FEBS *Letters*

FERM domain-containing protein FRMD5 regulates cell motility via binding to integrin β5 subunit and ROCK1

Jinxia Hu [a,b,c,d,e], Miaomiao Niu [a,b,c], Xueying Li [a,b,c], Danyu Lu [a,b,c], Jia Cui [a,b,c], Weizhi Xu [a,b,c], Gang Li [d], Jun Zhan [a,b,c], Hongquan Zhang [a,b,c,∗]

[a] Key Laboratory of Carcinogenesis and Translational Research, Ministry of Education, Peking University Health Science Center, Beijing 100191, China
[b] State Key Laboratory of Natural and Biomimetic Drugs, Peking University Health Science Center, Beijing 100191, China
[c] Laboratory of Molecular Cell Biology and Tumor Biology, Department of Anatomy, Histology and Embryology, Peking University Health Science Center, Beijing 100191, China
[d] Department of Biochemistry and Molecular Biology, Peking University Health Science Center, Beijing 100191, China
[e] Department of Biochemistry and Molecular Biology, Binzhou Medical University, Yantai 264000, Shandong, China

ARTICLE INFO

ABSTRACT

FRMD5 is a novel FERM domain-containing protein depicted in tumor progression. However, the mechanisms underlying FRMD5 inhibition of cell migration is largely unknown. Here, we show that FRMD5 regulates cell migration by interacting with integrin β5 cytoplasmic tail and ROCK1 in human lung cancer cells. FRMD5 promotes cell–matrix adhesion and cell spreading on vitronectin, and thus inhibits cell migration. Furthermore, FRMD5 interacts with ROCK1 and inhibits its activation that leads to the inhibition of myosin light chain phosphorylation and the actin stress fiber formation. Taken together, these findings demonstrate that the putative tumor suppressive protein FRMD5 regulates tumor cell motility *via* a dual pathway involving FRMD5 binding to integrin β5 tail and to ROCK1.

Structured summary of protein interactions:
integrin B5 binds to **FRMD5** by pull down (View interaction) **integrin B5** physically interacts with **FRMD5** by pull down (View interaction) **FRMD5** physically interacts with **integrin B5** by anti tag coimmunoprecipitation (View interaction) **FRMD5** physically interacts with **ROCK1** by anti tag coimmunoprecipitation (1, 2) **ROCK1** physically interacts with **FRMD5** by anti bait coip (1, 2) **Kindlin-2** physically interacts with **integrin B5** by anti tag coimmunoprecipitation (View interaction) **Talin** physically interacts with **integrin B5** by anti tag coimmunoprecipitation (View interaction)

1. Introduction

The protein 4.1R, ezrin, radixin, moesin (FERM) superfamily consists of over fifty proteins and all members contain a three-lobed 'cloverleaf' structure called FERM domain which binds a variety of cell-membrane associated proteins and lipids [1]. These proteins serve as the linker proteins between cell membrane and the cytoskeleton, and play an important role in the regulation of cancer cell migration and metastasis [2].

FRMD5 is a FERM domain-containing protein consisting of 570 amino acids. The N-terminal of FRMD5 contains a FERM domain and a FERM-associated domain (FA domain). However, the C-terminal of FRMD5 has no obvious domain structure. Little is known about FRMD5 structurally and functionally. Recently studies demonstrated that FRMD5 may be involved in metabolic regulation, as FRMD5-WDR76 loci was found associated with altered serum protein concentrations [3]. In addition, FRMD5 SNPs were found related with the postprandial chylomicron (CM) triacylglycerol (TG) response [4]. Interestingly, FRMD5 was also found as a target gene of mutant p53 (R273H) in U251 human glioblastoma cells in which p53 (R273H) downregulated FRMD5 expression by binding to intronic and intra-genic sequences of FRMD5, suggesting that FRMD5 may be a suppressive molecule during tumor progression [5]. We cloned the FRMD5 cDNA and examined the role of FRMD5 in tumor progression. We found that knockdown of FRMD5 in lung cancer cell H1299 promotes tumor formation in Nude mice. Furthermore, FRMD5 was found to inhibit the migration and

∗ Corresponding author at: Laboratory of Molecular Cell Biology and Tumor Biology, Department of Anatomy, Histology and Embryology, School of Basic Medical Sciences, Peking University Health Science Center, #38 Xue Yuan Road, Beijing 100191, China.
  E-mail address: Hongquan.Zhang@bjmu.edu.cn (H. Zhang).

invasion abilities of H1299 cells [6]. However, the mechanism behind these functions is largely unknown.

It was known that moderate cell–matrix adhesion and stress fiber formation are needed for cell movement [7]. Cell–matrix adhesion is mediated by the transmembrane receptors, the integrins, a family of heterodimeric receptors composed of α and β subunits. Integrins play important roles in the regulation of tumor migration and invasion [8]. FERM domain-containing proteins including Talin [9] and Kindlins [10] have been reported to regulate integrin activation through directly molecular interaction with the cytoplasmic tail of integrin β subunit [11].

ROCK1 is a downstream effector of the small GTPase RhoA and play major roles in a wide range of cellular activities including cell migration, invasion and metastasis. ROCK1 regulates cell motility through phosphorylation of the myosin light chain (MLC) and thus promotes stress fiber formation and enhances the capability of cell motility and migration [12]. In our previous study we found that FRMD5 interacts with p120-catenin, whereas other group reported that p120-catenin interacts with ROCK1 [13]. Therefore, there is a possibility that FRMD5 may also interact with ROCK1 and regulate the biological function of ROCK1.

In this study, we investigated the association of FRMD5 with integrin β5 and ROCK1. We identify a new mechanism for regulation of cancer cell migration by showing that FRMD5 coordinates integrin β5-mediated cell–matrix interaction and ROCK1-controlled actin stress fiber formation.

1.1. Plasmids and siRNA

Plasmids of GST-fusion proteins integrin β1, β3 and β5 were kindly provided by Dr. Staffan Strömblad (Karolinska Institutet, Sweden). The shRNA expressing plasmid targeting GGAACTGAG TGGTCAAACA of FRMD5 mRNA was constructed according to the manufacture protocol of pSUPER RNAi system. FRMD5 small interference RNA used in this study is si-718 which has the highest knockdown efficiency in the three siRNAs described in [6] and targets the same site of FRMD5 with shRNA above. An irrelevant control siRNA was obtained from Qiagen.

The Flag-FRMD5-N-ter and Flag-FRMD5-C-ter were constructed as described in [6], FRMD5 siRNA/shRNA-resistant mutant and Flag-FRMD5-ΔFA plasmid were constructed using Site-directed Gene Mutagenesis Kit (Beyotime Institute of Biotechnology) as described by the manufacturer. The MBP-FRMD5-FERM was constructed using pMAL-C2X vector (Supplementary Fig. 1A, Table).

