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Flotillin-1 promotes cell growth and metastasis in oral squamous cell carcinoma

P. XIONG[1], L. Y. XIAO[1], C. C. ZHAO[2], R. YANG[1], Q. GUO[1], Y. Q. ZHAO[1], W. LI[1,*], Y. SUN[2,*]

[1]State Key Laboratory of Oral Diseases, Sichuan University, Chengdu, 610041, China; [2]The Digital Dental Center of Chengdu Military Region Hospital, Chengdu, 610041, China

**Correspondence: leawei2000@126.com, stomatology_scu@163.com*

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Even to date, Oral squamous cell carcinoma (OSCC), which is one of the most common malignancies worldwide, is still a major public health problem. The cellular mechanisms underlying development of OSCC are poorly understood. Lipid rafts-associated proteins not only serve as a docking platform for protein sorting and membrane trafficking, but also coordinate signaling molecules at cell membrane to mediate intracellular responses, which makes them susceptible to be subverted by cancer cells. Although Flotillin-1 has been discovered for decades, its potential role in OSCC development is largely unknown. In current study, we demonstrate that Flotillin-1 is highly expressed in OSCC cell lines compared to normal oral epithelial cells. Modulation of Flotillin-1 expression via transfection with Flotillin-1 expression vector or shRNA showed that Flotillin-1 has a clearly positive impact on cell growth and motility in KB and/or Tca8113 cell lines. These observations were further supported by using mice or zebrafish tumor xenograft models. Mechanistic study indicated that Flotillin-1 expression activates NF-κB signaling pathway by enhancing phosphorylation of p65 and IκBα, and translocation of p65 into nucleus. Furthermore, inhibition of EGFR by AG1478 markedly repressed Flotillin-1-induced activation of NF-κB signaling pathway. Our studies suggested that Flotillin-1 plays an important role in OSCC development, and might be a potential therapeutic target for OSCC.

Key words: OSCC, Flotillin-1, proliferation, metastasis

Oral cavity cancer accounts for 24% of head and neck cancers and causes over 200,000 deaths annually [1]. OSCC, including tumors affecting the tongue, floor of the mouth, buccal mucosa and gingiva, is the most frequent malignancy of the head and neck [2]. Despite improvements in surgery and radiation, the prognosis of OSCC remains dismal because more than 50% of patients die within 5 years after diagnosis [3]. Surgical excision is commonly used in treating OSCC, however, it has significant side effects on swallowing, speech and other physical appearance. The additional chemotherapy treatment showed efficacy in organ preservation in some sites in oral cavity, but it resulted in limited improvement in survival rates, which is probably due to drug resistant, either intrinsic or acquired, after the initial round of treatment [4]. On the other hand, the presence of lymph node metastasis, which affects more than 50% of OSCC patients, is one of the most important prognostic indicators associated with poor outcome. Although detection of nodal metastasis, mainly based on physical examination of enlarged lymph nodes and imaging, is widely applied in OSCC diagnosis, there is still a high incidence (> 20%) of neck metastasis [5].

It is widely accepted that development of OSCC involves multiple steps, starting with pre-malignant lesions, dysplasia, tumor *in situ* and at last tumor spread in a distant organ [6]. As is the case with other tumor types, OSCC is a complicated genetic disease which is companied with increased genomic instability. Both gene mutation and amplification were demonstrated with respect to cancer development and drug resistance via activating oncogenes and inhibiting tumor suppressor genes [7]. For instance, genetic abnormity in p53 and p16/MTS1 has long been found in OSCC patients and were tightly associated with poor prognosis [8, 9]. However, recently it is clearer that, alterations in protein post-translational

Abbreviations: OSCC, oral squamous cell carcinoma; **SPFH,** stomatin-prohibitin-flotillin-HflK/C; **EMT,** Epithelial-mesenchymal transition; **MTT,** 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

modification, cellular compartment translocation and protein-protein interactions also play a crucial role in carcinogenesis and tumor metastasis; and these molecular events cannot be studied by simply detecting the amount of RNA or by analyzing nucleotide sequence [10].

Flotillin-1 and Flotillin-2, belonging to the highly conserved Flotillin protein family, share approximately 50% identity in their amino acid sequence [11]. Both of the two Flotillin proteins contain a stomatin-prohibitin-flotillin-HflK/C (SPFH) domain, a motif that is essential to form a heterooligomeric complex. Physical interaction of the two proteins was reported to play crucial roles in diverse cellular processes, including cell-cell adhesion, cytoskeleton assembly, phagocytosis and protein secretion [12]. Since the Flotillin complexes are detergent resistant, Flotillin proteins are considered as components of lipid rafts and were demonstrated to be involved in the initiation of signaling transduction [13]. It was documented that activation of Src tyrosine kinases induced Flotilins internalization which was important for tyrosine kinase–regulated endocytic process [14]. Further, Flotillins mediated recruitment of Cbl-CAP to lipid rafts upon insulin treatment, which was a key step for glucose uptake in adipocytes [15].

Recently, a small but increasing number of studies have shed a light on the potential role of Flotilin-1 in carcinogenesis [16-18], but the precise mechanism remains unclear. In this study, we found that Flotillin-1 was highly expressed in OSCC cell lines. Overexpression of Flotillin-1 enhanced proliferation and metastasis of OSCC cells.

Materials and methods

Cells culture. Human gingival epithelial cells were isolated from the normal gingival tissues of healthy donors under informed consent as described previously [19]. Human OSCC cell line Tca8113, Ca9-22, HSC4, SAS, H357 and KB were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) containing 10% fetal calf serum (Gibco, USA), penicillin (100 U/L) and streptomycin (10 mg/L). Cells were incubated in a humidified atmosphere containing 5% CO_2 at 37°C and passaged every 5 days at a split ratio of 1:4 using trypsin.

