Tumor Suppression of Ras GTPase-Activating Protein RASA5 through Antagonizing Ras Signaling Perturbation in Carcinomas

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HIGHLIGHTS
RASA5 is normally widely expressed but epigenetically silenced in multiple cancers

Epigenetic disruption of RASA5 is associated with tumor progression in patients

RASAS5 suppresses RAS signaling, depending on its RasGAP catalytic activity

RASA5 functions as a tumor suppressor through inhibiting EMT and metastasis

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Tumor Suppression of Ras GTPase-Activating Protein RASA5 through Antagonizing Ras Signaling Perturbation in Carcinomas

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SUMMARY
Aberrant Ras signaling activation is common in cancers with even few Ras mutations, indicating alternative dysregulation other than genetic mutations. We identified a Ras GTPase-activating gene RASA5/SYNAP1, at the common 6p21.3 deletion, methylated/downregulated in multiple carcinomas and different from other RASA family members (RASA1–RASA4), indicating its special functions in tumorigenesis. RASA5 mutations are rare, unlike other RASA members, whereas its promoter CpG methylation is frequent in multiple cancer cell lines and primary carcinomas and associated with patient's poor survival. RASA5 expression inhibited tumor cell migration/invasion and growth in mouse model, functioning as a tumor suppressor. RASA5 suppressed Ras signaling, depending on its Ras GTPase-activating protein catalytic activity, which could be counteracted by oncogenic HRas Q61L mutant. RASA5 knockdown enhanced Ras signaling to promote tumor cell growth. RASA5 also inhibited epithelial–mesenchymal transition (EMT) through regulating actin reorganization. Thus, epigenetic inactivation of RASA5 contributing to hyperactive Ras signaling is involved in Ras-driven human oncogenesis.

INTRODUCTION
Ras small GTPases are key regulators of multiple signaling cascades, controlling diverse cellular processes (Cully and Downward, 2008; Schubbert et al., 2007). Ras activity depends on its two forms through binding partners: active GTP-bound and inactive GDP-bound Ras (Bos et al., 2007). Around 30% of different types of human cancers contain mutant Ras genes (K-Ras, H-Ras, N-Ras) with persistently activated GTP-bound state (Bos, 1989), which results in constitutively activated Ras signaling even in the absence of extracellular stimuli. Oncogenic Ras signaling has been implicated in malignant transformation of cancers (Bos, 1989; Pylayeva-Gupta et al., 2011). The frequency of Ras mutations varies greatly among cancer types, with very low frequency detected in some tumors such as esophageal squamous cell carcinoma (ESCC) (Cancer Genome Atlas Research Network et al., 2017; Gao et al., 2014), nasopharyngeal carcinoma (NPC) (Li et al., 2017; Lin et al., 2014), and breast cancer (Cancer Genome Atlas Network, 2012; vonLintig et al., 2000). However, even in the absence of Ras mutations, aberrant activation of Ras signaling is still common and critical for these tumors.

A family of Ras GTPase-activating proteins (RasGAPs) stimulates the GTPase activity of wild-type RAS but not its oncogenic mutants and turns activated Ras GTP-bound state into its inactive GDP-bound state (Bos et al., 2007; King et al., 2013). RasGAPs inactivate Ras signaling and inhibit oncogenic transformation initiated by RAS. RasGAP family comprises of diverse proteins with different expression patterns in normal development and diseases. So far, 14 RasGAPs have been identified in mammals (Grewal et al., 2011), with tumor-suppressive functions, and epigenetic alterations have been documented for several RasGAPs in various cancer types (Maertens and Cichowski, 2014), including NF1 (Cichowski and Jacks, 2001), DAB2IP (DiMinin et al., 2014), RASAL1 (Jin et al., 2007), and RASAL2 (McLaughlin et al., 2013). Thus, alternative mechanisms like inactivation of negative regulators of Ras signaling might have contributed to uncontrolled Ras signaling in cancers with infrequent Ras mutations.

Among RasGAPs, the RASA subfamily consists of five RasGAPs (Maertens and Cichowski, 2014): RASA1/p120GAP, three GAP1 members (RASA2/GAP1m, RASA3/GAP1IP4BP, and RASA4/CAPRI), and RASA5/SynGAP. The RASA group, as negative regulators of Ras signaling, could act as tumor suppressors in cancer...
pathogenesis. RASA1 inactivating mutations, as driver mutation, defines a genetically novel subclass of non-small cell lung cancer, together with NF1 mutations (Hayashi et al., 2018; Kitajima and Barbie, 2018). Allelic loss of RASA1 is frequent in triple-negative breast cancer with TP53 mutations, and its downregulation leads to the malignant phenotype of mammary cells (Suarez-Cabrera et al., 2017). RASA2 inactivating mutations, as a melanoma driver, are associated with patient’s poor survival (Arafeh et al., 2015). RASA3 was identified as a functional tumor suppressor in gastric and colon cancers (Schumans et al., 2015). RASA4 hypermethylation is correlated with patient’s poor prognosis in resistant juvenile myelomonocytic leukemia (Poetsch et al., 2014).

In this study, we report our identification and characterization of RASA5 as a tumor-suppressive RasGAP for human cancers. We defined a hemizygous 6p21.3 microdeletion in carcinomas by array comparative genomic hybridization and identified RASA5 as a disrupted target at this deletion. RASA5 was originally identified as a brain-specific RasGAP modulating synaptic transmission and plasticity (Chen et al., 1998; Komiyama et al., 2002). Despite its known role in neuronal development, its pathogenic role, especially in tumorigenesis, has never been studied. We found that RASA5 was broadly expressed in multiple normal tissues, albeit at lower levels than brain tissue. We further investigated its tumor-suppressive functions in antagonizing Ras signaling in tumor cells. Our study elucidates the importance of epigenetic disruption of RASA5 as a RasGAP to drive aberrant Ras signaling activation in human cancers, especially those with infrequent Ras mutations.

**RESULTS**

**RASA5 as a Downregulated Target at the Identified 6p21.3 Deletion and Associated with Poor Clinical Outcome**

We detected a hemizygous microdeletion at 6p21.3 in carcinoma cell lines by 1-Mb array-CGH analysis (Figure S1A). Chromosomal regions with genetic loss likely harbor candidate tumor suppressor genes (TSGs). We thus further examined expression levels of 23 genes localized within this deletion by semi-quantitative reverse transcription (RT)-PCR in a panel of tumor cell lines (3x NPC, 4x ESCC, 2x CRC, and 1x NSCLC), with normal tissues (testis, larynx, and esophagus) as controls. Significant downregulation of RASA5 was detected in all cell lines, with downregulation of LEMD2 and IP6K3 also seen in some cell lines (Figure S1B and Table S1), suggesting that RASA5 is the major candidate TSG within this deletion with its downregulation related to tumorigenesis.

RASA5 expression was previously reported to be brain specific by traditional Northern blot assay. To further examine its expression pattern, we examined its expression at both mRNA and protein levels in multiple normal tissues. Results showed variable but ubiquitous expression of RASA5 RNA in human normal tissues, albeit most abundantly in the brain when fewer PCR cycles were used (Figure 1A). We also accessed the GeneCards database and identified basal-level expression of RASA5 in human tissues (Figure S2A). Furthermore, endogenous expression of RASA5 protein (~148 kDa) was detected in normal esophageal, trachea, breast, and testis tissues by Western blot, with the highest level in brain as expected (Figures 1B and S2). Immunohistochemistry (IHC) staining (Human Protein Atlas cohort) also showed RASA5 protein expression in most normal tissues, ranging from high to low levels, and located in the cell cytoplasm (Figures 1C and S2B). These results suggest that RASA5 is a brain-dominant but not brain-restricted protein, likely possessing important functions in other normal tissues as well.

Further bioinformatic analysis of the Oncomine database confirmed RASA5 downregulation in ESCC and breast tumor clinical samples, with significant reduction observed in benign breast neoplasm and breast tumor samples (Figure 1D). We also performed Kaplan-Meier meta-analyses to analyze the clinical significance of RASA5 deregulation in tumors using online databases (Protein Atlas version 18, bc-GenExMiner v4.1). RASA5 low expression was significantly associated with poor outcome of patients with head and neck squamous cell carcinoma (HNSCC) and breast cancer (Figure 1E), suggesting an important role of RASA5 disruption in tumorigenesis.

**RASA5 Expression Is Repressed by Promoter CpG Methylation in Human Cancers**

Loss-of-function mutations of RASA members such as RASA1 are well documented in human cancers; we thus investigated possible genetic alterations of RASA5 in cancers using the COSMIC tumor database. Surprisingly, only <2% of cases (upper aero-digestive track, esophagus, lung, and breast) had detectable
Figure 1. RASA5 is Expressed in Multiple Normal Tissues including Brain but Downregulated in Carcinomas

(A) Examination of RASA5 transcript in normal tissues by semi-quantitative RT-PCR, using different PCR cycles (28, 26, and 24 cycles), with GAPDH as a control. PBMC, peripheral blood mononuclear cells.

(B) RASA5 protein expression in normal tissues detected by Western blot, with brain tissue as the highest level. Anti-RASA5 antibody (Ab1, ThermoFisher, PA1-046) was used, with β-actin as a loading control (upper panel). Graph represents quantification of Western blots with fold change (lower panel). Data were presented as mean ± SEM of three independent experiments and representative data were shown.

