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Received: 28 June 2022 | Revised: 20 December 2022 | Accepted: 16 January 2023

DOI: 10.1002/ijc.34443

MOLECULAR CANCER BIOLOGY

IJC INTERNATIONAL JOURNAL of CANCER

Lipid raft protein flotillin-1 is important for the interaction between SOS1 and H-Ras/K-Ras, leading to Ras activation

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Funding information
National Research Foundation of Korea, Grant/Award Numbers: 2016R1A6A1A03007648, 2019R1A2C1009773

Abstract

Ras mutations have been frequently observed in human cancer. Although there is a high degree of similarity between Ras isomers, they display preferential coupling in specific cancer types. The binding of Ras to the plasma membrane is essential for its activation and biological functions. The present study elucidated Ras isoform-specific interactions with the membrane and their role in Ras-mediated biological activities. We investigated the role of a lipid raft protein flotillin-1 (Flot-1) in the activations of Ras. We found that Flot-1 was co-localized with H-Ras, but not with N-Ras, in lipid rafts of MDA-MB-231 human breast cells. The amino-terminal hydrophobic domain (1-38) of Flot-1 interacted with the hypervariable region of H-Ras. The epidermal growth factor-stimulated activation of H-Ras required Flot-1 which was not necessary for that of N-Ras in breast cancer cells. Flot-1 interacted with son of sevenless (SOS)-1, which promotes the conversion of Ras-bound GDP to GTP. Notably, Flot-1 was crucial for the interaction between SOS1 and H-Ras/K-Ras in breast and pancreatic cancer cells. Stable knockdown of Flot-1 reduced the in vivo metastasis in a mouse xenograft model with human breast carcinoma cells. A tissue microarray composed of 61 human pancreatic cancer samples showed higher levels of Flot-1 expression in pancreatic tumor tissues compared to normal tissues, and a correlation between K-Ras and Flot-1. Taken together, our findings suggest that Flot-1 may serve as a membrane platform for the interaction of SOS1 with H-Ras/K-Ras in human cancer cells, presenting Flot-1 as a potential target for Ras-driven cancers.

KEYWORDS
flotillin-1, H-Ras, K-Ras, son of sevenless 1, triple-negative breast cancer

What's new?

The binding of Ras mutant isoforms, which are frequently observed in human cancers, to the plasma membrane is essential for their biological functions. The molecular mechanisms

Abbreviations: ADAM12, a disintegrin and metalloproteinase domain-containing protein 12; CD44, homing cell adhesion molecule; c-Src, proto-oncogene tyrosine-protein kinase; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; FBS, fetal bovine serum; Flot-1, flotillin-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GEF, guanine nucleotide-exchange factor; GEPIA, Gene Expression Profiling Interactive Analysis; HRP, horseradish peroxidase; HVR, hypervariable region; IHC, immunohistochemical; IP, immunoprecipitation; NRGA, NOD/ShiLtJ-Rag2em1AMC Il2rgem1AMC; PBS, phosphate-buffered saline; PLA, proximity ligation assay; scr-shRNA, scrambled-shRNA; SDS, sodium dodecyl sulfate; shRNA, small hairpin RNA; SOS, son of sevenless; TMA, tissue microarray; WT, wild-type.

Hao Jin, Minsoo Koh and Hyesol Lim contributed equally to this work.

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underlying Ras isoform-specific activation however remain unclear. Our study showed that the membrane lipid raft protein Flotillin-1 was crucial for the interaction between SOS1 and H-Ras/K-Ras and subsequent Ras activation in human breast and pancreatic cancer cells. Flotillin-1 knockdown delayed breast cancer metastasis in a xenograft mouse model. The findings suggest that Flotillin-1 may serve as a membrane platform for SOS1 and H-Ras/K-Ras interactions and identify Flotillin-1 as a potential target in Ras-driven cancers.

1 | INTRODUCTION

The first identified oncogene, *ras*, is involved in multiple cellular signal transduction processes, leading to cell proliferation, invasion and metastasis.[1] H-Ras, N-Ras, K-Ras-4A and K-Ras-4B are the active mutant isoforms of Ras frequently observed in human cancers.[2] Ras isoforms share almost complete sequence homology except for the C-terminal hypervariable region (HVR), which consists of residues 166 to 189 that might affect their different cellular functions.[3,4]

Mounting evidence suggests Ras isoform-specific coupling in certain cancers. Among the Ras isoforms, K-Ras is predominantly mutated in many human cancers.[2] K-Ras mutations are frequently observed in pancreatic cancer.[5,6] N-Ras mutations are common in leukemias and cutaneous melanomas.[7,8] Oncogenic alterations in H-Ras are frequent in human cancers.[9] Although approximately 2% of breast cancer patients carry mutated *ras* genes, active mutated Ras proteins are associated with decreased survival in breast cancer patients.[10,11] Our previous studies demonstrated that H-Ras was involved in the malignant progression of breast cells.[4,12,13]

The activation of Ras requires son of sevenless (SOS) 1, a guanine nucleotide-exchange factor (GEF), which facilitates Ras activation in the membrane.[14] H-Ras is in dynamic equilibrium between lipid rafts and nonraft areas of the plasma membrane, which is regulated by Ras GTP loading.[15] H-Ras-GDP is primarily localized in lipid rafts. When activated, active H-Ras-GTP must exit lipid raft domains to induce the effective activation of Raf.[15,16] A cell-free system was used to demonstrate that N-Ras-GDP resided in nonlipid raft, whereas active N-Ras-GTP moved to lipid raft, showing an opposite pattern of membrane localization compared to H-Ras.[17] K-Ras-4A was localized in lipid raft,[18,19] whereas K-Ras-4B resided in nonlipid raft of plasma membrane.[20] Little is known about the micro-localization of the active state of K-Ras in the membrane. Although there is a key link between lipid rafts and Ras isoform-specific activation, the exact nature of the molecular interaction between lipid raft components and Ras remains unclear.

Flotillin-1 (Flot-1) is one of the scaffolding proteins in lipid rafts. Flot-1 plays important roles in signaling, cell adhesion, and the organization of the actin cytoskeleton.[21,22] Flot-1 has been associated with the poor prognosis of cancer patients in breast cancer and early-stage cervical cancer.[23,24] We previously demonstrated the role of Flot-1 in the epidermal growth factor (EGF)-induced activation of H-Ras using MDA-MB-231 human triple-negative breast cancer (TNBC) cells.[23] However, the detailed mechanism underlying the functional significance of Flot-1 has not yet been fully understood.

The aim of our study was to identify the role of Flot-1 in the activation of Ras isoforms at the molecular level. Our findings showed that Flot-1 was important for the interaction between SOS1 and H-Ras/K-Ras, which led to Ras activation in human breast and pancreatic cancer cells. Using tissue microarray and the Gene Expression Profiling Interactive Analysis (GEPIA) database, we demonstrated a correlation between K-Ras and Flot-1 expression in pancreatic cancer patients. These results revealed the potential mechanism of the crucial role of Flot-1 in H-Ras and K-Ras activation, suggesting that Flot-1 may be a potential therapeutic target in human cancer.

2 | MATERIALS AND METHODS

2.1 | Cell lines

MCF10A (RRID: CVCL_0598), H-Ras MCF10A and N-Ras MCF10A cells were established and cultured as previously described.[4,12] MDA-MB-231 (RRID: CVCL_0062), Hs578T (RRID: CVCL_0332), T24 (RRID: CVCL_0554) and AsPC-1 (RRID: CVCL_0152) cells were purchased from the Korean Cell Line Bank. MCF10A cells were purchased from the American Type Culture Collection (ATCC). MDA-MB-231, T24 and AsPC-1 cells were cultured in RPMI-1640 (Corning Life Sciences, Corning, NY) and Hs578T cells were cultured in Dulbecco's modified Eagle's medium (DMEM; HyClone; Thermo Fisher Scientific, Inc., Waltham, MA), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and incubated in a humidified incubator containing 5% CO_2 at 37°C. All cell-based experiments were performed using cells that had been tested and cultured for 4 weeks. All human cell lines have been authenticated using STR profiling within the last 3 years at the Korean Cell Line Bank. All experiments were performed with mycoplasma-free cells.