1.2. Cell culture

The human lung cancer cell lines, NCI-H1299 and A549, the human kidney epithelial cell line 293T and green monkey kidney liver cell line COS-7, were purchased from American Type Culture Collection. H1299 and A549 cells were cultured in RPMI 1640 medium (Invitrogen), and 293T and COS-7 cells were cultured in DMEM medium (Invitrogen). All cell culture media were supplemented with 10% (v/v) FBS (Gibco), penicillin (100 units/ml), and streptomycin (100 μg/ml) (Gibco). Cells were grown at 37 °C in humidified conditions with 5% CO_2.

1.3. GST pull-down experiments

GST-β1-tail, GST-β3-tail, GST-β5-tail chimeras, GST proteins and MBP, MBP-FRMD5-FERM were produced in *E. coli* BL21 cells. Cells were induced with 1 mM IPTG for 4 h at 37 °C. Bacteria were harvested and suspended in lysis buffer (PBS, 1% Triton X-100, 1 mM PMSF, 5 mM DTT, and anti-proteases), and sonicated on ice. GST-fusion or GST proteins were purified from cleared lysates by mixing with glutathione-sepharose 4B beads (GE HealthCare),

5 ml of cleared lysate/400 μl of beads, for 1 h at 4 °C. After extensive washing, the beads were incubated in binding buffer (50 mM HEPES, pH 7.5, 50 mM NaCl, 0.1% Nonidet P-40 with protease inhibitor mixture) at 4 °C for 1 h with COS-7 cell extracts overexpressing Flag-FRMD5 or MBP-fusion proteins. Equal amounts of GST or GST-fusion proteins were used in protein interaction experiments. In all cases, after extensive washing, bound proteins were eluted and analyzed by Western blotting.

1.4. Cell motility assay

Fifteen thousand cells were seeded in 60-mm tissue culture plates and incubated at 37 °C for 6 h. Then dynamics of cell movement was monitored using time-lapse confocal microscopy (Carl Zeiss LSM780, Germany). Cells were maintained in an incubator with humidified air (95%) and 5% CO_2 at 37 °C. 30 randomly-chosen cells from experimental group cell line were selected and scanned sequentially every 30 min for 20 h at 20× magnification. The obtained images were converted to video files using ZEN software. Cell tracking was followed by Image J software, and the graph was created using GraphPad Prism 5.

1.5. Adhesion and spreading assay

In a 48-well plate pre-coated with Vitronectin, 5×10^4 cells/well were seeded with 300 μl of adhesion buffer (RPMI 1640, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$ and 0.5% BSA). Cells were left to adhere for 2 h at 37 °C, and unattached cells were then aspirated. The remaining cells were gently washed three times to remove unbound cells. The relative number of attached cells was measured using WST-1 cell viability assay as described previously [14]. The samples were prepared in triplicates. And three independent experiments were analyzed.

For spreading assay, after attachment at 37 °C for 2 h as described above, cells were fixed with 4% formaldehyde for 15 min and typically 15 microscopic fields were randomly chosen to be photographed and analyzed.

1.6. Cell migration assay

Transwell chambers (Costar) with 8 μm pore size were used to perform the cell migration assay. The lower surface of the Transwell membrane was immersed to 600 μl PBS containing 6 μg/ml Vitronectin. 1×10^4 H1299 cells or 3×10^4 A549 cells were separately seeded on the upper surface of the Transwell membranes. After 6 h incubation in migration buffer (RPMI 1640, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.2 mM $MnCl_2$, and 0.5% bovine serum albumin) at 37 °C in humidified 5% CO_2, the Transwell membranes were fixed with 4% formaldehyde for 15 min and stained with Crystal Violet for 10 min. Finally, 15 microscopic fields were randomly chosen for analyses. Integrin blockade was performed using monoclonal antibodies directed against αvβ5-integrins (Chemi-Con, Rosemont, Illinois), inhibited ROCK1 kinase activity by Y27632 (EMD/Mil lipore, Billerica, MA). All assays were done in at least triplicates.

1.7. Co-immunoprecipitation (Co-IP) and Western blotting analysis

Co-IP and Western blotting were performed as described in [6]. The following antibodies: anti-FRMD5 (HPA011746, Sigma–Aldrich, St. Louis, MO), anti-ROCK1 (ab45171, Abcam, Cambridge, MA, USA), anti-MLC and anti-P-MLC (3672s and 3675s, Cell Signaling Technology, Danvers, MA), anti-integrinβ5 (D24A5, Cell Signaling Technology, Danvers, MA), anti-β-actin (TA-09, Zhongshan golden bridge, Beijing, China) and anti-MBP (SAB2104172,

Sigma–Aldrich, St. Louis, MO) were used to detect the corresponding proteins.

1.8. Immunofluorescence assay

Immunofluorescence for cells was performed as described in [6]. FITC-labeled phalloidin (1:1000; Invitrogen) was used to visualize filamentous actin. Hochest 33342 (Sigma–Aldrich, MO, USA) was used for nuclear staining. And mouse anti-human Flag polyclonal antibody was used to stain Flag-FRMD5 (1:100; Sigma–Aldrich, St. Louis, MO).

2. Results

2.1. Depletion of FRMD5 promotes cell motility

Given that FRMD5 is a membrane associated molecule [6] and it may involve in cell motility. To this end, we established H1299 cells stably expressing FRMD5-shRNA and FRMD5 was efficiently knocked down in these cells. For determine the specificity and efficacy of the shRNA, we constructed a FRMD5 mutant (Supplementary Fig. 1A, Flag-FRMD5-Re) that resists to the knockdown effect of siRNA and shRNA of FRMD5 used in this study (Fig. 1A). Western blot assay shown that the shRNA can knock down exogenous and endogenous expressed FRMD5, but cannot affect the expression of FRMD5-Re. Then we examined the role of FRMD5 in the regulation of cell motility using single cell trajectory assay. The trajectory paths of ten randomly-chosen cells were plotted to one origin for each experimental group (Fig. 1B). Furthermore, thirty cells were analyzed for observation of detailed cell motility parameters. We found that the average of migratory displacement and distance of cells from the H1299 FRMD5-shRNA group were longer than the other groups (Fig. 1C and D). Likewise, the average of cell migration velocity of FRMD5 knockdown group was faster than that of other groups (Fig. 1E). These results demonstrated that depletion of FRMD5 promotes cell motility.

