SCP Phosphatases Suppress Renal Cell Carcinoma by Stabilizing PML and Inhibiting mTOR/HIF Signaling

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Abstract

The tumor-suppressor protein promyelocytic leukemia (PML) is aberrantly degraded in multiple types of human cancers through mechanisms that are incompletely understood. Here, we show that the phosphatase SCP1 and its isoforms SCP2/3 dephosphorylate PML at S518, thereby blocking PML ubiquitination and degradation mediated by the prolyl isomerase Pin1 and the ubiquitin ligase KLHL20. Clinically, SCP1 and SCP3 are downregulated in clear cell renal cell carcinoma (ccRCC) and these events correlated with PMLS518 phosphorylation, PML turnover, and high-grade tumors. Restoring SCP1-mediated PML stabilization not only inhibited malignant features of ccRCC, including proliferation, migration, invasion, tumor growth, and tumor angiogenesis, but also suppressed the mTOR–HIF pathway. Furthermore, blocking PML degradation in ccRCC by SCP1 overexpression or Pin1 inhibition enhanced the tumor-suppressive effects of the mTOR inhibitor temsirolimus. Taken together, our results define a novel pathway of PML degradation in ccRCC that involves SCP downregulation, revealing contributions of this pathway to ccRCC progression and offering a mechanistic rationale for combination therapies that jointly target PML degradation and mTOR inhibition for ccRCC treatment. Cancer Res; 74(23): 6935–6. ©2014 AACR.

Introduction

The promyelocytic leukemia (PML) was identified in the patients with acute promyelocytic leukemia (1, 2) and elicits multiple tumor-suppressive functions, such as anti-proliferation, apoptosis, senescence, anti-migration, anti-invasion, and anti-angiogenesis (3–6). These PML functions are attributed to the regulation of a number of tumor promoting/suppressing pathways, including p53 (7, 8), PTEN/Akt (9, 10), and mTOR (3). PML knockout mice display an increased susceptibility to tumor formation and/or progression (11–14). Clinically, PML protein, but not mRNA, is frequently downregulated in a broad spectrum of human cancers (15). Evidence has emerged that the ubiquitin–proteasome pathway is a key mechanism for PML deregulation in tumors (16). For instance, a CK2/PIAS1-mediated PML ubiquitination pathway is hyperactivated in small cell lung cancer (12, 17), and PML expression inversely correlates with its ubiquitin ligase E6AP in Burkitt lymphoma (18). Recently, we identified a PML destruction pathway mediated by a Cullin 3 (Cul3) ubiquitin ligase complex with KLHL20 as the substrate-binding subunit (19). This pathway is hyperactivated in prostate cancers and contributes to tumor progression partly through a feedback potentiation of the hypoxia-inducible factor (HIF) pathway. Recruitment of PML to KLHL20 requires a two-step posttranslational event, that is, phosphorylation followed by Pin1-dependent prolyl-isomerization on the S518-P519 residues. Given the dynamic regulation of protein phosphorylation state, the PMLS518-specific phosphatase may govern PML stability in certain type of tumors. However, the identity of this phosphatase remains elusive.

Protein serine/threonine phosphatases can be divided into PPM (PP2C-related), PPP (PP1/PP2A/PP2B-related), and FCP/SCP families. The FCP/SCP family consists of TFIIF-associated C-terminal domain (CTD) phosphatase (FCP1) and three small CTD phosphatases (SCP1, SCP2, and SCP3) and relies on aspartic acid of the sequence motif DxD/T/V for catalysis (20–23). SCPs negatively regulate RNA polymerase II by dephosphorylating its CTD, which contributes to suppression of neuronal genes in non-neuronal cells (24) and attenuation of neuronal genes in non-neuronal cells (24) and attenuation of androgen receptor transcriptional activity (25). In addition, SCPs dephosphorylate the linker region of Smad1/2/3, thereby potentiating TGFβ and BMP pathways (26–28). Although SCPs are widely expressed in normal human tissues, deletion and missense/nonsense mutations of SCP3 (also known as HYA22)
gene are observed in several epithelial cancers (29). Furthermore, SCP3 overexpression in carcinoma cell lines suppresses proliferation and tumor formation.

Clear cell renal cell carcinoma (ccRCC) is the most frequent and malignant type of renal cancer (30, 31). ccRCC can occur sporadically (>96%) or hereditarily (<4%). Almost all hereditary ccRCC and most sporadic ccRCC are characterized by loss/inactivation of the von Hippel-Lindau (VHL) tumor-suppressor gene (32). VHL is a substrate-binding subunit of Cullin 2 ubiquitin ligase that targets HIF1/2α for degradation. VHL loss causes constitutive expression of HIF1/2α, which, in turn, activates the expression of genes involving in metabolic changes, angiogenesis, and other signaling events critical for the growth of renal tumors (33). These important roles of the VHL–HIF pathway in ccRCC pathogenesis laid the groundwork for the utilization of several antiangiogenic agents for ccRCC treatment (34). However, primary and acquired resistance to the antiangiogenic therapies occurs inevitably, indicating the need for improved management of this disease.