2.2 | Isolation of lipid rafts and membrane fractions

Detergent-resistant lipid rafts were isolated by the discontinuous sucrose gradient method as described previously.[25] Briefly, cells grown to confluence were washed with ice-cold phosphate-buffered saline (PBS) twice on ice, scraped and pelleted by centrifugation (12 000 rpm for 5 minutes). After homogenization, the lysates were brought to a final concentration of 40% sucrose by adding an identical volume of 80% sucrose. After centrifugation (38 000 rpm for 20 hours), the

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gradients were collected in 13 fractions (1 mL each) for subsequent analysis by immunoblotting. Isolation of the membrane fraction was performed as described previously.[26] Briefly, cells at ~80% confluence were homogenized. Cell homogenates were centrifuged at 3000 rpm for 10 minutes. After resuspending the pellet in a chelating buffer, the suspension was centrifuged at 12 000 rpm for 10 minutes. The resulting pellet was resuspended in an incubation buffer.

2.3 | Immunoblot analysis

Whole-cell lysates were prepared using sodium dodecyl sulfate (SDS) lysis buffer. Immunoblot analysis was performed as described previously.[13] After blocking with 10% nonfat milk for 30 minutes at room temperature and incubating with primary antibodies, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies and chemiluminescence reagents to quantify the relative expression levels, thereby detecting the immunoreactive bands. The Western Bright ECL kit (Advansta Inc., San Jose, CA) was used for band detection. The relative band intensities were determined by quantifying each band using FluorChem E (ProteinSimple, Inc., San Francisco, CA).

2.4 | Small hairpin RNA targeting Flot-1

Small hairpin RNA (shRNA) molecules targeting Flot-1 and control shRNA were purchased from Santa Cruz (Santa Cruz, CA). Cells cultured in a 6-well plate were transfected using Lipofectamine 2000 and OPTI-MEM reduced-serum medium (Invitrogen, Waltham, MA) following the manufacturer's instructions. Transfected cells were selected with puromycin (2.5 μg/mL) for 3 weeks.

2.5 | Construction of Flot-1 mutant

Expression vectors for full-length human Flot-1 and a truncated mutant lacking amino acids 39 to 427 were constructed using PCR. The amplified DNA was ligated into pcDNA3.1 (Invitrogen). Cells cultured in a 6-well plate were transfected with Flot-1 cDNA or control vector (pcDNA3.1) using TransIT-2020 transfection reagent.

2.6 | In vitro GEF assay

The in vitro GEF activity of SOS1 variants was examined using a GEF exchange assay biochemical kit (Cytoskeleton, Inc., Denver, CO) as described previously.[27] The exchange reaction mixtures were placed into a black-colored plate, and the fluorescence of each sample was measured. The exchange reaction was induced by adding SOS1 (aa 564-1049) to the H-Ras reaction mixtures. Flot-1 (full-length, Origen Technologies, Rockville, MD) was added to the reaction mixtures, and the relative fluorescence was monitored.

2.7 | In vitro invasion assay

The in vitro invasion assay was performed as described previously.[12] An indirect coculture invasion assay was performed using a 24-well Transwell unit (Corning Life Sciences, Corning, NY). The lower surface of the Transwell was coated with Type I collagen (Discovery Labware, Bedford, MA), and the upper surface of the Transwell was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ) and air-dried for 1 hour. AsPC-1 cells (2×10^4 cells/well) resuspended in serum-free medium were seeded in the Transwell. The cells were fixed with methanol and stained with 0.5% crystal violet. The absorbance was measured using a Synergy 2 Multi-Mode Reader (Biotek Instrument, Inc., Winooski, VT).

2.8 | Soft agar growth assay

Soft agar assays were performed in 6-well plates using a 3-mL basal layer of 0.6% agar in RPMI. A total of 5000 cells/well in 0.3% top agar were plated. Fresh top agar was overlaid every 5 days. After 2 weeks, positive colonies (>0.2 mm in diameter) were counted and the transformation efficiency was determined.

2.9 | Tissue microarray

We used tissue microarrays (TMAs) composed of 63 invasive pancreatic adenocarcinoma samples. TMAs for pancreatic adenocarcinoma and matched normal adjacent tissues (HPan-Ade120Sur-01) were purchased from US Biomax Inc. Immunohistochemical (IHC) analysis was performed using K-RAS or Flot-1 antibody as described as follows.

2.10 | IHC analysis

TMA tissue sections were treated with citrate buffer (pH 6.0) and autoclaved for 15 minutes. These sections were then blocked with 5% bovine serum albumin and 1% normal goat serum (Vector Laboratories, Newark, CA) in PBS for 1 hour. For peroxidase IHC analysis, endogenous peroxidase activity was blocked with 3.45% H_2O_2 (Samchun Chemicals, Seoul, Republic of Korea) for 15 minutes. Before incubating the sections with mouse primary antibody, mouse IgG was blocked using an M.O.M. Mouse IgG blocking kit (Vector Laboratories). Signals were analyzed by bright-field microscopy (Nikon; Melville; ECLIPSE 80i).

2.11 | Preparation and imaging of MDA-MB-231-derived metastasis model

Female NOD/ShiLtJ-Rag2em1AMC Il2rgem1AMC (NRGA) mice (7 weeks old) were obtained from JUNGAH BIO. All animal experiments were approved beforehand by the Institutional Animal Care

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and Use Committee of Duksung Women's University in accordance with the guidelines for the care and use of laboratory animals. NRGA mice were adapted for 1 week and randomly divided into two groups (n = 5/group). MDA-MB-231-luciferase cells or MDA-MB-231-shFlot1-luciferase cells (1×10^6 cells/mouse) were intravenously injected into the tail veins of NRGA mice. The progression of MDA-MB-231-derived metastasis was observed using an in vivo imaging system for noninvasive vital visualization. In vivo images were taken at 1, 3 and 5 weeks to compare luminescence intensity between the two groups. Each image was obtained by exposure to VISQUE InVivo Elite (Vieworks) for 10 minutes after the intraperitoneal injection of D-luciferin (PerkinElmer, Waltham, MA).

2.12 | Co-immunoprecipitation

Membrane fractions and whole lysates were incubated with the indicated antibodies overnight at 4°C. Protein G Agarose (Thermo Fisher Scientific) was added, resolved on SDS polyacrylamide gels and immunoblotted with the appropriate antibody.

2.13 | In situ proximity ligation assay

In situ PLA was performed using a Duolink-Detection Kit 613 with PLA-MIUNS and PLUS-Probes for rabbits and mice (Olink Bioscience, Uppsala, Sweden) as described previously.[28] The number of in situ PLA signals per cell was determined using a Carl Zeiss confocal fluorescence microscope (Thornwood) and counted by semi-automated image analysis (BlobFinderV3.0).

2.14 | Statistical analyses

All results are presented as the mean ± SD of three independent experiments. Multiple comparisons between groups were analyzed by one-way ANOVA (GraphPad Software, Inc., La Jolla, CA). For the TMA analysis of pancreatic cancer and matched normal adjacent tissues, statistical analyses were performed using GraphPad Prism 5 software. Associations between K-Ras and Flot-1 staining were evaluated by the Chi-squared test.[29] The statistical significance of differences was determined using the Student's *t*-test. Statistically significant *P* values are presented as $^*P < .05$ and $^{**}P < .01$.