(C) IHC of normal tissue microarrays (esophagus, nasopharynx, breast) from Human Protein Atlas (www.proteinatlas.org, version 17). Anti-RASA5/SYNGAP1 pAb (HPA038373, Sigma-Aldrich) was used at a 1:35 ratio for staining.

(D) RASA5 expression (Kim Esophagus)

(E) RASA5 expression (Curtis Breast)

(F) RASA5 mutations in cancers

(A) Examination of RASA5 transcript in normal tissues by semi-quantitative RT-PCR, using different PCR cycles (28, 26, and 24 cycles), with GAPDH as a control. PBMC, peripheral blood mononuclear cells.

(B) RASA5 protein expression in normal tissues detected by Western blot, with brain tissue as the highest level. Anti-RASA5 antibody (Ab1, ThermoFisher, PA1-046) was used, with β-actin as a loading control (upper panel). Graph represents quantification of Western blots with fold change (lower panel). Data were presented as mean ± SEM of three independent experiments and representative data were shown.

(C) IHC of normal tissue microarrays (esophagus, nasopharynx, breast) from Human Protein Atlas (www.proteinatlas.org, version 17). Anti-RASA5/SYNGAP1 pAb (HPA038373, Sigma-Aldrich) was used at a 1:35 ratio for staining.
RASA5 mutations, compared with its high-frequent (~8%) mutations in nerve system cancer (Figure 1F). Thus, genetic mutation of RASA5 is not common in human cancers.

Downregulation or loss of RASA5 was detected in multiple tumor cell lines but not immortalized normal cell lines (Figures 2A, 2B, S2C, and S3A and Table S2). Interestingly, in direct contrast to cancer-associated downregulation of RASA5, other RASA family members (RASA1, 2, 3, and 4) were broadly expressed and only occasionally silenced in tumor cell lines (Figures 2C and 3B), indicating that RASA5 has unique and important functions in tumorigenesis among the RASA family.

Further analysis using CpG Island Searcher (http://cpgislands.usc.edu/) identified a CpG island spanning RASA5 transcription start site and exon 1, suggesting possible silencing by promoter CpG methylation (Figure 2D). We thus performed methylation-specific PCR (MSP) to examine RASA5 promoter methylation and its correlation with expression levels. Results showed that cell lines with reduced or loss of RASA5 expression were frequently methylated in the promoter (Figures 2A and S3A). RASA5 is methylated in all ESCC and NPC cell lines (18m/18 and Sm/5, respectively) and 67% (6m/9) breast cancer cell lines but not methylated in any corresponding immortalized normal epithelial cell lines (Figures 2A and S2C and Table S2). To validate the MSP results, we employed high-resolution methylation analysis using bisulfite genomic sequencing (BGS) to analyze the methylation status of 13 CpG sites within the examined cell lines upon drug treatment, accompanied by increased unmethylated promoter alleles and decreased methylated alleles (Figure 2E). Genetic demethylation of a colon cancer cell line (HCT116) by knockout (KO) of either single DNMT1 (1KO cell line) or DNMT3B (3BKO cell line) restored RASA5 expression through abolishing methylation, and knockout of both DNMT1 and DNMT3B (DKO cell line) achieved highest RASA5 expression level and more unmethylated alleles (Figure 2E). Further high-resolution BGS analysis confirmed the pharmacologic and genetic demethylation of RASA5 promoter (Figure 2D). These results indicate that promoter methylation plays a critical and direct role in RASA5 silencing in human tumor cells.

**Promoter Methylation of RASA5 Is Common in Multiple Primary Tumors**

We further examined the methylation status of RASA5 promoter in multiple primary tumors and adjacent nonmalignant tissues. RASA5 methylation was detected in 35% (24m/69) of ESCC tumors (including 20 paired samples), whereas only two paired surgically marginal nonmalignant tissues showed weak methylation and no normal esophagus tissue had methylation detected (Figures 2F and S3C). Moreover, RASA5 promoter methylation was detected frequently in other tumor samples, including 82% (18m/22) of NPC, 68% (13m/19) of breast, 73% (8m/11) of colon, and 62% (32m/52) of gastric tumors (Figures 2F, S3D, and S3E and Table S2), in direct contrast to the absence of methylation in normal tissues. Detailed BGS analysis further confirmed the dense methylation of RASA5 promoter alleles in representative primary tumors but not normal tissues (Figure S3F).

We retrieved RASA5 methylation and mRNA expression data from 183 esophageal and 656 breast invasive cancer samples in The Cancer Genome Atlas database. Linear regression analysis revealed an inverse correlation between RASA5 promoter methylation and its mRNA expression in esophageal and breast cancer patients (Figure 2G). Moreover, esophageal and breast invasive cancer patients with lower methylation of RASA5 are associated with better overall survival (Figure 2H). These results suggest that
RASA5 promoter methylation is tumor-specific and responsible for its downregulation/silencing in primary tumors.

**RASA5 Functions as a Tumor Suppressor in Multiple Carcinoma Cells**

We next investigated the tumor biology function of RASA5 in cancer cells. Ectopic expression of RASA5 in methylated and silenced tumor cell lines (KYSE150, HNE1, and MB231) dramatically reduced their colony formation abilities to 17%-50% (*p < 0.05) together with decreased colony sizes, compared with control cells transfected with empty vectors (Figures 3A and 3B), indicating that RASA5 functions as a TSG in carcinoma cells.

We also assessed the role of RASA5 on tumor cell apoptosis. Chromatin condensation and nuclear rupture as hallmarks of apoptotic cells were observed in EGFP-RASA5 transfected KYSE150 and HNE1 carcinoma cells with DAPI staining, whereas the nuclei of vector control cells remained intact (Figure 3C). Western blot assays confirmed that RASA5 enhanced the protein levels of apoptotic markers including cleaved poly (ADP-ribose) polymerase (PARP) and cleaved caspase-3 (Figure 3D). We also examined the effect of RASA5 on caspase-3 cleavage and activation using an apoptosis reporter system. The caspase3-sensor protein can be translocated into cell nuclei, then show stronger fluorescence when caspase-3’s nuclear exclusion sequence is cleaved off and activated upon cell apoptosis. We observed many more carcinoma cells showing activated nuclei-localized caspase-3 after RASA5 expression than controls transfected with empty vectors (Figures S4A and S4B), indicating that RASA5 expression could induce carcinoma cell apoptosis.

To study RASA5 functions further in vivo, RASA5- and empty vector-transfected MB231 cells were injected subcutaneously into nude mice to generate xenograft tumor models. Results showed that tumor weight and sizes of RASA5-expressing xenografts were significantly decreased compared with controls (Figures 3E and 3F). These results provide direct evidence that RASA5 indeed functions as a TSG in carcinoma cells.

**RASA5 Inhibits Carcinogenesis through Antagonizing RAS Signaling**

Further analysis of cancer genomic database (http://www.cbioportal.org/) showed that RASA5 inactivating mutations appear to be mutually exclusive to the active mutations of KRAS, HRAS, and NRAS in ESCC, NPC, and breast tumors, although in general genetic mutations of these genes are rare in these carcinomas (Figure 4A). A similar trend was also observed in HNSCC tumors, which had a relatively higher Hras mutation rate (~6%) (Figure 4A).

RASA5 protein showed cytoplasmic subcellular localization with a punctuated distribution pattern in exogenously expressed COS7 and KYSE150 cells, as well as endogenously expressed BT549 and T47D cells, partially Golgi and/or endoplasmic reticulum(ER)-located (Figure 4B). This was further confirmed by co-localization of RASA5 with ER/Golgi compartment, as assayed with ER- or Golgi-specific fluorescent plasmid transfection (pDsRed-ER and pEYFP-Golgi, respectively) in RASA5 exogenously and
Figure 3. RASA5 Functions as a Tumor Suppressor in Carcinoma Cells

(A) Representative colony formation assay by monolayer culture of RASA5-expressing ESCC, NPC, and breast tumor cells. Quantitative analyses of colony numbers shown as values of mean ± SEM of three independent experiments using Student’s t test (lower panel), with colonies (>50 cells) in empty vector-transfected cells set as 100; *, p < 0.05.

(B) RASA5 expression was confirmed using both anti-Flag and anti-RASA5 (Ab1, ThermoFisher) by Western blot. The straight arrow represents the specific band of RASA5 detected by anti-Flag antibody. Graphs represent quantification of Western blots with fold change compared with controls (lower panel). Data were presented as mean ± SEM of three independent experiments via Student’s t test. ***, p < 0.001.

(C) Characteristic nuclear changes of apoptotic cells after RASA5 expression. Arrows indicate EGFP+ cells.

(D) Cleavage of apoptotic markers, PARP and caspase-3, in carcinoma cells transfected with Flag-tagged RASA5 construct. GAPDH served as a loading control. Graphs represent quantification of Western blots with fold change compared with controls (right). **, p < 0.01; ***, p < 0.001. Data were presented as mean ± SEM of three independent experiments via Student’s t test.

Figure 3A shows the results of colony formation assays with ESCC, NPC, and breast tumor cells. The bars represent the mean ± SEM of three independent experiments, with colonies in empty vector-transfected cells set as 100. The graph indicates a significant decrease in colony formation in RASA5-expressing cells, as marked by an asterisk (*, p < 0.05).

