

Rasip1 is a RUNX1 target gene and promotes migration of NSCLC cells

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Background: Runt-related transcription factor 1 (RUNX1), an essential regulator of hematopoiesis, is overexpressed in patients with nonsmall-cell lung cancer (NSCLC) and is correlated with enhanced metastatic ability. Ras-interacting protein 1 (*Rasip1*), a potential oncogene, is required for blood vessel formation, and recently, it has been shown that *Rasip1* is widely expressed in NSCLC patients. We noticed that *Rasip1* promoter contains several potential RUNX1-binding sequences. However, the relationship between *Rasip1* and RUNX1 in NSCLC is still unknown. In this study, the potential function of RUNX1 involving in *Rasip1* expression and the potential role of *Rasip1* in lung cancer cells were investigated.

Materials and methods: *Rasip1* and RUNX1 expressions were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and Western blotting in NSCLC cells lines. A549 and H1299 cells were transfected with plasmids or interfering RNA (siRNA) to upregulate or downregulate the expression of *Rasip1* and RUNX1. Cell motility was assessed by transwell and wound-healing assay. Location of *Rasip1* and RUNX1 was detected via immunofluorescence. Meanwhile, chromatin immunoprecipitation was done using an anti-RUNX1 antibody. *Rasip1* promoter was constructed, and cells were lysed for the analysis of luciferase activity.

Results: In this study, we showed that ectopic expression or knockdown of RUNX1 resulted in a significant increase or reduction in *Rasip1* expression, respectively. RUNX1 bound directly to a specific DNA sequence within *Rasip1* promoter and modulated its transcription. Furthermore, silencing of *Rasip1* inhibited the migration of RUNX1-overexpressing NSCLC cells through inactivation of Rac1 pathway. Moreover, we found that *Rasip1* was expressed ubiquitously in NSCLC cells lines and enhanced cell migration. In addition, EGFR signaling was involved both in the expression and the subcellular localization of *Rasip1*.

Conclusion: Our data indicated that *Rasip1* is regulated in part by the transcription factor RUNX1 and might be developed as a therapeutic target for NSCLC.

Keywords: *Rasip1*, RUNX1, EGF, migration, nonsmall-cell lung cancer

Introduction

Nonsmall-cell lung cancer (NSCLC) accounts for ~80% of all lung cancer cases, and its metastasis is a major cause of high mortality.¹ The EGFR tyrosine kinase inhibitors (TKIs) are an effective treatment for NSCLC patients with *EGFR*-activating mutations.² However, *EGFR* T790M and *K-Ras* mutations result in resistance to TKIs, which limit the effectiveness of chemotherapy.³ Therapies targeting Ras downstream effectors have been adopted in NSCLC patients.^{4,5} Thus, identification of novel Ras effectors as promising NSCLC therapeutic targets is needed.

Ras-interacting protein 1 (Rasip1), an emerging Ras effector, has been identified as the endothelial-restricted protein that contains a Ras-associating (RA) domain and a dilute (DIL) domain.⁶ A previous study indicated that Rasip1 is essential for vascular development and angiogenesis. Depletion of Rasip1 in mouse islet endothelial cells (MS1) inhibited angiogenesis and cell motility.⁷ Loss of Rasip1 in human umbilical vein endothelial cells impaired cell–cell attachment and increased basal permeability.⁸ In addition, elimination of Rasip1 in endothelial cell (ECs) reduced cell polarity, which was necessary for Rap1-induced cell spreading and endothelial barrier.^{9,10} Rasip1 mediated Cdc42 and Rac1 signaling during vascular tubulogenesis.⁹ Activated Rac1 is known to induce the lamellipodia formation or membrane ruffles, which plays a critical role for tumor metastasis.^{11,12} *Rasip1* has been found in the human lung tissue and NSCLC patients. However, the role of Rasip1 in NSCLC pathogenesis remains unknown.

Runt-related transcription factor 1 (RUNX1, also known as AML1), a member of the RUNX family, contains a conserved Runt domain that binds to core-binding factor subunit- β (CBF β) and specific DNA sequences (5'-TGTG-GTT-3'). RUNX1 is required for normal hematopoietic development, and the function of RUNX1 in leukemia is well established.¹³ It is now clear that RUNX1 plays an essential and paradoxical role in cancer development and progression. In hematopoietic diseases, RUNX1 mutations often lead to accelerated tumor development,¹⁴ whereas some level of wild-type (WT) RUNX1 activity is still necessary to promote the leukemogenic cell growth and survival.¹⁵ *RUNX1* has been identified as a downregulated gene in metastasis-prone solid tumors, acting as a tumor suppressor.¹⁶ However, the expression of RUNX1 is upregulated in patients with epithelial cancers and promotes tumor growth and metastasis.^{17–19} In lung adenocarcinoma, *RUNX1* is one of the significantly overexpressed genes and could be regarded as a biomarker for cancer diagnosis.^{17,20} Unfortunately, the RUNX1 target gene(s) in lung cancer is still unclear. To our interest, several potential RUNX1-binding sequences were found ubiquitously within *Rasip1* promoter. We are inspired to hypothesize that RUNX1 may act as a transcription factor of Rasip1.

In this study, we found that Rasip1 could enhance Rac1 activity and ERK phosphorylation, thereby promoting RUNX1-mediated migration in NSCLC cells lines. RUNX1 bound directly to *Rasip1* promoter and enhanced the expression of Rasip1. In addition, EGF could induce the plasma membrane translocation of Rasip1 and affect Rasip1 expression. These findings revealed RUNX1 as a transcriptional

regulator of Rasip1 and uncovered a critical role of Rasip1 in NSCLC metastasis.

Materials and methods

Cell lines and cell culture

Human BEAS-2B, MRC-5, H1299, A549, SPC-A-1, PC9 and NCI-H292 cell lines were purchased from the Cell Biology Institute of Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Biological Industries, Beit-Haemek, Israel) with 10% fetal bovine serum (FBS; Thermo Fisher Scientific, Waltham, MA, USA). The cells were serum-starved overnight, and then treated with EGF (R&D Systems, Inc., Minneapolis, MN, USA) and U0126 (Promega Corporation, Fitchburg, WI, USA) or CBF β -Runx1 Inhibitor II Ro5-3335 (EMD Millipore, Billerica, MA, USA) for various time periods as indicated before harvest.

Plasmids and siRNAs

Full-length cDNA of human Rasip1 was amplified from pDONRTM223-Rasip1 plasmid (Youbio, Hunan, China) using the following sequences: 5'-CGGAATTCGGCCAC-CATGCTGTCTGGTGAACG-3' (sense) and 5'-GGGG-TACCCAGGAGACGTGGCCACG-3' (antisense). *EcoRI*, *KpnI* restriction sites are underlined. The PCR products were cloned into the pEGFP-N1 vector (Clontech, Palo Alto, CA, USA). The primers for human *RUNX1* are as follows: 5'-CCGGAATTCATGCGTATCCCCGTAG-3' (sense) and 5'-CCGCTCGAGGTAGGGCCTCCACACG-3' (antisense). *EcoRI*, *XhoI* restriction sites are underlined. The PCR products were subcloned into the pCMV-C-HA vector (Beyotime, Nantong, China). A549 and H1299 cells were transfected with plasmids or siRNAs (GenePharma, Shanghai, China) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). The siRNA sequences targeting *Rasip1* are as follows: siRasip1-#1, 5'-GCAGCCUCUGUCAAGUCUUTT-3'; siRasip1-#2, 5'-CCAGGACUUUGUGGUGUAUTT-3'; and siRasip1-#3, 5'-GCGAACCUUGAAAUGUCACCT-3'. The siRNA sequences targeting *RUNX1* are as follows: siRUNX1-#1, 5'-CCAGGUUGCAAGAUUUAAUTT-3'; siRUNX1-#2, 5'-UCGAAGUGGAAGAGGGAAATT-3'; and siRUNX1-#3, 5'-GGCAGAAACUAGAUGAUCATT-3'. Those sequences targeting *RUNX2* are as follows: siRUNX2-#1, 5'-GGUCCUAUGACCAGUCUUATT-3'; siRUNX2-#2, 5'-CUCUGCACCAAGUCCUUUUTT-3'; and siRUNX2-#3, 5'-CCAGCCACCUUUACUUA-CATT-3'. Those sequences targeting *RUNX3* are as follows: siRUNX3-#1, 5'-UGACGAGAACUACUCCGCUTT-3';

siRUNX3-#2, 5'-CCUUCAAGGUGGUGGCAUUTT-3'; and siRUNX3-#3, 5'-CCCUGACCAUCACUGUGUUTT-3'.

