Epigenetic silencing of a Ca^{2+}-regulated Ras GTPase-activating protein RASAL defines a new mechanism of Ras activation in human cancers

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Ras has achieved notoriety as an oncogene aberrantly activated in multiple human tumors. Approximately 30% of all human tumors express an oncogenic form of this GTPase that is locked in an active conformation as a result of being insensitive to Ras GTPase-activating proteins (GAPs), proteins that normally regulate the inactivation of Ras by enhancing its intrinsic GTPase activity. Besides oncogenic mutations in Ras, signaling by wild-type Ras is also frequently deregulated in tumors through aberrant coupling to activated cell surface receptors. This indicates that alternative mechanisms of aberrant wild-type Ras activation may be involved in tumorigenesis. Here, we describe another mechanism through which aberrant Ras activation is achieved in human cancers. We have established that Ras GTPase-activating-like protein (RASAL), a Ca^{2+}-regulated Ras GAP that decodes the frequency of Ca^{2+} oscillations, is silenced through CpG methylation in multiple tumors. With the finding that ectopic expression of catalytically active RASAL leads to growth inhibition of these tumor cells by Ras inactivation, we have provided evidence that epigenetically silencing of this Ras GAP represents a mechanism of aberrant Ras activation in certain cancers. Our demonstration that RASAL constitutes a tumor suppressor gene has therefore further emphasized the importance of Ca^{2+} in the regulation of Ras signaling and has established that deregulation of this pathway is an important step in Ras-mediated tumorigenesis.

calcium | Ras GTPase-activating-like protein | tumorigenesis

As with other small GTPases, Ras switches between two distinct conformations, an inactive GDP-bound state and active GTP-bound complex. The rate of switching is controlled by two classes of proteins. Ras guanine nucleotide exchange factors (GEFs) drive activation by increasing the exchange of GDP for GTP, whereas Ras GTPase-activating proteins (Ras GAPs) enhance the intrinsic Ras GTPase activity thereby leading to inactivation through the conversion of GTP into GDP. Once in the active GTP-bound conformation, Ras engages a number of effectors that couple this GTPase to the regulation of multiple signaling cascades important for cellular proliferation, differentiation, and survival (1, 2).

Ras has achieved notoriety as an oncogene aberrantly activated in multiple human tumors (3). Approximately 30% of all human tumors express an oncogenic form of Ras that is locked in the active conformation as a result of being insensitive to Ras GAPs (3). Besides these oncogenic mutations, signaling through wild-type Ras is also frequently deregulated in tumors through aberrant coupling to activated cell surface receptors. In addition, evidence is emerging that deregulation of Ras GAPs (proteins that normally switch off Ras signaling) may constitute an additional mechanism by which aberrant Ras activation can lead to tumorigenesis (4). Having said this, only one Ras GAP, neurofibromin or neurofibromatosis type 1 (NF1), has so far been confirmed to function as a bona fide tumor suppressor gene. Neurofibromatosis type 1 is a common cancer predisposition syndrome where patients carrying mutations that disrupt the gene encoding for neurofibromin have an increased risk of developing benign and in some case malignant tumors (5).

Besides neurofibromin, various other mammalian Ras GAPs have been identified (6, 7), including $p120^{GAP}$, the SynGAPs (DAB2IP, nGAP, and Syn GAP), and, of particular interest here, the GAP1 proteins. This family comprises $GAP1^{m}$ (or RASA2), $GAP1^{IP4BP}$ (or RASA3), Ca^{2+}-promoted Ras inactivator (CAPRI, or RASA4), and Ras GTPase-activating-like protein (RASAL) or RASAL1 (8–12), and is characterized by a conserved basic domain structure, comprising N-terminal tandem C_2 domains, a central Ras GAP domain and a C-terminal pleckstrin homology domain that is associated with a Bruton's tyrosine kinase (Btk) motif (reviewed in 13). Although this domain structure is conserved, subtle variation in the function of each individual domain and the interaction between domains has a pronounced effect on the regulation of each protein (9, 11, 12, 14, 15). For $GAP1^{IP4BP}$ and $GAP1^{m}$, the association of phosphoinositides with their pleckstrin homology domains is an important determinant in targeting these proteins to the cytosolic leaflet of the plasma membrane where they inactivate Ras (11, 16, 17). In the case of CAPRI and RASAL, plasma membrane targeting is driven through their tandem C2 domains, which bind phospholipids upon an elevation in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) (12, 14, 15). Again the Ca^{2+}-dependent plasma membrane recruitment leads to a local increase of Ras GAP activity and hence leads to Ras inactivation. Interestingly, the activation of CAPRI and RASAL is uniquely sensitive to distinct aspects of the increase in $[Ca^{2+}]_i$. Thus, although CAPRI senses the amplitude of the Ca^{2+} signal by undergoing a sustained but transient association with the plasma membrane, RASAL decodes the frequency of Ca^{2+} oscillations through an oscillatory association with this membrane (12, 14, 15). The identification and characterization of RASAL and CAPRI has therefore established

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Abbreviations: BGS, bisulfite genomic sequencing; Btk, Bruton's tyrosine kinase; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CGI, CpG island; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; MSP, methylation-specific PCR.

