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Conserved Oncogenic Behavior of the FAM83 Family Regulates MAPK Signaling in Human Cancer

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Abstract

FAM83B (Family with sequence similarity 83, member B) was recently identified as a novel oncogene involved in activating CRAF/MAPK signaling and driving epithelial cell transformation. FAM83B is one of eight members of a protein family (FAM83) characterized by a highly conserved domain of unknown function (DUF1669), which is necessary and sufficient to drive transformation. Here, it is demonstrated that additional FAM83 members also exhibit oncogenic properties and have significantly elevated levels of expression in multiple human tumor types using a TissueScan Cancer Survey Panel PCR array and database mining. Furthermore, modeling the observed tumor expression of FAM83A, FAM83C, FAM83D, or FAM83E promoted human mammary epithelial cell (HMEC) transformation, which correlated with the ability of each FAM83 member to bind CRAF (RAF1) and promote CRAF membrane localization. Conversely, ablation of FAM83A or FAM83D from breast cancer cells resulted in diminished MAPK signaling with marked suppression of growth *in vitro* and tumorigenicity *in vivo*. Importantly, each FAM83 member was determined to be elevated in at least one of 17 distinct tumor types examined, with FAM83A, FAM83B, and FAM83D most frequently overexpressed in several diverse tissue types. Finally, evidence suggests that elevated expression of FAM83 members is associated with tumor grade and overall survival.

Implications—FAM83 proteins represent a novel family of oncogenes suitable for the development of cancer therapies aimed at suppressing MAPK signaling.

Keywords

MAPK; FAM83A; FAM83B; FAM83D; breast cancer; transformation

Introduction

During cancer development, growth factor signaling pathways are often aberrantly upregulated, which aids in the survival and proliferation of the cancerous cells (1).

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Dysregulation of growth factor signaling occurs due to the hyperactivation of growth factor receptors (due to amplification or mutational activation), overproduction of ligands, or the hyperactivation of downstream effectors. However, therapeutically targeting growth factor signaling has proven difficult due to the plasticity of cancer cells and the adaptive reprogramming of growth-promoting signals following targeted inhibition (1–5). Furthermore, since many targeted therapies inhibit key proteins that are also required for the proliferation and survival of non-cancerous cells, there can be dose-limiting toxicities associated with their use. The identification of novel cancer-specific proteins that are key regulators of growth-signaling would provide new therapeutic targets that may lead to more effective therapeutic strategies for treating human cancers.

We recently identified *FAM83B (Family with sequence similarity 83, member B)* using an innovative phenotypic forward genetic approach to screen for novel, putative oncogenes that drive the transformation of immortalized human mammary epithelial cell (HMEC; (6)). During our initial characterization of FAM83B in human cancer specimens, we noted elevated FAM83B expression in specific cancer subtypes and an association with increased tumor grade and decreased overall patient survival. Importantly, simply elevating FAM83B expression in non-transformed HMEC resulted in the hyperactivation of MAPK signaling and the acquisition of numerous tumorigenic properties. Our studies determined that FAM83B functionally interacts with CRAF, thereby increasing CRAF membrane localization and MAPK activation. Conversely, inhibition of FAM83B from breast cancer cell lines decreased CRAF membrane localization, decreased basal and EGF-stimulated MAPK activity, and suppressed tumorigenicity *in vivo* (6).

Importantly, FAM83B is one member of an 8-member family of proteins that shares a highly conserved N-terminal domain of unknown function (DUF1669). The DUF1669 of FAM83B is necessary and sufficient to bind to CRAF and promote HMEC transformation, suggesting that additional FAM83 members may also regulate MAPK signaling. Supplementary the idea that additional FAM83 members may also promote aberrant MAPK signaling, Lee et al., identified FAM83A using a distinct genetic screen for novel genes that confer resistance to EGFR tyrosine kinase inhibitors in tumorigenic mammary epithelial cells (7). Importantly, FAM83A also interacts with CRAF to promote MAPK activation. Here, we report that numerous FAM83 members exhibit oncogenic properties and have significantly elevated levels of expression in many human tumor types. The novel FAM83 members examined here co-precipitate CRAF, increase CRAF membrane localization following ectopic expression in non-transformed HMEC, and promote anchorage-independent growth (AIG). Conversely, ablation of FAM83 members from breast cancer cells results in a marked loss of MAPK signaling, as well as tumorigenicity. We propose that the FAM83 proteins represent a novel family of oncogenes that may provide new targets for the development of more effective cancer therapies.

Materials and Methods

Cell lines

HME1-hTERT cells were grown as described (8). MDA-MB-468, MDA-MB-231, MCF7, and 293T cells were grown in DMEM + 5% fetal bovine serum. HCC1937 cells were grown

in RPMI + 10% fetal bovine serum. Two dimensional and 3- dimensional growth assays were performed as described (6). Lentiviruses and retroviruses were produced by transient transfection of 293T or Phoenix-Ampho cells, respectively, as previously described (9).

Cloning FAM83 members

cDNAs encoding FAM83A, FAM83C, FAM83D, and FAM83E were acquired from Open Biosystems and sequence verified following PCR-based cloning into the retroviral vector LPCX (Clontech). The FAM83A cDNA (BC052300) was amplified using primers (5′GCGAATTCATCGGTGAGCCGGTCAAGGCACCTGGGCAAAATC 3′ and 5′ CCATCGATCCTGGGCCTGCGGAGGGCAGCAG 3′). The FAM83A PCR product was cloned into pCMV-FLAG2 (Sigma), and then subcloned into LPCX. The FAM83C cDNA (BC113483) was amplified using two primers (5′ GAAGATCTATGGACTACAAGGACGACGATGACAAGGTGTTCGGAGGCCCGGGG CCTGG 3′ and 5′ CCATCGATCTTTGGCTAGGACTCAAAGCGGCT 3′) and cloned directly into LPCX. The FAM83D cDNA (BC006553) was amplified using two primers (5′CGCGGATCCATGGACTACAAGGACGACGATGACAAGAGTCCGAGCGCCGCCA TGGCTCT 3′ AND 5′CCATCGATCGGAGCAGTTACTGATAGGAAGGATAAAG 3′) and cloned directly into LPCX. The FAM83E cDNA (BC111970) was amplified using two primers (5′ GAAGATCTATGGACTACAAGGACGACGATGACAAGGTGGCGGCCTCCCAGCTG GCGGCGC 3′ and 5′ CCATCGATGCTCCTGTTCAGGGTTG 3′) and cloned directly into LPCX.

shRNA Reagents

For the knockdown experiments, cells were transduced with viruses expressing pLKO-shGFP, pLKO-shFAM83A (SHCLNG-NM_032899, TRCN0000168628, TRCN0000172664), pLKO-shFAM83D (SHCLNG-NM_030919, TRCN0000159856, TRCN0000162279), or shFAM83B (6).