In this study, we report that SCPs dephosphorylate PML to block Pin1/KLHL20–mediated PML ubiquitination and degradation. The SCP–PML axis is downregulated in ccRCC, which contributes, in part, to HIF induction and ccRCC malignancy. We provide evidence indicating that the restoration of PML stability may be combined with mTOR inhibitor for treating ccRCC.

Materials and Methods

Cell culture and transfection

HeLa, 293T, and 293FT cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS). HK-2 cells were cultured in DMEM-F12 medium supplemented with 10% FCS. A-498 cells were cultured in Minimal Essential Medium containing 10% FCS and 1 mmol/L sodium pyruvate. 786-O cells were cultured in RPMI-1640 medium supplemented with 10% FCS, 10 mmol/L HEPES, and 1 mmol/L sodium pyruvate. PC-3 cells were cultured in DMEM high-glucose medium supplemented with 10% FCS, nonessential amino acid, 1 mmol/L sodium pyruvate, and 20 mmol/L L-glutamine. All cell lines were obtained from the American Type Culture Collection or Biosource Collection (Taiwan). For subcutaneous xenografts, mice were transplanted with 5.5×10⁶ 786-O cell derivatives mixed with PBS and Matrigel. For conditional SCP1-WT or SCP1-Dm expression, Flag-SCP1 and Flag-SCP1 Dm were subcloned to pAS4.1w.Ppuro-aOn obtained from National RNAi Core Facility, Taiwan.

Proliferation, migration, and invasion assays

Proliferation was assayed using Cell Proliferation ELISA BrdU kit (Roche). Transwell migration assay was performed as described previously (36). To distinguish migration effect from proliferation effect, the same number of cells was seeded on a regular culture plate. At the end of incubation, cells that had migrated onto the lower membrane surface of the Transwell plate were fixed by 4% formaldehyde, stained with DAPI, counted, and normalized with the number of cells appearing in the regular plate. For invasion assays, the Transwell membrane was coated with Matrigel.

Tissue specimens and IHC analysis

Tissue microarrays were obtained from Biomax Inc. and Pantomics Inc. Studies involving these tissues were approved by Institutional Review Boards at Academia Sinica (Taipei, Taiwan). For immunohistochemical (IHC) staining, endogenous peroxidase activity was blocked by incubation in 3% H2O2 at room temperature for 20 minutes. Antigen retrieval was performed by heat denaturation of paraffin sections with 10 mmol/L sodium citrate buffer (pH 6.0) for 30 minutes and nonspecific binding was blocked by PBS containing 10% goat serum or Avidin/Biotin Blocking kit (Invitrogen). The slides were incubated with antibody to SCP1 (1:150), SCP3 (1:100), PML (1:60), pS518PML (1:300), or KLHL20 (1:400) at 4°C for overnight. The bound antibody was detected by the Super-Picture Polymer Detection Kit (Invitrogen). The counterstaining was performed with hematoxylin. Protein expression was scored semiquantitatively as high or low expression based on staining intensity and percentage of cells staining positive.

For IHC analysis on xenograft tumors, paraffin-embedded tumors were cut to 5-μm sections, deparaffinized, hydrated, and rinsed with PBS. IHC staining was performed as described above with antibody to CD31 (1:100) or Ki67 (1:100). The positive signal was quantified by the ImageJ software.

Cell viability (MTS) assay

Cells were seeded at a density of 1×10⁵ cells in 96-well plates and treated with various inhibitors for 48 hours. CellTiter 96 AQueous One Solution Reagent (Promega) was added and incubated for 50 minutes at 37°C, followed by absorbance measurement at 490 nm.

Xenograft model

Six- to eight-week-old female BALB/c nude mice were purchased from National Laboratory Animal Center (Taipei, Taiwan). For subcutaneous xenografts, mice were transplanted with 5.5×10⁶ 786-O cell derivatives mixed with PBS and Matrigel. For conditional SCP1-WT or SCP1-Dm expression, mice at 2 weeks after transplantation were randomly grouped.
and given drinking water (containing 50 g sucrose/L) with or without 2 mg/mL doxycycline. Drinking water was exchanged every 3 days. For inhibitor treatment, once the transplanted tumors reached approximately 50 mm³, mice were selected and randomly divided into four groups for intraperitoneal injection daily with DMSO, temsirolimus (0.1 mg/kg), Juglone (1 mg/kg), or a combination of temsirolimus and Juglone in approximately 200 µL PBS with 0.5% DMSO. Body weight and tumor size of the drug-injected mice were measured every 2 or 3 days. All mice experiments were conducted with the approval from the Experimental Animal Committee, Academia Sinica. For additional details, refer to Supplementary Materials and Methods.