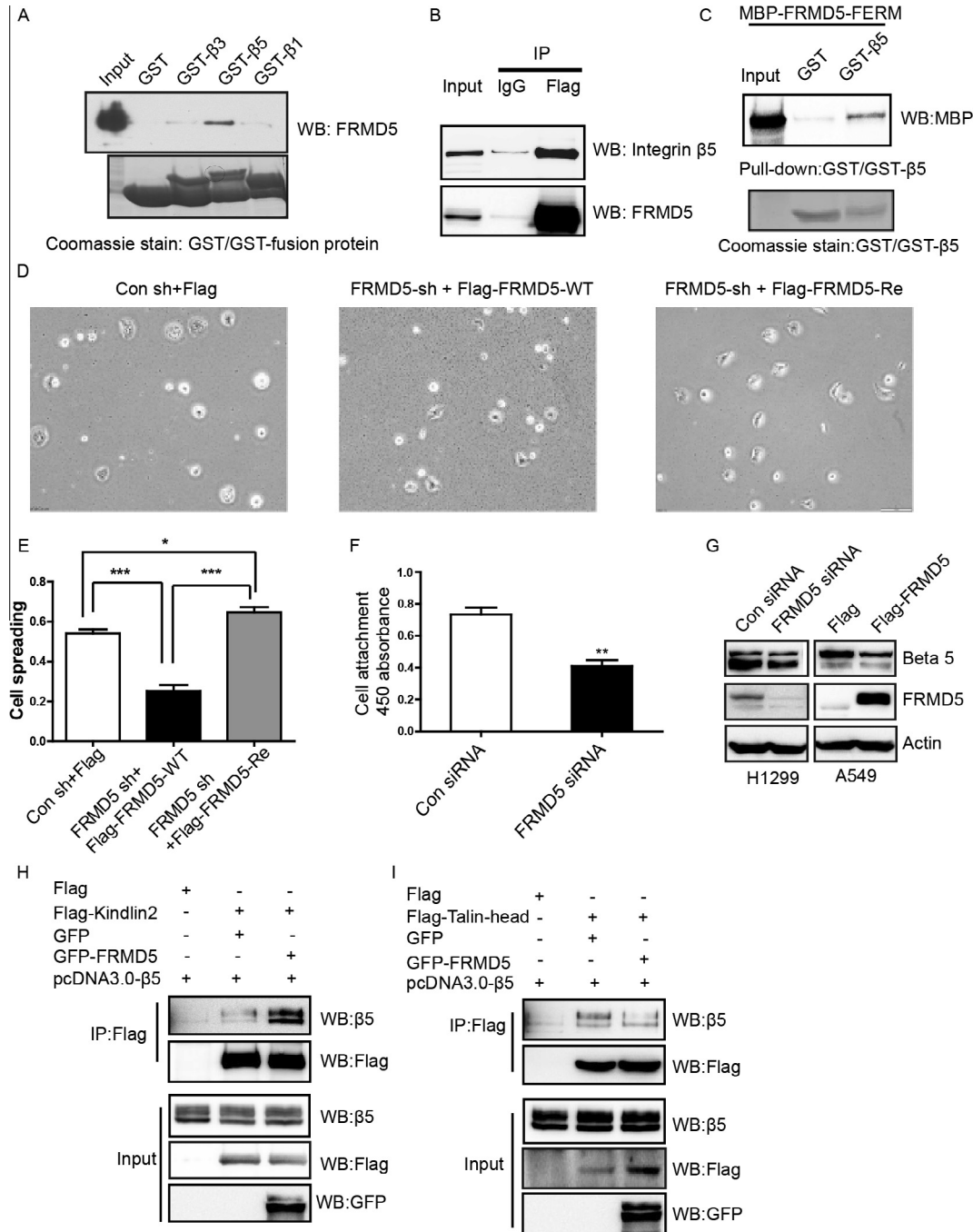### 2.2. FRMD5 interacts with integrin β5 cytoplasmic tail and is required for cell–matrix adhesion

A variety of FERM domain-containing proteins are known to interact with the cytoplasmic tails of integrin β subunit through
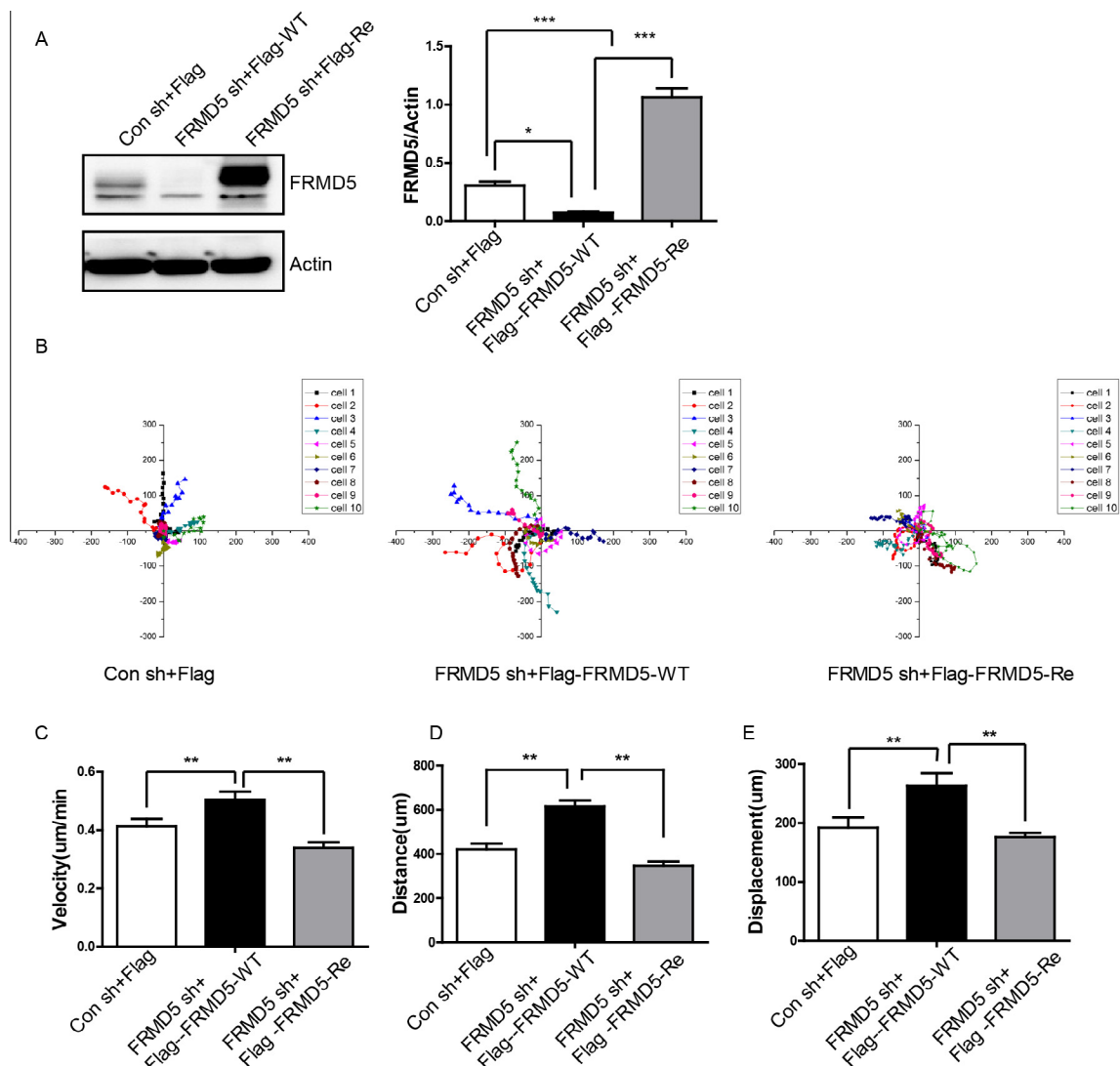
Fig. 1. Downregulation of FRMD5 promotes cell motility. (A) Western bolt was used to detect the expression of FRMD5. H1299 cells with FRMD5 stably knockdown were transiently transfected with Flag-FRMD5-WT or Flag-FRMD5-Re. H1299 cells with control shRNA were transfected with Flag vector as control. (B) Single cell tracking assay was performed after the cells were seeded for 6 h. Single cell motility was captured every 30 min for 20 at 37 °C under 5% CO_2 atmosphere. Paths of 10 cells of each group were shown. The displacement (C), distance (D), and velocity (E) of 30 randomly selected cells were analyzed by one way ANOVA assay, $^*P < 0.05$, $^{**}P < 0.01$. $^{***}P < 0.001$.

