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ORIGINAL ARTICLE

Dysregulation of RalA signaling through dual regulatory mechanisms exerts its oncogenic functions in hepatocellular carcinoma

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Abstract

Background and Aims: Ras-like (Ral) small guanosine triphosphatases (GTPases), *RalA* and *RalB*, are proto-oncogenes directly downstream of *Ras* and cycle between the active guanosine triphosphate-bound and inactive guanosine diphosphate-bound forms. RalGTPase-activating protein (RalGAP) complex exerts a negative regulation. Currently, the role of Ral up-regulation in cancers remains unclear. We aimed to examine the clinical significance, functional implications, and underlying mechanisms of RalA signaling in HCC.

Approach and Results: Our in-house and The Cancer Genome Atlas RNA sequencing data and quantitative PCR data revealed significant up-regulation of *RalA* in patients' HCCs. Up-regulation of RalA was associated with more aggressive tumor behavior and poorer prognosis. Consistently, knockdown of *RalA* in HCC cells attenuated cell proliferation and migration in vitro and tumorigenicity and metastasis in vivo. We found that RalA up-regulation was driven by copy number gain and uncovered that SP1 and ETS proto-oncogene 2 transcription factor cotranscriptionally drove RalA expression. On the other hand, *RalGAPA2* knockdown increased the RalA activity and promoted intrahepatic and extrahepatic metastasis in vivo. Consistently, we observed significant *RalGAPA2* down-regulation in patients' HCCs. Intriguingly, HCC tumors showing simultaneous down-regulation of *RalGAPA2* and up-regulation of *RalA* displayed a significant association with more aggressive tumor behavior in terms of more frequent venous invasion, more advanced tumor stage,

Abbreviations: ChIP, chromatin immunoprecipitation; CSC, cancer stem cell; EpCAM, epithelial cell adhesion molecule; ETS, ETS proto-oncogene transcription factor; GTPase, guanosine triphosphatase; IC_{50}, median inhibitory concentration; KD, knockdown; KO, knockout; mTOR, mammalian target of rapamycin; NTC, nontargeted control; NTL, nontumorous liver; qPCR, quantitative PCR; Ral, Ras-like; RalGAP, Ral GTPase-activating protein; RNA-seq, RNA sequencing; shRNA, short hairpin RNA; TCGA, The Cancer Genome Atlas.

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and poorer overall survival. Of note, Ral inhibition by a Ral-specific inhibitor RBC8 suppressed the oncogenic functions in a dose-dependent manner and sensitized HCC cells to sorafenib treatment, with an underlying enhanced inhibition of mammalian target of rapamycin signaling.

Conclusions: Our results provide biological insight that dysregulation of RalA signaling through dual regulatory mechanisms supports its oncogenic functions in HCC. Targeting RalA may serve as a potential alternative therapeutic approach alone or in combination with currently available therapy.

INTRODUCTION

Activation of Ras guanosine triphosphatase (GTPase) is one of the signature molecular alterations in human cancers.[1] Ras-like (Ral) GTPases, including RalA and RalB, are small GTPases first identified based on their significant sequence homology to Ras.[2] Acting as essential downstream signaling of Ras, Ral activation has been shown to induce the transformation of various types of human cancers and support tumor development.[3-5] Recently, increasing evidence has shown that Ral-mediated signaling is an emerging downstream pathway of Ras and supports the oncogenic development of multiple solid cancers. Although RalA shares more than 80% of sequence homology with RalB,[3] they may be functionally distinct from each other. For instance, RalA is required for the transformation of Ras mutation–driven pancreatic cancer cells as well as supporting the anchorage-independent growth in colorectal cancer,[6-8] whereas RalB was demonstrated to contribute to the promigratory functions, supporting cancer invasiveness and cancer progression.[7,9,10] However, the expression pattern, functional roles, and clinical relevance of Ral signaling are highly context-dependent, and their potential roles in cancer types with a low degree of Ras mutation, including HCC, are largely unknown and remain to be clarified.

Consisting of an alpha catalytic and a beta regulatory subunit, the dimeric RalGTPase-activating protein (RalGAP) complex functions as a specific negative regulator against Ral activity by accelerating its intrinsic guanosine triphosphate (GTP) hydrolysis.[11] Recently, a series of studies has suggested that dysregulation of the RalGAP complex is present in multiple solid cancers, including those of the buccal cavity, prostate, bladder, and colon.[12-15] In addition, functional inactivation of the RalGAP complex could promote cell migration in vitro and enhance cancer metastasis in vivo in an RalA-dependent manner.[12-14] Furthermore, the catalytic subunit of RalGAP has been suggested to be a previously undocumented critical tumor suppressor gene for liver carcinogenesis in a two-step forward short hairpin RNA (shRNA)-based library screening in mice.[16] Although these findings have aroused attention regarding the potential involvement of the RalGAP-RalA signaling axis in cancer development, the actual dysregulation of the molecular components of the RalGAP complex in HCC and its clinicopathological relevance are yet to be defined. More importantly, how one could exploit the potential dysregulation of the RalGAP complex to rationally design alternative strategies to target the active RalA signaling would be a question worthy to be addressed.

In this study, we systematically examined the expression of Ral and its negative regulator in HCC and showed their potential association with the clinicopathological features of HCC. On identifying the predominant forms of Ral and RalGAP that were expressed in human HCCs, we generated the corresponding genetic knockdown (KD), knockout (KO), and overexpressing HCC cells for functional characterization and underlying mechanistic studies of Ral signaling in HCC. We also built a model and delineated how Ral signaling could be up-regulated in HCC through transcriptional as well as posttranslational regulations and eventually supported the pro-oncogenic functions. Of significance, the anti-HCC effects of a specific Ral inhibitor, when applied alone or in combination with sorafenib, were also examined. Taken together, our results provide biological insights on the dysregulation of RalA signaling through dual mechanisms in HCC. Targeting RalA may serve as a potential alternative therapeutic approach alone or in combination with the currently available therapy for patients with HCC.

MATERIALS AND METHODS

Human tissue specimens

All human HCC and corresponding nontumorous liver (NTL) tissue samples were collected during surgical resection from the Department of Surgery at Queen Mary Hospital of Hong Kong. Specimens were collected with informed consent, and the use of human clinical samples was approved by the Institutional Review Board of the University of Hong Kong and the Hospital Authority Hong Kong West Cluster (UW 09-185 and UW 17-056).

All patients had surgical resection only; there was no transplantation or involvement of donation of organs.

Cell lines and cell culture

Human HCC cell lines, Hep3B and PLC/PRF/5, and hepatoblastoma cell line HepG2 were purchased from American Type Culture Collection. Human HCC cell line Huh7 was purchased from Japanese Collection of Research Bioresources Cell Bank. Metastatic human HCC cell line, MHCC97L, was a gift from Dr. Z. Y. Tang (Fudan University, Shanghai, China). The immortalized normal liver MIHA cells were a gift from Dr. J. R. Chowdhury, Albert Einstein College of Medicine, New York. Authentication of HCC cell lines used in this study was performed by short tandem repeat (STR) DNA profiling in March 2018, and no cellular cross-contamination was detected. The STR result for MHCC97L is provided in Figure S1A. "Xenome," using RNA sequencing (RNA-seq) data, estimated a negligible 0.04% to 0.42% (n = 3) for MHCC97L with mouse contamination and 0.15% to 0.40% for clinical human NTL and HCC samples (n = 6), thus indicating our MHCC97L cells do not contain cells of murine origin.[17] Furthermore, MHCC97L used in this study contains HBV integrated in *Telomerase reverse transcriptase* (*TERT*) locus of the genome.[18] All cell cultures were tested negative for mycoplasma contamination.

HepG2 and Hep3B and PLC/PRF/5 were cultured with Minimum Essential Medium, whereas the other cells were maintained in DMEM with high glucose (Gibco BRL). The working culture medium was supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL), 100 mg/ml penicillin G, and 50 μg/ml streptomycin (Gibco BRL). Additional 110 mg/l sodium pyruvate was also included for culturing HepG2, Hep3B, MHCC97L, and MIHA cells. The cells were kept in a humidified incubator with a constant supply of 5% CO_2 maintained at 37°C.

Plasmids

RalA (NM_005402.4) and its dominant active form (G23V) were amplified from HCC cell lines and subcloned into LentiBlast vector. For the RalGAPA2 (NM_020343.3) construct, GAP (1447-1873aa) was amplified from HCC cell lines and subcloned into LentiBlast vector. ETS Proto-oncogene transcription factor 2 (NM_005239) was amplified from normal human liver complementary DNA (cDNA) and subcloned into Flag-pcDNA3.1+neo plasmid. SP1 cDNA was amplified from SP1-pcDNA3.1 and subcloned into Flag-pcDNA3.1+neo plasmid. Wild-type (-1,000 to +20) RALA promoter was amplified from normal human liver DNA and subcloned into pGL3-basic vector. The mutant representing loss of putative SP1 binding site with double mutations at positions -168 nt (converting the 7 nucleotides from GGGGTGG to CTAAAAA at the corresponding site) and -117 nt (converting the 4 nucleotides from GCGG to AAAA at the corresponding site) of DNA was generated based on wild-type RALA promoter DNA and subcloned into pGL3-basic vector. The mutant representing loss of the putative ETS2 binding site with double mutations at positions -38 nt and +4 nt (convert the 4 nucleotides from GGAA to AAAA at the corresponding site) of DNA were generated based on wild-type RALA promoter DNA and subcloned into pGL3-basic vector. Furthermore, the mutant representing the loss of both putative SP1 and ETS2 binding site with 4 mutations at positions -168 nt, -117 nt, -38 nt, and +4 nt of the transcription start site of RALA mRNA was generated based on both SP1 mutant and ETS2 mutant of RALA promoter DNA and subcloned into pGL3-basic vector for reporter assay.