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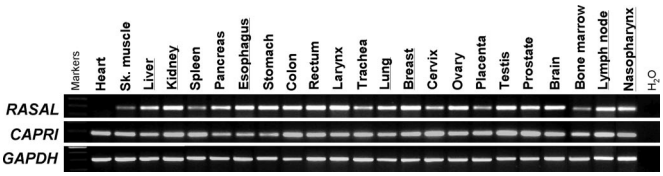

Fig. 1. *CAPRI* and *RASAL* showed broad tissue expression. *RASAL* and *CAPRI* expression in a panel of normal adult tissues was examined by semiquantitative RT-PCR, using *GAPDH* as a control. Normal tissues underlined represent tissues whose corresponding tumors have been studied in this report. Sk, skeletal.

a link between the temporal dynamics of Ca^{2+} signaling and the regulation of the Ras binary switch (18).

Here, we have extended previous reports that down-regulation of *RASAL* and *CAPRI* can induce cellular transformation *in vitro* (19, 20) by studying the expression of these Ras GAPs in a variety of tumors. This has revealed that although *CAPRI* expression is generally unperturbed, *RASAL* is down-regulated in multiple tumors by epigenetic silencing through CpG methylation. Importantly, ectopic expression of *RASAL*, but not its mutants that lack either Ras GAP activity or Ca^{2+} regulation, leads to growth inhibition of tumor cells by inactivation of wild-type Ras. Our study has therefore identified *RASAL* as a bona fide tumor suppressor gene that, through epigenetic silencing, is broadly disrupted in multiple tumors. In doing so, we have defined a physiologically important role for Ca^{2+}-mediated inactivation of Ras through the frequency decoder RASAL, and have established that deregulation of this pathway is an important step in Ras-mediated tumorigenesis.

Results

In Contrast to CAPRI, Reduced Levels of *RASAL* Expression Were Observed in Multiple Tumors. Previous data from Northern blot analysis and *in situ* hybridization have suggested that *RASAL* displays a limited expression pattern being highly expressed in thyroid and adrenal medulla with lower levels in brain, spinal cord and trachea (10). To examine its expression further and to correlate this with the expression of *CAPRI*, we performed more sensitive semiquantitative RT-PCR on a panel of normal human adult tissues (Fig. 1). This revealed a wide and variable expression profile for both *RASAL* and *CAPRI*, indicating that they have important functions within multiple tissue types. To investigate whether this expression profile was perturbed in human cancers, we initially

Table 1. Reduced expression of *RASAL* in tumors (data extracted from expression databases available online)

Tissue type	Sample	Median of expression	*P*	Ref.
Normal oral mucosa	13	0.581	3.8e−16	21
HNSCC	41	−0.178		
Nontumor brain	23	0.691	7.1e−20	55
Oligodendroglioma	50	−0.312		
Normal oral epithelium	4	1.361	1.5e−3	22
OSCC	16	−0.378		
Bladder	14	0.123	1.4e−3	56
Bladder carcinoma	40	−0.056		
Nevus	18	0.258	3.7e−10	23
Melanoma	45	−0.321		
HNSCC without LNM	50	0.048	2.3e−3	24
HNSCC with LNM	59	−0.117		
OSCC without LNM	7	0.593	5.5e−3	22
OSCC with LNM	7	−0.448		

HNSCC, head and neck squamous cell carcinoma; OSCC, oral squamous cell carcinoma; LNM, lymph node metastasis.