Western blotting and quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Western blotting was performed as previously described.²¹ The cells were lysed in RIPA buffer containing phenylmethane sulfonyl fluoride (PMSF) and protease inhibitor cocktail. The cellular lysates were detected by Western blotting with antibodies against Rasip1, RUNX1, RUNX3 (Abcam, San Francisco, USA), ERK, phospho-ERK, HA, phospho-RUNX1 (Cell Signaling Technology, Danvers, MA, USA), Rac1 (EMD Millipore), Rasip1, RUNX2 (Protein Tech Group, Wuhan, China) and GAPDH (Thermo Fisher Scientific). Total RNA was extracted by TRIzol reagent (Thermo Fisher Scientific). The reverse transcriptase reaction was performed using a HiScript II Q RT SuperMix (Vazyme, Nanjing, China). The qRT-PCR was conducted using the ABI StepOne™ real-time PCR system (Thermo Fisher Scientific) with AceQ qPCR SYBR Green Master Mix (Vazyme). Primers for *Rasip1*, *RUNX1*, *RUNX2*, *RUNX3* and *GAPDH* are listed in Table 1.

Immunofluorescence microscopy

Twelve hours after serum starvation, cells were treated with or without 50 ng/mL EGF for 30 minutes. Cells were fixed in 4% paraformaldehyde for 20 minutes and then permeabilized with 0.1% TritonX-100 before blocking in 1% BSA for 1 hour. Cells were incubated with anti-Rasip1 (Abgent, San Diego, CA, USA) or anti-HA (Cell Signaling Technology) antibodies overnight at 4°C. After several washes, cells were then incubated with Alexa-coupled secondary antibody (Thermo Fisher Scientific) and FITC-phalloidin (Sigma-Aldrich Co., St Louis, MO, USA). DAPI (Southern Biotech, Birmingham, AL, USA) was added and incubated with cells before observation. Images were acquired with an Olympus BX51 microscope.

Rac1 activation assay

Activation of Rac1 was detected according to the method previously described.²² Briefly, GST-PAK-CRIB fusion proteins

were expressed in BL21 bacteria and captured on MagneGST Glutathione Particles (Promega Corporation). Equal concentrations of total cellular protein were incubated with fusion proteins at 4°C for 60 minutes to pull down GTP-Rac1. After incubation, beads were washed and resuspended in 2× SDS sample buffer and boiled. Rac1 activation was analyzed by Western blotting with anti-Rac1 antibody.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed using the ChIP Assay Kit (Upstate; EMD Millipore), as previously described.²³ Nuclear DNAs were sonicated to an average length of about <1,000 bp and incubated with RUNX1 antibody or normal rabbit IgG (Abcam). The DNA bound to the antibody was extracted by using TIANamp Genomic DNA Kit (Tiangen, Beijing, China) and confirmed by PCR. PCR products were analyzed on a 1% agarose gel. Primers used for the *Rasip1* promoter are as follows: 5'-ACTTGTGTTCTGGATC-TATGGGCCT-3' (sense); 5'-AGTACAGGGGTGAGAA-GATCACA-3' (anti-sense).

Luciferase report assay

The 2.1 kb human *Rasip1* promoter fragment was amplified by using PCR Phanta® Super-Fidelity DNA Polymerase (Vazyme) and cloned into pGL3 Basic vector (Promega Corporation). The four short fragments of the *Rasip1* promoter containing different numbers of RUNX family binding sites were cloned using a DNA fragment and subcloned into pGL3 Basic vectors. *KpnI* and *HindIII* restriction sites were underlined. The primers are listed in Table 2. Cells growing in 24-well plates were transfected with *Rasip1* promoter reporter plasmid (full length or short fragments) and RUNX1 expression plasmid (wide-type or mutation) by using lipofectamine 2000 for 48 hours. The plasmid pRL-SV40 (Renilla luciferase) was used as an internal control to normalize for transfection efficiency. Cells were lysed, and luminescence was measured on a GloMax 20/20 luminometer (Promega Corporation) using the Dual-Luciferase Assay Kit (Promega Corporation).

Table 1 Primers for *Rasip1*, *RUNX1*, *RUNX2*, *RUNX3* and *GAPDH*

Primers	Sense	Antisense
<i>Rasip1</i>	CCTCGTCTTGACTGGCTAC	TAGTGGCTGAGCAGATGGTG
<i>RUNX1</i>	CTGCCCATCGCTTCAAGGT	GCCGAGTAGTTTTTCATCATTGCC
<i>RUNX2</i>	TGGTTACTGTGCATGGCGGGTA	TTCAGATCGTTGAACCTTGCTA
<i>RUNX3</i>	AGGCAATGACGAGAACTACTCC	CGAAGTTCGTTGAACCTGG
<i>GAPDH</i>	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

Abbreviation: Rasip1, Ras-interacting protein 1.

Table 2 Primers for five sections of the *Rasip1* promoter

Primers	Sense	Antisense
2,115	GGGGTACCCTGTAATCGCAGCACTTTGG	CCCAAGCTTGGAAAAGGCAAGAGGAAACC
1,954	GGGGTACCCAGGAGTTCCAGACCAGCC	CCCAAGCTTGGAAAAGGCAAGAGGAAACC
1,581	GGGGTACCTCTCTACAGACCTCTGGGGA	CCCAAGCTTGGAAAAGGCAAGAGGAAACC
850	GGGGTACCCACGGGGTGTGGCTTCCT	CCCAAGCTTGGAAAAGGCAAGAGGAAACC
538	GGGGTACCCTGTAATCGCAGCACTTTGG	CCCAAGCTTGGAAAAGGCAAGAGGAAACC

Abbreviation: Rasip1, Ras-interacting protein 1.

Mutagenesis

The pCMV-C-HA vector containing RUNX1 and the fragments of *Rasip1* promoter were used as templates. Mutations were generated by site-directed mutagenesis using the Fast Mutagenesis Kit V2 (Vazyme). All mutagenic primers are listed in Table 3.

Wound-healing assay

Twenty-four hours after transient transfection, wound-healing assay was carried out. Briefly, the cell monolayer was grown to confluence and scratched with a sterile P200 pipette tip. The cell monolayers were rinsed with PBS to remove nonadherent cells, and new medium was added. Images were collected immediately by an inverted microscope (Carl Zeiss Meditec AG, Jena, Germany). Cells were also visualized after 12 hours of incubation at 37°C for comparison of wound healing.

Transwell assay

After 24 hours of transfection, cells were isolated and suspended in DMEM without FBS. 4×10^4 cells were added in the upper chamber of the Transwell (EMD Millipore). The lower chamber was filled with 600 μ L of DMEM with 10% FBS. The cells were allowed to migrate for 12 hours to reach the lower surface of the membrane. Then, the cells were fixed in 4% formaldehyde and stained with 0.1% crystal violet. Nonmigrated cells were scraped off using a cotton swab, and migrated cells were counted under an inverted microscope (TS100; Nikon Corporation, Tokyo, Japan).

Statistical analysis

All experiments were repeated at least three times. All data are presented as mean \pm SD. Statistical analysis was performed using the Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Differences between two groups were analyzed by Student's *t*-test. One-way ANOVA was used to compare the differences among multiple groups. $P < 0.05$ was considered statistically significant.

Results

Rasip1 is involved in migration of NSCLC cells

Rasip1 mRNA was widely expressed in human and mouse tissues, whereas the protein was found only in lung tissue.²⁴ We searched the Cosmic database and found that *Rasip1* mRNA expression was increased in many cancers including NSCLC. Although Rasip1 protein has been detected in A549 cell, which is well known to harbor mutation in K-Ras,²⁵ its protein expression profile in NSCLC cells lines has not been clarified. Therefore, we examined the expression of Rasip1 in a panel of NSCLC cells lines and found that *Rasip1* mRNA and protein were expressed ubiquitously, particularly in A549 and NCI-H292 cells (Figure 1A). We also noted that the expression of Rasip1 in H1299 was lowest among the NSCLC cells lines. Therefore, we chose A549 and H1299 cell lines for functional study. To investigate the effect of Rasip1 on migration activity in NSCLC cells lines, the plasmid encoding Rasip1 tagged with GFP (Rasip1-GFP) was used to transfect the cells, and the migration rate of cells was detected. The cells were transfected with control vector and Rasip1-GFP plasmid, respectively. Western blotting was performed to assess the transfection efficiency (Figure 1B), and the overexpression of Rasip1 enhanced the migration ability of A549 and H1299 cells (Figures 1C, D and S1C). Immunofluorescence was also performed to check the transfection efficiency (Figure S1A). Then, interfering RNA (siRNA) was used to knockdown Rasip1 expression in A549 and H1299 cells. siRasip1 efficiently abolished the expression of Rasip1 (Figures 1E and S1B), and silencing Rasip1 dramatically impaired the migration ability of cancer cell (Figures 1F, G and S1D). In addition, we found that Rasip1 had no obvious effects on proliferation in NSCLC cells lines (data not shown). Previous study has shown that the levels of activated Rac1 and phosphorylated ERK (p-ERK) were markedly decreased in siRasip1-depleted ECs.⁹ To identify whether Rac1 and ERK were involved in Rasip1-mediated lung cancer migration, A549 and H1299 cells were transfected with siRasip1 and Rasip1-GFP plasmid,