Additional supporting information is included in Supporting Materials and Methods.

RESULTS

Frequent up-regulation of RalA in human HCCs

To examine the expression of Ral family genes in HCC, whole transcriptome sequencing (RNA-seq) analysis was performed on 16 pairs of human HCC tumor and NTL tissues. Of the two Ral genes, RalA but not RalB, was significantly up-regulated in HCC tumors (false discovery rate [FDR] = 0.004) (Figure 1A, upper panel). Consistently, RalA was also significantly up-regulated in the cohort of paired HCCs (n = 50) in The Cancer Genome Atlas (TCGA) database (FDR = 4.20E-4) (Figure 1A, lower panel). Moreover, the RalA expression showed a progressive stepwise increase along with the progression in tumor stages (Figure 1B) and tumor cellular differentiation grades (Figure 1C). Furthermore, HCCs with higher RalA expression were associated with poor prognosis with shorter patient overall survival (Figure 1D). RalA up-regulation was also independently confirmed by real-time quantitative PCR (qPCR) in our other patient cohort consisting of 90 pairs of HBV-associated HCC, with ≥2-fold up-regulation in 34.4% (31/90) of the HCC tumors (Figure 1E). With a cutoff of 2-fold up-regulation by qPCR, overexpression of RalA was significantly associated with more aggressive tumor behavior, namely, more frequent tumor microsatellite formation (p = 0.002), venous invasion (p = 0.007), and absence of tumor encapsulation (p = 0.0002) (Table 1). Taken together, RalA was frequently up-regulated in HCC and associated with more aggressive tumor behavior and poorer prognosis. Indeed, from TCGA, apart from HCC, RalA was also

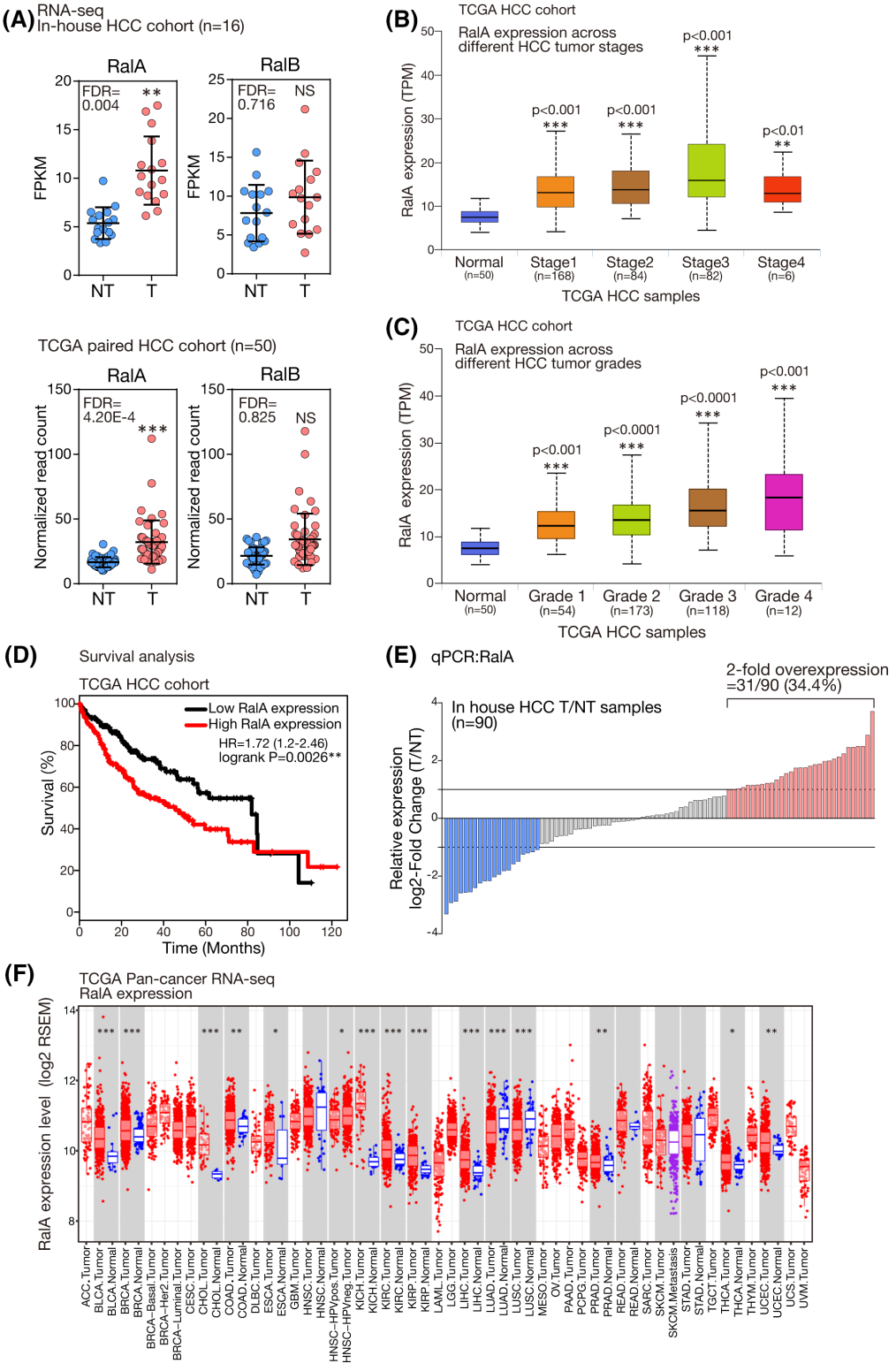


FIGURE 1 Up-regulation of RalA was associated with poor prognosis in human HCC. (A) RalA and RalB mRNA expression levels in tumors compared with nontumors in in-house HCC and TCGA paired HCC cohorts. (B) RalA expression across increasing HCC tumor stages and (C) cellular differentiation grades in TCGA HCC cohort. (D) Analysis of overall survival of patients with HCC with high or low RalA expression. (E) RalA mRNA expression in an independent cohort of 90 pairs of patients' HCCs. (F) RalA mRNA expression of tumours and non-tumourous tissues in different types of cancers

TABLE 1 Clinicopathological correlation of RalA overexpression in samples of patients with HCC

Parameters	RalA overexpression (*n* = 31)		RalA normal or underexpression (*n* = 59)		*p*[a]
Sex					0.800
Male	24	(26.7%)	43	(47.8%)	
Female	7	(7.8%)	16	(17.8%)	
Mean age (range)[b]	52.5	(28-70)	53.5	(24-74)	0.691
Tumor size					0.821
>5 cm	18	(20.2%)	37	(41.6%)	
≤5 cm	12	(13.5%)	22	(24.7%)	
Background liver disease					0.880
Normal	2	(2.2%)	4	(4.4%)	
Chronic hepatitis	11	(12.2%)	25	(27.8%)	
Cirrhosis	18	(20.0%)	30	(33.3%)	
Liver invasion					0.104
Presence	16	(19.3%)	19	(22.9%)	
Absence	13	(15.7%)	35	(42.2%)	
Tumor microsatellite formation					0.002[a]
Presence	24	(27.0%)	24	(27.0%)	
Absence	7	(7.9%)	34	(38.2%)	
Tumor encapsulation					0.0002[a]
Presence	2	(2.2%)	25	(28.1%)	
Absence	29	(32.6%)	33	(37.1%)	
Venous invasion					0.007[a]
Presence	24	(26.7%)	28	(31.1%)	
Absence	7	(7.8%)	31	(34.4%)	
Cellular differentiation					0.078
Edmondson grade I-II	18	(20.2%)	22	(24.7%)	
Edmondson grade III-IV	13	(14.6%)	36	(40.4%)	
Tumor-node-metastasis staging					0.059
I-II	6	(6.7%)	25	(28.1%)	
III-IV	24	(27.0%)	34	(38.2%)	

