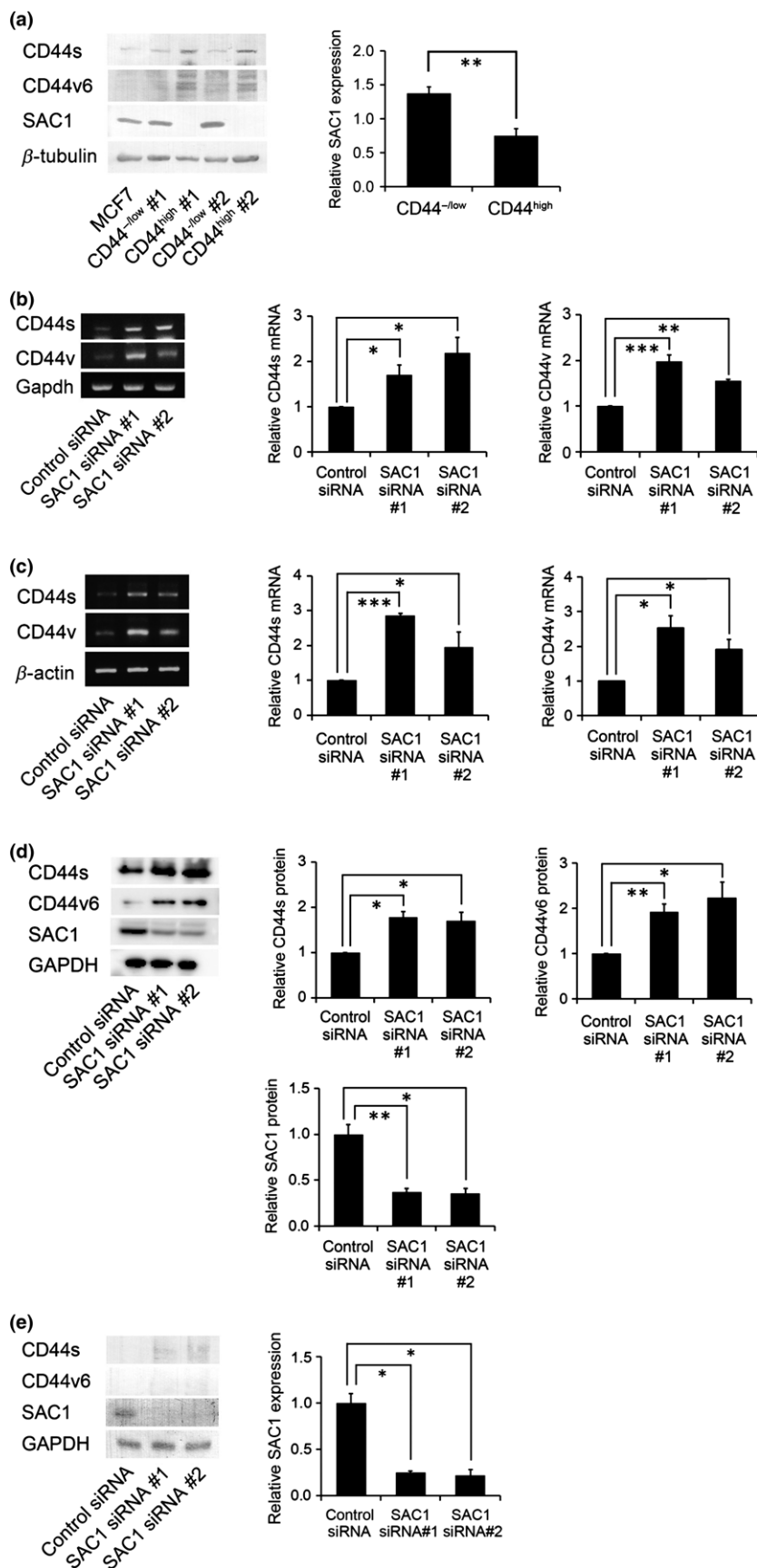
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**B**reast cancer is one of the most common cancers worldwide and its metastatic invasion is the primary cause of breast cancer mortality. It is classified by the expression of various molecular markers, including estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER-2). CD44 is a cell surface receptor for the components of the ECM and plays various roles in cancer invasion, metastasis, and tumor initiation. The gene encoding CD44 is subjected to alternative splicing.<sup>(1)</sup> The smallest CD44 (CD44s or standard CD44) is ubiquitously expressed, whereas the variant CD44 (CD44v) is mainly expressed in proliferating cells and cancer cells.<sup>(2)</sup> In human metaplastic and claudin-low type breast cancers, CD44v is expressed at a high level, with CD44v6 expressed at an extremely high level.<sup>(3)</sup> CD44 expression is associated with an invasive phenotype. Growth factors and primary cytokines induce the upregulation of CD44 variants and enhance adhesion.<sup>(4)</sup> Human breast cancer cell lines with high CD44<sup>+</sup>/CD24<sup>−</sup> populations, such as MDA-MB-231, are basal/mesenchymal in nature and are more invasive than other breast cancer cell lines, favoring bone and lung metastasis. In contrast, non-aggressive epithelial, predominantly luminal MCF7 and SK-BR-3 cells contain a small CD44<sup>+</sup>/CD24<sup>−</sup> subpopulation.

CD44, a transmembrane receptor, is expressed in the standard or variant form and plays a critical role in tumor progression and metastasis. This protein regulates cell adhesion and migration in breast cancer cells. We previously reported that phosphatidylinositol-4-phosphate (PI(4)P) at the Golgi regulates cell migration and invasion in breast cancer cell lines. In this study, we showed that an increase in PI(4)P levels at the Golgi by knockdown of PI(4)P phosphatase SAC1 increased the expression of standard CD44, variant CD44, and ezrin/radixin phosphorylation and enhanced the formation of focal adhesions mediated by CD44 and ezrin/radixin in MCF7 and SK-BR-3 cells. In contrast, knockdown of PI 4-kinase III $\beta$  in highly invasive MDA-MB-231 cells decreased these factors. These results suggest that SAC1 expression and PI(4)P at the Golgi are important in tumor progression and metastasis and are potential prognostic markers of breast cancers.

Many studies have evaluated the expression profile of MCF7 luminal breast cancer cells; although they contain a very small CD44<sup>+</sup>/CD24<sup>−</sup> subpopulation, these cells show relatively higher percentages of CD44<sup>+</sup> cells.<sup>(5,6)</sup> The induction of CD44s in MCF7 breast cancer cells promotes aggressive characteristics *in vitro* as well as metastasis to the liver,<sup>(7,8)</sup> whereas that of CD44s and CD44v6 in MDA-MB-468 breast cancer cells modulates cell adhesion, migration, and cell motility.<sup>(9)</sup> Ezrin–radixin–moesin (ERM) proteins are involved in many aspects of cancer cell adhesion, cell migration, and cell polarity. Ezrin is activated following phosphorylation at Thr-567,<sup>(10–13)</sup> resulting in the linking of the plasma membrane to the actin cytoskeleton. Once activated, ezrin can bind to CD44 and growth factor receptors at the plasma membrane,<sup>(14,15)</sup> and cells initiate a variety of signaling pathways such as activation of phosphatidylinositol (PI) 3-kinase and Rac1. Clinically, higher expression of Ezrin is observed in highly invasive breast cancers.<sup>(16,17)</sup> Ezrin regulates cancer cell invasion and metastasis by promoting focal adhesion turnover.<sup>(18)</sup> Focal adhesions are large integrin-based structures that connect the ECM to actin filaments known as stress fibers. The lifetime or dynamics of focal adhesions affects the dynamic organization of cell morphology, such as cell