Figure 3B illustrates the confirmation of RASA5 expression using Western blotting with anti-Flag and anti-RASA5 antibodies. The straight arrow indicates the specific band of RASA5, and the graphs show the fold change compared to controls, with significant differences indicated by triple asterisks (***, p < 0.001).

Figure 3C depicts the characteristic nuclear changes of apoptotic cells after RASA5 expression, denoted by arrows indicating EGFP+ cells.

Figure 3D presents the cleavage of apoptotic markers, PARP and caspase-3, in carcinoma cells transfected with Flag-tagged RASA5 construct. The graphs display the fold change compared to controls, with significant differences highlighted by double and triple asterisks (**, p < 0.01; ***, p < 0.001), and the data are expressed as mean ± SEM for three independent experiments.
endogenously expressed cells. Results showed that RASA5 signal was partially overlapped with that of ER (red) or Golgi (green) (Figure 4C). Moreover, some RASA5 proteins were obviously localized at the tips of actin tails in carcinoma cells (Figure 4C), whereas actin tail formation and retraction has been reported critically involved in cell migration.

We examined the effect of RASA5 on RAS signaling using luciferase reporters of SRE-Luc, AP-1-Luc, and TOPFlash for screening RASA5 downstream effectors. Results showed that ectopic expression of RASA5 significantly downregulated activities of SRE-Luc, AP-1-Luc, and TOPFlash in both HEK293 and KYSE150 cell lines (Figures 4D and 5A) and suppressed SRE-Luc reporter activities in a dosage-dependent manner (Figure 4D). We further examined effects of RASA5 on downstream MAPK/ERK and AKT signaling cascades by Western blot. Results showed dramatically reduced phosphorylation levels of key effectors of MAPK/ERK signaling (p-MEK1/2, p-ERK1/2) and AKT signaling (p-AKT) in RAS wild-type KYSE150 and HNE1 cells, as well as KRAS G13D-mutated MB231 cells (Figure 5A). Ras activity assay also showed substantially reduced amount of active GTP-bound Ras in RAS5-expressing tumor cells (Figure 5B). Moreover, introduction of oncogenic HRAS Q61L mutant abolished the suppression effects by RASA5 reconstitution on MAPK/ERK and AKT signaling effectors, through upregulating Ras-GTP activity (Figures 5A and 5B).

In addition, given that nuclear translocation was essential for activated ERK1/2 to exert its transcriptional regulations, the subcellular localization of ERK1/2 in cells with or without RASA5 re-expression was assessed. Results showed that RASA5 restoration in HONE1 cells markedly repressed the nuclear aggregation of phosphor-ERK1/2 (Figure 5C), which was also confirmed on the other hand by knockdown of endogenous RASA5 in carcinoma cells resulting in nuclear aggregation of phosphor-ERK1/2 (Figure 5D). Meanwhile, RASA5 caused reduction in expression levels of RAS target oncogenes MCL1 and c-MYC RNA, which could be reversed by ectopic expression of constitutively active HRAS Q61L mutant (Figure 5C).

The constitutively active HRAS Q61L mutant could rescue the suppression effect (to 40%–80%) of RASA5 over carcinoma cells in both anchorage-independent and -dependent growth, through monolayer colony and soft agar formation assays (Figures 6A and 6B). Furthermore, knockdown of endogenous RASA5 by two different siRNAs significantly increased colony formation abilities of T47D and BT549 cells (Figure 6C), consistent with the enhanced ERK phosphorylation levels (Figures 6D and S5). Treatment of specific MEK inhibitors (PD98059 and U0126) showed significant inhibition of phosphorylated ERK levels, but not its total protein levels, in cells with or without RASA5 knockdown (Figures 6D and S5). Treatment of MEK inhibitors also led to decreased tumor cell colony numbers and sizes. However, further knockdown of RASA5 in treated cells still slightly increased their colony numbers (Figure 6C). Collectively, these results demonstrate that RASA5 acts as a Ras-GAP to inhibit carcinoma cell proliferation through negatively regulating Ras signaling.

The RasGAP Domain Is Functionally Indispensable for RASA5 Activity

The human RASA5 encodes a 1343 amino acid (aa) protein, structurally consisting of 3 functional domains: an N-terminal PH (pleckstrin homology) (aa 27-253), a C2 domain (aa 263-365), and a GAP domain (aa 392-729). Protein sequence alignment of RASA5 from different species by ClustalX indicates that the RASA5 protein is evolutionarily well conserved (Figure S1). We generated three truncated variants, RASA5ΔGAP–G (aa 392–729), RASA5ΔGAP–L (aa 373–1343), and RASA5ΔC2–GAP (aa 259–1343), with deletion of GAP domain or GAP and C2-GAP domains, to analyze the importance of these domains in RASA5 functions toward Ras activity regulation (Figure 7A).

We first evaluated the effect of these domains in the subcellular localization of RASA5. Result showed altered diffused nuclear localization of RASA5ΔGAP–G, RASA5ΔGAP–L, and RASA5ΔC2–GAP mutants compared with the cytoplasmic distribution of wild-type RASA5, whereas the RASA5ΔC2–GAP mutant still partially retained cytoplasmic distribution (Figures 7B and 7E). The impact of these mutants on cell growth was also examined by clonogenic assay. The inhibition effect of colony formation by wild-type RASA5 was variably
Figure 4. RASA5 Is Partially Co-localized with ER/Golgi and Actin Tails in Carcinoma Cells

(A) RASA5 mutations in cancer databases (http://www.cbioportal.org/), together with KRAS, HRAS, and NRAS mutations.

(B) RASA5 subcellular localization stained by indirect immunofluorescence. Exogenous expression of RASA5 was examined by anti-Flag antibody (green) in COS7 and KYSE150 cells. Endogenous expression of RASA5 in BT549 and T47D cells was examined by two different anti-RASA5 antibodies (Ab1, ThermoFisher; Ab2, Epitomics, green), nuclei counterstained with DAPI (blue). Original magnification, 400x. Scale bar 200 μM.

(C) Co-localization of RASA5 with ER/Golgi compartments and actin tails. KYSE150 cells were co-transfected with Flag-RASA5 plasmid and either ER- or Golgi-specific plasmid (pDsRed-ER and pEYFP-Golgi, respectively). BT549 cells were transfected with ER- or Golgi-specific plasmid, respectively. Nuclei counterstained with DAPI (blue). Original magnification, 400x. Scale bar 200 μM.

(D) Promoter activities of SRE reporter were evaluated in RASA5-expressing cells by dual-luciferase assay, normalized to values of Renilla luciferase activity. Results were expressed as fold reduction of activity and shown as mean ± SEM of three independent experiments performed in triplicate analyzed by Student’s t test. Bottom panel: SRE-Luc reporter activity was reduced in KYSE150 cells with a RASA5 dosage-dependent manner; *, p < 0.05.
Figure 5. RASA5 Functions as a RasGAP and Antagonizes RAS Signaling

(A) Western blot of phosphor-MEK (pMEK), phosphor-ERK (pERK), and phosphor-Akt (pAKT) following RASA5 expression in RAS-wild-type (wt) KYSE150 and HNE1 cells, as well as KRAS-mutant (mut) MB231 cells. Ectopic expression of RASA5 downregulated phosphorylation levels of RAS signaling effectors, whereas active RAS mutant form (HRAS Q61L) counteracted this suppression. GAPDH used as a control. Quantification of the phosphor-immunoblots normalized to corresponding total protein levels (right). Data were presented as mean ± SEM of three independent experiments via Student’s t test and representative data were shown. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

(B) Active GTP-bound form of Ras was pulled down by Raf-RBD and subjected to Western blot analysis. RASA5 downregulated Ras-GTP level, but overexpression of oncogenic HRAS Q61L reversed this suppression. Graph represents quantification of Western blots with fold change compared with controls (right). Data were presented as mean ± SEM of three independent experiments via Student’s t test and representative data were shown. ***, p < 0.001.

(C) Semi-quantitative RT-PCR analyses of RASA5 inhibitory effect on Ras target oncogenes MCL1 and c-MYC at mRNA levels. Ectopic expression of HRAS Q61L mutant abrogated this inhibition. wt, wild-type; mut, mutant.
Figure 6. RASA5 Acts as a Tumor Suppressor via Its Effects on Ras Signaling

(A and B) Inhibition of anchorage-independent and -dependent growth by RASA5 in the presence of concomitantly expressed HRAS Q61L Ras-mutant in KYSE150 and HNE1 cells, by monolayer (A) and soft agar (B) colony formation assays. HNE1 and KYSE150 cells were transfected with vector or RASA5-expressing plasmid and selected with G418 at appropriate concentrations. Quantitative analyses of colony numbers are shown as values of mean ± SEM of three independent experiments using Student’s t test, with colonies (>50 cells) in empty vector-transfected cells set as 100; **, p < 0.01; ***, p < 0.001.
lost in cells expressing RASA5\(^{\Delta}\)GAPs (\(-\Delta\)G, \(-\Delta\)L) or RASA5\(^{\Delta\Delta}\)GAP mutant (Figures 7Ca and 7F). We next analyzed their effects on Ras activation by examining the phosphorylation levels of ERK1/2 and AKT. Both RASA5\(^{\Delta}\)GAPs (\(-\Delta\)G, \(-\Delta\)L) and RASA5\(^{\Delta\Delta}\)GAP mutants failed to suppress the phosphorylation of ERK1/2 and AKT, compared with wild-type RASA5 (Figures 7D and 7G). Together, our results suggest the RasGAP domain is functionally indispensable for the catalytic activity of RASA5.