Reagents. Rabbit-anti-Flotillin-1 (ab41927), Rabbit-anti-E-cadherin (ab53033), Mouse-anti-Vimentin (ab8069), Mouse-anti-Twist (ab135180), Rabbit-anti-α-tubulin (ab15246) and Rabbit-anti-β-actin (ab8227) were purchased from Abcam. Rabbit-anti-Phospho-NF-κB p65 (Ser536) (#3033), Mouse-anti-Phospho-IκBα (Ser32/36) (#9246), Rabbit-anti-Cyclin D1 (#2978), Mouse-anti-Histone H3 (#3638), Rabbit-anti-NF-κB p65 (#4764) and Rabbit-anti-IκBα (#9242) were obtained from Cell Signaling. AG1478 was purchased from Sigma.

Western blotting. Proteins were extracted in RIPA buffer (50 mM Tris base, 1.0 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF) and quantified by the DC protein assay kit (Bio-Rad). Samples were separated by 12% SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences). The membranes were blocked overnight with PBS containing 0.1% Tween 20 in 5% skimmed milk at 4 °C and subsequently probed by primary antibodies. Blots were incubated with the respective primary antibodies for 2 h at room temperature. After washing three times in TBS with Tween 20, the blots were incubated with secondary antibody (diluted 1:10,000; Santa Cruz Biotechnology) conjugated to horseradish peroxidase for 2 h at room temperature. Blots were visualized by enhanced chemiluminescence reagents (Amersham Biosciences).

RNA interferences. To repress expression of endogenous Flotillin-1, two distinct shRNA constructs were generated. The sequences were shFlotillin-1-1: CCC TCA ATG TCA AGA GTG AAA; shFlotillin-1-2: ACA GAG AGA TTA CGA ACT GAA; non-specific shRNA: AAU CAA CUG ACU CGA CCA CUA. The two oligonucleotides were cloned into the pSuper-retro-puro vector, respectively.

Cell proliferation assays. Cell proliferation was measured by means of MTT assay according to the manufacturer's instructions. 20 μl MTT (2 mg/ml, Sigma, St. Louis, MO) was added in the media and incubated for another 2 h. The media was removed and formazan precipitate was dissolved in 150 μl Dimethyl Sulfoxide (DMSO, Amresco, Solon, Ohio, USA). Ten minutes later, absorbance values were measured at 595 nm wavelength.

For colony formation assay, cells were seeded in 6-well plates at a density of 300 cells per well. Cells were transfected with plasmids or shRNAs for 48h, and then treated with cisplatin as described for another 24h. Cells were washed and then cultured for two weeks. Colonies were washed with PBS, fixed with methanol and stained with Crystal Violet (Sigma, St. Louis, MO, USA). Clones were counted under a microscope and a cell cluster with more than 50 cells was considered as a clone.

Cell motility assay. Cell migration assays were performed by scratch wound healing assay. Wounds were created in confluent cells by scraping the cell surface using a sterile pipette tip, and then the cells were washed with medium to remove free-floating cells and debris. Wound healing was measured by counting the number of cells migrated into scraped line.

Cell invasion was examined by using transwell 24-well chambers (Corning, USA). Matrigel (1:3, BD) was added to the transwell membrane chambers, incubated for 4 h. Cells in serum-free medium (2.5×10^4 cells per well) were added to the upper chamber. After 48 hours, the number of cells, which migrated to the lower chamber was counted.

***In vivo* tumor growth assay.** The Institutional Animal Care and Treatment Committee of Sichuan University approved all studies herein. KB cells that were stably transfected with mock vector or Flotillin1 expression plasmid were suspended in PBS, then injected subcutaneously in nude mice (2×10^6 cells/mouse). Tumor growth curves were plotted by measuring tumor volume at indicated times. Mice were sacrificed 25 days after injection.

Mouse lung metastasis model. Mouse lung metastasis model was established as previous reports [20]. KB cells that were stably transfected with mock vector or Flotillin1 expression plasmid were suspended in PBS, then injected in nude mice (1×10^6 cells/mouse) via the tail vein. Animals were sacrificed 45 days after injection. Lung metastasis was compared by counting the number of lung metastatic nodules and measuring the lung weight.

Zebrafish tumor metastasis model. Zebrafish tumor metastasis model was established following previous report [21]. At 48 h post-fertilization, zebrafish embryos of the Tg (fli1:EGFP) strain were dechorionated with help of a sharp tip forceps and anesthetized with 0.04 mg/ml of tricaine (MS-222, Sigma). Tumor cell were labeled with red fluorescent dye CM-DiI (DiI, Fluka, Germany). Approximately 100 cells were resuspended in serum-free DMEM (Sigma) and 5 nL of tumor cell solution were injected into the perivitelline cavity of each embryo using an Eppendorf microinjector (FemtoJet 5247, Eppendorf and Manipulator MM33-Right, Märzhäuser Wetziar). Embryos were kept at 28 °C after injection. Tumor cell metastasis was measured by counting the cells migrated to the posterior part by using a fluorescent microscope (Nikon Eclipse C1).

Preparation of nuclear and cytosolic protein samples. Nuclear and cytosolic protein samples were prepared by using ReadyPrep Protein Extraction Kit (Cytoplasmic/Nuclear). This kit was purchased from Bio-Rad.

Statistics analysis. Differences between two groups were assessed by Student's t test. *, P<0.05; **, P<0.01; ***, P<0.001.

Results

Flotillin-1 is expressed in OSCC cell lines. As an initial test, expression of Flotillin-1 in primary culture of normal oral epithelial cells and a series of OSCC cell lines, including KB, H357, SAS, Tca8113 Ca9-22, and HSC-4, were examined by immunoblot. As shown in Fig. 1, Flotillin-1 was expressed at a relatively low level in both the two strains of primary cultured oral epithelial cells. In contrast, Flotillin-1 was highly expressed in Tca8113, SAS, HSC-4 and H357 cell lines. While a moderate and low expression of Flotillin-1 was found in Ca9-22 and KB cell lines, respectively.