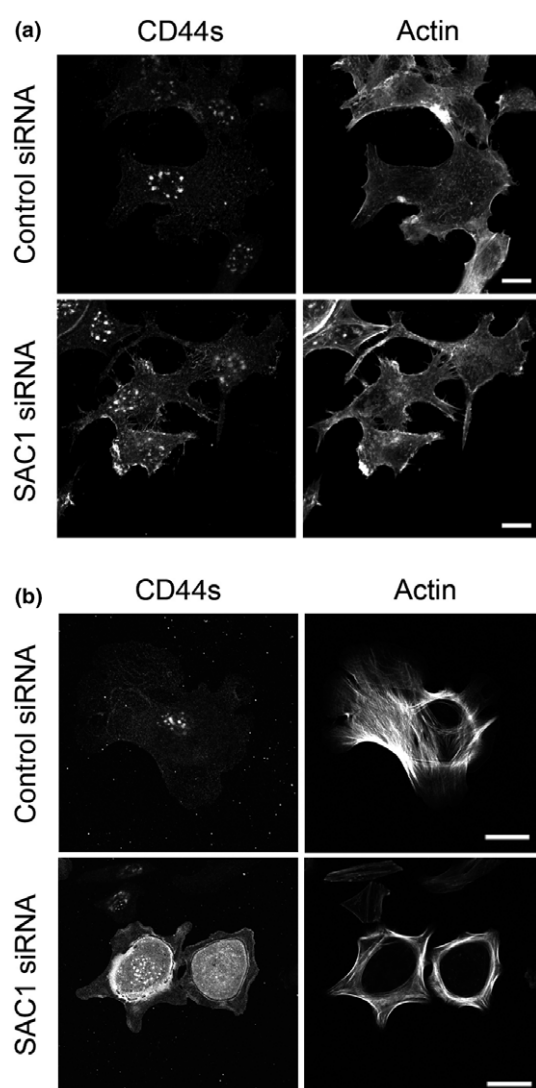


**Fig. 1.** Knockdown of SAC1 increased CD44 expression in luminal breast cancer cell lines. (a) Analysis of SAC1 expression in CD44<sup>+</sup> and CD44<sup>-</sup> fractions of MCF7 cells. Western blot analysis of the lysates obtained from the CD44<sup>+</sup> and CD44<sup>-</sup> fractions of MCF7 cells. The relative amounts of SAC1 expression in each fraction compared to unfractionated MCF7 cells are shown in the graph. The levels were quantified by densitometry, which were further normalized to β-tubulin levels. The results are presented as the mean ± SEM of three independent experiments. \*\**P* < 0.01. (b) Semiquantitative RT-PCR analysis of *CD44s*, *CD44v*, and *Gapdh* in MCF7 cells transfected with control or SAC1-targeted siRNAs. The relative amounts of these mRNAs were quantified by densitometry, which were further normalized to the amount of *Gapdh* mRNA. The results are presented as the mean ± SEM of four independent experiments. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001. (c) Semiquantitative RT-PCR analysis of *CD44s*, *CD44v*, and β-actin in SK-BR-3 cells transfected with control or SAC1-targeted siRNAs. The relative amounts of these mRNAs were quantified by densitometry, which were further normalized to the amount of *Gapdh* mRNA. The results are presented as the mean ± SEM of four independent experiments. \**P* < 0.05; \*\**P* < 0.01; \*\*\**P* < 0.001. (d) Changes in the expression of CD44 and CD44 variant 6 (CD44v6) proteins, induced by knockdown of SAC1 in MCF7 cells. MCF7 cells were transfected with control or SAC1-targeted siRNAs for 72 h before lysate collection. The relative amounts of SAC1, standard CD44 (CD44s), and CD44v6 expression were quantified by densitometry, which were further normalized to the amount of GAPDH. The results are presented as the mean ± SEM of four independent experiments. \**P* < 0.05; \*\**P* < 0.01. (e) Changes in expression of CD44 and CD44v6 proteins, induced by knockdown of SAC1 in SK-BR-3 cells. The relative amount of SAC1 expression was quantified by densitometry, which was further normalized to the amount of GAPDH. The results are presented as the mean ± SEM of three independent experiments. \**P* < 0.05.

Table 1. Effect of SAC1 knockdown on standard CD44 (CD44s) and variant CD44 (CD44v) mRNA levels in MCF7 and SK-BR-3 breast cancer cells

	CD44s $\Delta$ Cq (mean $\pm$ SEM)	Fold change in CD44s mRNA expression [ $2^{(-\Delta\Delta\text{Cq})}$ ] (mean $\pm$ SEM)	<i>P</i> -value ( <i>t</i> -test)	CD44v $\Delta$ Cq (mean $\pm$ SEM)	Fold change in CD44v mRNA expression [ $2^{(-\Delta\Delta\text{Cq})}$ ] (mean $\pm$ SEM)	<i>P</i> -value ( <i>t</i> -test)
MCF7						
Control siRNA	8.94 $\pm$ 0.18	1.00 $\pm$ 0.11	3.88 $\times$ e <sup>-4</sup>	13.26 $\pm$ 0.36	1.00 $\pm$ 0.15	0.018
SAC1 siRNA #1	8.10 $\pm$ 0.13	1.73 $\pm$ 0.15		12.69 $\pm$ 0.48	1.48 $\pm$ 0.36	
SAC1 siRNA #2	8.07 $\pm$ 0.26	1.83 $\pm$ 0.52		0.0160	12.06 $\pm$ 0.39	
SK-BR-3						
Control siRNA	10.16 $\pm$ 0.20	1.00 $\pm$ 0.16	0.0150	14.27 $\pm$ 0.64	1.00 $\pm$ 0.25	0.037
SAC1 siRNA #1	8.24 $\pm$ 0.22	3.79 $\pm$ 0.70		12.25 $\pm$ 0.54	3.52 $\pm$ 0.72	
SAC1 siRNA #2	7.64 $\pm$ 0.096	5.75 $\pm$ 0.41		0.0033	11.81 $\pm$ 0.25	

Each reaction contained cDNA derived from 10 ng total RNA. The results are presented as the mean  $\pm$  SEM of 11 independent experiments for MCF7 cells and of five independent experiments for SK-BR-3 cells, respectively.  $\Delta$ Cq, Cq (CD44s or CD44v) - Cq (Gapdh).



**Fig. 2.** Immunofluorescence analysis of CD44 in normal or SAC1-silenced MCF7 and SK-BR-3 cells. (a) Immunofluorescence analysis of endogenous CD44 in MCF7 cells transfected with control or SAC1-targeted siRNA. F-actin was visualized using Alexa Fluor 647-phalloidin. Scale bar = 20  $\mu$ m. (b) Immunofluorescence analysis of endogenous CD44 in SK-BR-3 cells transfected with control or SAC1-targeted siRNA. F-actin was visualized using Alexa Fluor 647-phalloidin. Scale bar = 20  $\mu$ m.