**RASA5 Inhibits Carcinoma Cell Migration and Invasion through Epithelial-Mesenchymal Transition (EMT)**

To fully characterize the oncogenic effect of RASA5 disruption, we assessed whether RASA5 might suppress carcinoma cell migration and invasion by wound healing or invasion assay. Scratch wound was made on confluent cell monolayer and observed 24 h later and cells transfected with empty vector got wound closed, where RASA5-expressing carcinoma cells remained unhealed (Figure 7A). Moreover, matrigel invasion assay showed much fewer RASA5-expressing tumor cells invaded through the membrane into lower chamber than controls (Figure 8A). These results suggest that RASA5 is able to suppress carcinoma cell migratory and invasion.

As EMT is important in cancer cell migration and invasion, we next examined the influence of EMT by RASA5 expression. The morphology of HNE1 and MB231 carcinoma cells changed from spindle-shaped mesenchymal cell to cobblestone-like epithelial cell type following the restoration of RASA5 (Figure 8B). On the other hand, RASA5-depleted T47D carcinoma cells by siRNA knockdown showed morphologic conversion from epithelial cell to mesenchymal cell-like (Figure 7B). Further epithelial or mesenchymal markers were examined as molecular indicators of the impact of RASA5 on carcinoma cell EMT. Ectopic expression of RASA5 upregulated epithelial marker E-cadherin and downregulated mesenchymal markers vimentin and fibronectin by Western Blot and immunofluorescence assays (Figures 8C and 9A). Conversely, knockdown of RASA5 in carcinoma cells led to upregulated fibronectin and vimentin but deceased E-cadherin expression (Figure 8D).

EMT is fundamentally linked to actin cytoskeleton reorganization; we further examined the impact of RASA5 on cytoskeletal reorganization. Carcinoma cells normally are with F-actin–accumulated leading edge (actin tails) for cell migration direction. However, carcinoma cells with RASA5 expression exhibited alterations in cell shape from polarized to roundish and lost the clearly defined leading edge protrusion (Figure 9B). Immunofluorescence staining with rhodamine-labeled phalloidin further showed that actin stress fibers formed in control cells after starvation treatment, whereas disassembled in RASA5-expressing cells (Figures 9B and 7C). RASA5 proteins appeared in some actin tail tips and thin, membranous tubes connecting COS-7 cells, highly similar to the tunneling nanotubes for actin-based cytoplasmic extensions (Figure 5D). Accordingly, phosphorylation of cofilin, a terminal effector of actin cytoskeletal rearrangement, was downregulated in RASA5-expressing cells, together with its upstream signaling molecules p-JNK and p-Rac (Figure 8C).

As EMT promotes invasiveness and stem cell-like features (stemness) of cancer cells, we extended our study to investigate whether RASA5 could affect carcinoma cell stemness. The expression of classic stem cell markers, including NANOG, NESTIN, OCT4, KLF4, CD44, and ABCG2, was downregulated in RASA5-expressing cells by semi-quantitative RT-PCR, whereas HRAS Q61L active mutant counteracted this inhibition effect (Figure 9C), suggesting that RASA5 regulates EMT/MET plasticity in carcinoma and in turn affects actin cytoskeleton reorganization, cell migration/invasion, and cell stemness.

**DISCUSSION**

In this study, we demonstrate an alternative mechanism of abnormally activating wild-type RAS signaling in carcinoma cells through epigenetic disruption of a RasGAP. We identified a RasGAP member, RASA5, epigenetically silenced in a tumor-specific manner in multiple carcinomas. Gain-of-function assay revealed...
Figure 7. RASA5 Exerts Its Tumor Suppressive Function Dependent on its GAP Domain

(A) Schematic view of full-length RASA5 (wild type) and three truncated mutants (RASA5ΔGAP-G, RASA5ΔGAP-L, RASA5ΔC2-GAP). ΔGAP-G: RASA5 with only RasGAP domain deleted; ΔGAP-L: RASA5 with PH and C2 domains remained; ΔC2-GAP: RASA5 with C2 and RasGAP domains deleted.

(B and E) Representative immunofluorescence analysis of subcellular localization of wild-type RASA5 and truncated mutants, RASA5ΔGAP-L and RASA5ΔC2-GAP mutants (B); RASA5ΔGAP-G mutant (E), labeled with anti-Flag antibody (green) or anti-RASA5 (Ab1, ThermoFisher). Nuclei marked by DAPI (blue). Original magnification, 400x. Scale bar 200 μM.
RasGAPs are supposed to be potential tumor suppressors owing to their capabilities to maintain Ras activity, Ras activation, and cell growth, at a slighter grade, when compared with RASA5 presented in a bar graph (data were mean ± SEM of three independent experiments, * p < 0.05, **, p < 0.01, ***, p < 0.001). Data were presented as mean ± SEM of three independent experiments by One Way ANOVA with multiple comparison post hoc analysis and representative data were shown.

ACCORDINGLY, by overexpression or knockdown of RASA5 in carcinoma cells, RASA5 was shown to exert tumor-suppressive functions through downregulating Ras-GTP level and further attenuating Ras signaling. These inhibitory effects could be abrogated in the presence of active Ras mutant or by deletion of RasGAP domain of RASA5. Furthermore, loss-of-function assay showed Ras activation, enhancement of carcinoma cell growth, and EMT upon loss of RASA5. These results demonstrate a causal role of RASA5 silencing in aberrant Ras activation during human carcinogenesis. This demonstrates a tumor suppressive role of RASA5 in Ras signaling perturbation in carcinomas, in addition to its classic role in neuronal development.

Loss of negative regulators of Ras signaling by genetic or epigenetic alterations may be a common oncogenic driver in human cancers. Since our group and others previously first reported the frequent epigenetic disruption of a RasGAP member, RASAL/RASAL1, as a functional TSG in multiple tumors (Calvisi et al., 2011; Jin et al., 2007; Liu et al., 2013; Ohta et al., 2009), other RasGAP members have also been identified as TSGs epigenetically inactivated, including RASL2 (Feng et al., 2014; Hui et al., 2017; McLaughlin et al., 2013; Olsen et al., 2017), DAB2IP (Dote et al., 2004; Duggan et al., 2007; Min et al., 2010), and NF1 (Cichowski and Jacks, 2001; Rodenhiser et al., 1993), highlighting the important contribution of epigenetic mechanism inactivating RasGAP members. Although only rare mutations of RASA5 were detected in human cancers, we identified a predominant role of promoter methylation accounting for RASA5 inactivation. We found that RASA5 was frequently downregulated in multiple human cancers with or without Ras mutations. Another important finding of this study is that, apart from its important role in neuronal/brain development and function, we found that RASA5 actually is broadly expressed across human normal tissues, albeit at lower and variable expression levels, and involved in the carcinogenesis of multiple carcinomas.

RASA5 belongs to SynGAP subfamily that structurally consists of an N-terminal PH domain, a C2 domain, and a GAP domain at the C-terminus. RASA5 has been shown to be a negative regulator of Ras regulating neuronal MAPK/ERK signaling in cultured neurons (Rumbaugh et al., 2006). Consistent with previous studies, we confirmed the GAP activity of RASA5 to Ras in carcinoma cells. Moreover, our assays using truncation mutants deficient of GAP domain or C2-GAP tandem demonstrated that the catalyzing activity of GAP domain is a functional prerequisite for RASA5. Meanwhile, we found that the C2 domain might also have some impact on GAP activity of RASA5, as RASA5GAP-C mutant seemed to affect RASA5 subcellular localization, Ras activation, and cell growth, at a slightly grade, when compared with RASA5GAP mutants (ΔG, ΔC).

RasGAPs are supposed to be potential tumor suppressors owing to their capabilities to maintain Ras proteins in inactive GDP-bound state (King et al., 2013; Maertens and Cichowski, 2014). RASA5 mutant mice died in the first postnatal week, indicating the importance of RASA5 for normal development, although the exact cause of death remains unclear (Jayabal and Clement, 2016; Ozkan et al., 2014). Here we demonstrated that ectopic expression of RASA5 in RASA5-deficient carcinoma cells decreased the levels of active GTP-bound Ras, whereas knockdown of endogenous RASA5 increased the levels of active form. Accordingly, by overexpression or knockdown of RASA5 in carcinoma cells, RASA5 was shown with multifaceted tumor suppressor functions involving cell growth, EMT, stemness, and migration/invasion. A mechanistic explanation for the constitutively activated states of oncogenic Ras mutant proteins was the loss of negative feedback regulation by RasGAPs. In agreement with this, we found a functional deficiency of RASA5 to wide-type Ras in that RASA5 failed to decrease the levels of GTP-bound Ras in carcinoma cells with ectopic expression of constitutively active oncogenic HRAS Q61L mutant. However, similar inhibitory effect was still observed in carcinoma cells with endogenous mutant K-RAS (Figures 5 and 7), which might be due to the presence of other intact wild-type Ras genes in the cell lines. Therefore,
Figure 8. RASA5 Restoration Suppresses Tumor Cell Invasion and Reverses Cell EMT Phenotype

(A) Matrigel invasion chamber assay of RASA5-expressing MB231 cells. Invaded cells at the lower surface of transwell filter were stained and counted. *p < 0.05. Scale bar 200 μM. Each bar represented mean ± SEM of three independent experiments with control cells as the baseline using Student’s t test.