Flotillin-1 promotes proliferation of OSCC cells. To investigate the impact of Flotillin-1 expression on OSCC cell proliferation, KB cell line was selected as *in vitro* model. Transfection with expression plasmid coding full-length Flotillin-1 markedly induced expression of exogenous Flotillin-1 in KB cells (Fig. 2A). As shown, overexpression of Flotillin-1 markedly enhanced proliferation ratio of KB cells, revealed by both MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and clonogenic formation assays. In MTT assay, though no difference was observed 24 h after transfection, the proliferation ratio in Flotillin-1 expressed KB cells was augmented by 35.10% at 48 h ($P < 0.05$) and 51.00% ($P < 0.01$) at 72 h posttransfection, compared to mock vector treated cells

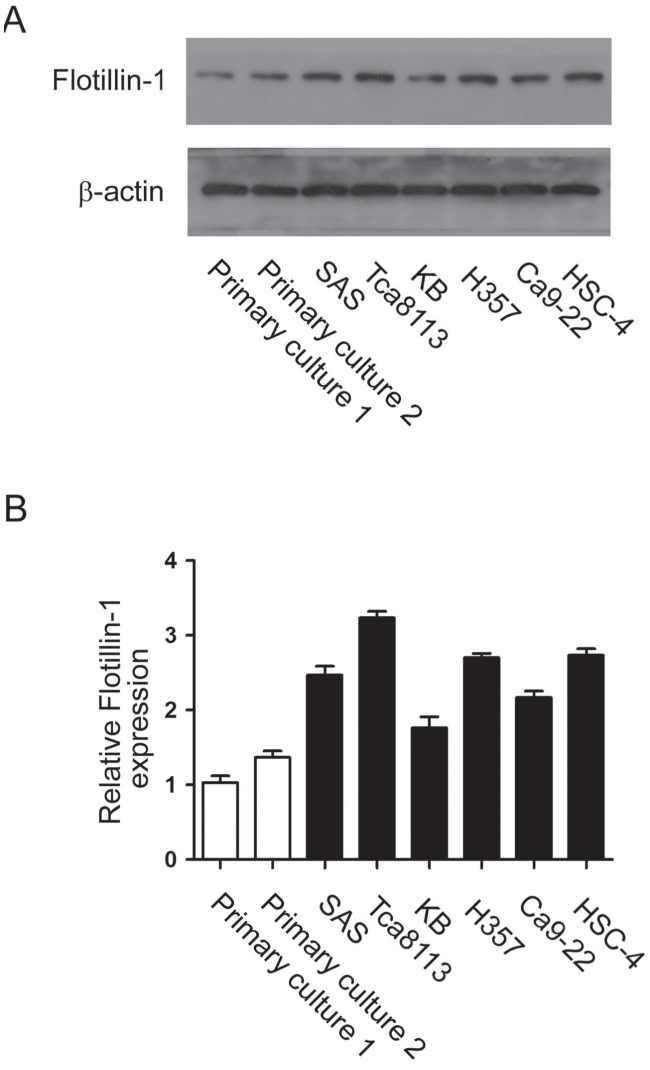

Figure 1. Flotillin-1 expression in OSCC cell lines and normal oral epithelial cells
Expression level of Flotilin-1 was assessed in SAS, Tca8113, KB, H357, Ca9-22, HSC-4 cell lines and two strains of primary culture of oral epithelial cells by immunoblot. Total protein was normalized by β-actin. Each blotting strip was determined by Quality-One software, and the x axis shows the average intensity of three parallel experimental runs.
All data were representative of three independent experiments.

(Fig. 2B). Similarly, in clonogenic formation assay, the number of clones was 126.70±12.12 in Flotillin-1 expressed KB cells versus 59.00±8.08 in mock control cells, increased by 114.00% ($P < 0.01$) (Fig. 2C).

To further support the observations in KB cells, expression of Flotillin-1 in Tca8113 cells were efficiently knocked down by two distinct shRNAs (Fig. 2D). As shown in Fig. 2E, MTT assay revealed that proliferation of Tca8113 cells was significantly depressed upon loss of Flotillin-1 expression, with the inhibition ratio of 52.40% for shFlotillin-1 ($P < 0.01$) and

