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Inactivation of Ras GTPase-activating proteins promotes unrestrained activity of wild-type Ras in human liver cancer

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Abstract

Background & Aims—Aberrant activation of Ras pathway is ubiquitous in human hepatocarcinogenesis, but the molecular mechanisms leading to Ras induction in the absence of Ras mutations remain underinvestigated. We defined the role of Ras GTPase activating proteins (GAPs) in the constitutive activity of Ras signaling during human hepatocarcinogenesis.

Methods—Mutation status of Ras genes and Ras effectors was assessed in a collection of human hepatocellular carcinoma (HCC). Levels of Ras GAPs (RASA1-4, RASAL1, nGAP, SYNGAP1, DAB2IP, and NF1) and the RASAL1 upstream inducer PITX1 were determined by real-time RT-PCR and immunoblotting. Promoter and genomic status of RASAL1, DAB2IP, NF1, and PITX1 were assessed by methylation assays and microsatellite analysis. Effects of RASAL1, DAB2IP, and PITX1 on HCC growth were evaluated by transfection and siRNA analyses of HCC cell lines.

Results—In the absence of Ras mutations, downregulation of at least one Ras GAP (RASAL1, DAB2IP, or NF1) was found in all HCC samples. Low levels of DAB2IP and PITX1 were detected mostly in a HCC subclass from patients with poor survival, indicating that these proteins control tumor aggressiveness. In HCC cells, reactivation of RASAL1, DAB2IP, and PITX1 inhibited proliferation and induced apoptosis, whereas their silencing increased proliferation and resistance to apoptosis.

Conclusions—Selective suppression of RASAL1, DAB2IP, or NF1 Ras GAPs results in unrestrained activation of Ras signaling in the presence of wild-type Ras in HCC.

Keywords

Ras GAPs; HCC; methylation

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Ras proteins are molecular switches for signalling cascades modulating many aspects of cell biology [1,2]. Two distinct conformations characterize *Ras* proteins: an inactive GDP-bound and an active GTP-bound state, which are controlled by *Ras* guanine nucleotide exchange factors (GEFs) and *Ras* GTPase-activating proteins (GAPs) [3]. *Ras* GEFs trigger activation of *Ras* by increasing the exchange of GDP for GTP, whereas *Ras* GAPs enhance the intrinsic *Ras* GTPase activity, leading to *Ras* inactivation through GTP into GDP conversion [3]. Approximately 30% of human tumors express an oncogenic form of *Ras* genes (*Ha-*, *K-*, and *N-Ras*), which is locked in the active conformation as a result of being insensitive to *Ras* GAPs [1,2]. Besides oncogenic mutations, aberrant activation of *Ras* cascade may also occur in the presence of wild-type *Ras* genes in cancer. Indeed, *Ras* pathway is often deregulated in tumors through mutations in upstream inducers and downstream effectors, or via inactivation of *Ras* inhibitors, including DAB2, RASSF1A, and SPRY2 [4]. Suppression of *Ras* GAPs may constitute an additional mechanism whereby aberrant *Ras* activation promotes tumorigenesis [5]. Various *Ras* GAPs have been identified, including p120GAP or RASA1, the SynGAPs (Disabled homolog 2 Interacting Protein or DAB2IP, nGAP, and SynGAP1), neurofibromin (NF1), and the GAP1 proteins (GAP1m or RASA2, $GAP1^{IP4BP}$ or RASA3, Ca^{2+}-promoted *Ras* inactivator or RASA4, and *Ras* GTPase-activating-like protein 1 or RASAL1) [3,5]. The role of *Ras* GAPs in carcinogenesis remains unexplored. Only NF1 was shown to be a *bona fide* oncosuppressor gene [5], whereas few studies were conducted on other *Ras* GAPs. Recently, DAB2IP downregulation was detected in human prostate cancer [6], and two genome-wide studies identified DAB2IP as a putative oncosuppressor gene in aggressive prostate adenocarcinomas [7]. In human hepatocellular carcinoma (HCC), DAB2IP is often epigenetically silenced [8], while RASAL1 is downregulated in HCC cells [9]. However, *Ras* GAPs status has not been comprehensively examined, and no functional studies on the role of *Ras* GAPs in liver cancer cells have been performed.

Here, we investigated the expression levels of *Ras* GAPs in a large collection of human HCC, determined the molecular mechanisms responsible for inactivation of *Ras* GAPs, and studied their biologic role in HCC cell lines. Our findings indicate that inactivation of *RASAL1*, *DAB2IP*, or pituitary homeobox 1 (*PITX1*; a *RASAL1* upstream inducer) is a major oncogenic event leading to unconstrained activation of wild-type *Ras* in human HCC.

Materials and methods

Human tissue samples

Ten normal livers, 88 HCCs and corresponding surrounding non-tumorous livers were used. Clinicopathological features of the patients are listed in Supplementary Table 1. HCCs were divided in two groups, HCC with poor prognosis (HCCP) and HCC with better prognosis (HCCB), which were characterized by a shorter (< 3 years) or longer (> 3 years) survival following liver partial resection, respectively [10]. Tissues were kindly provided by Dr. Z. Sung (National Laboratory of Molecular Oncology, Beijing, China) and the Liver Tissue Procurement and Distribution System (Minneapolis, MN; Pittsburgh, PA; Richmond, VA), funded by NIH Contract #N01-DK-9-2310. Institutional Review Board approval was obtained at participating hospitals and the National Institutes of Health.

Mutation analysis

Mutations at *Ha-RAS*, *Ki-RAS*, and *N-RAS*, *A-RAF*, *B-RAF*, *RAF-1*, and *EGFR* genes were assessed in the whole sample collection by direct DNA sequencing as described [11-13].