(B) Morphology changes of HNE1 and MB231 cells stably expressing RASA5. Original magnification, 400x. Scale bar 200 μM.

(C) Ectopic RASA5 expression upregulated E-cadherin, downregulated vimentin, fibronectin and phosphorylation levels of Rac, cofilin, and JNK1/2 by Western blot in HNE1 and MB231 cells. GADPH as a loading control. Graphs represent quantification of Western blots with fold changes compared with controls (right). Data were presented as mean ± SEM of three independent experiments via Student’s t test and representative data were shown. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

(D) Expression of EMT markers (E-cadherin, vimentin, and fibronectin) was examined by Western blot in BT549 and T47D cells with RASA5-knockdown through two different siRNAs (siRASA5-1, siRASA5-2). Right panel graphs represent quantification of Western blots with fold changes compared with controls (right). *p < 0.05, **, p < 0.01. Data were presented as mean ± SEM of three independent experiments via Student’s t test and representative data were shown.
our data suggest that RASA5 can serve as a negative regulator of wild-type RAS signaling and exert its tumor suppressor role in human cancers with wild-type Ras (Figure 9D).

In summary, our findings broadened the current understanding of the biological function of RASA5, from normal neuronal development to carcinogenesis, which could act as a tumor suppressor in cancers with wild-type Ras through inhibition of Ras activity and downstream signaling pathways. We further highlighted the important role of epigenetic silencing of RasGAPs in Ras-mediated carcinogenesis, further in line with our previous findings of RASAL/RASAL1 involvement in multiple tumorigenesis. Restoration of RASA5 activity through pharmacologic intervention might be further exploited as a therapeutic strategy.
Limitation of the Study
In this study, we identified a 6p21.3 gene RASAS5/SYNGAP1 antagonizing aberrant Ras signaling in human cancers, which was previously recognized as a RasGAP related to neural cell development and regulation. As RASA5 is frequently silenced by promoter CpG methylation in human cancers, unlike other RASA family members, further investigation of its tumor suppressive roles in other human cancers with more Ras mutations is needed. In addition to Ras signaling, whether other cross-talking signaling pathways may also be regulated by RASA5 during tumorigenesis should be studied more. RASA5 exerts significant suppression to EMT and stemness in tumor cells; in-depth mechanic study for its regulation of the self-renewal of cancer stem-like cells is also needed.

METHODS
All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.10.007.

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AUTHOR CONTRIBUTIONS
QT, LL, YF: conceptual design; YF, LL, XH, LZ, LS, JL, TX: performed experiments; ATC, WY, RLH: provided material and reviewed manuscript; LL, YF, XH, QT: drafted the manuscript; LL, QT: finalized the manuscript.

DECLARATION OF INTERESTS
The authors declare no conflict of interest.

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Supplemental Information

Tumor Suppression of Ras GTPase-Activating Protein RASA5 through Antagonizing Ras Signaling Perturbation in Carcinomas

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Supplementary Information

Transparent Methods

Array-Comparative Genomic Hybridization (aCGH)

Whole-genome arrays of 1-Mb resolution with 3,040 BAC/PAC clones were kindly provided by Dr C. Langford at the Wellcome Trust Sanger Institute, Cambridge, UK (http://www.sanger.ac.uk/Projects/Microarrays/), with clone details listed in Ensembl database (www.ensembl.org/Homo_sapiens/index.html). Array CGH was performed as described previously (Ying et al., 2006).

Cell lines, tumor samples and drug treatments

Multiple carcinoma cell lines and immortalized normal epithelial cell lines were purchased from ATCC and cultured under standard conditions. Human normal adult tissue RNA samples were purchased commercially. Archived DNA samples of paired Chinese ESCC (T) and adjacent surgical marginal tissues (N), NPC, normal nasopharynx and esophagus tissues, breast cancer tissues, and normal breast tissues were used (Jin et al., 2007).

For Aza treatment, cells were seeded at a density of 1×10^5 cells/ml and incubated overnight. Medium was then replaced with fresh medium containing Aza (Sigma-Aldrich, St Louis, MO) at a final concentration of 5-10 μM. Cells were allowed to grow for 72 h, with changing of Aza-containing medium every 24 h. For combined treatment of Aza with TSA, cells were treated with TSA (100 ng/ml in DMSO) for additional 24 h after Aza treatment. MEK inhibitor PD98059 (Sigma-Aldrich, St Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) to reach a concentration of 20 μM for cell treatment. MEK inhibitor U0126 (Cell Signaling, #9903) was dissolved in DMSO to reach a concentration of 10 μM for cell treatment.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). cDNA products were amplified with Go-Taq polymerase for 32 cycles (Promega, Madison, WI), with GAPDH as a control for RNA integrity. The sequences of primer sets and reaction systems was listed in Table S3, S4.

Bisulfite treatment and promoter methylation analysis
Bisulfite modification of DNA, MSP and BGS were carried out as previously described (Jin et al., 2007). The sequences of primer sets used for MSP and BGS were listed in Table S3. MSP was performed for 40 cycles using AmpliTaq Gold (Applied Biosystem, Foster City, CA). For BGS, amplified fragments were TA-cloned into PCR4-Topo vector (Invitrogen, CA) with 6-8 colonies randomly chosen and sequenced.

**Construction of plasmids and mutagenesis**

Primer sequences for cloning PCR were listed in Table S3. AccuPrime™ Taq DNA Polymerase was used for all PCR reactions. Full-length open reading frame (ORF) sequence of RASA5 was cloned from the adult brain cDNA library by PCR with a Flag-tag attached to the N-terminus. The product was then digested and ligated into the BamHI/XbaI site of pEGFP-C1 (Clontech) or pcDNA3.1 expression vector (Invitrogen, CA).

Three truncated variants: RASA5ΔGAP with deletion of GAP-G domain (aa 392-729), GAP-L domain (aa 373-1343) and RASA5ΔC2-GAP deficient of C2-GAP tandem (aa 259-1343) were generated by site-directed PCR with pcDNA3.1-Flag-RASA5 plasmid as the template. Sequences of each insert were confirmed by sequencing.

For RASA5 silencing, cells were transfected with two different siRNAs targeting RASA5 and scrambled control siRNAs using Lipofectamine 3000 (Invitrogen, CA) according to the manufacturer’s instruction. siRNA RASA5-1 was from Invitrogen: Stealth RNAi for RASA5 (Cat#: HSS113025) (UUA CAU AGA ACA GGU CUU UAC CAC C) with a Stealth RNAi negative control; siRNA RASA5-2 was purchased from OriGene (Cat#: SR305827).

**Immunofluorescence assay**

Cells were transfected with indicated plasmids using Lipofectamine™ 3000 reagent (Invitrogen). 24 h after transfection, cells were fixed in 4% paraformaldehyde in PBS and permeabilized with Triton X-100 at room temperature (RT). Cells were incubated with primary antibodies at 4 °C overnight. After three washes with PBS, rhodamine- or FITC, or Alexa Fluor® 488, or Alexa Fluor® 555-conjugated secondary antibodies were added and incubated for 1 h. Nuclear counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI) (Calbiochem, San Diego, CA). Coverslips
were mounted in DABCO and subjected to analyze with fluorescence microscope (Olympus, Japan). Antibodies used were listed in Table S5.

**Colony formation assay**

For monolayer colony formation assay, cells were plated in a 12-well plate ($2 \times 10^5$ cells/well). After overnight culture, cells were transfected with either RASA5-expressing plasmids or empty vectors, using Lipofectamine 3000 (Invitrogen, CA). 48 h after transfection, cells were replated with an appropriate density in 6-well plates with G418 (0.4 - 1.2 mg/ml) selection for 7-12 days. BT549 and T47D cells were plated for colony formation assay, and then transfected with RASA5 siRNAs or control siRNAs, and treated with the indicated inhibitors 20 µM PD98059 and 10 µM U0126. The culture medium with siRNAs and MEK inhibitors were incubated for an additional 10-14 days, changed with fresh medium containing siRNAs and MEK inhibitors every three days. Surviving colonies (≥ 50 cells per colony) were visualized with Gentian Violet staining (ICM Pharma, Singapore) and manually counted.

For colony formation assay in soft agar, transfections were undertaken as in monolayer colony formation assay. 48 h post-transfection, cells were resuspended in full medium containing 0.4 mg/ml G418 and 0.3% soft agar, and then seeded at a density of $4 \times 10^3$ cells into a 24-well plate. Colonies (>50 cells) were counted and photographed after 10-20 days. All the experiments were performed in triplicate wells and repeated for three times independently.

**Protein preparation and Western blot**

Cells were transfected as described above. 48 h post-transfection, cells were lysed in RIPA buffer. A total of 50 µg of protein extracts were separated by SDS-PAGE and transferred to Nitrocellulose membranes. After probed with primary antibodies, the membrane was incubated with secondary antibodies and was visualized using the ECL detection system (GE Healthcare). Antibodies used were listed in Table S5. All the images were cropped around the known molecular weight of the interested band and representative blots were shown. Each blot repeated independently three times with similar results. Image J was used to quantify blots and the quantitative data presented by the graph, with error bars and significance calculations.