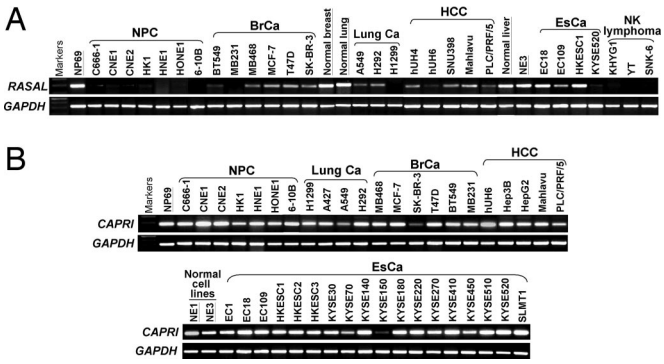

Fig. 2. In contrast to *CAPRI*, *RASAL* expression is frequently down-regulated in tumor cell lines. *RASAL* (*A*) and *CAPRI* (*B*) expression in a variety of tumor cell lines was examined by semiquantitative RT-PCR, using *GAPDH* as a control. Ca, carcinoma; NPC, nasopharyngeal carcinoma; BrCa, breast carcinoma; HCC, hepatocellular carcinoma; EsCa, esophageal carcinoma. Normal tissues and normal epithelial cell lines are underlined.

screened for *RASAL* expression in normal and tumor tissues, using gene expression databases with SAGE Genie (http://cgap.nci.nih.gov/SAGE) and Oncomine (www.oncomine.com). This revealed that *RASAL* expression appeared to be down-regulated in multiple tumors of different origins, including brain, skin, bladder, head, and neck (Table 1). To verify these database searches, we examined *RASAL* and *CAPRI* expression directly in a series of tumor cell lines, using semiquantitative RT-PCR. This revealed that whereas the expression of *CAPRI* was generally unperturbed, a significant reduction in *RASAL* expression was observed in multiple cell lines, notably carcinoma cell lines of nasopharynx [nasopharyngeal carcinoma (NPC)], breast, lung, liver, and esophagus [esophageal squamous cell carcinoma (ESCC)] (Fig. 2 *A* and *B*).

RASAL Down-Regulation Results from Promoter CpG Methylation. Given the frequency of *RASAL* down-regulation, we chose to focus our efforts on establishing the mechanism by which this occurred. Down-regulation of gene expression can result from either genetic or epigenetic mechanism. During our integrative genomic/epigenetic studies of tumor suppressor gene alterations in common carcinomas (25), we did localize the *RASAL* gene within a 1-Mb hemizygous deletion at 12q24.13 (111.6–112.6 Mb). However, only 3 of 20 carcinoma cell lines examined showed this deletion and no homozygous deletion was detected (Fig. 3 and Table 2). Such rarity of *RASAL* locus deletion argues that genetic deletion is, at least, not the main mechanism by which RASAL is down-regulated in tumors.

Recently, it has been established that the transcription factor PITX1, which has a lowered expression at both mRNA and protein level in colon cancer cell lines, prostate, and bladder tumor tissues,

Table 2. Summary of aCGH results in cell lines with 12q24 deletion

		aCGH signal ratio (log_2)	
Carcinoma type	Cell line	dJ363118	bA438N16
NPC	6-10B	−0.437*	
	HNE2		−0.298
ESCC	HKESC1	−0.358*	−0.639*
	HKESC2	−0.496*	−0.203
	HKESC3	−0.235	−0.207
	SLMT1	−0.258	

Only BAC clones with log_2 signal ratio ≤ -0.2 are shown. *, Significant deletion (at least hemizygous) was defined as log_2 signal ratio ≤ -0.4. NPC, nasopharyngeal carcinoma; ESCC, esophageal squamous cell carcinoma.