Real-time RT–PCR, methylation analyses, microsatellite analysis, imunoblotting, and immunoprecipitation

Real-time RT PCR, methylation and microsatellite analyses were performed as described (Supplementary Material) [4]. Primers for *Ha-Ras*, *Ki-Ras*, *N-Ras*, *RASA1-4*, *nGAP*, *SYNGAP1*, *hDAB2IP*, *RASAL1*, *NF1*, *PITX1*, and ribonucleic acid ribosomal 18S (*RNR-18*, internal control) genes were from Applied Biosystems (Foster City, CA). Primers to assess the promoter status of *PITX1* were designed using the MethPrimer software [14], and those for *RASAL1*, *DAB2IP*, and *NF1* genes were previously generated (Supplementary Table 3) [6,9,15]. Presence of promoter hypermethylation was defined as the amplification of the specific PCR product of the investigated gene when using methylated-specific primers. Tissue lysates were subjected to immunoblotting and immunoprecipitation as reported (Supplementary Material) [4].

Ras activation and Ras GEF assays, microvessel density (MVD)

Levels of activated Pan-, Ha-, Ki-, and N-*R*as (Pan-, Ha-, Ki-, and N-*R*as-GTP) were determined with the *R*as Activation Assay Kit (Millipore, Billerica, MA). *Ras* GEF activity and MVD were determined as described (Supplementary Material) [16,17].

Cell Lines, viability, apoptosis, and vascular endothelial growth factor-α secretion assays

Human HCC cell lines were subjected to either silencing or demethylating experiments as described in Supplementary Material.

Results

Activation of wild-type Ras during human hepatocarcinogenesis

Protein and mRNA levels of Pan-, Ha-, Ki-, and N-*Ras*, as assessed by immunoblotting and real-time RT-PCR, were significantly higher in non-neoplastic surrounding livers as compared with normal livers, with no significant differences between the two prognostic HCC subclasses. No further upregulation of Ki-*Ras* and N-*Ras* was detected in HCC, whereas an additional increase of Pan- and Ha-*Ras* levels occurred in HCC, predominantly HCCP (Fig. 1A, B and C). Levels of activated (GTP-bound) Pan-, Ha-, Ki-, and N-*Ras* were instead progressively upregulated from normal livers to HCCP (Fig. 1A and B), which was paralleled by an increase in *Ras* GEF activity (Fig. 1D). This suggests that Ki- and N-*Ras* overactivity is not due to transcriptional induction in HCC. Thus, we tested whether mutations in *Ha-*, *Ki-*, and N-*Ras*, *Ras* upstream inducers (*EGFR*) or downstream effectors (*A-Raf*, *B-Raf*, and *Raf-1*) were responsible for *Ras* activation. No mutations were detected, indicating that unconstrained activation of *Ras* proteins is independent of gene mutations in human hepatocarcinogenesis.

Frequent downregulation of DAB2IP and RASAL1 in human HCC

Since suppression of *Ras* GAPs may trigger uncontrolled activation of wild-type *Ras* [3,5], we investigated whether *Ras* GAPs expression was perturbed in human HCC by real-time RT-PCR. Based on the expression pattern, *Ras* GAP genes could be divided into three distinct categories (Fig. 2A). *RASA1-4*, *nGAP*, and *Syngap1* genes were progressively upregulated from normal livers to HCC; *NF1* expression was upregulated in most tumors and decreased in a small HCC subset (11/88, 12.5%); *RASAL1* and *DAB2IP* mRNA were almost ubiquitously downregulated (65/88, 73.8%; and 67/88, 76.1%, respectively) in HCC when compared with normal and surrounding non-neoplastic livers. Levels of *RASAL1*, *DAB2IP*, and *NF1* genes were higher in surroundings than in normal livers, while expression of *DAB2IP* was lowest in HCCP. Equivalent results were obtained when assessing RASAL1, DAB2IP, and NF1 levels by immunoblotting (Fig. 2B and Supplementary Fig. 1).

As a possible mechanism for *RASAL1*, *DAB2IP* and *NF1* silencing, the promoter methylation status of these genes was investigated using methylation-specific PCR and combined bisulphite restriction analysis (Fig. 3). No hypermethylation at *RASAL1*, *DAB2IP* and *NF1* promoters was observed in normal livers (not shown). Aberrant hypermethylation of *RASAL1* and *DAB2IP* promoters occurred both in non-tumorous surrounding livers and HCC, but at significantly higher frequency in tumors (56.8% vs. 13.6%, $P = 3.03 \times 10^{-10}$ and 76.1% vs. 29.5%, $P = 2.94 \times 10^{-10}$, respectively). *NF1* promoter hypermethylation occurred only in a small HCC subset (5/88, 5.7%). A significant downregulation of RASAL1, DAB2IP, and NF1 mRNA levels was detected in methylated when compared with unmethylated samples and normal livers (Supplementary Fig. 1). Strikingly, all HCCs exhibited promoter methylation of at least one of the three *Ras* GAPs (Fig. 3), underlining the selective pressure toward wild-type *Ras* unrestrained activity via *Ras* GAPs inactivation in hepatocarcinogenesis.

Subsequently, we examined the correlation between the clinicopathological data from the HCC patients and aberrant methylation status of *RASAL1*, *DAB2IP*, and *NF1* promoters. Aberrant *DAB2IP* promoter methylation was more frequent in HCCP than HCCB (100% vs. 48.8%, $P = 6.81 \times 10^{-10}$), suggesting that *DAB2IP* inactivation contributes to HCC aggressiveness. No other clinicopathological features correlated with *Ras* GAPs methylation status, including patient's age, sex, etiology, presence of cirrhosis, tumor size, Edmondson/Steiner grade, and alpha-fetoprotein levels.

