FRMD4A Upregulation in Human Squamous Cell Carcinoma Promotes Tumor Growth and Metastasis and Is Associated with Poor Prognosis

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Abstract

New therapeutic strategies are needed to improve treatment of head and neck squamous cell carcinoma (HNSCC), an aggressive tumor with poor survival rates. FRMD4A is a human epidermal stem cell marker implicated previously in epithelial polarity that is upregulated in SCC cells. Here, we report that FRMD4A upregulation occurs in primary human HNSCCs where high expression levels correlate with increased risks of relapse. FRMD4A silencing decreased growth and metastasis of human SCC xenografts in skin and tongue, reduced SCC proliferation and intercellular adhesion, and stimulated caspase-3 activity and expression of terminal differentiation markers. Notably, FRMD4A attenuation caused nuclear accumulation of YAP, suggesting a potential role for FRMD4A in Hippo signaling. Treatment with the HSP90 inhibitor 17-DMAG or ligation of CD44 with hyaluronan caused nuclear depletion of FRMD4A, nuclear accumulation of YAP and reduced SCC growth and metastasis. Together, our findings suggest FRMD4A as a novel candidate therapeutic target in HNSCC based on the key role in metastatic growth we have identified.

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Introduction

Squamous cell carcinoma (SCC) of the skin and oral cavity pose differing threats to health. Skin SCCs are very common and rarely metastasize; they can often be cured by surgery alone (1). SCCs in the oral cavity (head and neck SCC; HNSCC) are less common but tend to present at an advanced stage (2). Despite radical surgery and adjuvant therapy, HNSCC lesions often recur and spread to other body sites. Survival rates for oral SCCs are approximately 50% and have not improved for 30 years.

Both skin SCCs and HNSCCs arise from multilayered epithelia, which are maintained by stem cells residing in the basal epithelial layer, attached to an underlying basement membrane (3). Skin SCCs and HNSCCs exhibit cellular heterogeneity, with some cells expressing markers of the normal terminal differentiation process whereas others are undifferentiated. There is also good evidence for functional heterogeneity, both in terms of clonal growth of SCCs cells in vitro (4, 5) and in xenografts in immunocompromised mice (6). Thus, SCC is one of the tumor types for which there is experimental support for the existence of cancer stem cells (tumor-initiating cells; ref. 7).

Using single-cell gene expression profiling, we identified FERM domain containing 4A (FRMD4A) mRNA as a marker of cultured human epidermal stem cells (8). When we examined expression of a panel of 14 epidermal stem cell markers in cultured HNSCC cells, FRMD4A was the only marker that was consistently highly upregulated in HNSCC and thus emerged as a candidate HNSCC stem cell marker (5). FRMD4A was recently reported to connect the Par-3 complex to Arf6 guanine-nucleotide exchange factor and regulate assembly of adherens junctions in cultured mammary epithelial cells (9).

We have now characterized FRMD4A expression and function in SCCs and present evidence that it is an attractive target for novel therapies to control SCC growth and metastasis.

Materials and Methods

Analysis of FRMD4A levels in published data sets

Processed gene expression data from 2 published HNSCC studies (10, 11) were downloaded from NCBI GEO (accession numbers GSE10300 and GSE686). The X-Tile program (12) was used to determine the optimal cutoff point for Kaplan–Meier analysis conducted using the "survival" package for R while correcting for the use of minimum P statistics.

Human tissue specimens

Work with human material was carried out in compliance with the UK Human Tissue Act (2004) and approved by the National Research Ethics Service (08/H0306/30). Appropriate informed consent was obtained from patients diagnosed with...
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oral SCCs, before operation at Addenbrooke’s Hospital (Cambridge, UK). Small biopsy specimens were removed from freshly resected oral SCCs. Specimens of normal skin were obtained with informed consent from operations to remove excess skin.