Table 3 Primers for R174Q, S249A, *Rasip1* promoter mutants 1–4

Primers	Sense	Antisense
R174Q	ATGGCCCCAAGAACCCTCGAAGACATCGGCAGAAACT	CGAGGTTCTTGGGGCCCATCCACTGTGATTTTGATGG
S249A	ATCCAACCAGCCCCACCGTGGTCTACGATCAGTCTT	ACGGTGGGGCTGGTGGATCTGCCTTGTATCCTGCATCTGACTC
<i>Rasip1</i> promoter	ATTAGGTGCTCTCTACAGACCTCTGGGACCCCGTG	TGTAGAGACGACACCTAATAGACCACCTCTCTGGGTCA
Mutant 1		
<i>Rasip1</i> promoter	ACGGGGTGTGCGCTTCTGCCGGGGCGTGGCCTCTGG	GCAGGAAGCGACACCCCGTGCCCCGAAACCACACCCCA
Mutant 2		
<i>Rasip1</i> promoter	#1: ATTAGGTGCTCTCTACAGACCTCTGGGACCCCGTG	#1: TGTAGAGACGACACCTAATAGACCACCTCTCTGGGTCA
Mutant 3	#2: GTTGGGTGCTCTCGGGCACGGGTGTGGCTTCTT	#2: CCCCAGAACGACACCCCAACAGGAAGTACGCCCCCAT
<i>Rasip1</i> promoter	#1: ATTAGGTGCTCTCTACAGACCTCTGGGACCCCGTG	#1: TGTAGAGACGACACCTAATAGACCACCTCTCTGGGTCA
Mutant 4	#2: ACGGGGTGCTGCGCTTCTGCCGGGGCGTGGCCTCTGG	#2: GCAGGAAGCGACACCCCGTGCCCCGAAACCACACCCCA

Abbreviation: Rasip1, Ras-interacting protein 1.

respectively. Deletion of Rasip1 in NSCLC cells lines reduced Rac1 activity and ERK phosphorylation, while overexpression of Rasip1 enhanced Rac1 activity and increased p-ERK level (Figure 1H). Taken together, these findings suggested that Rasip1 could mediate the migration of NSCLC cells by activating Rac1 and ERK phosphorylation.

RUNX1 is required for the expression of Rasip1

To explore molecular mechanisms involved in regulation of Rasip1 expression, we analyzed the *Rasip1* promoter by the JASPAR CORE database and noted that RUNX may be a putative transcription factor. By using the Evolutionarily Conserved Region Browser, we found that 2.0 kb of the *Rasip1* promoter region was highly conserved among various species including human, mouse, rat and zebrafish. We aligned multiple *Rasip1* promoter regions and identified many potential RUNX-binding sites (Figure 2A). In mammals, there are three members of the RUNX family, including RUNX1, RUNX2 and RUNX3. To determine which member of the RUNX family regulated the expression of Rasip1, we examined their mRNA and protein expressions in NSCLC cells lines. The expression of RUNX1 was significantly higher when compared with RUNX2 and RUNX3 (Figure 2B and C). Knockdown of RUNX1 in NSCLC cells lines remarkably reduced Rasip1 expression compared with siRUNX2 and siRUNX3 (Figure 2D and E). These data suggested that RUNX1 was essential for Rasip1 expression. The structure of RUNX1 common point mutations and the protein phosphorylation sites are indicated by the bars and asterisk (Figure 2F). To rule out potential nonspecific effects of RUNX1, two RUNX1 mutant (R174Q and S249A) plasmids were constructed. The R174Q mutant could still dimerize with CBF β , but reduce DNA binding ability and transcription function. The S249A mutant failed to be phosphorylated, and its transcriptional activity was impaired. In A549 and H1299 cells, overexpression of RUNX1 resulted in a significant increase in Rasip1 expression, at both mRNA (Figure 2G) and protein levels (Figure 2H). However, overexpression of the R174Q and S249A plasmids in NSCLC cells lines had no significant effect on Rasip1 expression, at the mRNA and protein levels. These results confirmed that RUNX1 could be a key transcription factor of Rasip1.

RUNX1 acts as a transcription factor of Rasip1

To elucidate the molecular mechanism by which RUNX1 regulates expression of Rasip1, a 2.1 kb *Rasip1* promoter

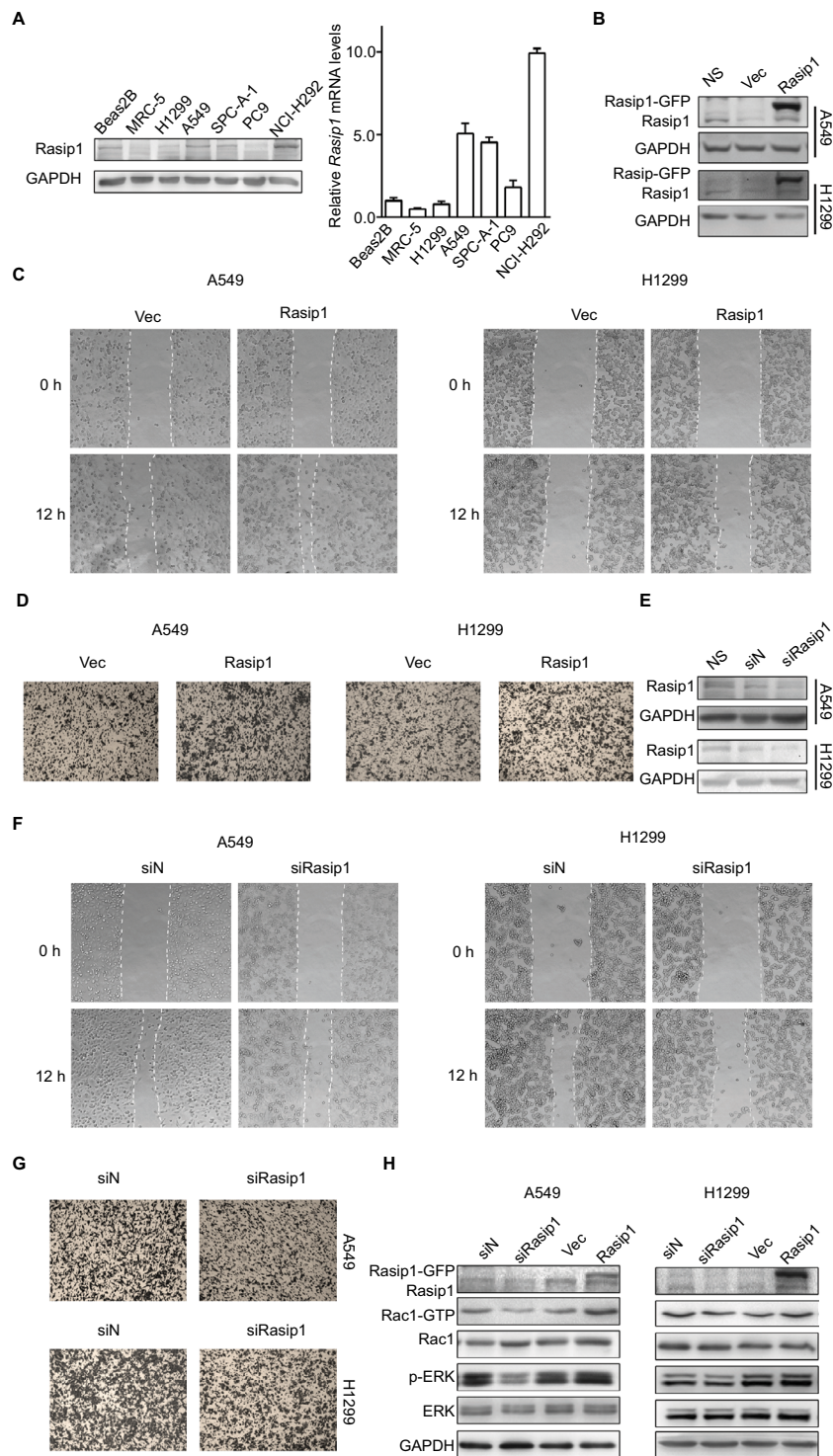


Figure 1 Rasip1 enhances migration of NSCLC cells.