Genomic status of *Ras* GAPs was further investigated using LOH analysis of *RASAL1*, *DAB2IP*, and *NF1* loci, by comparing HCCs with respective surrounding livers. LOH rate at *RASAL1*, *DAB2IP*, and *NF1* loci was 28.4%, 21.6%, and 12.5%, respectively. Noticeably, promoter hypermethylation and/or LOH for *DAB2IP* and *NF1* genes was paralleled by *DAB2IP* and *NF1* gene downregulation (not shown), suggesting that these molecular mechanisms are responsible for *DAB2IP* and *NF1* silencing in human HCC. Nevertheless, 15 out of 65 (23.1%) HCCs displaying *RASAL1* downregulation showed no promoter methylation or LOH, implying other mechanisms in the suppression of *RASAL1*.

Inhibition of PITX1 contributes to RASAL1 inactivation in human HCC

Previously, it was shown that the *PITX1* gene inhibits *Ras* activity via *RASAL1* transactivation [18]. To investigate the importance of PITX1 in modulating *RASAL1* expression, we assessed the levels of PITX1 by real-time RT-PCR and immunoblotting (Fig. 2A and B and Supplementary Fig. 2). PITX1 mRNA and protein levels were induced in non-neoplastic surrounding livers when compared with normal livers, whereas PITX1 was downregulated in 37 of 88 (42%) tumors, mainly HCCP (30/37, 81.1%). Interestingly, PITX1 was overexpressed in a subset of HCCB (10/88, 11.4%) displaying RASAL1 upregulation (not shown). Furthermore, all HCCs exhibiting low RASAL1 expression independent of its promoter hypermethylation showed downregulation of PITX1, suggesting that PITX1 modulates RASAL1 expression. To substantiate our hypothesis, PITX1 was suppressed by siRNA in HuH6 and Hep3B cell lines (expressing high PITX1 levels). As expected, PITX1 inhibition induced downregulation of RASAL1 (Fig. 2C and Supplementary Fig. 3A). Likewise, *PITX1* cDNA transfection into SNU449 cells (displaying low PITX1 and RASAL1 levels, but unmethylated RASAL1 promoter) triggered RASAL1 upregulation (Fig. 2D and Supplementary Fig. 3B). However, PITX1 cDNA transfection into PLC/Alexander cells (displaying low PITX1 and RASAL1 expression, the latter due to the RASAL1 promoter hypermethylation [9]) failed to restore RASAL1 expression (Fig. 2E and Supplementary Fig. 3C). Thus, PITX1 induction does not override RASAL1 epigenetic silencing. Next, we investigated the mechanisms responsible for PITX1 downregulation in HCC (Fig. 3). PITX1 promoter hypermethylation was rare (5/88, 5.7%) in non-tumorous surrounding livers, while it was found in 25 of 37 HCC (67.6%) with low PITX1 levels and

at significant higher frequency in HCCP (17/25, 68%). Furthermore, 6 HCC with PITX1 promoter hypermethylation displayed LOH at the PITX1 locus (Fig. 3). The role of methylation on PITX1 silencing was substantiated in Focus and SNU389 cell lines, harbouring PITX1 promoter hypermethylation (Calvisi DF, unpublished observation). Treatment of these cell lines with the demethylating agent zebularine caused a marked up-regulation of PITX1 levels (Supplementary Fig. 4A-D), corroborating the role of promoter hypermethylation in PITX1 transcriptional repression.

RASAL1, DAB2IP or PITX1 suppression promotes unrestrained Ras activity in human HCC

The downregulation of RASAL1, DAB2IP, and PITX1 in HCC with high levels of activated wild-type *Ras* suggests that either the loss of RASAL1, DAB2IP or PITX1 precludes the requirement for *Ras* mutation or, alternatively, that their expression is repressed by wild-type *Ras*. To test this hypothesis, we introduced the wild-type *Ha-Ras* gene into HuH6 and Hep3B cells (expressing RASAL1, DAB2IP, and PITX1). Transient transfection with *Ha-Ras* caused upregulation rather than downregulation of *RASAL1, DAB2IP* or *PITX1* genes, presumably as a consequence of Ha-*Ras* negative regulatory loop activation (Fig. 4A and B). Similarly, transfection with a dominant-negative mutant form of *Ha-Ras* gene (S17N) in SNU449 cells displaying low RASAL1, DAB2IP, and PITX1 expression, did not upregulate RASAL1, DAB2IP, and PITX1 (Fig. 4C and D). Thus, inactivation of RASAL1, DAB2IP, and PITX1 triggers uncontrolled activation of wild-type *Ras* in HCC.

Manipulation of DAB2IP, RASAL1, or PITX1 affects HCC cell growth in vitro

The role of DAB2IP, RASAL1, and PITX1 in HCC was investigated by their silencing (via siRNA) or overexpression in HCC cells. DAB2IP, RASAL1, or PITX1 suppression in HuH6 and Hep3B cells increased cell viability, with significant differences depending on the gene silenced. The most dramatic increase in HCC cell growth was detected when suppressing PITX1 or DAB2IP, whereas RASAL1 inhibition caused less growth promotion (Fig. 5A). Furthermore, inhibition of DAB2IP, RASAL1 or PITX1 induced a rise in *Ras* GEF activity regardless of the silenced gene (Supplementary Fig. 5A). Opposite results were obtained when overexpressing *DAB2IP*, *RASAL1*, or *PITX1* in SNU449 HCC cells. Indeed, the strongest reduction in cell viability and increase in apoptosis were observed following transfection of *PITX1* or *DAB2IP* (Fig. 5B and C). Combination of *DAB2IP*, *RASAL1*, or *PITX1* overexpression with the apoptosis inducer Staurosporine significantly intensified HCC cells apoptosis (Fig. 5D). *Ras* GEF activity was equally increased following overexpression of *DAB2IP*, *RASAL1*, or *PITX1* genes (Supplementary Fig. 5B).