For laser capture microdissection, tissue was frozen in optimum cutting temperature (OCT) and sectioned with a cryostat. Sections were briefly air-dried and stained with cresyl violet. Using the Zeiss/P.A.L.M. Laser Capture Microdissection System, the basal and granular epidermal layers were collected separately from 2 serial sections and material from both sections was pooled. RNA was isolated using RNasy (Qiagen), then reverse-transcribed with a Superscript III kit (Invitrogen).

Cell culture and lentiviral transduction

SCC13 and SCC25 (13) and keratinocytes from normal human epidermis (by passage 4) and oral mucosa (CRI-005 passage 4) were cultured as described previously (5, 8), except that SCC lines were grown without a feeder layer of J2-3T3 cells. SCC13 and SCC25 cells were passaged for less than 6 months following thawing. SCC25 are available from American Type Culture Collection and validated by short tandem repeat profiling. The culture medium comprised FAD [3 parts Dulbecco’s Modified Eagle’s Medium (DMEM), 1 part Ham’s F-12 medium, 1.8 × 10⁻⁷ mol/L adenine] supplemented with 10% fetal calf serum, 0.5 µg/mL hydrocortisone, 5 µg/mL insulin, 10⁻¹⁰ mol/L cholera toxin, and 10 ng/mL EGF. SG15 cells were derived from a SCC (pathologic stage T2N1M0; well-differentiated) on the lateral tongue of a 51-year-old female patient. Cells from the tumor were disaggregated in 0.25% trypsin/EDTA (Gibco) at 37°C and then passaged on feeders until they became feeder-independent.

The YFP/luciferase lentivirus construct was generated in-house. Short hairpin RNA (shRNA) constructs were purchased from Open Biosystems. Viruses were packaged by transient transfection of 293T cells (by passage 4) and used to infect SCC cells. Infected cells were selected in hygromycin (YFP/luciferase) or puromycin (shRNAs).

To stimulate keratinocyte differentiation, cells were grown in keratinocyte serum-free medium (KSFM; Gibco) supplemented with 30 µg/mL bovine pituitary extract and 0.2 ng/mL EGF for 2 to 3 days. Cells were then incubated in medium containing 10 µmol/L AG1478 (Calbiochem) and/or 200 ng/mL recombinant human BMP2/7 heterodimer (R&D Systems) for 48 hours.

For colony-forming assays, 100 cells were plated in triplicate in 10-cm plates without feeders. After 14 days, cultures were fixed and stained with 1% rhodamine B and 1% Nile blue (Acros Organic). Colony-forming efficiency was calculated as the percentage of plated cells that formed a colony of 3 or more cells.

For real-time imaging, 1,000 cells were plated in triplicate for each condition, in a 48-well plate without feeders and recorded using an IncuCyte. Wells were imaged every 3 hours for 7 days.

Matrigel invasion assays were conducted using Boyden chambers (BD BioCoat Matrigel invasion chambers) in 24-well plates. Cells transduced with YFP/luciferase were serum-starved in KSFM for 24 hours. About 10⁵ cells in KSFM were added per chamber in wells containing complete FAD medium. After 24 hours, the Matrigel was removed from the well, and luciferin was added to the medium. The chambers were scanned and quantified using the IVIS.

For sphere-forming assays, a 12-well plate was coated with poly-HEMA (2-hydroxyethyl methacrylate) to prevent adhesion of cells to the plastic. One hundred cells were plated in methylcellulose in each well, in triplicate. After 21 days, the wells were scanned and quantified using a Typhoon scanner.

Quantitative PCR

RNA was isolated and amplified as described previously (8). Quantitative PCR (Q-PCR) was carried out using TaqMan gene expression assays for FRMD4A and transglutaminase (Applied Biosystems). All data were normalized to 18S.

