



NHERF4 hijacks Mas-mediated PLC/AKT signaling to suppress the invasive potential of clear cell renal cell carcinoma cells

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ABSTRACT

The Mas receptor has been reported to promote migration and invasion of clear cell renal cell carcinoma (ccRCC) cells via Ang-(1–7)-dependent AKT signaling. However, the mechanism underlying the regulation of Mas function remains unknown. Here, eight PDZ domain-containing proteins were identified as Mas interactors using surface plasmon resonance (SPR) coupled to mass spectrometry (MS). NHERF4 was the only downregulated gene across multiple independent ccRCC datasets. GST pull-down and co-immunoprecipitation assays confirmed physical interaction between NHERF4 and Mas. Using NHERF4 overexpression and knockdown assays, we found that NHERF4 inhibited Mas-induced migration, invasion and *in vivo* metastasis of ccRCC cells. Mechanistically, NHERF4 suppressed Mas-stimulated AKT phosphorylation and the PLC/Ca²⁺ response. We further demonstrated that NHERF4 compromised Mas-mediated migration and invasion of ccRCC cells via regulation of the PLC/AKT signaling axis. Analysis of the ccRCC dataset revealed that low levels of NHERF4 expression were correlated with higher TNM stage, and independently predicted poor prognosis of ccRCC patients. Overall, our study identified NHERF4 as a novel regulator of ccRCC invasiveness, and a prognostic biomarker, which may be beneficial for determining optimal therapeutic strategies for ccRCC patients.

1. Introduction

Renal cell cancer (RCC) is derived from the renal epithelium and accounts for approximately 90% of kidney cancers. Clear cell RCC (ccRCC) is the most common subtype and is responsible for the majority of cancer-related deaths [1]. Despite surgical resection with curative intent, over 30% of patients with localized ccRCC eventually develop metastases, which require systemic therapies. Investigations into ccRCC have revealed that hyperactivation of vascular endothelial growth factor receptor (VEGFR) and mammalian target of rapamycin (mTOR) signaling pathways resulting from mutations of the von Hippel-Lindau (VHL) tumor suppressor gene and mTOR contributes to the pathogenesis of ccRCC [2]. Although targeted therapies against these pathways have been developed, the median survival of most patients was only 30 months [3], highlighting the need for a deeper understanding of the underlying biological processes associated with advanced ccRCC.

The ACE2/Ang-(1–7)/Mas axis is one of the main components of the

renin-angiotensin system (RAS) [4]. Mas belongs to the G protein-coupled receptor (GPCR) family and is highly expressed in human tissues including the kidney, heart and brain [5]. Mas was initially identified as a proto-oncogene due to its ability to transform NIH 3T3 cells [6]. Ang-(1–7) is generated in an ACE2-dependent manner and acts as an endogenous ligand for Mas [7,8]. Recently, accumulating evidences have revealed a deleterious role for this axis in the renal system. Treatment of renal tubular cells with Ang-(1–7) led to an epithelial-to-mesenchymal transformation (EMT)-like phenotype characterized by loss of typical morphology of epithelial cells and increased expression of mesenchymal markers [9]. The phenotypic switch of EMT is known to be a key driver of tumor metastasis [10]. Upregulation of ACE2 has been associated with the aggressiveness of ccRCC [11]. Moreover, our previous work has shown that Ang-(1–7)/Mas promoted migration and invasion of ccRCC cells through the AKT signaling pathway [12]. These findings suggest that Mas activation may be linked to the progression of ccRCC and may be useful as a therapeutic target.

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Several recent studies have indicated that PDZ domain-containing proteins may be involved in the regulation of Mas function. PDZ domains are conserved modules of approximately 90 amino acids that recognize C-terminal (CT) residues of binding partners, and mediate protein complex assembly to regulate a variety of biological processes including signal transduction and cell polarity [13]. Mas possesses a canonical PDZ-binding motif (PBM) at its C-terminus. We have found that the postsynaptic density-enriched PDZ protein PSD95 can increase stability of Mas via a physical interaction [14]. Others also reported that deletion of the PBM of Mas affected Mas-induced activation of the Gαq signaling pathway in HEK293 cells [15]. Given the known role of PDZ proteins, we hypothesized that they could play a potential role in regulating Mas-mediated malignant behaviors of ccRCC cells.

Here, we identified NHERF4 as a Mas-binding protein, which inhibited Mas-driven invasion of ccRCC cells via regulation of the PLC/AKT signaling axis. We also demonstrated that low expression of NHERF4 was strongly correlated with higher TNM stage and independently predicted poor outcome of ccRCC patients. Our findings highlight a novel regulatory mechanism involving the progression of ccRCC.

2. Materials and methods

2.1. Antibodies and reagents

The sources of antibodies were as follows: *anti-pS⁴⁷³AKT* and *anti-AKT* antibodies from Cell Signaling Technologies (Beverly, MA); *anti-Mas* antibodies from NOVUS Biologicals (Littleton, CO); *anti-NHERF4* antibody from Abcam (Cambridge, UK); *anti-Flag* antibody from Sigma-aldrich (St Louis, MO); *anti-HA* and *anti-His* antibodies from MBL (Nagoya, Japan); *anti-β-actin* antibody from Bioss (Beijing, China).

Ang-(1–7) and A779 were from BACHEM (Torrance, CA). U73122 was from Sigma-aldrich (St Louis, MO). AKT inhibitor AKTVIII was purchased from Selleck Chemicals (Houston, TX). Fluo-4/AM was from Invitrogen (Carlsbad, CA).

2.2. Plasmids and siRNA

The plasmids pCGN-HA-Mas and pCDNA3-NHERF4 were kindly provided by Dr Randy Hall (Emory University, GA). The construct of Mas-CT was generated as described previously [14]. The vectors encoding Flag-NHERF4, HA-NHERF4, PDZ domains of NHERF4, wild-type and ETVA mutant of Flag-Mas were amplified by PCR and verified by bidirectional sequencing.

siRNAs were obtained from Sango Biotech (Shanghai, China). Three independent NHERF4 siRNAs were tested and the one with the highest efficiency was used. The NHERF4 siRNA chosen for further study was 5'-GCUGUGCCACAUGAGUAAATT-3'. The control siRNA was 5'-UUCUCCGAACGUGUCACGUTT-3'.

2.3. Cell culture and transfection

HEK293, COS-7 and the human ccRCC cell lines 786-O and 769-P were obtained from Institute of Basic Medical Sciences, Cell resource center, China Academy of Medical Sciences (Beijing, China). Cells were maintained in DMEM (HEK293 and COS-7) or RPMI-1640 (786-O and 769-P) medium supplemented with 10% FBS and 1% antibiotics.

Cell transfection was performed using Lipofectamine 3000 (Invitrogen, Carlsbad, CA). For transient transfection experiments, further assays were carried out 48 h post-transfection. For establishment of stable NHERF4-expressing cells, the cells were selected with 0.8 mg/mL G418 at 24 h after transfection.

2.4. SPR fishing assay

The SPR experiments were performed using a Biacore T200 system (GE Healthcare, Uppsala, Sweden). All the SPR-based materials were

purchased from GE Healthcare. Standard buffer HBS-EP+ was used as running buffer. First, GST fusion proteins were immobilized on 4 flow cells of a CM5 chip using amine coupling kit according to the manufacturer's protocol. Next, lysates of HEK293 cells were diluted at 0.5 mg/mL and injected over the sensor surface at a flow rate of 5 µL/min for 180 s. Two µL of 0.5% TFA was injected and remained in the flow cells for 20 s. Finally, the solution containing the eluted proteins was deposited into 10 µL of 50 mM NH₄HCO₃. Recovery program consisted of 15 cycles of "inject and recover". Within one cycle, the sample injection and recovery sequence was repeated 5 times.