Notes: (A) The endogenous expression levels of Rasip1 in NSCLC cell lines were analyzed by Western blotting and qRT-PCR. Rasip1 mRNAs are normalized to gene expression levels in a human bronchial epithelial cell line (BEAS-2B). MRC-5, human fetal lung fibroblasts, is also a normal lung cell line. (B) The pEGFP-N1 vector (control) and Rasip1-GFP plasmid were overexpressed in H1299 and A549 cells, respectively. Forty-eight hours after transfection, Western blotting was performed to detect the expression of Rasip1 and GAPDH. (C) After 24 hours of transfection, wound-healing assay was performed to evaluate the migration of the cells. The images were taken at the 0 and 12th hour by an inverted microscope. (D) After 24 hours of transfection, cell motility was measured by transwell assay. Cells were counted at time point 12 hours. (E) A549 and H1299 cells were transfected with negative siRNA (control) or Rasip1 siRNAs. Forty-eight hours after transfection, knockdown efficiencies were assessed by Western blotting. (F) At 24th hour after Rasip1 knockdown, wound-healing assay was done to evaluate migration ability. (G) At 24th hour after deletion of Rasip1, transwell assay was performed to evaluate cell motility. (H) A549 cells and H1299 cells were transfected with negative siRNA, Rasip1 siRNAs, pEGFP-N1 vector and Rasip1-GFP plasmid, respectively. After 48 hours of transfection, lysates were detected by Western blotting with antibodies against Rasip1, Rac1, p-ERK, ERK and GAPDH. The GTP-bound and total Rac1 were detected with anti-Rac1 antibody. All the experiments were repeated at least three times.

Abbreviations: h, hours; NSCLC, nonsmall-cell lung cancer; qRT-PCR, quantitative reverse transcription polymerase chain reaction; Rasip1, Ras-interacting protein 1; p-ERK, phosphorylated ERK.

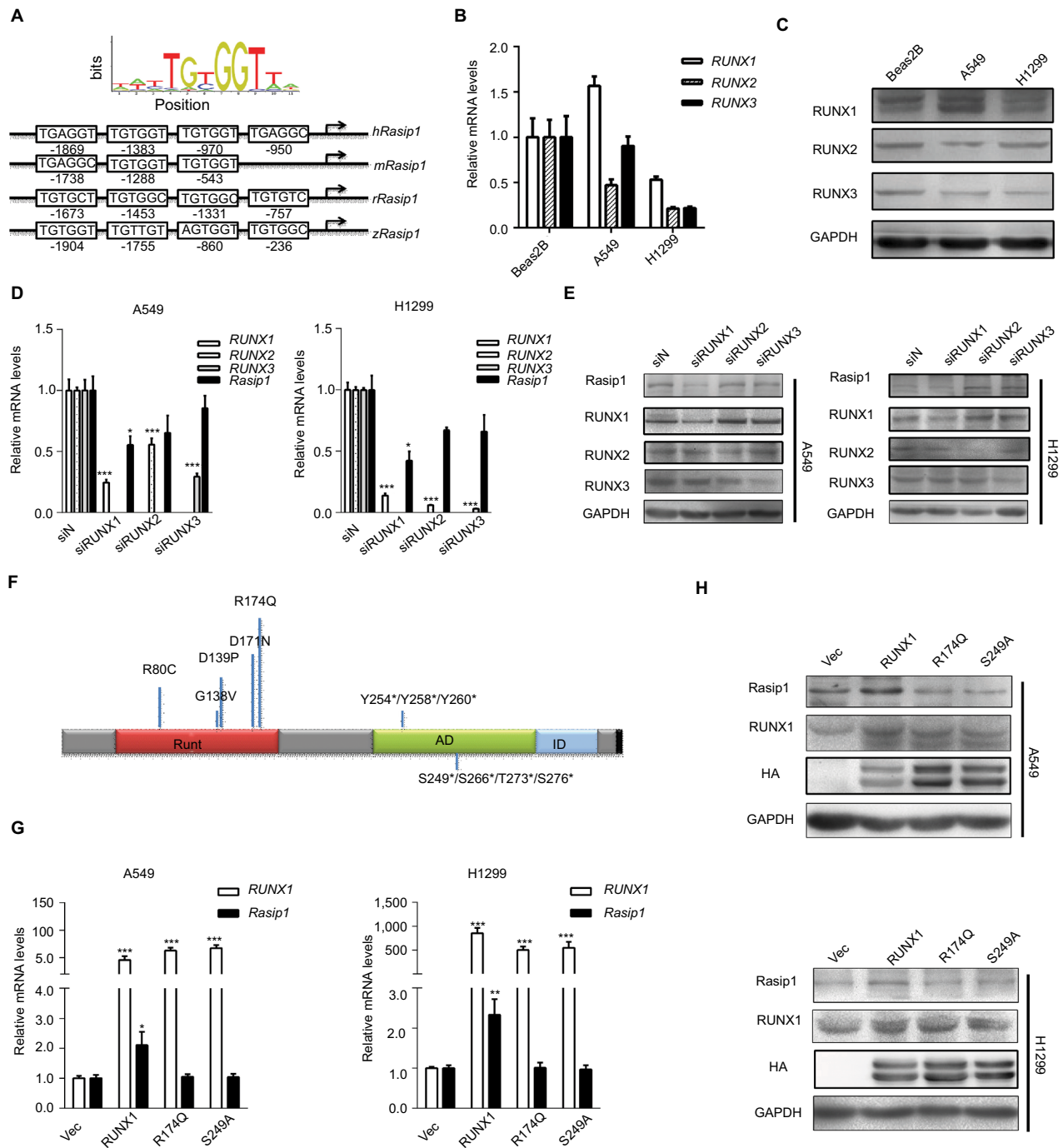


Figure 2 RUNX1 promotes Rasip1 expression.

Notes: (A) Schematic depiction of the sequence alignment of RUNX binding site from various species within the *Rasip1* promoter region. (B) The endogenous expression levels of *RUNX1*, *RUNX2* or *RUNX3* mRNA were measured by qRT-PCR in A549 cells and H1299 cells. Data are normalized to gene expression levels in BEAS-2B cell. (C) The *RUNX1*, *RUNX2* or *RUNX3* protein was measured by Western blotting in BEAS-2B, A549 and H1299 cells. (D) A549 cells and H1299 cells were transfected with negative siRNA, siRUNX1, siRUNX2 and siRUNX3, respectively. After 24 hours of transfection, qRT-PCR was performed to detect the expression of *Rasip1* mRNA. (E) After 48 hours of transfection, expression of *Rasip1* was detected by Western blotting. (F) The structure of *RUNX1* with somatic mutations at the RD and the protein phosphorylation sites. Numbers indicate positions of amino acid residues of *RUNX1* from the N terminus. Runt (the DNA- and CBF β -binding domain), AD (the activation domain) and ID (the inhibitory domain) are indicated. The bars above in each diagram represent the relative frequency of *RUNX1* point mutations in cancer, and the phosphorylation residues were indicated by the asterisk. (G) A549 and H1299 cells were transfected with *RUNX1*-HA, R174Q-HA, S249A-HA plasmid and pCMV-C-HA vector (control), respectively. *RUNX1* and *Rasip1* mRNA expression were analyzed by qRT-PCR. (H) Western blotting was performed by using anti-*RUNX1* and anti-*Rasip1* antibodies. All the experiments were repeated at least three times.

Abbreviations: h, hours; qRT-PCR, quantitative reverse transcription polymerase chain reaction; *Rasip1*, Ras-interacting protein 1; *RUNX1*, Runt-related transcription factor 1; RD, Runt domain.

was constructed and its transcriptional activity was detected. The 2.1 kb *Rasip1* promoter activity increased ~50% in RUNX1-overexpressed cells (Figure 3A). Moreover, the effects of RUNX1 on *Rasip1* promoter occupancy were tested by ChIP assay. Notably, an increased occupancy of RUNX1 in the region of *Rasip1* promoter was observed in EGF-treated cells (Figure 3B); these findings were consistent with the role of EGF in regulating RUNX1 transcriptional activity.²⁶ To define the key sequences in the *Rasip1* promoter required for transcription, a series of truncated promoters that contained putative RUNX1-binding sequences and the plasmid-encoding RUNX1 tagged with HA (RUNX1-HA) were constructed. 1,954 bp and 1,581 bp fragments had increased transcriptional activity (Figure 3C). There were three putative RUNX1-binding sequences in 1,954 bp fragment. To further identify the more important RUNX1-binding sites in the fragment, four mutation promoter constructs were generated (Figure 3D). The promoter activity dropped sharply to 30% when the -1,383 bp and -950 bp sites were both mutated (Figure 3E). Taken together, these observations indicated that RUNX1 could bind directly to *Rasip1* promoter to regulate its transcription.

To determine the molecular basis of the differences between RUNX1 and its mutants (R174Q and S249A) in regulating *Rasip1* expression, three truncated *Rasip1* promoters (1,954 bp and 1,581 bp) with high transcriptional activity were cotransfected with the RUNX1-HA or its mutant plasmids in NSCLC cells lines. The S249A mutant did not have a remarkable effect on the transcriptional activities of truncated promoters when compared with WT RUNX1. In contrast to a previous study,²⁷ the R174Q mutant still could enhance the transcriptional activity of three truncated promoters (Figure 3F). To explore how the R174Q mutant impairs its transcription function, immunofluorescence assay was performed. After EGF stimulation, the S249A mutant could be translocated from the cytoplasm to the nucleus, similar to WT RUNX1, whereas the R174Q mutant was still partially localized in the cytoplasm of transfected cells (Figure 3G). These results indicated that the R174Q disrupted its nuclear localization and consequently lost its transcription activity.

