RASSF1A Suppresses the Invasion and Metastatic Potential of Human Non–Small Cell Lung Cancer Cells by Inhibiting YAP Activation through the GEF-H1/RhoB Pathway

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Abstract

Inactivation of the tumor suppressor gene RASSF1A by promoter hypermethylation represents a key event underlying the initiation and progression of lung cancer. RASSF1A inactivation is also associated with poor prognosis and may promote metastatic spread. In this study, we investigated how RASSF1A inactivation conferred invasive phenotypes to human bronchial epithelial cells. RNAi-mediated silencing of RASSF1A induced epithelial–mesenchymal transition (EMT), fomenting a motile and invasive cellular phenotype in vitro and increased metastatic prowess in vivo. Mechanistic investigations revealed that RASSF1A blocked tumor growth by stimulating cofilin/PP2A-mediated dephosphorylation of the guanine nucleotide exchange factor GEF-H1, thereby stimulating its ability to activate the antimetastatic small GTPase RhoB. Furthermore, RASSF1A reduced nuclear accumulation of the Hippo pathway transcriptional cofactor Yes-associated protein (YAP), which was reinforced by RhoB activation. Collectively, our results indicated that RASSF1 acts to restrict EMT and invasion by indirectly controlling YAP nuclear shuttling and activation through a RhoB-regulated cytoskeletal remodeling process, with potential implications to delay the progression of RASSF1-hypermethylated lung tumors. Cancer Res; 76(6): 1–14. ©2016 AACR.

Introduction

One of the key events in the natural history of lung carcinomas is the early inactivation of the tumor suppressor gene RASSF1 [Ras association (RalGDS/AF-6) domain family member 1], in 30% of non–small cell lung cancer (NSCLC), by promoter hypermethylation (1). The RASSF1A protein, encoded by 1 of 8 splicing isoforms, termed 1A to 1H (2), is expressed in all normal human tissues, and carries several domains mediating protein–protein interactions with multiple partners, making RASSF1A a key regulator of apoptosis, cell-cycle progression, and genome integrity (2). It was also suggested that RASSF1A further plays a role in tumor cell adhesion and motility (3), though the molecular mechanisms are poorly understood. In Drosophila melanogaster, the dRASSF protein is thought to interfere with the Hippo pathway, composed of the Ste20-like Hippo kinase, (fly ortholog of human MST1/2), the AGC kinase Warts (Drosophila ortholog of human Lats1/2), and the terminal target Yorkie, a transcriptional cofactor (Drosophila ortholog of Yes-associated protein, YAP; ref. 4). In the nucleus of mammalian cells, active YAP interacts with TEAD transcription factors to regulate transcription of multiple genes, some of which have been shown to mediate epithelial-to-mesenchymal transition (EMT; ref. 5). YAP was recently reported to transduce mechanical signals from extracellular matrix (ECM), which are mediated by a Rho GTPase activity, and tension of actin–myosin cytoskeleton, independently of Hippo signaling (6). The Rho (A, B, and C) small GTP/GDP-binding proteins, are indeed well-known master regulators of cell adhesion and motility (7). Their activation state is highly regulated by guanine nucleotide exchange factors (GEF), which trigger the exchange of GDP for GTP (8). Once activated, RhoA, RhoC proteins mediate cell motility and tumor invasion, whereas RhoB is rather a migration-inhibiting protein, counteracting the action other Rho proteins (9). Rho proteins were reported to influence YAP subcellular localization, but the links connecting RASSF1A, YAP, and Rho signaling network remain elusive in human cells (5).

We previously reported that patients with stage I–II NSCLC and RASSF1A promoter gene methylation displayed a 3-fold decrease in 5-year overall survival, suggesting that RASSF1A inactivation could not only be involved in initial bronchial carcinogenesis, but...
A) RT-PCR

B) Western Blot

C) Immunofluorescence

D) 2D culture

E) ALI culture

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also in the acquisition of a metastatic phenotype, as patients ultimately die from metastasis spread (10). Here, we sought to decipher this putative RASSF1A specific role in the acquisition of invasiveness, by using RNAi to mimic RASSF1A loss of function in lung cancer cell lines, as in nontumorigenic, untransformed HBEC-3 bronchial cell lines (11). We found that RASSF1A knockdown, by inducing EMT and increasing cell motility and invasion capacities, favors in vivo lung metastasis process. Mechanistically, we reveal a connection between RASSF1A depletion, RhoB downregulation, and YAP activation, leading to the acquisition of tumorigenic and invasive hallmarks.