Real-time PCR reagents

The primer sequences used were as follows: FAM83A forward 5′-CTCGGACTGGAGATTTGTCC-3′; FAM83A-reverse 5′-GGAACTCCTCGTCAAACAGC-3′; FAM83B forward 5′-ACGTCCAGTGAGCTTCTACG-3′; FAM83B reverse 5′-AAGCAATGGACTAGACCTGC-3′; FAM83D forward 5′-AGAGCGGCAATTCCACTTCG-3′; FAM83D reverse 5′-TGCCAGAATGAAGGCCAAGG-3′; GAPDH forward 5′-TGCACCACCAACTGCTTAGC-3′; GAPDH reverse 5′-GGCATGGACTGTGGTCATGAG-3′.

Immunoblotting, immunoprecipitation, and subcellular fractionation

Whole cell lysates, Western blots, and FLAG immunoprecipitations were performed as previously (10). For Western analysis, antibodies to GAPDH (Calbiochem), FLAG (M2; Sigma-Aldrich), ERK1/2, P-ERK1/2 (Thr202/Tyr204), c-RAF (Cell Signaling); RAS

(RAS10; Millipore) were used. Rabbit polyclonal antiserum against FAM83A was kindly provided by Dr. Mina Bissell (Lawrence Berkeley National Laboratories, Berkeley, CA; (7)). The subcellular protein fractionation kit was purchased from Thermo Scientific and the standard protocol was used.

Real-time PCR and Northern Analysis

Total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen). Reverse transcription was performed using the iScript cDNA Synthesis Kit (BioRad). Real-time PCR was carried out using the iQ SYBR Green Supermix (BioRad). Northern blot analysis and TissueScan Cancer Survey Panel 384 - I (Origene) real-time PCR and analysis were carried out as previously described using primers described in the Supplementary materials (6).

Xenograft tumor formation

NCr (nude) mice were bred and maintained under defined conditions at the Athymic Animal and Xenograft Core facility at the Case Western Reserve University Case Comprehensive Cancer Center. For tumorigenicity studies, MDA-468 cells were infected with lentiviruses encoding FAM83A-shRNA or FAM83D-shRNA and selected in puromycin. Following selection, 2×10^6 MDA-468 cells were resuspended in 50% Matrigel in complete media and injected subcutaneously into both the right and left flank of 5 mice per group (10 injections total per group). After 10 weeks, tumors were removed and weighed.

Results

The FAM83 protein family represents a potential novel family of oncogenes upregulated in human cancers

Using a validation-based insertional mutagenesis (VBIM) forward genetic screen, we recently identified *FAM83B* as a novel gene capable of driving HMEC transformation (6). FAM83B is one of eight members of a protein family (FAM83), based solely on the presence of the DUF1669. A PRALINE amino acid alignment of the eight FAM83 members highlights the conserved amino-terminus DUF1669 (comprising the first ~300 amino acids) and the striking divergence of the family members beyond this domain (Figure 1; (11)). Importantly, the expression of all FAM83 proteins has been confirmed by mass spectrometry, and is often associated with receptor tyrosine kinase activity (including EGFR, c-MET, and other serum-stimulated kinases; Supplementary Table 1). Our recent studies have shown that the oncogenic properties of FAM83B are dependent upon the conserved DUF1669 (6). Therefore, we hypothesized that additional FAM83 family members may also exhibit oncogenic properties. To test this hypothesis, we cloned five FAM83 members (Fig. 2A), overexpressed each in untransformed HME1 cells (Fig 2B, additional expression analysis in Supplementary Figure 1, and Supplementary Table 2), and plated the FAM83-expressing HME1 cells into soft agar to measure AIG. Cells expressing each of the five FAM83 members exhibited significantly more AIG compared to control cells expressing GFP (Fig. 2B–2D), suggesting that each FAM83 protein functions as a putative oncogene.

Oncogenes often exhibit aberrant expression or activity in human tumors. To examine whether additional *FAM83* members are also aberrantly expressed in cancerous tissues, publicly available microarray data was examined. Indeed, *FAM83* member mRNAs were significantly elevated in a wide variety of tumor types, often correlating with (1) specific cancer subtypes, (2) increased tumor grade, and (3) decreased overall survival (Supplementary Table 3). To confirm these microarray studies, we examined the expression of *FAM83A, FAM83C, FAM83D, and FAM83E* in a panel of 17 different tumor types using quantitative real-time PCR. Of the 17 tissues examined, 9 had a statistically significant ($p<0.05$) increase in one or more of the *FAM83* mRNAs in cancer versus normal tissue specimens (Fig. 3A). Each of the five *FAM83* members tested were significantly elevated in at least one tumor type, with *FAM83A* and *FAM83D* elevated in three and eight cancer subtypes, respectively (Fig. 3A). Moreover, we recently reported that *FAM83B* is significantly elevated in six of the 17 tumor tissues examined, including breast, lung, ovary, cervical, testis, thyroid, bladder, and lymphoid cancers (6). Several cancer types had more than one family member significantly increased when compared to normal tissue, including breast (*FAM83B* and *FAM83D*), lung (*FAM83A*, *FAM83B*, and *FAM83D*), testis (*FAM83A*, *FAM83B*, and *FAM83D*), bladder (*FAM83A*, *FAM83B*, *FAM83C*, and *FAM83D*), thyroid (*FAM83B* and *FAM83D*), ovary (*FAM83B* and *FAM83E*), and lymphoid (*FAM83B* and *FAM83D*; Fig. 3A and (6)).