3 | RESULTS

3.1 | Flot-1 is crucial for the activation of H-Ras, but not that of N-Ras, in breast cancer cells

To provide a molecular mechanism for the selective role of Flot-1 in H-Ras and N-Ras activation, we first examined the membrane localization of Flot-1 and the active and total forms of H-Ras and N-Ras in MDA-MB-231 cells containing WT (wild-type) H-Ras/N-Ras.[30,31] Lipid raft fractions were determined by the presence of caveolin-1.[32] The lipid raft fractions are marked with boxes (Figure 1). In the absence of EGF (-EGF), total H-Ras was detected in both lipid raft and nonlipid raft fractions, whereas total N-Ras was only detected in the nonlipid raft fractions of control MDA-MB-231 cells (Figure 1A). Both H-Ras-GTP and N-Ras-GTP (circled) were detected in nonlipid raft fractions under EGF stimulation (Figure 1B). Notably, EGF-induced H-Ras-GTP (circled) almost disappeared after the stable knockdown of Flot-1 by shRNA (flot-1-shRNA; Figure 1C,D), whereas the N-Ras-GTP was unaffected. Treatment with EGF increased the interaction between Flot-1 and H-Ras in the lipid raft fraction (Figure 1E). These data demonstrated that EGF increased the interaction between H-Ras and Flot-1 in lipid rafts, which might be important for H-Ras activation. Conversely, the EGF-stimulated activation of N-Ras was independent of Flot-1. Flot-1 in lipid rafts might be involved in the conversion of the GDP-bound form of H-Ras to the GTP-bound active form in breast cancer cells under EGF stimulation.

We additionally investigated the binding of Flot-1 and H-Ras in two cell lines that contained an endogenously active mutant of H-Ras, Hs578T TNBC cells containing H-Ras G12D and T24 human bladder carcinoma cells containing H-Ras G12V. The binding between Flot-1 and H-Ras was observed in both cell lines as evidenced by Co-immunoprecipitation (IP) assay (Figure 1F).

3.2 | N-terminal amino acid residues (1-38) of Flot-1 is important for its interaction with the HVR of H-Ras

To investigate the interaction between Flot-1 and H-Ras/N-Ras, we performed an in situ PLA in H-Ras MCF10A and N-Ras MCF10A cells. As shown in Figure 2A, the average PLA dots between Flot-1 and H-Ras (749.3) was higher than that between Flot-1 and N-Ras (132.5). The interaction between Flot-1 and H-Ras was further confirmed by a Co-IP assay using H-Ras MCF10A cells (Figure 2B). The interaction between Flot-1 and N-Ras was not detected in N-Ras MCF10A cells.

To identify the sequences responsible for H-Ras-specific interaction with Flot-1, we used our previously established H-Ras and N-Ras chimeric constructs with switched HVR sequences, named Hn166-189 and Nh166-189.[33] Hn166-189, an H-Ras mutant containing the N-Ras HVR, did not interact with Flot-1 in whole-cell lysates or the membrane fractions (Figure 2C, left). Interaction between Ras and Flot-1 was observed in Nh166-189, a N-Ras mutant containing the H-Ras HVR (Figure 2C, right). These data demonstrate that the H-Ras HVR sequences were important for binding to Flot-1.

We next aimed to define the region of Flot-1 essential for binding to H-Ras. The first hydrophobic domain (residues 10-36) of Flot-1 is known to be important in targeting lipid rafts.[34] Thus, we generated a myc-tagged wild-type Flot-1 designated WT Flot-1-myc with 1 to 427 amino acid residues and a truncated Flot-1 mutant designated

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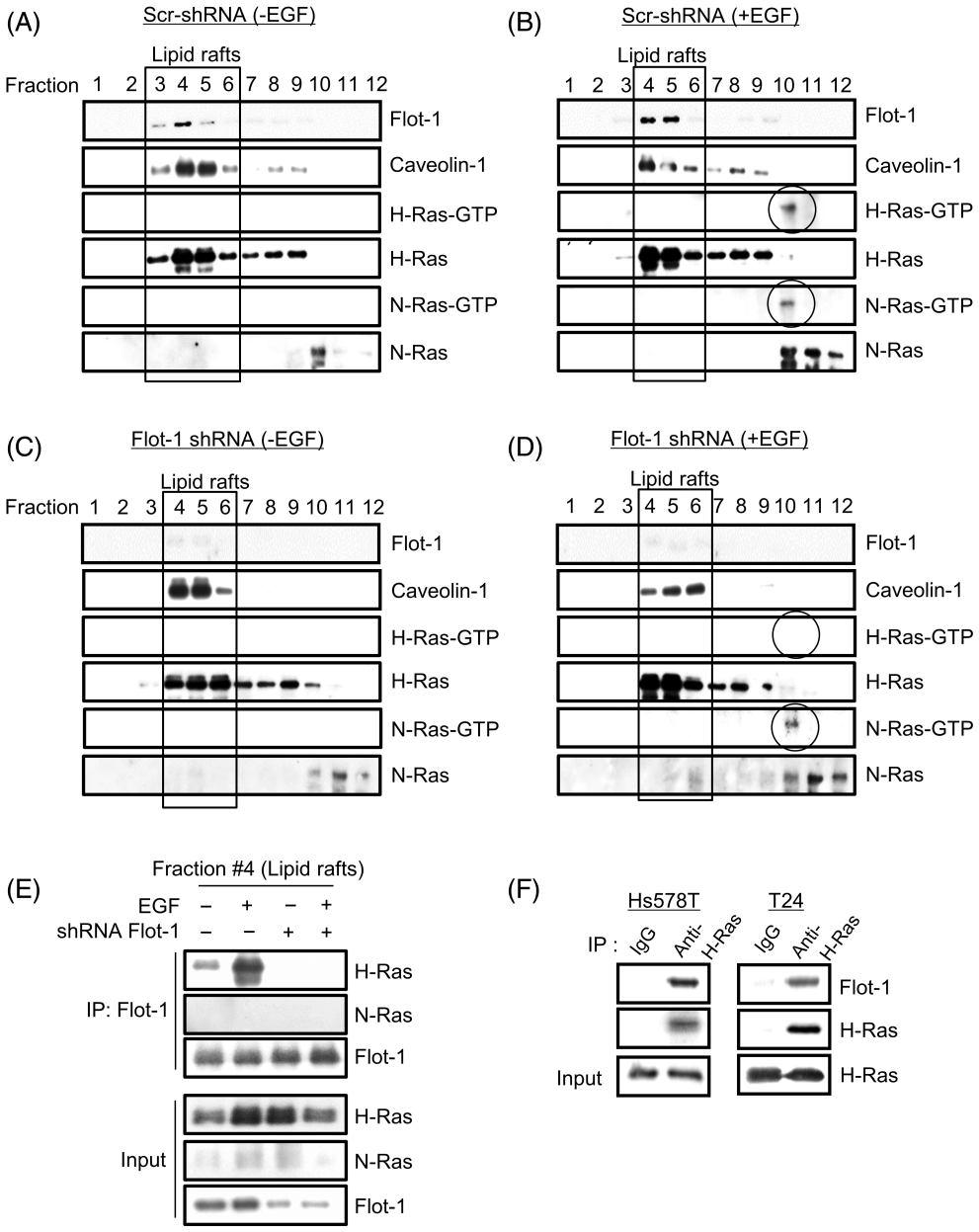

FIGURE 1 Flot-1 plays a crucial role in EGF-induced H-Ras activation. (A-D) MDA-MB-231 cells were stably transfected with scr-shRNA as a negative control (A and B) or shRNA targeting Flot-1 (C and D). The cells were cultured in serum-free media for 24 hours and exposed to 10 ng/mL EGF for 30 minutes (B and D). Membrane fractions of MDA-MB-231 cells were isolated by the sucrose density gradient method. The GTP-bound form of Ras was pulled down with glutathione S-transferase fusion proteins corresponding to the Ras-binding domain of Raf-1. The Ras-GTP proteins bound to the beads were identified using an anti-H-Ras or anti-N-Ras antibodies in immunoblot analysis. The protein levels were determined by immunoblot analysis. The marked box indicates lipid raft fractions. (E) MDA-MB-231 cells were stably transfected with scr-shRNA as a negative control or shRNA targeting Flot-1. The cells were incubated in serum-free media for 24 hours and exposed to 10 ng/mL EGF for 30 minutes. Fraction No. 4 (lipid raft fraction) of the cells was immunoprecipitated with anti-Flot-1 antibody for 17 hours and then immunoblotted with anti-H-Ras antibody. (F) Whole-cell lysates of Hs578T and T24 cells were immunoprecipitated with anti-H-Ras antibody and immunoblotted with anti-Flot-1 antibody. Three independent experiments were performed

Δ39Flot-1-myc with 39 to 427 amino acid residues. A strong interaction was observed between H-Ras and WT Flot-1-myc, while the interaction between H-Ras and Δ39Flot-1-myc was weak in both whole-cell lysates and membrane fractions (Figure 2D). These data suggest the involvement of the N-terminal residues (1-38) of Flot-1 in its interaction with the HVR of H-Ras.