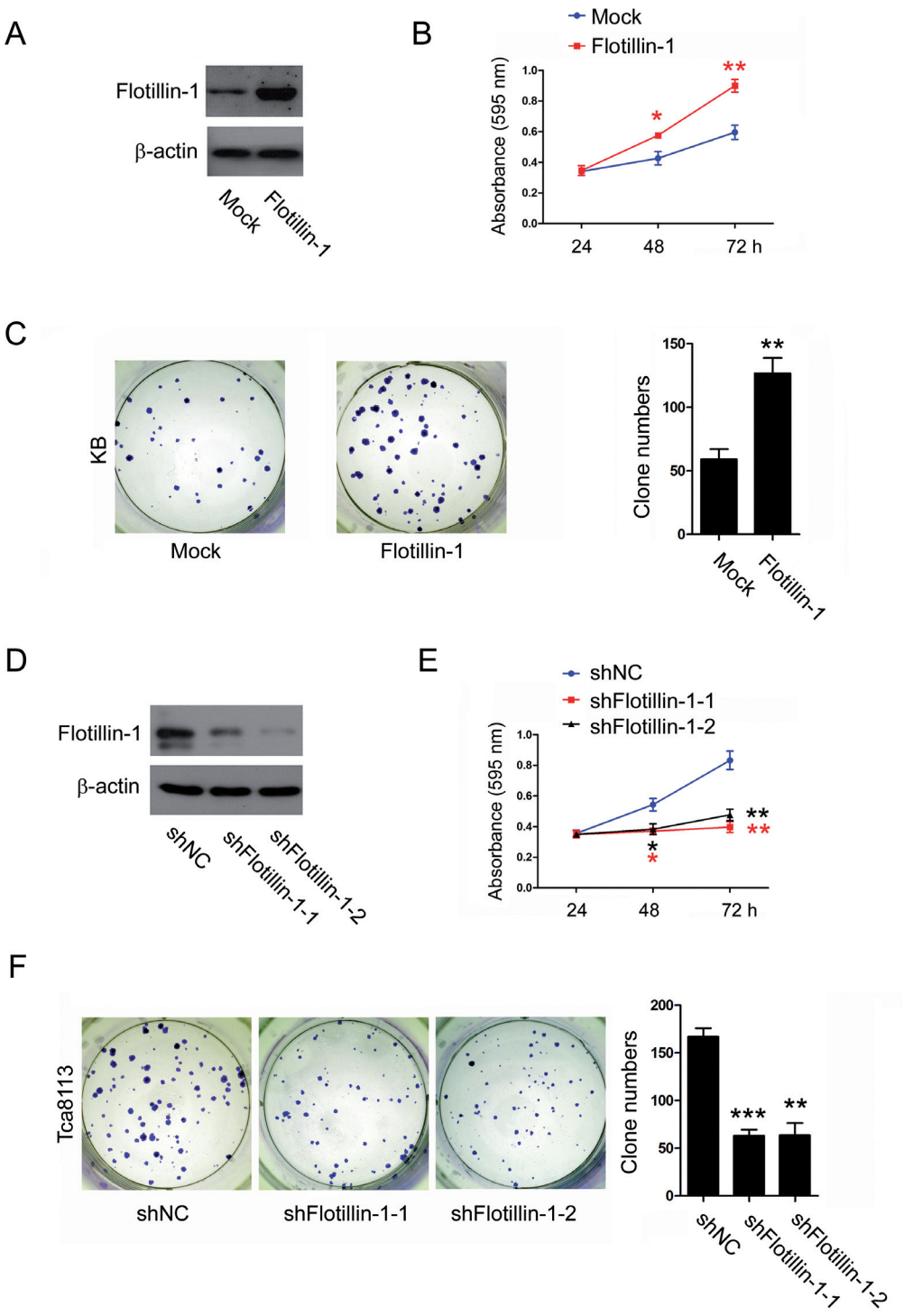

Figure 2. Flotillin-1 promotes OSCC cell proliferation

(A) KB cells were transfected with pcDNA3.1-Flotillin-1 plasmid or mock vector. Expression of Flotillin-1 was examined by immunoblot. Total protein was normalized by β-actin. (B) KB cells were transfected with pcDNA3.1-Flotillin-1 plasmid or mock vector. Proliferation of KB cells was examined by MTT assay at indicated time points. (C) KB cells were transfected with pcDNA3.1- Flotillin-1 plasmid or mock vector. Proliferation of KB cells was examined by colongenic formation assay. (D) Tca8113 cells were transfected with shNC, shFlotillin-1-1 or shFlotillin-1-2, respectively. Expression of Flotillin-1 was examined by immunoblot. (E) Tca8113 cells were transfected with shNC, shFlotillin-1-1 or shFlotillin-1-2, respectively. Cell proliferation was examined by MTT assay at indicated time points. (F) Tca8113 cells were transfected with shNC, shFlotillin-1-1 or shFlotillin-1-2, respectively. Proliferation of Tca8113 cells was examined by colongenic formation assay.

All data were representative of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001.