**Materials and Methods**

**Cell culture and transfection**

Isogenic HBEC-3 and HBEC-3-KRasV12 bronchial cells (Dr. White, UT Southwestern Medical Center, Dallas, TX; ref. 12), tumorigenic epithelial cell lines BEAS-2B, cancer-derived cell lines A549 and H1975 from ATCC, and BEAS-2B-KRasV12 from Dr. Pradines (9, 13) were grown in supplemented appropriate media (Supplementary Table S1), transfected at 30% confluence, using Lipofectamine RNAlMAX (Invitrogen) with siRNA, plasmid DNA, or control mimics (Dharmacon; Supplementary Tables S2 and S3). HBEC-3 and HBEC-3-KRasG12V were authenticated using standard karyotyping techniques as previously described (12). A549, H1975, BEAS-2B, were passaged for fewer than 6 months after receipt from ATCC. BEAS-2B cells transformed by KRasV12 by Dr. Pradines (UMR INSERM 1037-CRCT, Toulouse, France) were obtained by transfection with the pZIP–KRasV12 plasmid (9). KRas–mutant allele in HBEC-3-KRasV12 or BEAS2B-KRasV12 cells was checked by using a RT-PCR/RFLP method as described previously (12).

**RT-PCR**

After extraction, RT-PCR was done with each primer sets (Supplementary Table S4) as described previously (12). RT-PCR data were normalized to the human S16. Relative quantification was calculated using the ΔΔCt method.

**Antibodies and immunoblotting**

Antibodies against vimentin; ZO-1; β-catenin; Twist; Lats1; phospho-cofilin(Ser3); cofilin; phospho-LIMK(Thr508)/LIMK2 (Thr505); LIMK1; PDZK; ROCK1; Yap; RhoA; RhoB; GEFH1; phospho-GEFH1 (Ser858); calpain; PARP; phospho-PP1 (Thr505); LIMK1; Cell Signaling Technology); PP2AcAlpha (Millipore); and actin (Sigma-Aldrich), were used at 1:1,000 dilution. Whole cell protein extracts were prepared as described previously (14), and proteins detected by immunoblotting by enhanced chemiluminescence with ECL kit (Promega).

**Immunofluorescence and image analysis**

Immunofluorescence studies used E-cadherin and Yap primary antibodies from Cell Signaling Technology, syndecan-1 and syndecan-4 from R&D, CD44v6 from Santa Cruz Biotechnology, N-cadherin, and RASSF1A from ebioscience. The AlexaFluor555 and 486-labeled (Invitrogen) secondary antibodies were added for 1 hour. Coverslips were mounted with DAPI (Santa Cruz Biotechnology), and image captured with high-throughput confocal microscopy (FluoView FV1000, Olympus).

**Wound healing assay**

Transfected cells grown onto 24-well collagen IV or fibronectin-coated plates (BD Biocoat Matrigel Invasion Chamber) were pretreated with mitomycin C (1 μg/ml) 12 hours before an artificial "wound" created at 0 hour. Photographs were taken (×10) at 0 hour and 6 hours. The distances subtracted across the wound at 0 hour and 6 hours were expressed as μm/hour.

**Invasion**

A total of 25 × 10^3 cells in serum-free medium were added to the top invasion chambers of 24-well transwell plates containing cell culture insert with 8 μm pore size (BD BioCoat Matrigel Invasion Chamber, BD Biociences). Complete media were added to the bottom chambers. Cells were incubated 24 hours (migration assay) to 48 hours (invasion assay). Top (nonmigrating) cells were removed, bottom (migrating) cells were stained with crystal violet. Assays were performed in triplicate, and data presented as the average number of migrating/invasive cells.

**Cell adhesion**

A total of 2 × 10^4 transfected cells were seeded in 96-well plates coated with collagen IV or fibronectin (BD Biociences). After 1 hour incubation at 37°C, cells were washed and stained with crystal violet (30 minutes), washed again, and OD read at 590 nm.

**Soft-agar assay**

Base agar matrix (50 μL, Cell Biolabs) was seeded in 96-well plate and 3,000 cells/well layered on agar followed by 50 μL of 2× complete medium. Colonies were counted for each well 21 days later.

**Coimmunoprecipitation and GTP-Rho pull-down assay**

Cells were lysed in chilled immunoprecipitation buffer and the cleared lysate (500 μg) incubated with 3 μg of the indicated antibody and 30 μL of protein-A agarose beads (Repplagen) in 1 mL of IP buffer (13). Beads were resuspended in 30 μL of 2× Laemmli buffer and subjected to Western blotting. For GTP-Rho pulldown assays, cell lysates were incubated with beads glutathione-one-S-transferase (GST)–Rhotekin Rho binding domain (RBD) 45
minutes at 4°C and precipitated by Western blotting using RhoB or RhoA antibodies.