3.3 | Flot-1 participates in the GEF activity of SOS1

To elucidate the role of Flot-1 in the EGF-induced H-Ras activation, we investigated whether Flot-1 affected the interaction between SOS1 and H-Ras in MDA-MB-231 cells. The Co-IP analysis showed

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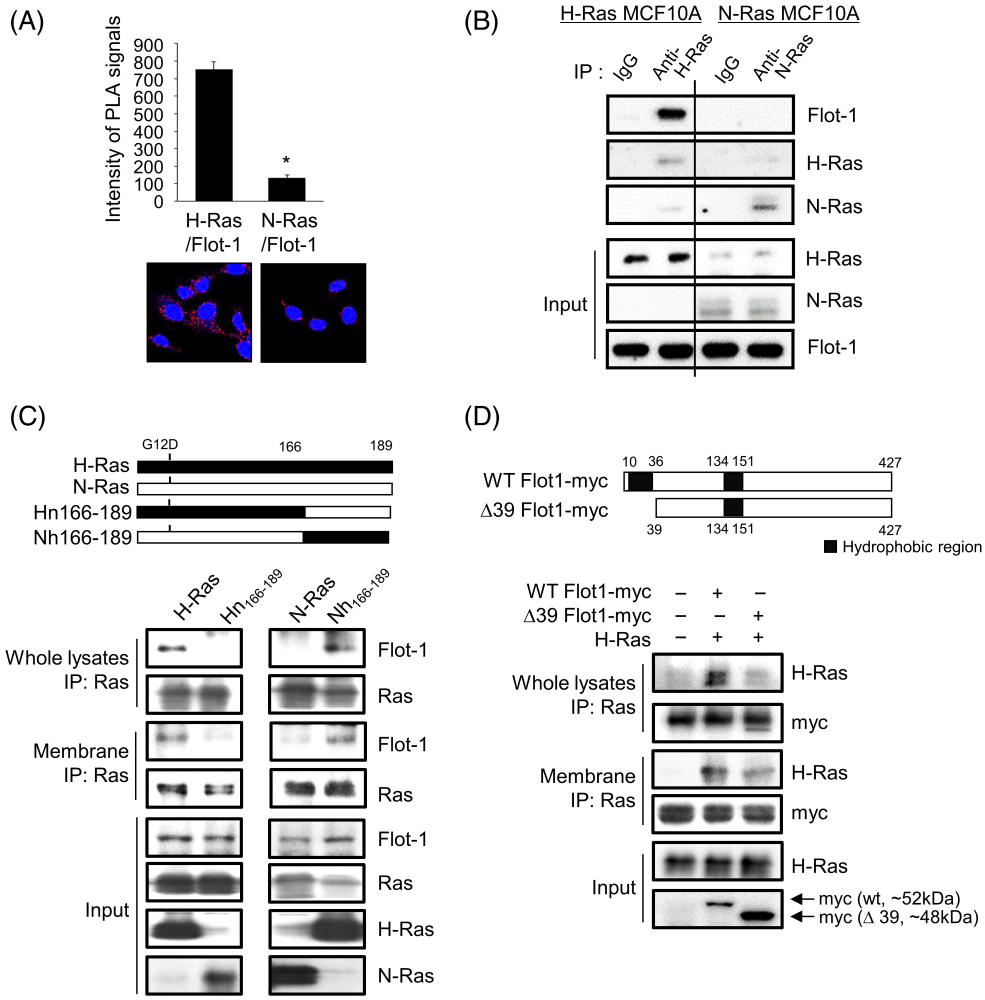

FIGURE 2 N-terminal amino acid residues[1-38] of Flot-1 interact with H-Ras HVR. (A) In situ PLA signals between H-Ras/N-Ras and Flot-1 in MCF10A cells were analyzed using BlobFinder software (lower). The intensity of the PLA signals was determined using ImageJ software (upper; *t*-test, **P* < .05). (B) Membrane fractions of H-Ras MCF10A and N-Ras MCF10A cells were immunoprecipitated with anti-H-Ras or N-Ras antibodies, respectively, and immunoblotted with anti-Flot-1 antibody. (C) Schematic outline of H-Ras/N-Ras chimera constructs. All of the Ras constructs contained an oncogenic mutation at codon 12 (G12D). Whole-cell lysates and membrane fractions of MCF10A cells stably expressing H-Ras, N-Ras or H-Ras/N-Ras chimera constructs were immunoprecipitated with anti-Ras antibody followed by immunoblot analysis for detecting Flot-1. (D) Schematic outline of the deletion mutant constructs of Flot-1. A black box indicates a hydrophobic region. Co-transfection was performed in MCF10A cells with H-Ras and myc-tagged wild-type Flot-1 constructs (WT Flot1-myc) or H-Ras and a myc-tagged truncated Flot-1 mutant (△39 Flot1-myc). Whole-cell lysates or membrane fractions were immunoprecipitated with anti-myc antibody followed by immunoblot analysis. Three independent experiments were performed [Color figure can be viewed at wileyonlinelibrary.com]

that the interaction between SOS1 and H-Ras was increased by EGF treatment (Figure 3A). Notably, stable knockdown of Flot-1 by shRNA markedly decreased the EGF-stimulated interaction between SOS1 and H-Ras.

An important role of Flot-1 in the EGF-induced interaction between SOS1 and H-Ras was further confirmed by the in situ PLA assay. As shown in Figure 3B (upper), treatment with EGF increased the interaction between SOS1 and H-Ras. Stable silencing of Flot-1 significantly inhibited this interaction as evidenced by decreased PLA signals. These data support the crucial role of Flot-1 in the interaction between SOS1 and H-Ras. The PLA signals between SOS1 and Flot-1 were also significantly increased by EGF stimulation, and decreased by Flot-1 knockdown (Figure 3B, lower). These data indicate that EGF-induced binding between SOS1 and H-Ras required Flot-1 in MDA-MB-231 cells.

We next examined the role of Flot-1 in the GEF activity of SOS1 by conducting a Ras-GEF assay. When an exchange domain of SOS1 protein (aa 564-1049) was mixed with full-length H-Ras protein, the fluorescence intensity representing GEF activity was increased compared to that of H-Ras protein alone (Figure 3C, left). The addition of Flot-1 protein (full-length) further enhanced the fluorescence intensity, indicating that Flot-1 could stimulate the GEF activity of SOS1 on H-Ras. The quantitative analysis of these data revealed that the addition of Flot-1 resulted in a significant increase (1.58-fold) in GEF activity, which was measured as the fold-change of released GDP compared to the H-Ras + SOS1 group (Figure 3C, right). These data