A more extensive expression analysis was performed on select *FAM83* members and in select tumor types. Given the increased expression of *FAM83A* in breast cancer recently reported by Lee et al. (7), and our pilot analysis indicating *FAM83D* is also upregulated in breast cancer, the mRNA level of each was quantified in an additional 161 breast tumors. The expression of both *FAM83A* and *FAM83D* was significantly increased in the breast cancer specimens compared to normal breast tissue (Fig. 3B). Based on our pilot data and publically available microarray data, we next examined *FAM83A, FAM83B*, *FAM83D*, and *FAM83E* in an additional 74 ovarian cancer specimens. The expression of all four *FAM83* members was significantly increased in the additional ovarian cancer specimens examined compared to normal tissue (Fig. 3B). *FAM83A*, *FAM83B*, *FAM83C*, and *FAM83D* were also further examined in additional bladder cancer specimens. The expression of *FAM83A* and *FAM83C*, but not *FAM83B* ($p=0.144$) or *FAM83D* ($p=0.257$), was significantly increased in the additional bladder cancer specimens examined compared to normal tissue (Fig. 3B). Finally, we examined *FAM83A, FAM83B*, and *FAM83D* in an additional 96 lung tumors. The expression of *FAM83* members was significantly increased in the additional lung cancer specimens compared to normal tissue (Fig. 3B). Moreover, *FAM83A* and *FAM83D* were elevated in both squamous cell carcinoma and adenocarcinoma lung cancer sub-types, whereas *FAM83B* was elevated only in squamous cell carcinoma (Supplementary Fig. 2). Of note, our finding that *FAM83A* is significantly elevated in lung cancer is in line with its identification as a lung cancer biomarker, where it has been reported to be elevated in ~70% of lung adenocarcinomas (12). Taken together, our data suggest that the expression of FAM83 member mRNA is common to diverse human malignancies. In addition to the cancer-associated expression of FAM83 members, the relative expression of each of the five *FAM83* members in the normal tissues examined during our analysis, and from publically

available data available on Oncomine is summarized in Supplementary Figure 3 and Supplementary Figure 4 (13).

Ablation of FAM83 family members in human breast cancer cells suppresses tumorigenicity

Each of the five FAM83 members tested in our studies was able to drive HME1 transformation and each was significantly upregulated in at least one cancer type. Given that *FAM83A* and *FAM83D* are most frequently upregulated, we focused on these *FAM83* members and their similarities to *FAM83B*. In our previous studies of FAM83B, we determined that the growth and tumorigenicity of tumor-derived cancer cells harboring elevated FAM83B could be suppressed following FAM83B ablation (6). To test whether ablation of FAM83A or FAM83D resulted in a similar suppression of tumorigenicity, five lentiviral shRNA constructs targeting each gene were obtained and tested. Each shRNA construct was tested in MDA468 cells, which harbor increased expression of both *FAM83A* and *FAM83D* relative to untransformed HMEC (Fig. 4A). The two most efficient shRNAs capable of targeting *FAM83A* (83A-2 and 83A-4) or *FAM83D* (83D-2 and 83D-4) were used in subsequent studies. Each *FAM83* shRNA, or a control shRNA targeting GFP, was delivered to MDA468 cells by lentiviral infection, the cells were selected in puromycin, and efficient knockdown was confirmed by Western or real-time PCR analysis (Fig. 4B). Following the suppression of FAM83A or FAM83D, cell number, AIG, acini formation in laminin-rich basement membrane (lrBM), and tumorigenicity *in vivo* were examined. Similar to our observations with FAM83B, the growth of MDA468 cells was significantly suppressed (by 80–95%) following FAM83A or FAM83D ablation (Fig. 4C). Furthermore, the growth of soft agar colonies and acini in lrBM were also significantly suppressed following FAM83A or FAM83D knockdown (Fig. 4D and 4E). Finally, sh*FAM83A*- and sh*FAM83D*-expressing MDA468 cells were injected into immunocompromised mice, and tumor formation was assessed. Again, suppression of either FAM83A or FAM83D resulted in a significant reduction in tumor weight when compared to shGFP-expressing MDA468 cells (Fig. 4F).

Our previous studies defined a key role for FAM83B in EGFR/RAS signaling. To determine whether transformed breast cells also require sustained FAM83A or FAM83D expression, we examined EGF-dependent HCC1937 breast cancer cells, MDA231 breast cancer cells (which harbor mutant RAS), and HME1 cells transformed by exogenous mutant RAS-G12V (HME1-Ras^{G12V}). HCC1937, MDA231, or HME1-Ras^{G12V} cells were infected with lentiviruses encoding sh*FAM83A* or sh*FAM83D*, selected in puromycin, and plated to evaluate proliferation, AIG, and growth in lrBM. Again, ablation of FAM83A or FAM83D suppressed the growth and AIG of each of the cancer cell lines, as well as the growth of HCC1937 in lrBM (Fig. 5A–E). A similar dependence for FAM83A and FAM83D was also observed with MCF7 cells (Supplementary Fig. 5). Taken together, our data suggest that sustained expression of FAM83A and FAM83D is required for breast cancer cells to retain their tumorigenic properties. Importantly, given the redundant phenotype observed following ablation of FAM83A, FAM83B, or FAM83D, we confirmed that the shRNAs used in our studies are specific for each FAM83 member (Supplementary Fig. 6).

FAM83 family members are key regulators CRAF and MAPK signaling

We previously demonstrated that elevated FAM83B expression stimulates aberrant activation of MAPK signaling by binding to CRAF and increasing CRAF membrane localization (6). The similar phenotypes observed following the suppression of FAM83A, FAM83B, or FAM83D expression suggest that each FAM83 member is involved in CRAF activation. Analysis of MAPK signaling/ERK phosphorylation was performed using MDA468 cells expressing *FAM83A* or *FAM83D* shRNAs. Indeed, the inhibition of FAM83A or FAM83D expression strongly suppressed ERK phosphorylation (Fig. 6A). Therefore, FAM83A, FAM83B, and FAM83D all appear to be important intermediaries in maintaining aberrant CRAF/MAPK activation in MDA468 breast cancer cells.