their FERM domain [15,16]. It is of interest to know that if FRMD5 can interact with integrin. In a GST pull-down assay we found that FRMD5 could be pulled down by GST-β5 tail but not integrin β1 or integrin β3 tails (Fig. 2A). This interaction was further confirmed in a co-IP assay using A549 cell lysate transiently transfected with Flag-FRMD5, and the result showed that endogenous integrin β5 interacts with FRMD5 (Fig. 2B). For answer if the interaction between FRMD5 and integrins is a direct one, GST and GST-β5 tail were used to pull down MBP-tagged FERM domain of FRMD5

expressed in bacteria. We found that MBP-FRMD5-FERM can be pulled down by GST-β5 tail but not GST, indicating that FRMD5 can directly interact with integrin β5 (Fig. 2C). Since integrin αvβ5 mainly mediates cell attachment and migration on Vitronectin (VN), we then examined the effect of FRMD5 on cell spreading and attachment in H1299 cells. H1299 cells were plated onto coverslides pre-coated with VN and cell spreading was examined. The results demonstrated that FRMD5 knockdown greatly affected cell spreading (Fig. 2D and E for quantification) and attachment


Fig. 2. FRMD5 strengthen cell–matrix adhesion via interaction with integrin β5. RMD5 interacts with integrin β5. (A) GST and GST-fusion cytoplasmic tail of integrin β1, β3 and β5 subunits were used to pull down Flag-FRMD5 in COS-7 cells, and only integrin β5 was found to interact with FRMD5. (B) A549 cells were transfected with Flag-FRMD5 for 48 h. Then co-IP was carried out using an anti-Flag antibody. Immunoprecipitates were probed using an anti-FRMD5 antibody and an anti-integrin β5 antibody separately. (C) GST and GST-integrin β5 cytoplasmic tail were used to pull down MBP-FRMD5-FERM expressed in bacterial. Anti-MBP antibody was used to detect the MBP-fusion protein in Western blot assay. (D) FRMD5 promotes cell spreading on VN. $^*P < 0.05$, $^{***}P < 0.001$, bar = 100 μm. (E) Knockdown of FRMD5 by siRNA inhibits cell attachment on VN. $^{**}P < 0.01$. (F) Western blot shown that downregulation or overexpression of FRMD5 cannot change integrin β5 expression. (G) Co-IP assay shown that FRMD5 promotes the interaction of Kindlin-2 with integrin β5. (H) FRMD5 inhibits Talin head interaction with integrin β5 in a co-IP assay.

(Fig. 2F) on VN, suggesting that downregulation of FRMD5 represses cell attachment to VN, and FRMD5 is required for integrin αvβ5-mediated cell–matrix adhesion on VN. However, downregulation or upregulation of FRMD5 do not influence the protein level of integrin β5 (Fig. 2G). It is known that Talin and Kindlin family members are FERM domain-containing proteins, and they all bind to the integrin β cytoplasmic tails. Therefore, it is of interest to examine if FRMD5 affects the binding of Kindlin or Talin with integrin

