VHL loss in renal cell carcinoma leads to up-regulation of CUB domain-containing protein 1 to stimulate PKCδ-driven migration

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A common genetic mutation found in clear cell renal cell carcinoma (CC-RCC) is the loss of the von Hippel-Lindau (*VHL*) gene, which results in stabilization of hypoxia-inducible factors (HIFs), and contributes to cancer progression and metastasis. CUB-domain-containing protein 1 (CDCP1) was shown to promote metastasis in scirrhous and lung adenocarcinomas as well as in prostate cancer. In this study, we established a molecular mechanism linking *VHL* loss to induction of the *CDCP1* gene through the HIF-1/2 pathway in renal cancer. Also, we report that Fyn, which forms a complex with CDCP1 and mediates its signaling to PKCδ, is a HIF-1 target gene. Mechanistically, we found that CDCP1 specifically regulates phosphorylation of PKCδ, but not of focal adhesion kinase or Crk-associated substrate. Signal transduction from CDCP1 to PKCδ leads to its activation, increasing migration of CC-RCC. Furthermore, patient survival can be stratified by CDCP1 expression at the cell surface of the tumor. Taken together, our data indicates that CDCP1 protein might serve as a therapeutic target for CC-RCC.

Kidney cancer is the third most common malignancy of the genitourinary system and is the sixth leading cause of cancer death in the United States. Clear cell renal cell carcinoma (CC-RCC) is the most common type of kidney cancer and is increasing in number in the United States, accounting for >8 of 10 cases. Standard means for treating most solid tumors, including radio- and chemotherapy, have consistently shown disappointing results in the treatment of CC-RCC, placing it among the most radio- and chemo-resistant cancers. Surgery is the primary treatment of choice for patients diagnosed with early stages of the disease. However, >30% of patients are diagnosed with metastatic disease, and one-third of initially metastasis-free patients develop metastasis after the initial surgery. No curative therapy exists for patients diagnosed with metastatic CC-RCC.

It is known that hypoxic tumor cells are especially aggressive, metastatic, and resistant to therapy (1). Hypoxia triggers activity of hypoxia-inducible factor (HIF) that regulates expression of a large number of target genes involved in tumor progression (2). In the presence of oxygen, HIF-1α and HIF-2α are hydroxylated on prolines 402/564 and 405/531, respectively, and are recognized by the von Hippel-Lindau tumor suppressor protein (pVHL), which mediates their degradation. Under hypoxic conditions, hydroxylation of HIF-1α and HIF-2α, and binding to pVHL decreases, HIF-1α and HIF-2α become stabilized, and each forms a heterodimer with aryl hydrocarbon receptor nuclear translocator (ARNT) to increase the expression of a large number of target genes involved in glycolysis, adhesion, migration, and angiogenesis (2, 3). The mechanisms underlying the metastatic properties of hypoxic cells have started to emerge in the last decade (4–6). Nevertheless, elucidation of hypoxia-regulated genes implicated in metastasis is extremely important to provide new therapeutic targets and overcome potential complications related to drug resistance.

CUB-domain-containing protein 1 (CDCP1) was first described as being expressed on the cell surface of metastatic cell lines (7). Later, CDCP1 was shown to increase the number of nodules formed by lung adenocarcinoma cells in lungs in tail vein injection experiments (8), enhance peritoneal dissemination of scirrhous adenocarcinoma (9), and to induce metastasis in the chicken embryo metastatic model (10). Although the role of CDCP1 in metastasis and its downstream signaling became the subject of investigation, the mechanism of its overexpression in multiple types of cancer was not explored. In this study, we established that the *CDCP1* gene is regulated by HIF-1 and HIF-2, providing a mechanism of CDCP1 overexpression in cell types, where HIF activity is stimulated by dysregulation of signaling pathways upstream of HIF, such as isocitrate dehydrogenase 1 (IDH1), phosphoinositide 3-kinase/Akt (PI-3K/Akt), mitogen-activated protein kinase (MAPK), and Von Hippel Lindau (VHL) pathways (11).

In this work, we investigated the role of CDCP1 in CC-RCC type of cancer, where *VHL* tumor suppressor gene is inactive in 80% of cases (2), leading to HIF stabilization under normoxic conditions as well as the expression of HIF target genes, including CDCP1. We further found that CDCP1 is heavily tyrosine phosphorylated in CC-RCC, is in a complex with Src family kinases (SFKs) and mediates signal transduction from SFKs to PKCδ, but not to other SFK substrates, like focal adhesion kinase (FAK) and Crk-associated substrate (CAS). Our additional findings show that *Fyn* is a HIF-1 target gene and PKCδ relocalizes to the cell membrane upon *VHL* loss, placing CDCP1 in a context for being constitutively active in CC-RCC. The metastatic process is known to manifest in increased cell motility and resistance to apoptosis in vitro. Thus, in this work we have investigated the promigratory role of CDCP1 in CC-RCC. Interestingly, we did not find a role for CDCP1 in protecting cells from anoikis in CC-RCC unlike published studies have reported for lung adenocarcinoma and scirrhous adenocarcinoma (8, 9). However, we did find a correlation of CDCP1 cell surface expression with patient outcome: 50% of patients positive for CDCP1 on the membrane die by 90 mo of the followup; >75% of patients with negative or cytoplasmic CDCP1 are alive at the end of the followup, which is 119 mo. Thus, our data suggest that CDCP1 expression might play a crucial role in

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poor outcome of CC-RCC patients through stimulation of migration and metastasis.

