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FAM123A Binds to Microtubules and Inhibits the Guanine Nucleotide Exchange Factor ARHGEF2 to Decrease Actomyosin Contractility

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The FAM123 gene family comprises three members: *FAM123A*, the tumor suppressor *WTX* (also known as *FAM123B*), and *FAM123C*. *WTX* is required for normal development and causally contributes to human disease, in part through its regulation of β -catenin-dependent WNT signaling. The roles of *FAM123A* and *FAM123C* in signaling, cell behavior, and human disease remain less understood. We defined and compared the protein-protein interaction networks for each member of the FAM123 family by affinity purification and mass spectrometry. Protein localization and functional studies suggest that the FAM123 family members have conserved and divergent cellular roles. In contrast to *WTX* and *FAM123C*, we found that microtubule-associated proteins were enriched in the *FAM123A* protein interaction network. *FAM123A* interacted with and tracked with the plus end of dynamic microtubules. Domain interaction experiments revealed a “SKIP” amino acid motif in *FAM123A* that mediated interaction with the microtubule tip tracking proteins end-binding protein 1 (EB1) and EB3—and therefore with microtubules. Cells depleted of *FAM123A* showed compartment-specific effects on microtubule dynamics, increased actomyosin contractility, larger focal adhesions, and decreased cell migration. These effects required binding of *FAM123A* to and inhibition of the guanine nucleotide exchange factor ARHGEF2, a microtubule-associated activator of RhoA. Together, these data suggest that the SKIP motif enables *FAM123A*, but not the other FAM123 family members, to bind to EB proteins, localize to microtubules, and coordinate microtubule dynamics and actomyosin contractility.

INTRODUCTION

The FAM123 gene family comprises three members: *FAM123A*, which is also known as *AMER2*; *FAM123B*, which is also known as *WTX*, *AMER1*, and *OSCS*; and *FAM123C*. The founding member, *FAM123B* (hereinafter referred to as *WTX*), plays fundamental roles in normal development and human disease. Mutations in *WTX* contribute to various diseases, such as Wilms tumor, a pediatric kidney cancer (1, 2), and osteopathia striata congenita with cranial sclerosis (OSCS), an X-linked developmental disorder that causes bone-related defects in females (3) and is lethal in males, often at embryonic or neonatal developmental stages. Mice lacking *WTX* display various developmental abnormalities in tissues of mesenchymal origin, such as increased bone mass and decreased adipose tissue (4). Cellular and molecular analyses of these tissues indicate a critical role for *WTX* in regulating cell differentiation programs in mesenchymal progenitors.

Mass spectrometry (MS)-based proteomic dissection of *WTX* protein complexes has revealed several core components of the β -catenin-dependent WNT signal transduction pathway, including β -catenin (encoded by *CTNNB1*), β TrCP2 (encoded by *FBXW11*), and adenomatous polyposis coli (APC) (5, 6). Subsequent functional studies in various cells and organisms demonstrated that *WTX* inhibits WNT pathway activity (4, 5, 7). In vitro studies and cell-based assays suggest that *WTX* promotes β -catenin ubiquitination and subsequent proteasomal degradation, perhaps by serving as a membrane-bound scaffold for the β -catenin phosphorylation complex (5–8).

Of the FAM123 family members, *WTX* and *FAM123A* share greatest homology, particularly in their N termini (6, 9, 10). Two conserved functional domains in *WTX* and *FAM123A* have been identified and characterized (6, 11). First, both proteins share an N-terminal phosphatidylinositol(4,5)-bisphosphate-binding domain that localizes these proteins to the plasma membrane and is required for *WTX*- and *FAM123A*-mediated inhibition of WNT signal transduction. Second, *WTX* and *FAM123A* directly bind to APC and regulate its subcellular distribution, recruiting it from the microtubule tip complex to the plasma membrane. Although the functional consequences of this redistribution are not completely understood, the role of APC in microtubule stabilization and maintenance of cell-cell junctions suggests that *WTX* and *FAM123A* may influence directional cell migration and polarity (6). Whether the more distantly related family member *FAM123C* also regulates WNT signaling, localizes to the plasma membrane, or binds APC remains unknown.

In contrast to *WTX*, the cellular, developmental, and disease contributions of *FAM123A* and *FAM123C* remain less understood. Thus, we defined and compared the protein-protein interaction networks for each

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member in the FAM123 family. Functional annotation of the resulting protein interaction network and comparative protein dynamic studies supports both conserved and divergent functions for the FAM123 family members. Here, we report a “family-unique” function for FAM123A in controlling communication between the microtubule and actomyosin cytoskeletal networks. We found that FAM123A binds the microtubule plus-end tracking proteins end-binding protein 1 (EB1) and EB3; moves on microtubules; and controls microtubule dynamics, actomyosin organization, and cell migration. We present a model wherein FAM123A binds to and inhibits guanine nucleotide exchange factor H1 (GEF-H1; encoded by *ARHGEF2*) to decrease actomyosin contractility.

RESULTS

Comparative proteomics of the WTX family reveals shared and unique interactions

To provide insight into the cellular functions of FAM123A and FAM123C, we defined their protein interaction networks by shotgun liquid chromatography–tandem MS of affinity-purified protein complexes. Integration of the resulting protein interaction networks with a previously defined WTX protein interaction network revealed shared and unique protein-protein interactions (Fig. 1A and table S1). Consistent with the homology relationships within the family, FAM123A and WTX shared several common

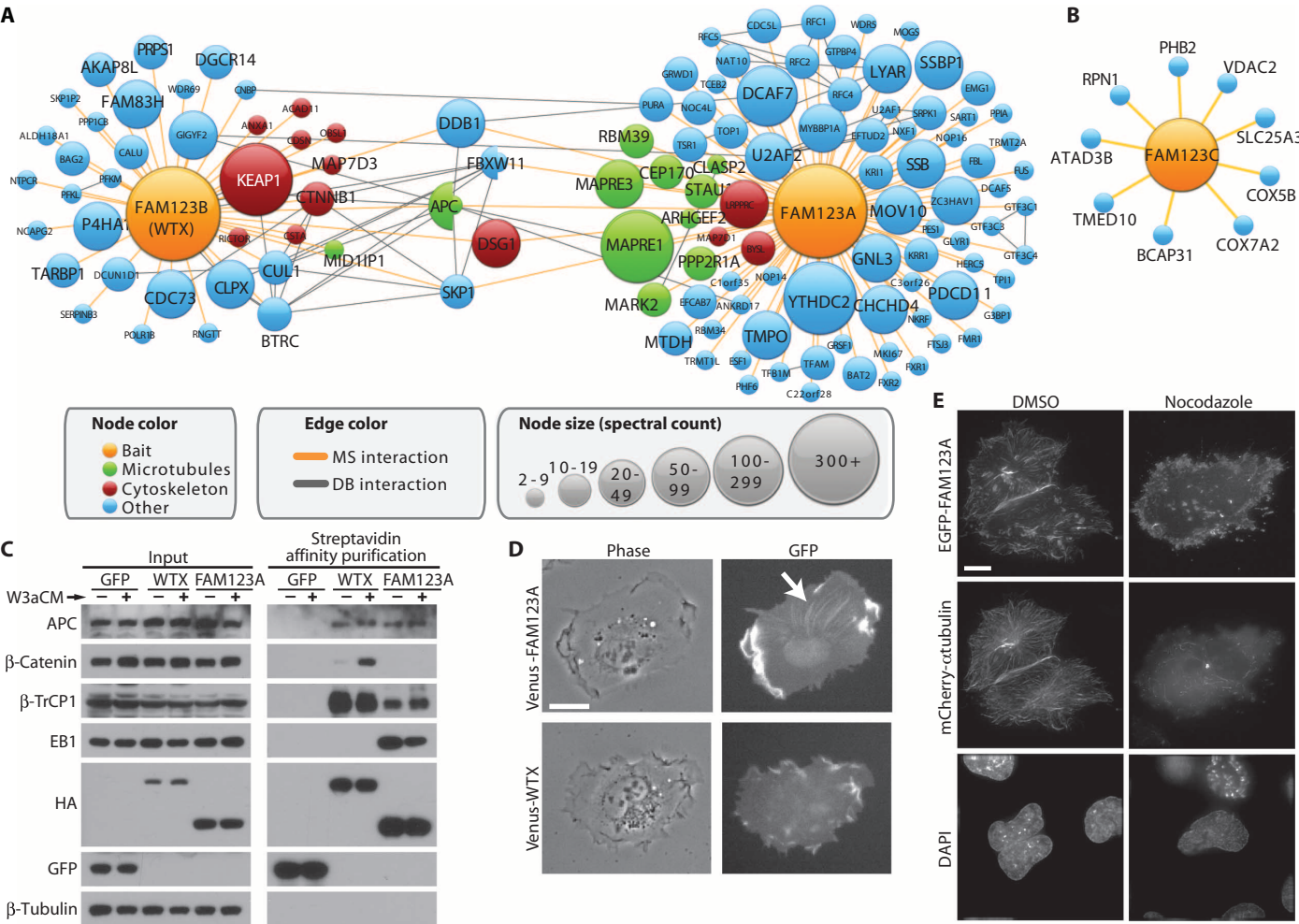


Fig. 1. FAM123A associates with a microtubule-enriched protein interaction network and moves on microtubules. **(A)** WTX and FAM123A protein interaction networks as defined by AP/MS. Proteins shown were represented by at least two unique peptides in at least two (of four) WTX experiments or at least two (of three) in FAM123A experiments. DB, database. **(B)** The FAM123C protein interaction network, as defined by proteins that were identified by at least two independent peptides in at least two of three experiments. In **(A)** and **(B)**, node size and coloring reflect spectral counts and gene ontology, respectively. **(C)** Streptavidin affinity-purified protein complexes from HEK293T cells stably expressing SBPHA-GFP, SBPHA-WTX, or SBPHA-FAM123A were analyzed by Western blot for the indicated

endogenous proteins (SBP, streptavidin binding peptide; HA, hemagglutinin). Data represent two biological replicates. **(D)** Images from movies of HT1080 cells that were transiently transfected with Venus-tagged FAM123A (movie S1) or WTX (movie S2) constructs. Data are representative of two independent biological replicates. Scale bar, 20 μ m. **(E)** HeLa cells were transfected with EGFP-FAM123A and mCherry-tagged α -tubulin, treated with dimethyl sulfoxide (DMSO) or nocodazole, fixed, and imaged by deconvolution microscopy. The FAM123A image is representative of five independent experiments imaged on five different days. Of 65 imaged cells, 39 exhibited long filamentous structures in cells expressing Venus-FAM123A that colocalized with microtubules. Scale bar, 5 μ m.

interacting proteins; in contrast, the FAM123C protein interaction network was distinct (Fig. 1B and table S1).