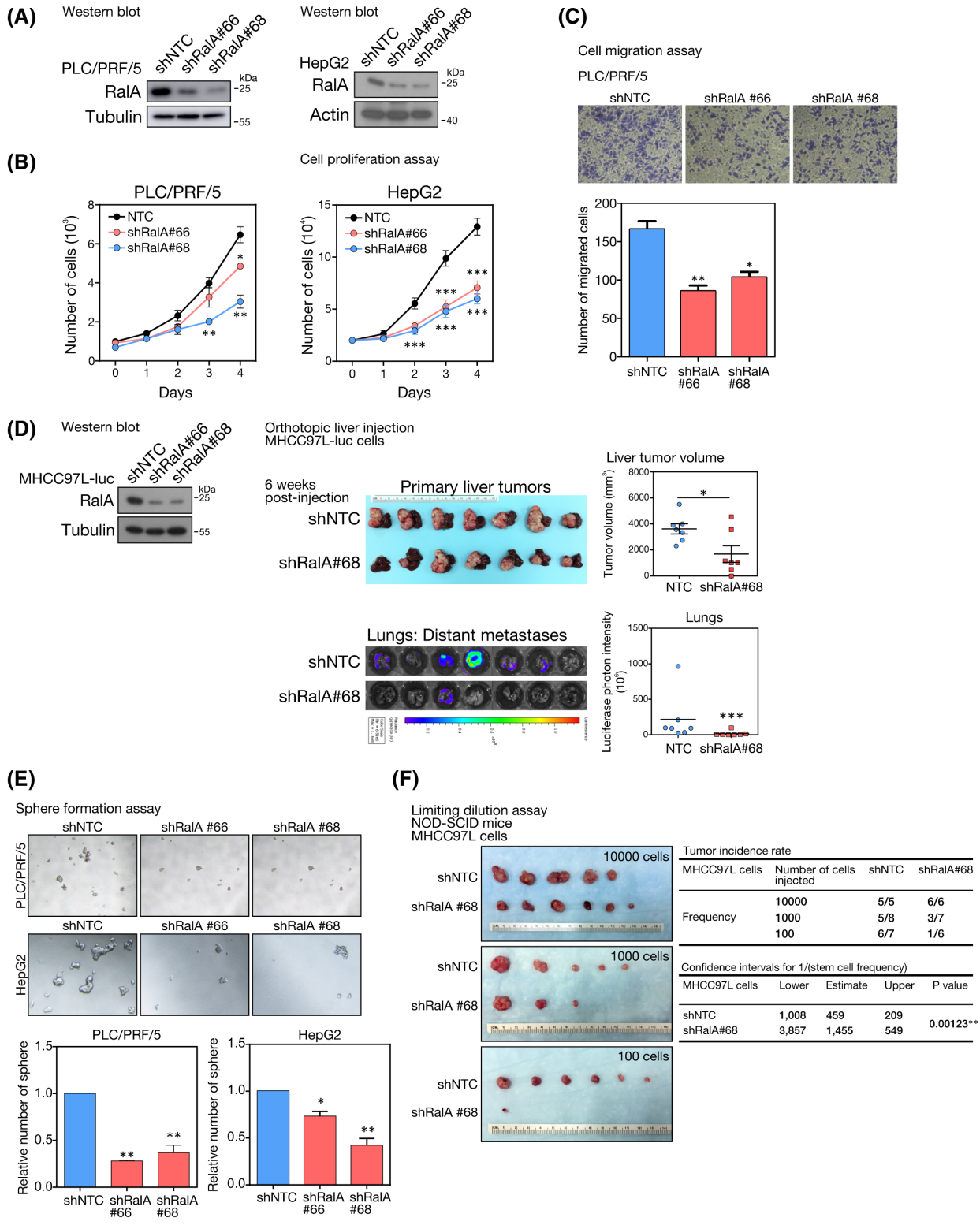
[a]Fisher's exact test.
[b]*t* test.

found to be significantly up-regulated in 12 other solid tumors (Figure 1F).

RalA enhanced HCC cell proliferation, self-renewal ability, and metastasis

To characterize the functional roles of RalA in HCC, we used both KD and overexpression approaches. RalA stable KD was established in PLC/PRF/5 and HepG2 cells with a lentiviral-based shRNA approach. These cell lines were selected from a panel of HCC cell lines as they expressed moderate levels of RalA transcript and protein (Figure S1B,C). Successful RalA KD was confirmed with western blotting (Figure 2A). RalA stable KD PLC/PRF/5 and HepG2 cells proliferated more slowly as compared with the nontargeted control (NTC) (Figure 2B). Moreover, RalA KD PLC/PRF/5 cells showed a significant decrease in migration rates (Figure 2C). Additionally, in an in vivo orthotopic liver injection model, the luciferase-labeled metastatic HCC cell line, MHCC97L-luc, with stable RalA KD (Figure 2D, left) showed significantly smaller tumor sizes as compared with the NTC (Figure 2D, upper right panel). Of significance, fewer lung metastases were detected in the lungs of the RalA KD group and with lower bioluminescence signals (Figure 2D, lower right panel). The results suggest that RalA KD may suppress HCC tumor formation and lung metastasis.

We questioned whether RalA might also play a role in contributing to the stemness properties of HCC. We performed the sphere formation assay to assess the



Tumor incidence rate

MHCC97L cells	Number of cells injected	shNTC	shRalA#68
Frequency	10000	5/5	6/6
	1000	5/8	3/7
	100	6/7	1/6

Confidence intervals for 1/(stem cell frequency)

MHCC97L cells	Lower	Estimate	Upper	P value
shNTC	1,008	459	209	0.00123**
shRalA#68	3,857	1,455	549	

FIGURE 2 RalA KD suppressed HCC progression and cell stemness properties. (A) Successful RalA KD in PLC/PRF/5 and HepG2 cells checked by western blotting. (B) The cell proliferation rate of RalA KD as compared with NTC in PLC/PRF/5 and HepG2 cells. (C) The cell migration rate of RalA KD as compared with NTC in PLC/PRF/5. (D) Size of primary liver tumors and presence of distant lung metastasis by orthotopic injection of MHCC97L-luc RalA KD cells. (E) Sphere formation ability of RalA KD as compared with NTC in PLC/PRF/5 and HepG2 cells. (F) The tumor incidence rate of RalA KD cells by limiting dilution assay using subcutaneous injection of MHCC97L cells

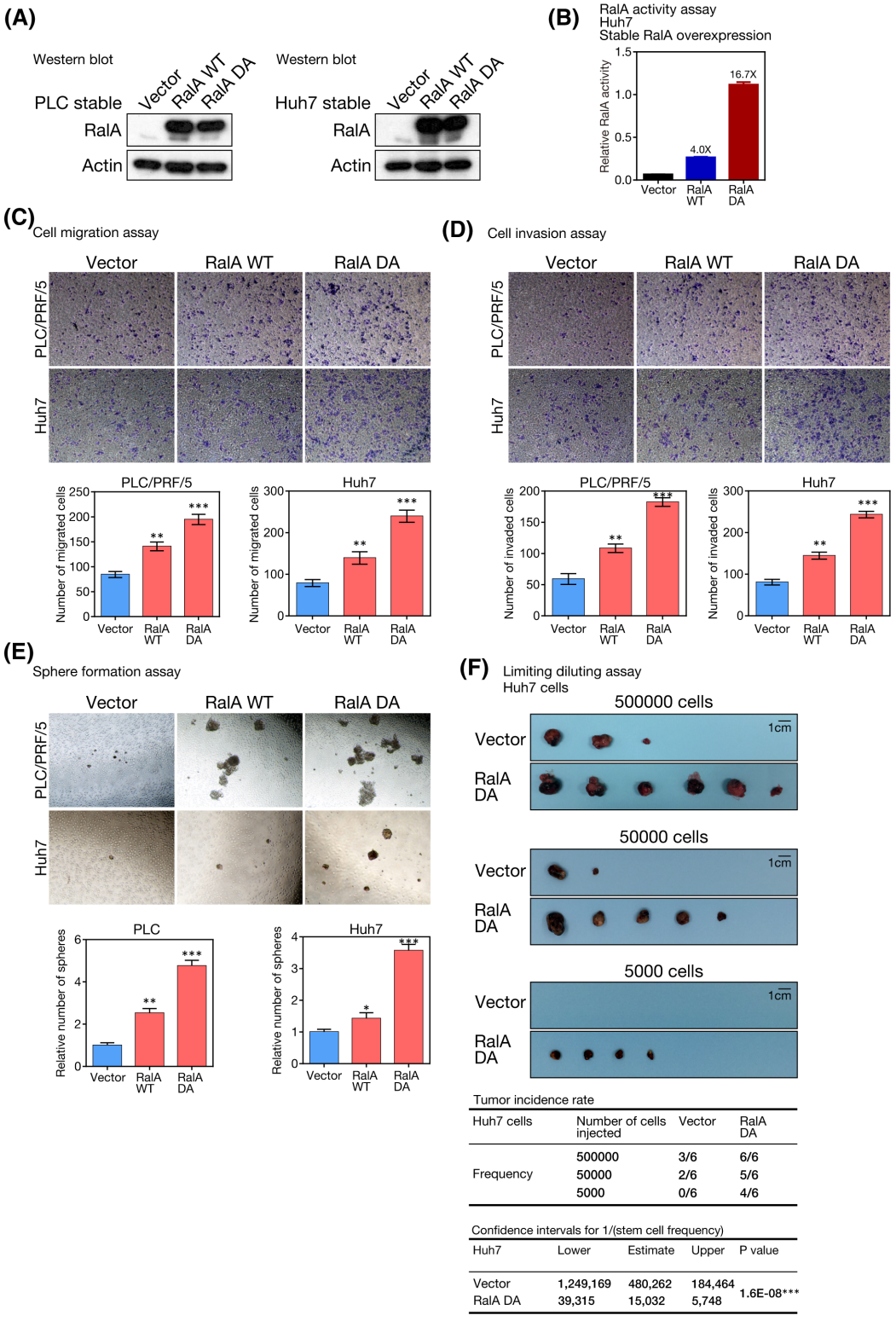
self-renewal ability in vitro. The sphere-forming ability was significantly suppressed on RalA KD in PLC/PRF/5 and HepG2 (Figure 2E). Furthermore, liver cancer stem cells (CSCs) with specific CSC markers have been reported to contribute to cancer stemness. Therefore, we performed a correlation analysis between the expression levels of RalA and different liver CSC markers in TCGA HCC cohort. RalA expression was found to be positively correlated with the expression of specific liver CSC markers, including CD24, CD47, and epithelial cell adhesion molecule (EpCAM; Figure S2A). Moreover, we examined the RNA-seq data of patients' HCC tumors, the cells of which were sorted by flow cytometry according to different CSC markers. The expression of RalA transcript was comparatively higher in HCC tumors with positive/high expression of CD24 (71.4%), CD47 (60.0%), and EpCAM (64.3%), as compared with those with negative/low CD24, CD47, and EpCAM expression (Figure S2B). To functionally demonstrate and confirm the critical role of RalA in supporting HCC stemness and tumor initiation, limiting dilution assay was performed by injecting different numbers of MHCC97L cells in nonobese diabetic/severe combined immunodeficiency (NOD-SCID) mice. Interestingly, knocking down RalA significantly attenuated tumor initiation ability ($p = 0.00123$) (Figure 2F).