Results

CDCP1 Is a Hypoxia-Inducible and pVHL-Regulated Gene. Using microarray technology, we compared mRNA expression levels between RCC4-VHL (stably transduced by wild-type *VHL*) and RCC4 ($VHL^{-/-}$) cells as well as between RCC4-VHL cells grown in normoxia (21% O_2) and hypoxia (0.5% O_2, 16 h) (12). We found that *CDCP1* mRNA was increased with *VHL* loss (RCC4 vs. RCC-VHL cells), and *CDCP1* expression was induced at least threefold upon hypoxic exposure of RCC4-VHL (Fig. S1). We confirmed that *CDCP1* mRNA was robustly induced by hypoxic exposure of RCC4-VHL cells (Fig. 1*A*). Additionally, we show that CDCP1 protein is induced upon hypoxic exposure (0.5% O_2) in kidney cancer cells with WT *VHL* (ACHN, SN12C, and Caki-1) (Fig. 1*B*), although SN12C and Caki-1 have considerable expression under normoxic conditions. Next, we screened for CDCP1 expression in a variety of CC-RCC cell lines, which are characterized by the *VHL* loss and matching cell lines where *VHL* was reexpressed by stable transfection. Fig. 1*C* shows that CDCP1 expression depends on *VHL* status and, accordingly, correlates with HIF expression.

Knowing that *CDCP1* expression increases upon *VHL* loss, we hypothesized that *CDCP1* would be overexpressed in CC-RCC cell lines. Microarray data available in the oncomine database comparing eight renal tumor cell lines to pooled tumor cell lines of different origin support this hypothesis by showing the highest level of *CDCP1* expression in cell lines derived from kidney cancer (Fig. 1*D*).

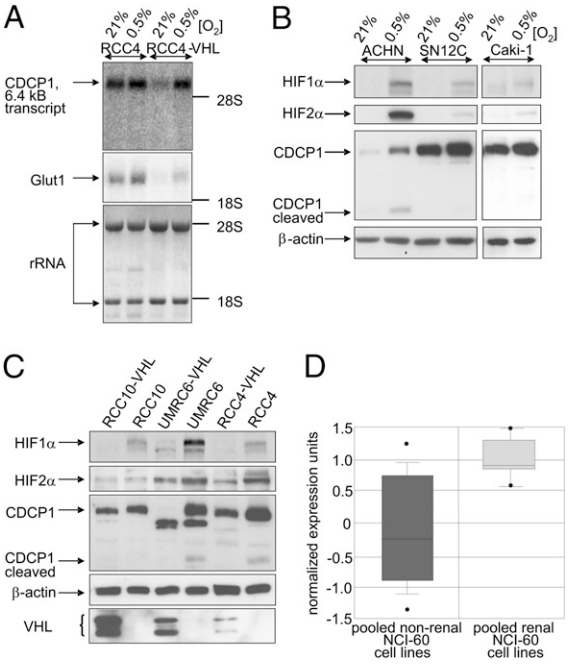

Fig. 1. CDCP1 expression depends on the oxygen level and *VHL* status in kidney cancer cell lines. (*A*) The RNA from RCC4 and RCC4-VHL exposed to 21% O_2 (normoxia) and 0.5% O_2 (hypoxia) for 16 h was extracted, and *CDCP1* expression was determined by Northern blot. Hypoxic induction of *Glut1* was used as a positive control. (*B*) Western blot showing that CDCP1 protein is induced in response to hypoxia (0.5%, 16 h) in ACHN, SN12C, and Caki-1 cells, which are WT for *VHL*. (*C*) CDCP1 expression depends on *VHL* status. Western blot was done on parental vector-transduced RCC4, UMRC6, and RCC10 cell lines, where the *VHL* gene is mutated or lost, or the same cell lines, where the *VHL* expression was reconstituted by transduction with *VHL*-expressing plasmid. (*D*) CDCP1 expression is up-regulated in kidney cancer cell lines. Oncomine expression data on *CDCP1* mRNA level in pooled non-renal cancer cell lines vs. renal cancer cell lines; *t* test: −7.093, *P* value: 9.4e-8.