Because of their homology, overlapping protein interaction networks, and shared function as inhibitors of WNT signaling, protein interactions from the WTX and FAM123A networks were validated. FAM123A or WTX protein complexes were isolated by streptavidin-affinity purification from human embryonic kidney (HEK) 293T cells, and associated endogenous proteins were detected by Western blot. Whereas both WTX and FAM123A proteins associated with APC and β TrCP1 (encoded by *BTRC*) and β TrCP2 (encoded by *FBXW11*), WTX specifically bound β -catenin, confirming the proteomic data (Fig. 1C). These results strengthen models in which WTX and FAM123A functionally regulate β -catenin-dependent WNT signaling at the level of the destruction complex, most likely through interactions with APC, β TrCP1, or β TrCP2.

We chose to further investigate the enrichment of microtubule-associated proteins in the FAM123A protein interaction network (Fig. 1A, green nodes), such as EB1 (encoded by *MAPRE1*), an obligate component of the microtubule plus-end tip tracking protein complex (12–14). Western blot analysis of affinity-purified protein complexes revealed an interaction between FAM123A and endogenous EB1 but not between WTX and EB1 (Fig. 1C). Additionally, several other proteins in the FAM123A network bind to the microtubule cytoskeleton: the microtubule affinity-regulating kinases MARK2 and MARK3, GEF-H1, EB3 (encoded by *MAPRE3*), ACF7 (actin-crosslinking factor 7; encoded by *MACF1*), CLASP2 (cytoplasmic linker protein-associated protein 2), and APC (15–20) (table S1). By affinity purification and mass spectrometry (AP/MS), these proteins are the frequently observed FAM123A interacting proteins, and are largely absent from the WTX protein interaction network (Fig. 1A).

FAM123A moves on growing microtubules

WTX and FAM123A, but not FAM123C, localize predominantly to the cytoplasmic face of the plasma membrane through interactions with phospholipids (fig. S1) (6). Given the relative enrichment of microtubule-associated proteins in the FAM123A protein interaction network, we hypothesized that a pool of FAM123A might localize to and move on microtubules. Imaging of Venus-FAM123A or Venus-WTX in live HT1080 human sarcoma cells (Fig. 1D) indicated that both proteins exhibited mainly membranous distribution, seemed to be enriched at cell ruffles, and induced cell death when overexpressed. In cells expressing low to moderate amounts, FAM123A (movie S1), but not WTX (movie S2), localized to filamentous structures resembling microtubules. This localization pattern was often polarized with respect to the direction of cell movement; of 142 cells with low to moderate expression of FAM123A, 94 cells showed FAM123A decorating filamentous structures polarized in the direction of cell movement. Colocalization of FAM123A and α -tubulin confirmed the microtubule localization suggested by live-cell imaging (Fig. 1E), which was abolished by the addition of the microtubule destabilizing drug nocodazole. By high-resolution live-cell imaging, EGFP (enhanced green fluorescent protein)-FAM123A was observed to predominantly move on segments of microtubules and was also observed as dots that moved on linear trajectories from the cell body toward the cell periphery (movie S3). Thus, both comparative proteomics and live-cell imaging demonstrate that FAM123A, but not WTX, associates with and moves on microtubules.

FAM123A interacts with APC and EB1 through distinct domains

MS and Western blot analysis revealed that FAM123A associated with the plus-end tracking protein EB1 (Fig. 1, A and C). Stably expressed hemagglutinin (HA)-tagged FAM123A was detected in protein complexes isolated by immunoprecipitation of endogenous EB1 (Fig. 2A). To demonstrate the

interaction between the endogenous proteins, we generated an antibody against FAM123A and confirmed its specificity with two nonoverlapping *FAM123A*-specific small interfering RNAs (siRNAs) (Fig. 2, B and C). In both HEK293T and HeLa cells, endogenous FAM123A was detected within endogenous EB1 immunopurified protein complexes (Fig. 2, D and E). Confocal Z-series images of HeLa cells expressing EGFP-FAM123A and immunofluorescently labeled for endogenous EB1 demonstrated basal colocalization of these proteins with increased intensity at the microtubule distal ends toward the leading edge (Fig. 2F).

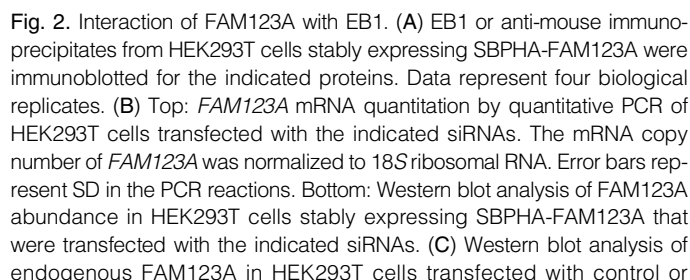
APC directly interacts with EB1 and FAM123A. To determine whether FAM123A indirectly binds EB1 through APC (21), we purified FAM123A from cells transfected with siRNAs targeting either *APC* or *EB1*, and found that FAM123A interacts with EB1 in the absence of APC, and similarly interacts with APC after silencing of EB1 (Fig. 3A). These results suggest that FAM123A independently binds APC and EB1. Although WTX associates with APC, it did not affinity-purify with EB1 (Fig. 1C). To map the domains of FAM123A that interacted with APC and EB1, we generated a series of FAM123A deletion mutants on the basis of homology within the WTX family and of secondary structure prediction for FAM123A (<http://robeta.bakerlab.org/>). Affinity purification of full-length FAM123A protein or the truncated fragments from cells expressing EB1 or APC indicated that FAM123A interacted with EB1 through its extreme C terminus, specifically amino acids 457 to 552 (Fig. 3B) and with APC through an N-terminal domain comprising amino acids 261 to 349 (Fig. 3C). Additionally, FAM123A interacted with β TrCP2 through a C-terminal region encompassing residues 261 to 470, which overlaps with the APC binding domain (Fig. 3D and fig. S2). These results demonstrate that FAM123A independently interacts with EB1 and APC through nonoverlapping domains.

FAM123A interacts with EB proteins through the EB binding motif “Ser-x-Ile-Pro”

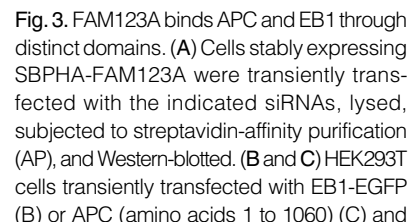
Many plus-end tracking proteins contain a characteristic EB-binding motif, which is defined by a Ser-x-Ile-Pro (SxIP) consensus (Fig. 4A) (22), and which enables direct binding to EB1 and EB3 and, consequently, localization to the growing microtubule plus-end. We found a SKIP^{487–490} and a TKIP^{518–521} motif within FAM123A, both of which are in the EB1 binding region identified by domain mapping (Fig. 3B). To determine whether the SKIP^{487–490} motif is required for binding to EB1 and EB3, we created a mutant in which Ile⁴⁸⁹ and Pro⁴⁹⁰ residues were changed to alanine (FAM123A-IPAA) (Fig. 4A). Affinity purification and Western blot analysis revealed that in contrast to wild-type FAM123A, the IPAA mutant did not pull down EB1 or EB3 (Fig. 4, B and C). These results demonstrate that the SKIP^{487–490} motif in FAM123A is necessary for association with EB1 and EB3; whether the TKIP^{518–521} motif contributes to binding in the presence of the SKIP^{487–490} motif remains to be tested.

Many plus-end tracking proteins containing the “SxIP motif” bind a coiled-coil domain within the C terminus of EBs, referred to as the EBH domain (17, 22). To determine whether FAM123A also binds to the EBH domain of EB1, we generated two EB1 deletion constructs that encoded the N-terminal microtubule-binding domain (amino acids 1 to 135) or the C-terminal EBH domain (amino acids 136 to 268). Affinity purification of FAM123A from cells coexpressing these EB1 truncations revealed an association between FAM123A and the EBH domain of EB1 (Fig. 4D). Thus, FAM123A interacts with the C-terminal region of EB1 through the consensus EB1 binding motif SxIP.

The SxIP motif targets functionally and structurally unrelated plus-end tracking proteins to growing microtubule ends in an EB-dependent manner (22). To determine whether FAM123A microtubule localization and plus-end tracking require EB association, we compared the protein



FAM123A-specific siRNA#2. (D) Endogenous EB1 protein complexes from HEK293T cells were immunoblotted for FAM123A. (E) Endogenous EB1 immunoprecipitates from HeLa cells were immunoblotted for FAM123A. (F) HeLa cells transfected with EGFP-FAM123A were fixed, stained for endogenous EB1, and imaged with confocal microscopy. A Z-projection for all captured 0.2- μ m slices and a single 0.2- μ m slice at the bottom of the cell is shown. The colocalization of EGFP-FAM123A with EB1 was analyzed in two independent experiments imaged on two different days. For 20 EGFP-FAM123A cells analyzed, all 20 showed colocalization with EB1. Scale bar. 10 μ m.