2.5. LC-MS/MS analysis

The recovered proteins were prepared using FASP method as previously described [16]. The extracted peptides were analyzed using an Orbitrap Fusion Lumos mass spectrometer equipped with an Easy nLC-1000 (Thermo Fisher Scientific).

MS raw data files were queried against human UniProt database using SEQUEST-HT search algorithm, via Proteome Discoverer (PD, V2.2, Thermo Fisher). The precursor mass tolerance for protein identification on MS was 15 ppm and the product ion tolerance for MS/MS was 0.6 Da. Carbamidomethylation (C) was set as a fixed modification, and oxidation (M) was specified as a variable modification. Full cleavage by trypsin was used, with up to two missed cleavages allowed. The peptides were filtered with 1% false discovery rate (FDR) at peptide level.

2.6. Dataset collection and ccRCC patient specimens

The GEO ccRCC datasets (GSE53757, GSE36895 and GSE16449) were used to screen differential expressed genes (DEGs). The oncomine ccRCC RNA-seq was retrieved from <http://www.oncomine.com/>. TCGA ccRCC RNA-seq and corresponding clinical data were collected from <http://www.synapse.org>. The gene set enrichment analysis (GSEA) was performed as previously described [17]. A false discovery rate <0.25 was considered statistically significant.

The ccRCC specimens were collected from the First Hospital of Shanxi Medical University of Taiyuan during 2015. The patients were included after signing written informed consents. The study was approved by the Ethics Committee of First Hospital of Shanxi Medical University.

2.7. GST pull-down, immunoprecipitation and immunoblotting

Purification of GST- or His-fused proteins, GST pull-down assay, lysis of cells and tumor samples, immunoprecipitation (IP) and immunoblotting (IB) were performed as described previously [12,14].

2.8. Transwell migration and invasion assays

The assays were performed using 24-well transwell insert pre-coated with fibronectin or matrigel as described previously [12].

2.9. In vivo metastasis assay

The study was approved by the Animal Use and Care Committee of Capital Medical University. At day 0, control vector or NHERF4-overexpressing 786-O cells (2.5×10^6 cells in 200 µL PBS) were inoculated into the lateral tail vein of 5-week-old male BALB/c nude mice (Vital River Laboratories, Beijing, China). At day 7, each group was allocated into two treatment subgroups respectively. Treatment consisted of either: a) daily subcutaneous (sc) injection of PBS for the control group, b) daily sc injection of 0.5 mg/kg Ang-(1–7). Treatment was administered for up to 60 days, after which time the animals were sacrificed. The livers were then removed and the tumor nodules were counted.

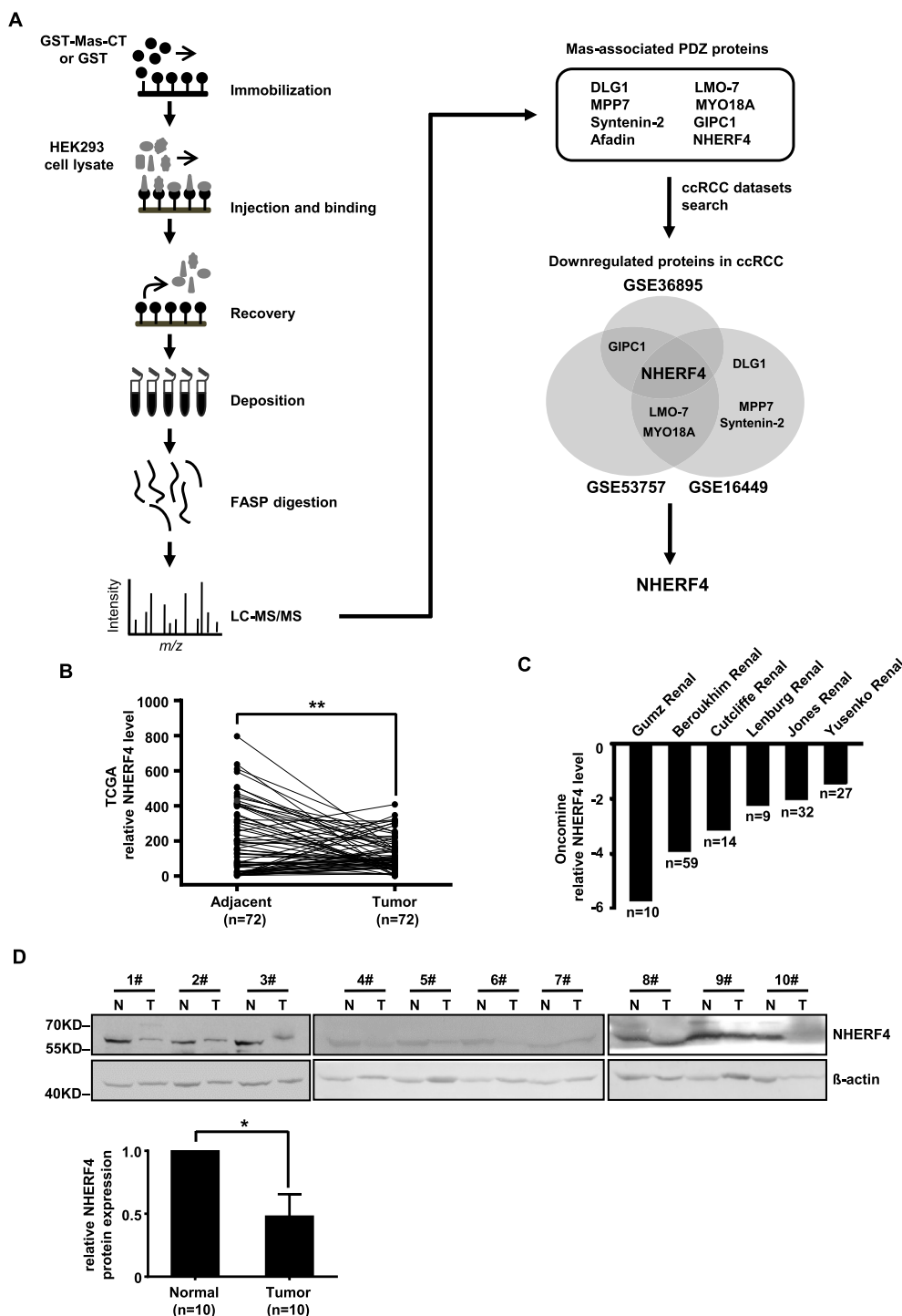


Fig. 1. NHERF4 is associated with Mas and is downregulated in ccRCC. (A) Schematic description of the workflow for screening of Mas-associated PDZ proteins from HEK293 cell lysate by SPR fishing combined with LC/MS, and profiling mRNA expression of the PDZ proteins in ccRCC datasets from GEO (GSE53757, GSE36895 and GSE16449). NHERF4 was identified to be downregulated across three datasets. (B) NHERF4 mRNA expression in 72 paired ccRCC tissues from TCGA dataset (** $P < 0.01$). (C) NHERF4 mRNA expression in tumor versus normal renal tissues from Oncomine analysis of six independent published ccRCC datasets. (D) IB analysis of NHERF4 protein expression in 10 paired tumor (T) and normal renal tissues (N) from ccRCC patients. Representative images were shown. β -Actin was used as a loading control. Data were expressed as fold change compared to normal renal tissues (* $P < 0.05$).