The DUF1669 is the only predicted functional domain within the FAM83 proteins, and is highly conserved between all 8 members. Moreover, the DUF1669 of FAM83B is critical for CRAF binding, AIG, and the increased phosphorylation of ERK1/2 (6). To test whether each of the FAM83 members capable of driving HME1 transformation can co-precipitate with CRAF, each FLAG-tagged FAM83 member was co-expressed with CRAF and the cell lysates subjected to immunoprecipitation with a FLAG antibody. Similar to our findings with FAM83B, each FAM83 member efficiently co-precipitated CRAF when compared to a control lysates expressing CRAF only (Fig. 6B). Moreover, cellular fractionation determined that CRAF membrane localization was increased in HME1 cells expressing FAM83A, FAM83B or FAM83D (Fig. 6C). In contrast to the increased CRAF activity, we observed no change in RAS activity in any of the FAM83-expressing cells (Supplementary Fig. 7).

Taken together, our studies suggest that each FAM83 member transforms HME1 cells using a similar mechanism of CRAF/MAPK activation. However, it is unclear why cancer cells would require the sustained expression of multiple FAM83 members if their functions were simply redundant. To determine whether individual tumors commonly harbor elevated expression of more than one FAM83 member, we further examined FAM83A, FAM83B, FAM83D, and FAM83E mRNA in individual tumors in the cancer panels described in Figure 3. Interestingly, we identified numerous ovarian, breast, bladder and lung cancers specimens in which two, three, or even all four FAM83 members examined were significantly elevated in the same tumor (Supplementary Fig. 8). We further confirmed that multiple *FAM83* members are overexpressed in the same tumor using matched normal and tumor lung specimens. Again, *FAM83A*, *FAM83B*, and *FAM83D* expression was significantly elevated in the tumor lung specimens when compared to the matched, associated normal lung tissue (Fig. 6D). A heatmap was generated to compare the expression of the three *FAM83* members in each tumor, which further confirmed that many tumors have elevated expression of more than one *FAM83* member (Fig. 6E). The number of tumors overexpressing *FAM83A*, *FAM83B*, and/or *FAM83D* by 30-fold or greater relative to matched normal tissue was quantified and is shown in Figure 6F. We conclude that, while all FAM83 members share the highly conserved DUF1669, they lack any significant homology beyond this domain and the frequent upregulation of multiple FAM83 members may suggest that each FAM83 member has additional, as yet unidentified, cellular functions.

Discussion

Using a phenotypic forward genetic approach, we recently identified FAM83B as a novel oncogene capable of driving the transformation of immortalized HMECs (6). Our study provided the foundation for understanding how this previously uncharacterized protein activates MAPK signaling. Moreover, our findings implicated FAM83B as an intriguing new target for therapies aimed at disrupting MAPK signaling in cancers harboring elevated EGFR/RAS signaling. Importantly, FAM83B is a member of an 8-member protein family, which shares a highly conserved N-terminal DUF1669. Given that FAM83B required the DUF1669 to drive HMEC transformation, we hypothesized that additional DUF1669-containing FAM83 members might also have oncogenic functions when overexpressed. We demonstrate here that 4 additional FAM83 members (FAM83A, FAM83C, FAM83D and FAM83E) can also promote HMEC transformation and that each is overexpressed in at least 1 of 17 tumor types examined. *FAM83A*, *FAM83B*, and *FAM83D* were among the most frequently upregulated in our analysis, with significant elevation in 5, 6, and 8 of 17 tumor types, respectively. Further analysis of FAM83A and FAM83D in breast cancer cells determined that shRNA-mediated ablation of either FAM83 member strongly suppressed MAPK effector signaling, AIG, and tumorigenicity, similar to our findings with FAM83B.

In breast cancer, *FAM83A*, *FAM83B* and *FAM83D* are significantly upregulated relative to normal breast tissue and associated with estrogen-receptor (ER) and progesterone-receptor (PR) negative tumors, with higher grade and poor outcome. ER/PR negativity is a key property that is used to define the more aggressive basal-like or triple negative breast cancer subtypes, which are less responsive to standard systemic chemotherapies. Given the recent finding that elevated ERK activation in basal-like breast cancer conferred elevated proliferation rates even following neoadjuvant therapy (14), we propose that the inhibition of key intermediaries involved in MAPK activation, such as the FAM83 proteins, may facilitate genotoxic killing of these difficult to treat cancers. In addition, we recently identified that elevated FAM83B expression also activates the PI3K/AKT signaling pathway and EGFR/PLD1 axis (15, 16). The combined activation of both MAPK and PI3K/AKT signaling resulted in a decreased sensitivity to numerous targeted therapies, including EGFR, PI3K, AKT, and mTOR inhibitors. If elevated FAM83 protein expression confers an intrinsic or selected resistance to tumor cells treated with either genotoxic or targeted therapies, then the development of new therapies that specifically target the FAM83 proteins may suppress both MAPK and PI3K/AKT signaling and serve to inhibit the further expansion of resistant tumor cells.

The overexpression of FAM83 family members in cancer compared to normal tissues may also offer an expanded therapeutic window when compared to direct MAPK or PI3K/AKT inhibition, since they are required for normal cellular homeostasis. However, additional analysis of FAM83 members in normal tissues should be examined further, as there may be specific tissues which normally express elevated FAM83 expression, which may impact the potential to target FAM83 members therapeutically. Our data suggest that disrupting FAM83 protein function in cancer cells would strongly interfere with the growth and survival of cancer cells harboring elevated FAM83 expression, yet would minimally affect the growth of normal tissues. Certain cancer cell lines appear to be “addicted” to elevated

FAM83 member expression, with some lines actually requiring the sustained expression of more than one FAM83 member. For example, MDA468 and HCC1937 cells require the sustained expression of FAM83A, FAM83B, and FAM83D, as demonstrated by the significant reduction of AIG and tumorigenicity observed upon shRNA-mediated ablation of any of the three. Whether the growth suppression observed following the knock-down of each FAM83 member is solely attributable to the FAM83-CRAF interaction and MAPK activation, or additional, undefined functions of these novel proteins still needs to be resolved. However, our data suggest that therapeutically targeting just one of the FAM83 family members elevated in a human tumor may effectively suppress tumor growth.