The effect of overexpressing DAB2IP, RASAL1, or PITX1 in SNU449 cells co-transfected with wild-type *Ha-Ras* was also evaluated. Forced overexpression of *DAB2IP*, *RASAL1*, or *PITX1* significantly decreased the growth of *Ha-Ras* co-transfected cells (not shown), and the levels of activated *Ha-Ras* (*Ras* GTP), ERK2, c-FOS, and c-JUN (Fig. 5E). In striking contrast, *DAB2IP*, *RASAL1*, or *PITX1* overexpression had a significantly less pronounced effect on HCC cell growth (not shown) and failed to inhibit ERK2, c-FOS, and c-JUN expression when co-transfected with *ERK2* (Fig. 5F). Thus, RASAL1, DAB2IP, and PITX1 inhibit wild-type *Ras* growth upstream of ERK in HCC cells.

DAB2IP expression directly correlates with ASK1-driven apoptosis and inversely with activation of VEGFR2/Akt/PLC-γ cascade in human HCC

DAB2IP (or AK1-interacting protein-1, AIP1) promotes apoptosis by activating Apoptosis signal–regulating kinase 1 (ASK1), an upstream inducer of Jun-amino-terminal kinase (JNK) and p38 mitogen-activated-protein-kinase (MAPK) proteins [19]. DAB2IP activates ASK1 by allowing its release from the inactive complex with 14-3-3 [20]. Furthermore, DAB2IP suppresses growth and pro-angiogenic signals driven by vascular endothelial

growth factor receptor 2 (VEGFR2)/Akt/phospholipase C-gamma (PLC-γ) axis via its binding to VEGFR2 and the p85 regulatory subunit of phosphoinositide 3-kinase (PI3K-p85) [21]. DAB2IP expression was highest in surrounding non-tumorous livers (Fig. 2A), where it was paralleled by low levels of phosphorylated ASK1, ASK1/14-3-3 complexes (signs of inactivated ASK1), and by high expression of activated (phosphorylated) JNK and p38 MAPK (Fig. 6A and Supplementary Fig. 6A). Activation of VEGFR2 cascade members, including VEGFR2, Akt, and PLC-γ, was low in surrounding non-tumorous livers, presumably due to high levels of DAB2IP bound to VEGFR2 and PI3K-p85. In contrast, DAB2IP expression was ubiquitously low in HCCP (Fig. 2A), where elevated amount of phosphorylated ASK1 and ASK1/14-3-3 and low levels of pJNK and p38 MAPK were detected (Fig. 6A and Supplementary Fig. 6A). Low levels of DAB2IP-VEGFR2 and DAB2IP-PI3K-p85 complexes were paralleled by high levels of activated VEGFR2, PLC-γ, and Akt in HCCP. A putative anti-proliferative and anti-angiogenic role of DAB2IP was further suggested by the inverse relationships between DAB2IP levels and MVD extent in HCC (Fig. 6B). Overexpression of DAB2IP into SNU449 cells resulted in ASK1 activation and suppression of the VEGFR2/Akt/PLC-γ axis, whereas opposite results where obtained when silencing DAB2IP in HuH6 cells (Fig. 6C and D, and Supplementary Fig. 6B and C). In addition, suppression of DAB2IP in HuH6 and Hep3B cells augmented VEGF-α secretion in the medium (Supplementary Fig. 7A), whereas DAB2IP overexpression in SNU449 cells dramatically inhibited VEGF-α secretion (Supplementary Fig. 7B). Altogether, these data envisage the involvement of DAB2IP in the modulation of ASK1-dependent apoptosis and VEGFR2/Akt/PLC-γ-driven angiogenesis in human HCC.

Ras mutations and Ras GAPs promoter hypermethylation are mutually exclusive in breast, lung, pancreatic, and colorectal tumors

Finally, we investigated the frequency of *Ras* mutations and hypermethylation of RASAL1, PITX1, and DAB2IP promoters in a collection of human breast, lung, pancreatic, and colorectal tumors. No somatic mutations at Ha-*Ras* and N-*Ras* genes were detected (not shown). Mutations of Ki-*Ras* were rare in breast (1/25, 4%), intermediate in lung (5/30, 16.7%), and elevated in colorectal (12/30, 40%), and pancreatic (17/30, 56.7%) tumors (Supplementary Fig. 8). Promoter hypermethylation of RASAL1, PITX1, and DAB2IP genes was inversely correlated with Ki-*Ras* mutations, being extremely high in breast (21/25, 84%), intermediate in lung (17/30, 56.7%), and low in colorectal (8/30, 26.7%) and pancreatic (5/30, 16.7%) tumors. Strikingly, with the exception of a lung tumor showing concomitant Ki-*Ras* mutation and DAB2IP promoter methylation, tumors displayed either Ki-Ras mutations or promoter hypermethylation of *Ras* GAPs (Supplementary Fig. 7). Thus, Ki-*Ras* mutations and *Ras* GAP methylation are mutually exclusive events in various human tumors.

DISCUSSION

In this study, we provide evidence that suppression of RASAL1, DAB2IP, and NF1 *Ras* GAPs, which are responsible for switching off *Ras* signalling in normal cells, and PITX1, a RASAL1 upstream inducer, results in aberrant activation of the wild-type *Ras* in human HCC. This alternative mode of *Ras* activation may be implicated in carcinogenesis of other organs not harbouring *Ras* mutations. Indeed, we show that mutation of Ki-*Ras* gene and inactivation of *Ras* GAPs by promoter hypermethylation are mutually exclusive events in breast, lung, colon, and pancreatic cancer.