**Luciferase assay**
Cells were transfected with a plasmid either empty or expressing RASA5, along with SRE-luc and Renilla luciferase plasmid (pRL-SV40). Cell lysate was assayed for determining the firefly and Renilla luciferase activities in succession using a Dual Luciferase kit (Promega, Madison, WI). Luciferase activity of firefly was normalized against that of Renilla. Each assay was performed in triplicate and repeated for three times.

**Wound healing assay**

Cells grown on 12-well plates were carefully wounded using a sterile pipette tip, washed once with fresh medium, and then supplemented with fresh medium. After incubation for 24 h and 48 h, wound closure was monitored under a phase contrast microscope. Each assay was performed in triplicate and all experiments were repeated for three times.

**Matrigel invasion assay**

In vitro Matrigel invasion assays were performed with BD BioCoat Matrigel Invasion Chambers (Transwell) (BD Biosciences, Heidelberg) in 24-well plates. Briefly, transfected cells were resuspended in serum-free medium at a density of $2.5 \times 10^4$ cells/ml. The GFR Matrigel inserts were rehydrated by incubating with 0.5ml warm culture medium for 2 hours, and then 0.5 ml of cell suspension was added to the inserts. Full medium with 10% serum was added to the bottom chamber as a chemoattractant. After incubation for 24 h at 37°C, cells were fixed and stained with Gentian Violet. Data represented the results of three independent experiments.

**Ras activity assay**

Active levels of Ras-GTP were determined by affinity precipitation using GST-Raf-RBD (Upstate Biotechnology, Inc., NY), a GST fusion protein containing the Raf/Ras-binding domain. Cells were transfected with pcDNA3.1-Flag-RASA5 plasmid or empty vector, together with or without an oncogenic active form of H-Ras cDNA (Q61L mutant) in pUSEamp (Upstate Biotechnology, Inc., NY), using Lipofectamine 2000 (Invitrogen, CA). 48 h after transfection, cells were incubated in medium containing 10% serum for 5 minutes following overnight serum starvation. Then cells were lysed and incubated with 10ul of Raf-1 RBD agarose for 30 minutes at 4°C with gentle rocking. Agarose beads were collected by pulsing and bound proteins were eluted with
SDS/PAGE sample buffer, resolved on 12% acrylamide gels, and subjected to Western blot analysis with specific Ras antibody.

**Statistical analysis**

All statistical analyses were performed in excel and using SPSS version 21.0. All data were generated from at least three independent experiments and represented as means ± SEM. Gene expression data were analyzed using log2 transformed and normalized. Log-rank test was used to compare the survival distributions of cancer patient groups with different levels of RASA5 expression or methylation. β-value ranging from 0 to 1 was a measure of DNA methylation level for completely unmethylated to completely methylated. The Spearman correlation coefficient between RASA5 methylation and gene expression were calculated in excel. Overall survival curves were plotted using Kaplan-Meier analysis. Unpaired Student’s t-test was used to compare two experiment groups and 1-way ANOVA (with Newman-Keuls post-hoc test) was used to compare groups of three or more. When $p < 0.05$, data were considered to be statistically significant for all experiments.
Supplementary figure legends

Figure S1. Identification of RASA5 as a target of the 6p21.3 deletion, related to Figure 1. (A) Representative results of 1-Mb array-CGH of CNE2 and 5-8F carcinoma cell lines. Normalized log2 signal intensity ratios from -1 to 1 are plotted. Each single BAC clone is represented by a dark blue colored dot. The BAC clone containing the RASA5 locus (bA175A4) is labeled with the red dot, and also indicated by red rectangle in the top cytoband of Chr. 6p. Middle panel, the 6p21.31-6p21.32 gene-rich region shown as in Ensembl Genome Browser (www.ensembl.org/index.html), with the RASA5 gene highlighted by red rectangle. (B) Expression pattern of all candidate genes within 6p21.31-6p21.32 in representative tumor cell lines by semi-quantitative RT-PCR, with 3 normal tissues (testis, larynx and esophagus) used as controls. ESCC, esophageal squamous cell carcinoma; NPC, nasopharyngeal carcinoma.

Figure S2. Analysis of RASA5 expression in human tissues, related to Figure 1 and Figure 2. (A) RASA5 mRNA expression in normal human tissues from GTEx, BioGPS, and CGAP SAGE databases. (B) Bar graph illustrating RASA5 expression levels by IHC in human normal tissues. Data were retrieved from Human Protein Atlas database (http://proteinatlas.org, version 17). N, not detected; L, low; M, medium; H, high. (C) RASA5 protein expression in normal tissues and tumor cell lines by Western blot using anti-RASA5 (Ab2).

Figure S3. RASA5 promoter is frequently methylated in primary carcinomas, related to Figure 2. (A) RASA5 was downregulated and methylated in carcinoma cell lines, detected by semi-quantitative RT-PCR and MSP, but expressed in immortalized non-transformed normal epithelial cell lines (with names green underlined). M, methylated; U, unmethylated; Ca, cancer; CRC, colon cancer. (B) Expression pattern of RASA family members in cancer cell lines as examined by semi-quantitative RT-PCR. GAPDH has been used as an internal control for RNA integrity (not shown here). Ca, cancer; CRC, colon cancer. (C, D, E) Representative MSP analyses of RASA5
promoter methylation in primary carcinomas. (F) BGS results confirmed the dense methylation of RASA5 in primary NPC and ESCC tumor samples but not in normal tissue samples. M, methylated; U, unmethylated. N, paired tumor-adjacent normal tissues; T, tumor.

Figure S4. RASA5 reconstitution triggers tumor cell apoptosis and suppresses Ras signaling as a RasGAP, related to Figure 3-6. (A) Re-expression of RASA5 induced the activation of caspase-3. HNE1 and KYSE150 carcinoma cells were co-transfected with EGFP-caspase-3-sensor reporter plasmid, together with either RASA5-expressing or empty vector and inspected 48 hours after co-transfection. Original magnification, 400x (upper), 100x (lower). Scale bar 200 μm. (B) Quantitative analyses of caspase-3-acitivated cells. Data were mean ± SEM (N=3), asterisk indicated p < 0.05). (C) Assessed by dual-luciferase reporter system. Ectopic expression of RASA5 in both HEK293 and KYSE150 cell lines significantly downregulated activities of TOPFlash and AP-1-Luc. Data were presented as mean ± SEM of three independent experiments via Student’s t test and representative data were shown. *, p < 0.05. (D) Altered subcellular localizations of p-ERK1/2 in HONE1 carcinoma cells with RASA5 re-expression. HONE1 cells were transfected with RASA5-construct or empty vector and immunolabeled for p-ERK1/2 (red). Original magnification, 400x. (E) Nuclear translocation of p-ERK1/2 in T47D cells depleted of RASA5 expression. T47D cells were transfected with control or RASA5 siRNA, and then immunoabeled for p-ERK1/2 (red). DAPI counterstaining (blue) represented cell nuclei. Original magnification, 400x. Scale bar 200 μm.

Figure S5. Knockdown of RASA5 enhances ERK phosphorylation levels, related to Figure 6. Graphs represent quantification of the phosphor-immunoblots normalized to corresponding total protein levels by Western blot in T47D and BT549 cells. Data were presented as mean ± SEM of three independent experiments via Student’s t test. *, p < 0.05; **, p < 0.01.

Figure S6. The RASA5 protein sequence is evolutionarily well conserved among
different species, related to Figure 7. RASA5 amino acid sequences from different species (Homo sapiens, Bos Taurus, Rattus norvegicus, and Xenopus (Silurana) tropicalis) were compared and aligned using ClustalX (http://www.clustal.org/clustal2/). The only major difference in its C-terminal tail is emphasized with a red rectangle.

Figure S7. RASA5 inhibits the migration and F-actin stress fiber formation of carcinoma cells, related to Figure 8-9. (A) Wound healing assay showed reduced wound closure rate in RASA5-expressing cells compared to controls 24 hours after scratch wound made on confluent cell monolayer. Dash lines indicated wound edge. (B) Knockdown of RASA5 induced morphology change of T47D carcinoma cells from cobblestone-like to spindle-like. Original magnification, 400x. Scale bar 200 μm. (C) HONE1 and MB231 cells were transiently transfected with RASA5-EGFP construct or empty vector and subjected to immunofluorescence staining of F-actin stress fiber with Phalloidin (red). Arrows indicated EGFP+ cells. DAPI counterstaining (blue) represented cell nuclei. Original magnification, 400x. Scale bar 200 μm. (D) Immunofluorescence staining showed that RASA5 is located in cell-cell interaction regions and actin tails under the fluorescence microscope. Left, FITC green fluorescence of Flag-tagged RASA5; middle, DAPI-stained cell nuclei; right, merged images. Original magnification, 400x. Scale bar 200 μm.
Suppl. fig.1

A

1-Mb aCGH

Log2T/R

6p21

NPC

5-8F

BAC clone: bA175A4

Chr. 6p21.32–p21.31

Ensembl Genes

Ensembl Homo sapiens version 82 (GRCh38.p12); chromosome 6: 30,872,395 - 30,907,386

B

Markers

BRD2, COL11A2, RXRβ, SLCA39A7, RING1, VPS52, B3GALT4, WDR46, RGL2, TAPBP, ZBTB9, WDR46, BAK1, CUTA, COL11A2, SLC39A7, HSD17B8, RING1, B3GALT4, RGL2, ZBTB9, WDR46, BAK1, CUTA