Results

SCP1/2/3 dephosphorylate PML at S518

Consistent with a crucial role of S518 phosphorylation in PML degradation (19), PMLS518A mutant was more resistant to proteasome inhibitor than wild-type PML (Supplementary Fig. S1A). To identify phosphatases targeting this residue, we undertook an expression screening strategy by transfecting cells with a panel of Ser/Thr phosphatases together with PML-I. Immunoblot with a pS518-specific antibody (19) revealed that SCP1, SCP2, and SCP3 significantly decreased PML-I S518 phosphorylation (Supplementary Fig. S1B and Fig. 1A). However, the catalytically inactive mutants (Dm mutants) of SCP1, SCP2, and SCP3 did not alter PML S518 phosphorylation (Fig. 1A). Because SCP1 is the prototype of this family of phosphatases, we mainly focused on SCP1 in the following studies. SCP1 also decreased S518 phosphorylation on endogenous PML (Fig. 1B). Furthermore, in vitro analysis revealed that recombinant GST-SCP1 readily dephosphorylated purified PML at S518, whereas SCP1 Dm did not elicit this effect (Fig. 1C). This result demonstrated a direct action of SCP1 on PML. Importantly, simultaneous depletion of SCP1, SCP2, and SCP3 elevated S518 phosphorylation on endogenous PML (Fig. 1D, top), even though the total level of PML was decreased (due to PML destabilization, see below; Fig. 1D, middle). Our data support a physiologic role of these SCPs in dephosphorylating PML at S518.

SCP1 interacts with PML

A bona fide phosphatase should interact with its substrate. Accordingly, immunoprecipitation analysis demonstrated the interaction of Flag-SCP1 with both PML-I and PML-IV (Fig. 1E). With a SCP1-specific antibody (Supplementary Fig. S2), we detected the interaction between endogenous PML and endogenous SCP1 by reciprocal immunoprecipitation (Fig. 1F). Furthermore, in vitro pull-down analysis showed that recombinant GST-SCP1 and GST-SCP1 Dm were able to bind purified PML-I (Fig. 1G). These findings indicate a direct and physical association of PML with SCP1, further substantiating SCP1 as the PMLS518 phosphatase.

SCP1 inhibits Pin1/KLHL20–mediated PML ubiquitination and degradation through PML S518

Our previous study indicates that PMLS518 phosphorylation is required for interaction with Pin1, which catalyzes PML prolyl-isomerization to facilitate PML recruitment to the KLHL20-containing ubiquitin ligase (19). Consistent with the role of SCP1 in PMLS518 dephosphorylation, overexpression of SCP1, but not SCP1 Dm, compromised PML interaction with Pin1 and KLHL20 (Fig. 2A and B) and suppressed PML-I ubiquitination mediated by KLHL20-based ubiquitin ligase (Fig. 2C). In addition, preincubation of purified PML-I with GST-SCP1 for dephosphorylation suppressed its ubiquitination in vitro by KLHL20-based E3 ligase (Fig. 2D). These data collectively indicate that SCP1-mediated PML dephosphorylation disrupts PML interaction with Pin1 and KLHL20, thereby attenuating KLHL20-mediated PML polyubiquitination. We next evaluated the role of SCP1 in PML stability. The turnover of PML was decreased by overexpression of SCP1, but not SCP1 Dm (Fig. 2E). Furthermore, the steady-state level of endogenous PML was elevated by SCP1 overexpression (Fig. 2B, lysate) and was decreased by SCP1/2/3 knockdown (Fig. 1D, middle). Consistent with our previous finding that KLHL20-dependent PML ubiquitination is responsible for hypoxia-induced PML destruction (19), overexpression of SCP1, but not SCP1 Dm, completely reversed hypoxia-induced PML destruction (Fig. 2F). Thus, SCP-mediated PML dephosphorylation stabilizes PML in both normoxia and hypoxic conditions.

Previous study revealed that Pin1 interacts with PML through multiple pS/T-P motifs (37), we thus evaluated whether the effect of SCP1 on PML is mediated specifically by S518 dephosphorylation. Importantly, the ubiquitination and abundance of PMLS518A mutant were not affected by SCP1 (Fig. 2G and H). Accordingly, SCP1 failed to regulate the turnover of PMLS518A (Fig. 2E), which had an elevated stability due to resistant to Pin1/KLHL20-mediated degradation (19). Our data support a pivotal and specific role of S518 dephosphorylation in SCP1-mediated PML stabilization.