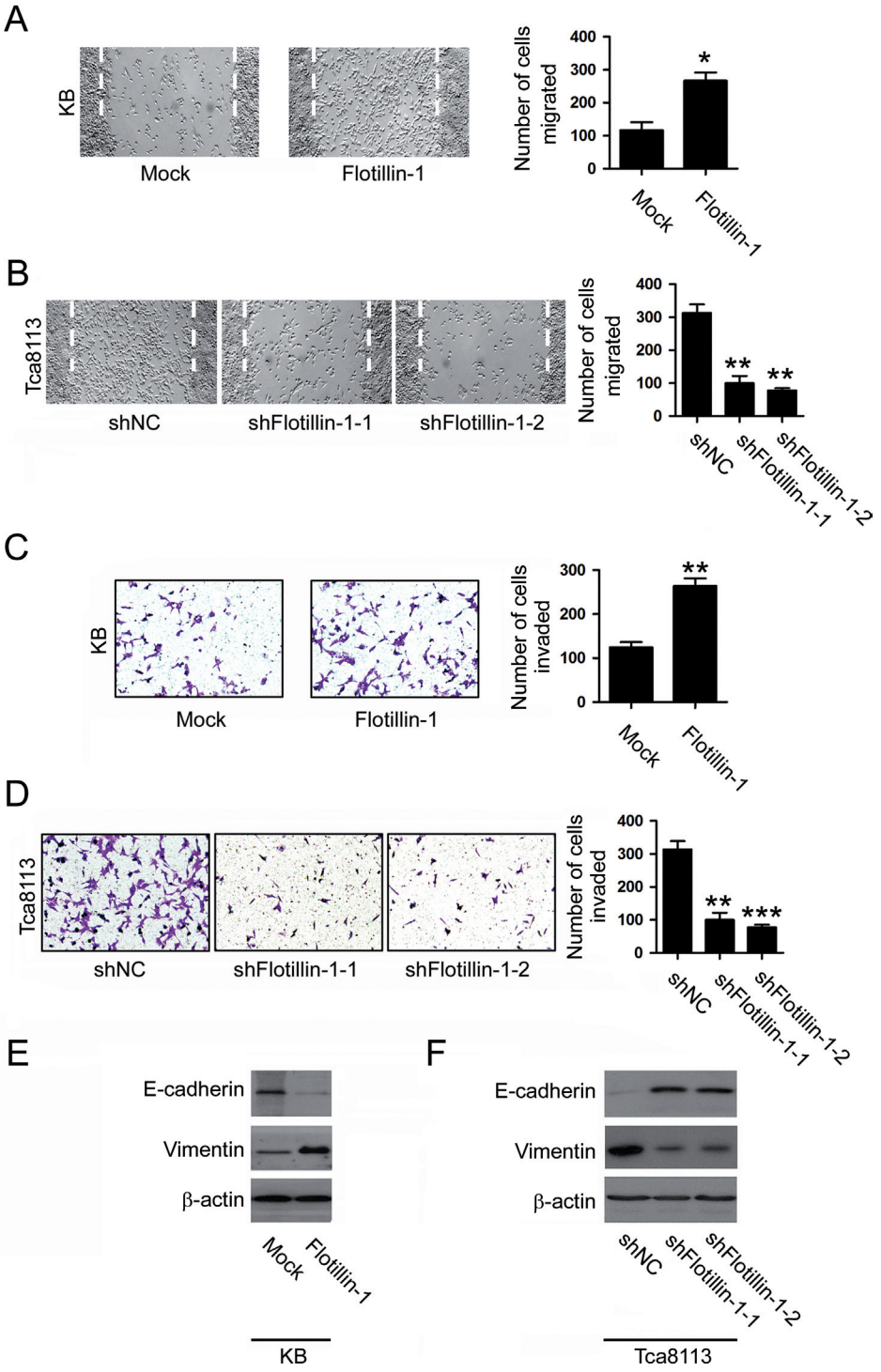

Figure 3. Flotillin-1 promotes OSCC cell metastasis and induces EMT

(A) KB cells were transfected with pcDNA3.1-Flotillin-1 plasmid or mock vector for 48h. Cell migratory capability was examined by wound scratch assay. (B) Tca8113 cells were transfected with shNC, shFlotillin-1-1 or shFlotillin-1-2 for 48 h, respectively. Cell migratory capability was examined by wound scratch assay. (C) KB cells were transfected with pcDNA3.1-Flotillin-1 plasmid or mock vector for 48 h. Cell invasive capability was examined by Matrigel assay. (D) Tca8113 cells were transfected with shNC, shFlotillin-1-1 or shFlotillin-1-2 for 48 h, respectively. Cell invasive capability was examined by Matrigel assay. (E) KB cells were transfected with pcDNA3.1-Flotillin-1 plasmid or mock vector for 48h. Expression of E-cadherin and Vimentin was examined by immunoblot. Total protein was normalized by β-actin. (F) Tca8113 cells were transfected with shNC, shFlotillin-1-1 or shFlotillin-1-2 for 48 h, respectively. Expression of E-cadherin and Vimentin was examined by immunoblot. Total protein was normalized by β-actin.

All data were representative of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001.

42.80% for Flotillin-2 ($P < 0.01$) 72 h after transfection. Further Knockdown of Flotillin-1 also repressed the clonogenic formation capability. As shown, the clone number of shNC treated Tca8113 cells was 167.00±8.88, while clone number of shFlotillin-1 or Flotillin-2-treated Tca8113 cells was 63.33±6.17 ($P < 0.001$) and 63.67±12.72 ($P < 0.01$), respectively (Fig. 2F). These results suggested a pro-proliferative role of Flotillin-1 in OSCC cells.

Flotillin-1 mediates metastasis of OSCC cells. To validate the pro-metastatic potential of Flotillin-1 in OSCC cells, the effect of Flotillin-1 on the migratory and invasive capability of OSCC cells was examined. In wound healing assay, the amount of Flotillin-1-expressed KB cells migrated into the wound area was much higher compared to the control cells ($P < 0.05$) (Fig. 3A). In contrast, shRNA-mediated Flotillin-1 repression markedly reduced the number of migrated Tca8113 cells (P < 0.01 for either shFlotillin-1-1 group or shFlotillin-1-2 VS. shNC group) (Fig. 3B). Consistent with this observation, for matrigel invasion assay, overexpression of Flotillin-1 resulted in about two fold more invaded KB cells ($P < 0.01$) (Fig. 3C); in contrast, the invasive capability of Tca8113 cells was decreased by 61.70% ($P < 0.01$, shFlotillin-1-1) or 74.50% ($P < 0.001$, shFlotillin-1-2) upon knockdown of Flotillin-1(Fig. 3D).

Epithelial-mesenchymal transition (EMT), which plays essential roles in development, was recently considered as an initial step of cancer metastasis [22]. Next, of our particular interest, we sought to determine whether EMT was involved in Flotillin-1-induced OSCC cell metastasis. As shown in Fig. 3E, overexpression of Flotillin-1 decreased the expression of epithelial cell marker, E-cadherin, and increased the expression of mesenchymal cell marker, vimentin, in KB cells. Accordingly, repression of Flotillin-1 by two distinct shRNAs substantially restored expression of E-cadherin and suppressed expression of Vimentin in Tca8113 cells. These data suggested that Flotillin-1 promotes OSCC cell metastasis by inducing EMT.

Flotillin-1 facilitates OSCC cell growth and metastasis *in vivo*. To extend the *in vitro* findings, animal tumor xenograft models was used to investigate the effect of Flotillin-1 on OSCC growth. To this end, KB cell clones stably expressing Flotillin-1 or mock vector were established (Fig. 4A) and subcutaneously transplanted in nude mice, respectively. Tumor growth was continuously monitored by measuring the tumor volume. As shown in Fig. 4B, overexpression of Flotillin-1 enhanced tumor growth in a duration-dependent manner. Though no significant difference was observed at 5 day post-transplantation, tumor volume was increased by 69.38% at 25 days post-transplantation (Fig. 4B).

To further validate the pro-metastatic effect of Flotillin-1 *in vivo*, both zebrafish and mice metastatic models were used. In zebrafish model, overexpression of Flotillin-1 notably increased KB cell dissemination to the posterior part of fish body (Fig. 4C). Similarly, in mice metastatic model, those KB cells stably expressing Flotillin-1 raised clearly more lung metastatic nodules and increased lung weight 15 days after injection, compared to the mock control (Fig. 4D). Therefore, these data suggested that Flotillin-1 played an important role in both proliferation and metastasis of OSCC cell *in vivo*.