Reciprocally, wild-type RalA and its constitutively active form G23V were overexpressed in PLC/PRF/5 and Huh7 cells. Successful overexpression of RalA protein in these two cell lines was confirmed by western blotting (Figure 3A). Also, the activity of wild-type RalA and its G23V form was confirmed by the elevation of active RalA (RalA-GTP) level in Huh7 cells by RalA activity assay (Figure 3B). Functionally, overexpression of RalA significantly accelerated HCC cell migratory and invasive abilities in these cells (Figure 3C,D). In addition, RalA overexpression drastically enhanced the sphere-forming ability of both cells in vitro (Figure 3E) and significantly increased the tumor incidence and tumor size in the limited dilution assay by subcutaneous injection of Huh7 cells in vivo (Figure 3F).

RalA expression correlated with copy number gain and was cotranscriptionally regulated and driven by SP1 and ETS2 in HCC

To examine the potential genetic alterations leading to this up-regulation, we analyzed the copy number variation of RalA in TCGA HCC cohort. Indeed, an increased copy number of RalA was found to be positively and significantly correlated with RalA expression in TCGA HCC cohort ($p < 0.0001$), suggesting that the copy number gain of the RalA gene may contribute to its up-regulation (Figure 4A).

Furthermore, to uncover how RalA expression was transcriptionally regulated, we retrieved the chromatin immunoprecipitation (ChIP)-sequencing data from the ENCODE portal to specifically sort out the potential transcription factors that could physically interact with the RalA promoter region (chr7:39,623,330-39,623,680). Among all the transcription factors, ETS1, GA binding protein transcription factor subunit alpha (GABPA), ETS variant transcription factor (ETV) 4, ETV6, E74 like ETS transcription factor (ELF)1, ELF4, and ETS transcription factor ELK1 (ELK1) from SP1 and ETS families of transcription factors were suggested to potentially interact with defined consensus sites in the RalA promoter region. To further narrow down the potential candidates in regulating RalA expression, the correlation between the mRNA expression of ETS transcription factors and RalA was first examined in 25 HCC cell lines in Cancer Cell Line Encyclopedia. Specifically, ETS2, GABPB2, and FLI1 were the top three transcription factors with their expression to be most positively correlated with RalA. Of note, ETS2 was also the dominantly expressed ETS transcription factor among these candidates. Additionally, examination of the RNA-seq data of the TCGA patient cohort with HCC revealed that SP1 (chr12:53775894-53777406:+) and ETS2 (chr21:40177849-40178044:+) are the two main transcription factors showing positive correlations with RalA mRNA expression (Figure 4B). To demonstrate SP1 and ETS2 indeed played functional roles in regulating RalA expression, wild-type RalA promoter-luciferase reporter was transiently coexpressed with either SP1 or ETS2 expression construct, followed by dual-luciferase reporter assay in Huh7 cells. Expression of SP1 or ETS2 significantly increased the RalA promoter activities by 42.3% and 26.8%, respectively, as compared with the empty vector control (Figure 4C). To pinpoint the putative SP1 and ETS2 binding sites in the RalA promoter, we employed the IN-silico SEarch for Co-occurring Transcription factors 2.0 (INSECT 2.0) tool for more precise *in silico* prediction.[19] In brief, two putative SP1 binding sites were predicted at -168 nt and -117 nt upstream of the RalA promoter, whereas two putative ETS2 binding sites were predicted at -38 nt upstream and +4 nt downstream of the RalA transcription start site. To validate these predicted SP1 and ETS2 binding sites, specific mutations were introduced into the corresponding positions in RalA promoter-luciferase reporter and in the indicated combinations, followed by luciferase reporter assay (Figure 4D, left panel). Mutations of either the two SP1 binding sites or the two ETS2 binding sites reduced the reporter activity by 15.3% and 89.3%, respectively. More importantly, mutations of all four binding sites that attenuated both SP1 and ETS2 binding drastically reduced the luciferase reporter activity by 95%, suggesting that SP1 and ETS2 cotranscriptionally regulate and drive RalA expression (Figure 4D, right panel). The actual interactions between SP1 and ETS2 with the RalA promoter region were further confirmed by ChIP assay and



Tumor incidence rate

Huh7 cells	Number of cells injected	Vector	RalA DA
Frequency	500000	3/6	6/6
	50000	2/6	5/6
	5000	0/6	4/6

Confidence intervals for 1/(stem cell frequency)

Huh7	Lower	Estimate	Upper	P value
Vector	1,249,169	480,262	184,464	1.6E-08***
RalA DA	39,315	15,032	5,748	

FIGURE 3 RalA overexpression enhanced cell migratory ability and cancer stemness features. (A) Overexpression of RalA wild-type (WT) or dominant active (DA) form in PLC/PRF/5 and Huh7 cells. (B) RalA activity on expression of RalA WT and DA in Huh7 cells. (C) Cell migration rate and (D) cell invasion rate of RalA WT or DA overexpression in PLC/PRF/5 and Huh7 cell lines. (E) Sphere-forming ability was enhanced with RalA WT or DA overexpression in PLC/PRF/5 and Huh7 cell lines. (F) Evaluation of tumor incidence rate of RalA DA-overexpressing Huh7 cells as compared with the control cells by subcutaneous injection of limiting diluted cell suspension

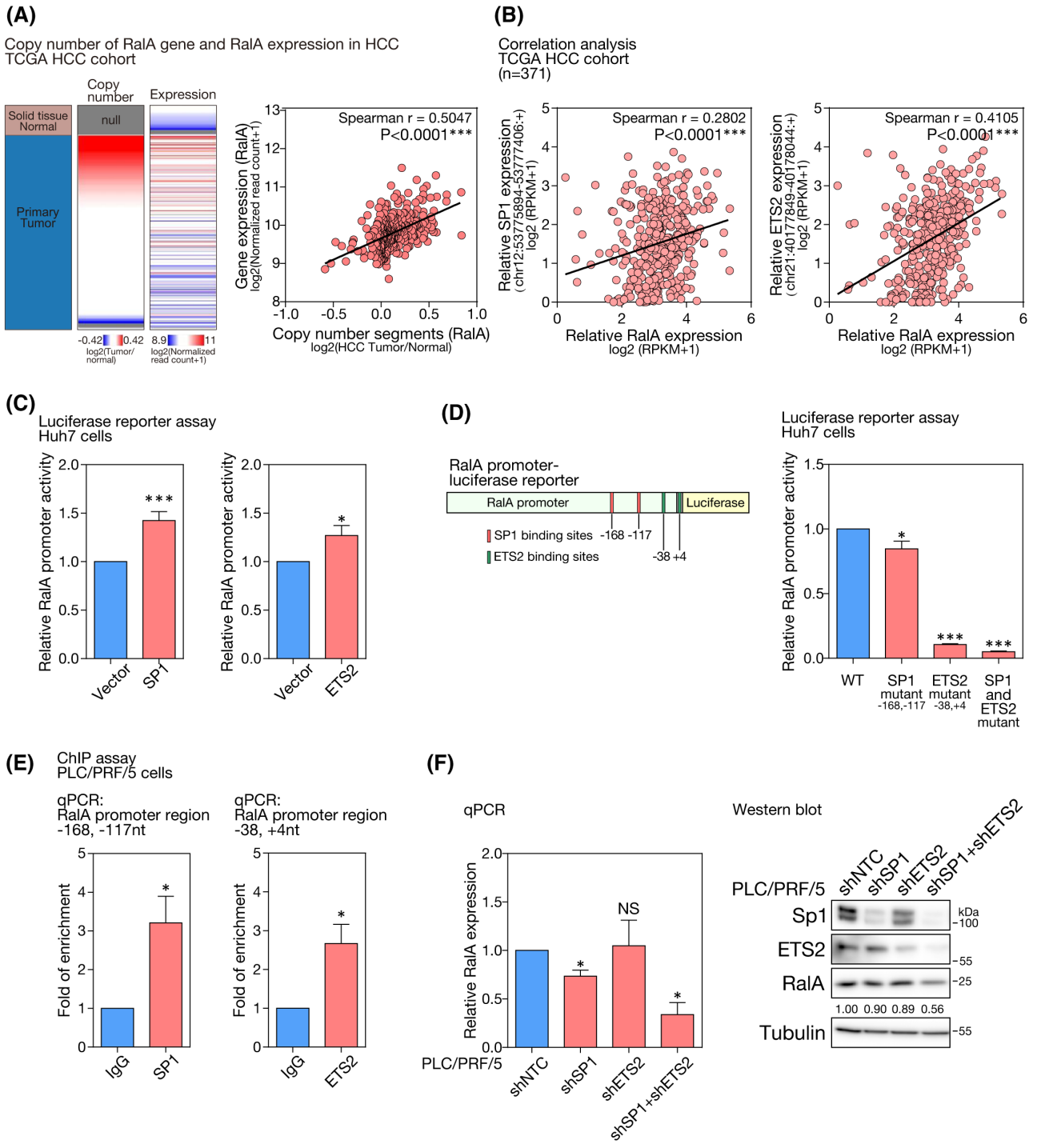


FIGURE 4 Transcriptional regulation of RalA expression by SP1 and ETS2 in human HCC. (A) Copy number variation of RalA was examined in the TCGA HCC cohort. Increased copy number of RalA was found to be positively and significantly correlated with RalA expression. (B) Correlation analysis of SP1 or ETS2 with RalA expression in TCGA HCC cohort. (C) RalA promoter activity was detected by luciferase reporter assay using overexpression of either SP1 or ETS2. (D) RalA promoter activity was assessed by luciferase reporter assay using mutant forms of SP1 and/or ETS2, which are defective in binding RalA promoter. (E) ChIP assay verified the physical interaction of RalA promoter with SP1 and/or ETS2. (F) KD of SP1 and ETS2 reduced both RalA mRNA and protein expression in PLC/PRF/5 cells