Since *Ras* GAPs negatively modulate the *Ras* cascade, their potential oncosuppressor role has been hypothesized [3,6]. Here, we demonstrate that re-expression of *RASAL1*, *DAB2IP*, or *PITX1* strongly restrains HCC cell growth *in vitro* by reducing proliferation and increasing apoptosis. RASAL1 anti-neoplastic function resides in its ability to facilitate *Ras*

GTP hydrolysis in a Ca^{++}-dependent manner [3,5], and its expression is reduced in several human tumors [9]. Recently, RASAL1 was found to be downregulated in aggressive colorectal carcinomas but not in adenomas, suggesting a prognostic role for RASAL1 in colon cancer [22]. This finding differs from the almost ubiquitous inactivation of RASAL1 in HCC, suggesting that RASAL1 downregulation has a different impact on tumor development and progression depending on cell/tissue type. Furthermore, predominant inactivation of DAB2IP and PITX1 in HCCP supports their role in HCC progression. Accordingly, PITX1 is among the genes frequently silenced by promoter hypermethylation in moderately/poorly-differentiated HCC [23]. Overexpression of *PITX1* or *DAB2IP* caused a stronger growth restraint than overexpression of *RASAL1* although all three genes showed equal ability to reduce *Ras* GEF activity in HCC cell lines. This finding suggests that PITX1 and DAB2IP function may be directed toward targets other than *Ras*. Ongoing studies are investigating PITX1 molecular targets in human HCC. As concerns to the DAB2IP, previous data and present work suggest that its oncosuppressor role may also reside in the ability to activate the ASK1/JNK/p38 MAPK cascade and to inhibit the VEGFR2/Akt/PLC-γ axis [6,19-21].

Nonetheless, our data indicate that RASAL1-, DAB2IP- or PITX1-mediated suppression of *Ras* occurs upstream of ERK, implying that ERK deregulation cannot be effectively counteracted by RASAL1, DAB2IP or PITX1 overexpression. This finding, together with the observed inactivation of RAF/ERK inhibitors, including SPRY2 and NORE1A [4], substantiates the existence of a redundant group of genes regulating the *Ras* pathway at various steps that must be inactivated to achieve unconstrained activation of *Ras* cascade in hepatocarcinogenesis.

Our data also suggest that not all *Ras* GAPs possess anti-growth properties in liver. Although most *Ras* GAPs were upregulated in HCC, their induction did not compensate for the loss of RASAL1, DAB2IP or PITX1 and did not reduce *Ras* activation and Ras GEF levels. Furthermore, we cannot exclude that some *Ras* GAPs possess paradoxical growth properties in cancer. Indeed, RASA1 was found to be required for activation of the Akt pathway in normal cardiomyocytes and lung carcinoma cells [24].

In summary, we provide evidence that inactivation of *RASAL1*, *DAB2IP* and *PITX1* putative oncosuppressors has a causative role in promoting unrestrained activation of wild-type *Ras* in human HCC (Supplementary Figure 9). Our results emphasize the importance of *Ras* pathway in hepatocarcinogenesis and speak in favour of therapeutic approaches aimed at reactivating RASAL1, DAB2IP and PITX1 and/or inhibiting *Ras* in human HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper:

AIP1	ASK1-interacting protein 1
ASK1	apoptosis signal–regulating kinase 1

CAPRI	Ca^{2+}-promoted *Ras* inactivator
DAB2IP	disabled homolog 2 interacting protein
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
HCC	hepatocellular carcinoma
HCCB	hepatocellular carcinoma with better prognosis
HCCP	hepatocellular carcinoma with poor prognosis
JUNK	Jun-amino-terminal kinase
MAPK	mitogen activated protein kinase
NF1	neurofibromin
PITX1	pituitary homeobox 1
PLCγ	phospholipase C-gamma
PP2A	protein phosphatase 2A
RASAL1	*Ras* GTPase-activating-like protein 1
VEGFR2	vascular endothelial growth factor receptor 2

References

1. Barbacid M. *Ras* oncogenes: their role in neoplasias. Eur J Clin Invest 1990;20:225–235. [PubMed: 2114981]
2. Karnoub AE, Weinberg RA. *Ras* oncogenes: split personalities. Nat Rev Mol Cell Biol 2008;9:517–531. [PubMed: 18568040]
3. Iwashita S, Song SY. *Ras* GAPs: a crucial regulator of extracellular stimuli for homeostasis of cellular functions. Mol Biosyst 2008;4:213–222. [PubMed: 18437264]
4. Calvisi DF, Ladu S, Gorden A, Farina M, Lee JS, Conner EA, et al. Mechanistic and prognostic significance of aberrant methylation in the molecular pathogenesis of human hepatocellular carcinoma. J Clin Invest 2007;117:2713–2722. [PubMed: 17717605]
5. Bernards A, Settleman J. GAPs in growth factor signalling. Growth Factors 2005;23:143–149. [PubMed: 16019436]
6. Wang Z, Tseng CP, Pong RC, Chen H, McConnell JD, Navone N, et al. The mechanism of growth-inhibitory effect of DOC-2/DAB2 in prostate cancer. Characterization of a novel GTPase-activating protein associated with N-terminal domain of DOC-2/DAB2. J Biol Chem 2002;277:12622–12631. [PubMed: 11812785]
7. Duggan D, Zheng SL, Knowlton M, Benitez D, Dimitrov L, Wiklund F, et al. Two genome-wide association studies of aggressive prostate cancer implicate putative prostate tumor suppressor gene DAB2IP. J Natl Cancer Inst 2007;99:1836–1844. [PubMed: 18073375]
8. Qiu GH, Xie H, Wheelhouse N, Harrison D, Chen GG, Salto-Tellez M, et al. Differential expression of hDAB2IPA and hDAB2IPB in normal tissues and promoter methylation of hDAB2IPA in hepatocellular carcinoma. J Hepatol 2007;46:655–663. [PubMed: 17258345]
9. Jin H, Wang X, Ying J, Wong AH, Cui Y, Srivastava G, et al. Epigenetic silencing of a Ca(2+)-regulated Ras GTPase-activating protein RASAL defines a new mechanism of Ras activation in human cancers. Proc Natl Acad Sci U S A 2007;104:12353–12358. [PubMed: 17640920]
10. Lee JS, Chu IS, Heo J, Calvisi DF, Sun Z, Roskams T, et al. Classification and prediction of survival in hepatocellular carcinoma by gene expression profiling. Hepatology 2004;40:667–676. [PubMed: 15349906]