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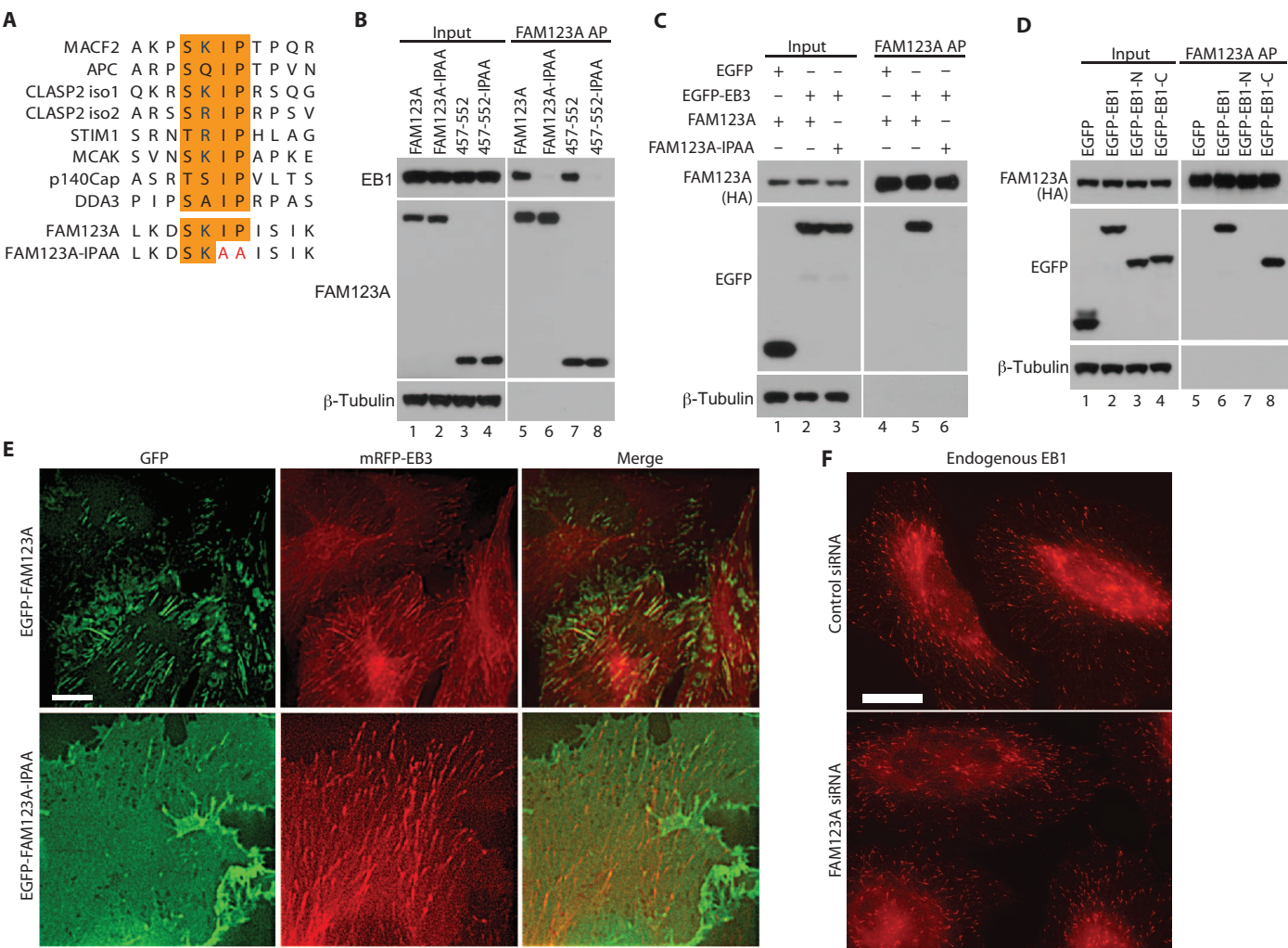


Fig. 4. EB1 association is required for FAM123A microtubule localization. (A) Protein sequence alignment of FAM123A and the SKIP domains of various plus-end tracking proteins. (B and C) Western blot analysis of streptavidin-affinity pulldowns from HEK293T transfected with EB1-EGFP (B) or EB3-EGFP (C) and the indicated SBPHA-FAM123A constructs. Data represent three biological replicates. (D) Western blot analysis of streptavidin-affinity pulldown assays followed by Western blot analysis of SBPHA-FAM123A cells transfected with EGFP, EB1-EGFP, EGFP-EB1-N, or EGFP-EB1-C. Data represent two biological replicates. (E) Images from live-cell imaging of HeLa cells expressing EGFP-FAM123A (movie S4) or EGFP-FAM123A-IPAA (movie S5). The colocalization of EGFP-FAM123A was compared to

distribution and dynamics of FAM123A and FAM123A-IPAA by live-cell imaging (Fig. 4E and movies S4 and S5). Wild-type FAM123A coated the distal ends of the microtubule and cotracked with EB3 in the cell body (movie S4). In contrast, FAM123A-IPAA exhibited decreased EB3-associated plus-end tip tracking and microtubule decoration, coating very few microtubule stretches at the cell periphery (movie S5). A C-terminal fragment of FAM123A that contains the SKIP⁴⁸⁷⁻⁴⁹⁰ EB-interaction motif did not decorate the microtubule lattice but rather behaved as a classic plus-end microtubule-binding protein showing robust EB3-associated tip tracking (fig. S3 and movie S6). Mutation of the SKIP⁴⁸⁷⁻⁴⁹⁰ motif com-

pletely abolished microtubule colocalization and EB3-associated tip tracking (fig. S3 and movie S7). Despite the autonomous microtubule-binding capability of EB proteins in vitro, FAM123A could influence EB1 loading or distribution on microtubules ex vivo. However, depletion of FAM123A in HeLa cells did not affect the subcellular distribution of endogenous EB1 (Fig. 4F). To complement the loss-of-function approach, we localized EB1 in cells stably overexpressing FAM123A (fig. S4). Forced expression of FAM123A, but not of FAM123A-IPAA, relocalized EB1 to the microtubule lattice at the bottom of the cell and to the plasma membrane more apically.

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Although consistent with its ability to bind EB1, it remains to be seen whether EB1 redistribution after FAM123A overexpression occurs normally. Together, these data demonstrate that (i) FAM123A is a microtubule-associated protein with EB-dependent plus-end tip tracking capabilities; (ii) FAM123A binds EB proteins through the SKIP^{487–490} motif; (iii) FAM123A predominantly decorates the microtubule lattice at the cell periphery in a largely EB-dependent fashion; and (iv) FAM123A silencing does not affect EB1 subcellular localization.

EB-protein association is not required for FAM123A regulation of Wnt signaling

Of the three FAM123 family members, only FAM123A contains an SxIP motif, which is consistent with the lack of association between WTX and EB1 (Fig. 1, A and C). Given this unique protein interaction within the FAM123 family, we predicted that the EB1 association would be dispensable for the common functions of WTX and FAM123A, such as regulation of β -catenin-dependent WNT signaling (5, 7, 11). Indeed, siRNA-based knockdown of FAM123A or WTX increased the activity of a β -catenin reporter gene (fig. S5A). In a gain-of-function approach, overexpression of FAM123A or FAM123A-IPAA reduced WNT3A-dependent reporter activation in a concentration-dependent manner (fig. S5, B and C). These data suggest that the ability of FAM123A to modulate the Wnt- β -catenin pathway is independent of its interaction with EB1 and microtubules.

FAM123A controls microtubule organization and growth rates

Because FAM123A bound EB proteins and moved on microtubules, we tested whether its loss affected microtubule organization and dynamics. siRNA-mediated silencing of *FAM123A* in HeLa cells induced disorganization of the microtubule network with excessively curved microtubules and increased microtubule density (Fig. 5, A and B). In contrast, siRNA-mediated silencing of WTX yielded a distinct microtubule organization (Fig. 5A). We used total internal reflective fluorescence (TIRF) microscopy to image GFP-tagged EB3 in siRNA-transfected HeLa cells, and found that FAM123A promotes microtubule growth within the cell body because cells lacking FAM123A had significantly slower microtubule polymerization (Fig. 5, C and D). In contrast, in the absence of FAM123A, microtubules demonstrated less dynamic movement near F-actin bundles, particularly near adhesion complexes (Fig. 5D). Thus, FAM123A regulates microtubule dynamics and the overall organization of the microtubule network.

FAM123A inhibits actin contractility by suppressing the GEF-H1-RhoA-ROCK-MLC pathway

In addition to the altered microtubule organization, we noticed that FAM123A-depleted cells had phase dark cortical membranes (Fig. 6A). These observations and the increase in cortical F-actin detected by TIRF microscopy (Fig. 5D) suggested involvement of the signaling pathway that mediates cross talk between the microtubule and actomyosin cytoskeletal networks (15, 23). Specifically, microtubule depolymerization induces actin stress fiber formation and cell contractility through activation of the Rho-specific exchange factor GEF-H1, which subsequently activates RhoA, Rho kinase (ROCK), and myosin light chain (MLC) (Fig. 6B) (24, 25). In *FAM123A*-silenced HeLa cells, thick and short actin stress fibers were disposed in a nonparallel arrangement, and microtubule depolymerization induced by nocodazole treatment resulted in several morphological changes, including thick bundles of contracted actin stress fibers frequently localized at the cell center, kidney-shaped nuclei shifted to the cell periphery, and cytoplasmic pools of free tubulin confined to a small area close to the nucleus or near the cell center (or both) (Fig. 6D). Thus,

FAM123A-depleted cells display an altered microtubule network and increased actomyosin contractility that is exacerbated with nocodazole treatment.

We used multiple experimental approaches to determine whether FAM123A-induced actomyosin contractility requires the GEF-H1-RhoA-ROCK-MLC2 pathway. First, treatment of cells with the ROCK inhibitor Y-27632 (26) abolished actin stress fiber formation and cell contractility induced by FAM123A knockdown (Fig. 6C). Second, siRNAs targeting GEF-H1 reversed the actomyosin contractility induced by FAM123A depletion but did not rescue the disordered microtubule phenotype induced by *FAM123A* silencing (Fig. 6D). By blinded quantitation, 78% of FAM123A-depleted cells were scored as displaying increased actomyosin contractility; in the absence of GEF-H1, this was reduced to 38% of cells (Fig. 6E). As was previously reported, silencing of *GEF-H1* resulted in decreased actin bundling (fig. S6) (24). Third, activation of RhoA was higher in cells depleted of FAM123A than in control siRNA-transfected cells, an effect that required GEF-H1 (Fig. 6F). Fourth, phosphorylation of myosin was increased in *FAM123A*-silenced cells but not in cells in which both FAM123A and GEF-H1 were depleted (Fig. 6, G to I). By immunofluorescence, phosphorylated MLC localized primarily to stress fibers near the cell periphery after FAM123A loss (Fig. 6I), compared with the more uniform distribution seen in control siRNA-transfected cells. Finally, we used a serum response factor (SRF) transcriptional activity assay as an indirect readout for RhoA activity and actomyosin contractility (27). *FAM123A* silencing induced SRF reporter activity in a GEF-H1-dependent fashion (Fig. 6J). FAM123A overexpression repressed SRF-mediated transcription induced by both nocodazole and GEF-H1 overexpression (Fig. 6K). Together, these data demonstrate that FAM123A decreases actin contractility by inhibiting the GEF-H1 pathway and that, epistatically, FAM123A functions upstream of GEF-H1.