RUNX1 promotes lung cancer cell migration in a *Rasip1*-dependent manner

RUNX1 regulates a list of target genes and is linked to metastasis of lung adenocarcinoma.^{20,28} It has been reported that knockdown of RUNX1 in H1299 cell inhibited cell invasion.²⁹ To investigate whether *Rasip1* was involved in RUNX1-mediated cell migration, RUNX1-HA plasmid and

si*Rasip1* were cotransfected into NSCLC cells lines. RUNX1 remarkably increased the migration ability of NSCLC cells, which was reduced by depletion of *Rasip1* (Figures 4A and S2A). Similar results were obtained using the transwell assay (Figures 4B and S2B). To determine the functional impact of *Rasip1* on RUNX1-mediated cell migration, we examined the downstream molecules. Overexpression of RUNX1 in A549 and H1299 cells also increased the Rac1 activity, whereas si*Rasip1* treatment attenuated these changes (Figure 4C). Collectively, these data suggested that *Rasip1* might be a downstream mediator of RUNX1 in NSCLC cells lines.

EGF stimulates *Rasip1* expression and induces the plasma membrane translocation of *Rasip1*

EGFR is commonly overexpressed and mutated in NSCLC patients, resulting in an increased cell proliferation, survival and metastasis.² RUNX1 was phosphorylated by ERK at S249 and S266 residues, and its transcriptional activity was enhanced.²⁶ However, phosphorylated RUNX1 (p-RUNX1) was susceptible to degradation by the ubiquitin-proteasome pathway.¹³ To explore whether the expression of *Rasip1* is influenced by EGF stimulation, we treated NSCLC cells lines with EGF, and a time course of *Rasip1* expression was detected. EGF stimulation led to a rapid increase in the *Rasip1* expression. *Rasip1* mRNA increased to the first peak after 2 hours of EGF treatment and was followed by a decrease in its initial level after 4 hours (data not shown). ERK inhibitor (U0126) and RUNX1 inhibitor (Ro5-3335) completely suppressed EGF-induced *Rasip1* mRNA expression (Figure 5A). Subsequently, we found that *Rasip1* protein also increased after 2 hours of EGF stimulation (Figure 5B). In accordance with the previous studies, EGF induced the phosphorylation of RUNX1 at Ser249. Considering that the phosphorylation of RUNX1 promoted its degradation in protein level, we analyzed expression of total RUNX1 and found that it decreased after EGF stimulation. As we have mentioned earlier, *Rasip1* could increase the phosphorylation of ERK. Together, these results suggested that this complexity feedback may be a mechanism for keeping *Rasip1* overexpression in NSCLC cells lines.

Recently, we have reported that EGF could induce directional migration of NSCLC cells through regulating the localization of RUSC2.³⁰ Previous study has shown that *Rasip1* could bind directly to Ras proteins, which are the downstream effectors of EGFR signaling pathway.⁶ To investigate how *Rasip1* is regulated under EGF stimulation, we transfected NSCLC cells lines with *Rasip1*-GFP plasmid,

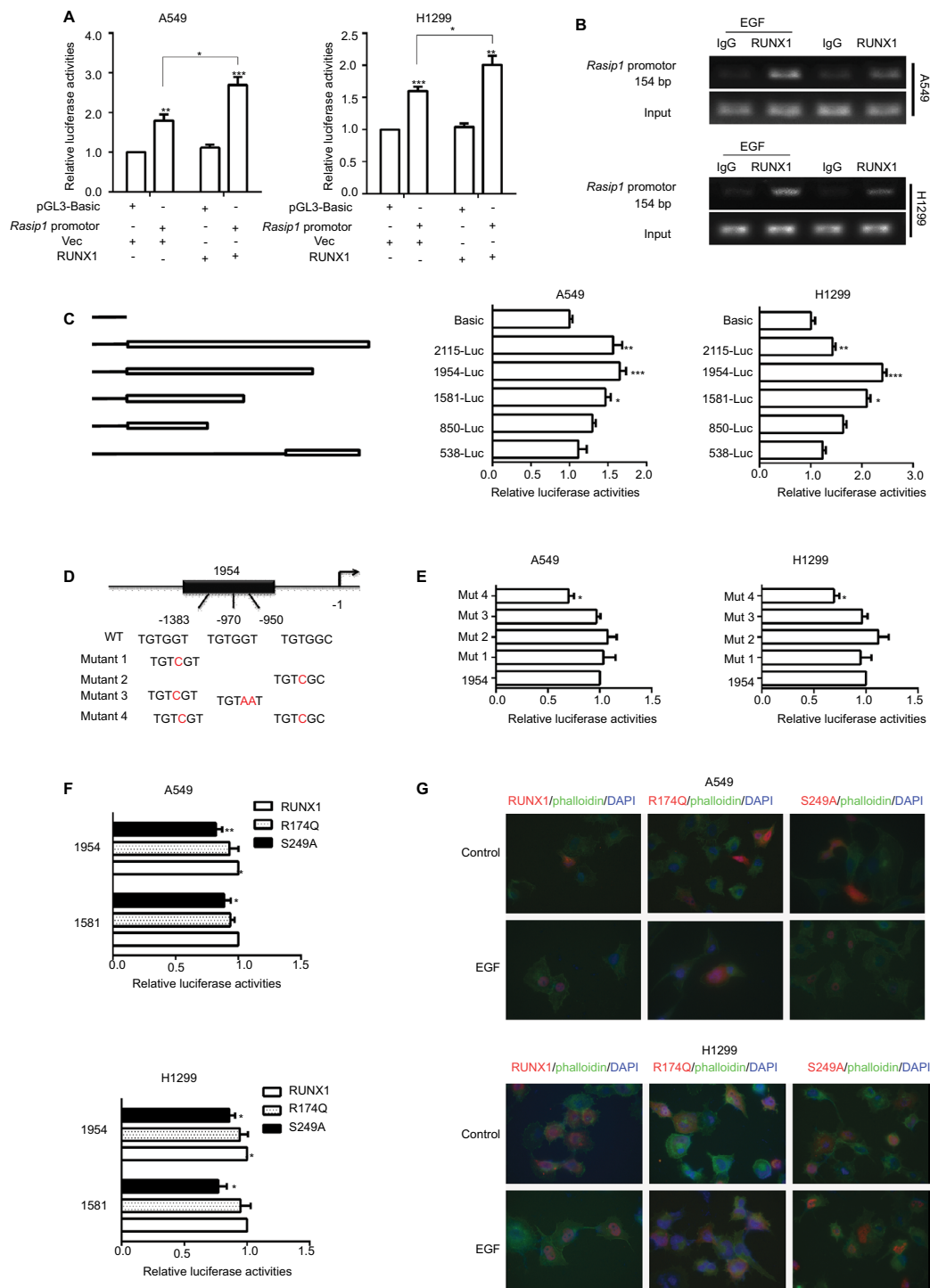


Figure 3 RUNX1 binds to *Rasip1* promoter and increases *Rasip1* transcription.

Notes: (A) A549 and H1299 cells were cotransfected with the pGL3-Basic (control) or Rasip1-Luc reporter and RUNX1-HA plasmid or pCMV-C-HA vector, respectively. Forty-eight hours later, cell extracts were analyzed for luciferase activity. (B) A549 and H1299 cells that were either treated or untreated with EGF (50 ng/mL) for 2 hours. ChIP was performed to analyze the RUNX1 occupancy within the *Rasip1* promoter. Normal rabbit IgG was used as negative controls. PCR amplification was performed using primers corresponding to the *Rasip1* promoter sequence. (C) Different alignments of the Rasip1-Luc reporter were illustrated to the right. A549 and H1299 cells were transfected with the Rasip1-Luc reporter and RUNX1-HA plasmid for 48 hours. Cells were lysed for the analysis of luciferase activity. (D) Schema of the 1,954 bp fragment with positioning of the putative RUNX1-binding sequences. (E) The putative RUNX1-binding site in 1,954 bp-Luc reporter was mutated by site-directed mutagenesis. H1299 and A549 cells were cotransfected with the mutagenized promoter constructs and RUNX1-HA plasmid for 48 hours and then analyzed for luciferase activity. The 1,954-Luc (WT) reporter was used as the control. (F) A549 and H1299 cells were cotransfected with 1,954 bp/1,581 bp-Luc reporter and the WT RUNX1-HA or mutants (R174Q-HA and S249A-HA) plasmid for 48 hours. Then, the cells were lysed to analyze luciferase activity. (G) A549 and H1299 cells were transfected with the RUNX1-HA, R174Q-HA or S249A-HA plasmid, respectively, and stimulated with EGF for 1 hour. Then, photos of the cells were taken and analyzed for the cellular localization of RUNX1. RUNX1/R174Q/S249A-HA, red; phalloidin, green and nucleus (DAPI), blue. All the experiments were repeated at least three times.

Abbreviations: Rasip1, Ras-interacting protein 1; RUNX1, Runt-related transcription factor 1; WT, wild type.