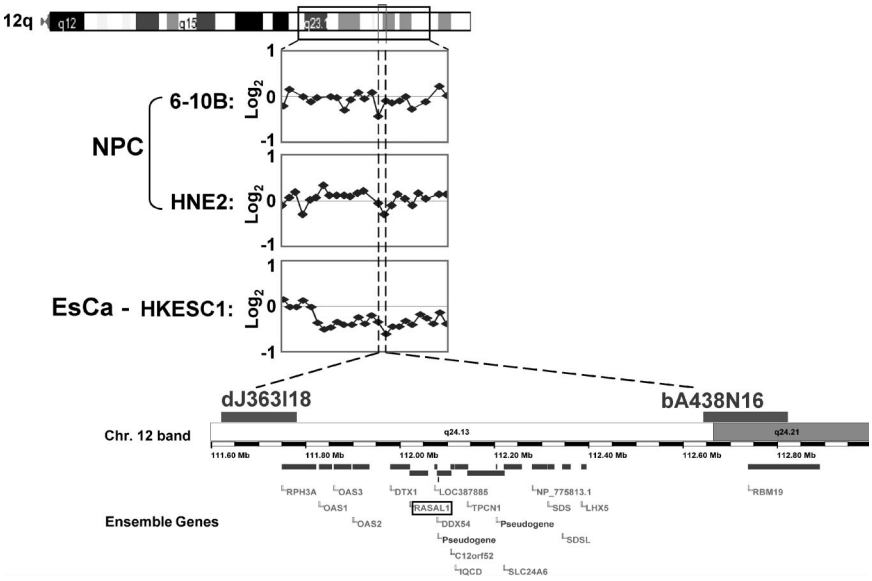

Fig. 3. Infrequent deletion of 12q24 in tumor cell lines. Representative results of 1-Mb array comparative genomic hybridization show a small hemizygous deletion including the *RASAL* (*RASAL1*) locus in some cell lines. Cytoband of 12q is shown in *Top*. Normalized $\log_2$ signal intensity ratios from −1 to 1 are plotted. Each black dot represents a single BAC clone. Two BAC clones closest to the *RASAL* locus (dJ363I18 and bA438N16) are indicated with two vertical dashed lines. The *RASAL* locus is shown in *Lower* as in Ensemble Human Contigview (www.ensemble.org/).

functions as a tumor suppressor by regulating the expression of *RASAL* (19). Thus, in cells expressing wild-type Ras, the reduction of PITX1 lowers *RASAL* expression and therefore decreases the efficiency of GAP-mediated Ras inactivation. However, in the current study, we did not detect an obvious reduction of PITX1 mRNA levels in any of the cell lines examined [supporting information (SI) Fig. 8].

Another key mechanism responsible for gene silencing is CpG island (CGI) methylation, i.e., epigenetic mechanism. To examine this possibility, we used CpG Island Searcher (http://ccnt.hsc.usc.edu/cpgislands2) to identify likely CGI in *RASAL*. This revealed the presence of a typical CGI near the exon 1 of RASAL and hence suggested that *RASAL* may be susceptible to methylation-mediated silencing (Fig. 4*A*). To address this, we initially performed methylation-specific PCR (MSP). This revealed that, although the *RASAL* CGI was not methylated in immortalized but nontransformed epithelial cell lines NE1 and NE3 (esophageal) and NP69 (nasopharyngeal), methylation was detected in all cell lines with silenced *RASAL* (Fig. 4*B*). Supporting this, detailed methylation analysis, using bisulfite genomic sequencing (BGS) also confirmed *RASAL* methylation in the silenced cell lines (Fig. 4*C*). Importantly, in a large cohort of primary tumors, *RASAL* methylation was also detected in a variety of carcinomas and nasal NK/T cell lymphoma but not in normal tissues, normal NK cells, or normal PBMC samples (Table 3 and Fig. 5).

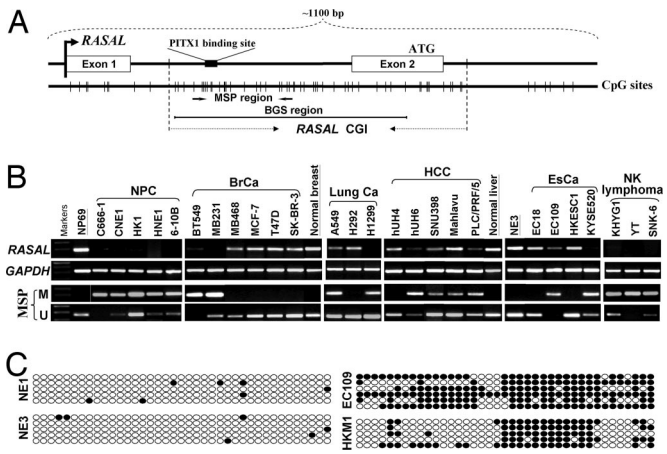

Fig. 4. Epigenetic down-regulation of *RASAL* in tumor cell lines. (*A*) The structure of the *RASAL* gene. The CGI, exon 1 and 2, the PITX1 binding site, MSP, and BGS primer regions are labeled. Each short vertical line represents a CpG site. (*B*) The methylation status of the *RASAL* CGI was analyzed by MSP. M, methylated; U, unmethylated. NP69 is an immortalized but nontransformed normal nasopharyngeal epithelial cell line, and NE1 and NE3 are immortalized normal esophageal epithelial cell lines. For clarity, some RT-PCR data from Fig. 2*A* are reproduced in this figure. (*C*) Methylation of the *RASAL* CGI was confirmed by BGS in esophageal (EC109) and NPC cell lines (HKM1), but not in normal esophageal epithelial cell lines (NE1, NE3). One row of circles represents an individual allele of the *RASAL* CGI analyzed. One circle indicates one CpG site. Filled circles represent methylated CpG sites.