Because cellular morphology and cytoskeletal organization are particularly sensitive to transfection and siRNA-based off-target effects, we further validated the specificity of the FAM123A phenotype. HeLa cells expressing one of two siRNAs that targeted the 3' untranslated region of *FAM123A* (fig. S7A) showed an exacerbated actomyosin contractility phenotype after nocodazole treatment, similar to the open reading frame-directed FAM123A#1 and #2 siRNAs. This phenotype was rescued by forced expression of a FAM123A-GFP fusion protein (fig. S7B). Together, these data confirm that FAM123A decreases actomyosin contractility.

FAM123A binds to and inhibits GEF-H1 activity

Proteomic analysis of the FAM123A protein complex identified GEF-H1 as a potential interacting protein, suggesting a molecular mechanism by which FAM123A controls actomyosin contractility (Fig. 1A). First, we confirmed the GEF-H1-FAM123A interaction by Western blot analysis of affinity-purified FAM123A protein complexes or immunopurified GEF-H1 protein complexes (Fig. 7, A and B). Endogenous GEF-H1 was detected in affinity-purified FAM123A protein complexes (Fig. 7A), and FAM123A immunopurified with endogenous GEF-H1 (Fig. 7B). The FAM123A-IPAA mutant, which lacks the ability to bind EB proteins, also associated with GEF-H1 (Fig. 7, A and B). Additionally, we detected the endogenous forms of the microtubule-associated proteins CLASP2 and MARK2 in affinity-purified FAM123A protein complexes, as was suggested by the FAM123A proteomic analyses (Fig. 7A).

As a first test of the functional relationship between FAM123A and GEF-H1, we assessed the activation status of GEF-H1 in FAM123A-depleted cells by purifying active GEF-H1 with a nucleotide-free mutant of RhoA, RhoA-G17A coupled to glutathione *S*-transferase (GST) (28). Western blot analysis of RhoA-G17A pulldowns revealed a significant increase in active GEF-H1 after FAM123A depletion (Fig. 7C). We attempted

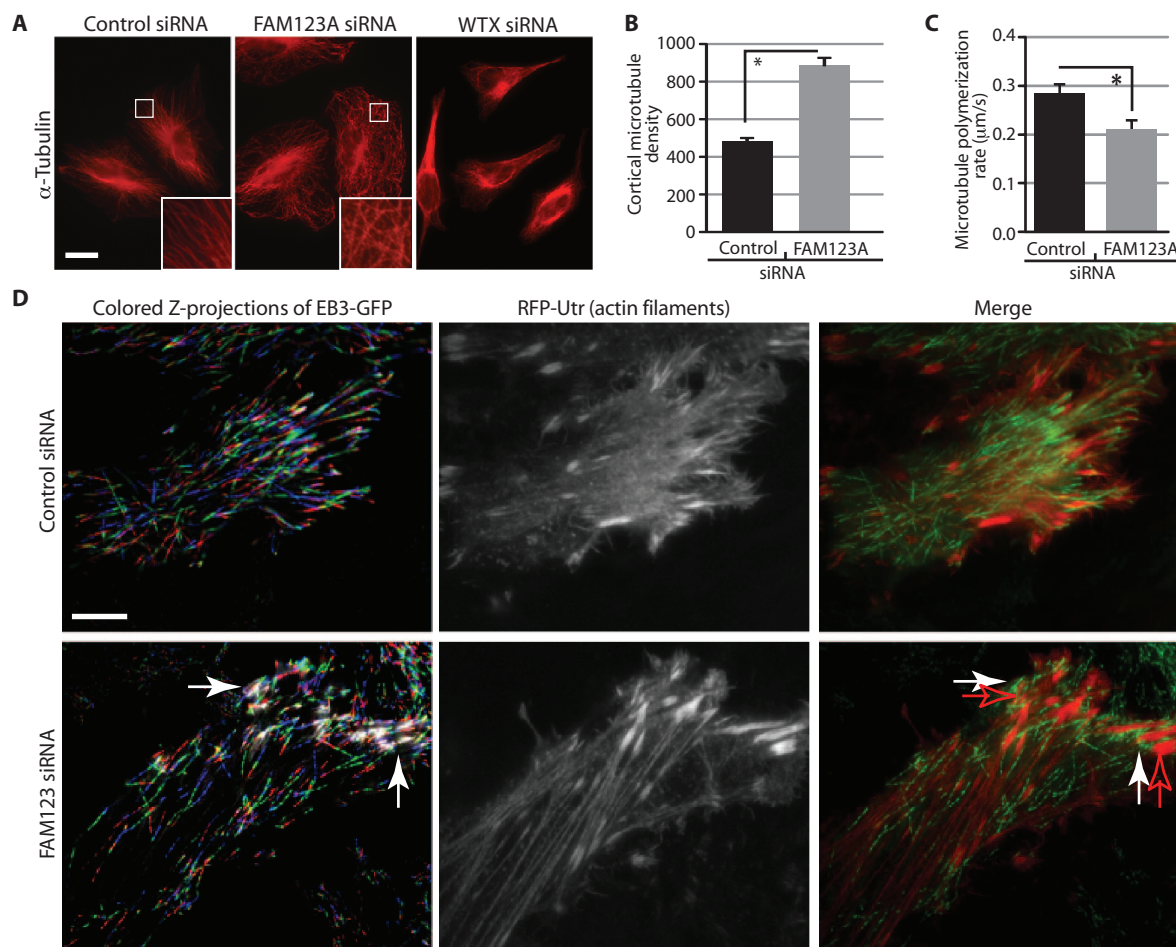


Fig. 5. FAM123A depletion results in altered microtubule organization and increased actomyosin contractility. **(A)** HeLa cells were transfected with the indicated siRNAs and stained with an anti- α -tubulin antibody. Images are representative of three independent biological replicates. **(B)** Plots of fluorescence intensity of α -tubulin staining in cells from **(A)**. The integrated intensity was measured within a $5\text{-}\mu\text{m}^2$ box at $5\text{-}\mu\text{m}$ distance from the cell periphery, in three different regions per cell for 23 control and FAM123A siRNA-transfected cells ($*P < 0.0001$, Welch-corrected t test). **(C)** Microtubule polymerization rates in siRNA-transfected HeLa cells. Ten microtubules were measured in each cell. Nine cells were analyzed for each siRNA

($*P = 0.0129$, Welch-corrected t test). **(D)** TIRF movies of siRNA-transfected HeLa cells expressing EB3-GFP (left) and RFP-Utr (middle). Left: RGB-colored 5-s Z-projections of EB3-GFP-labeled growing microtubule ends. Growing filaments are labeled either red, green, or blue and overlapping stationary microtubule ends are white. Regions in cells lacking FAM123 where the microtubule ends are stationary (not assembling) are indicated (white arrows). Middle: Expressed RFP-Utr shows the filamentous actin near the cell substratum. Right: A 15-frame (75 s) Z-stack of EB3-GFP (green) and RFP-Utr (red) over time. Stationary microtubule ends (white arrows) are in close apposition to actin bundles at the cell periphery (red open arrows).

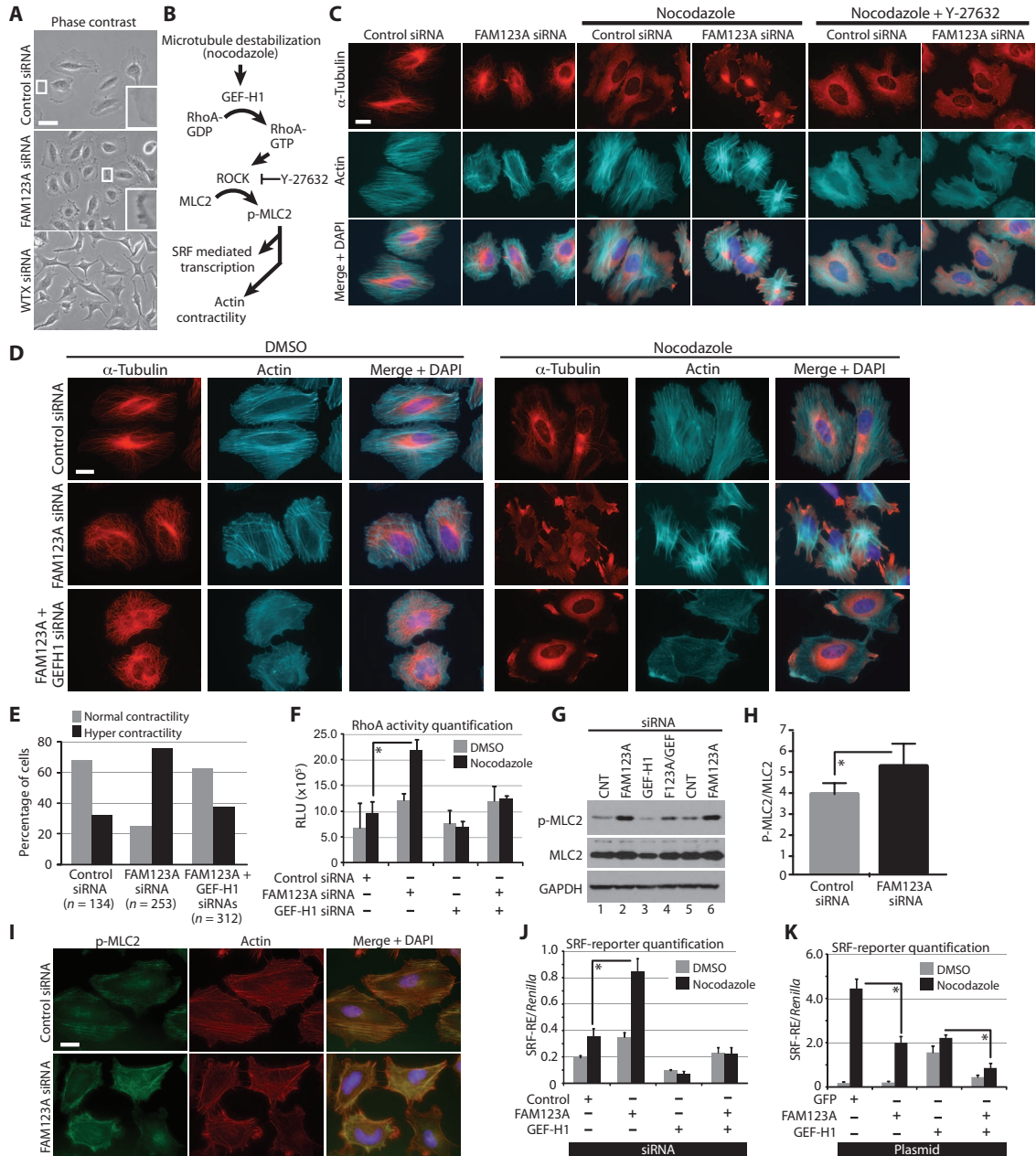
to examine the subcellular localization of GEF-H1 after FAM123A overexpression or knockdown; however, we were unable to visualize microtubule localization of GEF-H1 with various commercially available antibodies and fixation techniques. We reasoned that an effect of FAM123A on GEF-H1 activity could be indirectly assessed by global interrogation of the GEF-H1 protein interaction network. For this approach, we used SILAC (stable isotope labeling with amino acids in cell culture)-based quantitative proteomics of immunopurified endogenous GEF-H1 protein complexes from parental cells or cells overexpressing FAM123A. Averaging the SILAC ratios from biological replicates identified GEF-H1 protein interactions that were increased by FAM123A expression, such as the association between GEF-H1 and DSTN, an F-actin-depolymerizing protein (29, 30), and those that were decreased by FAM123A expression, such as the association between GEF-H1 and SLK, a kinase involved in

actin and microtubule organization (31–33). Together, these data demonstrate that FAM123A controls GEF-H1 activity and GEF-H1 protein-protein interactions.