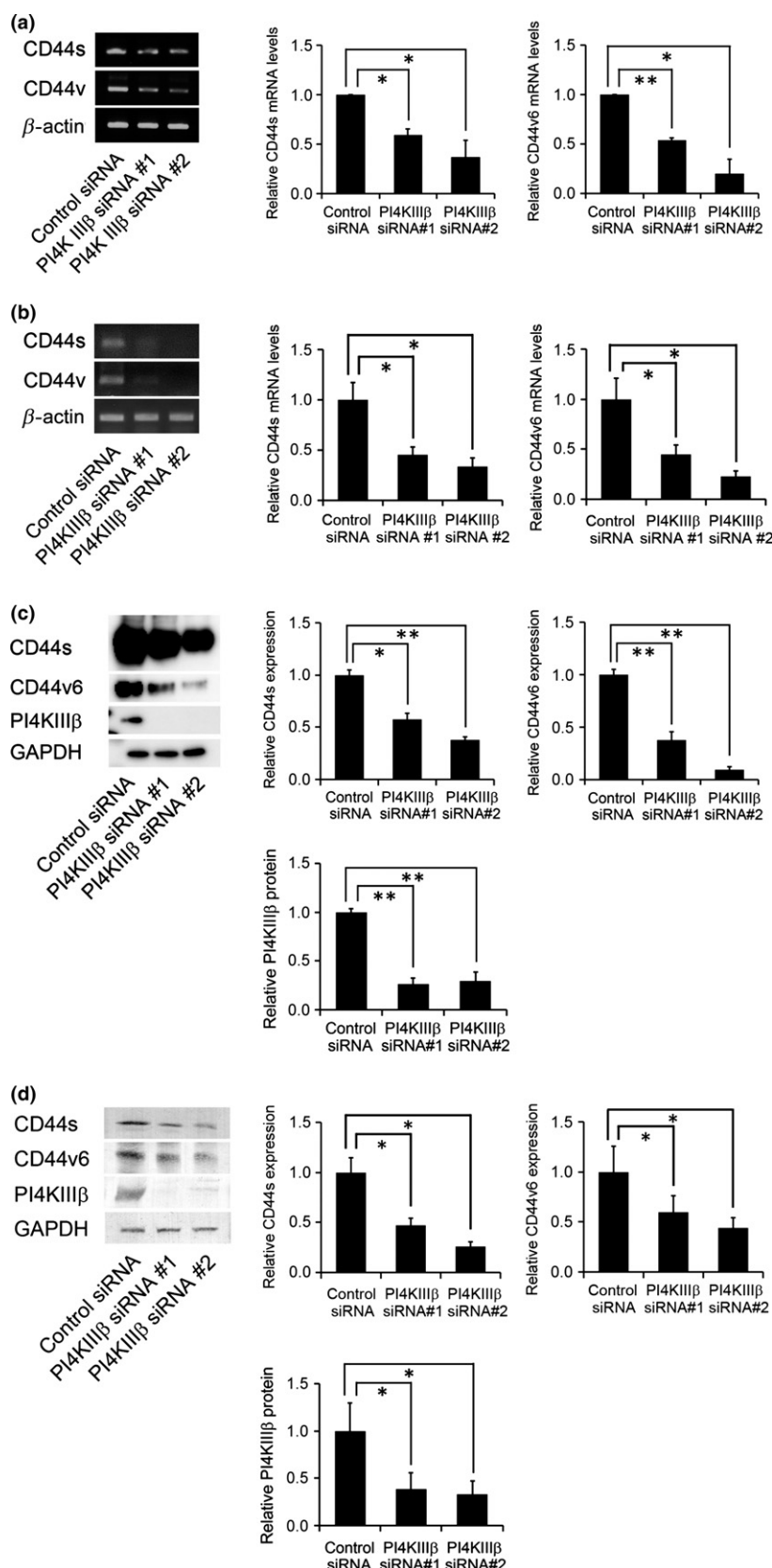
motility. In the lamellipodia of migrating cells, nascent adhesions, consisting of integrin, focal adhesion kinase, and vinculin are formed. These structures are short-lived and undergo rapid turnover. Thus, a number of focal adhesions is involved in cancer cell migration.

Our recent finding suggested a strong correlation between phosphatidylinositol-4-phosphate (PI(4)P) at the Golgi and the invasive phenotype of breast cancer cell lines.<sup>(19)</sup> Phosphatidylinositol-4-phosphate is enriched at the trans-Golgi and is required for trafficking from the Golgi to the plasma membrane.<sup>(20)</sup> In highly invasive breast cancer cell lines (MDA-MB-231 and Hs578t), Golgi PI(4)P levels are significantly higher than those in weakly invasive (MCF7 and T-47D) cell lines. Moreover, the expression of PI 4-kinase III $\beta$  (PI4KIII $\beta$ ), which generates PI(4)P at the Golgi, is higher in late-stage metastatic human breast cancer tissues (stages III and IV) than in early-stage tissues (non-metastatic, stages I and IIa). In contrast, SAC1 expression is decreased in human metastatic breast cancer tissues at stages III and IV.<sup>(19)</sup> In accordance with these findings, knockdown of SAC1 in MCF-7 cells results in decreased cell-cell adhesion and a more invasive phenotype, whereas knockdown of PI4KIII $\beta$ , which generates PI(4)P in the Golgi, in MDA-MB-231 cells increases cell-cell adhesion and decreases cancer cell migration. In these cells, PI(4)P levels at the Golgi and SAC1 expression are co-related to an invasive phenotype.<sup>(19)</sup> In this study, we identified the mechanism underlying these changes in the invasive phenotype. Phosphatidylinositol-4-phosphate generation at the Golgi resulted in altered expression of breast cancer stem cell markers, including CD44, and increased focal adhesion formation.

## Materials and Methods

**Cell lines.** MCF-7, MDA-MB-231, and Hs578t cells were cultured as described previously.<sup>(19)</sup> SK-BR-3 cells were cultured in DMEM supplemented with 10% FBS.

**Materials.** Mouse anti-PI4KIII $\beta$  mAb was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Mouse anti-vinculin mAb was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-CD44s pan mouse mAb (clone #2C5) and CD44v6 mouse mAb (clone #2F10) were purchased from R&D Systems (Minneapolis, MN, USA). Anti-phospho ezrin (Thr-567)-radixin (Thr-564)-moesin (Thr-558) and anti- $\beta$ -tubulin rabbit polyclonal antibodies were from Cell Signaling Technologies (Danvers, MA, USA). Anti-ezrin rabbit polyclonal, anti-radixin rabbit monoclonal, and anti-SAC1 rabbit polyclonal antibodies