In addition to our discovery of FAM83B in a forward genetic screen for genes capable of driving HMEC transformation, Lee et al. recently identified FAM83A using a distinct genetic screen for genes that confer resistance to EGFR receptor tyrosine kinase inhibitors (TKIs) in tumorigenic mammary epithelial cells (7). Similar to our findings with FAM83B, overexpression of FAM83A conferred resistance to EGFR inhibition whereas ablation of FAM83A phenotypically normalized the malignant cells, resulting in the reacquisition of TKI sensitivity and a basal polarity in lrBM that is characteristic of non-tumorigenic cells. *FAM83A* is frequently co-amplified with the *MYC* proto-oncogene in both breast cancer cell lines and primary breast tumors and its expression correlates with poor prognosis (7). Our analysis confirms that breast cancer, as well as lung, bladder, testis and ovarian cancer have significantly elevated FAM83A levels (as well as FAM83B and FAM83D) when compared to normal tissue. Our analysis of FAM83 in lung cancer confirms a report by Li et al. showing that *FAM83A* may serve as a putative lung cancer biomarker (12). Their study demonstrated that *FAM83A* is significantly upregulated in greater than 50% of human lung cancers. Moreover, *FAM83A* could be detected by RT-PCR of peripheral blood mononuclear cells (including circulating cancer cells) from lung cancer patients, but not from healthy controls (17). Lung cancers as early as Stage I/Stage II could be identified by this analysis. Such an assay could be an important step toward screening for tumors harboring high *FAM83A* expression. Currently, it is unclear whether this will work for other *FAM83* members or other cancers, but we propose the elevated expression of the *FAM83* genes (not mutation) may make this approach feasible. Taken together, our studies and those of Lee et al. provide the first evidence that elevated FAM83 expression in cancer cells plays an important role in aberrant EGFR and RAS signaling and therapeutic resistance. The separate identification of two FAM83 members in distinct genetic screens, the elevated expression of each FAM83 member in a diverse set of cancer types, and the involvement of each FAM83 member in CRAF/MAPK signaling provides the basis for the further pursuit of novel therapeutics that target FAM83 members in a broad array of cancers.

While all 8 FAM83 members share the highly conserved DUF1669, they also vary greatly in size (ranging from 434–1179 amino acids) and otherwise lack any significant homology beyond the DUF1669. The DUF1669 of FAM83B is sufficient to drive transformation, and interact with CRAF and EGFR (6, 15). Furthermore, FAM83A is the smallest FAM83 member at 434 amino acids, and is comprised mainly of the DUF1669. Thus, we propose that the DUF1669 is the oncogenic portion of the FAM83 proteins, and therefore the most attractive target for therapy. Based on the divergent sizes of the FAM83 proteins, we

hypothesize that each may have additional, DUF1669-independent cellular functions. Consistent with this hypothesis, our analysis identified numerous primary tumors that had selectively upregulated more than one FAM83 members. One would predict that the elevated expression of FAM83 members would be mutually exclusive if they simply had redundant functions. Additional evidence for the divergent functions of FAM83 members is provided by the observation that FAM83D binds to the chromokinesin KID and localizes to the spindle during mitosis to regulate spindle maintenance (18). Importantly, the FAM83D-KID interaction occurs independently of the FAM83D DUF1669, which has no significant similarity with other FAM83 proteins (Figure 1). Deciphering the additional, non-redundant cellular functions of FAM83 members during tumorigenesis and the mechanism underlying FAM83 family overexpression in human cancer cells will be the focus of future studies. In conclusion, our significant discovery of a novel family of oncogenes as critical mediators of EGFR/RAS signaling provides important new insight into how RAS effector signaling is activated during tumorigenesis, and provides a solid basis for pursuing new therapeutics to target FAM83 members in a broad array of cancers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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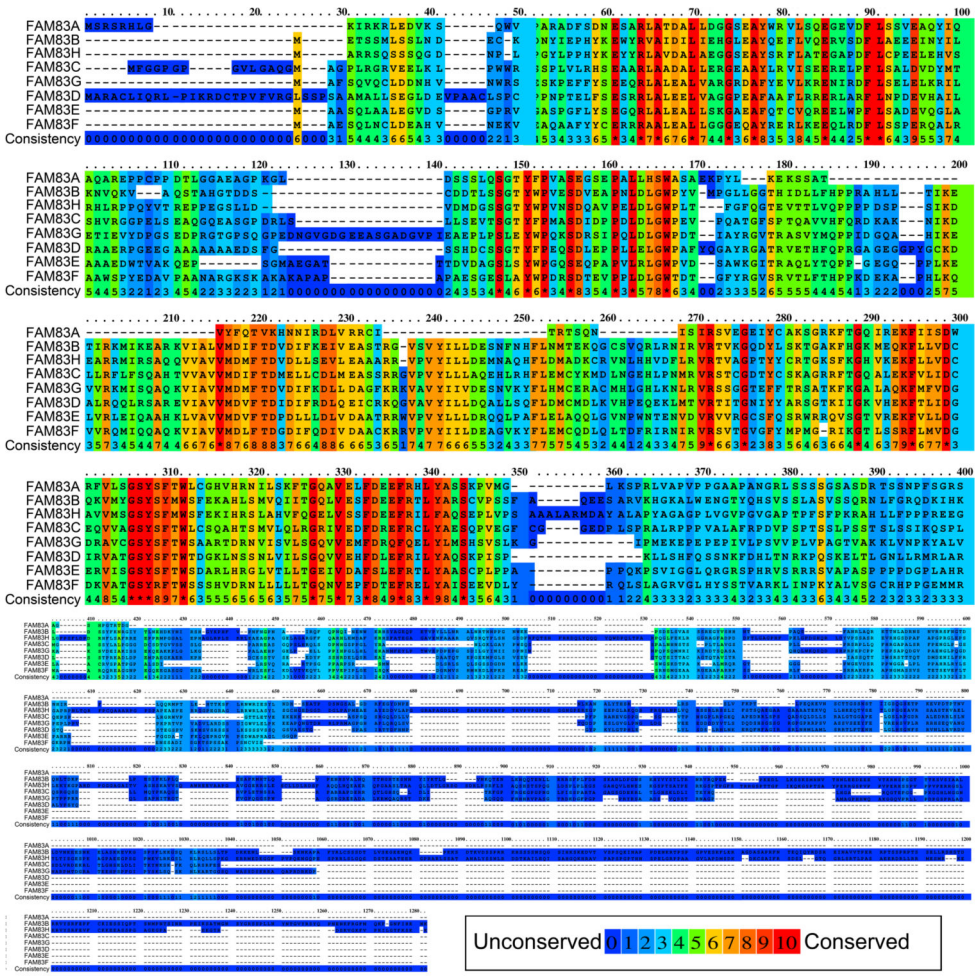