11. Jiang W, Kahn SM, Guillem JG, Lu SH, Weinstein IB. Rapid detection of *Ras* oncogenes in human tumors: applications to colon, esophageal, and gastric cancer. Oncogene 1989;4:923–928. [PubMed: 2666911]
12. Fransen K, Klintenas M, Osterstrom A, Dimberg J, Monstein HJ, Söderkvist P. Mutation analysis of the BRAF, ARAF and RAF-1 genes in human colorectal adenocarcinomas. Carcinogenesis 2004;25:527–533. [PubMed: 14688025]
13. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res 2004;64:8919–8923. [PubMed: 15604253]
14. Li LC, Dahiya R. MethPrimer: designing primers for methylation PCRs. Bioinformatics 2002;18:1427–1431. [PubMed: 12424112]
15. Rübben A, Bausch B, Nikkels A. Somatic deletion of the NF1 gene in a neurofibromatosis type 1-associated malignant melanoma demonstrated by digital PCR. Mol Cancer 2006;5:36. [PubMed: 16961930]
16. Li BQ, Kaplan D, Kung H, Kamata T. Nerve growth factor stimulation of the Ras-Guanine Nucleotide Exchange Factor and GAP activities. Science 1992;256:1456–1459. [PubMed: 1604323]
17. Tanigawa N, Lu C, Mitsui T, Miura S. Quantitation of sinusoid-like vessels in HCC: its clinical and prognostic significance. Hepatology 1997;26:1216–1223. [PubMed: 9362365]
18. Kolfschoten IG, van Leeuwen B, Berns K, Mullenders J, Beijersbergen RL, Bernards R, et al. A genetic screen identifies PITX1 as a suppressor of RAS activity and tumorigenicity. Cell 2005;121:849–858. [PubMed: 15960973]
19. Min W, Lin Y, Tang S, Yu L, Zhang H, Wan T, et al. AIP1 recruits phosphatase PP2A to ASK1 in tumor necrosis factor-induced ASK1-JNK activation. Circ Res 2008;102:840–848. [PubMed: 18292600]
20. Zhang R, He X, Liu W, Lu M, Hsieh JT, Min W. AIP1 mediates TNF-alpha-induced ASK1 activation by facilitating dissociation of ASK1 from its inhibitor 14-3-3. J Clin Invest 2003;111:1933–1943. [PubMed: 12813029]
21. Zhang H, He Y, Dai S, Xu Z, Luo Y, Wan T, et al. AIP1 functions as an endogenous inhibitor of VEGFR2-mediated signaling and inflammatory angiogenesis in mice. J Clin Invest 2008;118:3904–3916. [PubMed: 19033661]
22. Ohta M, Seto M, Ijichi H, Miyabayashi K, Kudo Y, Mohri D, et al. Decreased expression of the *Ras*-GTPase activating protein RASAL1 is associated with colorectal tumor progression. Gastroenterology 2008;136:206–216. [PubMed: 18992247]
23. Gao W, Kondo Y, Shen L, Shimizu Y, Sano T, Yamao K, et al. Variable DNA methylation patterns associated with progression of disease in hepatocellular carcinomas. Carcinogenesis 2008;29:1901–1910. [PubMed: 18632756]
24. Pamonsinlapatham P, Hadj-Slimane R, Lepelletier Y, Allain B, Toccafondi M, Garbay C, et al. P120-Ras GTPase activating protein (RasGAP): a multi-interacting protein in downstream signaling. Biochimie 2009;91:320–328. [PubMed: 19022332]

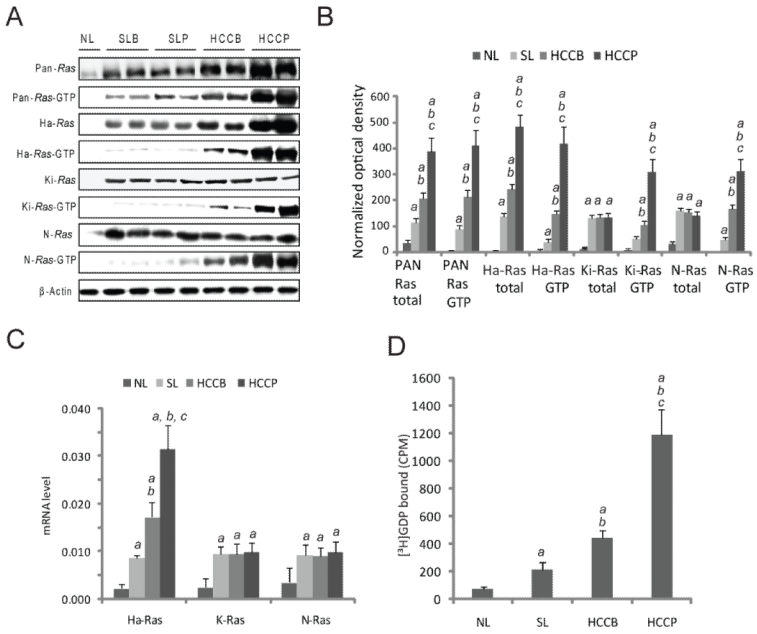

Figure 1. Activation of *Ras* proteins in human HCC

(*A*) Whole cell lysates were prepared from normal livers (*NL*), surrounding livers with better (*SLB*) or poor (*SLP*) prognosis, and HCC with better (*HCCB*) or poor (HCCP) prognosis and immunoblotted with indicated antibodies. Representative immunoblotting shown. (*B*) Optical densities were normalized to β-actin values and expressed in arbitrary units. Each *bar* represents mean ± SD. (*C*) Expression levels of Ha-, Ki-, and N-Ras in normal livers, HCC and corresponding non-tumorous surrounding livers by real-time RT-PCR. N-Target (NT) = $2^{-\Delta Ct}$; ΔCt = RNR18-Ct target gene. Each *bar* represents mean ± SD. (*D*) Assessment of Ras GEF activity in normal livers, HCC and matching non-tumorous surrounding livers. Tissue lysates were added to the unlabeled GDP Ras-agarose complex in the presence of 10 μCi of [3H]GDP. The amount of bound Ras-[^{3}H]GDP was determined by a scintillation counter. Addition of 2 mmol/L GTP-γ-S to the mix of [^{3}H]GDP, tissue lysates, and unlabeled GDP Ras-agarose complex, abolished the exchange reaction. Each *bar* represents mean ± SD. SLB and SLP data did not show statistical differences, and are thus merged and indicated as surrounding livers (SL) in *B, C*, and *D*. Multiple comparison test (Kruskal-Wallis) $p < 0.05$ *a*, *vs.* NL; *b vs*. SL; *c*, *vs*. HCCB.