β cytoplasmic tails. To this end, we co-transfected 293T cells with GFP-FRMD5, Flag-Kindlin-2 and pcDNA3.0-β5 integrin or co-transfected 293T cells with GFP-FRMD5, Flag-Talin-Head and pCDNA3.0-β5, we then performed a co-IP assay and found that FRMD5 promoted Kindlin 2 interaction with integrin β5 but inhibited the interaction between Talin head and integrin β5 (Fig. 2H and I). These findings indicated that FRMD5 interferes with the interaction of integrin β cytoplasmic tails with their binding partners.
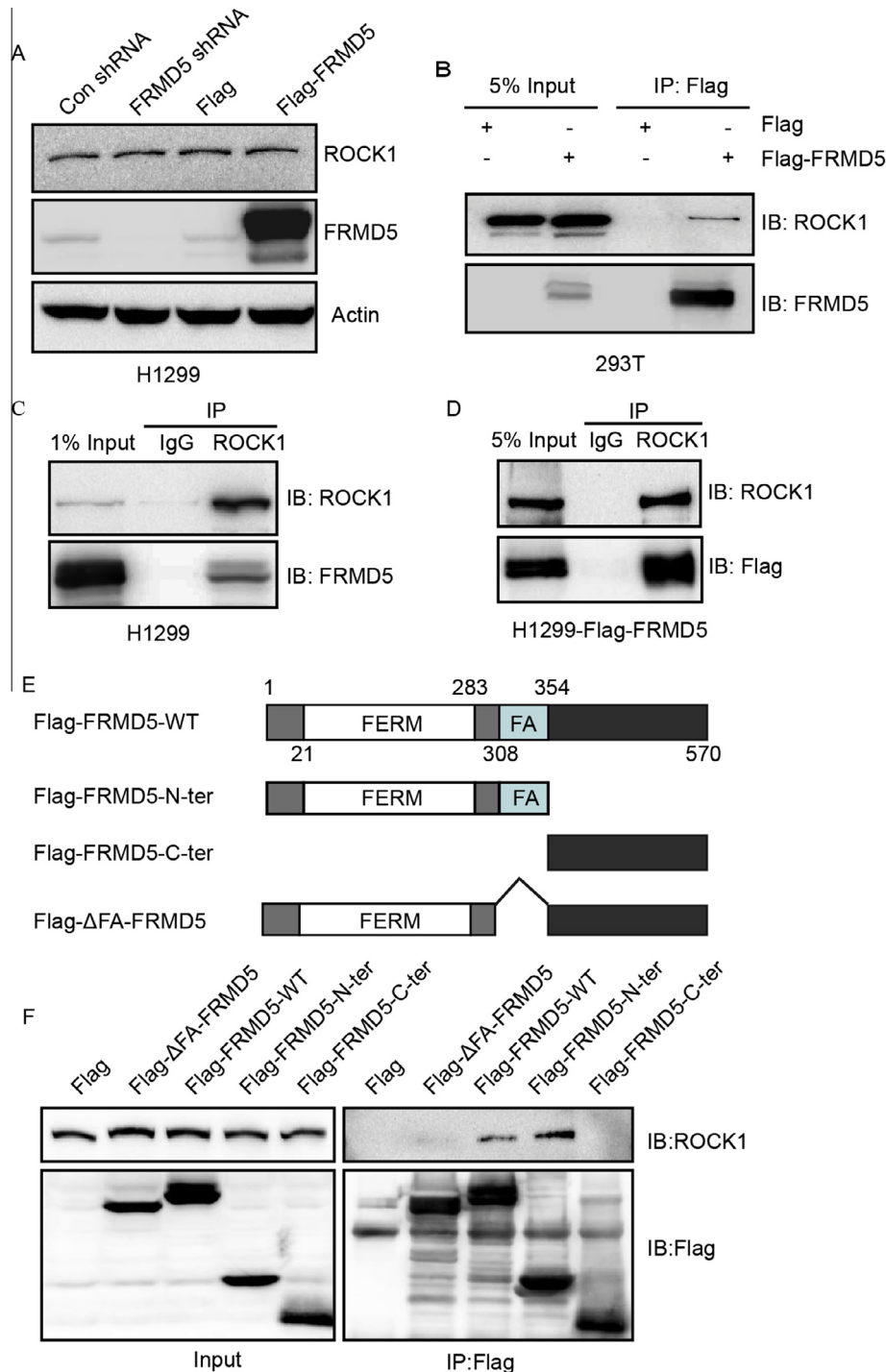
Fig. 3. FRMD5 interacts with ROCK1 without affecting the expression of ROCK1. (A) ROCK1 expression was detected by Western blot in H1299 cells stably expressing FRMD5 shRNA or con-shRNA, or H1299 cells transiently transfected with Flag or Flag-FRMD5 vectors. (B) 293T cells were transfected with Flag or Flag-FRMD5 vectors, then co-IP was performed with an anti-Flag mAb. FRMD5 and ROCK1 were detect using an anti-FRMD5 Ab and an anti-ROCK1 Ab. (C and D) Both endogenous FRMD5 and exogenous Flag-FRMD5 interact with ROCK1 in H1299 cells in co-IP assay. (E and F) Different FRMD5 constructs were cloned to determine the FRMD5-ROCK1 binding site. FA domain of FRMD5 was found necessary for FRMD5-ROCK1 interaction.
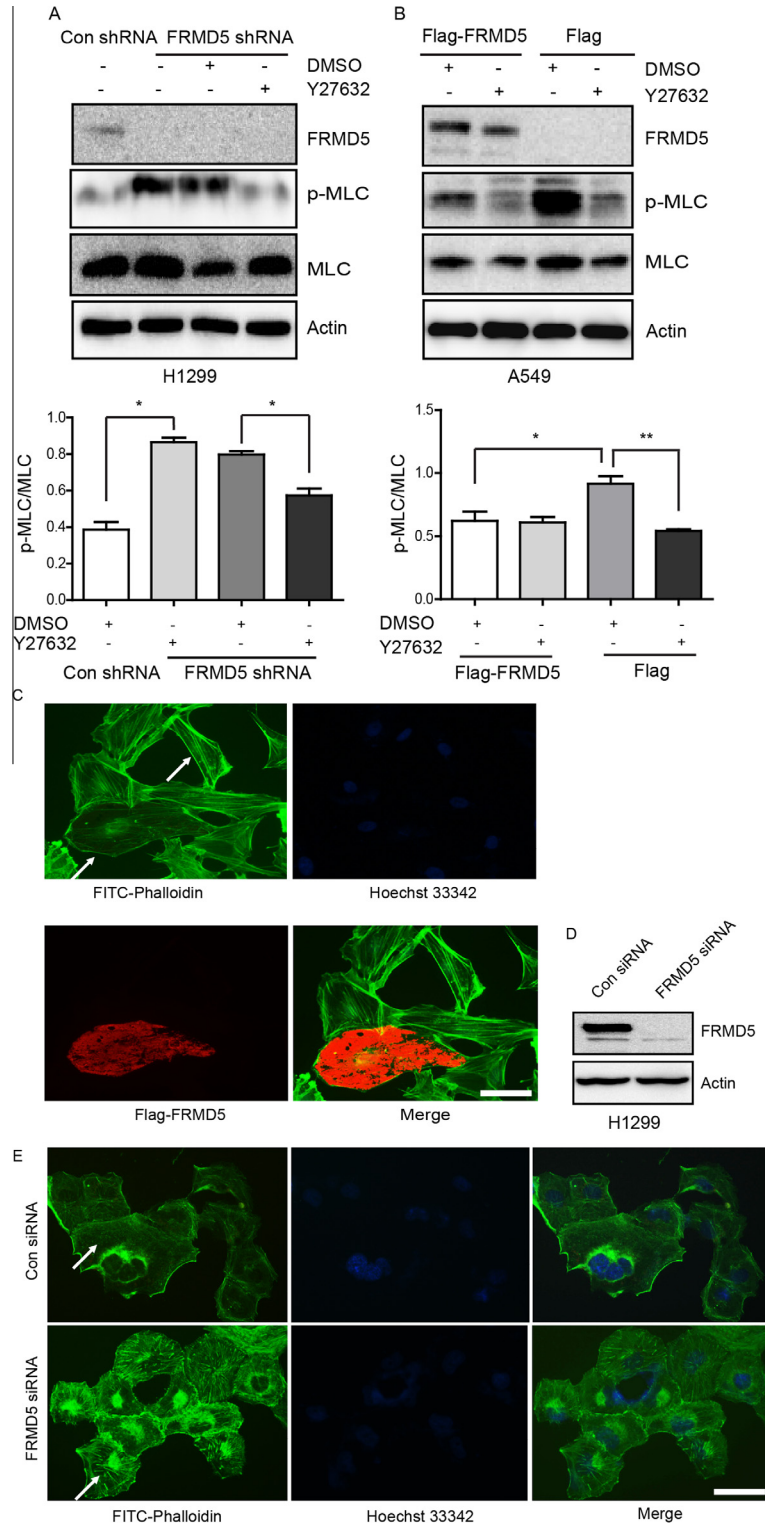
Fig. 4. FRMD5 inhibits the formation of stress fiber by suppressing ROCK1 activity. (A) A549 cells were transfected with Flag or Flag-FRMD5 expression vectors for 48 h. Before Western blot analysis cells were treated with DMSO or Y27632 for 1 h. The expression of related proteins was detected by Western blot using indicated antibodies. (B) H1299 cells stably expressing FRMD5 shRNA or control shRNA were used to detect the expression of p-MLC after treated with Y27632 or DMSO as described above. (C) A549 cells were transiently transfected with Flag-FRMD5 for 48 h, then the cells were fixed and F-actin was stained by FITC-Phalloidine (Green), FRMD5 was stained using an anti-Flag antibody (red), and the nuclei (blue) were stained by Hoechst 33342, bar = 50 nm. (D) H1299 cells were transiently transfected with FRMD5 siRNA or control siRNA for 72 h. Western blot was then performed to detect the expression of FRMD5. (E) Immunofluorescence was carried out to detect the stress fiber rearrangement as above, F-actin were stained by FITC-Phalloidine and the nuclei were stained by Hoechst 33342, bar = 50 nm.