To determine whether CpG methylation directly suppresses *RASAL* expression in tumor cells, we compared *RASAL* expression in tumor cells before and after treatment with the DNA methyltransferase inhibitor 5-aza-2′-deoxycytidine with or without the combination of histone deacetylase inhibitor Trichostatin A (TSA). These treatments have the effect of removing CpG methylation thereby de-repressing gene transcription. Under these conditions, a dramatic induction of *RASAL* expression was observed and this correlated with reduced methylation after drug treatment even in the absence of TSA (Fig. 6). Such data thus directly establish that in these cell lines, down-regulation of *RASAL* occurs directly through CpG methylation.

Ectopic Expression of Catalytically Active RASAL Suppresses the Malignant Phenotype of Tumor Cells with Silenced RASAL. Previous work has established that in a transformation model of human fibroblasts that contain ectopic expression of hTERT and SV40 small T-antigen and short-hairpin RNA vectors to suppress p53 and $p16^{INK4A}$ expression (26), *RASAL* suppression can induce cellular transformation (19). Given that *RASAL* is down-regulated in a number of tumor cell lines, we sought to establish whether ectopic expression of RASAL could revert the malignant phenotype. The anchorage-dependent and -independent growth of three cells lines with reduced or silenced *RASAL* expression (CNE2 and HONE1, NPC cell lines and EC109, an ESCC cell line) was dramatically inhibited by ectopic expression of *RASAL* (Fig. 7*A* and SI Fig. 9*A*). Such growth inhibition depended on the Ras GAP activity of *RASAL* as ectopic expression of RASAL(Q507N), a site-directed mutant that lacks Ras GAP activity (27), failed to show such an inhibition (Fig. 7*A* and SI Fig. 9*A*). Moreover, expression of RASAL(D202A), a C2B domain mutant that is unable to undergo Ca^{2+}-induced recruitment to the plasma membrane (14), also failed to inhibit cell growth (Fig. 7*A* and SI Fig. 9*A*). These observations

Table 3. Frequencies of *RASAL* CGI methylation in tumors and normal tissues

Tissue type	Cell lines	Tissue samples
Tumor		
Nasopharyngeal carcinoma (NPC)	100% (8/8)	53% (26/49)
Lung carcinoma	50% (2/4)	
Esophageal squamous cell carcinoma (ESCC)	31% (5/16)	
Hepatocellular carcinoma (HCC)	31% (4/13)	25% (1/4)
Breast carcinoma (BrCa)	22% (2/9)	21% (3/14)
Nasal NK/T-cell lymphoma (NL)	100% (2/2)	51% (12/21)
Renal cell carcinoma (RCC)	40% (2/5)	
Nontumor		
Nasopharyngitis		0% (0/7)
Normal nasopharynx		0% (0/3)
Surgical margin tissues of HCC		0% (0/6)
Normal $CD3^{-}CD16^{+}CD56^{+}$ NK cells		0% (0/1)
Normal PBMCs from healthy individuals		0% (0/10)
Surgical margin tissues of breast carcinoma (including two tumor-adjacent tissues)		0% (0/6)

are consistent with ectopic *RASAL* expression reverting the transformed phenotype through a Ca^{2+}-dependent inactivation of wild-type Ras. In agreement with this, in *RASAL*-down-regulated cells, the level of the active GTP-bound form of Ras was greatly reduced upon ectopic expression of wild type but not the mutated forms of RASAL (Fig. 7*B*). Confirming the need to inactivate Ras to revert the transformed phenotype of tumor cells with down-regulated *RASAL*. Ectopic expression of a constitutively active, oncogenic mutant form of Ras [Ras(Q61L)] overcame the growth inhibition induced upon ectopic *RASAL* expression (Fig. 7*C*). Thus, in cells in which endogenous *RASAL* has been epigenetically silenced, ectopic expression of *RASAL* can inhibit tumorigenesis of cells harboring wild-type but not oncogenic forms of Ras.

Discussion

The Ras gene is commonly mutated in many but not all human tumors, indicating that alternative mechanisms of aberrant Ras activation may be involved in tumorigenesis (3). Here, we have described that RASAL, a Ca^{2+}-regulated Ras GAP that decodes the frequency of Ca^{2+} oscillations (14), is silenced through CpG methylation in multiple tumors. With the finding that ectopic expression of catalytically active RASAL leads to growth inhibition of these tumor cells by Ras inactivation, we have provided evidence that epigenetically silencing of this Ras GAP represents a new mechanism of aberrant Ras activation in certain cancers.