2.10. Measurement of intracellular calcium signaling

The cells were seeded into a 96-well clear bottom black plate (2×10^4 cells per well) and cultured for 20 h. After loaded with $2 \mu\text{M}$ Fluo-4/AM, the cells were maintained in $50 \mu\text{L}$ of Ca^{2+} -free HBSS buffer [20 mM HEPES (pH 7.4), 120 mM NaCl, 5.4 mM KCl, 1 mM MgCl_2 , 10 mM glucose, 0.2 mM EGTA] for 10 min at 25°C . The fluorescence intensity was measured at 2 s intervals at excitation/emission wavelength of 500/535 nm using EnVision multilabel reader (PerkinElmer, Waltham, MA). During the recording, $50 \mu\text{L}$ of Ca^{2+} -free HBSS buffer containing Ang-(1–7) was added using an automatic dispenser instrument. The base-line readings were collected for 10 s prior to stimulation. In some cases, the

cells were pre-treated with indicated inhibitors for 10 min. The fluorescence values were calculated by subtracting readings of the non-stained cells from those of corresponding stained cells.

2.11. Statistical analysis

The statistical analyses were performed using the GraphPad Prism 7.0 (La Jolla, CA) and SPSS statistic 18.0 (SPSS Inc. Chicago, IL). A value of $P < 0.05$ was considered statistically significant.

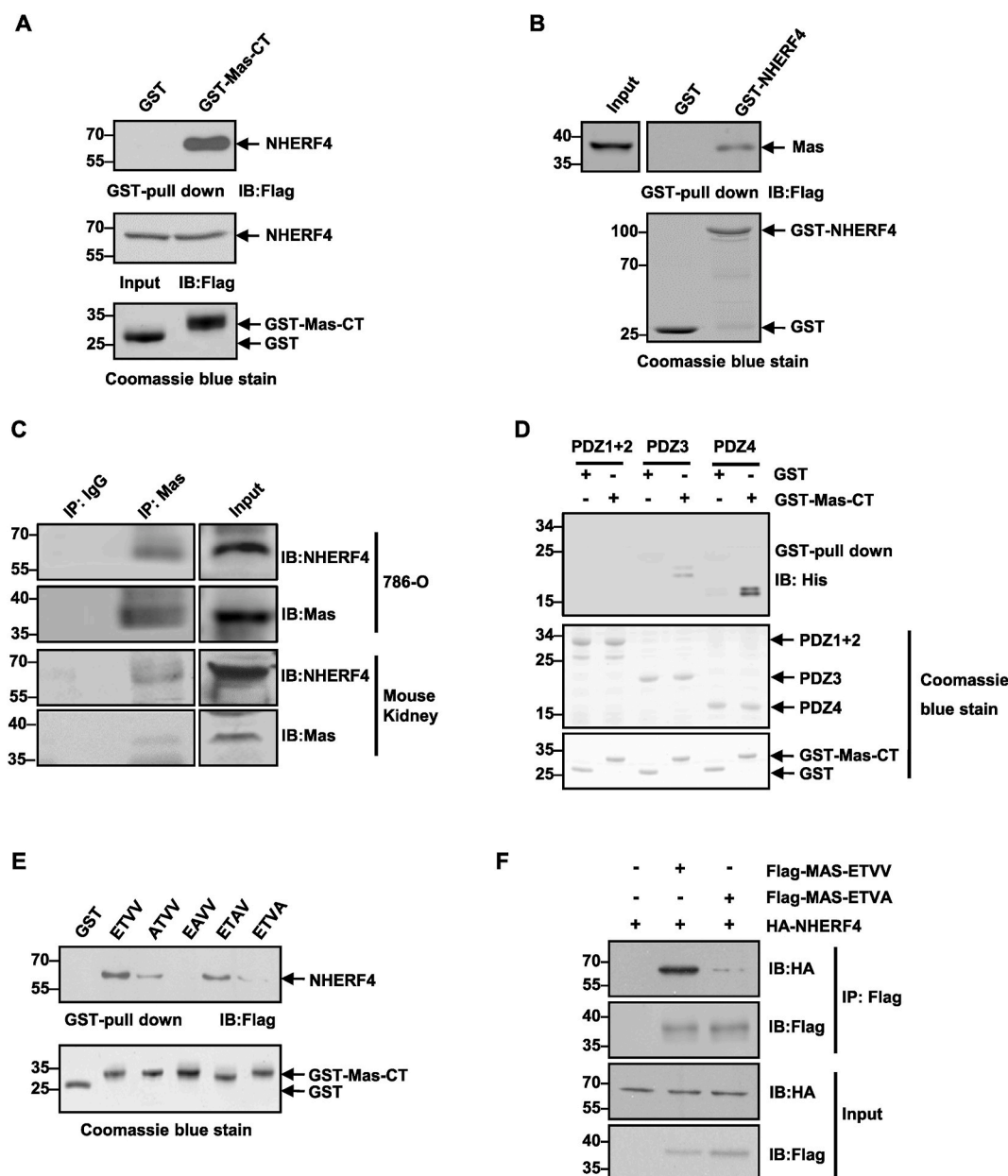


Fig. 2. NHERF4 physically interacts with Mas. **(A, B)** Association of NHERF4 with Mas *in vitro*. **(A)** GST or GST-fused Mas-CT were used to pull down NHERF4 from transfected COS-7 cell extract. **(B)** GST or GST-fused NHERF4 were used to pull down Mas from transfected COS-7 cell extract. The pulled down proteins were detected by IB using indicated antibodies. Coomassie blue staining of GST-fused proteins was shown. **(C)** Interaction between endogenous Mas and NHERF4. Lysates from 786-O cells or mouse kidney tissue were prepared and co-IP assays were performed using anti-Mas antibody. Immunocomplexes were immunoblotted with *anti*-NHERF4 antibody. **(D)** Mapping the interface in NHERF4 required for the interaction with Mas. GST-fused Mas-CT was used to pull down His-fused PDZ domain constructs of NHERF4. The pull-down complexes were immunoblotted with anti-His antibody. Coomassie blue stainings of GST/His-fused proteins were shown. **(E)** Identification of structural determinants of Mas-CT interaction with NHERF4. GST-fused wild-type Mas-CT (indicated as the last four amino acids ETVV) or its point mutants (mutations in each of the amino acids) were used to pull down NHERF4 from lysates of NHERF4-transfected COS-7 cells. The pull-down complexes were then immunoblotted with *anti*-NHERF4 antibody. **(F)** Specificity of interaction between Mas and NHERF4 in cells. COS-7 cells were co-transfected with HA-NHERF4 and wild-type Flag-Mas-ETVV or its point mutant Flag-Mas-ETVA. Cell lysates were prepared and co-IP assay was performed using anti-Flag antibody followed by IB with indicated antibodies.