FAM123A regulates cell adhesion and cell migration

Actin stress fibers and focal adhesions are physically and functionally tethered; increases in cellular contractility or application of external mechanical force induces simultaneous growth of focal adhesions and the attached actin stress fibers (34, 35). To determine whether actomyosin contractility induced by FAM123A loss affected focal adhesions, we visualized vinculin as a marker of focal adhesions (Fig. 8A). Consistent with higher contractility, FAM123A-depleted cells had larger adhesions than control cells (Fig. 8A). To assess cell adhesion and spreading assay, we performed real-time quantitation of electrical impedance during cell spreading, which

Fig. 6. FAM123A regulates actomyosin contractility through GEF-H1. (A) HeLa cells transfected with siRNAs were imaged by phase-contrast microscopy. Images represent more than three biological replicates. Cell morphologies were confirmed with three different FAM123A siRNAs and two different WTX siRNAs. Scale bar, 50 μ m. (B) Major signaling proteins connecting microtubule destabilization to actin contractility. (C) HeLa cells were transfected with control or FAM123A siRNA. Where indicated, cells were treated with the indicated drug before costaining with an anti- α -tubulin antibody, Alexa Fluor 647-conjugated phalloidin, and DAPI. Images are representative of three independent biological replicate experiments. Scale bar, 20 μ m. (D) siRNA-transfected HeLa cells were treated with nocodazole and costained with an anti- α -tubulin antibody, Alexa Fluor 647-conjugated phalloidin, and DAPI. Images are representative of three biological replicates. (E) Quantification of cell phenotypes in (D). Samples were scored in a blinded fashion by three independent investigators. *n*, number of cells scored. (F) siRNA-transfected HeLa cells were serum-starved, treated with nocodazole, and analyzed for RhoA activity ($P = 0.002$, Student's *t* test of biological triplicate experiments). (G) siRNA-transfected HeLa cells were immunoblotted for the indicated proteins. Data are representative of five independent biological experiments. (H) Total MLC2 and phosphorylation of MLC2 at Ser¹⁹ from biological triplicate experiments were quantitated by LI-COR and plotted ($*P = 0.0159$, Student's *t* test). (I) siRNA-transfected HeLa cells were stained with phospho-MLC2 (Ser¹⁹) antibody, Alexa Fluor 647-conjugated phalloidin, and DAPI. Images are representative of three biological replicates. (J)



(K) Quantitation of luciferase activity in HeLa cells that were transiently transfected with the indicated expression construct(s), SRF firefly luciferase reporter, and a CMV-driven *Renilla* luciferase reporter ($*P < 0.05$, Welch-corrected *t* test of biological triplicates).

revealed no effect of FAM123A silencing during the early stages of cell attachment and spreading (Fig. 8B). However, FAM123A-depleted cells had higher impedance values than control cells after the first hour of cell plating, indicating increased adhesion (Fig. 8C), a phenotype that required

GEF-H1 (Fig. 8, B and C). We also examined the formation of adhesion complexes by immunostaining during cell spreading (Fig. 8D and fig. S8). Consistent with the results from the cell spreading assay, FAM123A-depleted and control cells showed phenotypically similar formation of

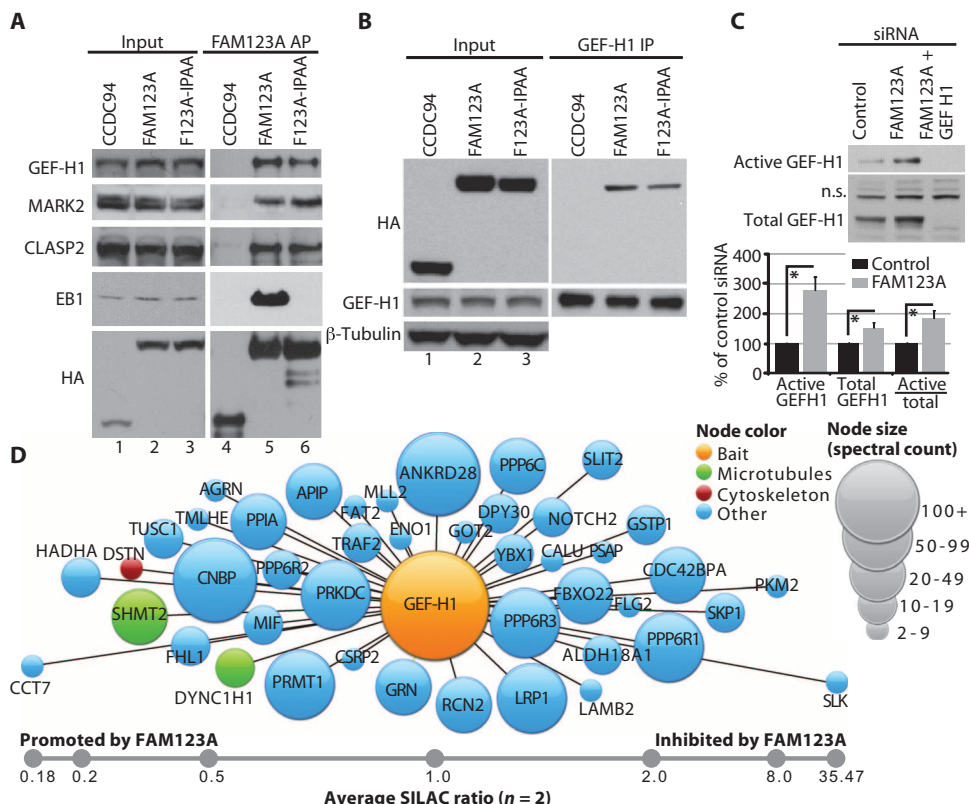


Fig. 7. FAM123A binds to GEF-H1 and regulates its activity. (A) Streptavidin-affinity pulldowns of HEK293T cells stably expressing SBPHA-tagged CCDC94, FAM123A, or FAM123A-IPAA were immunoblotted for endogenous associated proteins. (B) With the HEK293T stable cell lines described in (A), endogenous GEF-H1 was immunoprecipitated and protein complexes were analyzed by Western blot. (C) Active GEF-H1 and total cellular GEF-H1 were determined by densitometry ($*P < 0.05$, paired Student's *t* test of six biological replicate experiments; error bars represent SE). (D) The GEF-H1 protein interaction network, illustrating the FAM123A-sensitive interactions as determined by quantitative IP/MS. The average SILAC ratios from biological duplicate experiments are shown below the network.

adhesion and actin stress fibers in the early stages of cell spreading. However, after the first hour, the adhesions in FAM123A-depleted cells were larger and had thicker actin stress fibers, effects that were GEF-H1-dependent because cells depleted of both FAM123A and GEF-H1 or GEF-H1 alone resembled the control siRNA-transfected cells. Given these cytoskeletal and adhesion phenotypes, we tested whether FAM123A silencing affected cell migration. HeLa cells transfected with either control or FAM123A siRNAs were plated to confluency, scratched, and imaged by live-cell microscopy. Compared with control siRNA-transfected cells, FAM123A depletion resulted in a 20% decrease in cell migration, which may have resulted from increased adhesion (Fig. 8E).

DISCUSSION

We performed unbiased protein-protein interaction screens to discover new functions for members of the FAM123 protein family. Here, we have characterized a family-unique physical and functional relationship between FAM123A and the microtubule and actomyosin cytoskeletal networks. We found that FAM123A binds EB proteins and interacts with dynamic microtubules through its SKIP⁴⁸⁷⁻⁴⁹⁰ motif. FAM123A knock-

down resulted in compartment-specific effects on microtubule dynamics, a globally disorganized microtubule network, increased GEF-H1 activity, increased actomyosin contractility, increased cell adhesion, and decreased cell migration.

Members of the FAM123 gene family control β -catenin-dependent WNT signaling

FAM123A inhibits β -catenin-dependent WNT signaling (11). Our data both confirm this findings and provide additional mechanistic insight. First, comparative protein-protein interaction studies of WTX and FAM123A revealed a robust association between β -catenin and WTX but not between β -catenin and FAM123A. Although a low affinity or transient interaction between FAM123A and β -catenin may occur, we interpret our data to suggest that association with β -catenin is not required for regulation of WNT signaling by WTX (or FAM123A). Consistent with this notion, the REA repeats in WTX that are responsible for mediating direct interaction with β -catenin are found only in mammalian orthologs of WTX, although WTX inhibits WNT signaling in zebrafish and *Xenopus* (7). On the basis of our findings, it is likely that WTX and FAM123A inhibit WNT signaling through interactions with APC or β TrCP1/2 (or both), which associate with both WTX and FAM123A.

Our data demonstrate that the FAM123A-EB interaction is dispensable for WNT regulation, at least with respect to nonpolarized cells grown in two dimensions. In contrast to WTX, which is uniformly distributed across human tissues, FAM123A is largely restricted to neuronal tissues (9). These disparities in distribution may provide an explanation as to why the ability to control cytoskeletal dynamics is specific to FAM123A, which may have evolved to remodel the cytoskeleton during neuronal migration (11). Because the FAM123C protein interaction network did not share common protein associations with FAM123A or WTX, our data suggest that FAM123C lacks regulatory functions over WNT signaling, although this remains to be formally demonstrated.

FAM123A tracks growing microtubules

Plus-end tracking proteins comprise a structurally and functionally diverse group of microtubule-associated proteins that accumulate at the ends of growing microtubules (15, 17, 19, 36). Many plus-end tracking proteins have a conserved SxIP motif that directly associates with EB proteins and thus mediates localization to the microtubule plus-end (22). In at least two ways, however, our protein dynamic studies also differentiate FAM123A from other plus-end tracking proteins.