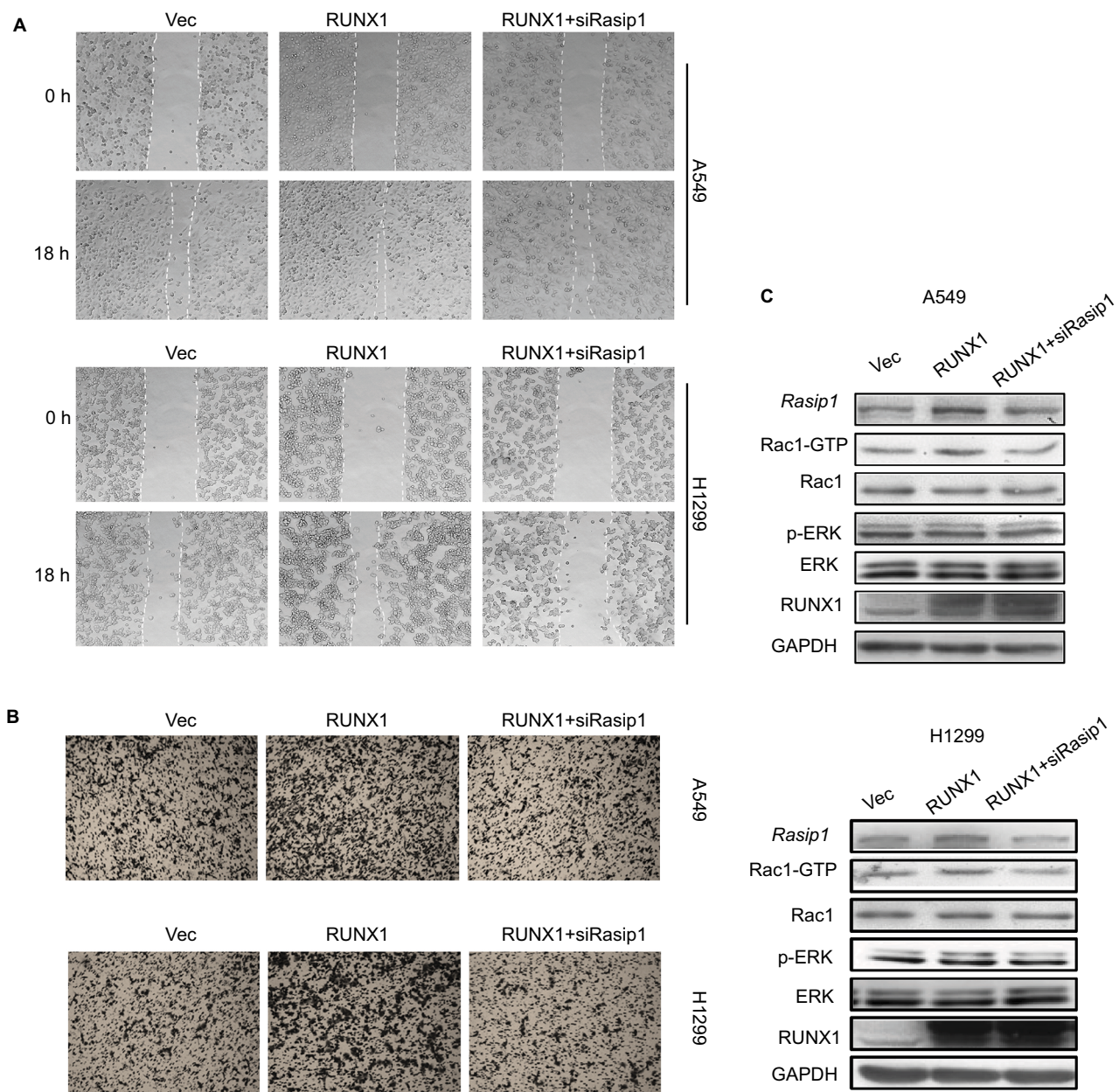


Figure 4 Deletion of Rasip1 blocks RUNX1-mediated migration.

Notes: (A) pCMV-C-HA vector or RUNX1-HA plasmid and negative siRNAs or Rasip1 siRNAs were cotransfected into H1299 and A549 cells, respectively. Wound-healing assay was performed, and images were taken at 0 and 18th hour by an inverted microscope. The empty vector was used as the control. (B) After 24 hours of transfection, transwell assay was performed, and cells were counted at the 12th hour after plating. (C) After 48 hours of transfection, cells were lysed and the lysates were detected by Western blotting for the Rasip1, Rac1, p-ERK, ERK, RUNX1 and GAPDH. The Rac1-GTP was pulled down by GST-PAK-CRIB fusion proteins. All the experiments were repeated at least three times.

Abbreviations: Rasip1, Ras-interacting protein 1; RUNX1, Runt-related transcription factor 1; p-ERK, phosphorylated ERK.

and the subcellular localization of Rasip1 was determined by immunofluorescence assay. Under resting conditions, Rasip1 was predominantly localized in the cytoplasmic compartment. After EGF stimulation, Rasip1 was recruited to the plasma membrane (Figure 5C). Our findings implied that EGF might activate downstream molecules, to form a complex with Rasip1, and mediate the translocation of Rasip1 to the plasma membrane.

Discussion

Metastasis is a very complex phenomenon, which is responsible for death of NSCLC patients. Targeting metastasis pathways is a potential new therapeutic strategy and has received more and more attention.³¹ However, the molecular mechanisms underlying metastasis in NSCLC were not fully known. From the database, we noticed that Rasip1, a pan-endothelium marker, was overexpressed in NSCLC patients.

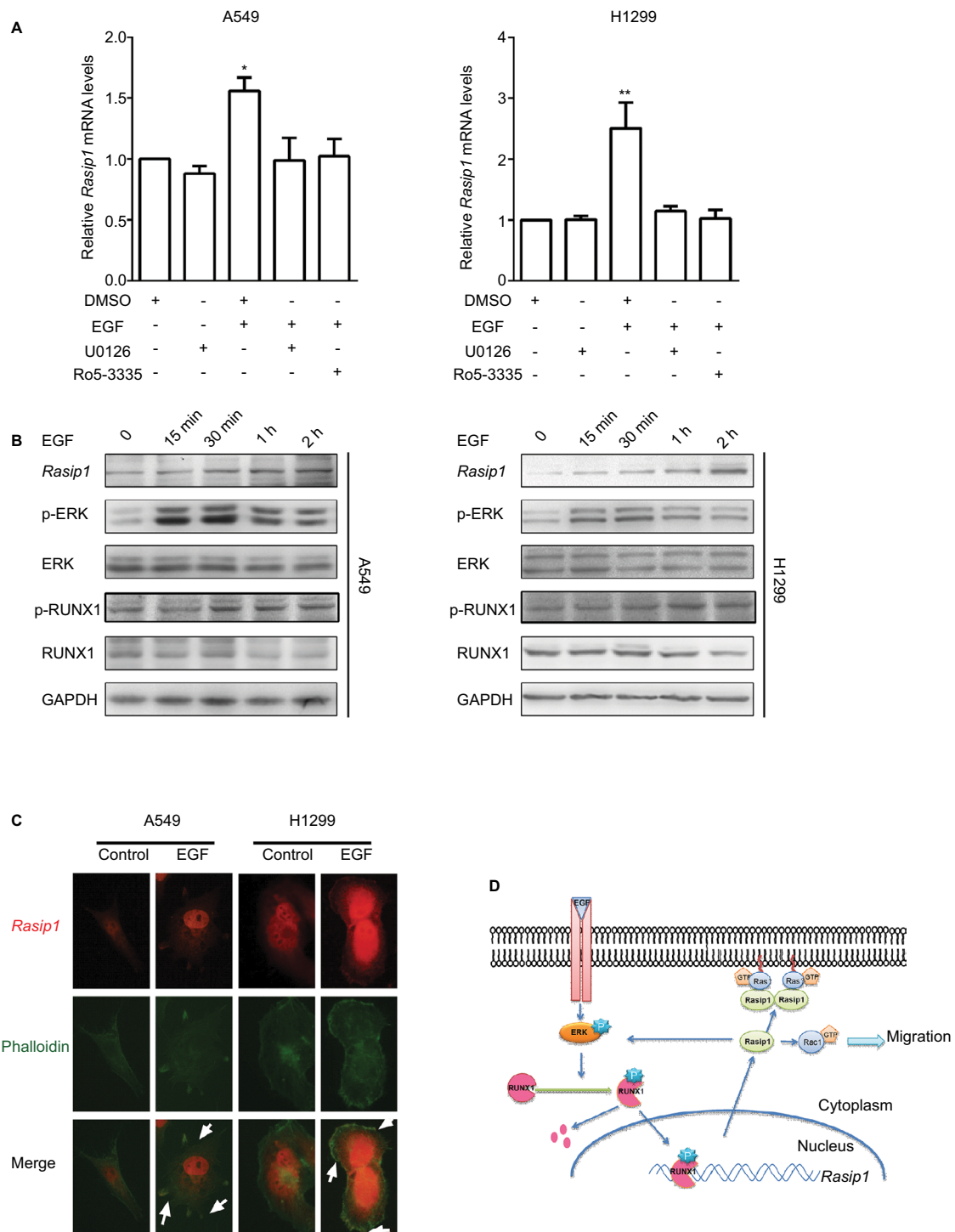


Figure 5 EGF regulates the expression and subcellular localization of Rasip1.