2.3. FRMD5 interacts with ROCK1

It is known that reorganization of actin cytoskeleton is a prerequisite for cell motility. ROCK1 is the major effector of a small GTPase RhoA, and a key molecule that regulates stress fiber formation during cell migration. The downstream protein and substrate of ROCK1 is myosin light chain, which can be phosphorylated by ROCK1 at Ser-19. Phosphorylated ROCK1 induces actin polymerization and provide tension needed for cell movement [17].

In addition, ROCK1 has been reported as a candidate p120 binding partner that recruits ROCK1 and is correlated tightly with AJ integrity [13]. Given that our previous study demonstrated that FRMD5 interacts with p120-catenin and co-localizes with several adherent junction molecules [6], we therefore examined if FRMD5 affects the expression of ROCK1 or associates with ROCK1 in H1299 cells to regulate cell motility. Our results showed that ROCK1 expression was not changed either by knockdown or overexpression

of FRMD5 (Fig. 3A). Interestingly, we found that ROCK1 interacts with exogenous FRMD5 (Fig. 3B and D) as well as endogenous ROCK1 (Fig. 3C) in 293T and H1299 cells in co-IP assays.

To determine the binding region between FRMD5 and ROCK1, a panel of FRMD5 deletion mutants were constructed (Fig. 3E). Then a co-IP assay was performed and ROCK1 was found not interact with FRMD5 without the FA domain (Fig. 3F), suggesting that the FA domain is required for the interaction between FRMD5 and ROCK1. We also noted that the N-terminal of FRMD5 has a higher ability to associate with ROCK1 than the full-length FRMD5 (Fig. 3F left panel). These data indicated that FRMD5 interacts with ROCK1 through its FA domain with the ROCK1N-terminal domain.

2.4. FRMD5 inhibits ROCK1 activity and stress fiber formation

Aforementioned data indicated that FRMD5 interacts with ROCK1. Therefore, it is of interest to know if this interaction affects
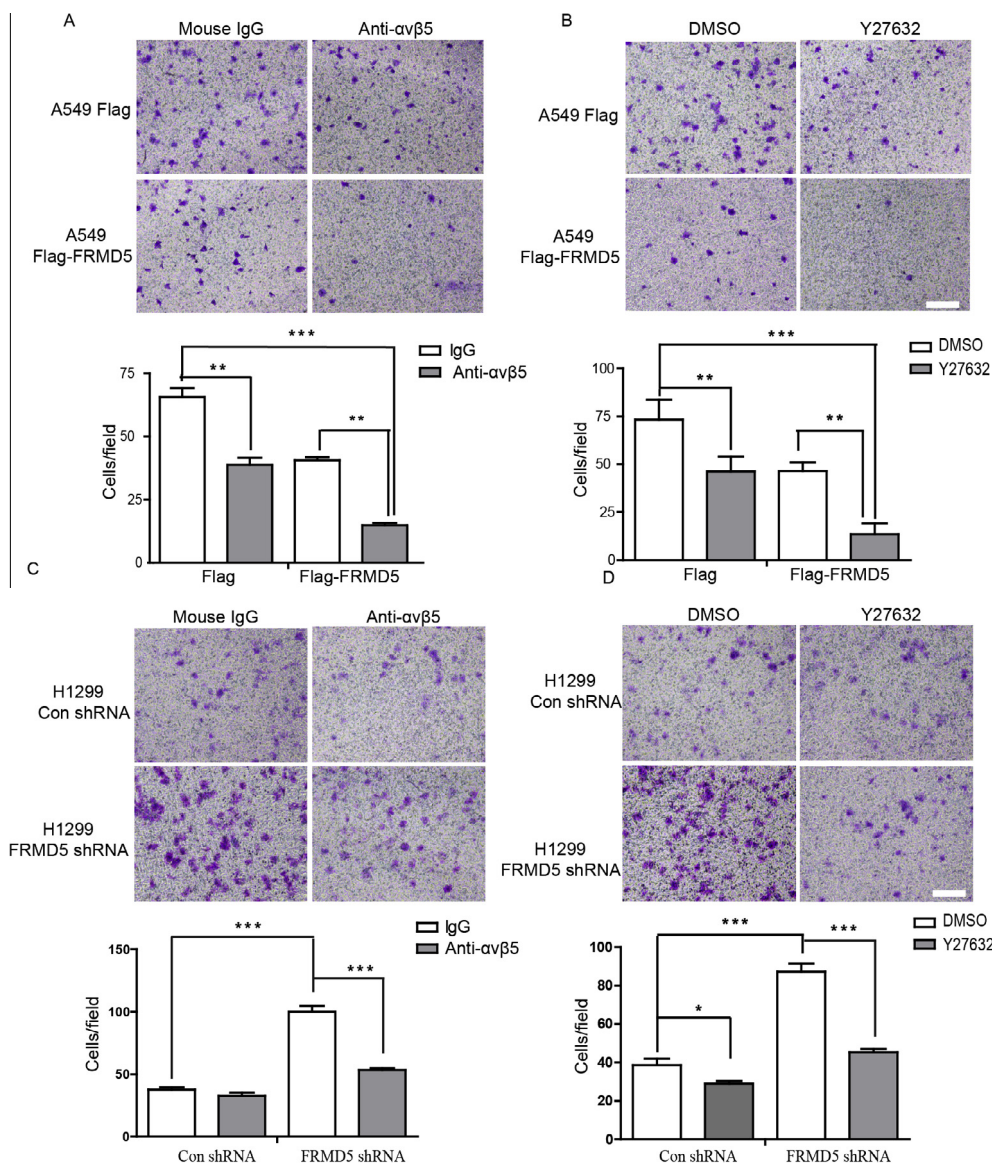
Fig. 5. FRMD5 inhibits tumor cell migration. (A) A549 cells were transfected with Flag or Flag-FRMD5 vectors, respectively. After 48 h, cells were harvested and pre-treated with IgG or $\alpha v \beta 5$ specific blocking antibody (50 μg/ml) for 0.5 h, then the cells were added to the upper chamber of Transwell (3×10^4 cells/well), and vitronectin (50 ng/ml) was add to the lower-chamber. 6 h later, the cells migrated were fixed and stained by crystal violet. (B) A549 cells transfected with Flag or Flag-FRMD5, respectively, after 48 h, then treated the cells with Y27632 (10 μM) for 1 h. Next the cells were harvested and counted, migration assay was performed as in A. (C, D) Downregulation of FRMD5 enhances cell migration.1 × 10⁴ H1299 cells stably expressing FRMD5 shRNA or con shRNA were used to for migration assay as described above, treated the cells with functional blocking antibody (C) or Y27632 (D), respectively. Student's t-test was used in the statistical analyses (*P < 0.05, **P < 0.01, ***P < 0.001).