In addition to the epigenetic silencing described in the present study, previous work has defined that down-regulation of *RASAL* can also be achieved in certain tumors through transcriptional control driven by PITX1 (19). Although we were unable to confirm this in the present study, such data support the conclusion that the Ca^{2+}-mediated regulation of *RASAL* is an important tumor suppressor pathway in multiple cancers. The importance of RASAL as a Ca^{2+}-regulated tumor suppressor is further emphasized because down-regulation of CAPRI [another Ca^{2+}-regulated Ras GAP (12, 15)] was not observed in the vast majority of tumor cell lines studied. Having said this, the *CAPRI* gene is located at 7q22.1, a chromosomal segment where loss of heterozygosity is commonly observed in malignant myeloid diseases (28), and so CAPRI may have tumor suppressor functions in these tissue types. Indeed, we did observe the down-regulation of *CAPRI* in some of the cell lines in our study (Fig. 2*B*). Why silencing of *RASAL* occurs with a high frequency in these cell lines relative to *CAPRI* remains unclear. One possibility is that this stems from the fact that RASAL is a Ras GAP that is remarkably sensitive to even small oscillations in $[Ca^{2+}]_i$, and hence

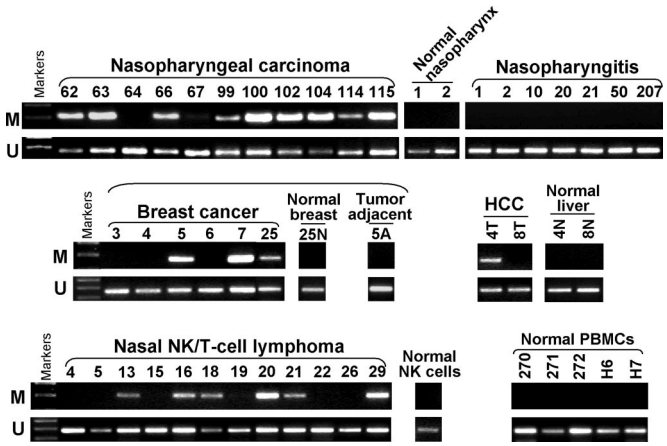

Fig. 5. *RASAL* CGI is also methylated in different primary tumors (carcinomas and lymphoma) but not in nontumor tissues or normal cells. The methylation status of the *RASAL* CGI was analyzed by MSP. M, methylated; U, unmethylated. Representative results are shown.

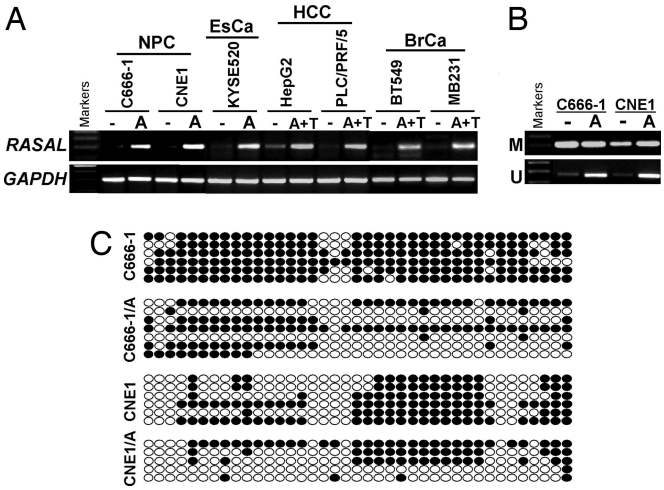

Fig. 6. Demethylation reactivates *RASAL* expression. (*A*) Cell lines were treated with 5-aza-2′-deoxycytidine, with (A+T) or without (A) TSA, and the levels of *RASAL* expression were determined by semiquantitative RT-PCR. Methylation status of the *RASAL* CGI was analyzed by MSP (*B*) and BGS (*C*). −, not treated.