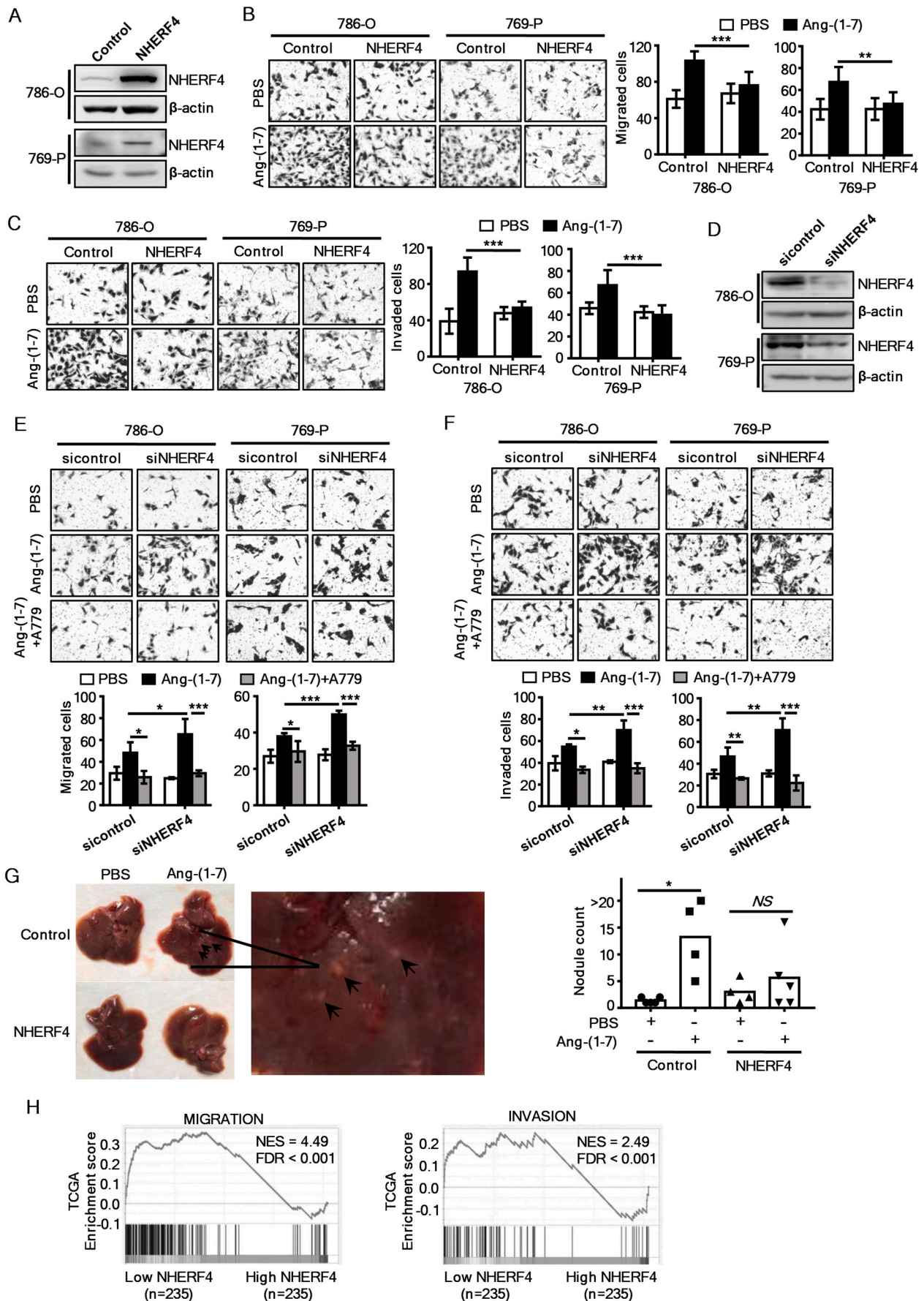
3. Results

3.1. NHERF4 is associated with Mas and is downregulated in ccRCC

To screen for PDZ proteins that associate with Mas, we used GST-Mas-CT as bait and employed the SPR fishing technique (Fig. 1A). Chip-bound proteins from human embryonic kidney cells HEK293 were collected by an “injection and recover” program (Supplementary Fig. S1A). MS/MS analysis showed that all PDZ protein hits were

exclusively presented in samples from SPR fishing with GST-Mas-CT (Supplementary Table S1-2). GST-Mas-CT, but not GST, associated with eight PDZ proteins including DLG1, LMO-7, MPP7, MYO18A, Syntenin-2, GIPC1, Afadin and NHERF4 (Fig. 1A, Supplementary Fig. S1B and Table S3).

To determine whether these PDZ proteins had a significant role in ccRCC, we analyzed their mRNA expression patterns in three publicly available independent ccRCC datasets from GEO (GSE53757, GSE36895 and GSE16449). We found that *NHERF4* was the only DEG and its mRNA



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Fig. 3. NHERF4 inhibits Mas-mediated migration and invasion of ccRCC cells *in vitro* and *in vivo*. (A) IB analysis of NHERF4 expression in 786-O or 769-P cells stably expressing NHERF4 or control vector. (B, C) Transwell migration (B) and transwell invasion (C) assays were performed in cells as in (A). The cells were treated with PBS or 1 μ M Ang-(1–7). (D) IB analysis of NHERF4 expression in 786-O or 769-P cells transfected with siNHERF4 or sicontrol. (E, F) Transwell migration (E) and transwell invasion (F) assays were performed in cells as in (D). The cells were treated with PBS, 1 μ M Ang-(1–7) or 1 μ M Ang-(1–7)/10 μ M A779. (B, C, E, F) Images are representative of 3 independent experiments and data are expressed as mean $\pm$ SD. * P < 0.05, ** P < 0.01, *** P < 0.001. (G) 786-O cells stably expressing NHERF4 or control vector were injected intravenously through the tail veins of 5-week-old male BALB/c nude mice. Treatment of PBS or Ang-(1–7) initiated 7 days after ccRCC cells injection. Left, images of the excised liver tissues at day 60. Arrows indicate tumor nodules in liver. Right, quantification of metastatic nodules in liver. Data are expressed as mean $\pm$ SD. * P < 0.05; NS, not significant. (H) Enrichment plots for genes associated with migration and invasion pathways according to NHERF4 mRNA expression levels by GSEA of TCGA ccRCC dataset. FDR, false discovery rate; NES, normalized enrichment score.

level was downregulated in tumor tissues compared with adjacent normal tissues across the three datasets (Supplementary Fig. S2A). This finding was confirmed by data from both TCGA and Oncomine ccRCC datasets (Fig. 1B–C). Consistent with these findings, immunoblotting analysis of paired ccRCC specimens also showed decreased NHERF4 protein levels in tumor tissues (Fig. 1D).

3.2. NHERF4 physically interacts with Mas

To examine the interaction of NHERF4 with Mas, a GST pull-down assay was performed. We observed a robust association between Mas-CT and exogenous Flag-NHERF4 (Fig. 2A). Reciprocally, GST-NHERF4 interacted with exogenous Flag-Mas (Fig. 2B). Furthermore, the endogenous Mas/NHERF4 complex was observed in the ccRCC cells, 786-O and 769-P, as well as in mouse kidney tissue by co-IP (Fig. 2C and Supplementary Fig. S3).

NHERF4 contains four PDZ domains. To determine which domain could contribute to its interaction with Mas, each PDZ domain of NHERF4 was subjected to a separate GST pull-down assay. PDZ domains 3 and 4 bound to Mas-CT, with domain 4 showing stronger binding than domain 3 (Fig. 2D). Next, to map the interaction interface of Mas-CT with NHERF4, the last four residues ETVV of PBM were individually mutated to alanine (ETVA, ETAV, EAVV and ATVV) and these mutants were expressed as GST-fused proteins. COS-7 cells were transfected with Flag-NHERF4. GST pull-down analysis showed that these point mutations reduced or abolished binding to NHERF4 (Fig. 2E). The binding specificity was further validated by co-IP in COS-7 cells, which had been co-transfected with HA-NHERF4 and wild-type Flag-Mas-ETVV or point mutant Flag-Mas-ETVA (Fig. 2F).