Testis

Larynx

Esophagus

C666-1

HK1

HNE1

EC18

HKESC1

KYSE410

KYSE510

HCT116

LOVO

H1299

Normal tissues

NPC cell lines

ESCC cell lines

Colon Ca cell lines

Lung Ca cell line
### A RASA5 RNA expression in multiple normal tissues (from GeneCards human gene database)

| Tissue          | RNAseq (100×+FPKM)^1 | STEx | Microarray | SAGE | CGAP TAS TTTAATAAAC |
|-----------------|------------------------|------|------------|------|---------------------|
| Bone Marrow     |                        |      |            |      |                     |
| Whole Blood     |                        |      |            |      |                     |
| White Blood Cells|                       |      |            |      |                     |
| Thymus          |                        |      |            |      |                     |
| Brain           |                        |      |            |      |                     |
| Cerebellum      |                        |      |            |      |                     |
| Retina          |                        |      |            |      |                     |
| Spinal Cord     |                        |      |            |      |                     |
| Tidal Nerve     |                        |      |            |      |                     |
| Heart           |                        |      |            |      |                     |
| Artery          |                        |      |            |      |                     |
| Smooth Muscle   |                        |      |            |      |                     |
| Skeletal Muscle |                        |      |            |      |                     |
| Small Intestine |                        |      |            |      |                     |
| Colon           |                        |      |            |      |                     |
| Adipocyte       |                        |      |            |      |                     |
| Kidney          |                        |      |            |      |                     |
| Liver           |                        |      |            |      |                     |
| Lung            |                        |      |            |      |                     |
| Spleen          |                        |      |            |      |                     |
| Stomach         |                        |      |            |      |                     |
| Esophagus       |                        |      |            |      |                     |
| Bladder         |                        |      |            |      |                     |
| Pancreas        |                        |      |            |      |                     |
| Thyroid         |                        |      |            |      |                     |
| Salivary Gland  |                        |      |            |      |                     |
| Adrenal Gland   |                        |      |            |      |                     |
| Pituitary       |                        |      |            |      |                     |
| Breast          |                        |      |            |      |                     |
| Skin            |                        |      |            |      |                     |
| Ovary           |                        |      |            |      |                     |
| Uterus          |                        |      |            |      |                     |
| Placenta        |                        |      |            |      |                     |
| Prostate        |                        |      |            |      |                     |
| Testis          |                        |      |            |      |                     |

#### Major Tissues

- Immune
- Nervous
- Muscle
- Internal
- Secretory
- Reproductive

### B RASA5 protein expression in multiple normal tissues (from Human Protein Atlas version 17)

#### Score

- High
- Medium
- Low
- Not detected

### C Adult normal tissues

- **RASA5 (Ab2)**
- **α-Tubulin**

#### Tissues

- Testis
- Trachea
- Esophagus
- Brain

#### Breast Ca

- BT549
- MB468
- MCF7
- T47D
- YCC-B1

#### ESCC

- EC1
- EC18
- HKESC1

#### NPC

- C665-1

#### RASA5 methyl:

- m
- m/m
- u

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Suppl fig2
| Gene     | Description                                                                 | Predicted function                                                                                      |
|----------|-----------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------|
| BRD2     | Bromodomain containing 2                                                     | Involved in transcription complexes and acetylated chromatin during mitosis.                             |
| RPL32P1  | Ribosomal protein L32 pseudogene 1                                          | No CCDS                                                                                                 |
| COL11A2P | Collagen, type XI, alpha 2 pseudogene                                        | No CCDS                                                                                                 |
| COL11A2  | Collagen, type XI, alpha 2                                                   | Mutations in this gene are associated with type III Stickler syndrome.                                   |
| RXRB     | Retinoid X receptor, beta solute carrier family 39 (zinc transporter), member 7 | Increasing both DNA binding and transcriptional function.                                               |
| SLC39A7  | Solute carrier family 39 (zinc transporter)                                  | Zinc cannot passively diffuse across cell membranes and requires specific transporters, such as SLC39A7.|
| HSD17B8  | Hydroxysteroid (17-beta) dehydrogenase 8                                    | Regulating the concentration of biologically active estrogens and androgens.                            |
| RING1    | Ring finger protein 1                                                        | Acting as a transcriptional repressor interacts with the polycomb group proteins.                      |
| ZNF314P  | Zinc finger protein 314 pseudogene                                          | No CCDS                                                                                                 |
| HTATSF1P | HIV-1 Tat specific factor 1 pseudogene                                      | No CCDS                                                                                                 |
| VPS52    | Vacuolar protein sorting 52 homolog (S. cerevisiae)                         | Involved in tetrameric Golgi-associated retrograde protein complex.                                     |
| RPS18    | Ribosomal protein S18                                                       | S13P family of ribosomal proteins.                                                                      |
| WDR46    | WD repeat domain 46                                                         | Possessing WD40 domain, found in a number of eukaryotic proteins that cover a wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly. |
| PFDN6    | Prefoldin subunit 6                                                          | A subunit of the heteromeric prefoldin complex that chaperones nascent actin and alpha- and beta-tubulin chains pending their transfer to the cytosolic chaperonin containing TCP1 complex. |
| RGL2     | Ral guanine nucleotide dissociation stimulator-like 2                       | Possessing Ras guanyl-nucleotide exchange factor activity.                                              |
| ZBTB22   | Zinc finger and BTB domain containing 22                                     | Interacting with BDP1, a subunit of TFIIIB.                                                             |
| B3GALT4  | UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 4            | Encoding type II membrane-bound glycoproteins with diverse enzymatic functions using different donor substrates. |
| DAXX     | Death-domain associated protein                                              | Interacting with apoptosis antigen Fas, centromere protein C, and transcription factor erythroblastosis virus E26 oncogene homolog 1. |
| TAPBP    | TAP binding protein (tapasin)                                                | Transporting antigenic peptides across the endoplasmic reticulum membrane.                             |
| PHF1     | PHD finger protein 1                                                         | Zinc finger-like PHD (plant homeodomain) finger.                                                        |
| RPL12P1  | Ribosomal protein L12 pseudogene 1                                          | No CCDS                                                                                                 |
| RPL35AP4 | Ribosomal protein L35a pseudogene 4                                         | No CCDS                                                                                                 |
| KIFC1    | Kinesin family member C1                                                     | Important in a cargo-transport system.                                                                   |
| CUTA     | CutA divalent cation tolerance homolog (E. coli)                            | Undergoing an unusual transfer into the secretory pathway and affecting the folding, oligomerization, and secretion of acetylcholinesterase. |
| ZBTB9    | Zinc finger and BTB domain containing 9                                      |                                                                                                         |
| GGNBP1   | Gametogenetin binding protein 1                                              | No CCDS                                                                                                 |
| BAK1     | BCL2-antagonist/killer 1                                                     | Localized to mitochondria, inducing apoptosis; interacting with the tumor suppressor P53 after exposure to cell stress. |
| ITPR3    | Inositol 1,4,5-triphosphate receptor, type 3                                | Encoding a receptor for inositol 1,4,5-trisphosphate, a second messenger that mediates the release of intracellular calcium. |
| IP6K3    | Inositol hexaphosphate kinase 3                                              | Encoding a protein that belongs to the inositol phosphokinase family.                                   |
| LEMD2    | LEM domain containing 2                                                      | Regulating kinase signaling in myoblast differentiation.                                              |
| MLN      | Motilin                                                                     | Regulating gastrointestinal contractions and motility; producing the mature peptide and a byproduct referred to as motilin-associated peptide. |
Table S2. Summary of RASA5 methylation in carcinomas and normal cell lines, related to Figure 2.

| Samples                                      | Promoter methylation (%) |
|----------------------------------------------|--------------------------|
| **Carcinoma cell lines**                     |                          |
| Esophageal                                   | 18/18                    |
| Nasopharyngeal                               | 5/5                      |
| Hypopharyngeal                               | 1/1                      |
| Breast                                       | 6/9 (67%)                |
| Lung                                         | 6/8 (75%)                |
| Gastric                                      | 15/16 (94%)              |
| Colon                                        | 9/10 (90%)               |
| **Immortalized normal epithelial cell lines**|                          |
| Het1A, NE1, NE3, NE083, NP460                | 1/9 (11%)                |
| HMEC, HMEpC, NL20, CCD841con                 |                          |
| **Primary carcinomas**                       |                          |
| ESCC paired primary tumor (T)                | 12/20 (60%)              |
| ESCC (II#)                                   | 12/49 (25%)              |
| NPC (OCT#)                                   | 18/22 (82%)              |
| Breast                                       | 13/19 (68%)              |
| Colon (I#)                                   | 8/11 (73%)               |
| Gastric Ca (I#)                              | 6/14 (43%)               |
| Gastric Ca (II#)                             | 26/38 (68%)              |
| **Normal tissues**                           |                          |
| Normal esophageal epithelial tissues         | 0/7                      |
| ESCC paired normal tissues (N)               | 2w/20 (10%)              |
| Normal nasopharyngeal tissues                | 0/8                      |
| Normal breast tissues                        | 0/7                      |
| Breast adjacent tissues                      | 0/5                      |