Antibodies

SGO-3 and SGO-4 antibodies were raised by injecting FRMD4A peptide PPPQSLERQLRMHYHRNDYDKC into rabbits. The antibodies were affinity-purified on AminoLink Plus Coupling Resin columns (Thermo Scientific). The following antibodies were also used: 06 integrin (GoH3; BD Pharmingen), E-cadherin (HECD-1; prepared in-house), GFP (Abcam), Ki67 (Dako), cleaved caspase-3 (R&D Systems), YAP1 (Abcam), LATS1 (Abcam), MST1 (Abcam), Involution (513; prepared in-house), CD44 (BD Pharmingen), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Invitrogen). AlexaFluor-488- or -555-conjugated and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Invitrogen.

Histology and in situ hybridization

Tissue was either fixed in 10% neutral-buffered formalin and embedded in paraffin or frozen in OCT-embedding matrix (Raymond A. Lamb). Epitope retrieval was conducted on paraffin sections by boiling in citrate buffer for 10 minutes. Blocking buffer contained 10% fetal calf serum, 4% bovine serum, 2.5% fish skin gelatin, and 0.05% Tween-20. Staining was conducted essentially as described previously (16). Immunofluorescent staining was imaged using a Leica Tandem Confocal microscope. Ki67-stained sections were photographed and analyzed using an Ariel SL-50 system (Applied Imaging, Corp.), as described previously (16). In situ hybridization was conducted on sections of paraffin-fixed human foreskin, essentially as described previously (17).

Western blotting

Cells were disaggregated using trypsin/EDTA and collected by centrifugation. Cell pellets were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors (16) and subjected to PAGE on 4% to 12% gradient gels. Proteins were transferred to nitrocellulose membranes and blocked in Tris buffer, pH 8.0, containing 2.5% skimmed milk powder and 0.05% Tween-20. Blots were incubated with primary and secondary antibodies and visualized by enhanced chemiluminescence (16). Antibodies to FRMD4A and YAP were used at 1:200 dilution; anti-GAPDH was diluted at 1:1,000.
Xenografts

Experiments were subject to CRUK ethical review and carried out under the terms of a U.K. Government Home Office license. NOD.Cg-Pkdcsid I2rgtm1Wji/Sdj (NSG) mice were purchased from the Jackson Laboratories and bred in-house. Mice were subject to inhalation anesthesia during SCC implantation and IVIS imaging.

SCC cells were introduced into the anterior dorsal mucosa of the tongue with a 30-g needle and 1-mL syringe. For tail vein injections, 2.5 × 10^5 cells in 50 µL culture medium were introduced per mouse. To form tumors in back skin, silicone chambers were first implanted into a full thickness wound, as described previously (18). SCC cells were injected into the chamber, and 1 week later, the top of the chamber was cut off. After a further week, the whole silicone chamber was removed.

Tumor size was determined by scanning mice in an IVIS 200 series imaging system (Caliper Life Sciences) following intraperitoneal injection of 15 mg/mL L-/luciferin (potassium salt) dissolved in PBS (10 µL/g). Tumor size was recorded weekly. To induce FRMD4A knockdown with doxycycline, mice were fed chow containing the drug (IPS Product Supplies).

To determine where FRMD4A (genome accession number NM_018027) is expressed in normal human epidermis, we conducted in situ hybridization with a 35S-labeled probe to exon 25 (Fig. 1A). FRMD4A transcripts were detected throughout the epithelial compartment of human HNSCC. As predicted from the in situ hybridization and Q-PCR data, FRMD4A was expressed in the epidermal basal layer and downregulated in the suprabasal layers (Fig. 1C). We next stained 13 tumors of the alveolar ridge, tongue, and floor of mouth (6 well-differentiated; 4 moderately differentiated; 3 poorly differentiated). FRMD4A protein was expressed throughout the epithelial compartment of human HNSCCs and was upregulated regardless of pathologic grade (Fig. 1C).

To further evaluate the significance of FRMD4A upregulation in HNSCCs, we examined published gene expression data from 2 collections of tumors (10, 11) using the X-Tile program (12). Kaplan–Meier plots from both data sets showed a highly significant correlation between high levels of FRMD4A and risk of relapse (Fig. ID).