**Fig. 3.** Knockdown of phosphoinositide 4-kinase III $\beta$  (PI4KIII $\beta$ ) attenuated the expression of CD44 variants (CD44v) in MDA-MB-231 cells. (a) Semiquantitative RT-PCR analysis of *CD44s*, *CD44v*, and *Gapdh* in MDA-MB-231 cells transfected with control or PI4KIII $\beta$ -targeted siRNAs. The relative amounts of these mRNAs were quantified by densitometry, which were further normalized to the amount of *Gapdh* mRNA. The results are presented as the mean  $\pm$  SEM of four independent experiments. \* $P$  < 0.05; \*\* $P$  < 0.01. (b) Semiquantitative RT-PCR analysis of *CD44s*, *CD44v*, and  $\beta$ -actin in Hs578t cells transfected with control or PI4KIII $\beta$ -targeted siRNAs. The relative amounts of these mRNAs were quantified by densitometry, which were further normalized to the amount of  $\beta$ -actin mRNA. The results are presented as the mean  $\pm$  SEM of three independent experiments. \* $P$  < 0.05; \*\* $P$  < 0.01. (c) Changes in the expression of CD44 and CD44v6 proteins induced by the knockdown of PI4KIII $\beta$  in MDA-MB-231 cells. MDA-MB-231 cells were transfected with control or PI4KIII $\beta$  siRNAs for 72 h before lysate collection. The relative amounts of PI4KIII $\beta$ , CD44, and CD44v6 expression were quantified by densitometry. The results are presented as the mean  $\pm$  SEM of four independent experiments. \* $P$  < 0.05. (d) Changes in the expression of CD44 and CD44v6 proteins induced by the knockdown of PI4KIII $\beta$  in Hs578t cells. MDA-MB-231 cells were transfected with control or PI4KIII $\beta$  siRNAs for 72 h before lysate collection. The results are presented as the mean  $\pm$  SEM of four independent experiments. \* $P$  < 0.05.

were purchased from Abcam (Cambridge, UK), and Proteintech (Chicago, IL, USA), respectively. All fluorescent-conjugated secondary antibodies (Alexa Fluor 647-phalloidin, and Alexa Fluor 488- and 568-conjugated goat anti-rabbit or anti-mouse

secondary antibodies) were purchased from Life Technologies (Carlsbad, CA, USA).

**Cell culture and transfection of siRNA.** For the knockdown of SAC1 and PI4KIII $\beta$ , Stealth siRNAs were purchased from Life



Technologies.<sup>(19)</sup> Twenty nanomoles of Stealth siRNAs were transfected into each cell line using Lipofectamine RNAiMAX (Life Technologies); experiments were carried out 72 h after transfection.

**Separation of CD44<sup>+</sup> and CD44<sup>-</sup> MCF7 cells.** MCF7 cells were incubated with labeled CD44 microbeads at 4°C for 15 min. After brief washing with washing buffer containing PBS (pH 7.2), 1% BSA, and 2 mM EDTA, the cells were separated using a MultiMACS Cell Separator Plus (Miltenyi Biotec, Bergisch Gladbach, Germany) with CD44 microbeads (Miltenyi Biotec).

**Semiquantitative RT-PCR and real time RT-PCR.** The one-step RT-PCR kit from Life Technologies was used to synthesize cDNA from the RNAs purified from MCF7, SK-BR-3, MDA-MB-231, and Hs578t cells. Semiquantitative RT-PCR was undertaken using the following primer sets:  $\beta$ -actin forward, 5'-TCACCCACACTGTGCCCATCTACGA-3' and reverse, 5'-ATACTCTGCTTGCTGATCC-3' (product size 611 bp); GAPDH forward, 5'-CTCTGGTAAAGTGGATATTG-3' and reverse, 5'-CTCCCCCTGCAAATGAG-3'; CD44s forward, 5'-CAGAACGAATCCTGAAGAC-3' and reverse, 5'-GTGTCTTGGTCTCTGGTAG-3' (product size 198 bp); and CD44v forward, 5'-CAGAACGAATCCTGAAGAC-3' and reverse, 5'-GTGTCTTGGTCTCTGGTAG-3'. For quantitative real-time PCR, the Dynamo ColorFlash SYBR Green qPCR Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used with a PikoReal Real-Time PCR System (Thermo Fisher Scientific) and analyzed using PikoReal 2.0 software. The reaction conditions were as follows: incubated at 95°C for 7 min, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. Each sample was run in duplicate for each gene using the GAPDH gene as an internal reference. Quantification cycle values (Cq) were calculated using PikoReal software (Thermo Fisher Scientific) for all PCRs. Delta Cq ( $\Delta$ Cq) represents the different expression levels of CD44 genes. Relative expression of the CD44s and CD44v genes were calculated using the  $\Delta\Delta$ Cq method. Using the  $\Delta\Delta$ Cq method, the starting copy number of the samples was determined:  $\Delta$ Cq represents the mean Cq value of each sample and was calculated for GAPDH. The starting copy number of the unknown samples was determined relative to the known copy number of the calibrated sample using the following formula:  $\Delta\Delta$ Cq = [ $\Delta$ Cq GAPDH (control) -  $\Delta$ Cq CD44s or CD44v (control)] - [ $\Delta$ Cq GAPDH (sample) -  $\Delta$ Cq CD44s or CD44v (sample)]. The relative gene copy number was calculated using the expression  $2^{-\Delta\Delta$ Cq.

**Western blot analysis.** Cell lysates were lysed with lysis buffer (40 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-

100, 2 mM EDTA, 1 mM NaF, and 1 mM Na<sub>3</sub>VO<sub>4</sub>). The lysates were centrifuged at 15 000  $\times$  g at 4°C for 5 min. The proteins were separated by SDS-PAGE, and then transferred to nitrocellulose membranes. The membranes were incubated with primary antibodies for 1 h after blocking with blocking solution (5% skim milk, 1% BSA). The membrane was washed with Tris-buffered saline containing 0.1% Tween 20 before incubation with the secondary antibody. The immunoreactive bands were visualized by chemiluminescence.

**Immunofluorescence analysis.** Cells were fixed with 3.7% formaldehyde in PBS and then permeabilized with 0.2% Triton X-100 in PBS. They were blocked with 1.0% BSA in PBS and incubated with primary antibodies in blocking buffer. After five washes with PBS, the cells were incubated with the appropriate secondary antibodies in blocking buffer. After brief washing with PBS, coverslips were mounted onto slides using PermaFluor Mounting Medium (Thermo Fisher Scientific) and observed using a FluoView 1000-D confocal microscope (IX81; Olympus, Tokyo, Japan) equipped with 473-, 568-, and 633-nm diode lasers through an objective lens (60 $\times$  oil immersion objective, NA 1.35; Olympus) using FluoView software. Acquired images were processed using Photoshop (Adobe, San Jose, CA, USA).

**Quantification of focal adhesions.** Focal adhesions were quantified as described previously.<sup>(21)</sup> Cells were fixed and stained with anti-vinculin antibody and Alexa Fluor 647-phalloidin. Images were obtained by confocal microscopy. The focal adhesions were quantified by counting vinculin plaques. Forty cells obtained from three independent experiments were analyzed. All image processing was carried out using ImageJ software (NIH, Bethesda, MD, USA).