CDCP1 Expression Is Regulated by HIF-1 and HIF-2. We hypothesized that *CDCP1* gene expression might be induced by HIF, based on the fact that hypoxic exposure results in activation of HIF (2, 13) and *VHL* loss leads to the stabilization of HIF (14, 15). To address this hypothesis, we decided to elucidate whether CDCP1 is a target gene for HIF-1α, HIF-2α, or both. Although HIF-1α and HIF-2α have largely overlapping sets of target genes, growing evidence indicates that these two transcription factors also activate distinct hypoxia-inducible genes (16, 17). We transiently transfected RCC4 cells, which have lost *VHL* expression and have both HIF-1α and HIF-2α stabilized under normoxia, with siRNAs targeting *HIF-1*α, *HIF-2*α, both *HIF-1*α and *HIF-2*α, *ARNT*, or scrambled siRNA. Being a common subunit in HIF-1α and HIF-2α transcriptional complexes, ARNT down-regulation by siRNA was used to inactivate both HIF-1α and HIF-2α (18). Quantitative RT-PCR (QRT-PCR) analysis showed that the appropriate target gene expression was significantly down-regulated in these cells after siRNA transduction (Fig. S2*A*); phosphoglycerate kinase 1 (*PGK1*) gene expression (a known HIF-1 target; refs. 16 and 17) was abrogated when *HIF-1*α, both *HIF-1*α and *HIF-2*α, or *ARNT* were down-regulated (Fig. 2*A*, *i*); *CDCP1* gene expression in *VHL*-deficient cells was significantly inhibited when siRNAs to *HIF-1*α or *HIF-2*α were used, and was inhibited to a larger extent when siRNAs to both *HIF-1*α and *HIF-2*α, or *ARNT* were used, showing that *CDCP1* is a target of both HIF-1 and HIF-2 (Fig. 2*A*, *ii*). These results were confirmed in a similar experiment done with RCC4-VHL cells, which were exposed to 21% O_2 or 0.5% O_2 for 16 h (Fig. S2*B* and Fig. 2*B*). We also compared CDCP1 expression at the protein level in RCC4 cells transfected with the siRNAs targeting *HIF-1*α, *HIF-2*α, both *HIF-1*α and *HIF-2*α, *ARNT*, or nontargeting control siRNA (Fig. 2*C*, *i*) and in 786-0 cells transduced with either a nontargeting siRNA pool or siRNA targeting *HIF-2*α (Fig. 2*C*, *ii*). The 786-0 cells are characterized by loss of *VHL* and *HIF-1*α and express just one HIF isoform (*HIF-2*α). Fig. 2*C* shows a reduction in CDCP1 protein level when *HIF-1*α and *HIF-2*α, or *ARNT* are down-regulated in RCC4 cells, or *HIF-2*α is down-regulated in 786-0 cells. These results strongly suggest that CDCP1 is a target gene of both HIF-1 and HIF-2 in renal cells that have lost *VHL* or renal cells that possess WT *VHL* and are exposed to hypoxic conditions.

CDCP1 Signaling Pathway Is Constitutively Active in CC-RCC and Involves Overexpression of CDCP1, Fyn, and Relocalization of PKCδ to the Membrane Leading to PKCδ Tyr^{311} Phosphorylation. Although CDCP1 is overexpressed in CC-RCC, the important question of whether CDCP1 signaling is active in the CC-RCC context remains. CDCP1 is a phospho-protein that has multiple tyrosine (Tyr) residues in its intracellular domain and is a substrate of Src family kinases (SFKs) (8, 19, 20). Fyn and Yes, members of the Src family, have been reported to be in a complex with CDCP1 and promote its phosphorylation, potentiating its downstream signaling (8, 20). Our immunoprecipitation experiments in RCC4 cells showed that CDCP1 is tyrosine-phosphorylated and is complexed with all three SFKs: Src, Fyn, and Yes in CC-RCC (Fig. 3*A*).

Interestingly, *Fyn* kinase expression at the mRNA level was up-regulated in CC-RCC tumor samples characterized by *VHL* loss when compared with a set of CC-RCC samples with WT *VHL* in microarray analysis conducted by Gordan et al. (21). Additionally, the oncomine database shows that *Fyn* mRNA expression is up-regulated in CC-RCC compared with other types of kidney cancer and normal kidney tissue (Fig. S3). Despite the correlation of *Fyn* mRNA expression with *VHL* status, the causal relationship between *VHL* loss and *Fyn* up-regulation has not been explored. We established a mechanistic link between *VHL* and *Fyn* by examining changes in mRNA (Fig. 3*B*) and protein (Fig. 3*C*) expressions in CC-RCC cell lines, which are characterized by *VHL* loss, and matching cell lines where the *VHL* was reexpressed. Indeed, *VHL* loss leads to up-regulation of Fyn. We did not see regulation of Yes