We conclude that in normal epidermis, FRMD4A expression is confined to the basal layer, where the stem cells reside; that FRMD4A is widely expressed throughout the tumor mass in HNSCCs, and that high FRMD4A levels are correlated with poor prognosis in HNSCCs.

FRMD4A knockdown reduces cell–cell adhesion, growth, and invasion in culture

We used SCC13 cells and a second human SCC line, SCC25 (derived from a tongue SCC; ref. 13), to examine the effects of reduced FRMD4A expression on SCC cell behavior in vitro. In untransduced SCC13 (SCC13-WT) and SCC13 transduced with a scrambled shRNA control (SCC13-SCR), FRMD4A was localized predominantly in the cytoplasm, with some localization to the nucleus and partial colocalization with E-cadherin at cell–cell borders (Fig. 2A). FRMD4A knockdown (SCC13-A7) led to...
Figure 1. FRMD4A is expressed in basal cells of human epidermis and upregulated in HNSCC. A, *in situ* hybridization of FRMD4A and β-actin in neonatal human foreskin. Dark- and bright-field images of the same sections are shown. B, Q-PCR of transglutaminase 1 (TG1) and FRMD4A mRNA levels (relative to 18S mRNA) in different layers of adult human epidermis obtained by laser capture microdissection (left) and in cultured human keratinocytes treated with the differentiation inducing agents shown (right). Data are means ± SEM of 3 technical replicates (laser capture) or triplicate samples (cultured cells). C, detection of FRMD4A protein. Predicted domain structure of FRMD4A protein and location of SGO-3, 4 antibody epitopes. Western blots were probed for FRMD4A (SGO-3) or GAPDH (loading control). WT, control SCC13; SCR, SCC13 transduced with scrambled control shRNA; B9, A7, A8, A9, A10: independent shRNAs to FRMD4A; EK, epidermal keratinocytes; OK, oral keratinocytes. Molecular mass markers (kDa) are indicated. Sections of human abdominal skin and human tongue SCC were labeled with antibodies to FRMD4A (green; SGO-4) and α6-integrin (red) with 4′,6-diamidino-2-phenylindole (DAPI) nuclear counterstain. Scale bars, 50 μm. D, Kaplan–Meier plots from 2 independent data sets (LHS, ref. 10; RHS, ref. 11). Blue indicates low and green represents high FRMD4A expression. The number of tumors in each group is given in parentheses. The optimum cutoff point was determined using the method of Camp and colleagues (12).
loss of FRMD4A in all locations and to a marked reduction in E-cadherin staining, which coincided with reduced cell–cell contact and elongated cell morphology (Fig. 2A). Live cell imaging confirmed that whereas SCC13-WT and SCC13-SCR cells formed colonies with stable intercellular adhesions, SCC13-A7 cells did not (Supplementary Fig. S1).
FRMD4A knockdown in SCC13 and SCC25 using several different shRNAs resulted in a reduction in colony-forming efficiency, a surrogate readout of stem cell abundance (ref. 5; Fig. 2B). The reduction in colony formation correlated with an overall reduction in growth rate, as evaluated by live cell imaging (Fig. 2C; Supplementary Fig. S1). In addition, FRMD4A knockdown led to reduced SCC invasiveness in Boyden chamber assays and inhibition of anchorage-dependent growth, as determined by sphere formation in suspension (Fig. 2D).