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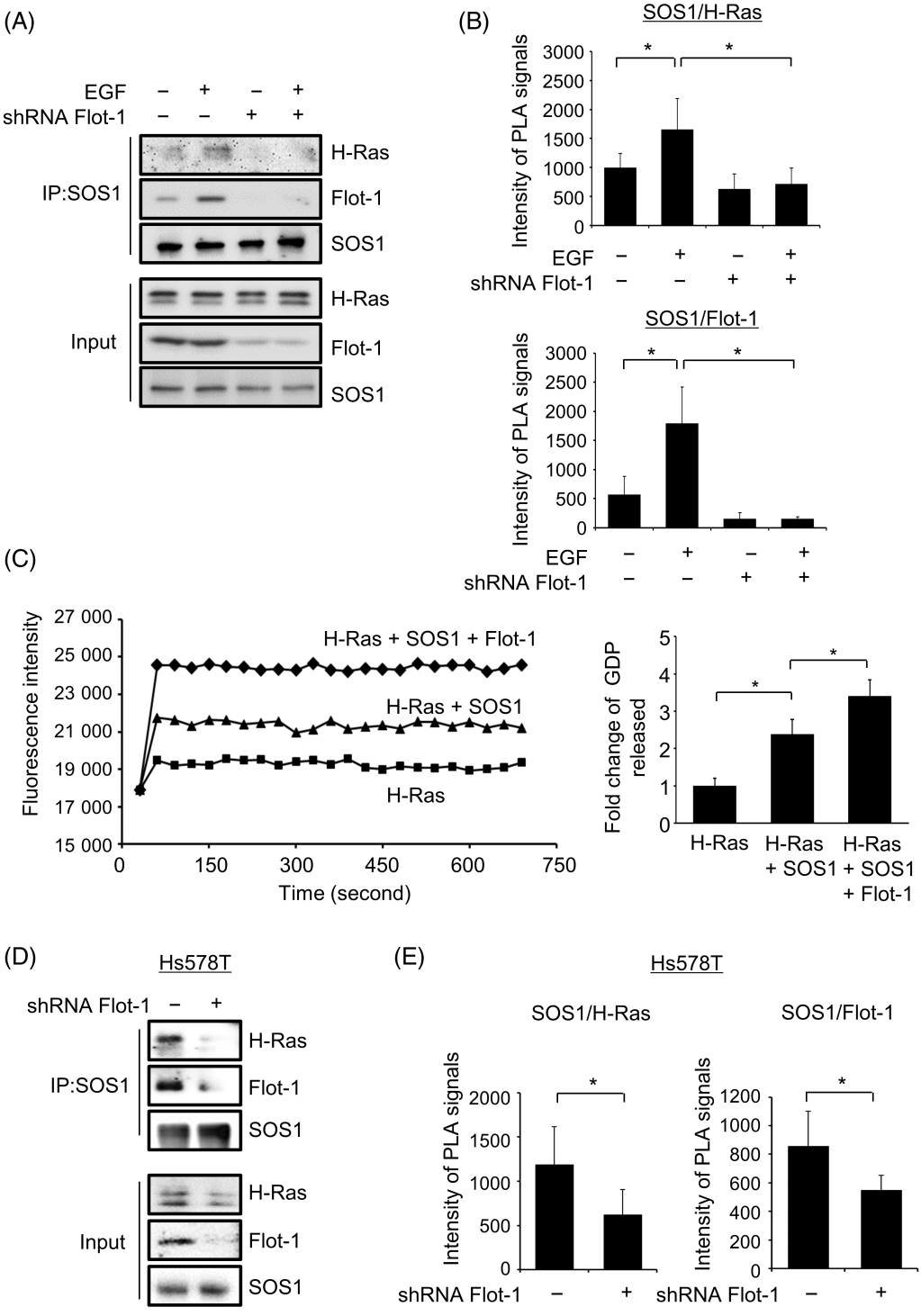

FIGURE 3 Flot-1 is crucial for the Ras-specific GEF activity of SOS1 by interacting with SOS1. (A) MDA-MB-231 cells were stably transfected with control scr-shRNA or shRNA targeting Flot-1. Whole-cell lysates were immunoprecipitated with anti-SOS-1 antibody followed by immunoblot analysis. (B) Interaction between SOS1 and H-Ras (top) or SOS1 and Flot-1 (bottom) was examined by in situ PLA (one-way ANOVA, $^*P < .05$). The PLA signal intensity was determined using ImageJ software. (C) The Ras-specific GEF activity of SOS1 was determined in vitro in the absence or presence of Flot-1 by fluorescence spectroscopy using a GEF exchange assay kit. The relative fluorescence intensity data from one representative experiment are shown (one-way ANOVA, $^*P < .05$). (D) Hs578T cells were stably transfected with scr-shRNA or shRNA targeting Flot-1. Whole-cell lysates of the cells were immunoprecipitated with anti-SOS1 antibody followed by immunoblot analysis for detecting H-Ras and Flot-1. (E) The in situ PLA was performed to investigate the interaction between SOS1 and H-Ras (left) or SOS1 and Flot-1 (right). The in situ PLA intensity was analyzed using ImageJ software (*t*-test, $^*P < .05$)

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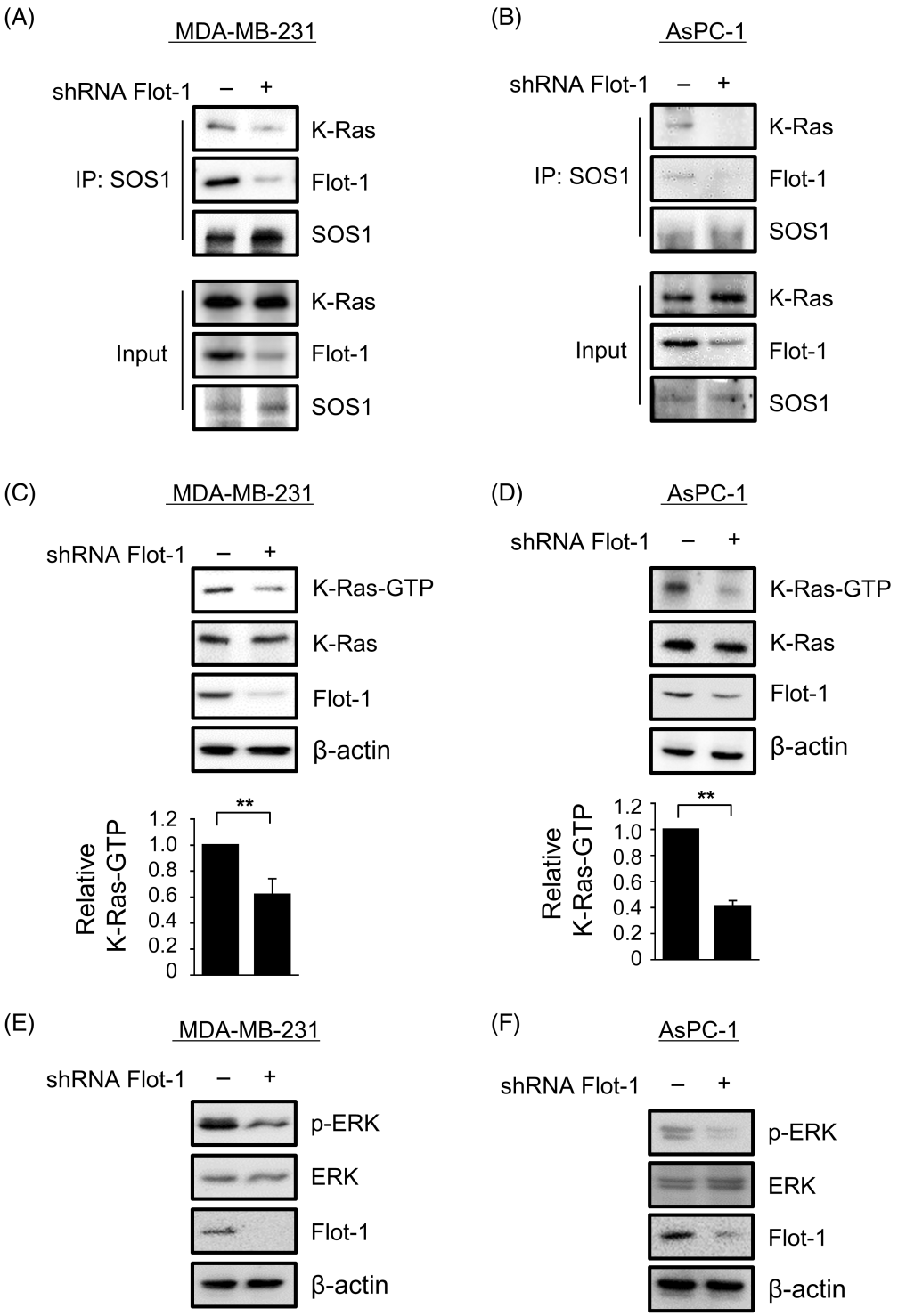

FIGURE 4 Flot-1 regulates K-Ras activation by interacting with K-Ras and SOS1 in human breast and pancreatic cancer cells. (A and B) MDA-MB-231 (A) and AsPC-1 (B) cells were stably transfected with scr-shRNA or shRNA targeting Flot-1. Whole-cell lysates were immunoprecipitated with anti-SOS-1 antibody followed by immunoblot analysis for detecting Flot-1 and K-Ras. (C and D) A pull-down assay was conducted using the whole-cell lysates of MDA-MB-231 (C) and AsPC-1 (D) cells transfected with scr-shRNA or shRNA targeting Flot-1. The GTP-bound form of Ras was pulled down with glutathione S-transferase fusion proteins corresponding to the Ras-binding domain of Raf-1. The Ras-GTP proteins bound to the beads were identified using an anti-K-Ras antibody in immunoblot analysis. Three independent experiments were performed and band intensities were quantitated by densitometric analysis (*t*-test, $^{**}P < .01$). (E and F) Immunoblot analysis was performed to detect the phosphorylated and total forms of ERK in MDA-MB-231 (E) and AsPC-1 (F) cells transfected with scr-shRNA or shRNA targeting Flot-1

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suggest that Flot-1 may play an important role in the GEF activity of SOS1, leading to the activation of H-Ras by interacting with SOS1.