Figure 1. The FAM83 proteins have a conserved N-terminal Domain of Unknown Function (DUF1669)

PRALINE amino acid sequence alignment analysis was performed on all 8 FAM83 members (A–H; (11)). The legend indicates the range of conservation from blue (unconserved) to red (conserved), and highlights the highly conserved amino terminus.

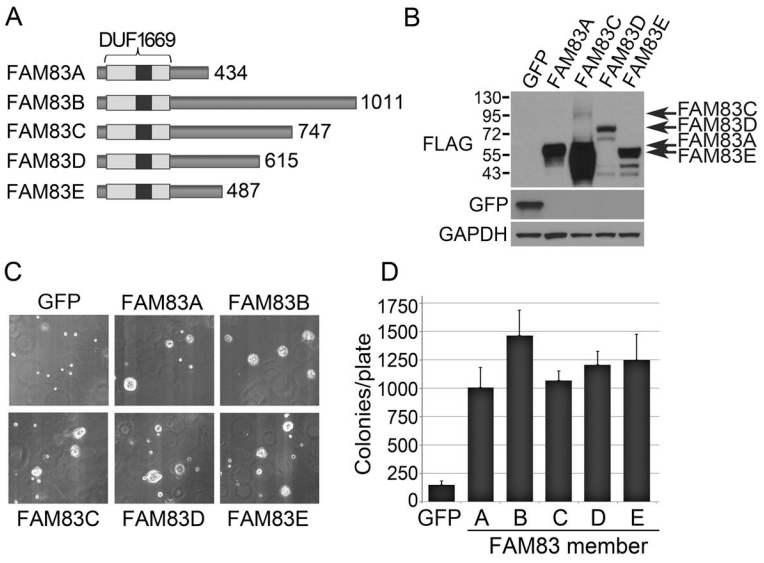

Figure 2. Numerous FAM83 members can drive the transformation of HME1 cells

(**A**) Schematic diagram of the five FAM83 members studied here, illustrating the conserved Domain of Unknown Function (DUF1669), and the number of amino acids encoding each protein. (**B–D**) HME1 cells were infected with a retrovirus encoding FAM83A-FAM83E, or a control retrovirus (Vector). Immunoblot analysis confirmed the expression of each FAM83 member **(B)**. The FAM83-expressing HME1 cells were plated into soft agar to assess AIG. Images of the colonies were acquired after 3 weeks **(C)**, and the number of colonies was quantified **(D)**. Error bars represent the mean +/− standard deviation for a representative experiment performed in triplicate.