In addition, the subcellular localization of Mas and NHERF4 was examined in HEK293 cells transfected with Flag-Mas and/or HA-NHERF4 by fluorescence confocal microscopy. In cells transfected with Mas alone, the receptors were localized predominantly in the plasma membrane. However, when Mas was co-expressed with NHERF4, a significant proportion of Mas was found in the cytoplasm, where it co-localized with NHERF4 (Supplementary Fig. S4). These findings suggest that the interaction between NHERF4 and Mas may affect membrane localization of the Mas receptor.

3.3. NHERF4 inhibits Mas-mediated migration and invasion of ccRCC cells

Previously, Mas has been shown to promote migration and invasion of ccRCC cells [12]. However, the mechanisms underlying the regulation of Mas function are largely unknown. We therefore investigated whether NHERF4 could regulate Mas function to affect cell phenotype. NHERF4 overexpression and knockdown experiments were performed to evaluate its effect on migration and invasion of the ccRCC cell lines, 786-O and 769-P. NHERF4 overexpression significantly suppressed Ang-(1–7)-driven cell migration and invasion, but had little effect in the absence of Ang-(1–7) (Fig. 3A–C). Conversely, NHERF4 silencing had an inverse effect, which was completely abolished when treated with A779, a selective Mas antagonist (Fig. 3D–F and Supplementary Fig. S5).

To determine whether NHERF4 would play a similar role *in vivo*, we evaluated the metastatic potential of control or NHERF4-overexpressing 786-O cells in mice. As anticipated, control cells readily formed

metastatic nodules in the livers of mice administered with Ang-(1–7) daily. In contrast, the invasive ability of tumor cells was dramatically compromised upon NHERF4 overexpression, as evidenced by reduced metastatic foci formation in the livers (Fig. 3G). Accordingly, GSEA on ccRCC datasets also revealed negative correlation of NHERF4 expression with both cell migration and invasion processes (Fig. 3H and Supplementary Fig. S6A–B).

3.4. NHERF4 regulates the Mas/PLC/AKT signaling axis in ccRCC cells

Our previous study implicated AKT signaling in Mas-mediated migration and invasion of ccRCC cells [12]. To identify the molecular mechanisms of NHERF4 function, we first analyzed Ang-(1–7)-induced AKT activation upon NHERF4 overexpression or knockdown in 786-O or 769-P cells. NHERF4 overexpression significantly attenuated Ang-(1–7)-induced phosphorylation of AKT in ccRCC cells (Fig. 4A), whereas NHERF4 deficiency led to a further increase in this signaling, which was suppressed by A779 (Fig. 4B and Supplementary Fig. S7A). These findings indicate that NHERF4 could downregulate AKT signaling by specifically targeting Mas.

GPCRs have been reported to activate AKT signaling in a PLC-dependent manner [18]. To determine the role of PLC in AKT activation in ccRCC cells, we treated ccRCC cells with the PLC inhibitor, U73122. Ang-(1–7)-induced AKT phosphorylation was significantly suppressed in either sicontrol or siNHERF4-transfected 786-O cells when treated with U73122 (Fig. 4B and Supplementary Fig. S7A). These data suggest that Mas might activate the PLC/AKT signaling axis, which is regulated by NHERF4 in ccRCC cells.

Ang-(1–7) has been reported to stimulate the PLC signaling pathway in non-cancerous cells [19,20]. To further determine whether NHERF4 affected the Ang-(1–7)/Mas/PLC signaling pathway in ccRCC cells, we used NHERF4 overexpression and knockdown to analyze the intracellular Ca^{2+} response, which is a well-established downstream event of PLC activation [21]. First, we observed that 1 μ M Ang-(1–7) robustly increased Ca^{2+} signaling in both 786-O or 769-P control cells (Fig. 4C–D), which were sensitive to treatment with either A779 or U73122 (Fig. 4D). Second, NHERF4 overexpression attenuated Ang-(1–7)-induced Ca^{2+} response (Fig. 4C), whereas depletion of NHERF4 further enhanced this signaling, which was suppressed by either A779 or U73122 (Fig. 4D). Taken together, our findings demonstrate that Ang-(1–7) predominantly signals through Mas to activate the PLC signaling pathway, which is attenuated by NHERF4. Consistent with these findings, GSEA on ccRCC datasets confirmed a negative correlation between NHERF4 expression, and Ca^{2+} and AKT signaling in ccRCC samples (Fig. 4E and Supplementary Fig. S7B).

3.5. PLC and AKT inhibitors abolish the effect of NHERF4 on Mas-mediated migration and invasion of ccRCC cells

We next explored whether ablation of the PLC/AKT signaling pathway was sufficient to block the effect of NHERF4 on Mas-mediated cell functions. We first found that Ang-(1–7)-driven cell migration and invasion were sensitive to treatment with U73122 or AKTVIII in sicontrol-transfected 786-O and 769-P cells (Fig. 5A–B), confirming the key role of the PLC/AKT signaling pathway in Mas-mediated cell functions. Meanwhile, U73122 or AKTVIII suppressed the compensatory