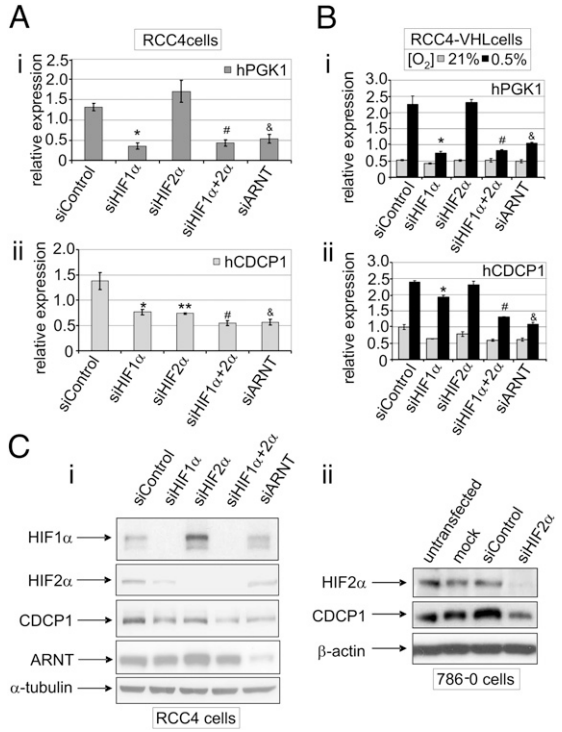

Fig. 2. CDCP1 expression is regulated by HIF-1 and HIF-2. (*A*) RCC4 cells were transiently transfected with the siRNAs targeting *HIF-1α*, *HIF-2α*, *HIF-1α+HIF-2α*, *ARNT*, or nontargeting Control#2 siRNA pool. Forty-eight hours after transfection RNA was extracted and cDNA was analyzed by QRT-PCR. *PGK1* expression was used as a control (*i*). *CDCP1* gene expression was significantly abolished when siRNAs to *HIF-1α*, *HIF-2α*, *HIF-1α+HIF-2α*, or *ARNT* were used, showing that *CDCP1* is a target of both HIF-1 and HIF-2 (*ii*). (*B*) RCC4-VHL cells were transfected with siRNAs to *HIF-1α*, *HIF-2α*, *HIF-1α+HIF-2α*, *ARNT*, or Control#2 nontargeting siRNA pool and 24 h after transfection exposed to 21% O_2 or 0.5% O_2 for 16 h. cDNA was analyzed by QRT-PCR. *PGK1* expression was used as a control (*i*). *CDCP1* mRNA is induced by hypoxia when either HIF-1α or HIF-2α are functional, and the induction is reduced when *ARNT* or both *HIF-1α+HIF-2α* are down-regulated (*ii*). Data in *A* and *B* represent the average of three independent transfections analyzed in triplicate ± SEM. The actual *P* values are listed in *Actual P Values* in the SI. (*C*) RCC4 cells were transiently transfected with the siRNAs targeting *HIF-1α*, *HIF-2α*, *HIF-1α+HIF-2α*, *ARNT*, or nontargeting Control#2 siRNA pool (*i*); 786-0 cells were transfected with the siRNAs targeting *HIF-2α*, nontargeting siRNA pool, or mock-transfected (*ii*). Ninety-six hours after transfection CDCP1 expression was assessed by Western blot.

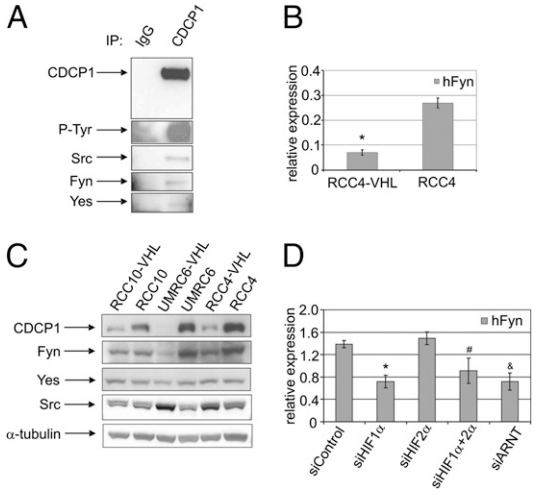

Fig. 3. SFKs coimmunoprecipitate with CDCP1, including Fyn, which expression depends on *VHL* status in CC-RCC. (*A*) Src, Fyn, and Yes are in the complex with CDCP1 in CC-RCC cancer cells, which is heavily tyrosine-phosphorylated. RCC4 lysates were subjected to immunoprecipitation with anti-CDCP1 antibody and analyzed by Western blot with antibodies recognizing CDCP1, Src, Fyn, Yes, and P-Tyrosine. (*B*) QRT-PCR showing that *Fyn* expression goes up upon *VHL* loss in RCC4 cells. Data represent average of three independent experiments analyzed in triplicate ± SEM. $*P < 0.001$. (*C*) Western blot, showing that Fyn expression along with CDCP1 expression is up-regulated in CC-RCC compared with CC-RCC-VHL, but not Src and Yes. (*D*) QRT-PCR showing that *Fyn* is a HIF-1 target gene. The cDNA from the experiment described in Fig. 2 was used. The actual *P* values are listed in *Actual P Values* in the SI.

by *VHL* loss. At the same time, we saw that Src is rather down-regulated upon *VHL* loss. Those observations bring us to a conclusion that *VHL* loss causes the change in the ratio of SFKs in the pool. The investigation of the HIF dependence of *Fyn* expression leads us to conclude that *Fyn* is a HIF-1 target gene, because inhibition of HIF-2α had no effect on *Fyn* expression (Fig. 3*D*). Thus, we have shown that *VHL* loss leads to the up-regulation of two proteins (CDCP1 and Fyn) in the triple complex, which contains CDCP1, Fyn, and PKCδ.