Notes: (A) A549 and H1299 cells were starved and were incubated for 2 hours with either 10 mmol/L U0126 or 25 mmol/L Ro5-3335 prior to EGF treatment (50 ng/mL for 2 hours). Total RNA was extracted, and the *Rasip1* mRNA was analyzed by qRT-PCR. (B) Serum-starved cells were stimulated with 50 ng/mL EGF (0, 15 minutes, 30 minutes, 1 hour and 2 hours), and lysates were detected by Western blotting for expression of Rasip1, p-ERK, ERK, p-RUNX1, RUNX1 and GAPDH. (C) A549 cells and H1299 cells cultured on coverslips were starved for 12 hours and treated or untreated with 50 ng/mL EGF for 30 minutes. The cells were fixed and incubated with anti-Rasip1 antibody followed by goat anti-Rabbit Alexa594 secondary antibody. The images were captured by an inverted immunofluorescence microscope. Original colors were Rasip1, red and phalloidin, green. Accumulation of Rasip1 on the plasma membrane was indicated by the arrow. (D) The schematic diagram illustrates the complex feedback of Rasip1 expression and the cellular localization of Rasip1 after EGF treatment. Activation of ERK by EGF stimulation promotes the phosphorylation of RUNX1 at Ser249, increases its transcription activity and, hence, induces its degradation. Phosphorylated RUNX1 binds to a specific DNA sequence within *Rasip1* promoter and increases its transcription. In turn, Rasip1 promotes Rac1 and ERK activation, thereby increasing cell migration. Together, this system formed a positive and negative feedback loop to regulate Rasip1 expression. With the extension of EGF treatment, Rasip1 could form a complex with Ras or other protein and be recruited to the plasma membrane. All the experiments were repeated at least three times. * $P < 0.05$ when compared to other groups. ** $P < 0.01$ when compared to other groups.

Abbreviations: h, hours; min, minutes; Rasip1, Ras-interacting protein 1; RUNX1, Runt-related transcription factor 1; qRT-PCR, quantitative reverse transcription polymerase chain reaction; p-ERK, phosphorylated ERK; p-RUNX1, phosphorylated RUNX1.

However, the expression and function of Rasip1 in NSCLC cells lines are still unknown. In this study, it was identified that Rasip1 was ubiquitously expressed in NSCLC cells lines and that it promoted cell migration through activation of Rac1 and ERK pathways. Rasip1 was colocalized with activated Ras proteins and initially identified around the Golgi apparatus.⁶ Recent study has highlighted a novel localization of Rasip1; Rasip1 activation in EC led to the translocation of Rasip1 to the plasma membrane.³² EGFR is frequently over-expressed and mutated in NSCLC, and its activation initiated intracellular signaling through Ras proteins.³³ In this study, we found that Rasip1 could be recruited to the plasma membrane by EGF stimulation. Therefore, we hypothesized that the EGFR pathway spatially controls the cellular localization of Rasip1 and thereby promotes the cell migration. Further studies are needed to focus on the contribution of Rasip1 on the plasma membrane.

The RUNX family is essential for cell survival, proliferation and differentiation. Depletion of RUNX1 in zebrafish abrogates the development of both blood cells and vessels; RUNX2 deficiency in mice arrests osteoblast differentiation; RUNX3 null mice exhibit an asthma-like phenotype, gastric mucosa hyperplasia, neural disorders, as well as abnormalities in CD4 expression.^{34–36} RUNX genes play essential roles in lung cancer, acting as either tumor suppressors or oncogenes. RUNX2 regulated Snail expression, thereby enhancing lung cancer migration and epithelial-to-mesenchymal transition.³⁷ RUNX3 inactivation by promoter hypermethylation is crucial for the development of lung adenocarcinoma.³⁸ Our results indicated that *RUNX1* mRNA was highly expressed in various NSCLC cells lines. Besides hematopoietic diseases, RUNX1 also plays a central role in many solid tumor development and progression. Increased expression of RUNX1 in epithelial cells is required for tumor formation. RUNX1, acting as a skin oncogene, stimulated Stat3 signaling and promoted tumor cell proliferation.³⁹ Elevated RUNX1 expression was associated with poor survival in prostate cancer and triple negative breast cancer.^{40,41} In serous epithelial ovarian carcinoma, RUNX1 was overexpressed and related to cell proliferation, migration and invasion.¹⁹ Depletion of the expression of RUNX1 in H1299 cell inhibited cell invasion. However, different results of RUNX1 gene expression have been reported in lung adenocarcinoma, involving cancer pathogenesis.^{16,20} Our data confirmed that RUNX1 is involved in the migration of NSCLC cells. Moreover, we identified that Rac1 was activated in RUNX1-mediated cell migration.

As a transcription factor, RUNX1 directly binds to a specific DNA sequence to repress or activate target gene transcription. Identification of target genes of RUNX1 is necessary for understanding the cellular function of this transcriptional factor. Several studies have revealed the target genes of RUNX1 in patients with leukemia, which include *MDR-1*, *GM-CSFR*, *c-fos*, *TCR β enhancer* and *IL-3*.²⁸ RUNX1 directly bonded to the functional sites within *PKC β* promoter and regulated its expression, which links RUNX1 to apoptosis in myeloid cells.⁴² *PU.1* has been identified as a target gene of RUNX1 in adult mouse hematopoiesis.⁴³ Downregulation of *NR4A3*, another RUNX1 target gene, contributed to hematopoiesis deregulation in FPD/AML.⁴⁴ The target genes of RUNX1 have effect on the cellular programs, including proliferation, differentiation, apoptosis and migration.⁴⁵ However, little is known about the target genes regulated by RUNX1 in other tissues. In this study, several potential RUNX-binding sites were identified within *Rasip1* promoter and silencing of RUNX1 suppressed the Rasip1 expression. In contrast, RUNX1 overexpression significantly enhanced the expression of Rasip1. These findings invoked the possibility that RUNX1 participates in the control of Rasip1 transcription. Indeed, our data indicated that RUNX1 bound directly to *Rasip1* promoter. To rule out the transcriptional activity of other RUNX family members, RUNX2 and RUNX3 were knocked down in NSCLC cells lines. The results showed that deletion of RUNX3 had a marginal effect on expression of *Rasip1* mRNA, but had no effect on protein expression. However, RUNX2 deletion had no influence on expression of both mRNA and protein of Rasip1. In addition, Rasip1 deletion inhibited RUNX1-mediated migration in NSCLC cells lines. Therefore, it was identified that RUNX1 had the ability to activate Rasip1 transcription in this study.

Given that RUNX1 is a weak transcription factor, its functions are reinforced through posttranslational modifications.^{46,47} Ser-249/Ser-266 residues of RUNX1 could be phosphorylated by ERK, which activates the interaction of RUNX1 with p300 and increased its transcriptional activity. Our data showed that expression of Rasip1 was increased, and the occupancy of RUNX1 in *Rasip1* promoter was enhanced by EGF stimulation. These changes were accompanied by increased RUNX1 phosphorylation after EGF treatment. However, phosphorylated RUNX1 was prone to be degraded by ubiquitin-mediated pathway. We found that the total RUNX1 and Rasip1 expression was reduced by long-term EGF stimulation. As we have noted, Rasip1 could mediate ERK phosphorylation. This multiple feedback loop might

lead to upregulation of the expression of Rasip1 in NSCLC cells lines (summarized in Figure 5D).

The R174Q and S249A mutants are well described, which lack transcriptional activity.^{48,49} Consistent with previous study, the S249A mutant impaired its transcriptional activities. Recent studies demonstrated the presence of R174Q in AML, myelodysplastic syndromes/AML, acute lymphoid leukemia, Familial platelet disorder/AML, as well as breast cancer.⁵⁰ This study had confirmed that the R174Q plasmid had no significant effect on Rasip1 expression. Interestingly, although Arg174 was reported critical for DNA binding,²⁷ the results of luciferase report assay showed that the R174Q mutant still retained the transcription activity, similar to WT RUNX1. We speculated that another mechanism should contribute to the function of R174Q mutant. As reported, the cellular localization is different between R174Q and WT RUNX1. We found that after EGF stimulation, unlike WT RUNX1, the R174Q mutant was still partially localized in the cytoplasm of transfected cells. Evidence was accumulating that the R174Q mutant might lose its ability to regulate Rasip1 expression through disrupting its nuclear localization. Although RUNX1 was considered to be associated with migration of many cancers, it should be noted that drugs target RUNX1 have not been proven in clinical trials.