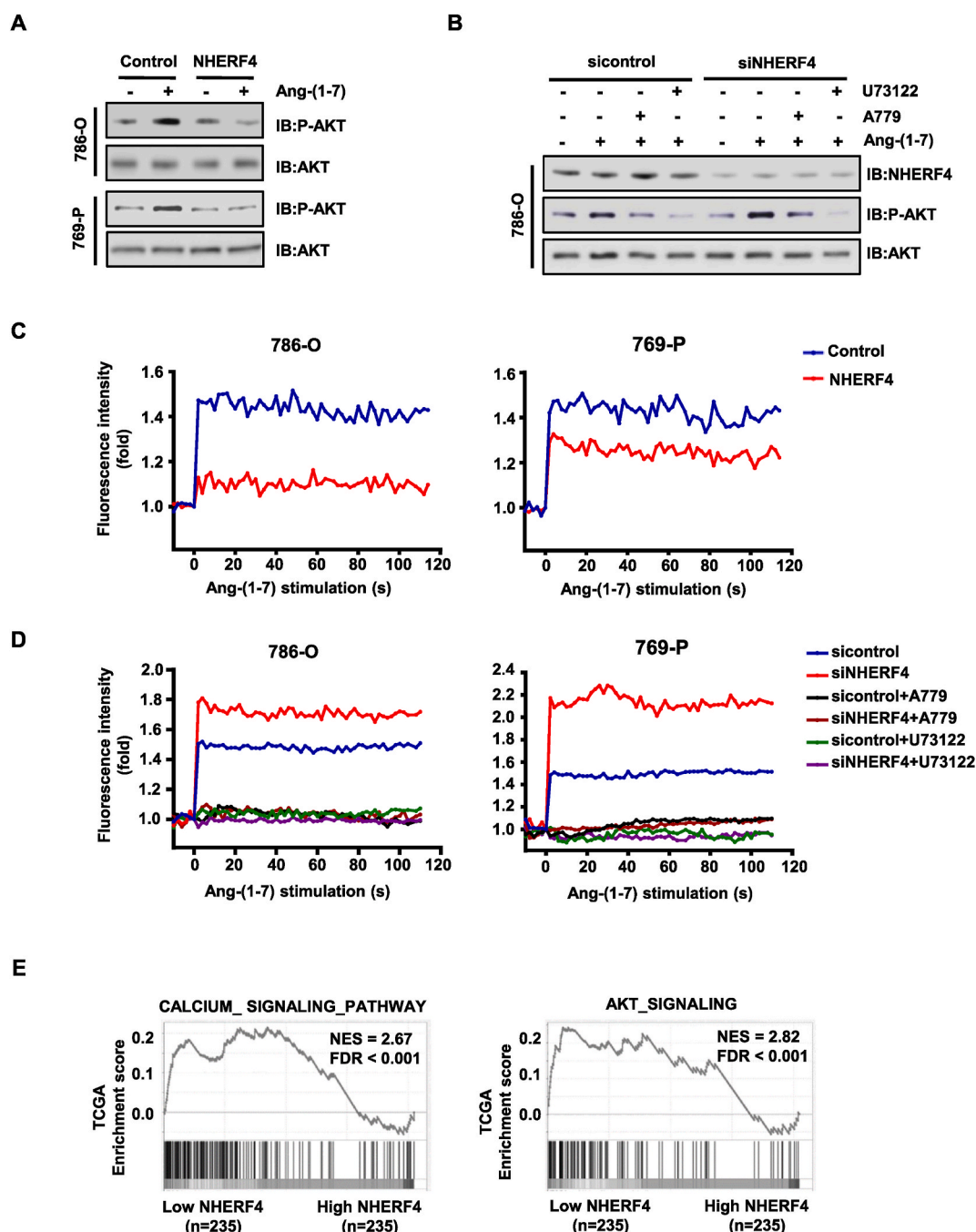


Fig. 4. NHERF4 suppresses Mas-mediated PLC/AKT signaling axis in ccRCC cells. (A) Effect of NHERF4 overexpression on Ang-(1-7)-stimulated AKT activation in 786-O and 769-P cells stably expressing NHERF4 or control vector. Cells were serum starved and treated with PBS or 1 μ M Ang-(1-7) before lysis. Phosphorylation of AKT was analyzed by IB. Total AKT was used as loading control. (B) Effect of NHERF4 depletion on Ang-(1-7)-stimulated AKT activation in 786-O cells transfected with siNHERF4 or sicontrol. Serum-starved cells were treated with PBS, 1 μ M Ang-(1-7), 1 μ M Ang-(1-7)/10 μ M A779 or 1 μ M Ang-(1-7)/0.1 μ M U73122. IB analysis was performed with indicated antibodies. (C) NHERF4 overexpression attenuated Ang-(1-7)-induced Ca^{2+} signaling in cells as in (A). The cells were loaded with Fluo-4/AM prior to stimulated with 1 μ M Ang-(1-7). (D) NHERF4 knockdown enhanced Mas-mediated Ca^{2+} response in cells as in (B). The cells were loaded with Fluo-4/AM, treated with 10 μ M A779 or 0.1 μ M U73122 and then stimulated with 1 μ M Ang-(1-7). (C, D) Values are presented relative to fluorescence intensity recorded at the time of Ang-(1-7) injection. Results shown are representative of 3 independent experiments. Data are expressed as mean. (E) Enrichment plots for genes associated with Ca^{2+} or AKT signaling pathway according to NHERF4 mRNA expression levels by GSEA of TCGA ccRCC dataset.

enhanced migration and invasion of NHERF4-depleted ccRCC cells (Fig. 5A–B). These data suggest that NHERF4 could inhibit Mas-mediated cell functions through the suppression of the PLC/AKT signaling pathway (Fig. 6).

3.6. Low NHERF4 expression is associated with tumor invasion and independently predicts poor prognosis of ccRCC patients

The clinical relevance of NHERF4 was next examined using TCGA ccRCC dataset. Low NHERF4 expression was strongly associated with the extent of primary tumor invasion, lymphatic involvement and metastasis (Fig. 7A–C), indicating the regulatory role of NHERF4 in

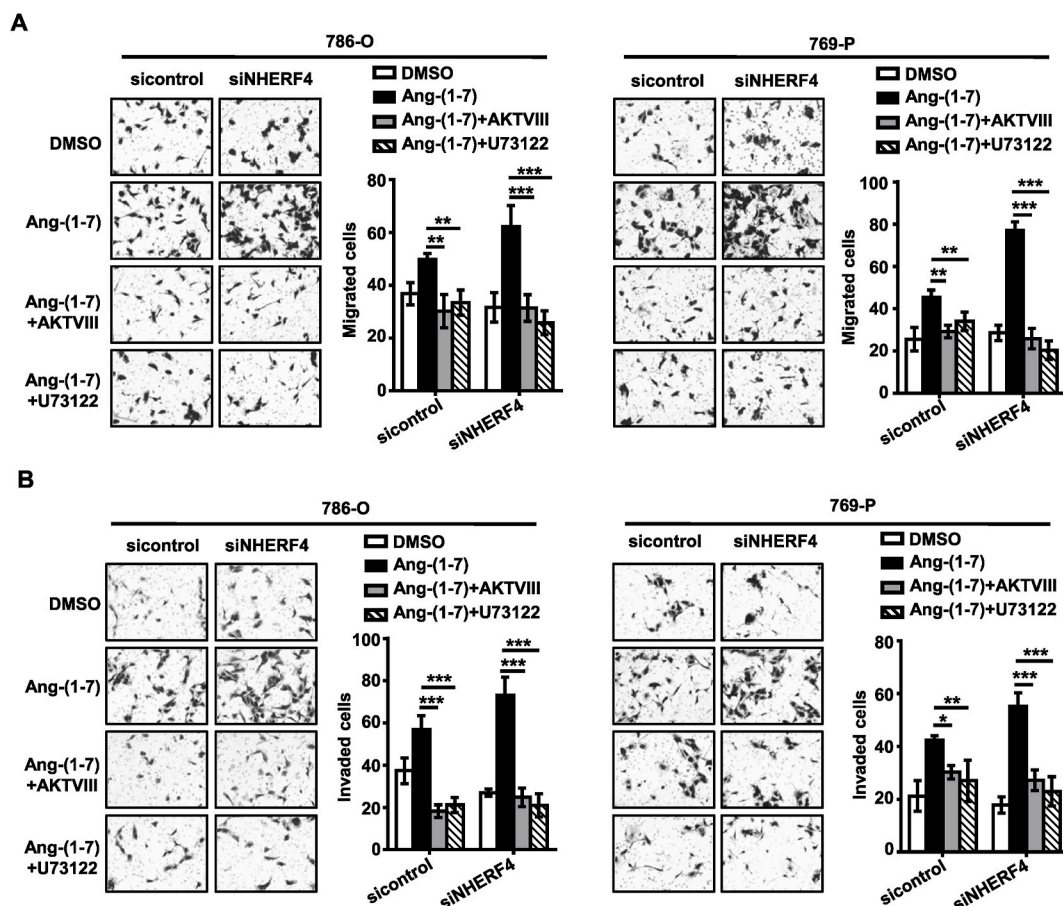


Fig. 5. PLC and AKT inhibitors abolish the effect of NHERF4 on Mas-mediated migration and invasion of ccRCC cells. 786-O and 769-P cells were transfected with sicontrol or siNHERF4. Transwell migration (A) and transwell invasion (B) assays were performed in the presence of DMSO, 1 μ M Ang-(1-7), 1 μ M Ang-(1-7)/1 μ M AKTVIII or 1 μ M Ang-(1-7)/0.1 μ M U73122. Images are representative of 3 independent experiments and data are expressed as mean $\pm$ SD. ** P < 0.01, *** P < 0.001.