First, unlike EB1 and many plus-end tracking proteins that localize to microtubule tips throughout the cell body, FAM123A predominantly decorates the distal ends of microtubules oriented in the direction of cell movement, a polarized distribution also seen for CLASPs, APC, and

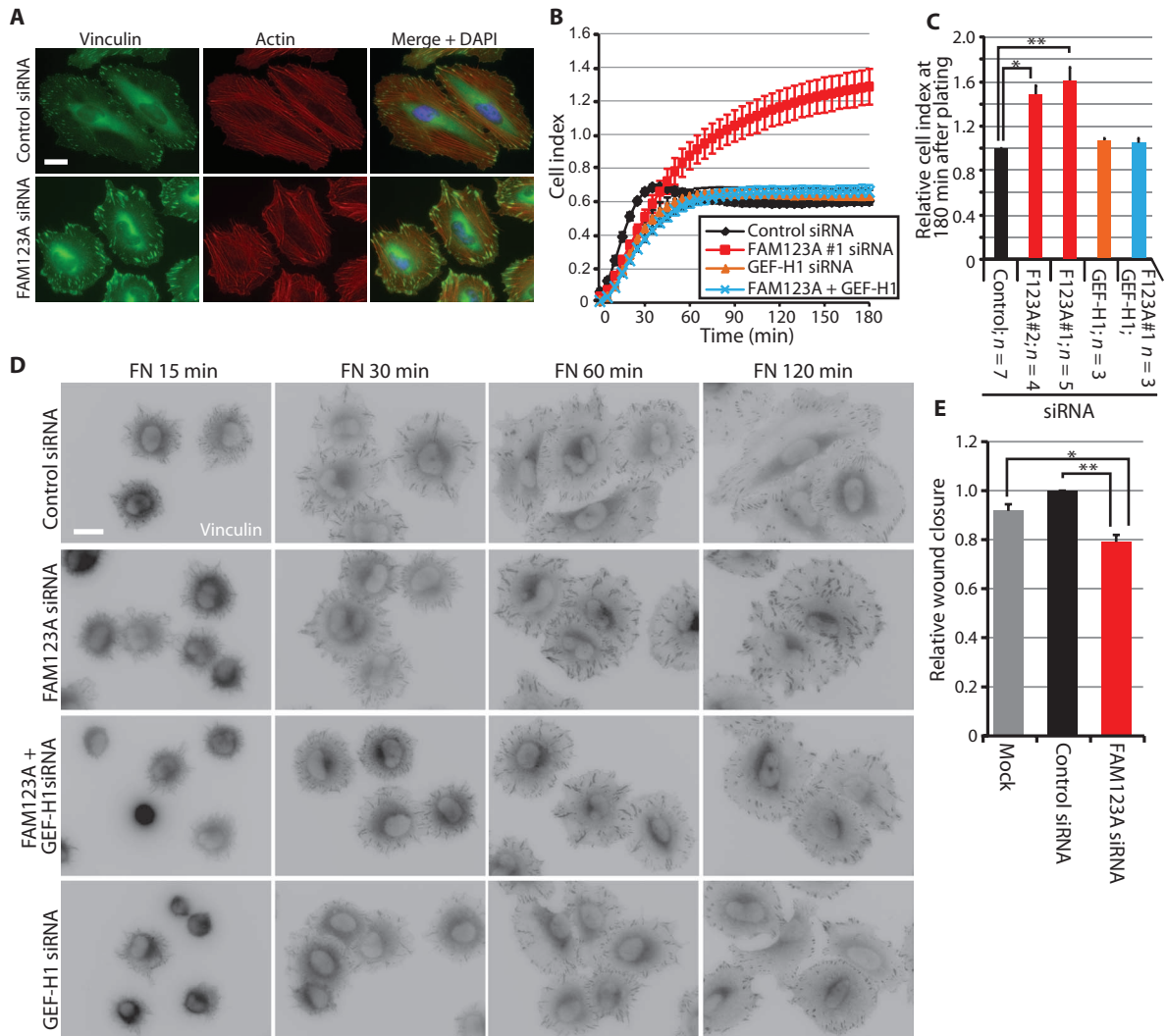


Fig. 8. FAM123A regulates cell adhesion and migration. (A) siRNA-transfected HeLa cells were fixed and stained with an anti-vinculin antibody, Alexa Fluor 594–phalloidin, and DAPI. Data represent four biological replicates. (B) Adhesion quantification of siRNA-transfected HeLa cells that were subjected to cell attachment with real-time acquisition of electrode impedance. (C) Histogram of relative impedance index 180 min after cell plating. Error bars represent SE across the biological replicates. (* P =

0.005 and ** P = 0.005, paired Student's t test; n , biological replicates). (D) siRNA-transfected HeLa cells were allowed to attach to fibronectin-coated coverslips for the indicated times before fixation and immunostaining with an anti-vinculin antibody. FN, fibronectin. (E) HeLa cells transfected with control or FAM123A siRNA were scratch-wounded and imaged over 24 hours. (* P = 0.03 and ** P = 0.018 by Student's t test). n = 3 biological replicates.

CDK5RAP2 (37–40). Through asymmetric distribution to the leading edge, these plus-end tracking proteins modulate microtubule dynamics and consequently promote cell polarization and directional migration (41, 42). We found that FAM123A loss differentially affects microtubule dynamics in different subcellular compartments (Fig. 5). Therefore, it is possible that FAM123A functions to regulate spatially confined microtubule stability, which presumably contributes to the establishment, maintenance, or modulation of asymmetric cell behavior.

Second, although they bind EB proteins for plus-end tracking, many plus-end tracking proteins can also directly associate with microtubules. By contrast, FAM123A microtubule tracking and localization was largely attenuated in the absence of EB association; one possibility is that the remaining microtubule localization is due to bridging proteins that tether the

N terminus of FAM123A to the microtubule, such as APC. Moreover, the subcellular distribution of full-length FAM123A and the C-terminal fragment are substantially different, although both bind EB proteins and track with assembling microtubule ends. Whereas the C terminus colocalizes with EB comets, full-length FAM123A exhibits slower microtubule tracking near the cortex, frequently coating the microtubule lattice. These data suggest that FAM123A may be involved in coupling assembling microtubules to membrane signaling pathways.

FAM123A regulates microtubule dynamics and organization

Like many other EB-dependent plus-end tracking proteins, we found that FAM123A depletion results in a disorganized microtubule network.

Whereas FAM123A asymmetrically localized to microtubules in the direction of cell movement, its depletion affected microtubule architecture throughout the entire cell body. FAM123A depletion resulted in decreased microtubule polymerization rates within the cell body and less dynamic and stabilized microtubules in apposition to actin adhesion complexes. Although untested, it is possible that the decreased microtubule polymerization rates occur secondarily to the increased cortical stability because of reprogramming overall tubulin homeostasis. For example, decreases in free tubulin dimers as a result of increased stability in some microtubules may result in global effects on microtubule dynamics (43). It also remains possible that actomyosin contractility induced by FAM123A silencing functions in a feed-forward loop to globally influence microtubule dynamics (44). Although our data do not provide an exact mechanism, we propose that FAM123A may control microtubule dynamics by regulating cortical capture through the tethering of microtubules to the plasma membrane. That is, FAM123A complexes with several plus-end tracking proteins that are thought to stabilize microtubules at the leading edge, such as EB1, EB3, CLASPs, APC, and ACF7 (15). It is possible that in association with these plus-end tracking proteins, FAM123A asymmetrically tracks microtubules in the direction of cell movement and promotes both cortical microtubule stabilization and polarized cargo delivery to the cortical membrane. Because WTX and FAM123A bind phospholipids and localize predominantly to the cytoplasmic membrane, an elaboration on this model might have FAM123A “jumping” off microtubules onto a phospholipid landing pad, perhaps delivering its associated proteins, such as GEF-H1 and APC, to the leading edge. In agreement with this model, FAM123A recruits APC to the plasma membrane and drives EB1 to the membrane when overexpressed (fig. S4) (6, 11).

FAM123A inhibits actomyosin contractility, thereby regulating adhesion and cell migration

Our data suggest that FAM123A binds to and inhibits GEF-H1, a guanine nucleotide exchange factor that localizes to microtubules, is activated by microtubule depolymerization, and activates RhoA-dependent actomyosin contraction (24, 25). We show that FAM123A depletion results in GEF-H1-dependent increased actomyosin contractility, enlarged focal adhesions, increased cellular adhesion, and decreased cell migration (Fig. 8). These observations demonstrate that FAM123A is similar to p21-activated protein kinase 1 (PAK1), PAK4, calpain-6, MARK2, and extracellular signal-regulated kinase (ERK), all of which regulate GEF-H1 to control spatiotemporal regulation of the actin cytoskeleton (45–54). That said, several important questions remain regarding the precise molecular mechanism by which FAM123A regulates GEF-H1.

First, our data demonstrate that FAM123A is epistatically upstream of GEF-H1 with respect to RhoA, ROCK, MLC2, actomyosin contractility, and cell adhesion but not to microtubule organization (Fig. 6). Therefore, actomyosin contractility likely underlies the FAM123A-dependent cell adhesion phenotype, rather than the altered microtubule dynamics. Given the limitations of interpreting cause and effect from siRNA-based experiments, additional studies are needed to more precisely define the role of FAM123A in microtubule dynamics and to understand how that effect is communicated through GEF-H1 to the actomyosin network. Second, as mentioned, the precise mechanism by which FAM123A inhibits GEF-H1 remains to be established. Although we show that ectopic FAM123A binds endogenous GEF-H1 (and vice versa), it remains to be determined whether the endogenous proteins associate. Moreover, we were unable to demonstrate GEF-H1 subcellular colocalization with FAM123A. Third, we found that FAM123A inhibits GEF-H1 in the presence and absence of microtubules. Specifically, the IPAA mutant form of FAM123A, which does not bind microtubules, associates with GEF-H1 (Fig. 7), and FAM123A

knockdown exacerbates actin contraction in the absence of microtubules (Fig. 6). These data suggest that FAM123A functionally affects GEF-H1 independently of microtubule polymerization status, similar to tumor necrosis factor- α -mediated GEF-H1 activation in tubular epithelia (48, 49). Finally, we found that FAM123A altered the GEF-H1 protein interaction network, which we interpret as further corroborative evidence that FAM123A controls GEF-H1 activity or subcellular localization. The biological implications of these GEF-H1 interactions and their control by FAM123A await further study.