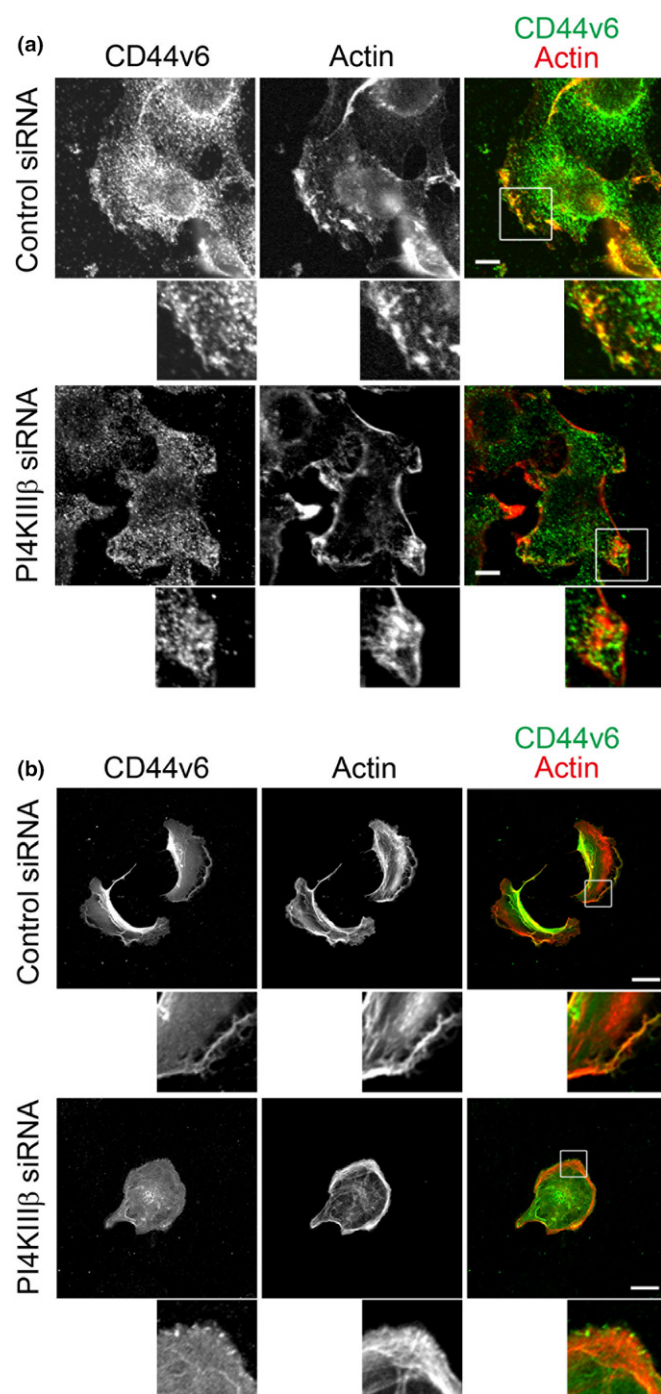
## Results

Knockdown of SAC1 increased the expression of CD44 in luminal breast cancer cells. MCF7 cells were originally suggested to show the luminal-epithelial phenotype, some portion of which express CD44 but does not show the CD44<sup>+</sup>/CD24<sup>-</sup> cell phenotype.<sup>(5,6)</sup> To obtain CD44<sup>-low</sup> cells from MCF7 cells, we undertook cell separation using an antibody that recognized both CD44s and CD44v. Western blot analysis showed that SAC1 expression was lower in the CD44<sup>+</sup> fraction than in the CD44<sup>-low</sup> fraction (Fig. 1a). Although MCF7 cells predominantly express CD44s,<sup>(22)</sup> CD44 variants, including CD44v6-10, v2-10, and v3-10, are also expressed.<sup>(23)</sup> Among these variants, the expression of CD44v6-10 is much higher

**Table 2.** Effect of phosphoinositide 4-kinase III $\beta$  (PI4KIII $\beta$ ) knockdown on standard CD44 (CD44s) and variant CD44 (CD44v) mRNA levels in MDA-MB-231 and Hs578t breast cancer cells

	CD44s $\Delta$ Cq (mean $\pm$ SEM)	Fold change in CD44s mRNA expression [2 <sup>(-<math>\Delta\Delta</math>Cq)</sup> ] (mean $\pm$ SEM)	P-value (t-test)	CD44v $\Delta$ Cq (mean $\pm$ SEM)	Fold change in CD44v mRNA expression [2 <sup>(-<math>\Delta\Delta</math>Cq)</sup> ] (mean $\pm$ SEM)	P-value (t-test)
MDA-MB-231						
Control siRNA	3.21 $\pm$ 0.13	1.00 $\pm$ 0.086		7.36 $\pm$ 0.29	1.00 $\pm$ 0.21	
PI4KIII $\beta$ siRNA #1	4.00 $\pm$ 0.22	0.58 $\pm$ 0.079	0.0460	8.92 $\pm$ 0.59	0.34 $\pm$ 0.14	0.020
PI4KIII $\beta$ siRNA #2	3.90 $\pm$ 0.087	0.62 $\pm$ 0.039	0.0062	8.10 $\pm$ 0.28	0.60 $\pm$ 0.13	0.034
Hs578t						
Control siRNA	1.31 $\pm$ 0.13	1.00 $\pm$ 0.085		5.77 $\pm$ 0.14	1.00 $\pm$ 0.093	
PI4KIII $\beta$ siRNA #1	2.55 $\pm$ 0.27	0.42 $\pm$ 0.069	0.0037	6.92 $\pm$ 0.47	0.45 $\pm$ 0.13	0.0498
PI4KIII $\beta$ siRNA #2	3.07 $\pm$ 0.35	0.30 $\pm$ 0.074	1.06 $\times$ e <sup>-5</sup>	7.76 $\pm$ 0.49	0.25 $\pm$ 0.082	0.0097

Each reaction contained cDNA derived from 10 ng total RNA. The results are presented as the mean  $\pm$  SEM of five independent experiments.  $\Delta$ Cq, Cq (CD44s or CD44v) - Cq (Gapdh).



**Fig. 4.** Immunofluorescence analysis of CD44 variant (CD44v) in phosphoinositide 4-kinase III $\beta$  (PI4KIII $\beta$ )-silenced MDA-MB-231 and Hs578t breast cancer cells. (a) Immunofluorescence analysis of endogenous CD44v6 in MDA-MB-231 cells transfected with control or PI4KIII $\beta$  siRNA. F-actin was visualized using Alexa Fluor 647-phalloidin. Enlarged images of boxed areas are shown in the lower panels. Yellow indicates the region of localization at the plasma membrane. Scale bar = 20  $\mu$ m. (b) Immunofluorescence analysis of endogenous CD44v6 in Hs578t cells transfected with control or PI4KIII $\beta$  siRNA. F-actin was visualized using Alexa Fluor 647-phalloidin. Enlarged images of boxed areas are shown in the lower panels. Yellow indicates the region of localization at the plasma membrane. Scale bar = 20  $\mu$ m.

than other variants in MCF7 cells.<sup>(24)</sup> Semiquantitative PCR showed that, among these variants, the expression of CD44s and CD44v mRNA was increased by SAC1 knockdown in