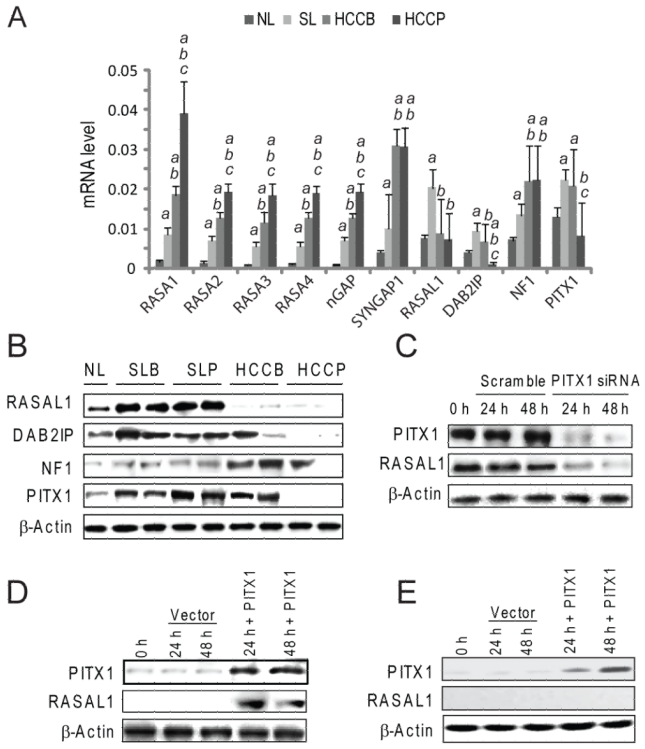

Figure 2. Levels of Ras GAPs in normal livers (*NL*), surrounding livers (*SL*), and HCC with better (*HCCB*) or poor (HCCP) prognosis, as determined by real-time RT-PCR (*A*) and immunoblotting (*B*)

(*A*) mRNA levels determined by real-time RT-PCR. N-Target (NT) = $2^{-\Delta Ct}$; ΔCt = RNR18-Ct target gene. Each *bar* represents mean ± SD. Multiple comparison test (Kruskal-Wallis): $P < 0.05$ *a, vs.* NL; *b vs.* SL; *c, vs.* HCCB. (*B*) Representative immunoblotting shown. (*C*) Effect of PITX1 silencing via siRNA in HuH6 cells on RASAL1 levels. (*D*,*E*) Effect of forced overexpression of PITX1 by transient transfection on RASAL1 levels in SNU449 (*D*) and PLC/Alexander cell lines (*E*), harbouring RASAL1 unmethylated and methylated gene promoter, respectively, as assessed by imunoblotting. Following transfection, PITX1 induces RASAL1 upregulation in SNU449 but not in PLC/Alexander cells (*D*,*E*). Experiments were repeated at least 3 times in triplicate, and results shown in (*C*) were reproduced in Hep3B cells (not shown).

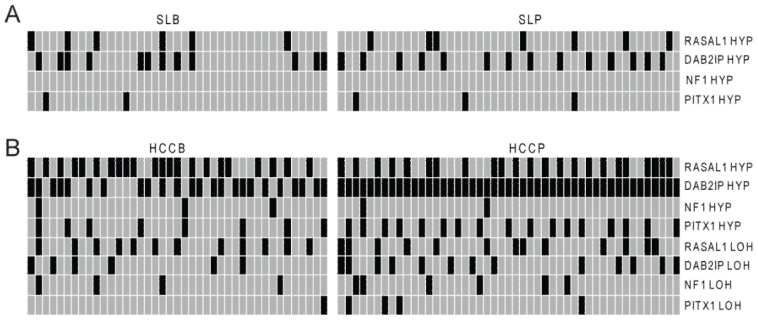

Figure 3. Frequency of promoter methylation (*HYP*) and loss of heterozygosity (*LOH*) at RASAL1, DAB2IP, NF1, and PITX1 genes in human non-tumorous surrounding livers (*A*) and corresponding HCC (*B*)

Normal livers did not exhibit any altered profile and, therefore, are not shown. Promoter methylation and genomic status of RASAL1, DAB2IP, NF1, and PITX1 genes was determined by methylation-specific PCR, combined bisulphite restriction analysis, and microsatellite analysis. Presence of promoter hypermethylation and/or LOH or absence of the same alterations is indicated by black and grey squares, respectively.

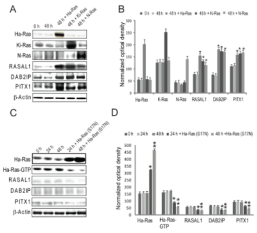

Figure 4. Expression of RASAL1, DAB2IP or PITX1 is not repressed by *Ras*

(*A*) Wild-type *Ha-Ras* was overexpressed by transient transfection in HuH6 cells and effect on RASAL1, DAB2IP, and PITX1 was evaluated by immunoblotting. (*B*) Densitometric analysis of data showed in (*A*). (*C*) Transfection of a dominant negative mutant form of *Ha-Ras* (S17N) did not rescue RASAL1, DAB2IP, and PITX1 levels in PLC/Alexander cells. (*D*) Densitometric analysis of data showed in (*C*). Experiments were repeated at least 3 times in triplicate, and results shown in (*A*) were reproduced in Hep3B cells (not shown). (*B, D)* each *bar* represents mean ± SD. Kruskal-Wallis test $p < 0.05$: (*B*) *, different from 48 h; (*D*) * and ** different from 24 and 48 h, respectively.