**Generation of an orthotopic xenograft model of human HNSCC**

In HNSCCs, death is frequently due to organ failure as a result of distant metastasis, rather than growth of the primary tumor (2). However, HNSCC growth in xenograft models is generally evaluated by the size of the primary tumor generated by grafting cells in a heterotopic site, the dorsal skin (6). To generate an improved xenograft model, we transduced cultured SCC cells with a lentiviral construct that expressed a YFP/luciferase fusion protein so that tumor growth could be evaluated both macroscopically and microscopically (Fig. 3A). Cells were either injected into a silicone chamber surgically implanted in the back skin (18) or directly injected into the tongue of NSG mice (Fig. 3B). Primary tumors were evaluated in live mice using an IVIS 200 series imaging system (Caliper Life Sciences) following intraperitoneal injection of luciferin (Fig. 3B). Organs were scanned post-mortem with an IVIS to detect metastatic disease (Fig. 3B).
The tumorigenicity of SCCs depended on the location of the graft: chamber-grafted SCCs required a minimum of $10^6$ cells to successfully form a tumor in 100% of mice, whereas tongue-grafted tumors developed after injection of only 100 cells (based on cohorts of 5 mice per group). The average survival times for mice injected with $10^6$ SCC13 in the dorsal skin (73d) or 100 SCC13 cells (70d) in the tongue were similar, although the maximum survival time was greater in the tongue (153d vs. 76d). In contrast, both the average (90d vs. 61d) and maximum survival times (157d vs. 73d) of SCC25 were greater in skin than in tongue. Thus, each SCC line was more malignant when grafted into the location from which it was derived.

Histologic analysis of skin xenografts revealed growth of the tumor throughout the host dermis and invasion of the panniculus carnosus muscle, with ulceration of the overlying skin (Fig. 3C). Oral SCCs often present as an ulcerating lesion on the culcus carnosus muscle, with ulceration of the overlying skin tumor throughout the host dermis and invasion of the panniculus carnosus muscle, which was recapitulated in the orthotopic tongue (Fig. 3C). Oral SCCs often present as an ulcerating lesion on the culcus carnosus muscle, with ulceration of the overlying skin.

Stable knockdown of FRMD4A reduces the growth rate of human SCCs and increases host survival

To evaluate the effect of FRMD4A knockdown on tumor growth, we used 2 independent strategies, one involving a conventional shRNA approach and the other involving an inducible vector (Fig. 4). We compared the effects on SCC13 and SCC25 cells growing in back skin or tongue. All cells were transduced with the YFP/knifeaerse construct shown in Fig. 3A so that tumor growth could be measured in living mice.

We first evaluated the growth as tongue xenografts of control SCC25 (SCC25-WT), SCC25 transduced with a scrambled shRNA (SCC25-SCR), and SCC25 in which FRMD4A had been knocked down (SCC25-A7). SCC25 cells were compared with SCC13 and SJG15 (recently derived from an aggressive tongue SCC; Fig. 4A). About $10^5$ cells were injected into 25 mice (3 per cell type), and primary tumor growth was evaluated by luciferase activity at intervals. SCC13, SJG15, SCC25-SCR, and SCC25-WT grew at a similar rate, and mice had to be sacrificed between days 45 and 135 because their weight had dropped by 20% or their general condition had deteriorated (Fig. 4A). In contrast, growth of SCC25-A7 tumors leveled-off after approximately 3 weeks and mice showed excellent long-term survival (Fig. 4A). Stable knockdown of FRMD4A...
increased the minimum number of SCC25 cells required to form a tongue tumor from 100 to $10^4$.

To evaluate the effect of FRMD4A knockdown in established tumors, SCC13 cells were infected with a tetracycline-on doxycycline-inducible FRMD4A shRNA or the empty vector control (EV) and then injected into chamber grafts on the back of NSG mice. Three weeks later, mice were given doxycycline-rich chow to induce FRMD4A knockdown. Before the change of diet, SCC13-EV and SCC13-FRMD4A tumors were increasing in size (Fig. 4B). However, on addition of doxycycline, SCC13-FRMD4A tumors grew significantly more slowly (Fig. 4B). Survival was significantly longer in mice in which FRMD4A had been knocked down than in control mice (Fig. 4B).

We also evaluated the effect of stable FRMD4A knockdown on SCC13 following injection into the tail vein of NSG mice (Fig. 4C). As expected, primary tumors developed in the lungs. Mice were sacrificed after 10 days and their lungs scanned. As in the case of skin and tongue tumors, FRMD4A knockdown reduced tumor growth.