Several groups have demonstrated the key role of CDCP1 in transducing signal to PKCδ, supporting its phosphorylation on Tyr^{311} by SFKs (8, 19). We infected 786-0 cells with lentiviruses expressing shRNAs targeting *CDCP1* or green fluorescent protein (GFP, control) and acquired stable cell lines, which we analyzed by Western blot for PKCδ phosphorylation (Fig. 4 *A* and *B*). Tyr^{311} phosphorylation of PKCδ gets ablated when CDCP1 expression is down-regulated by shRNA in the 786-0 cell line (Fig. 4*B*), showing that the same signaling pathway as reported for lung adenocarcinoma and scirrhous adenocarcinoma is active in kidney cancer. The specificity of CDCP1 shRNA action on PKCδ phosphorylation was confirmed by using independent siRNA pool targeting a different region of *CDCP1* mRNA (Fig. S4). FAK is another molecule known to be downstream of SFKs and has been reported to associate with Src and Fyn (22, 23). Importantly, FAK phosphorylation at multiple sites did not change in response to CDCP1 down-regulation (Fig. 4*C*). In addition, the phosphorylation of another SFK substrate Crk-associated substrate (CAS) (24, 25), at Tyr^{165} and Tyr^{249}, was found to be CDCP1-independent in RCC4 cells (Fig. 4*D*). This result reinforces the specificity in CDCP1 signal transduction from SFKs to PKCδ.

An interesting twist of PKCδ signaling, particularly in kidney cancer, comes from the link of PKCδ with pVHL. The pVHL protein was reported to directly interact with PKCδ, leading to its retention in cytoplasm and blocking the association between PKCδ and the IGF-I receptor for downstream signaling (26, 27). Our immunofluorescence study of PKCδ and CDCP1 localization in RCC4 and RCC4-VHL cells shows that CDCP1 is localized on the membrane and in the cytoplasm of RCC4 cells, and the staining intensity in RCC4-VHL cells is much lower compared with RCC4 (Fig. S5). PKCδ localizes mainly in the nucleus in both cell lines. However, we detected PKCδ on the membrane, colocalizing with CDCP1, in RCC4 cells, but not in RCC4-VHL cells. Thus, *VHL* loss might be of particular importance for stimulation of PKCδ signaling leading to PKCδ translocation to the membrane as well as CDCP1 and Fyn up-regulation and subsequent PKCδ phosphorylation at Tyr^{311}.

CDCP1 Signaling Pathway Promotes Migration of CC-RCC. To address the mechanism of CDCP1 action in CC-RCC, we investigated a number of different biological endpoints. We sought to determine whether CDCP1 might be involved in apoptosis, cell adhesion, migration, epithelial–mesenchymal transition (EMT), and cell growth based on the known properties of metastatic cells (8–10, 20, 28, 29).

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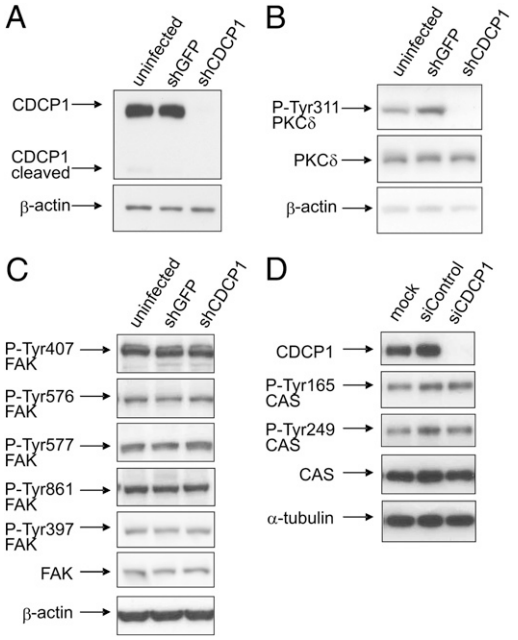

Fig. 4. CDCP1 signaling pathway is active in CC-RCC. (*A*) Western blot showing the CDCP1 down-regulation in 786-0 cells after shCDCP1 infection and selection on puromycin. (*B*) Western blot showing that CDCP1 down-regulation in 786-0 cells by stable infection with shRNA leads to ablation of the phosphorylation of PKCδ at Tyr^{311}. (*C*) Western blot showing that FAK phosphorylation at multiple Tyr phosphorylation sites is independent on CDCP1 expression in 786-0 cells. (*D*) Western blot showing that CAS phosphorylation at two Tyr phosphorylation sites is independent on CDCP1 expression in RCC4. Cells were transfected by using Dharmafect1 and siRNA targeting *CDCP1*, or nontargeting Control#1 siRNA. Mock refers to Dharmafect1-only transfection. Western blot was performed 48 h after transfection. Protein loading was normalized to β-actin or α-tubulin expression as indicated.