Conclusion

The results of this study indicated that *Rasip1* is a downstream target gene of RUNX1 and is essential for cell migration of NSCLC. Furthermore, we observed that EGF regulated the localization and expression of Rasip1. Thus, our findings pointed out the relationship between RUNX1 and *Rasip1* and generated a novel insight into cell migration in NSCLC. Given that Rasip1 also plays a critical role in angiogenesis, targeting Rasip1 may provide a new therapeutic treatment in NSCLC patients.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

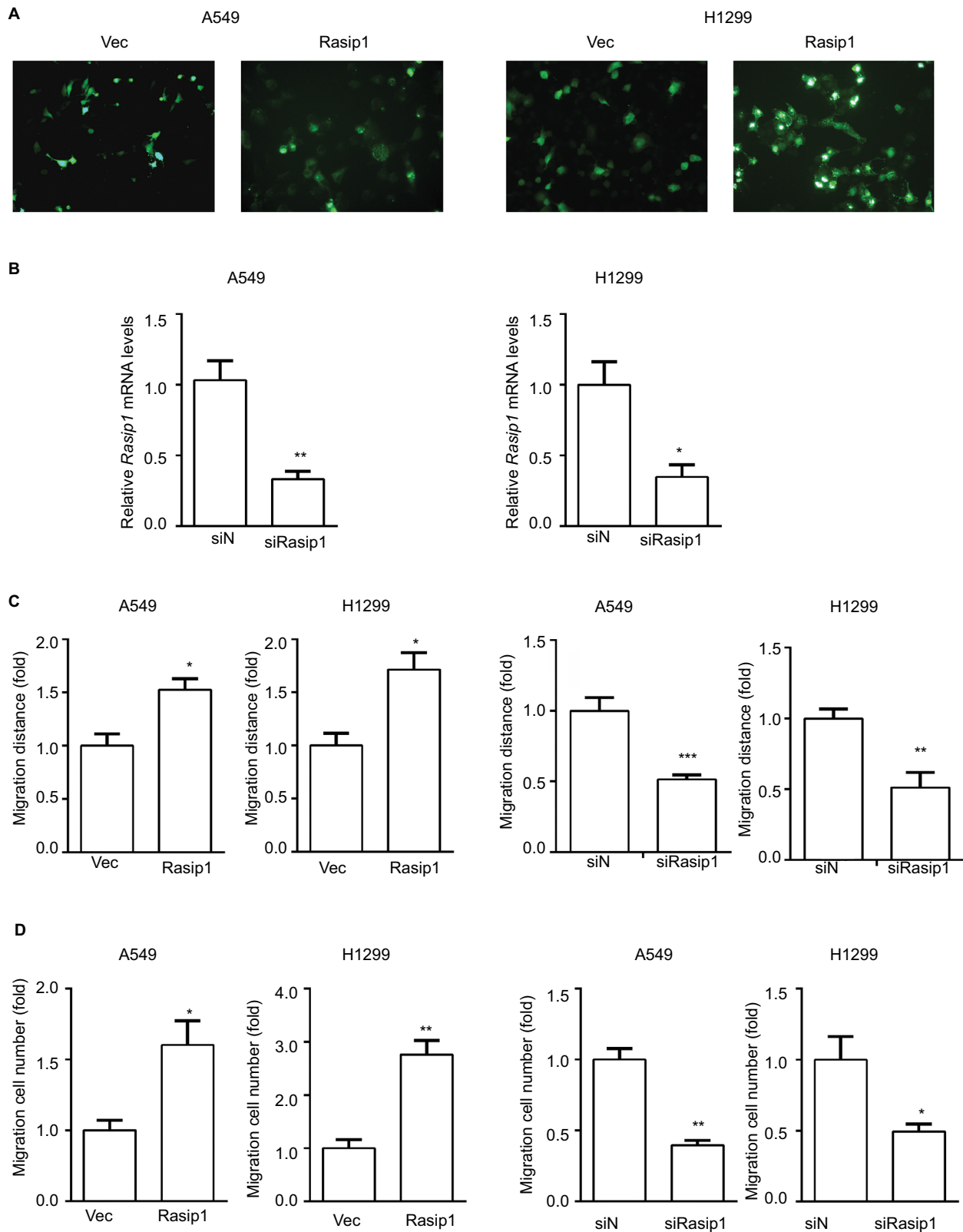


Figure S1 Rasip1 promotes migration of NSCLC cells.

Notes: (A) The pEGFP-N1 vector (control) and Rasip1-GFP plasmid were overexpressed in H1299 and A549 cells, respectively. The images were captured by an inverted immunofluorescence microscope. (B) A549 cells and H1299 cells were transfected with negative siRNA and siRasip1, respectively. After 24 hours of transfection, qRT-PCR was performed to detect the expression of *Rasip1* mRNA. (C) Wound-healing assay results were quantified and statistically analyzed. (D) Transwell assay results were quantified and statistically analyzed. Data are presented as mean \pm SD, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$.

Abbreviations: NSCLC, nonsmall-cell lung cancer; qRT-PCR, real-time quantitative PCR; Rasip1, Ras-interacting protein 1.

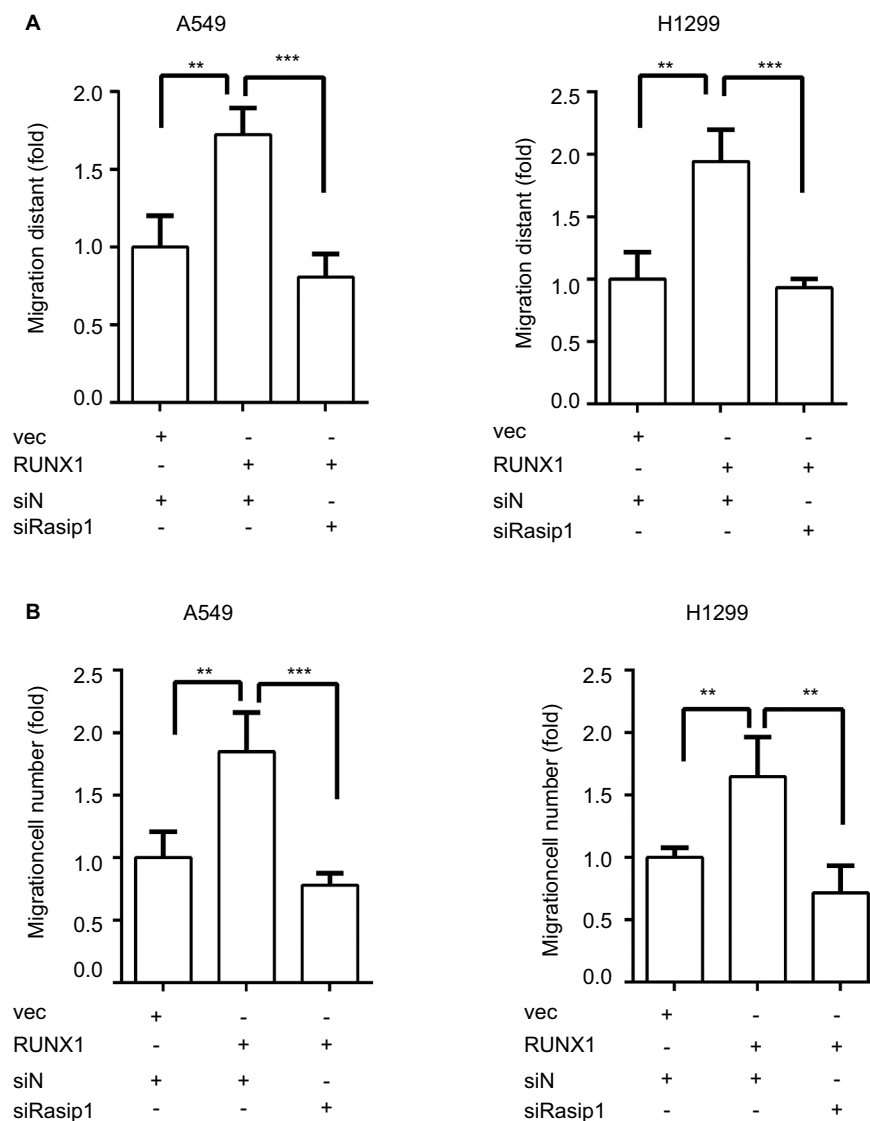


Figure S2 Rasip1 mediates RUNX1-induced migration.

Notes: (A) pCMV-C-HA vector or RUNX1-HA plasmid and negative siRNAs or Rasip1 siRNAs were cotransfected into H1299 and A549 cells, respectively. Wound-healing assay was quantified and statistically analyzed. (B) After 24 hours of transfection, transwell assay was performed and statistically analyzed. Data are presented as mean \pm SD, ** $P < 0.01$, *** $P < 0.005$.

Abbreviations: Rasip1, Ras-interacting protein 1; RUNX1, Runt-related transcription factor 1.

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