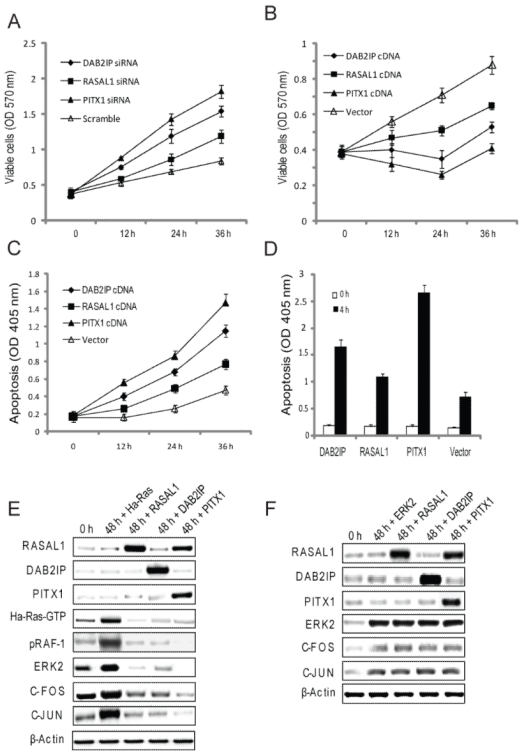

Figure 5. Effect of manipulating RASAL1, DAB2IP, and PITX1 expression in human HCC cell lines

(*A*) Silencing of RASAL1, DAB2IP or PITX1 via siRNA increased cell growth in HuH6 cells. Overexpression of RASAL1, DAB2IP or PITX1 by transient transfection induced growth restraint (*B*) and apoptosis (*C*) in SNU449 cells. The pro-apoptotic effect of RASAL1, DAB2IP, or PITX1 genes was further amplified with the use of Staurosporine (*D*). (*E*) SNU449 cells were transfected with wild-type *Ha-Ras* cDNA and co-transfected with *RASAL1*, *DAB2IP*, or *PITX1* cDNA. Overexpression of *RASAL1*, *DAB2IP*, or *PITX1* reduced the levels of activated *Ha-Ras* (*Ha-Ras*-GTP), and ERK2, c-FOS, and c-JUN effectors. (*F*). Overexpression of *RASAL1*, *DAB2IP*, or *PITX1* in SNU449 cells by transient transfection had no effect on ERK2, c-FOS, and c-JUN expression when co-transfected with *ERK2*. Experiments were repeated at least 3 times in triplicate. Kruskal-Wallis test, treated *vs*. scrambled: (*A,B,C*) $p < 0.05$ at 36 h. Student *t*-test (*D*), 4 *vs*. 0 h, $p < 4.69 \times 10^{-9}$.

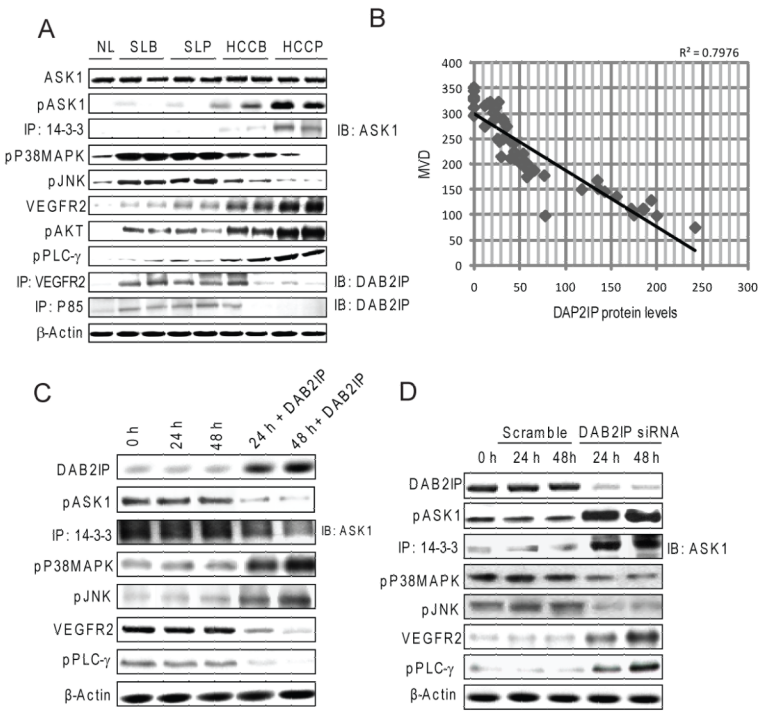

Figure 6. DAB2IP modulates the activation of the ASK1/P38MAPK/JNK and VEGFR2/AKT/PLC-γ pathways

(*A*) Whole cell lysates were prepared from normal livers (*NL*), surrounding livers with better (*SLB*) or poor (*SLP*) prognosis, and HCC with better (*HCCB*) or poor (HCCP) prognosis and immunoblotted with indicated antibodies. Representative immunoblotting shown. (*B*) Inverse relationship between DAB2IP protein levels and HCC microvessel density. (*C*) Effect of DAB2IP induction by transient transfection on ASK1/P38MAPK/JNK and VEGFR2/AKT/PLC-γ cascades in SNU449 cells. (*D*) Effect of DAB2IP silencing via siRNA in HuH6 cells on ASK1/P38MAPK/JNK and VEGFR2/AKT/PLC-γ cascades. All experiments were repeated at least 3 times in triplicate, and results shown in (*D*) were reproduced in Hep3B cells (not shown).