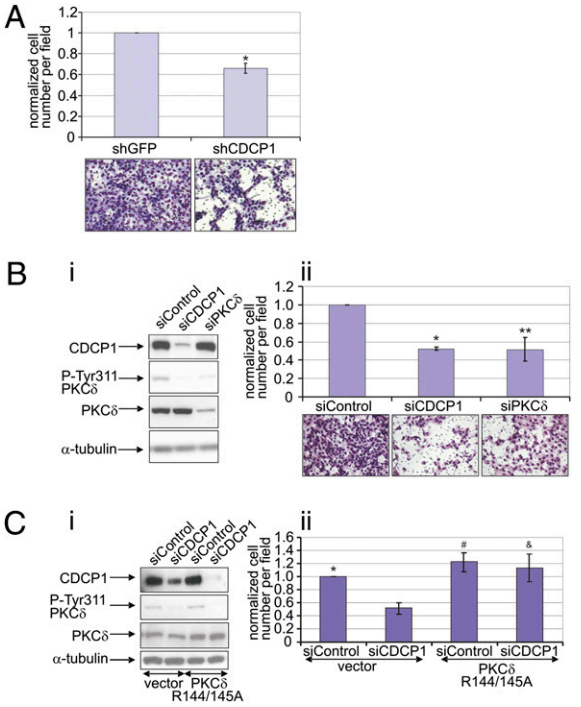

Fig. 5. CDCP1 protein induces PKCδ-dependent migration of CC-RCC. (*A*) Stable CDCP1 down-regulation with shRNA in 786-0 cells reduces the amount of migrated cells through the transwells. $*P < 0.001$. (*B*) PKCδ knockdown phenocopies the effect of CDCP1 knockdown on migration. The 786-0 cells were transfected by using Dharmafect1 and siRNAs targeting *CDCP1*, *PKCδ*, or nontargeting Control siRNA#1. The knockdowns were confirmed by Western blot (*i*), and the migration assay was performed 48 h after transfection (*ii*). (*C*) CC-RCC migration depends on CDCP1 signaling to PKCδ. The 786-0 cells were infected with LZRS-myc-PKCδR144/145A and LZRS-linker (vector control) lentiviruses, and stable cell lines were selected on puromycin. Both cell lines were transfected with siRNAs as in *B*, followed by Western blot (*i*) and migration through the transwells (*ii*). Overexpression of R144/145A constitutively active mutant of PKCδ rescued the reduction in migration caused by CDCP1 knockdown. The results represent the normalized number of migrated cells per field in minimum three independent experiments ± SEM and include representative photos of the membranes with the migrated cells. The number per field of migrated 786-0 cells expressing shGFP (*A*) or transfected with siControl (*B* and *C*) was designated as 1. The actual *P* values are listed in *Actual P Values* in the SI.

Cell migration is important for the cancer cells to spread beyond the primary tumor site because cells need to intravasate into blood vessels, then extravasate from them and colonize a distant organ. We assessed the migratory capacity of a number of CC-RCC cell lines in transwells, including 786-0, RCC4, RCC10, and UMRC6, and 786-0 cells proved to be the most suitable for this in vitro assay (Fig. S6). We compared the amount of migrated cells through transwells coated with fibronectin by using shGFP-infected and shCDCP1-infected 786-0 cells. We observed a 40% reduction in migratory ability of 786-0 cells when CDCP1 was down-regulated (Fig. 5*A*).

At the same time, our experiments showed no involvement of CDCP1 into apoptosis in suspension (anoikis) [no difference in soft agar growth (Fig. S7*A*); colony formation on culture dishes (Fig. S7*B*); Caspase 3 cleavage after incubation on low-adhesion plates (Fig. S7*C*)], EMT [no changes in classic EMT markers such as *N-cadherin*, *Lysyl oxidase (LOX)*, and *Snail1* as juged by QRT-PCR (Fig. S7*D*); and Western blot for *N*-cadherin (Fig. S7*E*)], cell growth (Fig. S7*F*), and cell adhesion [no difference in adhesion to fibronectin, fibrinogen, collagen I and IV, except increased adhesion to laminin when CDCP1 was down-regulated in 786-0 cells (Fig. S7*G*)].