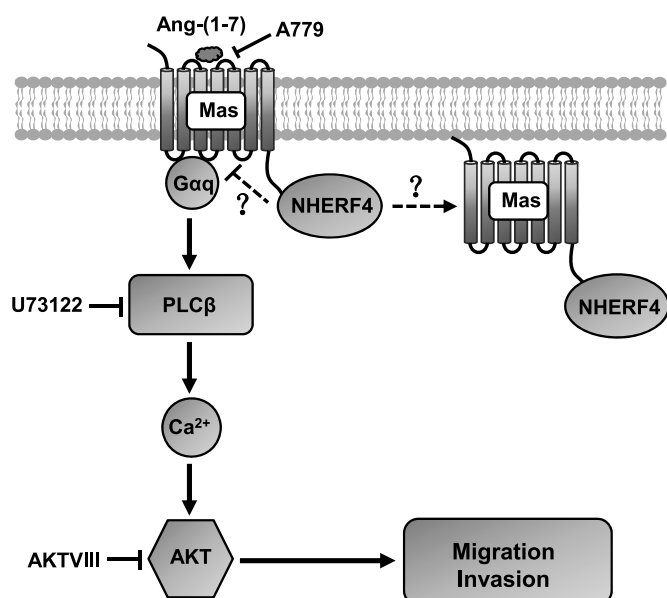


Fig. 6. A proposed model for NHERF4 regulating migration and invasion of ccRCC cells.

tumor invasion.

To further assess whether NHERF4 expression levels could stratify ccRCC patient survival, we performed Kaplan-Meier and log-rank test analyses on the data obtained from TCGA dataset. The results demonstrated that worse overall survival (OS) and disease-free survival (DFS) rates were associated with ccRCC patients who had low NHERF4 expression levels (Fig. 7D–E). The impact of NHERF4 expression on OS and DFS rates was further determined by univariate and multivariate analyses. Univariate analysis confirmed the correlation of NHERF4 levels with the survival of ccRCC patients (OS: HR 0.625, P = 0.006; DFS: HR 0.646, P = 0.031). When adjusted for other variables including age, sex, grade, tumor size, nodal and metastatic status in the multivariate analysis, NHERF4 expression level maintained its clinical significance with respect to survival (OS: HR 0.639, P = 0.009; DFS: HR 0.594, P = 0.011) (Table 1). Together, the data suggest that low NHERF4 expression in ccRCC may be an independent prognostic indicator for poor outcome in ccRCC patients.

4. Discussion

Hyperactive proto-oncogenes and loss of tumor suppressor genes are major factors involved in tumorigenesis. Activation of Mas by Ang-(1-7) can potentiate the migratory and invasive abilities of ccRCC cells [12]. Our bioinformatics analysis data demonstrated that there was no significant difference in Mas mRNA expression between paired ccRCC/normal tissues from the GSE66272 database (Supplementary Fig. S2C). Therefore, attenuated regulation of Mas activity may

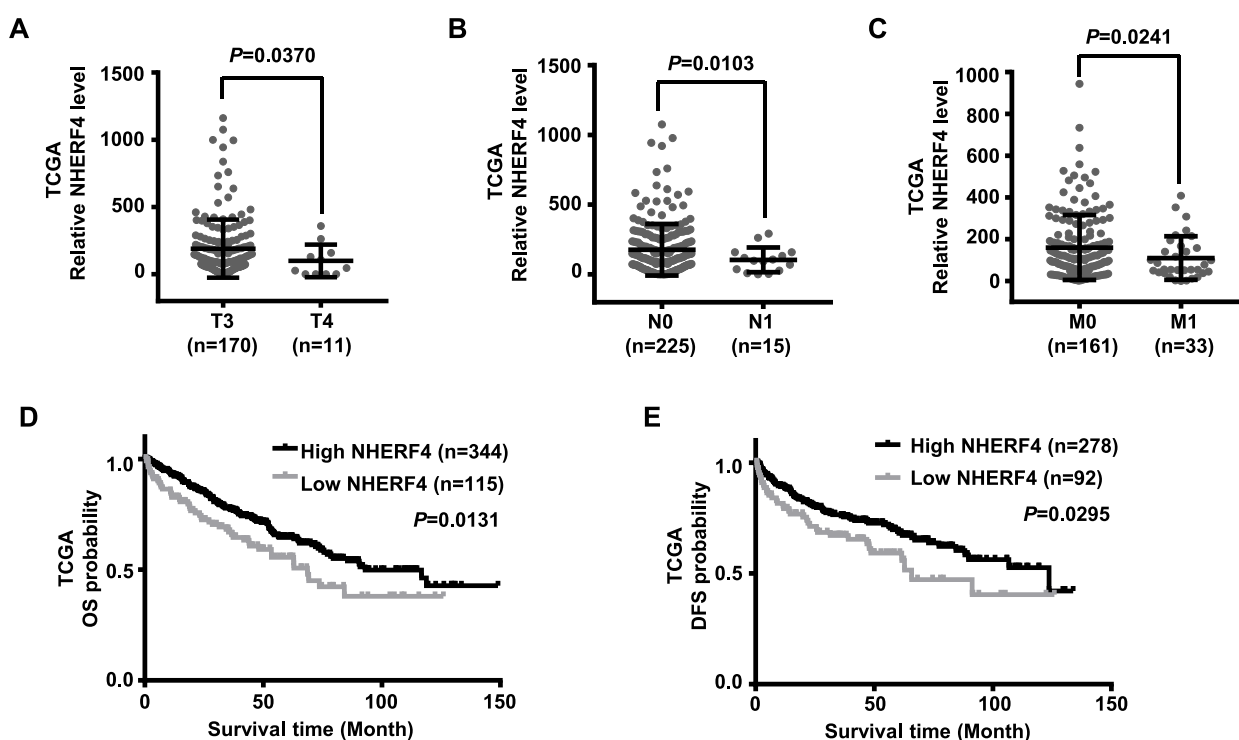


Fig. 7. Low NHERF4 expression is associated with tumor invasion and poor prognosis in ccRCC. (A–C) mRNA expression level of NHERF4 in ccRCC patients with stage T3 versus T4 (A), stage N0 versus N1 (B) and stage M0 versus M1 (C). (D, E) Overall survival (OS) or disease-free survival (DFS) analyses of ccRCC patients stratified by low (bottom quartile) and high (rest) NHERF4 mRNA expression levels in TCGA.

contribute to the invasiveness of ccRCC cells. In this study, we identified NHERF4 as a negative regulator of Mas function in ccRCC, as supported by: (i) downregulation of NHERF4 expression in ccRCC; (ii) physical interaction of NHERF4 with Mas; (iii) suppressive effect on Ang-(1–7)-induced migration, invasion and *in vivo* metastasis of ccRCC cells; (iv) inhibitory impact on the Ang-(1–7)/Mas/PLC/AKT signaling axis; and (v) its correlation with tumor invasion and its ability to independently predict survival of ccRCC patients.