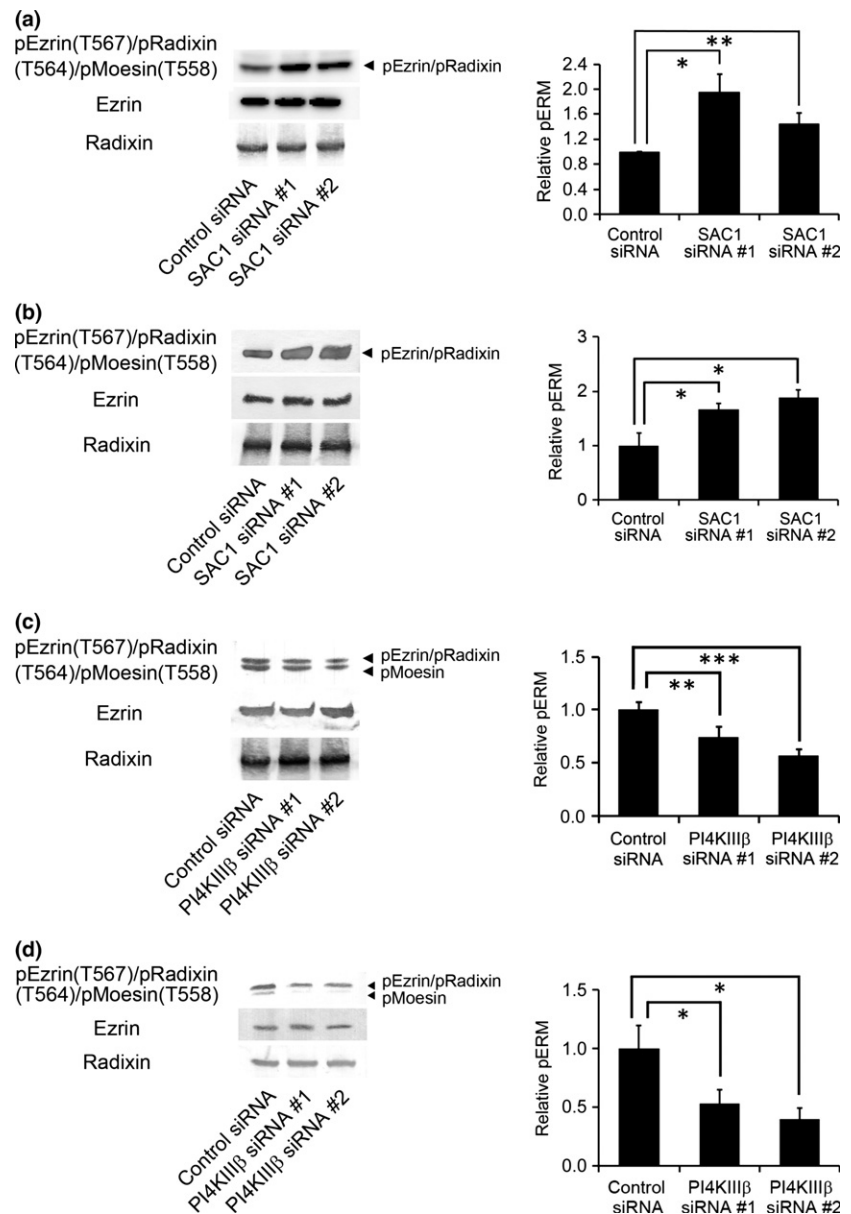
MCF7 (Fig. 1b) and SK-BR-3, another luminal-type, HER-2-enriched breast cancer cell (Fig. 1c). Similar results were obtained using quantitative real-time PCR (Table 1). We confirmed that the amplified RT-PCR products for CD44 variants were CD44v6-10 by sequencing. The levels of CD44s and CD44v6 proteins were increased when SAC1 was knocked down in MCF7 cells (Fig. 1d). The expression of these proteins following knockdown of SAC1 in SK-BR-3 cells was also examined. Expression of SAC1 protein was decreased by 75% by the knockdown of SAC1 compared to control siRNA-transfected cells (Fig. 1e). However, these proteins were very weakly detected by Western blot analysis, which might be caused by the very low CD44 expression in these cells (Fig. 1e). SAC1 depletion reduces cell-cell adhesion.<sup>(19)</sup> Although non-specific immunoreactivities in the nuclei were observed, immunofluorescence analysis showed that CD44s were localized at the cell surface by SAC1 knockdown in MCF7 (Fig. 2a) and SK-BR-3 cells (Fig. 2b).

Knockdown of PI4KIII $\beta$  decreased CD44 expression in basal breast cancer cells. Decreased PI(4)P at the Golgi mediates the suppression of migration and invasion of basal-mesenchymal MDA-MB-231 and Hs578t breast cancer cells.<sup>(19)</sup> These cells originally abundantly expressed CD44s and most cells showed the CD44<sup>+</sup>/CD24<sup>-</sup> phenotype. The mRNA expression of CD44s and CD44v6 was markedly decreased by the knockdown of PI4KIII $\beta$  in these cells (Fig. 3a,b, Table 2). Protein expression of CD44s and CD44v6 was also decreased by PI4KIII $\beta$  knockdown (Fig. 3c,d). Knockdown of PI4KIII $\beta$  promoted an increase in cell-cell adhesion in MDA-MB-231 cells.<sup>(19)</sup> Immunofluorescence analysis of these cells revealed that the expression of cell surface CD44v6 was also decreased by PI4KIII $\beta$  knockdown (Fig. 4). These results indicate that Golgi PI(4)P mediates the expression of CD44 isoforms such as CD44s and CD44v6.

Golgi PI(4)P regulated focal adhesion formation. The ERM family proteins act as linkers between the actin cytoskeleton and CD44 transmembrane receptor. Among them, ezrin is reportedly involved in changes in cell motility.<sup>(25)</sup> Phosphorylation of ezrin at Thr-567 by Rho-kinase triggers its association with CD44 at the plasma membrane, which subsequently enhances the formation of focal adhesions. Our previous results showed that Golgi PI(4)P regulates the activity of a small GTPase Rho.<sup>(19)</sup> Knockdown of SAC1 in MCF7 and SK-BR-3 cells caused a slight increase in ERM phosphorylation at Thr-567 and radixin phosphorylation at Thr-564 (Fig. 5a,b). In contrast, these phosphorylations were decreased by PI4KIII $\beta$  knockdown in MDA-MB-231 and Hs578t cells (Fig. 5c,d). Next, we evaluated the effect of SAC1 knockdown on focal adhesion formation because phosphorylated proteins from the ERM family regulate the formation of focal adhesions by cross-linking CD44 and the actin cytoskeleton. MCF7 and SK-BR-3 cells normally form a small number of vinculin-positive focal adhesions; this number was increased by SAC1 knockdown (Fig. 6a-d). Highly invasive MDA-MB-231 cells form vinculin-positive focal adhesions. However, their number was decreased by PI4KIII $\beta$  knockdown in MDA-MB-231 and Hs578t cells (Fig. 6e-h). These results suggest that Golgi PI(4)P regulates the formation of focal adhesions in breast cancer cell lines.

## Discussion

Phosphatidylinositol-4-phosphate at the Golgi is required for lipid and protein sorting. It is under the control of PI4KIII $\beta$  and SAC1 to coordinate secretion with cell growth.<sup>(26)</sup> We