a significant fold of enrichment of the corresponding DNA binding motif was observed (Figure 4E). To confirm the role of SP1 and ETS2 in positively regulating RalA expression, SP1 and ETS2 were silenced either individually by specific shRNAs or in combination in PLC/PRF/5 cells, followed by the detection of RalA expression at transcript and protein levels. Consistently, co-knocking down of SP1 and ETS2 produced the most prominent effects in

suppressing the RalA transcript ($p = 0.0327$) and protein expression (Figure 4F).

RalGAPA2, the negative regulator of ral, was down-regulated in HCC

RalA activity is tightly regulated by a dimeric RalGAP complex, consisting of a catalytic alpha subunit and a regulatory beta subunit. Of the two types of alpha subunits, RalGAPA2 but not RalGAPA1 is predominantly expressed in HCC tissues, as supported by the RNA-seq data. Similar observations were seen in our panel of HCC cell lines RNA-seq data and western blotting (Figure 5A). The clinical significance of RalGAPA2 in human HCC has not been reported. To this end, we examined RalGAPA2 mRNA expression with real-time qPCR in 90 pairs of HBV-associated HCC. Interestingly, RalGAPA2 showed significant down-regulation in the HCC tumors as compared with the NTLs (Figure 5B). The 37.8% (34/90) of HCC tumors showed down-regulation of RalGAPA2 mRNA expression at 2-fold cutoff (Figure 5C). Consistent down-regulation of RalGAPA2 was observed in HBV-HCC cohort from TCGA database (Figure S3A). Down-regulation of RalGAPA2 at the protein level was also observed in representative paired HCC tissues (Figure 5D). Survival analysis further showed that HCC tumors with lower RalGAPA2 expression were associated with poorer overall survival rates ($p = 0.045$) (Figure 5E). Similarly, we found patients with HBV-associated HCC from TCGA cohort with low RalGAPA2 expression in their tumors displayed shorter 2-year overall survival rates (Figure S3B). Also, RalGAPA2 down-regulation was correlated with poorer cellular differentiation ($p = 0.034$) (Supplementary Table 1). Furthermore, an integrative analysis combining the RalA and RalGAPA2 mRNA expression in a common set of patients with HCC cohort allowed us to interrogate the potential clinical association between RalA and RalGAPA2 expression. Intriguingly, HCC tumors showing simultaneous down-regulation of RalGAPA2 and up-regulation of RalA displayed a significant association with more aggressive tumor behavior, namely, presence of venous invasion ($p = 0.001$) and more advanced tumor stage ($p = 0.007$) (Figure 5F). Taken together, dysregulation of RalGAPA2 might also serve as a critical mechanism in sustaining RalA activity in supporting HCC development.

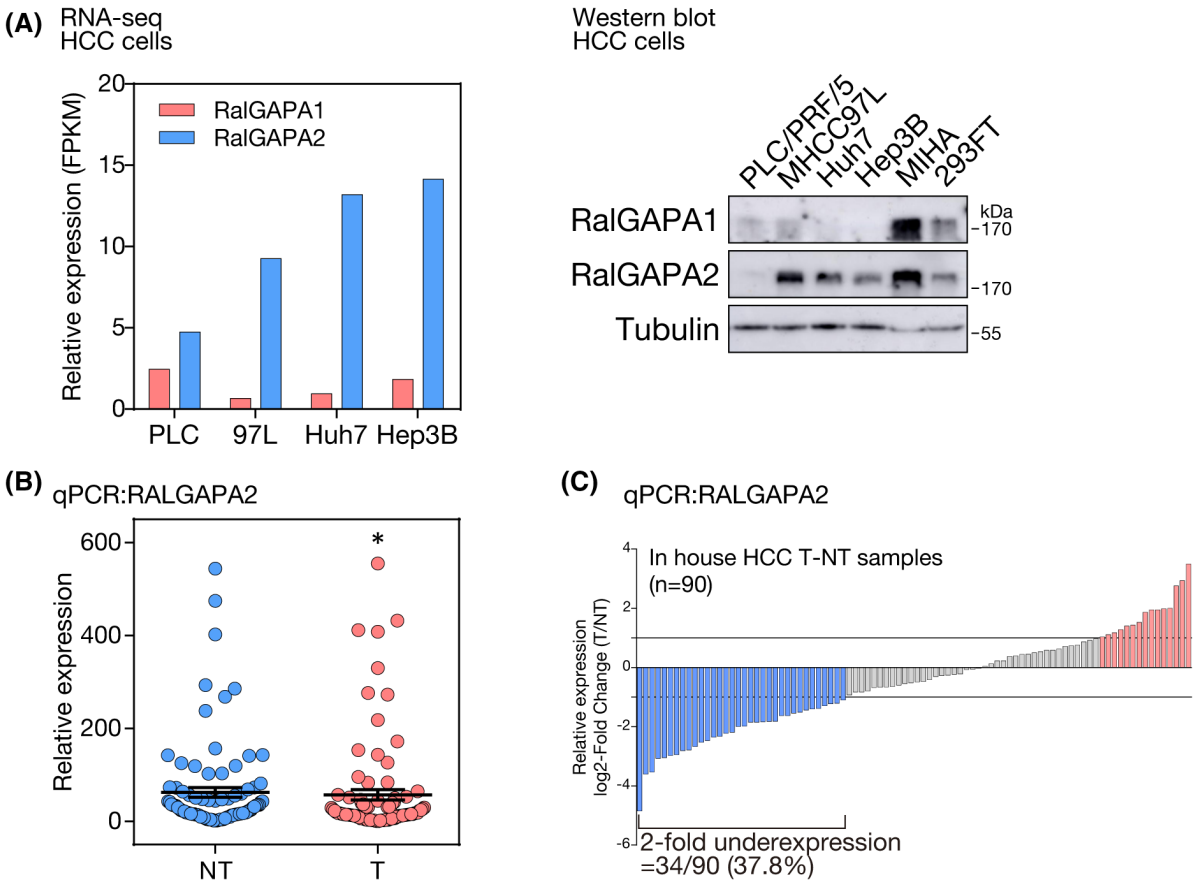
RalGAPA2 negatively regulated RalA activity and suppressed cell migration in vitro and HCC metastasis in vivo

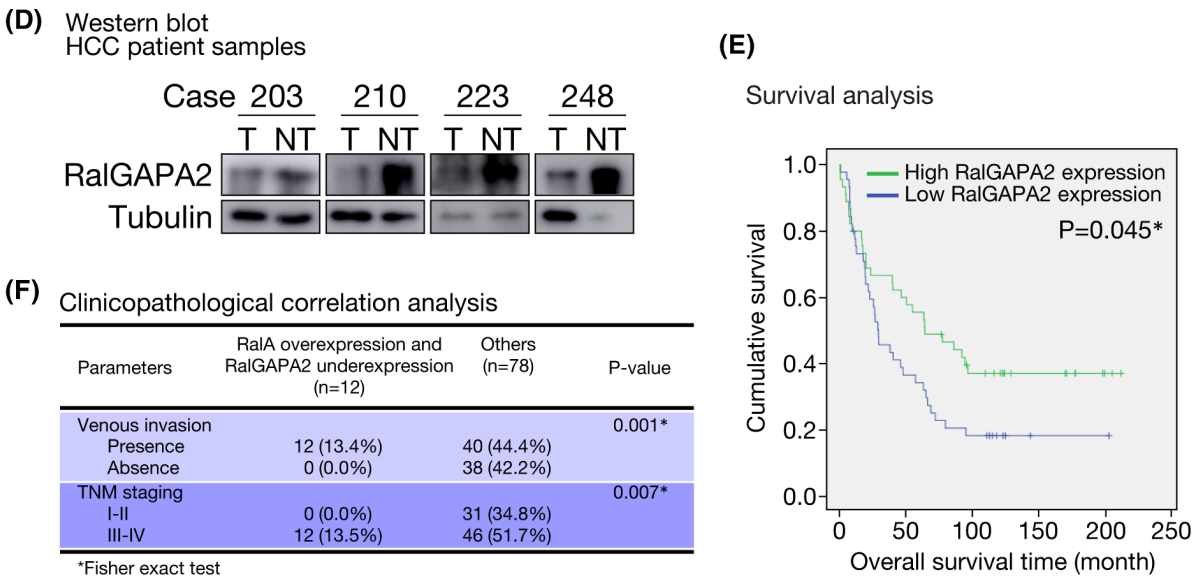
To functionally characterize RalGAPA2 in HCC, we silenced RalGAPA2 expression by stable shRNA-mediated KD. RalGAPA2 silencing significantly increased the migratory abilities of MHCC97L-luc (Figure 6A) and Huh7 cells (Figure S3C, upper panel). Similarly, CRISPR/Cas9-mediated RalGAPA2 KO in the same HCC parental cells also produced the promigratory effects (Figure 6B and Figure S3C, lower panel). In these KD and KO MHCC97L-luc and Huh7 cells, along with the promigratory effects, there was up-regulation of the RalA activity as revealed by the RalA activity assay, whereas the total RalA level remained unchanged (Figure 6C and Figure S3D). To further consolidate the negative regulation of RalGAPA2 toward RalA, we further depleted RalA expression in RalGAPA2 KD cells (Figure S3E). We observed that the promigratory effect result from RalGAPA2 KD was partially abolished when RalA was further suppressed. Also, the RalA activity was decreased compared with RalGAPA2 KD cells (Figure 6D), supporting that RalGAPA2 is a bona fide tumor suppressor through inactivating RalA signaling.