Flotillin-1 activates NF-κB signaling pathway. Next, we set out to explore the downstream signaling cascades of Flotillin-1. It is reported that Flotillin-1 increased proliferation, anchorage-independent growth, and invasive activity in esophageal squamous cell carcinoma cells via activation of NF-κB pathway [16]. Therefore, we have particular interest to determine whether similar mechanisms exist in OSCC cells. As shown, overexpression of Flotilin-1 in KB cells enhanced phosphorylation of IκBα and p65, and translocation of p65 into nucleus (Fig. 5A-B), suggesting activation of NF-κB pathway. While shRNA-mediated silence of Flotillin-1 markedly abolished phosphorylation of IκBα and p65, and reduced the nuclear content of p65 in Tca8113 cells (Fig. 5C-D). Furthermore, expression of two NF-κB targets, Cyclin D1 and Twist1 [23, 24], was upregualted in Flotillin-1-overexpressed KB cells. In contrast, knockdown of Flotillin-1 markedly repressed expression of the two proteins in Tca8113 cells (Fig. 5E). These data suggested that NF-κB pathway was probably involved in Flotillin-1-induced proliferation and metastasis of OSCC cells.

EGFR is involved in Flotillin-1-mediated activation of NF-κB signaling. It is reported that NF-κB activation is one of the downstream events in EGFR signaling [25]. Further, Flotillin-1 was capable to form a molecular complex with EGFR, and triggers EGFR-dependent activation of downstream signaling cascades [26]. Thus we asked whether EGFR was involved in Flotillin-1-mediated activation of NF-κB signaling. As shown in Fig. 6, Flotillin-1-induced phosphorylation of IκBα and p65 was notably attenuated upon treatment with AG1478, an inhibitor of EGFR [27], suggesting that EGFR was responsible for Flotillin-1-mediated activation of NF-κB signaling.

Discussion

Even to date, OSCC which is one of the most common malignancies worldwide, is still a major health problem. The cellular and biochemical mechanisms underlying development of OSCC are poorly understood [28]. Unrestricted growth and distant spread of OSCC cells require multiple cellular events, including disruption of cell cycle, metabolic homeostasis and cell-cell adhesive contacts [29]. Therefore, a more detailed analysis of these molecular events must have a favorable impact on early diagnosis and anti-cancer treatment of OSCC.

The dynamic organization of the cell membrane is involved in diverse biological processes, such as phagocytosis, protein secretion and signal transduction [30]. Lipid rafts, detergent-resistant membrane domains, not only serve as a docking platform for protein sorting and membrane trafficking, but also coordinate signaling molecules at cell membrane to mediate intracellular responses, which makes them susceptible to be subverted to cancer cells [31]. Recently, Flotillin-1, a lipid raft-associated protein, was found to be abnormally expressed