To evaluate whether FRMD4A knockdown affected metastasis as well as primary tumor growth, organs from each mouse in the experiments shown in Fig. 4B were scanned in an IVIS immediately post-mortem. Lungs and liver were selected for quantification, as they are the distant organs most affected by metastases in the human disease (2). In both organs, greater levels of metastatic disease were found in mice engrafted with SCC13 expressing the empty vector control than mice in which FRMD4A was knocked down (Fig. 4D). In doxycycline-treated mice injected with SCC13-EV, 5 of 5 had lung and 4 of 5 had liver metastases, whereas in mice injected with SCC13-FRMD4A, 1 of 5 had lung and 2 of 5 had liver metastases.

**FRMD4A knockdown reduces proliferation, stimulates differentiation, and induces apoptosis of SCC cells**

The xenograft experiments show that knockdown of FRMD4A decreased tumor growth and metastasis. To investigate the underlying mechanisms, histologic sections of primary and secondary tumors from the experiment shown in Fig. 4B were examined (Fig. 5). FRMD4A-knockdown tumors showed a decrease in proliferative, Ki67-positive cells (Fig. 5A) and exhibited extensive expression of the apoptosis marker cleaved caspase-3, which was not detected in control tumors (Fig. 5B). This correlated with an increase in differentiation, as evaluated both by conventional histology (Fig. 5A and data not shown) and by increased expression of the terminal differentiation marker involucrin (Fig. 5D). As predicted from the *in vitro* experiments (Fig. 2), E-cadherin levels were reduced in FRMD4A-knockdown tumors (Fig. 5C). Labeling with SGO-3 confirmed successful knockdown of FRMD4A in tumors (Fig. 5D).

We conclude that the reduced growth of tumors in which FRMD4A is knocked down reflects a reduction in proliferation, increased apoptosis and differentiation, and a decrease in levels of E-cadherin.

**FRMD4A influences SCC growth by modulating the Hippo pathway**

FERM domain–containing proteins of the ezrin/radixin/moesin (ERM) subfamily lie downstream of receptors that
mediate cell–cell and cell–extracellular matrix adhesion, including CD44, and regulate the mammalian Hippo signaling pathway (20–23). Given the effects of FRMD4A on E-cadherin levels in cultured cells and tumors, we investigated whether FRMD4A regulates Hippo signaling. The primary effector of the Hippo pathway is the transcriptional co-activator YAP. Phosphorylation of YAP by LATS1/2 maintains YAP in the cytoplasm, whereas removal of LATS1/2 allows unphosphorylated YAP to enter the nucleus and associate with DNA-binding transcription factors of the TEAD/TEF family. LATS1/2 is activated through phosphorylation by a complex containing the mammalian Hippo homologs MST1 and 2 (21).

Cultured SCC13-WT, SCC13-SCR, and SCC13-A7 were stained for YAP1, LATS1, and MST1 (Fig. 6A and data not shown). SCC13-WT and SCC13-SCR cells showed generalized cytoplasmic staining for YAP, with minimal nuclear accumulation (Fig. 6A). In contrast, on knockdown of FRMD4A, YAP had a predominantly nuclear localization (Fig. 6A). FRMD4A knockdown had little effect on MST localization, but staining of LATS was greatly reduced (Fig. 6A). Western blotting revealed an overall increase in levels of YAP on FRMD4A knockdown (Fig. 6B). The *in vitro* observations were confirmed *in vivo*: tumors in which FRMD4A expression was knocked down had increased nuclear YAP and reduced levels of LATS when compared with control tumors (EV; Fig. 6C and data not shown). In HNSCCs, the most highly differentiated tumors had the highest proportion of nuclear YAP1 (Fig. 6B).