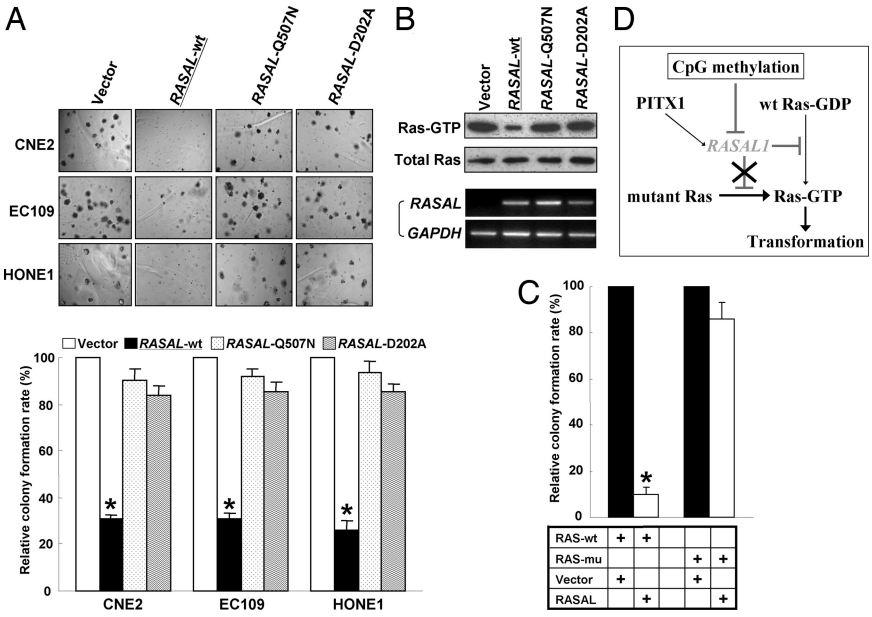

Fig. 7. Ectopic *RASAL* expression inhibits tumor cell growth through Ras inactivation. (*A*) The effect of ectopic expression of *RASAL* wild type (*RASAL*-wt) and its mutants on tumor cell growth as investigated by soft agar assay. Quantitative analyses of colony numbers are shown in *Lower* as values of mean ± standard deviation. *P* values were calculated with Student's *t* test. The asterisk indicates statistical significance ($P \leq 0.01$). (*B*) The active form of Ras was pulled down by Raf-RBD and subjected to Western blot analysis after SDS/PAGE. The expression levels of *RASAL* and its mutants were determined by RT-PCR. (*C*) The effect of *RASAL* expression on cellular growth of CNE2 cells in the presence of wild-type (wt) or mutant (mu) *RAS*(Q61L) was investigated by soft agar assay. Quantitative analysis of colony numbers is shown as in *A*. (*D*) Proposed model that CpG methylation-mediated silencing of *RASAL* contributes to *RAS*-mediated tumorigenesis. wt, wild type.

may play a more important role than CAPRI in defining the basal level of Ras activation (14).

Because Ras GAPs switch off Ras signaling, they have always been considered as potential tumor suppressor genes (4). However, with the exception of neurofibromin (neurofibromatosis type 1), until recently no clear evidence has emerged for their role in Ras-driven tumorigenesis. Few studies have detected mutation in the classic Ras GAP $p120^{GAP}$ (29), although heterozygous mutations of the $p120^{GAP}$ gene do promote abnormal angiogenic remodeling and cause the disease capillary malformation-arteriovenous malformation (30). More recently, in addition to the RNAi screens that have proposed tumor suppressor functions for RASAL and CAPRI (19, 20), DAB2IP (human DAB2 interactive protein), a Ras GAP that associates with the disable gene family member DAB2 (31), has been demonstrated to be silenced in prostate, breast, lung, and gastrointestinal tumors through aberrant promoter CpG methylation (32–36). Unfortunately, it remains to be established exactly how DAB2IP is regulated *in vivo* upon receptor activation. However, when taken with the data presented in the current study, it would appear that for some, but certainly not all Ras GAPs, epigenetic silencing of their expression may be a common feature in a wide variety of human tumors that carry nononcogenic forms of Ras.

It has been known for some considerable time that increases in $[Ca^{2+}]_i$ can influence cell proliferation and differentiation (37, 38). Initial evidence for a link between Ca^{2+} and Ras-signaling came from the observation that oncogenic forms of Ras could bypass certain aspects of Ca^{2+}-dependent cell cycle control (39). More recently, the molecular characterization of Ca^{2+}-regulated Ras GEFs and Ras GAPs has firmly established the link between Ca^{2+} and Ras signaling (40–42). Indeed, it is increasingly evident that most of the potential mammalian Ras GEFs and Ras GAPs are either directly or indirectly regulated by Ca^{2+} and/or diacylglycerol (41). This includes $p120^{GAP}$, which by interacting with the Ca^{2+} sensor annexin A6, undergoes Ca^{2+}-mediated association with the plasma membrane from where it negatively regulates Ras (43). Furthermore, in addition to controlling the binary Ras switch, by binding to calmodulin Ca^{2+} directly modulates the localization and signaling of K-Ras (44). Our demonstration that *RASAL* constitutes a tumor suppressor gene has therefore emphasized further the importance of Ca^{2+} in the regulation of Ras signaling, and has established that deregulation of this pathway is an important step in Ras-mediated transformation and tumorigenesis (Fig. 7*D*).