The metastatic potential of luciferase-labeled RalGAPA2 KD cells was further evaluated in vivo by intrasplenic injection in immunodeficient nude mice followed by the detection of hepatic metastases 10 weeks postinjection with bioluminescence imaging. At the experimental endpoint, RalGAPA2 KD cells showed a comparatively higher rate of hepatic metastasis of 60% as compared with no detectable hepatic metastasis in the control group (Figure 6E). Consistently, in an orthotopic liver injection model in immunodeficient nude, RalGAPA2 KO cells also showed a trend of forming larger liver tumors and having a higher rate of distant pulmonary metastasis (100% vs. 40% in the control group) (Figure S3F). Reciprocally, stable overexpression of the c-terminus GAP domain of RalGAPA2 (Figure S3G) significantly reduced the migratory abilities of HCC cells (Figure 6F). Taken together, our results supported that RalGAPA2 functions as a metastatic suppressor in HCC cells by inhibiting the RalA activity.

Pharmacological inhibition of RalA suppressed cell proliferation and migration of HCC cells

RBC8 is a specific Ral inhibitor and inhibits both RalA and RalB activities without cross affecting Ras or RhoA activity.[20] Because RalA overactivation promoted proliferation and metastasis in HCC, we questioned whether the pharmacological inhibition of RalA by RBC8 could reverse the phenotypes associated with aggressive HCC. RBC8 treatment in different HCC cell lines showed that, except PLC/PRF/5, the HCC cell lines were more sensitive to RalA inhibition as compared with the normal immortalized liver cells, MIHA (Figure 7A and Figure S4A), and RalA activity in HCC cells was positively correlated with the sensitivity toward RBC8 treatment. RBC8 significantly suppressed cell proliferation and





(F) Clinicopathological correlation analysis

Parameters	RalA overexpression and RalGAPA2 underexpression (n=12)	Others (n=78)	P-value
Venous invasion			0.001*
Presence	12 (13.4%)	40 (44.4%)	
Absence	0 (0.0%)	38 (42.2%)	
TNM staging			0.007*
I-II	0 (0.0%)	31 (34.8%)	
III-IV	12 (13.5%)	46 (51.7%)	

*Fisher exact test

FIGURE 5 Down-regulation of RalGAPA2 was associated with poorer overall survival rates in HCC. (A) The transcript expression of the two isoforms of RalGAP catalytic subunit, RalGAPA1 and RalGAPA2, in a panel of HCC cell lines by RNA-seq (left panel). The protein expression of RalGAPA1 and RalGAPA2 in HCC cell lines and immortalized normal liver cell line MIHA by western blotting (right panel). (B) RalGAPA2 mRNA expression by qPCR in our cohort of 90 paired HCC samples. (C) 37.8% (34/90) of the HCC tumors showed down-regulation of RalGAPA2 mRNA expression at 2-fold cutoff. (D) RalGAPA2 protein levels in representative tumors and nontumors of HCC samples from our patients' HCCs. (E) Survival analysis of patients with HCC with high and low RalGAPA2 expression. (F) Clinicopathological correlation of combined RalA overexpression and RalGAPA2 underexpression subgroup with others

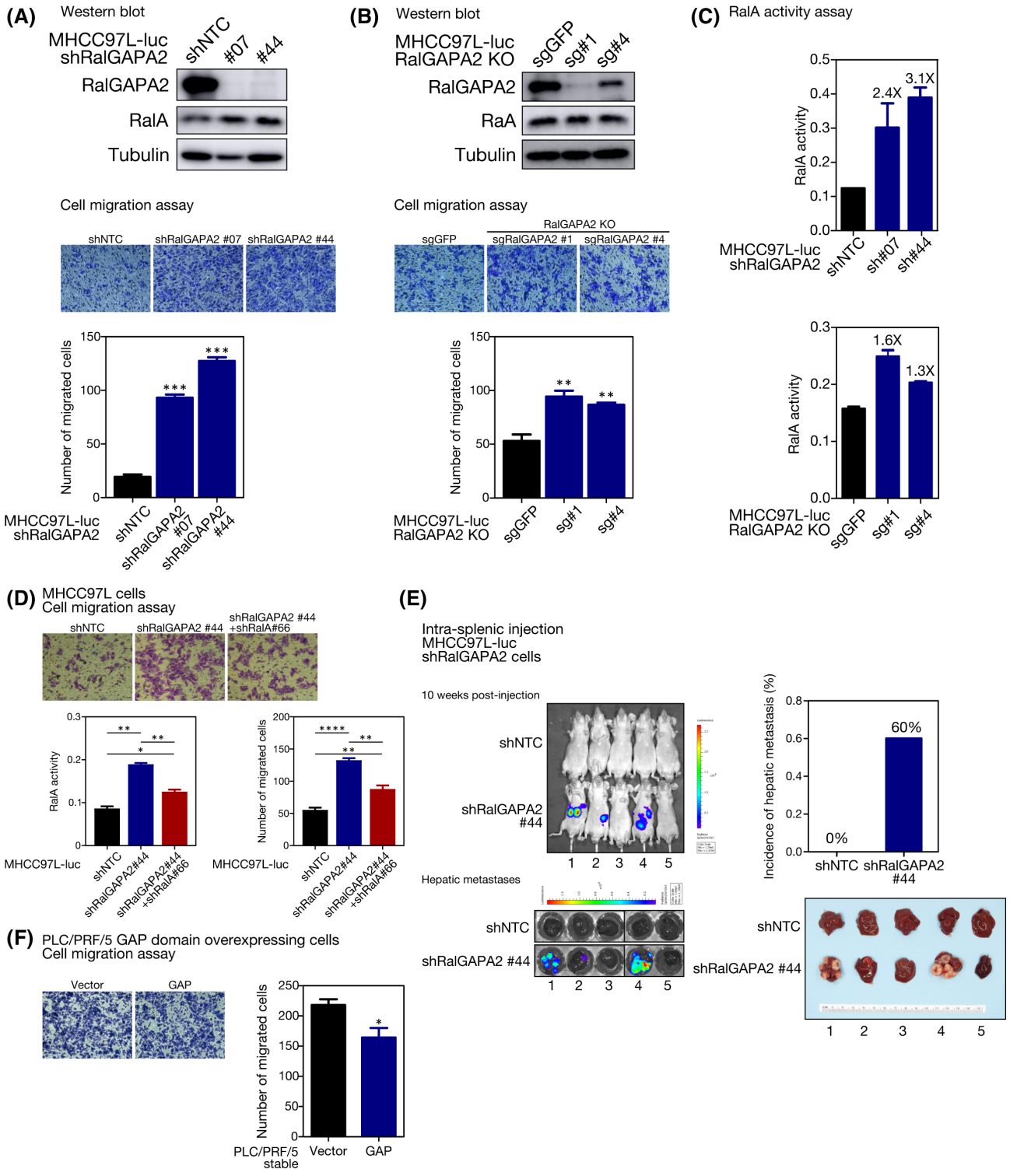


FIGURE 6 RalGAPA2 suppressed tumor metastasis through down-regulating RalA activity (A) shRNA-mediated RalGAPA2 KD checked by western blotting (upper panel) and corresponding cell migration assay (lower panel) in MHCC97L-luc cells. (B) Single guide RNA (sgRNA)-mediated RalGAPA2 KO checked by western blotting (upper panel) and corresponding cell migration assay (lower panel) in MHCC97L-luc cell line. (C) Detection of RalA activity in RalGAPA2 KD (upper panel) and RalGAPA2 KO (lower panel) MHCC97L-luc cell line. (D) Cell migration and RalA activity of RalGAPA2 and RalA double KD cells. (E) Hepatic metastases resulting from intrasplenic injection of RalGAPA2 KD and control cells. (F) Cell migration assays using RalGAPA2 construct containing GAP domain in PLC/PRF/5 cells

migration in a dose-dependent manner in PLC/PRF/5 and MHCC97L-luc cells (Figure 7B). Among different HCC cell lines, MHCC97L-luc and Huh7, which possessed a higher basal RalA activity, had a lower median inhibitory concentration (IC_{50}) value of RBC8 as compared with Hep3B and MIHA cells, which had