the kinase activity of ROCK1. Myosin light chain (MLC) was known as a downstream molecule of ROCK1, which regulates cell motility. Phosphorylation of MLC is an indicator of ROCK1 activity. To this end, we examined the effect of FRMD5 on MLC phosphorylation. Endogenous FRMD5 was stably knocked down in H1299 cells with FRMD5 shRNA expression vector, whereas Flag-FRMD5 was over-expressed in A549 cells transiently transfected with Flag-FRMD5 expression vector. An antibody that specifically recognizes the Ser-19 phosphorylation of MLC (p-MLC) was used to measure the ROCK1 activity. Our data indicated that knockdown of FRMD5 leads to increased phosphorylation of MLC (Fig. 4A upper and down panels), an indication of ROCK1 activation. On the contrary, overexpression of FRMD5 inhibited the phosphorylation of MLC (Fig. 4B upper and down panels). To ensure that the MLC phosphorylation was caused by ROCK1 kinase activity, we applied a ROCK1 specific inhibitor Y27632. As seen in Fig. 4A and B, the ROCK1 kinase activity was specifically blocked by Y27632. These data indicated that FRMD5 is a novel regulator of ROCK1 kinase activity. Meanwhile the possible role of FRMD5 on stress fiber formation was also examined. As shown in Fig. 4C, overexpression of FRMD5 apparently inhibited stress fiber formation (as indicated by arrow), whereas the FRMD5 non-expressed cells displayed strong actin-based stress fiber. On the opposite, knockdown of endogenous FRMD5 expression by siRNA enhanced stress fiber formation in H1299 cells transfected with FRMD5 siRNA (Fig. 4D and E, arrowed). Collectively, these data indicated that FRMD5 suppresses actin stress fiber formation by inhibition of ROCK1 kinase activity in cells.

2.5. FRMD5 regulates tumor cell migration by controlling the activity of ROCK1 and integrin β5 – matrix interaction

Given that FRMD5 interacts with integrin β5 and ROCK1 and regulates integrin-mediated cell–matrix adhesion and ROCK1-regulated actin polymerization, and inhibits cell migration (Supplementary Fig. 1B). To uncover the role of FRMD5 in tumor cell migration, in a gain of function experiment A549 cells were transiently transfected with Flag-FRMD5 expression vector controlled by an empty vector. After 48 h, cells were treated separately with integrin αvβ5 specific blocking antibody or ROCK1 inhibitor Y27632 for 0.5 h. Cell migration assays were then performed. As shown in Fig. 5A and B, overexpression of FRMD5 led to a decrease in cell migration. Importantly, the migratory ability of cells overexpressing Flag-FRMD5 or the control vector can be further inhibited by addition of an integrin αvβ5 functional blocking antibody (Fig. 5A upper and lower panels) or ROCK1 inhibitor Y27632 (Fig. 5B upper and lower panels) (P < 0.01). These data suggested that FRMD5 interacts with both integrin αvβ5-mediated and ROCK1-dependent processes to regulate cancer cell migration. Furthermore, in a loss of function experiment H1299 cells stably transfected with FRMD5-shRNA and the control vector were assayed for cell migration with or without integrin αvβ5 functional blocking antibody or ROCK1 inhibitor Y27632. As shown in Fig. 5C and D, we found that knockdown of endogenous FRMD5 by shRNA significantly promoted cell migration compared with the control (P < 0.001). However, the promoted effects by FRMD5 knockdown can be obviously reversed by αvβ5 functional blocking antibody or ROCK1 inhibitor Y27632 (P < 0.001). Interestingly, the cell migratory abilities in the control groups were only slightly reduced upon addition of αvβ5 functional blocking antibody or ROCK1 inhibitor Y27632, suggesting that FRMD5 plays an important role in integrin αvβ5 and ROCK1 regulated cancer cell migration. Taken together, these findings indicated that FRMD5 inhibits tumor cell migration through controlling integrin αvβ5-mediated cell–matrix interaction and ROCK1 kinase activity.

3. Discussion

Integrins play important roles in tumor cell migration and invasion. It was reported that expression of integrin αvβ5 is highly related with histological subtypes, TNM stages and lymph node metastases of lung cancer [18]. Abnormal activation of integrin αvβ5 was also reported in other types of cancer. Thus, targeting integrins is considered very promising in cancer therapy [19,20]. In this study, we identify a new function of FRMD5 by showing that FRMD5 regulates lung cancer cell migration. We here demonstrated for first time that FRMD5 interacted with integrin β5 cytoplasmic tail. This FRMD5 – integrin β5 interaction may stabilize cell–matrix structure and limits cell movement.

A variety of FERM domain-containing proteins including Talin, Kindlins and FAK have been found to interact and regulate integrin-mediated cellular functions [21]. Integrin β5 associates with Talin at the membrane proximal NPxY motif and Kindlins at the distal NXXY motif [22]. Therefore, identifying the site that FRMD5 binding to integrin is important for understanding the role of FRMD5 in integrin-mediated cellular functions. In the present study we made an important observation that FRMD5 promotes interaction between Kindlin2 and integrin β5 while inhibits Talin association with integrin β5. To look at the structure basis of this observation, we analyzed the 3D structure of integrin β3-talin chimera using Phyre2 (Supplementary data) and found that FRMD5 FERM domain has a similarity with the crystal structure of integrin β3-talin chimera, so we considered that FRMD5 influences integrin activation by competing with Talin in binding integrin β cytoplasmic tail, affecting the inside-out signal transduction, then enhances cell adhesion and promotes cell spreading on extracellular matrix, accordingly limits cell movement. However, molecular detail of FRMD5 promotion of Kindlin2 interaction with integrin β5 is an interesting topic and worthy of further study.

In addition, we did not show a direct interaction between FRMD5 and actin (Supplementary Fig 1C). However, we did find that FRMD5 regulates actin-based cytoskeletal rearrangements by modulating the kinase activity of ROCK1. It was known that a variety of proteins and microRNAs regulates tumor invasion and metastasis through control of ROCK1 expression or its kinase activity [23–25]. The ROCK1 composed of three parts, the N-terminal catalytic domain, the C-terminal Rho-binding domain, and the central coiled-coil region. As a serine/threonine kinase, ROCK1 can be activated by a variety of molecules including small GTPases. For example, Rho A associates with the C-terminal of ROCK1 and enhances its kinase activity [7]. However, Rho E inhibits the activation of ROCK1 by binding to its kinase domain [26]. Meanwhile, Rho E can also be phosphorylated by ROCK1 at several sites. In this study, we demonstrated for the first time that the FERM domain-containing protein FRMD5 interacts with ROCK1 through the FA domain of FRMD5, and inhibits ROCK1 kinase activity without affecting the protein level of ROCK1. We therefore presumed that the FRMD5-FA domain may contain putative phosphorylation sites of ROCK1 considering the fact that other FERM domain-containing proteins can be phosphorylated at the similar FA region by AGC protein kinase family [27]. In fact, ROCK1 do phosphorylates some of the FERM domain-containing proteins [28]. Therefore, ROCK1 phosphorylation of FRMD5-FA remains an interesting question for future experiment. The structure of FA domain modeled using Phyre2 displayed one sole α helix (residues 321–333) in FRMD5. On the other hand, the coiled-coil region of ROCK1 has a hinge that is a target binding site of ROCK1 inhibitor [29]. Based on structural analysis we predict that the hinge might be the region that binds to the α helix of FRMD5-FA domain. However, this hypothesis requires further investigations to confirm. Furthermore, the mechanism accounting for FRMD5 inhibition of ROCK1 activation is unclear and warrants further investigations.