Methods

Cell Lines and Tissue DNA/RNA Samples. A number of tumor cell lines and DNA/RNA samples of primary tumors (carcinomas of nasopharynx, breast, lung, liver, esophagus and kidney, and nasal NK/T cell lymphoma), and their corresponding normal tissues were used in this study, as described (25, 45–52).

Semiquantitative RT-PCR. Total RNA was extracted by using TriReagent (Molecular Research Centre, Cincinnati, OH). RNA was reverse-transcribed by using MuLV Reverse Transcriptase and random hexamer, and RT-PCR was performed as previously described, using the GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA) (47), using GAPDH as a control. The input of RNA for RT-PCR was 0.125 μg per reaction. Primers used were as follows: RASALF, 5′-actgcctagtgaaagtggac and RASALR, 5′-cgagatcttgccgatgatgt for *RASAL*; and CAPRIF, 5′-agcgcagctcgctgtacatc and CAPRIR, 5′- ggcaggtgcacttggtactc for *CAPRI*. RT-PCR was performed for 35 cycles, using AmpliTaq Gold (Applied Biosystems). The specificity of *RASAL* RT-PCR products was confirmed by direct sequencing of PCR products from two normal tissues (trachea, breast) and one normal (NP69) and two tumor (NPC, ESCC) cell lines.

Array Comparative Genomic Hybridization. Whole-genome arrays of 1-Mb resolution with 3,040 BAC/PAC clones were kindly provided by C. Langford (Wellcome Trust Sanger Institute, Cambridge, U.K.). Clones details are listed in the Ensembl database (www.ensembl.org/Homo sapiens/index.html). Array comparative genomic hybridization was performed as described in ref. 25. Briefly, sample DNA (600 ng) was labeled with Cy5-dCTP (Amersham Pharmacia, Piscataway, NJ), whereas reference DNA of normal PBMCs from healthy Chinese donors was labeled with Cy3-dCTP. Hybridized slides were scanned by using an Axon 4000B scanner (Axon Instruments, Union City, CA) and analyzed with the GenePixPro 4.0 image analysis software.

MSP and BGS. Bisulfite modification of DNA was carried out as previously described (48). For MSP (46, 47), bisulfite-modified DNA was amplified by using methylation-specific or unmethylation-specific primer pair: RASALm11, 5′-gtgtatttttgttttcgtcgttc and RASALm2, 5′-caacgaactcttaccgaaacg; and RASALU3, 5′-aatttattaggagttagtggttat and RASALU2, 5′-cacaacaaactcttac-

caaaaca. MSP primers were confirmed previously for not amplifying any not bisulfited DNA and thus specific. For BGS (25, 53), bisulfite-treated DNA was amplified by using BGS primers RASALBGS1, 5′-gtttaatgttaatttattaggagtt and RASALBGS3, 5′-ctaaccacaaactttccaaacaa and cloned into pCR4-TOPO vector (Invitrogen, Carlsbad, CA). At least five colonies were randomly chosen for sequencing.

Colony Formation Assay. Anchorage-dependent growth of tumor cells was investigated by monolayer colony formation assay (25). Cells were cultured overnight in a 12-well plate (1.0×10^5 per well) and transfected with various plasmids, using FuGENE 6 (Roche Diagnostics, Indianapolis, IN). Forty-eight hours later, the transfectants were replated in triplicate and cultured for 16–20 days with complete RPMI medium 1640 containing G418 (500 μg/ml). Surviving colonies were stained with Gentian Violet after methanol fixation, and visible colonies ($\geq$50 cells) were counted.

Soft Agar Assay. Anchorage-independent growth of tumor cells was determined by soft agar assay as described in ref. 54. The transfection was carried out as in colony formation assay. Forty-eight hours after transfection, cells were resuspended in complete medium containing G418 (500 μg/ml). With 1/10 volume of heated 3.3% soft agar added, 5×10^3 cells were seeded into each well of a 24-well plate. The colonies were counted after 10 days.

Ras Activity Assay. The purification of GST-Raf-RBD and detection of active Ras were performed as described in ref. 54. Cell lysates of CNE2, stimulated by 5-min incubation of 10% serum after overnight serum starvation, were incubated with GST-Raf-RBD prebound to glutathione-Sepharose for 30 min at 4°C with gentle rocking. Bound proteins were eluted with SDS/PAGE sample buffer, resolved on 12% acrylamide gels, and subjected to Western blot analysis.

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