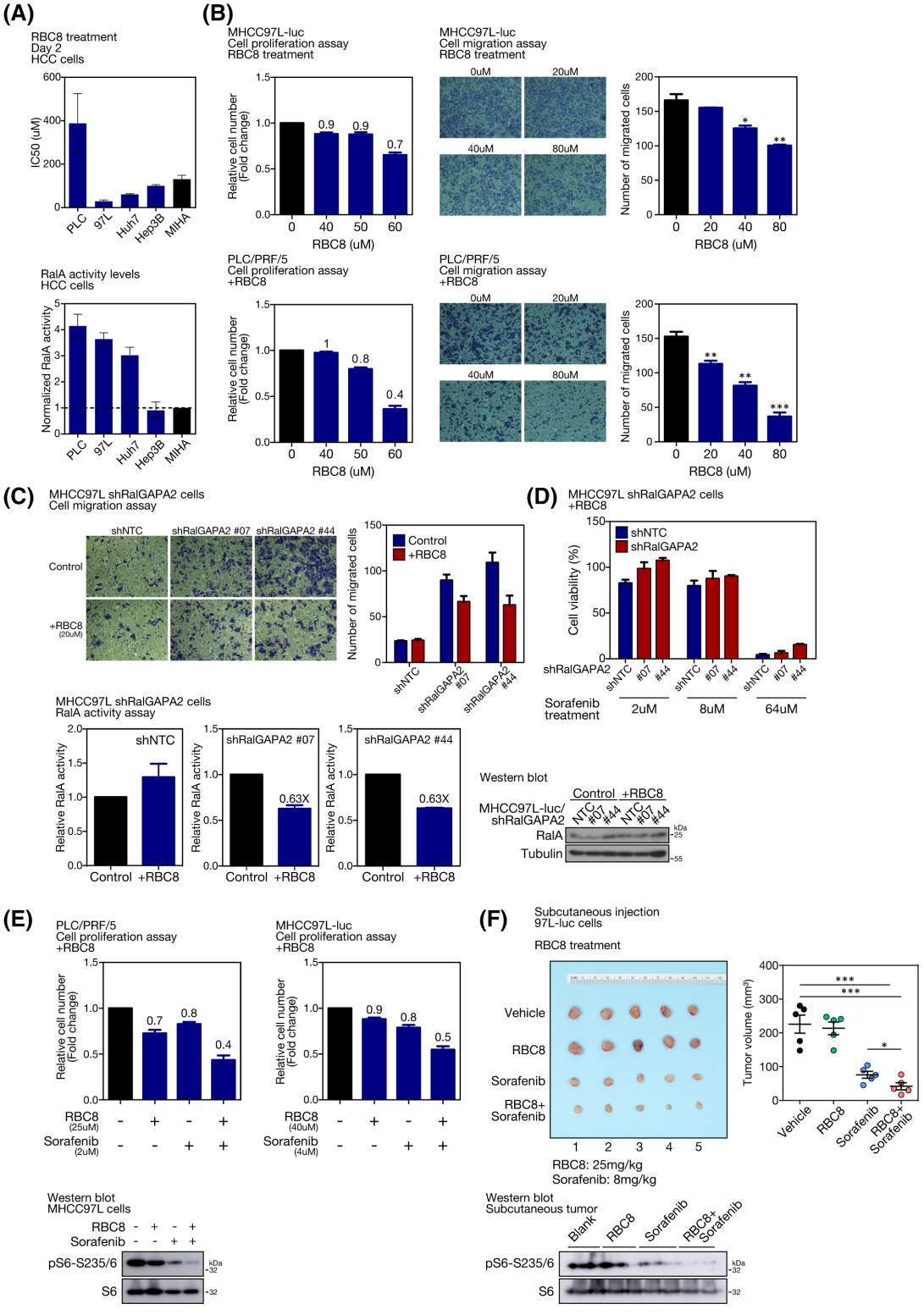


FIGURE 7 RBC8 and sorafenib synergistically inhibited cell proliferation underlying suppressing phosphorylation of S6. (A) IC_{50} values of RBC8 (upper panel) and RalA activity levels (lower panel) in our HCC cell line panel. (B) Cell proliferation (left panel) and cell migration rates (right panel) of MHCC97L-luc and PLC/PRF/5 cells under different doses of RBC8 as compared with the vehicle control. (C) Cell migration rates (upper panel) and the corresponding RalA activities (lower panel) of RalGAPA2 KD MHCC97L-luc cells and the control cells with or without the RBC8 treatment. (D) Cell viability of RalGAPA2 KD and control cells in three different dosages of sorafenib treatment. (E) The relative numbers of PLC/PRF/5 and MHCC97L-luc cells after the RBC8 and/or sorafenib treatment (upper panel) and the underlying changes of S6 phosphorylation by western blotting (lower panel). (F) The volume of subcutaneous tumors with RBC8 and/or sorafenib treatment (upper panel) and the underlying changes of S6 phosphorylation by western blotting (lower panel)

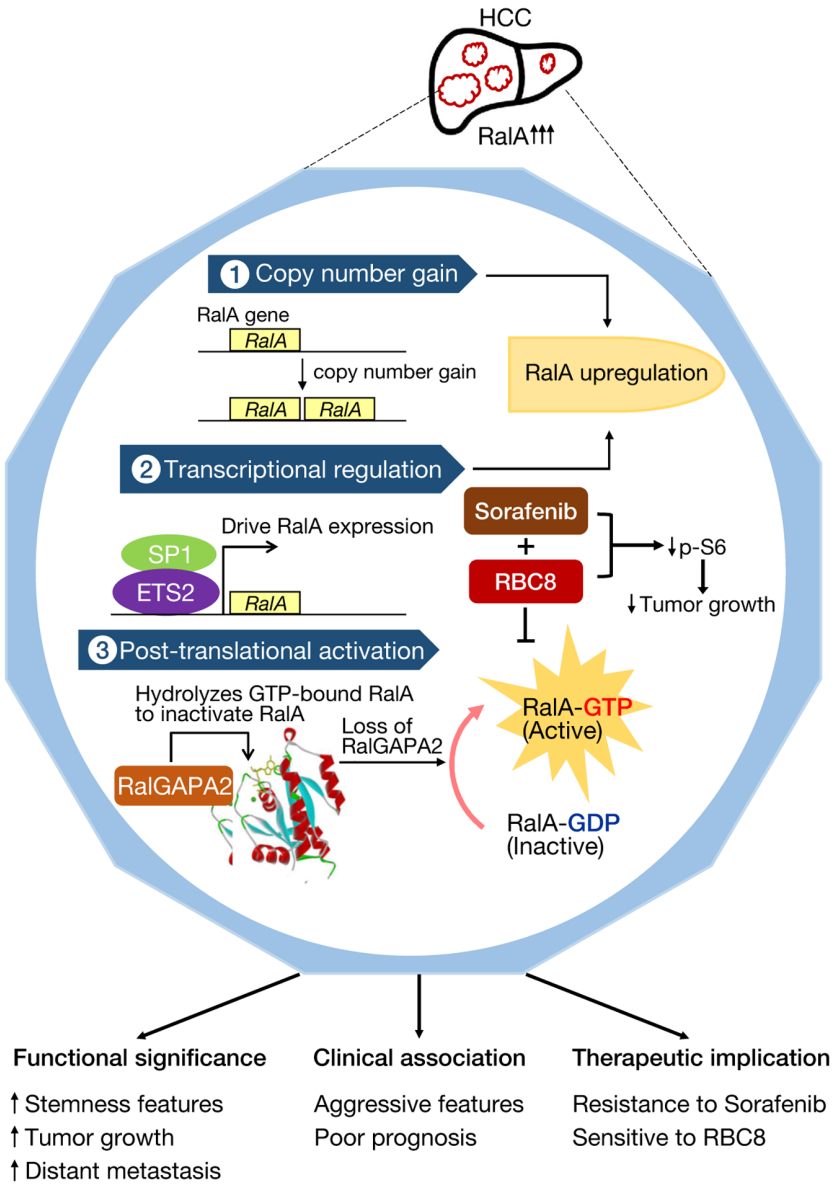


FIGURE 8 Overall summary of the study. Copy number gain of RalA gene and transcriptional regulation by SP1 and ETS2 contribute to RalA elevation in HCC. Loss of RalGAPA2 relieves its suppression on RalA activity and sustains HCC cells in active state. The up-regulation and elevation of RalA support HCC stemness features and favor tumor growth and metastasis. Clinically, patients with HCC with higher RalA expression are associated with aggressive features and poor prognosis. The Ral inhibitor RBC8 and sorafenib synergistically suppress tumor growth underlying enhanced suppression on phospho-ribosomal protein S6 (p-S6)

a relatively lower RalA activity. More importantly, the elevation of basal RalA activity brought by RalGAPA2 silencing could significantly render the cells more prone to RBC8 treatment. This was demonstrated by the significant down-regulation of the cell migration rate and the RalA activity of RalGAPA2 KD cells in the presence of RBC8 but not the control KD cells (Figure 7C, upper and lower panel).