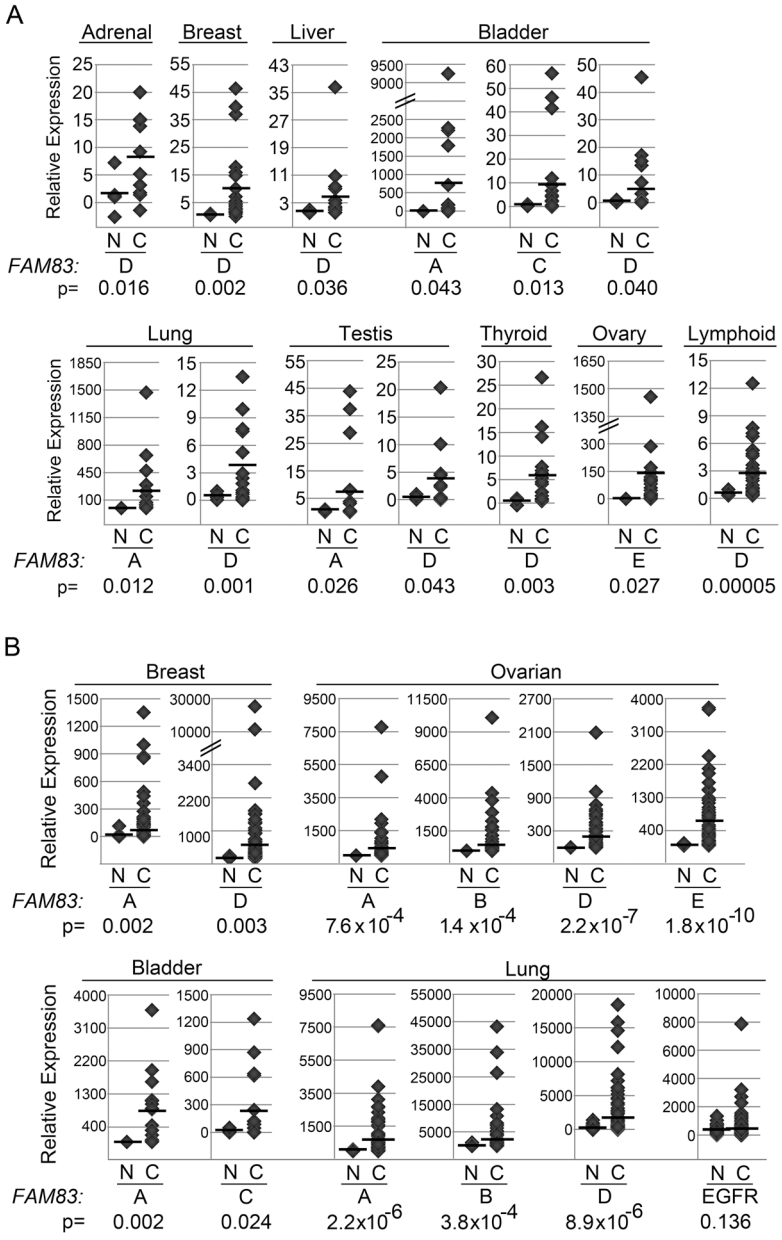

Figure 3. The *FAM83* members are overexpressed in diverse human cancers

(A) Origene TissueScan Cancer Panels (384-well multi-cancer survey panel) were analyzed by Real-Time PCR for *FAM83A, FAM83B FAM83C, FAM83D, and FAM83E*. The sample sizes for each tissue type are as follows: Adrenal: N=2, C=22; Breast: N=2, C=23; Bladder: N=2, C=22; Lung: N=4, C=19; Testis: N=6, C=19; Thyroid: N=3, C=18; Ovary: N=3, C=21; Lymphoid: N=3, C=34. **(B)** *FAM83* members overexpressed in the TissueScan Cancer Panels or in Oncomine microarray data were further examined in additional tissue specific TissueScan Cancer Panels. The sample sizes for each tissue type are as follows: Ovarian: N=13, C=76; Breast: N=16, C=191; Lung: N=38, C=98; Bladder: N=3, C=18. The relative expression of each *FAM83* member that had a statistically significant difference

between normal, associated (N) and cancerous (C) tissues is presented. The statistical significance was determined using a Welch's t-test.

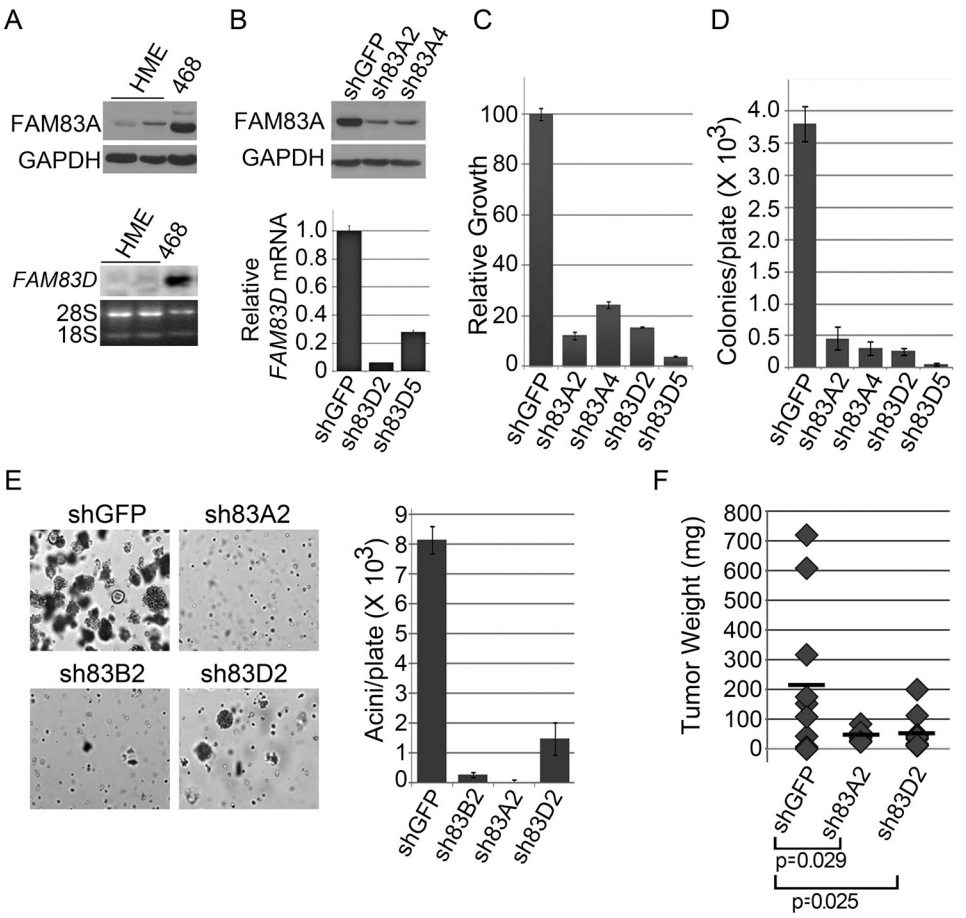

Figure 4. Ablation of FAM83A or FAM83D expression suppresses the growth of breast cancer cells

(A) Western analysis (upper panel) or Northern analysis (bottom panel) confirmed elevated expression of FAM83A and *FAM83D* in MDA468 cancer cells compared to two normal HMEC cell lines (HME). **(B)** MDA468 cells were infected with lentiviruses encoding an shRNA targeting GFP or two different shRNAs targeting either *FAM83A* (sh83A2 or sh83A4) or *FAM83D* (sh83D2 or sh83D5). Western analysis (upper panel) and Real-time analysis (lower panel) confirmed the suppression of FAM83A protein and *FAM83D* mRNA. (**C–E**) MDA468 cells expressing control, *FAM83A*, or *FAM83D* shRNA were plated to assess relative growth **(C)**, AIG **(D)**, or acini formation in lrBM **(E). (F)** MDA468 cells expressing control, *FAM83A*, or *FAM83D* shRNA were injected subcutaneously into immunocompromised mice to assess tumor formation. Error bars represent the mean +/− standard deviation for a representative experiment performed in triplicate.