CDCP1 Promotes CC-RCC Migration Through PKCδ Activation. Our data indicate that CDCP1 is absolutely necessary for signal transduction from SFKs to PKCδ Tyr^{311} and not to other SFK targets. Furthermore, PKCδ was reported to induce migration of RCC (30). To address the question whether CDCP1-mediated PKCδ signaling promotes migration of CC-RCC, we used siRNAs targeting CDCP1, PKCδ, or nontargeting control siRNA. The knockdowns were confirmed by immunoblotting (Fig. 5*B*, *i*), and migration assays were performed 48 h after transfection (Fig. 5*B*, *ii*). The 786-0 cells when transiently transfected with siRNA pool targeting CDCP1 exhibited a 50% reduction in migration (Fig. 5*B*). The results clearly show that PKCδ down-regulation by RNA interference leads to a 50% reduction in CC-RCC cell migration, which phenocopies the effect of CDCP1 siRNA. To further confirm the role of PKCδ in signal transduction from CDCP1 leading to enhanced migration, we rescued the reduction in migration caused by CDCP1 knockdown by overexpressing constitutively active mutant of PKCδ (R144/145A) (31) (Fig. 5*C*). Those results emphasize the importance of CDCP1 in migration and provide an in vitro mechanism of CDCP1 action in CC-RCC through PKCδ activation.

CDCP1 Expression on the Cell Surface of Tumor Cells Correlates with the Poor Survival of Patients Diagnosed with Kidney Cancer. Four studies assessed the possibility of using the level of CDCP1 expression as a prognostic factor for survival of lung adenocarcinoma, conventional RCC, pancreatic cancer patients, and endometrioid adenocarcinoma (29, 32–34). All of them indicated that elevated CDCP1 expression correlates with low overall survival.

We used an antibody that recognizes the extracellular domain of CDCP1 (R&D Systems) and stained a kidney cancer tissue microarray (TMA), which contains 50 kidney cancer tissue cores, most of the primary tumor origin. The specificity of the antibody was confirmed by immunoblotting of 786-0 cells transduced with shRNAs to CDCP1 or GFP (Fig. S8). Sixty-seven percent of samples had membrane and/or cytoplasmic staining of CDCP1, and 29% of samples had membrane staining only. Representative pictures of tumors scored as negative (*i*), positive cytoplasmic (*ii*), and positive membrane (*iii*) are shown in Fig. 6*A*. This TMA was accompanied with patient survival data, and we conducted Kaplan–Meier survival analysis of kidney cancer patients based on CDCP1 staining. In this analysis, group one represents patients totally negative for CDCP1 staining pooled with the patients with CDCP1 staining in the cytoplasm, whereas group two consists of patients with CDCP1 staining on the membrane. By this analysis, the survival curves are statistically significant with a *P* value of 0.0163. The median survival for patients with CDCP1 staining on the membrane is 90 mo (Fig. 6*B*).

Discussion

Our data show that CDCP1 is a downstream target of HIFs and plays a critical role in kidney cancer migration (Fig. 7). Importantly, *VHL* loss, which occurs in 80% of CC-RCC kidney cancer cases, results in HIF-dependent CDCP1-mediated signaling to PKCδ, leading to PKCδ translocation to the membrane and subsequent PKCδ phosphorylation at Tyr^{311}, which manifests in increased migration. We assume that *CDCP1* gene regulation can be induced by HIF via (*i*) direct binding and activation of the *CDCP1* gene promoter, or (*ii*) indirect activation of *CDCP1* transcription through a HIF-dependent pathway. The analysis of the *CDCP1* promoter region and the first intron showed that there are multiple HIF consensus binding sites. Further studies are needed to define the putative HIF binding site within CDCP1 promoter.

Although there are multiple tyrosine phosphorylation sites on PKCδ, Tyr^{311} located between the regulatory and catalytic domains has been linked to increased kinase activity in cells treated with H_2O_2 (35, 36). Experiments from a number of laboratories now support the idea that PKCδ is a positive regulator of breast cancer growth and metastasis (37–40) and promotes cell survival

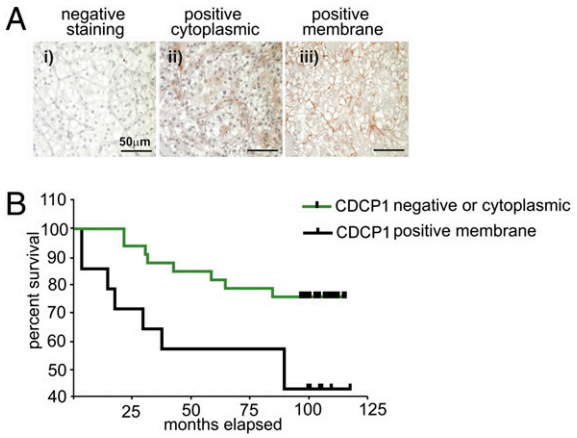

Fig. 6. CDCP1 expression is up-regulated in a subset of kidney cancer patients. (*A*) Tissue microarray stained for CDCP1 identifies two localizations of CDCP1: cytoplasmic and membrane. Representative pictures of tumors scored negative (*i*), positive cytoplasmic (*ii*), and positive membrane (*iii*) are shown. (*B*) Kaplan–Meier survival analysis of kidney cancer patients divided into two groups accordingly to CDCP1 staining. The first group represents patients totally negative for CDCP1 staining pooled with the patients with CDCP1 staining in the cytoplasm; the second group consists of patients with CDCP1 staining on the membrane. The survival curves are significantly different with $P = 0.0163$.