NHERF4, together with NHERF1, NHERF2 and NHERF3, belongs to the evolutionarily conserved NHERF family. These epithelial-enriched proteins were initially reported to interact with a variety of transporters and ion channels, indicating their critical role in water and salt homeostasis [22,23]. Following identification of cancer-related binding partners such as PDGFR, EGFR, ER α , LPA2, PTEN, β -catenin and YAP, NHERF proteins (NHERF1, NHERF2 and NHERF3) have recently emerged as key regulators in multiple cancers including breast, colon, biliary and gastric cancers [24–29]. Compared to its family members, NHERF4 has relatively restricted tissue distribution, being found at significant levels only in kidney and intestine [23]. Although NHERF4 shares considerable homology and a limited number of binding partners with other NHERF members, its role in the pathophysiology of cancer is less well understood. Here, our findings provided the first evidence of the relationship between NHERF4 and cancer. We demonstrated that NHERF4 specifically inhibited Mas-mediated migration and invasion of ccRCC cells and independently predicted prognosis of ccRCC patients. Interestingly, our recent studies revealed that NHERF3 had a similar expression profile and function in ccRCC, such as downregulated expression, inhibitory effect on malignant phenotypes of tumor cells and independent prediction for prognosis [30,31]. NHERF3 and NHERF4 shared the most similarity in structure among the family members. Although both proteins had redundant roles in ccRCC, their underlying molecular mechanisms appeared to be divergent. Our previous study demonstrated that NHERF3 functioned as a tumor suppressor by blocking SHP-1 phosphorylation via inhibition of the SHP-1/PLC β 3 interaction, resulting in delayed AKT phosphorylation and increased

STAT5 phosphorylation [31]. Here, we showed that the inhibitory effect of NHERF4 could be attributed to its ability to block the Mas-mediated PLC/AKT signaling axis through its interaction with Mas (Fig. 6). Strikingly, NHERF3 was not associated with Mas by either SPR fishing or GST-pull down assays (data not shown). Thus, it seems likely that NHERF4 and NHERF3 suppress AKT activation via independent pathways and different mechanisms. The cooperation between NHERF4 and NHERF3 in ccRCC appears worthy of further investigation.

GPCRs can lead to mobilization of Ca²⁺ from intracellular stores through PLC activation, which has been linked to the migratory phenotype of a variety of cancer cells, and may contribute to its critical role in tumor invasion [29,32,33]. Our findings supported previous studies demonstrating that PLC-Ca²⁺ is a major signaling pathway mediated by Mas [19,34,35]. Importantly, our findings extended this work by showing that inhibition of the Ang-(1–7)-induced Ca²⁺ response and AKT phosphorylation upon treatment of either a Mas antagonist or PLC inhibitor dramatically impaired the migratory and invasive potentials of ccRCC cells (Fig. 5A–B). These findings suggest a causal role of Ang-(1–7)-dependent Mas/PLC/AKT signaling axis in the progression of ccRCC. Moreover, it should be noted that most earlier studies on Ang-(1–7)/Mas-mediated PLC signaling were performed using the Mas-overexpressing HEK293 cell model [15,19]. Interestingly, Ang-(1–7) appears to be a more potent inducer to activate PLC signaling in ccRCC cells than in other cell types. A higher concentration (up to 1 mM) was required for activation of PLC signaling in HEK293 cells [19], whereas 1 μ M was sufficient in 786-O and 769-P cells (Fig. 4C). This could be due to a difference in cell-specific signaling, through a defective regulatory mechanism in ccRCC cells. This hypothesis is supported by our findings showing that NHERF4 depletion elicited compensatory activation of Ang-(1–7)-induced PLC/AKT signaling, thereby enhancing the invasive potential of ccRCC cells.

GPCR-interacting PDZ proteins have been shown to modulate GPCR function via multiple mechanisms such as stability or trafficking of receptor, and coupling of receptor to G proteins. For example, PSD95 was shown to interact with Mas to block its degradation [14]. NHERF4

Table 1
Univariate and multivariate analyses of NHERF4 mRNA level for survival.

Variable	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value	HR (95% CI)	P value
OS (n = 459)				
Age (years)				
≤50 (n = 98)				
>50 (n = 361)	2.141 (1.341–3.418)	0.001	1.934 (1.197–3.124)	0.007
Gender				
Female (n = 159)				
Male (n = 300)	0.929 (0.677–1.276)	0.650		
Grade				
G1 or G2 (n = 203)				
G3 or G4 (n = 256)	2.495 (1.761–3.535)	0.000	1.726 (1.189–2.505)	0.004
AJCC_Tumor				
T1 or T2 (n = 281)				
T3 or T4 (n = 178)	3.188 (2.326–4.369)	0.000	1.726 (1.186–2.513)	0.004
AJCC_Nodes				
N0 or NX (n = 445)				
N1 (n = 14)	3.426 (1.802–6.515)	0.000	2.84 (1.459–5.529)	0.002
AJCC_Metastasis				
M0 (n = 383)				
M1 (n = 76)	4.03 (2.933–5.537)	0.000	2.268 (1.572–3.271)	0.000
NHERF4				
Low (n = 115)				
High (n = 344)	0.625 (0.448–0.872)	0.006	0.639 (0.456–0.896)	0.009
DFS (n = 370)				
Age (years)				
≤50 (n = 92)				
>50 (n = 278)	1.39 (0.888–2.178)	0.150		
Gender				
Female (n = 121)				
Male (n = 249)	1.279 (0.857–1.908)	0.228		
Grade				
G1 or G2 (n = 173)				
G3 or G4 (n = 197)	3.146 (2.066–4.791)	0.000	2.270 (1.462–3.523)	0.000
AJCC_Tumor				
T1 or T2 (n = 237)				
T3 or T4 (n = 133)	4.695 (3.201–6.887)	0.000	3.216 (1.428–3.438)	0.000
AJCC_Nodes				
N0 or NX (n = 358)				
N1 (n = 12)	5.479 (2.744–10.937)	0.000	3.476 (1.705–7.008)	0.001
AJCC_Metastasis				
M0 (n = 317)				
M1 (n = 53)	8.212 (5.617–12.005)	0.000	4.911 (3.210–7.513)	0.000
NHERF4				
Low (n = 92)				
High (n = 278)	0.646 (0.435–0.961)	0.031	0.594 (0.397–0.889)	0.011

HR, hazard ratio; CI, confidence interval; AJCC, American Joint Committee on Cancer.

interacted with the I prostanoid receptor, enhancing its membrane expression and agonist-induced endothelial cell migration [36]. NHERF1 has been associated with the CCR5 chemokine receptor, increasing its recruitment of arrestin 2 and enhancing its internalization [37]. Furthermore, NHERF1 and NHERF2 were found to interact with parathyroid hormone receptor 1 (PTH1R) and regulate receptor-mediated signaling via direct modulation of receptor coupling to G proteins [38]. In the current study, NHERF4 overexpression exerted little effect on Mas protein expression in 786-O and 769-P cells (Supplementary Fig. S8A–B). Co-localization analysis suggested that NHERF4 might reduce membrane expression of Mas (Supplementary Fig. S4). However, it remains unclear whether NHERF4 has a similar function to NHERF1 and NHERF2 in mediating receptor coupling to G proteins (Fig. 6). Future studies are required to examine the effect of NHERF4 on the Mas receptor and elucidate the relationship between NHERF4, Mas and G proteins.

CRedit authorship contribution statement

Ying Yang: Methodology, Validation, Investigation, Data curation, Writing – original draft, Visualization, Writing – review & editing.

Jihuan Liang: Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. **Chunjuan Zhao:** Methodology, Formal analysis, Validation, Resources. **Yibin Chen:** Validation, Resources. **Siyu Gu:** Resources. **Xiaomei Yang:** Resources. **Hua Liu:** Resources. **Qiong Qin:** Resources, Writing – review & editing. **Haibo Wang:** Resources. **Zhuoli Yang:** Resources. **Xuedi Cao:** Resources. **Duiping Feng:** Resources. **Junqi He:** Conceptualization, Methodology, Validation, Supervision, Project administration, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2021.06.021>.

NHERF4 interacts with c-terminus of Mas and inhibits Mas-mediated PLC/Ca²⁺/AKT signaling axis, and thereby reduces invasive potentials of ccRCC cells. NHERF4 exerts regulatory function through the possible mechanisms involving promotion of Mas internalization and disruption of Mas/G protein interaction.

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