We examined whether Flot-1 could interact with activated H-Ras and SOS1 using Hs578T cells with active mutant (G12D) of H-Ras. The Co-IP analysis showed that the stable knockdown of Flot-1 markedly decreased the interaction between SOS1 and H-Ras in Hs578T cells (Figure 3D). As shown in Figure 3E, the PLA signals between SOS1 and H-Ras were significantly decreased by the stable silencing of Flot-1.

Knockdown of SOS1 by siRNA markedly decreased the EGF-stimulated H-Ras-GTP and N-Ras-GTP levels (Figure S1), indicating that the activation of both H-Ras and N-Ras are dependent on SOS1 activity. The results suggest that SOS1 may convey Flot-1-independent pathway(s) for N-Ras activation.

Taken together, these results suggest that Flot-1 may act as a platform for the interaction of SOS1 with H-Ras (both WT and active forms) in the human breast carcinoma cell lines MDA-MB-231 and Hs578T.

3.4 | Flot-1 is crucial to the interaction between SOS1 and K-Ras, as well as K-Ras activation in human breast and pancreatic cancer cells

We investigated whether Flot-1 affected the interaction between K-Ras and SOS1 in MDA-MB-231 cells containing an active mutant of K-Ras.[31] The Co-IP analysis revealed that Flot-1-shRNA inhibited the interaction between SOS1 and K-Ras (Figure 4A). Since the oncogenic K-Ras mutation has been associated with pancreatic cancer,[5,6] we examined the role of Flot-1 in the human pancreatic cancer cell line AsPC-1, in which K-Ras is endogenously activated.[35] Flot-1 knockdown markedly suppressed the interaction between SOS1 and K-Ras (Figure 4B). We next investigated whether Flot-1 affected K-Ras activation in these K-Ras-mutated cancer cells. The stable knockdown of Flot-1 significantly decreased the level of K-Ras-GTP both in MDA-MB-231 and AsPC-1 cells (Figure 4C,D, respectively). CRISPR/Cas9-mediated Flot-1 deletion (see Supporting Information Materials and methods) markedly suppressed the level of K-Ras-GTP in MDA-MB-231 cells (Figure S3A). These data suggest that Flot-1 may act as a scaffolding molecule to regulate interactions between K-Ras and SOS1.

Thus far, we have shown the functional significance of Flot-1 in the activation of H-Ras and K-Ras. ERK is a major downstream molecule of the Ras signaling pathway that causes tumorigenicity and cancer development.[36] The stable knockdown of Flot-1 significantly inhibited the phosphorylation of ERK in both MDA-MB-231 and in AsPC-1 cells (Figure 4E,F, respectively).

We next examined if Flot-1 knockdown affected the regulators of lipid raft function such as proto-oncogene tyrosine-protein kinase (c-Src), homing cell adhesion molecule (CD44) and a disintegrin and metalloproteinase domain-containing protein (ADAM) 12.[37-39] As shown in Figure S2, the protein levels of c-Src, CD44 and ADAM12 were markedly decreased by stable knockdown of Flot-1 in both MDA-MB-231 and AsPC-1 cells. These results suggest an important role of Flot-1 in lipid raft function in breast and pancreatic cancer cells.

3.5 | Flot-1 plays a role in the transforming ability and invasive phenotype of MDA-MB-231 and AsPC-1 cells

We investigated the role of Flot-1 in anchorage-independent growth by performing a soft agar assay. As shown in Figure 5A, the stable knockdown of Flot-1 significantly reduced the number of colonies compared to control scrambled-shRNA (scr-shRNA). Our previous report showed an important role of Flot-1 in the invasiveness of MDA-MB-231 cells.[23] The invasive phenotype of AsPC-1 cells was significantly inhibited by Flot-1-shRNA (Figure 5B). Similarly, CRISPR/Cas9-mediated Flot-1 deletion significantly inhibited the invasive ability of MDA-MB-231 cells (Figure S3B). These results demonstrated that Flot-1 was required for the transforming ability and invasive phenotype of MDA-MB-231 and AsPC-1 cells.

3.6 | Flot-1 is important in lung and tailbone metastases of human breast carcinoma cells in a xenograft mouse model

We examined the role of Flot-1 in the in vivo metastatic potential of MDA-MB-231 cells. To this end, we established a xenograft mouse tumor model carrying scr-shRNA or Flot-1-shRNA MDA-MB-231 cells that stably expressed constitutive luciferase, enabling metastasis to be monitored by a noninvasive in vivo imaging system. As shown in Figure 5C, all animals in both groups exhibited comparable bioluminescence initially and no significant difference in photon flux at 7 days. However, after 35 days, lung and tailbone metastases were significantly reduced in mice carrying Flot-1-shRNA MDA-MB-231 cells compared to the control mice with scr-shRNA. These results indicate that Flot-1 may be involved in the in vivo metastatic ability of MDA-MB-231 human breast carcinoma cells.

3.7 | Flot-1 correlates with K-Ras expression in human pancreatic cancer

To predict the clinical relevance of our findings in pancreatic cancer, we analyzed the immunohistochemical expression levels of Flot-1 and K-Ras using a tissue microarray of 61 human pancreatic cancer samples. As shown in Figure 6A, the representative immunohistochemistry images showed significantly higher levels of membrane and cytoplasmic expression of Flot-1 in pancreatic tumor tissues than in normal tissues. K-Ras was also highly expressed in tumor tissues. Quantitative analysis confirmed that the Flot-1 expression level in tumor tissues (72%) was higher than that in normal tissues (28%; Figure 6B). Higher expression of K-Ras (93%) was observed in tumor