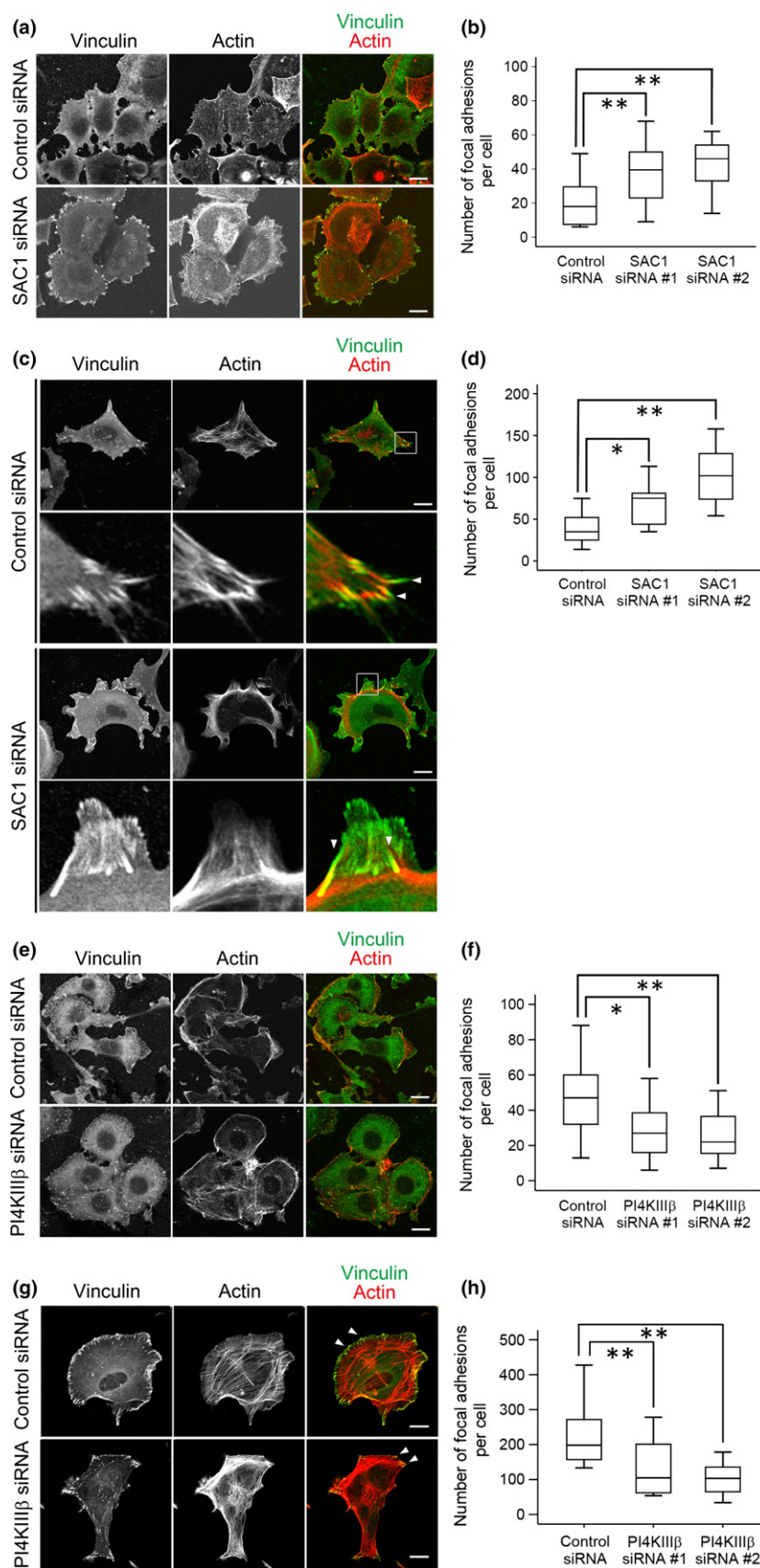
**Fig. 5.** Ezrin/radixin phosphorylation was under the control of Golgi phosphatidylinositol-4-phosphate (PI(4)P). (a,b) Effect of SAC1 knockdown on the phosphorylation of ezrin–radixin–moesin (ERM) protein in MCF7 (A) and SK-BR-3 (b) breast cancer cells. Values were normalized to total ezrin protein content. (c,d) Effect of phosphoinositide 4-kinase IIIβ (PI4KIIIβ) silencing on the phosphorylation of ERM in MDA-MB-231 (c) and Hs578t (d) cells. Values were normalized to total ezrin protein content. All results are presented as the mean ± SEM of four independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . p, phosphorylated.

recently proposed a model for breast cancer cell progression in which the Golgi PI(4)P apparatus affects cell motility and invasion in breast cancer cell lines.<sup>(19)</sup> Increased PI(4)P at the Golgi by knockdown of SAC1 in MCF7 cells increased cell motility, whereas decreased PI(4)P by PI4KIIIβ silencing in MDA-MB-231 cells decreased invasive phenotypes.<sup>(19)</sup> Cancer cell properties such as migration, adhesion, and invasion are characterized by the activation and expression of cell surface markers such as CD24 and CD44.<sup>(4)</sup> Recent studies have suggested that the expression of CD44 variants plays a role in adhesion, motility, and invasion of breast cancer cells.<sup>(9,27–29)</sup> To identify the subsequent changes induced by PI(4)P at the Golgi, we evaluated the expression of CD44 variants. The CD44<sup>+</sup> subpopulation of MCF7 cells showed lower expression of SAC1 than the CD44<sup>−</sup> subpopulation did, suggesting that SAC1 expression is inversely proportional to CD44 expression. Increased PI(4)P at the trans-Golgi by the knockdown of SAC1 in MCF7 cells resulted in the expression of CD44s and CD44v6, whereas decreased PI(4)P by the knockdown of

PI4KIIIβ in MDA-MB-231 cells decreased them (Fig. 7). Although the mechanisms underlying CD44 expression are poorly understood, nuclear factor-κB is known to be involved in the regulation of CD44 expression in breast cancer cell lines.<sup>(30)</sup> Golgi phosphoprotein 3 (GOLPH3), defined as an oncoprotein, is a PI(4)P effector at the Golgi. An increasing number of studies have implicated GOLPH3 in several cancers, including hepatocellular carcinoma,<sup>(31)</sup> glioblastoma,<sup>(32,33)</sup> and breast cancer.<sup>(34)</sup> The PI(4)P-binding ability of GOLPH3 is essential for breast cancer cell metastasis *in vitro* and *in vivo*.<sup>(19)</sup> Given that the expression of GOLPH3 in hepatocellular carcinoma cell lines promotes the activation of nuclear factor-κB,<sup>(35)</sup> it is not surprising that PI(4)P at the Golgi regulates CD44 expression in breast cancer cells.

Phosphatidylinositol-4-phosphate is abundant at the trans-Golgi compartment and a small portion was detected at the plasma membrane and nuclear envelope.<sup>(36)</sup> The molecular function of PI(4)P is the regulation of COPII vesicles fusion with the Golgi and trafficking proteins from the endoplasmic