**Modulating FRMD4A and the Hippo pathway to inhibit the growth of SCCs**

Having established that FRMD4A levels regulate the Hippo pathway, we next investigated whether treatment of SCC cells with the CD44 ligand hyaluronan (23) had any effect on the subcellular localization of FRMD4A. When SCC13 cells were...
seeded on culture dishes that had been precoated with hyaluronan, there was a reduction in colony-forming efficiency, colony area, and overall growth rate (Fig. 7A). This correlated with a dramatic loss of nuclear FRMD4A staining, increased CD44 labeling of apical cell surface microvilli, and an increase in nuclear YAP (Fig. 7B).

To test whether hyaluronan may have a potential role as a therapeutic agent in vivo, mice were xenografted with SCC13 into their back skin. Tumors were injected weekly with hyaluronan or PBS, and tumor size was measured using a Xenogen IVIS. Hyaluronan injected primary tumors were smaller than control tumors injected with PBS (Fig. 7C), and there was also a reduction in total metastatic disease quantitated as a decrease in the combined luciferase signal from liver and lungs (Fig. 7C).

Recent preclinical and clinical trials of HSP90 inhibitors have shown varying efficacy in the treatment of several cancer types, and it has been suggested that they act by depletion of LATS1/2 in the Hippo pathway, thereby increasing nuclear YAP (24). Following injection of SCC13 cells into the tongue, NSG mice received weekly intraperitoneal injections of the HSP90 inhibitor 17-DMAG (alvespimycin) or vehicle control. 17-DMAG–treated mice showed a reduction in growth of the primary tumors and decreased metastatic disease compared with controls (Fig. 7C). 17-DMAG–treated tumors not only showed increased nuclear YAP but also a striking loss of nuclear FRMD4A (Fig. 7D).

Discussion

We originally identified FRMD4A through gene expression profiling of single human epidermal stem cells (8) and selected it for further analysis because it was consistently upregulated in human SCC cell lines (5). By raising antibodies to the protein, we have now shown that in human epidermis, FRMD4A is only expressed in the basal cell layer, where the stem cells reside. In contrast, FRMD4A is upregulated in HNSCCs, regardless of differentiation status, and high levels correlate with poor prognosis. Our functional studies place FRMD4A downstream of CD44 in the Hippo pathway and suggest that targeting FRMD4A is an effective treatment for skin and oral SCCs.

FERM domains act as adaptors or scaffolds that interact with multiple protein partners (20). The reduction in E-cadherin–mediated adhesion that occurs on FRMD4A knockdown is consistent with the observation that FRMD4A regulates assembly of adherens junctions (9) and is also in keeping with the role of the ERM proteins in epithelial membrane organization (20). FRMD4A differs from ERM proteins (20) in having potential nuclear localization and nuclear export sequences (NetNES 1.1 prediction software; ref. 25) and a leucine zipper motif (amino acids 27–48; ref. 19). In support of the sequence predictions, we detected FRMD4A not only in the cytoplasm and at cell–cell borders but also in the nucleus. If, like ERM proteins, FRMD4A activity is regulated by phosphorylation-dependent interaction between the FERM domain and the C-terminus (20), unfolding could potentially reveal the NLS and leucine zipper motifs, allowing both nuclear translocation and homo- or heterodimerization. CD44 ligation with hyaluronan resulted in loss of nuclear FRMD4A, indicating that transmembrane receptor signaling regulates FRMD4A localization. In addition, FRMD4A binding to cytohesin-1 and Par-3 is required for its membrane association (9). Whether there is a direct functional interconnection between FRMD4A, CD44, and Hippo signaling, for example involving binding of FRMD4A to the CD44 cytoplasmic domain, remains to be investigated. In addition, the dependence of E-cadherin–mediated intercellular adhesion on FRMD4A suggests that FRMD4A may affect multiple signaling pathways and protein complexes.