**ShRASSF1A, SCID mice xenograft, and transgenic mice**

The experiments were performed according to the European Convention for the Protection of Vertebrates Used for Scientific Purposes. Groups of 7, strain 250, 6-week-old, female Fox Chase SCID<sup>−/−</sup> Beige mice from Charles River Laboratory were injected subcutaneously with ShRASSF1A-777- or ShRASSF1A-779- infected HBE-3 or H1975 cell suspension (1 × 10<sup>6</sup> cells/0.1 mL) in the right flank of each animal (see Supplementary Table S3 for si sequences). Mice were monitored for tumor growth twice a week and tumors were allowed to grow to 1,000 mm<sup>3</sup> before euthanization of the mice. The postmortem examination included macroscopic description of lungs and liver. Tumor xenografts and lungs were rapidly removed and dissected into two parts: one fragment was snap-frozen in nitrogen for RNA extraction and qRT-PCR, the other fragment fixed in PFA 4% for histologic analysis. Lung-specific tetracycline-inducible human EGFR<sup>L858R</sup> bitransgenic mice (CCSP-rf1A; tetOEGR<sup>L858R</sup>) crossed into rhob null (rhob<sup>-/-</sup>), or wild-type (rhob<sup>+/-</sup>) strains, have been described previously (14). Five- to 6-week-old mice were fed ad libitum with several layers of enlarged elongated cells, and focal features of dysplasia, such as a significant increase of binucleated cells (6-fold vs. normal HBE-3 cells, P < 0.01, Fig. 1D). Electron microscopy, with quantification performed in three independent ALI cultures, with 50 to 70 cells for each condition (Supplementary Fig. S1), confirmed that RASSF1A-depleted cells exhibited a complete loss of tight junctions, a significant decrease in dense adherens junctions, replaced by more diffuse adherens structures (Fig. 1E, c, sRASSF1A and AC).

RASSF1A depletion induces epithelial to EMT

We examined the expression of several EMT markers in bronchial cell lines, after RASSF1A depletion (Fig. 2). In RASSF1A-depleted cells, several epithelial markers such as E-cadherin (Fig. 2A and E), miR-200b/miR-200c (Fig. 2B and C), syndecan-1 (Fig. 2F), and ZO-1 (Fig. 2I), decreased, whereas various mesenchymal markers, such as miR-21 (Fig. 2D), N-cadherin (Fig. 2G), syndecan-4 (Fig. 2H), CD44v6 (Fig. 2I), Twist1 (Fig. 2K), β-catenin (Fig. 2L), and vimentin (Fig. 2M) increased.

To ensure that the EMT induced by RASSF1A knockdown was not the result of a siRNA off-target effect, HBE-3 cells were cotransfected with si-RASSF1A and a siRNA-resistant RASSF1A-encoding expression plasmid. Significantly, restoration of RASSF1A expression (Supplementary Fig. S3A) in RASSF1A-knockdown HBE-3 cells decreased vimentin and Twist1 reexpression (Supplementary Fig. S3B).

RASSF1A knockdown causes invasiveness and cellular transformation of HBE-3 cells in vitro