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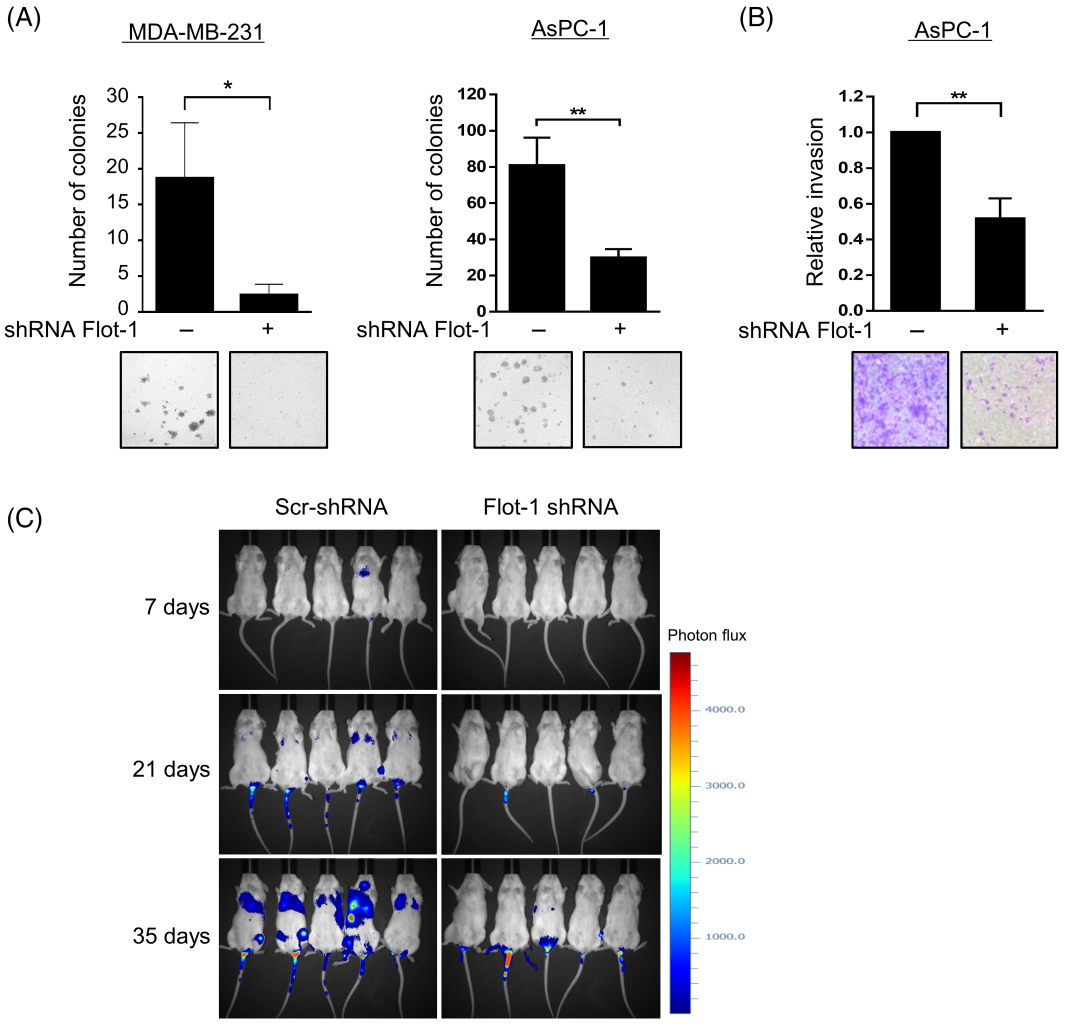

FIGURE 5 Knockdown of Flot-1 prevents progression and metastasis in breast and pancreatic cancer. (A) MDA-MB-231 (left) and AsPC-1 (right) cells were stably transfected with scr-shRNA or shRNA targeting Flot-1. A soft agar assay was conducted and the number of colonies was counted (*t*-test, $^{*}P < .05$, $^{**}P < .01$). Representative images are shown (original magnification, 100×). (B) AsPC-1 cells were stably transfected with scr-shRNA or shRNA targeting Flot-1. An in vitro invasion assay was performed (*t*-test, $^{**}P < .01$), and representative images are shown (original magnification, 100×). (C) Tumor-bearing mice were divided into two groups (n = 5): the control group (MDA-MB-231-luciferase cells transfected with scr-shRNA) and the Flot-1 knocked-down group (MDA-MB-231-luciferase cells transfected with shRNA targeting Flot-1). Cells suspended in 100 μL of PBS (pH 7.4) were intravenously injected into the tail veins of NRGA mice and the mice were monitored for 5 weeks. The mice were imaged for metastatic spreading on the 7th, 21st and 35th days. Bioluminescent imaging was conducted using VISQUETM InVivo Elite. The colored bar represents fluorescence intensity [Color figure can be viewed at wileyonlinelibrary.com]

tissues than in normal tissues (33%; Figure 6C). These results indicate an association of Flot-1 with K-Ras in human pancreatic cancer. As shown in Figure 6D, a strong correlation between Flot-1 and K-Ras was determined by analysis of a tissue microarray.

Next, we analyzed the correlation of K-Ras expression with Flot-1 expression using Gene Expression Profiling Interactive Analysis (GEPIA) database in pancreatic cancer patients. As shown in Figure 6E, there was a significant positive correlation between mRNA expression of Flot-1 and K-Ras (Pearson correlation coefficient of .45, $P = 3.9e{-}10$) in pancreatic cancer patients. Analysis of the Bioportal database (www.cbioportal.org) from K-Ras mutant human pancreatic cancer patients revealed a positive correlation between K-Ras and Flot-1 (Pearson correlation coefficient of .35, $P = 3.256e{-}5$; Figure 6F). Taken together, these data suggest that Flot-1 might contribute to the oncogenicity of K-Ras in human pancreatic cancer.

4 | DISCUSSION

Activation, the recruitment of effectors, and the biological activities of Ras are inseparably related to its membrane association.[40] The Ras HVR contains membrane-targeting or anchor domain-containing residues and thus, is responsible for the difference in the membrane micro-localization of Ras isoforms.[41] We previously showed that the H-Ras HVR affected the H-Ras-specific invasive phenotype of breast cells.[33] However, the Ras isoform-specific interactions with

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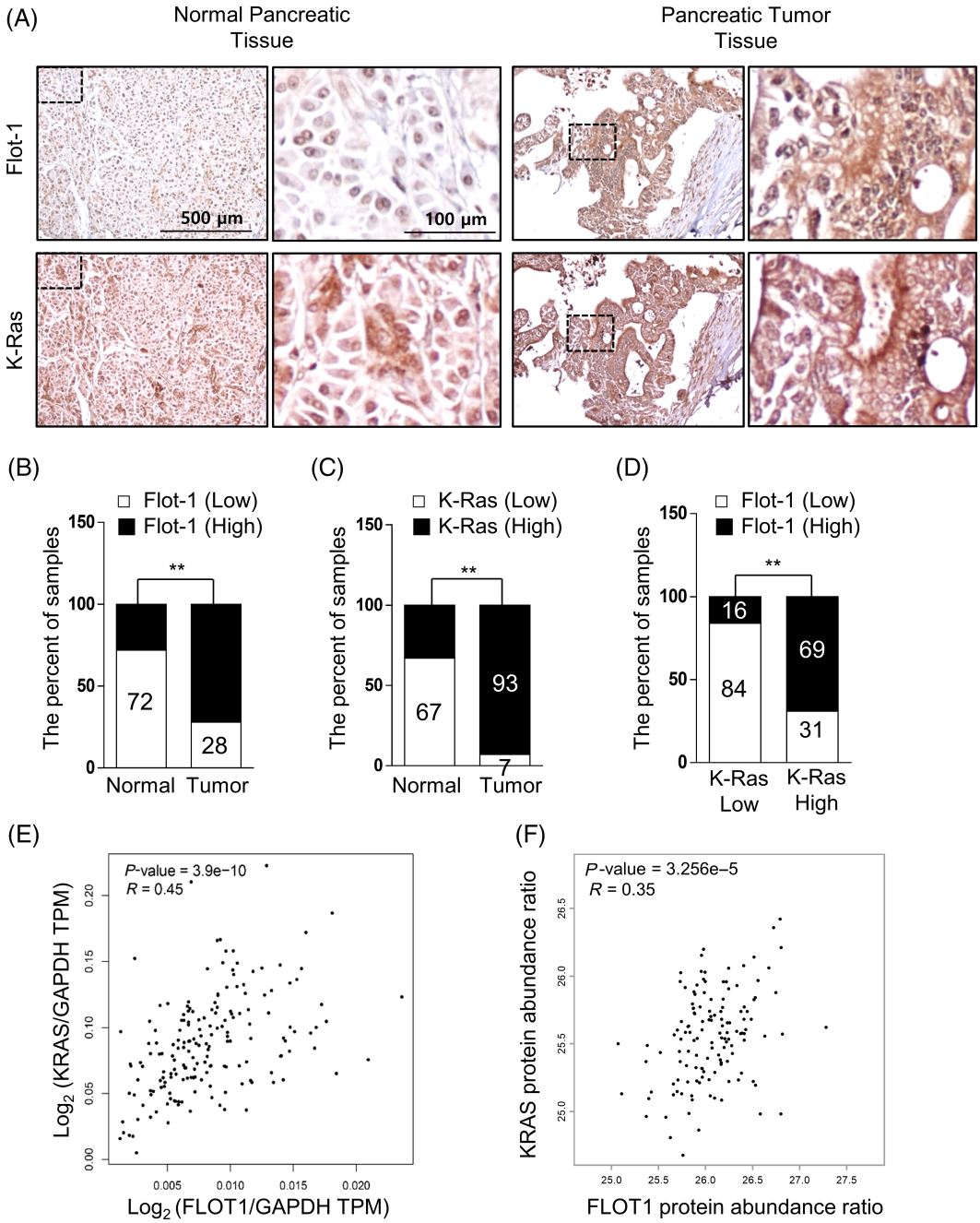