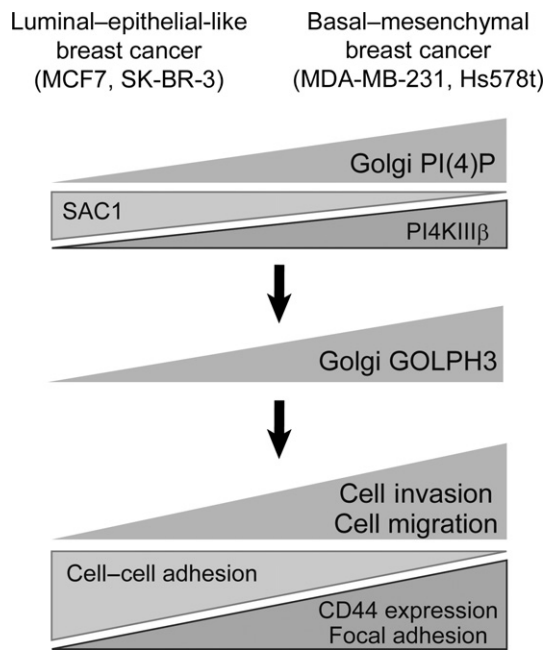




**Fig. 6.** Role of phosphatidylinositol-4-phosphate (PI(4)P) in the formation of focal adhesions in breast cancer cells. (a) Immunofluorescence analysis of vinculin in MCF7 cells transfected with control or SAC1-targeted siRNA. F-actin was visualized using Alexa Fluor 647–phalloidin. Scale bar = 20  $\mu$ m. (b) Vinculin-positive focal adhesion in MCF7 cells transfected with control or SAC1-targeted siRNA. The number of adhesions was determined using the images captured in 40 cells by confocal microscopy. (c) Immunofluorescence analysis of vinculin in SK-BR-3 cells transfected with control or SAC1-targeted siRNA. F-actin was visualized using Alexa Fluor 647–phalloidin. Enlarged images of boxed areas are shown in the lower panels. Arrowheads indicate focal adhesions. Scale bar = 20  $\mu$ m. (d) Vinculin-positive focal adhesion in SK-BR-3 cells transfected with control or SAC1-targeted siRNA. The number of adhesions was determined using the images captured in 40 cells by confocal microscopy. (e) Immunofluorescence analysis of endogenous vinculin in MDA-MB-231 cells transfected with control or phosphoinositide 4-kinase III $\beta$  (PI4KIII $\beta$ )-targeted siRNA. F-actin was visualized using Alexa Fluor 647–phalloidin. Scale bar = 20  $\mu$ m. (f) Vinculin-positive focal adhesion in control- or PI4KIII $\beta$ -depleted MDA-MB-231 cells. Focal adhesions were analyzed in more than 40 cells. (g) Immunofluorescence analysis of endogenous vinculin in Hs578t cells transfected with control or PI4KIII $\beta$ -targeted siRNA. F-actin was visualized using Alexa Fluor 647–phalloidin. Arrowheads indicate focal adhesions. Scale bar = 20  $\mu$ m. (h) Vinculin-positive focal adhesion in control- or PI4KIII $\beta$ -depleted Hs578t cells. Focal adhesions were analyzed in 40 cells. All data are presented as box and whisker plots with boxes representing 25th–75th percentile range and whiskers representing 10th–90th percentile range. \* $P$  < 0.05; \*\* $P$  < 0.01.

reticulum to the plasma membrane.<sup>(37)</sup> Immunofluorescence analysis of breast cancer cell lines showed that SAC1 and PI4KIII $\beta$  regulate the localization of CD44s and CD44v6 at

the plasma membrane. Golgi phosphoprotein 3 has pivotal roles in vesicle trafficking from the Golgi, and thus PI(4)P at the Golgi may affect the membrane trafficking of CD44s and



**Fig. 7.** Schematic illustration of regulatory cross-talk occurring between Golgi phosphatidylinositol-4-phosphate (PI(4)P), CD44 expression, and cell adhesion in breast cancer cell invasion and motility. PI(4)P at the Golgi plays a critical role in cancer progression. Changes in the Golgi PI(4)P levels altered the expression and localization of CD44, focal adhesion formation, cell-cell adhesion formation, cell migration/invasion, and metastasis in breast cancer cell lines. Regulation of the localization of the PI(4)P effector Golgi phosphoprotein 3 (GOLPH3) at the Golgi was predicted to cause these phenotypes. The involvement of PI(4)P levels in molecular subclassification of breast cancer cells is shown.

CD44v. A recent study showed that the interactions among GOLPH3, PI(4)P, and sialyltransferases efficiently regulate sialylation on several target proteins expressed on the cell surface, including integrins and some receptor tyrosine kinases, and the resulting glycoproteins cooperatively enhanced integrin-mediated cell migration and activated the PI 3-kinase signaling pathway.<sup>(38)</sup> Glycosylation of CD44 has been implicated in CD44-mediated cell adhesion to hyaluronan.<sup>(39)</sup> Taken together, our results suggest that PI(4)P at the Golgi regulates the expression, localization, and glycosylation status of CD44, and determines cancer cell characteristics (Fig. 7).

The ERM protein family plays a role in cytoskeletal organization in the plasma membrane.<sup>(40)</sup> Among them, ezrin plays an important role in morphogenesis, cell migration, tumor metastasis, and cell adhesion.<sup>(11–13,41)</sup> CD44, particularly CD44v6, recruits ezrin, which is phosphorylated at Thr-567 and becomes activated. Activated ezrin triggers the activation of focal adhesion kinase and the subsequent formation of focal adhesions. When SAC1 was knocked down in MCF7 and SK-BR-3 cells, which are luminal-type breast cancer cells, ezrin phosphorylation and the number of focal adhesions were increased, whereas knockdown of PI4KIIIβ in MDA-MB-231 cells decreased these phenomena. Given that the formation of

focal adhesions is a critical step for developing the invasive-metastatic characteristic of breast cancer cells, PI(4)P at the Golgi likely mediates these signaling pathways. Immunofluorescence analysis revealed the accumulation of cell surface CD44s by SAC1 knockdown in MCF7 cells. Among the phosphoinositides, (PI(4,5)P<sub>2</sub>) has been reported to bind to ERM family proteins and increase the binding affinity of ERM family proteins to CD44.<sup>(42,43)</sup> This increase promotes a link between filamentous actin and the receptors. Together with our previous findings that PI(4)P promotes an increase in RhoA activity,<sup>(19)</sup> we suggest that PI(4)P at the Golgi modulates the expression of CD44 (CD44s and CD44v) and promotes the formation of vinculin-positive focal adhesions; thus, it regulates the invasive phenotype of breast cancer cell lines. CD44 signaling acts on the Hippo-YAP/TAZ signaling pathway, which mediates cancer progression, cell survival, cell survival, metastasis, and stem cell maintenance of stem cell properties.<sup>(44,45)</sup> Hippo pathway mutations give rise to tumors, and deregulation of this pathway is frequently observed in human cancers.<sup>(46,47)</sup> Downregulation of CD44 or knockdown of RhoA decreases YAP expression, which promotes cell apoptosis and inhibits cell proliferation.<sup>(45)</sup> Phosphatidylinositol-4-phosphate at the Golgi may modulate this signaling by regulating CD44 expression and its localization at the cell surface.

Increased CD44 expression is one of the characteristics of cancer stem cells. The presence of cancer stem cells has been observed in a variety of cancers, including leukemia, colon cancer, ovarian cancer, glioma, and breast cancer.<sup>(48,49)</sup> Cancer stem cells are more resistant to chemotherapy and radiotherapy than other cancer cells within the same tumors. In addition, they are responsible for tumor recurrence, invasion, and metastasis. Breast cancer stem cells constitute only a small population of breast tumor cells, with the characteristic CD44<sup>+</sup>/CD24<sup>-low</sup> phenotype. Recently, the expression of another phosphoinositide phosphatase, SHIP2, was reported to be correlated with the size of the CD44<sup>+</sup>/CD24<sup>-low</sup> subpopulation.<sup>(50)</sup> Although the precise mechanisms underlying the changes in breast cancer phenotype remain unclear, our results showed that PI(4)P affects the molecular subtypes of breast cancer cell lines. Understanding these mechanisms will provide further insight into the acquisition of invasive potential by cancer cells and provide a foundation for therapeutic intervention.

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## Disclosure Statement

The authors have no conflict of interest.

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