EMT enhances tumor cell migration and invasion (16). To test whether RASSF1A deficiency induced an invasive phenotype in HBE-3 cells, we measured the expression of 84 genes involved in invasiveness (Supplementary Table S5). There was a significant reduction (>2-fold) in the expression of MMP9, mDia1, and RhoB, whereas MMP2, MMP14, RhoA, RhoC, RhoE, coflin, and integrinβ2 mRNAs increased (>2-fold) in RASSF1A-depleted HBE-3 cells. RASSF1A depletion could thus promote the acquisition of motile and invasive properties, by altering the Rho-family proteins expression, actin dynamic regulators, a cell-adhesion protein (ITGβ2), or matrix-metalloproteases. Accordingly, wound-healing assays revealed that RASSF1A-depleted HBE-3 cells migrated faster than controls (Fig. 3A). Similar results were observed when RASSF1A was reduced in several other untransformed and transformed bronchial cell lines (Supplementary Fig. S4), namely, HBE-3-Rasv12 (12), BEAS-2B-RasV12, or H1975 (Supplementary Table S1). This effect was independent of the adhesion substrate tested such as collagen I (Fig. 3A), collagen IV (Fig. 3A, Supplementary Fig. S4B), fibronectin (Supplementary Fig. S4B). HBE-3 two-dimensional migration induced by RASSF1A knockdown showed features evoking both collective migration and multicellular streaming (Supplementary Movie S1; ref. 16). Cell migration involves rearrangements of adhesion structures, and upon RASSF1A knockdown, talin, paxillin, moesin, and vinculin mRNAs expression levels actually decreased (Supplementary Fig. S5A). Consistently, RASSF1A depletion in HBE-3 cells reduced adhesion to laminin--(Supplementary Fig. S5B) and fibronectin-coated plates (Supplementary Fig. S5C). Thus, RASSF1A knockdown affects cell adhesion to substrate, cell–cell contacts (Fig. 1), EMT markers (Fig. 2), and cell motility (Fig. 3A). The siRNA impact on cell migration was specific as expression of a
Figure 2. RASSF1A depletion induces epithelial–mesenchymal transition. A–M, HBEC-3 cells transfected with siNeg or siRASSF1A. Experiments were performed 48 hours after transfection. A, representative images for E-cadherin stained ALI cultures. Scale bar, 20 µm. B–D, expression level of mir200b, mir200c, and mir21 using RNU48 as an internal control. E–I, quantification of E-cadherin, syndecan-1, N-cadherin, syndecan-4, and CD44v6 expression intensity by immunofluorescence, with DAPI for DNA. Scale bar, 50 µm. J–M, quantification of ZO-1, β-catenin, Twist1, and vimentin protein levels using actin as internal control. n = 3; *, P < 0.05; **, P < 0.01; and ***, P < 0.001, ANOVA followed by Dunnett test.
siRNA-resistant RASSF1A actually reversed the cell motility to normal levels (Supplementary Fig. S3C). The increased cell motility of RASSF1A-silenced cells translates into increased cell invasion capacity, as ascertained in matrigel invasion (Fig. 3B) and in transendothelial migration assays (Supplementary Fig. S4A). Similar results were observed upon RASSF1A depletion, in other bronchial cell lines (Supplementary Fig. S4B and S4C). The stimulating effect on migration of RASSF1A depletion was not rescued by cotransfection of a RASSF1C-encoding expression plasmid in HBE-3 cells, which conversely reversed the inhibiting effect on migration obtained by depleting RASSF1C by siRASSF1AC (Supplementary Fig. S3C). Besides, codepletion of both RASSF1A and RASSF1C using siRASSF1AC (Fig. 1C), had variable effects on cell migration, depending on substrate and cell types (Fig. 3, Supplementary Fig. S4). To further assess the differing requirements for RASSF1A and RASSF1C in migration, we used A549 cells with hypermethylated RASSF1A gene and no RASSF1A expression. In A549 cells, restoring RASSF1A expression, or depleting RASSF1C by siRASSF1AC, decreased cell invasion, whereas overexpression of RASSF1C increased cell migration through Matrigel (Fig. 3D).

We next evaluated anchorage-independent growth capacities of HBE in soft-agar colony formation assays (Fig. 3C). RASSF1A depletion provided HBE-3 and HBE-3 RasV12 cells anchorage-independent growth, whereas control HBE-3 and HBEC3 RasV12 cells failed to form colonies in soft agar (Fig. 3C and Supplementary Fig. S4D). The anchorage-independent growth of BEAS-2B was also stimulated by RASSF1A depletion (Supplementary Fig. S4D).

RASSF1A loss confers metastatic properties to H1975 cells in vitro

We infected tumorigenic EGFR-mutated lung cancer H1975 cells expressing wild-type RASSF1A, and untransformed HBE-3 cells, with lentivirus expressing shRNAs targeting RASSF1A (17). qRT-PCR (not shown) or Immunofluorescence analysis (Supplementary Fig. S6A) confirmed that RASSF1A expression decreased by 30% to 50% with either shRNA construct. Despite this modest silencing, shRASSF1A cells exhibited significantly increased migration, invasion, and growth in soft agar when compared with control cells (Supplementary Fig. S6B and S6C). When injected in SCID mice, normal and RASSF1A-depleted HBE-3 cells failed to form tumors after 6 months of observation (not shown), suggesting that RASSF1A depletion alone does not support xenograft growth. Conversely, parental tumorigenic H1975 showed the first signs of tumors at subcutaneous injection sites 12 to 21 days after injection, whereas tumors formed by RASSF1A-depleted H1975 cells emerged as soon as 9 days after injection (Fig. 4A). Tumor growth was significantly accelerated (2.8-fold) upon RASSF1A knockdown compared with controls (Fig. 4A). Using qRT-PCR (not shown) and Western blotting, we confirmed that shRASS1A-infected primary tumors still exhibited a significant decrease of RASSF1A (Fig. 4B). Finally, RASSF1A-depleted H1975 cells formed significantly more lung metastatic tumor foci than native cells (Fig. 4C and D), confirming that increased cell migration and cell invasion induced by RASSF1A loss, led in vivo to increased metastatic potential.