In summary, we found, using comparative proteomic analyses of the FAM123 family, that FAM123A associates with numerous microtubule-binding proteins. Subsequent protein dynamic studies and functional interrogation revealed that FAM123A controls microtubule polymerization rates, actomyosin contractility, and, consequently, cell adhesion and cell migration.

MATERIALS AND METHODS

Constructs

FAM123A isoform 2 complementary DNA (cDNA) was obtained by polymerase chain reaction (PCR) amplification from clone BCO32653 (Open Biosystems). The mutant FAM123A-IPAA (Ile⁴⁸⁹-Pro⁴⁹⁰ mutagenized to Ala⁴⁸⁹-Ala⁴⁹⁰) was created by standard PCR-based mutagenesis. The WTX constructs were previously described (5). The EB3-pEGFPN1 and EB3-RFP (red fluorescent protein) constructs were provided by A. Akhmanova (Erasmus Medical Center, Rotterdam, Netherlands). The EB1-pEGFPN1 construct was provided by L. Cassimeris (Lehigh University, Bethlehem, PA). EB1-pEGFPN2 and the deletions were obtained by amplifying EB1 by PCR from pEGFPN1-EB1 construct. The mCherry-tubulin plasmid was made by replacing GFP at the Bsr GI and Bam HI sites in pEGFP-Tub with mCherry. The β -catenin-activated firefly reporter (pBAR) and pcDNA3.1-Flag-APC (amino acids 1 to 1060) were previously described (5). The SRF-RE reporter pGL4.34[luc2P/SRF-RE/Hygro] was obtained from Promega. GFP-GEF-H1 was kindly provided by R. Garcia-Mata (UNC-Chapel Hill).

Reagents

Wnt3A and control conditioned media were produced with mouse fibroblasts (L cells) according to the American Type Culture Collection protocol. Nocodazole and Y-27632 were purchased from Sigma-Aldrich Corporation (catalog nos. M1404 and Y0503).

Tissue culture, transfections, and reporter assays

All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, in a 37°C humidified incubator with 5% CO₂. Transient transfections of siRNAs were performed with Lipofectamine RNAiMAX, as directed by the manufacturer (Invitrogen). All siRNAs were used at a final concentration of 20 nM and for 72 hours unless otherwise stated. siRNAs targeting human forms of WTX (siRNA#1 and #2), APC, axin1/2, and β -catenin have been previously published (5). The following siRNAs were used: Stealth M2 (Invitrogen) as control; EB1, AAGUGAAAUCCAAGC-UAAGC (42); FAM123A siRNA#1, GCCGGCUCUGUCUAAAAAG[dT] [dT]; FAM123A siRNA#2, GAGAUUAUUAUGCAGACCAAGAGG; FAM123A siRNA#3, CCUACGUUCAGUUGUUAGAUAGCA; FAM123A siRNA#4, CCUCUAGAUAAGUCCUGAGAAU; GEF-H1 siRNA#1, AGCAGGCCACGGAACUGGCAUUACU; GEF-H1 siRNA#2, UCCAUAACACGCUUCCUCAGCCAGCU; WTX siRNA#3, AAAGGCAGUCAUCUCCAGGUGGAGA. All siRNAs were synthesized by Invitrogen as Stealths, except for EB1 and FAM123A#1. Expression

constructs were transiently transfected into HEK293T cells with Lipofectamine 2000 (Invitrogen) or into HeLa cells with FuGENE 6-HD (Roche), as directed by the manufacturer. For gain-of-function reporter assays, cells were seeded in 48-well plates before transfection with a firefly luciferase reporter, *Renilla* luciferase control reporter, and effector plasmids. Cells stably engineered with these reporters were used for the loss-of-function experiments. Luciferase activity was quantitated with the Dual-Glo Luciferase Assay System (Promega).

Affinity purification and MS

Tandem affinity purification of FAM123A (isoform 2) was performed as previously described (55, 56), with minor modifications. Protein complexes were eluted from the streptavidin beads with 5 mM biotin in the absence of TEV protease. PPS Silent Surfactant (0.1%; Protein Discovery) was included in the final elution from the calmodulin beads. Before MS, PPS was acid-cleaved at 37°C for 30 min. Flag-based AP/MS of FAM123C was performed in triplicate as previously described (57). Quantitative immunoprecipitation and mass spectrometry (IP/MS) of endogenous GEF-H1 was performed in duplicate via SILAC (K6/R10) labeling. Briefly, protein lysates (~150 mg) from the following HEK293T-derived cell lines were compared at low confluency: parental cells (light) and Flag-FAM123A (heavy) or GFP (light) and HA-FAM123A (heavy). Cells were lysed in 50 mM Hepes-NaOH (pH 8.0), 150 mM NaCl, 10% glycerol, 0.1% NP-40, 2 mM EDTA, 2 mM dithiothreitol, and protease and phosphatase inhibitor cocktails (Roche) and subjected to immunoprecipitation with 10 µg of GEF-H1 antibody (A301-929A, Bethyl Labs). Following an on-beads digestion with FASP Protein Digestion Kit (Protein Discovery), tryptic peptides were cleaned up with C18 spin column (Thermo Scientific), then separated by reversed-phase nano-high-performance liquid chromatography with a nanoAquity UPLC system (Waters Corp.). Peptides were first trapped in a 2-cm trapping column [75-µm inside diameter (ID), Michrom Magic C18 beads of 5.0-µm particle size, 200-Å pore size] and then separated on a self-packed 25-cm column (75-µm ID, Michrom Magic C18 beads of 3.0-µm particle size, 100-Å pore size) at room temperature. The flow rate was 200 nL/min over a gradient of 1% buffer B (0.1% formic acid in acetonitrile) to 30% buffer B in 180 min. Then, a following wash raised buffer B to 70%. The identity of the eluted peptides was determined with an in-line LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). The ion source was operated at 2.0 to 2.4 kV with the ion transfer tube temperature set at 275°C. Full MS scan [300 to 2000 mass/charge ratio (*m/z*)] was acquired in Orbitrap at 60,000 resolution setting; data-dependent MS² spectra were acquired in LTQ by collision-induced dissociation with the 20 most intense ions. Precursor ions were selected on the basis of charge states (1, 2, or 3) and intensity thresholds (above 2000) from the full scan; dynamic exclusion (one repeat during 30 s, with a 60-s exclusion time window) was also taken into account. The polysiloxane lock mass of 445.120030 was used throughout spectral acquisition.

Protein identification and quantification

All raw data were converted to mzXML format before a search of the resultant spectra with Sorcerer-SEQUEST (build 4.0.4, Sage-N Research) and the Trans-Proteomic Pipeline (TPP v4.3.1). Data were searched against the human UniProtKB/Swiss-Prot sequence database (Release 2011_08) supplemented with common contaminants, such as porcine (Swiss-Prot P00761) and bovine (P00760) trypsin, and further concatenated with its reversed copy as a decoy (40,494 total sequences). Search parameters used were a precursor mass between 400 and 4500 atomic mass units (amu), up to 2 missed cleavages, a precursor-ion tolerance of 3 amu, accurate mass binning within PeptideProphet (58), semi-tryptic digestion, a static carbamidomethyl cysteine modification, variable methionine oxida-

tion, and additional variable modifications of R10 and K6 for SILAC experiments. SILAC ratios were calculated with TPP's Xpress (59), and protein abundance ratios were first normalized by the bait's ratio, then combined from replicate experiments by taking a weighted average using the number of quantified spectra for the protein in each replicate. False discovery rates (FDRs) were determined by ProteinProphet (58), and minimum protein probability cutoffs resulting in a 1% FDR were selected individually for each experiment. Further filtering of identified proteins was accomplished using the following criteria: at least two unique peptides were identified for the protein in each of at least two (of four) WTX experiments, at least two (of three) for proteins in FAM123C experiments, two (of two) for ARHGEF2, and two (of three) for FAM123A. Common contaminants were removed at the authors' discretion on the basis of previous experiments, such as keratins, ribosomal, and DEAD box proteins. Unfiltered data are provided in table S1 and may be downloaded from ProteomeCommons.org Tranche using the following hash: SG+Lb+X7PAO-mf9pdlyQREvVmA7sOZX/iwKCV7zQBvmofGaBNxvXg8D9dZkUs5Cg-biDMIBp6JlNW1fh0EgeWb8SOaaxMAAAAAAAAAADcA==.

Bioinformatics

PeptideProphet/ProteinProphet results for each AP/MS experiment were stored in a local ProHits database (60). ProHits performed the mapping of UniProtKB accession identifiers to Entrez Gene IDs. These results were then imported into Cytoscape v2.8.2 (61) for network visualization. Gene Ontology annotations were imported from the National Center for Biotechnology Information Entrez Gene through Cytoscape. Known protein-protein interactions were extracted from the following databases: BIND, BioGRID, DIP, HPRD, IntAct, MINT, and Reactome—downloaded on 15 August 2010.

Affinity pulldowns, immunoprecipitation, and Western blotting

In all biochemical experiments, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, and protease and phosphatase inhibitor cocktails (Roche). For streptavidin-affinity purification, cleared lysates were incubated for 1 hour with streptavidin resin (GE Healthcare) and subsequently washed and eluted. Immunoprecipitation of endogenous GEF-H1 was performed with anti-GEF-H1 antibodies (A301-929A, Bethyl Labs). Detection of proteins by Western blot was performed with the following antibodies: anti-Flag M2 monoclonal (Sigma-Aldrich Corporation), anti-HA polyclonal (1867423, Roche), anti-GFP polyclonal (ab290, Abcam), anti-β-catenin polyclonal (9562, Cell Signaling Technology), anti-β-TrCP1 monoclonal (37-3400, Invitrogen), anti-β-tubulin monoclonal (T7816, Sigma-Aldrich Corporation), anti-GEF-H1 (A301-928A, Bethyl Labs), anti-MARK2 (Roche), anti-CLASP2 (A302-155A, Bethyl Labs), anti-APC polyclonal (A300-180A, A300-981A, Bethyl Labs), anti-EB1 monoclonal (610534, BD Transduction Laboratories), anti-MLC2 (3672, Cell Signaling Technology), and anti-phospho-MLC2 (Ser¹⁹) (no. 3671, Cell Signaling Technology). For detection of endogenous FAM123A, a custom monoclonal antibody was produced (Abmart).