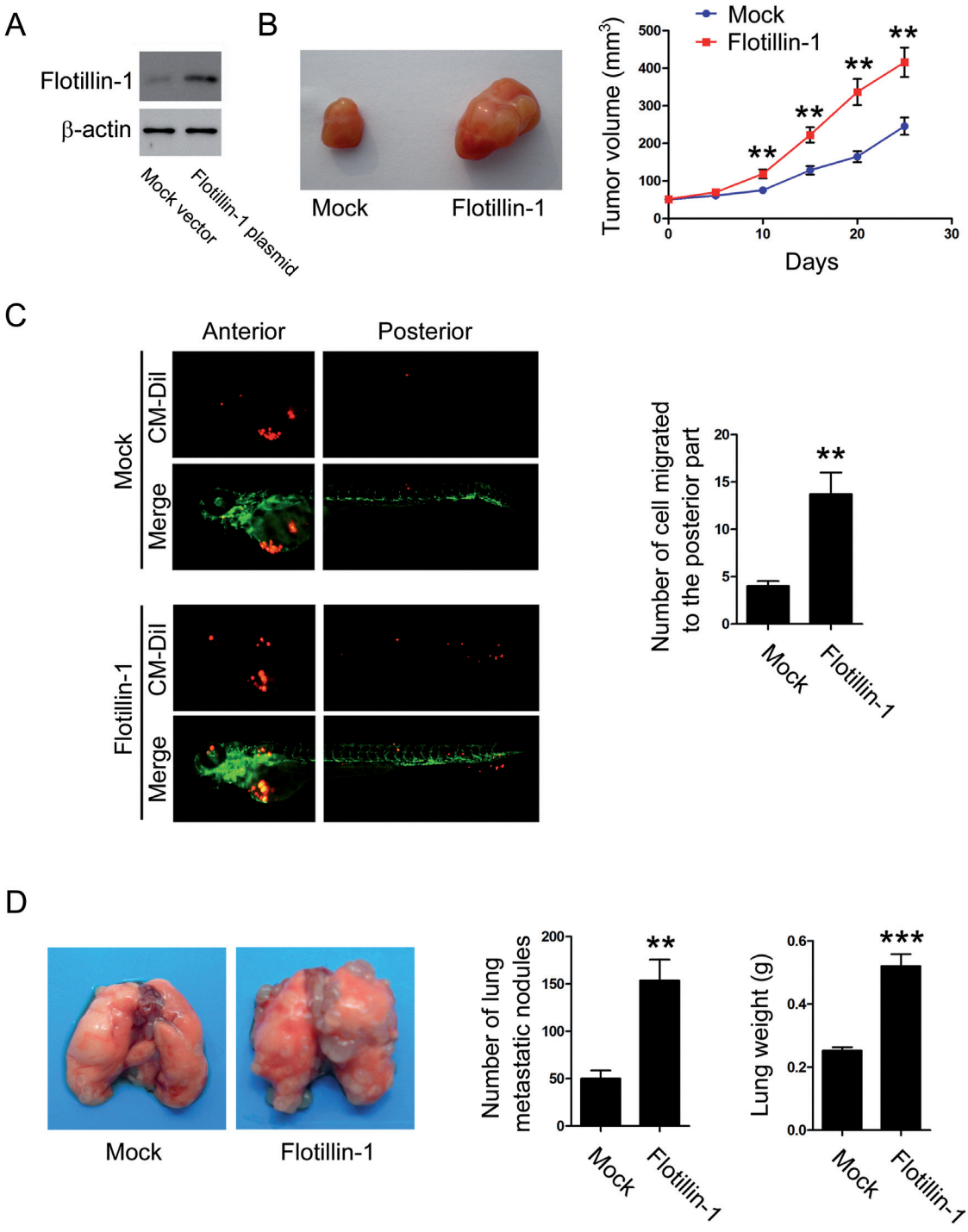

Figure 4. Flotillin-1 facilitates OSCC growth and metastasis *in vivo*

(A) KB cells were stably transfected with mock vector or pcDNA3.1-Flotillin-1 plasmid. Flotillin-1 expression in stable clones was examined by immunoblot. (B) KB cells that were stably transfected with mock vector or pcDNA3.1-Flotillin-1 plasmid were subcutaneously transplanted in nude mice. Representative image of tumors from Flotillin-1-expressed or mock control groups was shown (left two panels). The tumor volume was measured every five days. (C) KB cells that were stably transfected with mock vector or pcDNA3.1-Flotillin-1 plasmid were labeled with red fluorescent dye CM-DiI, and then injected in the perivitelline space of 48 h post-fertilization zebrafish embryos. Tumor cell dissemination was measured by counting the number of the cells migrated to the posterior part of the fish body. (D) KB cells that were stably transfected with mock vector or pcDNA3.1-Flotillin-1 plasmid were intravenously injected in nude mice. Mice were killed by cervical vertebra dislocation 40 days after injection. Representative image of lungs from Flotillin-1-expressed or mock control groups was shown (left two panels). Lung metastasis was determined by measuring the number of lung metastatic nodules or lung weight (right two panels). All data were representative of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001.