YAP mediates the EMT-like phenotypes induced by RASSF1A knockdown

The transcriptional coactivator YAP is involved in EMT regulation and connected with RASSF1A (18, 19). Indeed, upon RASSF1A depletion, we observed an increased expression of ITGB2, a YAP-transcriptional target (Supplementary Table S5; ref. 20). We further showed that active nuclear YAP was significantly increased upon RASSF1A depletion (>2-fold; Fig. 5A and B) as shown by nuclear/cytoplasmic fractionation experiments (Fig. 5A), immunofluorescence studies (Fig. 5B), and the concomitant upregulation of two other YAP target genes (6), besides ITGB2 (Supplementary Table S5): ANKRDI and CTGF (Fig. 5C). RASSF1A siRNA transfection also induced YAP nuclear accumulation in ALI HBEC-3 cultures (Fig. 5D). A time-course evaluation of YAP nuclear accumulation upon RASSF1A depletion further showed an increase of nuclear YAP, as soon as 12 hours posttransfection (Supplementary Fig. S7), when 30% decrease in RASSF1A protein content was already detectable (not shown), whereas both nuclear and cytoplasmic YAP were detected from 24 hours to 36 hours posttransfection. Cotransfection of siRNA-resistant RASSF1A (Supplementary Fig. S3D) or Yap-RNAi (Fig. 5B) reverted this phenotype.

The simultaneous silencing of RASSF1A/YAP reverted (i) the EMT-like phenotype due to RASSF1A deficiency [epithelial marker syndecan-1 (Fig. 5E) and mesenchymal vimentin (Fig. 5F) were reverted to normal levels as observed in controls], (ii) the increased cell invasion (Fig. 5G), but failed to prevent colony formation of RASSF1A-depleted HBEC-3 (Fig. 5H). YAP seems thus to be crucial for the EMT-like and cell migration/invasion phenotypes in RASSF1A-depleted cells, but not for anchorage-independent growth of RASSF1A-depleted cells.

RASSF1A controls activation of the coflin pathway

The coflin pathway is a key regulator of actin dynamics (21). Coflin mRNA expression increased upon RASSF1A depletion (Supplementary Table S5) and coflin phosphorylation significantly decreased, suggesting RASSF1A depletion increased coflin activity (Fig. 6A). In support of this finding, active phospho-LIMK, which suppresses coflin activity, was reduced, contrasting with the increase in global LIMK (Fig. 6A), whereas expression of the coflin-specific phosphatases PDXP/chronophin (22) increased (Fig. 6A). In addition, expression of Rock1, the kinase activating LIMK (23) was also reduced (Fig. 6A), whereas activation of Lats1 kinase, which is able to inhibit LIMK was increased as mentioned above. To our knowledge, these findings provide the first in vitro evidence that RASSF1A regulates the coflin pathway.

Figure 3.
RASSF1A depletion increases cell migration, invasion, and anchorage-independent growth. A–C, HBEC-3 cells transfected with siNeg or –RASSF1A. Experiments performed 48 hours after transfection. A, migration speed (μm/h) of transfected HBEC-3 cells assessed by the wound repair assay. Scale bar, 80 μm. B, invasion capacity of transfected HBEC-3 cells on BioCoat Matrigel Invasion Chamber. Relative invasion normalized to that of the cells transfected with siNeg. Scale bar, 80 μm. C, colony quantification of transfected HBEC-3 cells grown in soft agar. Scale bar, 20 mm. n = 3; **, P < 0.01; and * *, P < 0.001, ANOVA followed by Dunnett test. D, invasion capacity of A549 cells transfected with siNeg, si-RASSFIAC, RASSF1A, or RASSF1C on BioCoat Matrigel Invasion Chamber. Relative invasion normalized to that of the cells transfected with siNeg. Scale bar, 80 μm.
RhoB GTPase blocks invasiveness induced by RASSF1A depletion

LIMK controls cytoskeleton dynamics by regulating Rho family GTPases (24). We described (Supplementary Table S5) that mRNA expression of some Rho proteins was either upregulated (RhoA, RhoC), or downregulated (RhoB), upon RASSF1A depletion. We focused on RhoB because of its antimigratory role (9). We first confirmed the decrease of RhoB mRNA upon RASSF1A depletion using different RhoB mRNA primer pairs (Fig. 6B). We then checked whether reduced RhoB mRNA expression upon RASSF1A depletion led to a decrease of active GTP-bound RhoB protein. We actually found a significant decrease of endogenous active RhoB-GTP in RASSF1A-depleted HBEC-3 cells (Fig. 6C), but not of endogenous RhoA-GTP levels (Supplementary Fig. S8A). Expression of siRNA-resistant RASSF1A or constitutively active RhoBV14 restored normal RhoB-GTP levels (Fig. 6C).