Both ROCK1 and integrin αvβ5 regulates cell attachment and cytoskeletal rearrangements, suggesting that there is a functional overlapping between ROCK1 and integrin αvβ5. However, before this report there is no indication on how ROCK1 and integrin αvβ5 coordinate to regulate cell migration. Therefore, this report may represent an interpretation on the functional coordination between integrin αvβ5 and ROCK1.

In conclusion, putative tumor suppressive protein FRMD5 inhibits tumor cell migration through binding with integrin αvβ5 to regulate cell–matrix interaction and inhibition of ROCK1 kinase activity to control actin stress fiber formation. This way FRMD5 may play a tumor suppressive role in cancer progression.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.10.012.

References

[1] Tepass, U. (2009) FERM proteins in animal morphogenesis. Curr. Opin. Genet. Dev. 19, 357–367.

[2] Bosanquet, D.C., Ye, L., Harding, K.G. and Jiang, W.G. (2014) FERM family proteins and their importance in cellular movements and wound healing (Review). Int. J. Mol. Med.

[3] Franceschini, N. et al. (2012) Discovery and fine mapping of serum protein loci through transethnic meta-analysis. Am. J. Hum. Genet. 91, 744–753.

[4] Desmarchelier, C. et al. (2014) The postprandial chylomicron triacylglycerol response to dietary fat in healthy male adults is significantly explained by a combination of single nucleotide polymorphisms in genes involved in triacylglycerol metabolism. J. Clin. Endocrinol. Metab. 99, E484–E488.

[5] Brazdova, M. et al. (2009) Modulation of gene expression in U251 glioblastoma cells by binding of mutant p53 R273H to intronic and intergenic sequences. Nucleic Acids Res. 37, 1486–1500.

[6] Wang, T., Pei, X., Zhan, J., Hu, J., Yu, Y. and Zhang, H. (2012) FERM-containing protein FRMD5 is a p120-catenin interacting protein that regulates tumor progression. FEBS Lett. 586, 3044–3050.

[7] Parri, M. and Chiarugi, P. (2010) Rac and Rho GTPases in cancer cell motility control. Cell Commun. Signal. 8, 23.

[8] Bendas, G. and Borsig, L. (2012) Cancer cell adhesion and metastasis: selectins, integrins, and the inhibitory potential of heparins. Int. J. Cell Biol. 2012, 676731.

[9] Kim, C., Ye, F., Hu, X. and Ginsberg, M.H. (2012) Talin activates integrins by altering the topology of the beta transmembrane domain. J. Cell Biol. 197, 605–611.

[10] Moser, M., Legate, K.R., Zent, R. and Fassler, R. (2009) The tail of integrins, talin, and kindlins. Science 324, 895–899.

[11] Brahme, N.N. and Calderwood, D.A. (2012) Cell adhesion: a FERM grasp of the tail sorts out integrins. Curr. Biol. 22, R692–R694.

[12] Liu, S. (2011) The ROCK signaling and breast cancer metastasis. Mol. Biol. Rep. 38, 1363–1366.

[13] Smith, A.L., Dohn, M.R., Brown, M.V. and Reynolds, A.B. (2012) Association of Rho-associated protein kinase 1 with E-cadherin complexes is mediated by p120-catenin. Mol. Biol. Cell 23, 99–110.

[14] Gao, J., Khan, A.A., Shimokawa, T., Zhan, J., Stromblad, S., Fang, W. and Zhang, H. (2013) A feedback regulation between Kindlin-2 and GLI1 in prostate cancer cells. FEBS Lett. 587, 631–638.

[15] Zhang, H. et al. (2004) Myosin-X provides a motor-based link between integrins and the cytoskeleton. Nat. Cell Biol. 6, 523–531.

[16] Liu, Y., Guan, L., Zhan, J., Lu, D., Wan, J. and Zhang, H. (2014) FERM domain-containing unconventional myosin VIIA interacts with integrin beta5 subunit and regulates alphavbeta5-mediated cell adhesion and migration. FEBS Lett. 588, 2859–2866.

[17] Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y. and Kaibuchi, K. (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J. Biol. Chem. 271, 20246–20249.

[18] Bai, S.Y., Xu, N., Chen, C., Song, Y.L., Hu, J. and Bai, C.X. (2014) Integrin alphavbeta5 as a biomarker for the assessment of non-small cell lung cancer metastasis and overall survival. Clin. Respir. J.

[19] Burvenich, I. et al. (2008) Monoclonal antibody 14C5 targets integrin alphavbeta5. Mol. Cancer Ther. 7, 3771–3779.

[20] Yao, R., Sui, A., Wang, Z., Liu, S., Zhou, Q., Liu, X. and Zhang, H. (2012) Induction of non-small cell lung carcinoma apoptosis using soluble RGD-TRAIL by targeting the integrin receptor of tumor cells. Mol. Med. Rep. 6, 1355–1360.

[21] Tancioni, I. et al. (2014) FAK inhibition disrupts a beta5 integrin signaling axis controlling anchorage-independent ovarian carcinoma growth. Mol. Cancer Ther.

[22] Singh, S., D'Mello, V., van Bergen en Henegouwen, P. and Birge, R.B. (2007) A NPxY-independent beta5 integrin activation signal regulates phagocytosis of apoptotic cells. Biochem. Biophys. Res. Commun. 364, 540–548.

[23] Sun, J., Zhang, D., Zheng, Y., Zhao, Q., Zheng, M., Kovacevic, Z. and Richardson, D.R. (2013) Targeting the metastasis suppressor, NDRG1, using novel iron chelators: regulation of stress fiber-mediated tumor cell migration via modulation of the ROCK1/pMLC2 signaling pathway. Mol. Pharmacol. 83, 454–469.

[24] An, L., Liu, Y., Wu, A. and Guan, Y. (2013) MicroRNA-124 inhibits migration and invasion by down-regulating ROCK1 in glioma. PLoS ONE 8, e69478.

[25] Wang, Y., Zhao, W. and Fu, Q. (2013) MiR-335 suppresses migration and invasion by targeting ROCK1 in osteosarcoma cells. Mol. Cell. Biochem. 384, 105–111.

[26] Riento, K. and Ridley, A.J. (2006) Inhibition of ROCK by RhoE. Methods Enzymol. 406, 533–541.

[27] Baines, A.J. (2006) A FERM-adjacent (FA) region defines a subset of the 4.1 superfamily and is a potential regulator of FERM domain function. BMC Genomics 7, 85.

[28] Hebert, M., Potin, S., Sebbagh, M., Bertoglio, J., Breard, J. and Hamelin, J. (2008) Rho-ROCK-dependent ezrin-radixin-moesin phosphorylation regulates Fas-mediated apoptosis in Jurkat cells. J. Immunol. 181, 5963–5973.

[29] Patel, R.A. et al. (2012) RKI-1447 is a potent inhibitor of the Rho-associated ROCK kinases with anti-invasive and antitumor activities in breast cancer. Cancer Res. 72, 5025–5034.