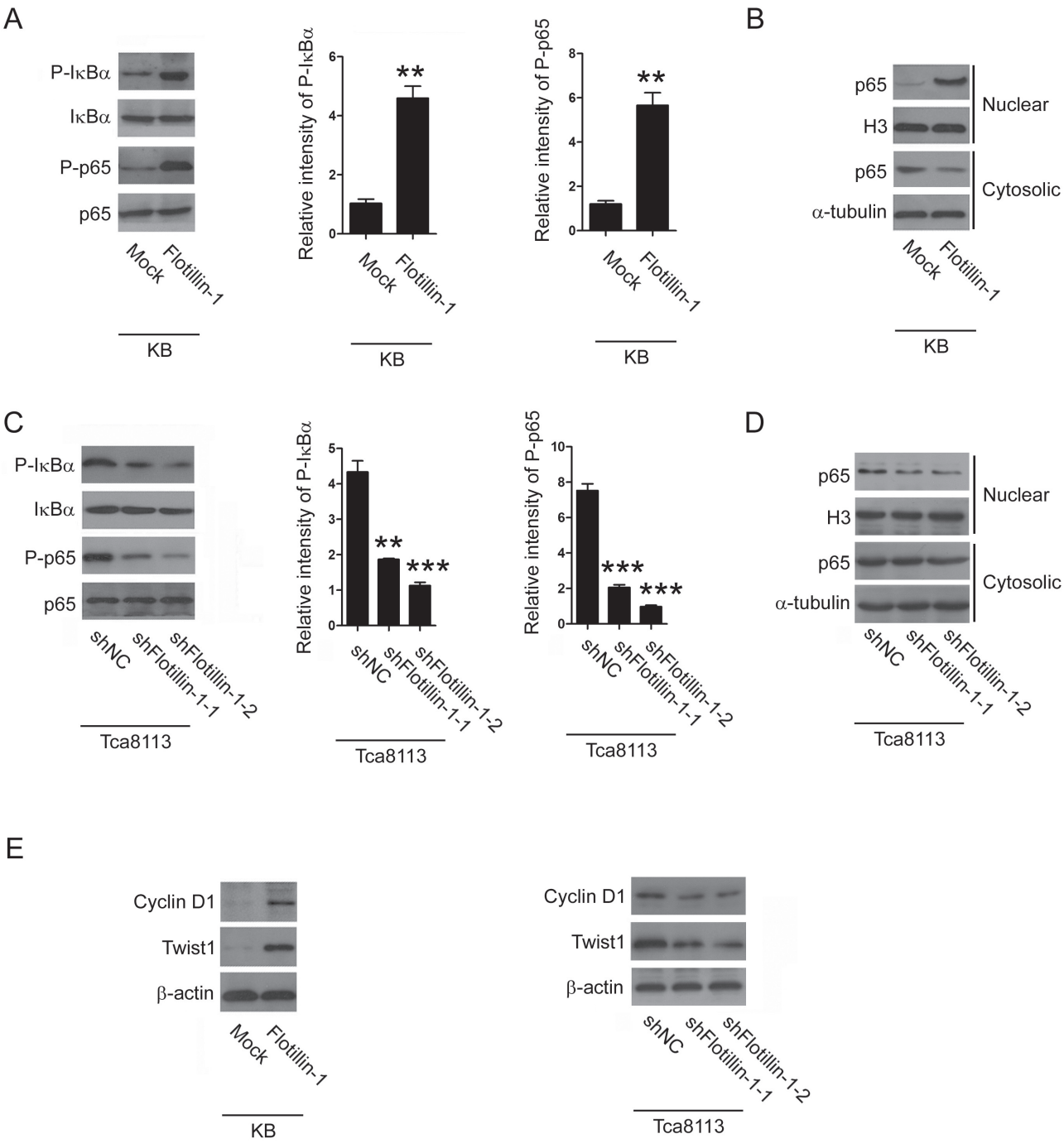

Figure 5. Flotillin-1 activates NF-κB signaling pathway

(A) KB cells were transfected with pcDNA3.1-Flotillin-1 plasmid or mock vector for 48 h, respectively. Phosphorylation of IκBα and p65 was examined by immunoblot. Total IκBα and p65 was used as internal control, respectively. Relative intensity of phosphorylated IκBα or p65 was normalized by total IκBα or p65, respectively, by using Quantity One software. (B) KB cells were transfected with pcDNA3.1-Flotillin-1 plasmid or mock vector for 48 h, respectively. Nuclear or cytosolic level of p65 was determined by immunoblot. Histone H3 or α-tubulin was used as internal control for nuclear or cytosolic fraction, respectively. H3, Histone H3. (C) Tca8113 cells were transfected with shNC, shFlotillin-1-1 or shFlotillin-1-2 for 48 h, respectively. Phosphorylation of IκBα and p65 was examined by immunoblot. Total IκBα and p65 was used as internal control, respectively. Relative intensity of phosphorylated IκBα or p65 was normalized by total IκBα or p65, respectively, by using Quantity One software. (D) Tca8113 cells were transfected with shNC, shFlotillin-1-1 or shFlotillin-1-2 for 48 h, respectively. Nuclear or cytosolic level of p65 was determined by immunoblot. Histone H3 or α-tubulin was used as internal control for nuclear or cytosolic fraction, respectively. H3, Histone H3. (E) KB cells were transfected with pcDNA3.1-Flotillin-1 plasmid or mock vector for 48 h, respectively. Tca8113 cells were transfected with shNC, shFlotillin-1-1 or shFlotillin-1-2 for 48 h, respectively. Expression of Cyclin D1 and Twist1 was examined by immunoblot. All data were representative of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001.

in several types of tumors [16-18]. In this study, we demonstrated that Flotillin-1 was highly expressed in a serial of OSCC cell lines while only a low level of Flotillin-1 expression was detected in primary culture of oral epithelial cells. Since these observations are based on *in vitro* cell culture, more work is still needed to validate Flotillin-1 expression with clinical samples.

Although Flotillin family proteins have been discovered for decades, the functional role of Flotillin-1 in carcinogenesis is relatively unknown [32]. To investigate the potential role of Flotillin-1 in OSCC development, we exogenously expressed Flotillin-1 in KB cells or Flotillin-1 shRNAs in Tca8113 cells, and compared proliferative and metastatic capability of tumor cells with high or low Flotillin-1 expression. We showed here that Flotillin-1 has a clearly positive impact on OSCC cell growth and motility either *in vitro* or *in vivo*. Our data are in line with previous reports that overexpression of Flotillin-1 facilitated prostate cancer cell proliferation, nevertheless, knockdown of Flotillin-1 restrained tumorigenic capability of breast cancer cells [18, 33]. The present data are also in agreement with the observation that upregulation of Flotillin-1 confers an aggressive phenotype on ESCC cells and promoted ESCC cell metastasis *in vivo* [16]. Further work will be conducted to validate the oncogenic property of Flotillin-1 by using other animal models, such as mice *in situ* OSCC model or Flotillin-1 KO mice.

Accumulating evidences have shown a pivotal role of NF-κB in tumor growth and metastasis. It is reported that NF-κB play a crucial role in neoplastic transformation in prostate and colon epithelial cells mediated by oncogenes such as Ras, Pim-2 or HTLV Tax [34-36]. In addition, continuous activation of NF-κB was also involved in oncogenic transformation of mammary cells [37]. Further, NF-κB is capable to survive cancer cell from DNA damage or oxidative stress by activating a serial of anti-apoptotic factors, including Bcl-XL and survivin [38]. It is also documented that TNF-α treatment enhanced cell motility in several cancer cell lines via NF-κB-dependent overexpression of CXCR4 and MCP-1 [39]. In contrast, Norcantharidin inhibited metastasis of hepatocellular carcinoma cell by repressing NF-κB -mediated MMP-9 transcription [40]. Since a large body of EMT-associated proteins have been found to be direct or indirect NF-κB targets, including Snail and MMPs, the impact of NF-κB on tumor metastasis is likely based on its regulatory effect on EMT, [41, 42]. In current study, overexpression of Flotillin-1 resulted in activation of NF-κB signaling and upregulation of NF-κB target genes in KB cell line. While shRNA-mediated Flotillin-1 silence markedly inhibited NF-κB signaling in Tca8113 cells. Notably, we also demonstrated that EGFR played a crucial role in Flotillin-1-induced activation of NF-κB signaling. Considering the previous report showing that Flotillin-1 activated NF-κB signaling via promoting TNFR translocation to lipid rafts [16], more work will be conducted to determine whether the Flotillin-1/EGFR complex is required for TNFR recruitment.

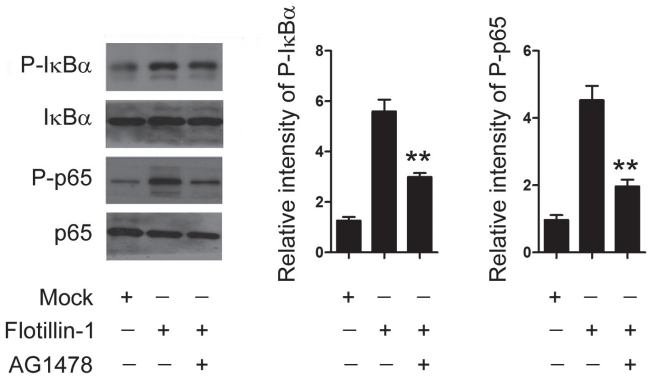

Figure 6. EGFR is involved in Flotillin-1-induced activation of NF-κB signaling pathway

KB cells were transfected with pcDNA3.1-Flotillin-1 plasmid or mock vector for 48 h in presence or absence of 10 μM AG1478. Phosphorylation of IκBα and p65 was examined by immunoblot. Total IκBα and p65 was used as internal control, respectively. Relative intensity of phosphorylated IκBα or p65 was normalized by total IκBα or p65, respectively, by using Quantity One software.

All data were representative of three independent experiments. *, P<0.05; **, P<0.01; ***, P<0.001.

In summary, our data provide new insights for the biological function of Flotillin-1 in OSCC development. Hopefully, the present data also suggested that targeting Flotillin-1, combined with Radio/chemo-therapy and surgical resection might pave a new road for OSCC treatment.

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