Significantly, coexpression of siRASSF1A with wild-type RhoB, or constitutively active RhoBV14, strongly reduced HBEC-3 cells invading capacity, whereas the dominant-negative RhoBN19 had no effect (Fig. 6D). Concurrent RASSF1A and RhoB depletion, as RhoB depletion alone, also increased HBEC-3 cells invasion (Supplementary Fig. S9A). Conversely, RhoA wild-type or RhoAV14 failed to inhibit invasion of RASSF1A-depleted HBEC-3 cells expressing shRASSF1A or shRNA-control. D, excised mice lungs and histologic photographs of lung metastases after subcutaneous injection with HBEC-3 cells expressing shRASSF1A or shRNA-control. Scale bar, 40 μm. *, P < 0.05; **, P < 0.01; ***, P < 0.001. ANOVA followed by Dunnett test.

To understand mechanistically how RASSF1A knockdown affected RhoB-GTP levels, we examined three well-defined Rho-GFs (including RhoA-specific GEFs), strongly inhibited invasion of RASSF1A-depleted HBEC-3 and HBC3-RasV12 cells (Supplementary Fig. S8B). By using Narciclasine, a Rho activator (25), in RASSF1A-depleted HEBC-3 cells, we revealed that Rho proteins sustained activation impaired cell migration and invasion. As we had showed that active RhoAV14 had no effects on cell migration upon RASSF1A depletion, the Narciclasine effect could then mainly due to RhoB reactivation (Supplementary Fig. S8C). Collectively, our results suggest that RASSF1A depletion specifically alters RhoB-inhibiting functions on migration.

Figure 4. RASSF1A depletion increases the metastatic potential of H1975. ShRASSF1A-777- or shRASSF1A-779-infected HBEC-3, or H1975 cell suspension (1 × 10^7 cells in 0.1 mL) were injected subcutaneously in female Fox Chase SCID /Beige mice. A, xenograft tumor size [length (L)/width (W)/thickness (E)] monitored twice a week. The carcinoma is the mean ± SEM of the air under the curves of xenograft size over time variation from each mouse from each group measured using GraphPad software. B, representative xenografts obtained after subcutaneous injection of sh, shRASSF1A777, or shRASSF1A778 cells. C, quantification of lung microscopic nodules metastases, for xenografted H1975 cells expressing shRASSF1A or shRNA-control. Scale bar, 40 μm. *, P < 0.05; **, P < 0.01; ***, P < 0.001. ANOVA followed by Dunnett test.
further supporting a role for RhoB inactivation in RASSF1A depletion-promoted cell invasion. Given that (i) murine GEFH1 was reported as binding partner of RhoBV14 in a Yeast two-hybrid screen (32), (ii) GEFH1 exhibits a Rho-specific activity in vitro, but is unable to catalyze GDP exchange on Rac (33), and (iii) GEFH1 was identified in dendritic cells as the major protein associated and colocalized with the upregulated RhoB, upon lipopolysaccharide stimulation (29), we focused on GEF-H1.

We found that RASSF1A knockdown increases Ser885-phosphorylation and inactivation of GEFH1 (Fig. 6G). Moreover, similar to RASSF1A depletion (Fig. 6H), GEFH1 knockdown in HBEC-3 cells decreased the activated RhoB-GTP level (Fig. 6H). Conversely, GEFH1 overexpression in RASSF1A-depleted cells increased RhoB-GTP levels (Fig. 6H). Significantly, GEFH1 depletion alone increased the HBEC-3 cells invasion (Fig. 6I), and codepletion of RASSF1A and GEFH1 additively elevated cell