The potential interrelationship between RalA activity and sorafenib resistance

Sorafenib is one of first-line molecularly targeted drugs for advanced HCC. The survival benefit of sorafenib is limited, as resistance usually develops due to the rewiring of the underlying molecular pathways to compensate for the critical oncogenic signaling inhibition. We observed that HCC cells with higher RalA activity were more tolerant toward sorafenib treatment. In our HCC cell line panel, the IC_{50} values of sorafenib had a trend of positive correlation with RalA activity (Figure S4B, left panel), whereas RalGAPA2 KD cells possessing relatively higher RalA activity survived better with increasing dosage of sorafenib from 2 μM up to 64 μM (Figure 7D). In addition, we found that RalA overexpression rendered PLC/PRF/5 and Huh7 cells more resistant to sorafenib-induced apoptosis, whereas RalA KD sensitized HepG2 toward sorafenib-induced cell death (Figure S4B, middle and right panel). We questioned

whether inhibition of RalA activity by RBC8 would alleviate sorafenib resistance. We first determined the specific dosages of sorafenib and RBC8 in PLC/PRF/5 and MHCC97L-luc cells (data not shown) with minimal effects on cell viability. Interestingly, RBC8 sensitized HCC cells toward sorafenib treatment, and this enhanced sensitization was observed in both PLC/PRF/5 and MHCC97L-luc cells (Figure 7E, upper panel). Furthermore, synergistic suppression of the tumor growth was observed when RBC8 was given in combination with sorafenib in vivo (Figure 7F, upper panel). Mechanistically, individual or combined sorafenib and RBC8 only exerted a modest suppression on phospho-extracellular-signal-regulated kinase (p-ERK) (Figure S4C) and RalA activity (Figure S4D). In contrast, suppression of phosphorylation of S6 was observed in transient exposure to RBC8 (Figure S4E) as well as prolonged sorafenib treatment (Figure 7E, lower panel). Of note, RBC8 and sorafenib showed synergistic suppression on phospho-ribosomal protein S6 (p-S6) both in vitro and in vivo (Figure 7E,F, lower panels), implicating that RBC8 enhances mammalian target of rapamycin (mTOR) inhibition mediated by sorafenib. Furthermore, in the xenograft tumors, the phosphorylation status of the other components in the mTOR pathway did not show any changes (Figure S4F). Taken together, inhibition of the RalA activity could sensitize sorafenib treatment, possibly through an enhanced suppression of mTOR activity.

DISCUSSION

In this study, we systematically examined and showed that RalA but not RalB was significantly up-regulated in our patients' HBV-associated HCC tumors with both RNA-seq analysis and qPCR assessment in independent cohorts, and this was also validated in TCGA database. Importantly, RalA up-regulation was associated with more aggressive tumor behavior, including multiple metastatic features, as well as more advanced tumor stages and poorer prognosis in patients with HCC. Consistently, functional characterization of RalA using RalA-specific KD and overexpressing HCC cells in vitro and in vivo further demonstrated that RalA enhanced HCC cell proliferation, self-renewal ability, and metastasis.

The findings from our limiting dilution experiments suggest that RalA expression was pivotal in HCC tumor initiation in vivo, and this provides a potential functional link between RalA and cancer stemness properties. Liver CSCs are recognized as a distinct cell population in primary liver tumor and are capable of self-renewal as well as propagation and initiating the formation of the whole tumor bulk.[21] RalA expression was reported to be enriched in CD44 highly expressed nonsmall cell lung cancer cells,[22] whereas a relatively higher RalA activity was detected in CD133-positive HCC cells.[23] However, in the current study, we found that RalA expression correlated more with CD24, CD47, and EpCAM expression in a patient cohort with HCC as well as in CD24-, CD47- and EpCAM-sorted HCC cells. Our findings are in line with the previous observations in bladder cancer cells[24] and a hamster model,[25] in which the expression of specific cancer stemness markers such as CD24 could be driven and up-regulated by RalA.

The molecular activation of RalA in HCC cells can take effect at transcriptional as well as posttranslational level. Although RalA has been reported to activate specific downstream transcriptional events,[26, 27] the transcriptional regulation of the RalA gene itself is unclear. By comprehensively examining the promoter region of the RalA gene followed by a series of relevant luciferase reporter and ChIP assays, we identified SP1 and ETS2 as the two transcription factors that cotranscriptionally regulated and drove RalA expression. Conversely, co-knocking down of SP1 and ETS2 produced the most prominent effects in suppressing the RalA transcript and protein expression. These findings add an important layer of positive regulation of RalA expression in addition to the negative regulation of the RalGAP complex, exemplifying that RalA, being an important gene in controlling cellular functions, is tightly and closely regulated.

Recently, the indispensable role of Ral in promoting hepatocarcinogenesis has been revealed in in vivo shRNA library screening for potential HCC candidate genes in mice.[16] Functional loss of RalGAPA2 by shRNA KD significantly promoted HCC formation underlying Ral signaling activation. RalGAP complex consists of either one of the catalytic subunits, namely RalGAPA1 or RalGAPA2, and a common regulatory subunit RalGAPB[11] and shares an overall similar molecular architecture with the tuberous sclerosis complex (TSC), the GAP complex against Rheb GTPase. Dysregulation of RalGAP complex 1 has been reported to cause neurodevelopmental defects[28] and also potentially distorted the balance of glucose metabolism in skeletal muscle and adipose tissues.[29,30] On the other hand, RalGAP complex 2 was reported to be inactivated in multiple malignancies including oral,[13] prostate,[14] bladder,[12] and colon cancers.[15] As we observed in this study, of the two catalytic subunits, RalGAPA2 but not RalGAPA1 was predominantly expressed in HCC cells at both transcript and protein levels. We further found that RalGAPA2 was frequently and significantly down-regulated in HCC tumors and, more importantly, this down-regulation was associated with poorer overall survival rates and poorer cellular differentiation. Interestingly, HCC tumors showing simultaneous down-regulation of RalGAPA2 and up-regulation of RalA were more significantly associated with the presence of venous

invasion and more advanced tumor stage. RalGAPA2 ablation in HCC cells significantly promoted cell migration in vitro and metastasis in vivo, with underlying elevated active RalA levels. Our observations were in line with the observed phenotypic changes after RalGAPA2 was depleted in certain non-HCC cancer models[12-14] and provided additional evidence that down-regulation of RalGAPA2 indeed also plays a critical role in driving HCC development.

With the recent advancement of DNA sequencing technologies, genetic alterations underlying the HCC development in patient samples have been examined at ultra-high resolution. These findings have revealed that HCC carcinogenesis is mainly driven by the loss of tumor suppressor genes coupled with distinct sets of oncogenic events that are specific to HCC.[31,32] Functioning as critical negative molecular switches in multiple oncogenic signal transduction pathways, GAP proteins have been recurrently found to be inactivated in HCC, and their functional loss could significantly promote HCC formation and progression. For instance, inactivation of the TSC complex was sufficient to drive HCC formation,[33,34] and loss-of-function mutations were recurrently found in human HCC tumors.[35,36] In addition, genetic loss of a RhoGAP, Deleted in Liver Cancer 1,[37] and the suppression of a RasGAP, Neurofibromatosis type 1,[38] were sufficient to drive HCC tumorigenesis. Although it is uncommon and challenging to restore these GAP proteins in patients' tumors as a therapeutic approach to retard their growth and survival, understanding the dysregulations of these GAPs could generate useful insight to identify key oncogenic signaling pathways and their effectors that could serve as targets for deriving alternative therapeutics. For instance, the critical role of the RalGAP-RalA axis led us to question the possibility of targeting this pathway. RBC8 is a Ral-specific inhibitor against both RalA and RalB and does not cross-affect Ras or RhoA activity.[20] Functionally, we observed RBC8 treatment could suppress cell proliferation and migration in a dose-dependent manner. Although RBC8 could induce these antitumor effects in HCC cell lines, the underlying decrease of RalA activity was only modest in the parental HCC cells. This could partly be due to the difference in the basal expression and activity levels of RalA in different cell lines, and similar findings were also observed in nonsmall cell lung cancer cell lines.[20] However, RalGAPA2 ablation still rendered HCC cells more prone to RBC8 treatment, possibly due to the significant up-regulation of RalA activity.

We also examined whether targeting RalA could improve the treatment efficacy of sorafenib, which is one of the alternative first-line drugs for advanced HCC.[39,40] HCC cells having a relatively higher RalA activity were found to be more resistant to sorafenib treatment. Similarly, RalA KD or overexpressing cells were more sensitive or resistant, respectively, toward sorafenib treatment. Interestingly, sorafenib treatment somehow increased the endogenous RalA protein level and RalA activity. In this case, supplementation of RBC8 together with sorafenib enhanced the chemosensitivity of HCC cells toward sorafenib and reduced the potential chemoresistance due to the possible compensatory mechanism mediated by RalA. Furthermore, prolonged sorafenib treatment has been reported to provoke the activation of Akt and mTOR pathways and contribute to chemoresistance.[41,42] Treatment of RBC8 together with sorafenib synergistically inhibited cell proliferation as indicated by the decreased phosphorylation of S6, the direct mTOR substrate. In other words, inhibition of RalA activity could potentiate the sorafenib treatment, possibly through an enhanced suppression of mTOR activity. Taken together, our results provide biological insights on the dysregulation of RalA signaling through dual regulatory mechanisms in HCC. Targeting RalA may serve as a potential alternative therapeutic approach alone or in combination with the currently available therapy for patients with HCC (Figure 8).

CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Lo-Kong Chan and Irene Oi-Lin Ng provided the study concept and design. Lu Tian, Luqing Zhao, Karen Man-Fong Sze, Daniel Wai-Hung Ho, Lo-Kong Chan, and Irene Oi-Lin Ng interpreted and analyzed the data. Lu Tian, Luqing Zhao, Karen Man-Fong Sze, Charles Shing Kam, Vanessa Sheung-In Ming, Xia Wang, Vanilla Xin Zhang, Daniel Wai-Hung Ho, and Lo-Kong Chan performed the experiments. Irene Oi-Lin Ng and Tan-To Cheung collected the patients' samples. Lu Tian, Luqing Zhao, Karen Man-Fong Sze, Lo-Kong Chan, and Irene Oi-Lin Ng wrote the manuscript. All authors approved the final version of the manuscript.

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SUPPORTING INFORMATION

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