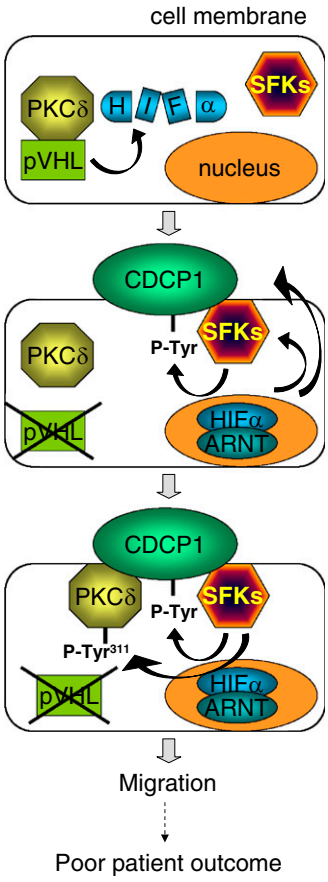

Fig. 7. Model of molecular events in CC-RCC leading to enhanced migration. In the presence of pVHL, HIF gets degraded by the proteasome, PKCδ is retained in the cytoplasm, and CDCP1 and Fyn expressions are low. *VHL* loss causes HIF stabilization, translocation to the nucleus, dimerization with ARNT, and activation of transcription. CDCP1 and Fyn expression up-regulates, CDCP1 and SFKs form complex on the membrane, and CDCP1 gets phosphorylated. PKCδ relocalizes to the membrane and forms a complex with CDCP1 and SFKs, gets phosphorylated by Tyr^{311} and activated, leading to increased cell migration.

and chemotherapeutic resistance of nonsmall cell lung cancer cells (41). PKCδ has also been reported to induce migration of RCC, prostate cancer, and pancreatic cancer cells through poorly elucidated mechanism (29, 30). Signal transduction from CDCP1 to PKCδ Tyr^{311} was described in several cancer cell types (8, 19, 29), and was linked to anoikis in A549 (8) and to cell migration in BxPC3 and Capan1 cells (29), but the direct evidence of the PKCδ involvement in those processes has not been demonstrated. Our study provides a direct link between CDCP1 and migration through activation of PKCδ in CC-RCC, showing that the reduction in migration in the absence of CDCP1 can be rescued by overexpression of constitutively active PKCδ mutant. At the same time, we did not find any role of CDCP1 in protecting CC-RCC from anoikis, which can be explained by multiple redundant apoptosis resistance mechanisms in play in CC-RCC, activated as a result of HIF stabilization upon *VHL* loss.

In this study, we have demonstrated the possibility of using cell surface CDCP1 expression as a prognostic factor for survival of kidney cancer patients. Interestingly, CDCP1 localization was heterogeneous in the TMA, CDCP1 was localized in the cytoplasm, or cell membrane, or both cytoplasm and cell membrane. Surprisingly, 67% of specimens were CDCP1-positive, but just 29% had cell surface expression of CDCP1. However, several kidney cancer cell lines analyzed by fluorescence-activated cell

sorting (786–0, RCC4, RCC10, UMRC6, ACHN; Fig. S9) have revealed the presence of CDCP1 on the cell surface. These results suggest an additional mechanism for CDCP1 regulation in vivo by changing its localization.

The data presented in this article suggest that blocking CDCP1 might be beneficial to prevent migration and, subsequently, metastatic spread. Moreover, CDCP1 expression on the cell surface of kidney cancer cells represents an ideal target for antibody- and small molecule-based therapeutics. Indeed, the antibody mAb41-2, which targets CDCP1, was reported to have antimetastatic activity toward CDCP1 overexpressing HeLa cells and PC3 cells (10). To date, our knowledge of CDCP1 function in normal tissues is limited. Thus, generation of CDCP1 knockout mice would be important to shed light on CDCP1 function under physiologic conditions.

Methods

Hypoxic Treatment. Cells were plated in glass dishes 12–24 h before hypoxic treatment and subjected to hypoxia in chambers [Invivo$_2$ 400 Hypoxic workstation (0.5%), Biotrace]. For Western blot analysis, cells were lysed inside the chamber.

Cell Transfections with siRNAs. Cells were transfected with nontargeting siControl#1, #2, or siControl pool, siHIF-1α, siHIF-2α, siARNT, siCDCP1, and siPKCδ smart pools (Dharmacon) by using Dharmafect1 reagent (Dharmacon) according to manufacturer's instructions.

Migration Experiments. All migration experiments were done on cells dislodged with cell dissociation buffer (Gibco), which were allowed to migrate through fibronectin-coated transwells (Costar) for 24 h.

shRNA Expression Constructs, Lentivirus Packaging, and Infection of Target Cells. Lentiviral plasmids pLKO.1shCDCP1 (Open Biosystems) and pLKO.1shGFP, and retroviral plasmids LZRS-myc-PKCδR144/145A (31) and LZRS-linker were used for viral production as described in ref. 42.

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