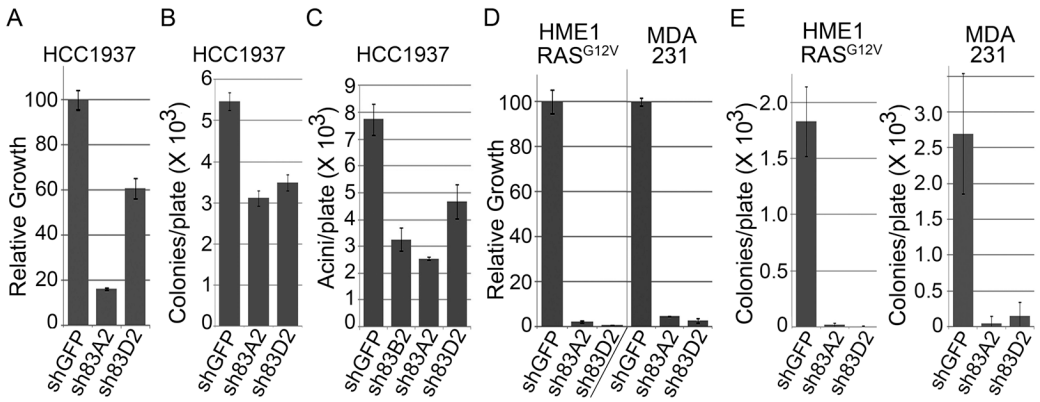

Figure 5. Suppression of FAM83A or FAM83D expression inhibits the growth of breast cancer cells and RAS-transformed HMEC

(A–C) HCC1937 cells were infected with lentiviruses encoding an shRNA targeting GFP, *FAM83A* (sh83A2), *FAM83D* (sh83D2), or *FAM83B* (sh83B2). The HCC1937 cells expressing control, *FAM83A*, or *FAM83D* shRNA were plated to assess relative growth **(A)**, AIG **(B)**, or acini formation in lrBM **(C). (D–E)** MDA231 breast cancer cells or HME1 cells transformed by exogenous mutant RAS-G12V (HME1-Ras^{G12V}) were infected with lentiviruses encoding an shRNA targeting GFP, *FAM83A* (sh83A2) or *FAM83D* (sh83D2) and plated to assess relative growth or AIG.

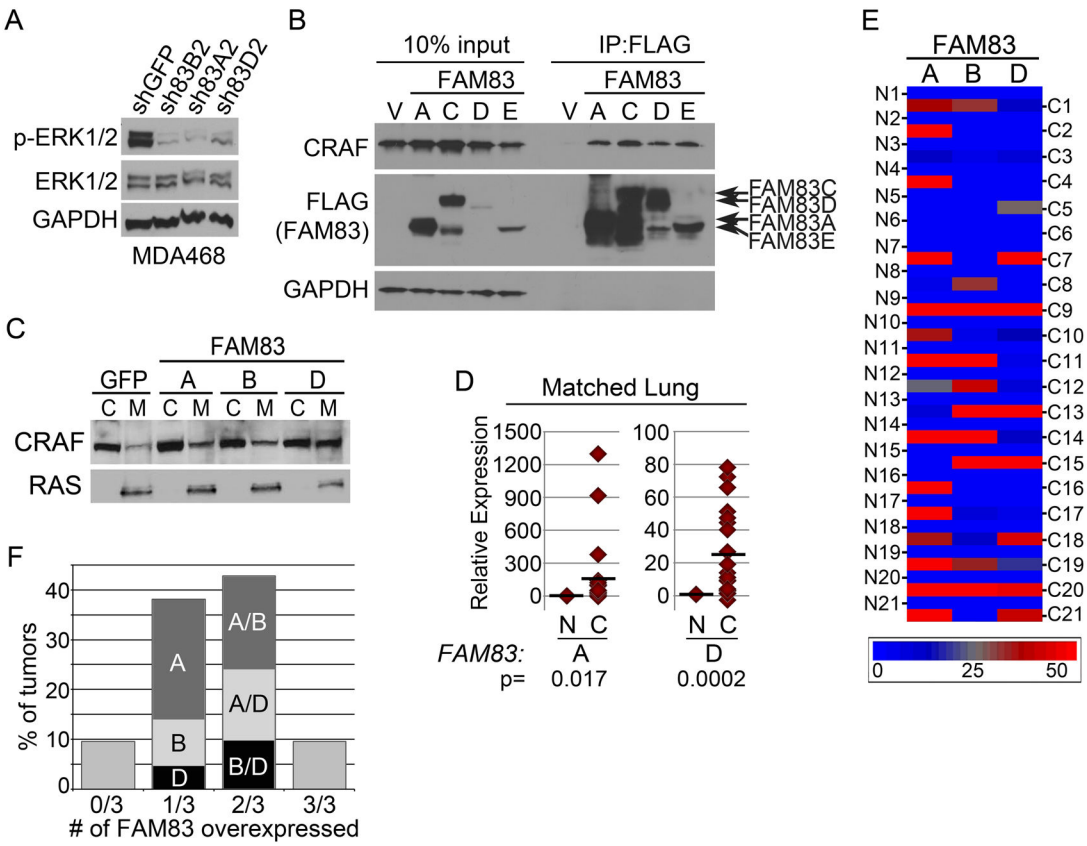

Figure 6. FAM83 members regulate CRAF-MAPK signaling

(A) MDA468 cells were infected with lentiviruses encoding shRNA targeting GFP, *FAM83A* (sh83A2), *FAM83D* (sh83D2), or FAM83B (sh83B2), and Western analysis of phospho-ERK1/2, ERK1/2, and GAPDH was performed. **(B)** FAM83 members co-precipitate CRAF. 293T cells were transfected with an expression constructs encoding CRAF, FAM83A, FAM83C, FAM83D, FAM83E, or a non-coding vector control (V) as indicated. Immunoprecipitation was performed using a FLAG antibody, and precipitated proteins analyzed by Western analysis to determine the amount of CRAF bound to each FAM83 member. **(C)** Subcellular protein fractionation was performed to isolate cytoplasmic (C) and membrane (M) fractions from HME1 cells expressing GFP, FAM83A, FAM83B, or FAM83D. Normalized portions of each extract were analyzed by Western blotting using antibodies against RAS and CRAF. **(D)** The relative expression of *FAM83A* and *FAM83D* was examined in a panel of 21 matched normal, associated lung (N) and lung cancer (C). Each cancer specimen is normalized to the corresponding normal, which is set equal to 1. **(E)** Heatmap showing the relative expression of *FAM83A, FAM83B, and FAM83D* in each tumor from the panel of 21 matched normal, associated lung (N) and lung cancer (C) shown in E. **(F)** Quantification of the number of tumors overexpressing 0, 1, 2, or all 3 of the *FAM83* members by greater than 30-fold is shown for the matched lung dataset. The distribution of each *FAM83* member, or pair of *FAM83* members is shown within each bar of the graph.