W: weak methylation.
Table S3. Sequences of other primers used in this study, related to Figure 2, 5 and 9.

| PCR         | Primers | Sequence (5’-3’)                      | Cycles |
|-------------|---------|---------------------------------------|--------|
| RT-PCR      | NANOGF  | ATGAGTGTGGATCCAGCTTG                  | 30     |
| NANOGR      | TGATGCACATGGAGACGTC                     | 30     |
| NESTINR     | ACGATCTGGCTGTGAG                        | 30     |
| ABCG2F      | CAGTGTCCAAGGAAACACC                     | 30     |
| ABCG2R      | GAGACCAGTTCATAGCC                      | 30     |
| OCT4F       | AAGGAGAGCTGGAGCA                       | 30     |
| OCT4R       | GAGGGTTCTGGCAT                          | 30     |
| MCL1F       | AGTTGTACCAGCTGC                        | 30     |
| MCL1R       | CTAGGTCTCTCATGAGG                      | 30     |
| c-MYCF      | CTCTCCGCTGCCAGTC                      | 30     |
| c-MYCR      | GCCTCAGCAGAAGGTATG                      | 30     |
| KLF4F       | TCCCCATCTCTCCACGTC                     | 30     |
| KLF4R       | TCCAGGAGATCTGTAACG                     | 30     |
| CD44F       | TGGACAAAGTGGTGAC                      | 30     |
| CD44R       | GGTCTATGAAAGCCGTGCC                    | 30     |
| GAPDHF      | GATGACCTGGCAGACGCT                     | 23     |
| GAPDHR      | ATCTCTGCCCCTCTCTGGGA                  |        |
| MSP         | RASA5m4                              | GCTTTTTTTTTTTTTTTGTTGCC                    | 40     |
| RASA5m2     | CTAAAAAAACATAAAAACATCGC                |        |
| RASA5u11    | GCGGTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT | 40     |
| RASA5u22    | GCGCATAAAAAACATAAAAACATCAC            |        |
| BGS         | RASA5BGS5                             | TTAGGGGTGGGGGGGTAG                        | 40     |
| RASA5BGS6   | CCTCTAAAAAACATAAAAACATC               |        |
| Cloning     | RASA5CF                              | GCTGAGATCCGACGACAGATGGATTACAGGATGAGGACGAGCAGATAAGGAGCAGTCTCGAGGCCTCC | 15     |
| RASA5CR     | GATGTCTGACTAGTGGGTCTGGTGGTGGGC        |        |
| RASA5ΔGAP-LF| GCTGAGATCCGACGACAGATGGATTACAGGATGAGGACGAGCAGATAAGGAGCAGTCTCGAGGCCTCC |        |
| RASA5ΔGAP-LR| GATGTCTGACTAGTGGGTCTGGTGGGAG         |        |
| RASA5ΔGAP-GF| TACGGCGACTGATCTCGAGCGCCACATGGATTACAGGATGAGGACGAGCAGATAAGGAGCAGTCTCGAGGCCTCC |        |
| RASA5ΔGAP-GR| TACGGCGACTGATCTCGAGCGCCACATGGATTACAGGATGAGGACGAGCAGATAAGGAGCAGTCTCGAGGCCTCC |        |
| RASA5ΔC2-GAPF| GCTGAGATCCGACGACAGATGGATTACAGGATGAGGACGAGCAGATAAGGAGCAGTCTCGAGGCCTCC |        |
| RASA5ΔC2-GAPR| GATGTCTGACTAGGCGGGCTGTGGTCCTGGTGG  |        |
Table S4. Primers for screening 6p21.3 genes by semi-quantitative RT-PCR, related to Figure 1.

| Gene    | Forward (5’-3’)                              | Reverse (5’-3’)                          |
|---------|----------------------------------------------|------------------------------------------|
| RASA5   | GTCTATGCCCCTTCAGAG                         | GTCCTATGCCCCCTTCAGAG                    |
| BRD2    | CTTGCGGTCAAGATGCTGCA                       | TTGGGATTGGACACCTCCG                      |
| COL11A2 | ATGGAGCGGTGCAGCCGC                        | GCCACATCAGCTGGACAGAT                    |
| RXRB    | AGAAATGCATTGTGGGGTCG                       | AAGGGATGGAGCTGGTGAAG                    |
| SLC39A7 | GATCTGCAAGAGGACTTCCA                      | GCTGCTGAGACTGACACTGT                   |
| HSD17B8 | TCTCAGCTCAGAACACCGACT                      | ACTTTCTCAGACAGACACTCT                  |
| RING1   | ACTGAGTCTGTATGAGCTGC                      | CAGTCAGAGCAGAAATCTGT                   |
| VPS52   | CTCAGATATGGAGAGGAAG                      | CATCCACCTTTTCCAGAAGAT                   |
| RPS18   | GCGAGTACCAACACAACA                       | TTGGTGAGGTCAATGTCTGC                    |
| WDR46   | GACAAACTTCAGACACCAAGAG                    | CCTAGAGATCCAGAGACTCTT                  |
| PFDN6   | TACCTTCCAGAGGTGACAGAC                    | GTGCTTCAGGTTCTGCC                      |
| RGL2    | GAGTCTGACTGAGCAGCCTTC                     | GGATCAAGAGGTCGATATTG                    |
| ZBTB22  | AGGGTTGCTGCTCTGCGCG                      | CTGCAGACGCTGCTGTTA                     |
| B3GALT4 | AACCAGGAAGCTTGGCAGTGG                    | CCACGCTCTCAGACAGTTCA                    |
| DAXX    | AGAAGATGAAGCAGCTGG                        | GAGAGTGGGCACCTGTG                      |
| TAPBP   | ATGAAGTCCCTCAGCTCTGCT                     | ACACTGAGATAGAGCTCAG                    |
| PHF1    | TCAAGATGTGCTGGGCCAGAT                    | TCTCAGAGCGACAGACAA                     |
| KIFC1   | TTCTCTTCACCTCGCCTCC                      | GGCCATCCCTTGCCTGTG                     |
| CUTA    | GTCTCTGACGCTTTTGTAC                     | CTCAGTCTCCCTCCGATCT                    |
| ZBTB9   | AGACCTCTGCTGGCCAGCTGT                    | AGGCACAGCTAACAAGCTG                    |
| BAK1    | AGGAGTGCGGAGAGCCTG                        | GAGGTAAGGTGACCATCTCT                   |
| ITPR3   | GTCAATGGGGTTCATCAGAC                    | TCCTGTCCTGCTTAAGTCTG                    |
| IP6K3   | GCACTCATGGACGCTGAGTAAGT                    | GACTCTGGAGAGCTTGAA                     |
| LEMD2   | CTCTCTCGGCTTCTGCTCT                      | TCTCACAGTCCACTGGAAT                    |
| MLN     | AAGATGGTATCCCGTAAGGC                    | CTCCGCAGGGTTCTACAGG                    |
Table S5. Antibodies used in this study, related to Figure 1-3 and Figure 5-8.

| Antibodies                                      | Source       | Identifier  |
|-------------------------------------------------|--------------|-------------|
| anti-mouse IgG F(ab)2 antibody                  | DAKO         | F0313       |
| anti-mouse IgG-Alexa Fluor 555-F(ab')2 antibody | Cell Signaling| 4409        |
| anti-rabbit IgG-Alexa Fluor 555-F(ab')2 antibody | Cell Signaling| 4413        |
| anti-mouse IgG-Alexa Fluor 488-F(ab')2 antibody | ThermoFisher  | A-11059     |
| anti-rabbit IgG-Alexa Fluor 488-F(ab')2 antibody | ThermoFisher  | A-11070     |
| anti-mouse IgG-HRP                              | DAKO         | P0161       |
| anti-rabbit IgG-HRP                             | DAKO         | P0448       |
| α-tubulin                                       | Lab Vision   | MS-581      |
| AKT (pan)                                       | Cell Signaling| 4691       |
| b-actin (AC-74)                                 | Sigma-Aldrich| A2228       |
| cleaved caspase-3                               | Cell Signaling| 9661       |
| cleaved PARP                                    | Cell Signaling| 9541       |
| E-Cadherin                                      | Cell Signaling| 4065       |
| ERK1/2 (p44/42 MAPK)                            | Cell Signaling| 4695       |
| Fibronectin                                     | Santa Cruz   | sc-9068     |
| Flag                                            | Sigma-Aldrich| F3165       |
| GAPDH                                           | Millipore    | MAB374      |
| MEK1/2                                          | Cell Signaling| 4694       |
| phosphor-AKT (Ser473)                           | Cell Signaling| 4060       |
| phosphor-Cofilin (Ser3)                         | Cell Signaling| 3313       |
| phosphor-ERK1/2 (Thr202/Tyr204)                 | Cell Signaling| 9101       |
| phosphor-MEK1/2 (Ser217/221)                    | Cell Signaling| 9121       |
| phosphor-SAPK/JNK (Thr183/Tyr185)               | Cell Signaling| 9251       |
| phospho-Rac1/cdc42 (Ser71)                      | Cell Signaling| 2461       |
| RASA5 (Ab1)                                     | ThermoFisher  | PA1-046     |
| RASA5 (Ab2)                                     | Epitomics    | 2477-S      |
| Rhodamine phalloidin                            | Invitrogen   | R415        |
| Vimentin                                        | Sigma-Aldrich| V6630       |
Reference

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