Our in vivo studies not only indicate that targeting FRMD4A is a promising new approach for treating SCC but also suggest possible therapeutic options, such as HSP90 drugs (24, 26) and injection of hyaluronan (27). Whereas multiple alternatively spliced forms of CD44 are expressed in SCCs (23, 28), hyaluronan is consistently downregulated (29), suggesting that much of the CD44 on the surface of tumor cells is unligated. HSP90 inhibitors and hyaluronan could potentially be beneficial in patients with HNSCCs, either where further management by surgery or adjuvant therapies is not possible or concurrently with standard treatment modalities.

A case can also be made for directly targeting FRMD4A, as this would potentially be more effective and specific than targeting upstream or downstream components in the signaling cascade. Two other FERM domain–containing proteins, focal adhesion kinase (FAK) and proline-rich tyrosine kinase 2 (Pyk2), have long been of interest as potential therapeutic targets in cancer (30). In the past, the focus has been on inhibiting their tyrosine kinase activity, but targeting the FAK FERM domain blocks phosphorylation of the activation loop (31). There is growing evidence that targeting protein–protein interactions is achievable (32) and compounds that target the Pyk2 FERM domain have been reported (30).

We observed a striking reciprocal relationship between nuclear accumulation of FRMD4A and YAP, both in cultured cells and in tumors, and in those contexts increased nuclear YAP correlated with reduced growth and increased differentiation. This is unexpected, as the Hippo pathway is upregulated in many solid tumors (21), and YAP positively regulates proliferation of epithelial stem cells (33, 34). In trying to reconcile these differing observations, it is worth noting that Hippo signaling is not simply a growth regulator (35). Its role as a cell density sensor may be particularly important in the context of multilayered epithelia (33). In mice, epidermal deletion of MST1/2 or LATS1/2 does not phenocopy YAP1 deletion, indicating that epidermal YAP functions independently of the canonical Hippo pathway. Instead, YAP1 forms a complex with α-catenin and 14-3-3 protein (33). Given the effect of FRMD4A knockdown on E-cadherin containing adherens junctions, it is tempting to speculate that FRMD4A may also impact on noncanonical Hippo signaling.

One final issue of interest is whether FRMD4A, a marker of normal epidermal stem cells (8), is also a cancer stem cell marker. FRMD4A is upregulated in HNSCCs and knockdown stimulates terminal differentiation. It is widely expressed by cells throughout the tumor mass, and while this could potentially reflect expansion of stem cells bearing oncogenic
Figure 7. Modulating FRMD4A and HIPPO via hyaluronan or 17-DMAG reduces SCC growth in vitro and in xenografts. A, SCC13 cells grown at clonal density on culture dishes coated with PBS or hyaluronan. Representative dishes stained with rhodamine blue are shown. Percentage of colony formation, colony area, and colony staining intensity (readout of cell density per unit colony area) were quantitated. Data are means ± SEM of triplicate dishes. B, SCC13 cells grown on coverslips coated with PBS or hyaluronan and labeled with antibodies to FRMD4A (green) and CD44 (red) or YAP1 (red). Left and second from left are pairs of images showing same fields. C, Effect of hyaluronan or 17-DMAG treatment on primary tumor growth (SCC13 in the case of hyaluronan; SCC25 in the case of 17-DMAG) and total metastatic disease (combined values for liver and lungs) in tongue xenografts. Quantitative data are means ± SEM (n = 5). D, sections of primary tumors were labeled with antibodies to FRMD4A (green) or YAP1 (red). Scale bars, 10 μm (B), 20 μm (D). HA, hyaluronan.
mutations, it is surprising that it does not correlate with differentiation status. The same has been observed for another marker of normal epidermal stem cells, the β1-integrin subunit (3). Thus, rather than defining specific subsets of tumor cells, 2 stem cell markers, FRMD4A and β1-integrin, show a generalized upregulation in HNSCCs.

Disclosure of Potential Conflicts of Interest
F.M. Watt is a paid editor of eLife. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: S.J. Goldie, F.M. Watt
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.J. Goldie, K.W. Mulder, D.W.-M. Tan, S.K. Lyons
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