Figure 5.
YAP mediates the EMT-like properties of RASSF1A-depleted HBEC-3 cells. A–I, HBEC-3 cells transiently transfected with siNeg or siRASSF1A in combination or not with siYap. Experiments performed 48 hours after transfection. A, quantification of Yap in total/cytosolic or nuclear fraction of HBEC-3 cells. B, quantification of YAP nuclear localization by immunofluorescence with DAPI for nucleus. Scale bar, 50 μm. C, ANKDR1 and CTGF mRNA quantification using actin as an internal control. D, representative images for Yap-stained ALI cultures. Scale bar, 20 μm. E, syndecan-1 expression quantification by immunofluorescence with DAPI for nucleus. Scale bar, 50 μm. F, vimentin protein levels quantification using actin as internal control. G, RASSF1A/yap depleted HBEC-3 cells invasion normalized to that of the cells transfected with siNeg. H, colony quantification of transfected HBEC-3 cells grown in soft agar. Scale bar, 20 μm. n = 3; *, P < 0.05; **, P < 0.01 and ***, P < 0.001, ANOVA followed by Dunnett test.
Figure 6.
Cofilin and RhoB contribute to the EMT-like phenotype induced by RASSF1A knockdown in HBEC-3 cells. A–M, HBEC-3 cells transfected with siNeg or siRASSF1A in combination or not with indicated siRNA or plasmid. Experiments performed 48 hours after transfection. A, quantification of phospho-cofilin/cofilin, PDXP, phospho-LIMK/LIMK, Rock1. Protein levels normalized using actin as internal control. (Continued on the following page.)
The opposite of RhoA effect, as overexpression of RhoAwt or RhoAV14 significantly increased nuclear YAP, whereas DNRhoAN19 plasmid transfection did not significantly alter YAP localization, but drastically reduced nuclear YAP in RASSF1A-depleted cells (Supplementary Fig. S8D). Conversely, YAP depletion could increase RhoB-GTP active form (Fig. 7E), suggesting a specific reciprocal interaction between RhoB and YAP. Significantly, RhoB depletion alone as codepletion of RhoB and RASSF1A also increased cell invasion (Supplementary Fig. S9A). This final set of data suggests that the RASSF1A–GFP–H1–RhoB axis not only controls cell motility and shape, but also regulates the cytoplasmic nuclear shuttling of YAP. We raised the question whether such YAP/RhoB regulation loop can occur in vivo by analyzing YAP nuclear expression in a previously described transgenic mouse model. This model was obtained by crossing rhoB wild-type or null mice, with mice prone to develop lung neoplastic changes through an inducible lung-specific EGFRL858R mutation (14). These RhoB-deficient mice develop focal or diffuse epithelial hyperplasia, atypical adenomatous hyperplasia, multifocal lung adenomas, and adenocarcinomas. Here, we intended to analyze 10 tumors from each of 5 rhoB−/− mice, and 10 tumors from each of rhoB+/- mice, that the RhoB-deficient mice exhibited 2.3-fold more tumors with strong nuclear YAP staining (54.2% vs. 23.4%, P < 0.05; Fig. 7D). Collectively, these findings support a novel function for RASSF1A in controlling the level of active RhoB through regulating the activity of its exchange factor GEF-H1, and further identify RhoB as a main determinant for regulation of YAP cytoplasmic nuclear shuttling.

Discussion

Here, we demonstrate a critical role for RASSF1A in the bronchial epithelial phenotype maintenance, reporting that RASSF1A is required to restrict EMT, cell migration, cell invasion, and metastasis of HBECl lines. To our knowledge, this work is the first to describe RASSF1A as a metastasis suppressor in lung carcinogenesis. Such promigratory effect could be attributed in part to the increase of YAP transcriptional activity in RASSF1A-depleted cells. YAP promigratory effects have been already reported in ovarian cancer cells treated by LPA (34) or in Drosophila border cells migration (35). In line with such data, we report here that RASSF1A knockdown promotes a nuclear accumulation of active YAP. A regulation of YAP has been previously reported through a Trio/Rho/Rac signaling circuitry, downstream Cdc42 activation (36). ECM rigidity and mechanical stress were also shown to regulate YAP transcriptional activity, via the tension the actomyosin cytoskeleton (37), and a Rho GTPase activity (6). However, the specific Rho protein involved in such process remained unknown (38).

(Continued)