For phospho-MLC detection, HeLa cells were transfected with siRNA at 10 nM for 48 hours. Cells were lysed in the well with UPX sample buffer (Protein Discovery) supplemented with protease and phosphatase inhibitors. Samples were boiled for 3 min before the addition of 4× sample buffer and sonication. Proteins were detected by Western blot and quantified with Odyssey Imager and Software by LI-COR Biosciences.

Immunofluorescence

For immunofluorescence, HeLa sarcoma cells were plated on fibronectin (10 µg/ml)-coated coverslips in DMEM supplemented with 10% FBS and

allowed to attach and spread. Cells were fixed in 4% paraformaldehyde (PFA; Electron Microscopy Sciences) in cytoskeletal buffer [5 mM Pipes (pH 6), 137 mM NaCl, 5 mM KCl, 1.1 mM sodium phosphate buffer, 0.4 mM KH_2PO_4 , 0.4 mM MgCl_2 , 0.4 mM NaHCO_3 , 2 mM EGTA, 50 mM glucose] for 15 min and permeabilized with 0.1% Triton in phosphate-buffered saline (PBS) for 5 min. After being blocked in 1% bovine serum albumin/PBS for 1 hour, cells were incubated with primary antibodies at 4°C overnight, followed by incubation with appropriate secondary antibodies, RRX-conjugated donkey anti-mouse immunoglobulin G (IgG) and fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories), at room temperature for 1 hour. For staining of endogenous EB1, cells were fixed in methanol at -20°C for 5 min. Staining of proteins was performed with the following antibodies: monoclonal anti- α -tubulin (clone DM1A, T9026, Sigma-Aldrich Corporation), monoclonal anti-vinculin (clone nVIN-1, V9131, Sigma-Aldrich Corporation), and anti-EB1 monoclonal (610534, BD Transduction Laboratories). Actin was stained with Alexa Fluor 647-phalloidin or Alexa Fluor 594-phalloidin (Invitrogen). Coverslips were mounted to slides with the ProLong Gold Antifade reagent (Invitrogen). Staining was analyzed with an Olympus IX 81-ZDC inverted fluorescence microscope (Olympus Corporation of the Americas) equipped with a 60 \times /1.42 Oil PlanApo objective lens and a Hamamatsu C10600-10B camera (OrcaR2, Hamamatsu Photonics Ltd.).

For subcellular localization of FAM123A, HeLa cells were grown on 18-mm glass coverslips coated with poly-L-lysine (Sigma-Aldrich Corporation) and cotransfected with EGFP-FAM123A and mCherry-tubulin. Cells were fixed in 1% PFA in pure -20°C methanol for 2 min and mounted with ProLong Gold Antifade with DAPI (Invitrogen). The imaging was performed on a DeltaVision deconvolution microscope (Applied Precision). For costaining of FAM123A-GFP and EB1, cells were fixed similarly and EB1 was stained with anti-EB1 antibody (BD Transduction Laboratories), followed by incubation with donkey anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories). Fixed cells were imaged with a complete Z-series in 0.2- μm sections, deconvolved, and projected with Adobe Photoshop CS and ImageJ software.

For comparing the distribution of FAM123A family members, HEK293T transiently expressing fluorescent-tagged proteins were plated on fibronectin-coated coverslips, fixed, and mounted as described above and imaged with a Zeiss LSM5 Pascal confocal laser scanning microscope equipped with a 63 \times /1.42 Oil PlanApo objective lens.

Live-cell imaging

For low-resolution imaging analysis, HT1080 sarcoma cells were plated onto fibronectin (5 $\mu\text{g}/\text{ml}$)-coated MatTek dishes (MatTek Corporation) in DMEM supplemented with 10% FBS and allowed to attach and spread. Cells were transfected with Venus-FAM123A or Venus-WTX with FuGENE HD (Promega) according to the manufacturer's instructions. Cells were imaged the next day in contrast phase and GFP fluorescence on a Nikon Biostation (every 5 min for 10 hours), with a 20 \times objective, at 37°C and 5% CO_2 .

For dynamic analysis of fluorescence-tagged proteins at higher magnification, HT1080 were plated onto fibronectin (5 $\mu\text{g}/\text{ml}$)-coated Delta T dishes (Bioprotech Inc.) in DMEM supplemented with 10% FBS and transfected with EGFP-FAM123A with FuGENE HD (Promega). The next day, cells were imaged at 37°C and 5% CO_2 with an Olympus IX 81-ZDC inverted fluorescence microscope (Olympus Corporation of the Americas) equipped with a Delta T Open Dish System (Bioprotech Inc.) with a heated lid. Time-lapse images were captured every 10 s with a heated 60 \times /1.42 Oil PlanApo objective lens and a Hamamatsu C10600-10B camera (OrcaR2, Hamamatsu Photonics Ltd.). Data acquisition was carried out with Velocity (version 5.5.1, PerkinElmer), and image processing was performed with ImageJ and Adobe Photoshop CS software.

HeLa cells were transfected with either EGFP-FAM123A, EGFP-C-terminal FAM123A, or EGFP-C-terminal FAM123A-IPAA and in some cases cotransfected with EB3-RFP with Nucleofector II (Amaxa Biosystems) and plated onto poly-D-lysine-coated MatTek dishes. After 24 hours of expression, the dually transfected cells were imaged at 37°C at a rate of 1 frame/5 s on a DeltaVision RT microscope (Applied Precision). Cells transfected with only one construct (movies S6 and S7) were imaged at a rate of 1 frame/2 s.

For microtubule dynamic studies, HeLa cells were transfected with EB3-GFP and RFP-Utr expressing DNA constructs and plated onto fibronectin-coated MatTek filming dishes; RFP-Utr encodes an RFP-fused calponin homology domain of the utrophin protein (62). The cells were transfected for 48 hours with either control siRNA or siRNA directed against FAM123A. The cells were then imaged with a Personal DeltaVision microscope custom-outfitted with TIRF light paths and a 60 \times Olympus TIRF objective. Images were collected at 5-s intervals. Z-projections were made using successive 5-frame intervals for a total of 15 frames. Each 5-frame projection was colored red, green, or blue to visualize the assembling microtubule ends. With this regimen, stationary microtubule ends consisting of RGB overlap are depicted as white.

For quantitation of cell migration, HeLa cells were transfected with 10 nM siRNA and plated onto a 12-well plate (4×10^5 cells per well) in complete growth medium 48 hours after transfections. After 12 hours, the monolayer of cells was wounded by manual scratching with a pipette tip, washed with PBS, and placed into complete growth medium. Time-lapse phase-contrast images were acquired every 7 min for 18 hours with an Olympus IX70 inverted fluorescence microscope, enclosed within an environmental chamber controlled for temperature, relative humidity, and CO_2 , and equipped with a 4 \times 0.13 Uplan FL PhL lens and a Hamamatsu Orca C4742-95 charge-coupled device camera. Data were acquired with Velocity (version 5.5.1, PerkinElmer). For calculation of relative migration, the scratch area was determined from movie-derived images at time 0 and at 6 hours. The difference in area between $t = 0$ and $t = 6$ at three locations along the scratch for each of the biological triplicate experiments was used to plot the relative rate of wound closure.

RhoA and GEF-H1 activity assays

Purified GST-RhoA(G17A) was provided by C. Guilluy and K. Burridge (UNC-Chapel Hill). Affinity precipitation of exchange factors with the nucleotide-free RhoA mutant (G17A) was performed as previously described (28). For quantitation, Western blot data from six biological replicate experiments were analyzed by densitometry. Data were first normalized to a loading control and then to 1 before averaging and plotting. Paired Student's *t* test was calculated using raw data. For RhoA activity quantitation, the G-LISA RhoA Activation Assay Biochem Kit (Cytoskeleton Inc.) was used. Specifically, 4×10^5 HeLa cells were plated in 60-mm plates. Cells were transfected overnight with siRNA and allowed to recover in 10% FBS/DMEM for 8 hours. Cells were then washed with PBS and starved in DMEM for 48 hours with one medium change at 24 hours. After 1-hour treatment with nocodazole, cells were lysed in 140 μl of cell lysis buffer. Plates were read on a Synergy HT microplate reader from BioTek.

Cell adhesion and spreading assay

Cell adhesion and spreading were measured as changes in impedance with the RT-CES system (ACEA Biosciences Inc.). The 16-well E-plates were coated with fibronectin (10 $\mu\text{g}/\text{ml}$) for 1 hour at 37°C. HeLa cells were transfected with 10 nM siRNAs and subjected to cell spreading assay about 65 hours after transfection. For measurements, the same number of cells (4×10^3) was added to each well. The E-plates containing HeLa cells were incubated at room temperature for 10 min before being placed

on the device station in the incubator for continuous recording of impedance-based cell index (every 30 s, for 3 hours). Additionally, siRNA-transfected cells were plated on fibronectin-coated coverslips, allowed to attach and spread for various periods, fixed, and stained for vinculin according to the methods described earlier.

SUPPLEMENTARY MATERIALS

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Fig. S1. FAM123C does not localize to the cytoplasmic membrane.

Fig. S2. FAM123A binds FBXW11.

Fig. S3. Mutation of the SKIP motif in FAM123A abrogates its microtubule localization and tracking.

Fig. S4. FAM123A overexpression alters the subcellular localization of EB1.

Fig. S5. EB1 and microtubule association is dispensable for the FAM123A control of β -catenin-dependent WNT signaling.

Fig. S6. GEF-H1 silencing alters microtubule organization and actomyosin contractility.

Fig. S7. The increased actin contractility observed in FAM123A siRNA-transfected cells is due to the specific silencing of FAM123A.

Fig. S8. FAM123A controls maturation of focal adhesions during cell spreading.

Table S1. Affinity purification and MS data.

Movie S1. Venus-fused FAM123A localizes to the cell membrane and to filamentous structures in the cell lamellipodium.

Movie S2. Venus-fused WTX localizes predominantly to the cell membrane.

Movie S3. EGFP-fused FAM123A moves on presumed microtubules toward the cell cortex.

Movie S4. EGFP-fused FAM123A tracks the tips and distal ends of EB3-associated microtubules.

Movie S5. FAM123A-IPAA shows decreased EB3-associated plus-end tip tracking and decoration of distal ends of microtubules.

Movie S6. The C-terminal fragment of FAM123A, which contains the SKIP EB-interaction motif, behaves as a classic +TIP showing robust microtubule tip tracking, but does not decorate the microtubule lattice.

Movie S7. The C-terminal fragment of FAM123A with a mutated SKIP motif does not show colocalization or tip tracking of microtubules.

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