FIGURE 6 Flot-1 is correlated with the expression of K-Ras in human pancreatic cancer. (A-D) Immunohistochemical analysis was performed using a tissue microarray composed of 63 cases to detect Flot-1 and K-Ras expression in human pancreatic normal and tumor tissues. (A) Representative images are shown (original magnification, 500×). The tissue samples were divided into two groups: high or low levels of Flot-1 (B) and high or low levels of K-Ras (C) in normal human pancreatic and tumor tissues. D, The tissue samples were divided into two groups: high or low levels of Flot-1 and high or low levels of K-Ras of human pancreatic tumor tissue. (E) The correlation between Flot-1 and K-Ras in human pancreatic cancer tissues. The gene expression was analyzed using Pearson's correlation coefficient in the GEPIA database. (F) The correlation between Flot-1 and K-Ras protein abundance ratio in human K-Ras mutant pancreatic cancer tissues. The data were analyzed using Pearson's correlation coefficient in the bioportal database [Color figure can be viewed at wileyonlinelibrary.com]

membrane components have not yet been fully explored. The results of the present study revealed that H-Ras interacted with Flot-1 and this interaction was important for H-Ras activation in breast epithelial cells. Both N-Ras-GDP and N-Ras-GTP were detected in the nonlipid rafts of MDA-MB-231 cells. Importantly, H-Ras activation required Flot-1, whereas Flot-1 was not crucial to N-Ras activation in MDA-MB-231 cells. It was shown that the active N-Ras-GTP was dependent on cholesterol in MDCK and COS-7 cells.[42] In contrast, Shishina et al. reported that N-Ras-GDP was detected in lipid rafts, and associated with the nonlipid rafts when bound to a GTP mimic.[43] Our data

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showed that N-Ras-GTP was detected in nonlipid raft fractions upon EGF stimulation. Given that the localization of H-Ras was shown to be cell type-dependent,[44] N-Ras localization may also be cell type-dependent. Further studies are required to clarify this issue. These data indicate a cellular microenvironment-dependent association of Ras isoforms with the lipid raft protein Flot-1.

SOS1, the first identified GEF for Ras in humans, binds to Ras-GDP at a catalytic domain or to Ras-GTP at an allosteric site.[14,45] The binding of Ras-GTP to the allosteric site of SOS1 enhanced the GEF activity of SOS1, and then, the binding continuously activated Ras.[46] A recent study suggests that inhibition of GEFs by SOS inhibitor may represent a feasible approach for targeting Ras-driven cancers.[47] Our results demonstrated that Flot-1 played a key role in the GEF activity of SOS1, and the activation of H-Ras. Flot-1 was important in SOS1-H-Ras interaction, not only upon EGF stimulation in MDA-MB-231 cells but also in Hs578T cells containing endogenously activated H-Ras.

K-Ras mutation accelerates pancreatic intraepithelial neoplasia formation to progress to metastatic pancreatic cancer.[48] Although K-Ras is not as imperative in breast cancer as in pancreatic cancer, breast cancer patients with high levels of K-Ras showed lower relapse-free survival rates.[49] The overexpression of active mutant K-Ras enhanced the migration of MCF10A breast epithelial cells.[50] Our data showed that stable knockdown of Flot-1 decreased K-Ras-GTP levels and the interaction between K-Ras and SOS1 in K-Ras-mutated MDA-MB-231 breast and AsPC-1 pancreatic cancer cells. These results imply that Flot-1 is involved in the activation of H-Ras/K-Ras. Modulating the interaction between SOS1 and K-Ras has been suggested as a promising strategy against cancer. In oncogenic K-Ras-harboring cancer cells, the inhibition of SOS1 reduced ERK phosphorylation.[51] The activation of WT Ras-induced by oncogenic K-Ras was mediated by SOS1, conferring tumorigenesis.[52] A recent study showed that the pharmacological inhibition of SOS1-K-Ras interaction suppressed cell growth in Ras-mutated cancers.[53] Our present study showed that Flot-1 was crucial for the interaction between SOS1 and K-Ras in MDA-MB-231 breast carcinoma and AsPC-1 pancreatic cancer cells.

The inhibition of Ras activation has been focused on inhibiting the oncogenic Ras mutant, Ras-GEF interaction, the membrane association, Ras oligomerization and downstream pathways.[54] Here, we suggest that targeting Flot-1 may be an effective strategy to inhibit SOS1-mediated Ras activation. Flot-1 promoted cell proliferation and tumorigenicity in breast cancer.[55] We have previously demonstrated that tumor growth was suppressed by the knockdown of Flot-1 in a TNBC xenograft tumor mice model.[23] The present study showed that Flot-1 knockdown reduced the invasive phenotype and anchorage-independent growth of human cancer cells with the endogenously active mutant K-Ras. Moreover, stable silencing of Flot-1 significantly inhibited the lung, tail vein and tail bone metastasis in a mice model xenografted with human breast cancer cells. Analysis of the GEPIA, bioportal and tissue microarrays from human pancreatic cancer patients revealed the positive correlation between K-Ras expression and Flot-1 expression.

Lipid raft proteins are highly expressed in cancer cells compared to normal cells, serving as a centralized platform for classifying different signaling processes and regulating many cancer-related signaling.[56] A significant number of proteins that are associated with lipid rafts, such as cholesterol, Flot-1 and caveolin, are involved in cancer development. They have received much attention as clinical biomarkers of cancer.[57,58] Flot-1 amplification has been observed in several solid tumors including breast and pancreatic cancers.[23,59] Although pancreatic cancer and breast cancer differ substantially in the molecular environment and clinical behavior, our results suggest that Flot-1 may be a potential target for these two cancers.

Taken together, our study revealed the role of the lipid raft protein Flot-1 in Ras activation by regulating the interaction with Ras and SOS1 in cancer. The detailed mechanism of SOS1 recruitment to the membrane has not been well-understood. It is possibility that Flot-1 may induce the association of SOS1 with Ras by providing a membrane platform, affecting its GEF activity.

A recent study suggests that inhibition of GEFs by SOS inhibitor may represent a viable approach for targeting Ras-driven cancers.[51] Our findings suggest that Flot-1 may be a potential target for Ras-driven cancers by interaction with Ras and SOS1. Further studies investigating the detailed molecular mechanisms underlying the role of Flot-1 in regulating the GEF activity of SOS1 might provide valuable insight into Ras-driven therapeutic targets in cancer.

AUTHOR CONTRIBUTIONS

Hao Jin, Minsoo Koh, Hyesol Lim, Hae-Young Yong, and Aree Moon conceived and designed the study. Hao Jin, Minsoo Koh, Hyesol Lim, Hae-Young Yong, Eun-Sook Kim and Sun Young Kim performed the experiments. Hao Jin, Minsoo Koh, Hae-Young Lim, Hae-Young Yong and Aree Moon analyzed and interpreted the data. Won-Ji Ryu and Kang-Yell Choi performed the TMA experiments and analysis. Kyoungmee Kim and Joohee Jung completed the in vivo experiments and analysis. Hao Jin, Minsoo Koh and Hyesol Lim contributed to manuscript drafting and writing. Hao Jin, Minsoo Koh, Hyesol Lim and Aree Moon reviewed and edited the manuscript. All authors read and approved the manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work was appropriately investigated and resolved. Aree Moon supervised the study. All the work reported in the paper has been performed by the authors, unless clearly specified in the text.

ACKNOWLEDGEMENT

The present study was supported by the National Research Foundation of Korea (Nos. 2016R1A6A1A03007648 and 2019R1A2C1009773).

CONFLICT OF INTEREST

The authors have no conflicts of interest relevant to our study to disclose.

DATA AVAILABILITY STATEMENT

The data that support the findings of our study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Jin H, Koh M, Lim H, et al. Lipid raft protein flotillin-1 is important for the interaction between SOS1 and H-Ras/K-Ras, leading to Ras activation. *Int J Cancer*. 2023;1-14. doi:10.1002/ijc.34443

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