RhoB modulates nuclear localization of YAP

Finally, we wondered whether the effects on YAP and RhoB signaling, upon RASSF1A depletion, were connected. We tested whether manipulation of RhoB levels/activity would alter YAP cytosol to nucleus translocation. Expression of dominant-negative RhoBN19 increased active YAP nuclear accumulation (Fig. 7A). Similar results were obtained with siRNA-RhoB (Supplementary Fig. S9B). Expression of RhoB-WT, RhoBV14 or individual expression of the three Rho-GEFs (Fig. 7C) suppressed nuclear accumulation of YAP upon RASSF1A knockdown. Expression of constitutively active RhoBV14 mutant, or RhoB wild-type alone, decreased YAP transcriptional activity, and was able to counteract YAP activity increase in RASSF1A-depleted cells, on the transcription of both ANKRD1 and CTGF genes (Fig. 7B). The RhoB influence on YAP nuclear accumulation and activation was
Figure 7.
RhoB activity modulates nuclear localization of Yap. A–C, HBEC-3 cells transfected with siNeg or siRASSF1A-1 in combination with A and B plasmids coding for RhoB, RhoBN19, or RhoBV14 (C) plasmids coding for Ect2, GEFH1, Vav2. Experiments were performed 48 hours after transfection. YAP nuclear localization (A and C) assayed by immunofluorescence, and YAP activity (B) by quantifying CTGF and ANKDR1 mRNA expression. Scale bar, 50 μm. n = 3; *, P < 0.05; **, P < 0.01; and ***, P < 0.001, ANOVA followed by Dunnett test. D, representative staining (right) and quantification of nuclear YAP intensity (left) in lung tumors of rhob+/+ (n = 5) and rhob−/− (n = 5) mice. E, active RhoB-GTP bound form quantification upon RASSF1A depletion or RASSF1A and YAP codepletion. F, model for RASSF1A/RhoB/GEFH1 multiprotein complex regulating YAP shuttling.
We previously reported that RASSF1A depletion dramatically altered tubulin cytoskeleton (10) as previously reported in A549 cells (39). We also found that pachitaxel, a tubulin-interacting drug, actually impaired cell invasion induced by RASSF1A depletion (data not shown). We show here that RASSF1A could alter cell migration by specifically activating actin cytoskeleton-regulating coflin. Besides, in our hands, coflin depletion reduced the RASSF1A-induced YAP nuclear localization, whereas Aragona and colleagues previously reported that YAP/TAZ nuclear localization and activity were upregulated upon coflin depletion in dense cells, grown on stiff substrate ECM (37). This discrepancy could result from coflin different functions according to different contexts: mechanotransduction and RASSF1A-dependent transformation. Aragona and colleagues showed coflin was required to limit YAP/TAZ activity in cells experiencing low mechanical stress (low density or soft substrate), while we show here that coflin is required to increase nuclear YAP in cells lacking RASSF1A expression.

Our data strongly suggest that among Rho actin–regulating proteins, RhoB could be targeted by RASSF1A and lead to the activation of the actin-depolymerizing factor coflin. Three main results led us to this hypothesis. First, in RASSF1A-depleted cells, RhoB mRNA expression is decreased, which paralleled a decrease of active RhoB-GTP protein. Second, overexpressed RhoB increased inactive phospho-coflin, whereas a dominant negative, nucleotide-free, RhoBN19 mutant, did not impact coflin regulation. Third, we showed that cell motility induced by RASSF1A depletion could be efficiently inhibited by GEFs activating RhoB, while dominant negative RhoBN19 mutant or siRhoB were able to increase cell motility. Noteworthily, in our settings, overexpressing RhoB-GEF, GEF-H1, restrained RASSF1A-induced cell migration, whereas previous studies had assigned a promigratory role to GEF-H1 (29, 40). Conversely, GEF-H1 depletion (Fig. 6I), as RhoB depletion or RhoBN19 dominant-negative mutant transfection, increased HBEc-3 cells migration, in line with the cell spreading phenotype observed in PC3 cells depleted of GEF-H1 or RhoB (41). We confirmed that GEF-H1 was phosphorylated upon RASSF1A depletion, thus presumably inactivated (42). Mechanistically, RASSF1A depletion, by inducing a substantial decrease in PP2A activity, could downregulate GEF-H1 and subsequently RhoB activity (Fig. 7F). This model is consistent with a previous report showing PP2A and RhoB physical interaction (13), and we show here, that actually, RASSF1A levels can alter the composition of a multiprotein complex composed of GEFH1, RhoB, and PP2ACA. We report that overexpression of RhoB (wild-type or constitutively GTP-bound), inhibited the YAP nuclear accumulation upon RASSF1A depletion. Conversely, dominant-negative RhoBN19 or RhoB depletion led to YAP nuclear accumulation. Finally, in vivo, lung tumors observed in transgenic RhoB-deficient mice exhibited increased nuclear YAP. An opposite effect for RhoA and Rac was recently suggested, favoring YAP activation (36, 43). Thus, if control of YAP nuclear entrance was first linked to the promigratory RhoA/Rac proteins, we unravel here how RASSF1A and RhoB could act as cell migration and metastasis suppressors, by opposing to YAP nuclear entrance.

Conclusion

Our results enlightened that RASSF1A is a master-organizer of epithelial cell differentiation maintenance and migration-inhibiting genetic program. In lung cancer, RASSF1A inactivation by promoter methylation, could therefore contribute cancer invasiveness. The prognostic effect of RASSF1A methylation in early lung cancer could result from the removal of a physiologic program, involving RhoB and its regulator GEF-H1, restricting YAP activity and the ability to form distant metastases (Fig. 7F). Thus, YAP pharmacologic targeting in RASSF1A-methylated tumors, could be efficient in preventing metastasis spread, and improve lung cancer overall survival by delaying tumor progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Other (many discussions, interpretations of data, modelling): J. Camonis

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