Deregulation of SCP–PML axis in ccRCC correlates with disease progression

Next, we investigated the relevance of SCP-dependent PML regulation to human cancers. IHC analysis on human tissue microarray derived from multiple types of patients with cancer revealed a possible downregulation of SCP1 in ccRCC (Supplementary Table S1). Furthermore, SCP3 downregulation was reported in certain RCC cell lines (29). We therefore accessed the expression of SCP1, SCP3, PML, and pS518PML in 36 ccRCC tissues and their matched adjacent normal tissues. Although SCP1, SCP3, and PML displayed high expression in normal tissues, they were all downregulated in ccRCCs (Fig. 3A and B and Supplementary Fig. S3). Conversely, PMLS518 phosphorylation was increased in ccRCCs. Furthermore, SCP1 and SCP3 expressions correlated positively with PML expression and negatively with PMLS518 phosphorylation in this cohort of normal and tumor tissues (Fig. 3C and D), supporting the existence of SCP–PML axis. Consistent with the activation of HIF1 in ccRCC, high expression of KLHL20, a HIF1 effector (19), was observed in the majority (88.9%) of ccRCC tissues (Fig. 3A and B). However, the expression of KLHL20 did not show an inverse correlation with that of PML and pS518PML (Fig. 3C and D). These findings indicate that SCP1 and SCP3 play more important roles than KLHL20 in determining the expression of SCP–PML Axis Suppresses ccRCC and mTOR/HIF Signaling
PML in ccRCC and therefore underscore the clinical relevance of SCP–PML axis to the pathogenesis of ccRCC. To determine the correlation of SCP1, SCP3, PML, and pS518PML expression with tumor stages, we performed IHC analysis on another cohort of ccRCC patients, which contains certain patients in advanced disease stages. By combining data from both cohorts, we found that downregulations of SCP1, SCP3, and PML and upregulation of pS518PML were more frequently observed in stage III/V patients than stage I/II patients (Fig. 3E). Our data indicate that downregulations of SCP1 and SCP3 are associated with PMLS518 phosphorylation, PML downregulation, and high-grade ccRCCs.

**SCP1 stabilizes PML in ccRCC to suppress ccRCC malignant characters**

Because the clinical data support a role of SCP–PML axis in ccRCC, we evaluated its impact on tumor-related features by using ccRCC cell lines. Overexpression of SCP1, but not SCP1 Dm, in ccRCC cell lines 786-O and A-498 reduced PMLS518 phosphorylation and increased endogenous PML expression (Fig. 4A). Similar results were obtained with SCP2 and SCP3 (Fig. 4B). Furthermore, proteasome inhibitor induced a greater PML upregulation in control or SCP1 Dm-expressing cells than in SCP1-expressing cells, supporting a role of SCP1 in blocking PML proteasomal degradation (Fig. 4C). Conversely, knockdown of SCP1/2/3 in HK-2 normal renal epithelial cells increased PMLS518 phosphorylation and reduced PML expression (Fig. 4D). Thus, the SCP–PML axis is manifested in ccRCC. To investigate the effect of SCP–PML axis on ccRCC malignancy, we established 786-O and A-498 stable lines carrying doxycycline-inducible SCP1 or SCP1 Dm. Then, PML shRNA or control shRNA was introduced to SCP1-inducible cells (Fig. 4E). These cells were used for assaying several malignant traits of ccRCC. Although...
Figure 2. SCP1 inhibits KLHL20- and Pin1-mediated PML ubiquitination and degradation through PML-S518. A, immunoprecipitation analysis of the interaction between PML-I and Pin1 in 293T cells transfected with indicated constructs. B, immunoprecipitation analysis of the interaction between KLHL20 and endogenous PML in HeLa cells transfected with indicated constructs. C and G, His-PML-I (C) or His-PML-I-S518A (G) purified under denaturing conditions by Ni-NTA agarose from 293T cells transfected with indicated constructs was analyzed by immunoblot for its ubiquitination. D, baculovirally purified PML-I was preincubated with GST or GST-SCP1 protein in the dephosphorylation reaction followed by in vitro ubiquitination assay and immunoblot analysis. E, immunoblot analysis of PML-I or PML-I-S518A stability in HeLa cells transfected with indicated constructs and treated with cycloheximide for indicated time points. The expression of SCP1 or its mutant was shown on the upper right panel. F, immunoblot analysis of PML expression in HeLa cells transfected with indicated constructs and cultured in normoxia or hypoxia. H, immunoblot analysis of PML-I or PML-I-S518A expression in 293T cells transfected with indicated constructs.
expression of SCP1 Dm in 786-O and A-498 cells did not elicit any significant effect, SCP1 expression suppressed proliferation, migration, and invasion (Fig. 4F and G). Importantly, these tumor-promoting functions were all abrogated by PML knockdown. To test the effects of SCP–PML axis on tumor growth in vivo and tumor angiogenesis, we subcutaneously injected 786-O derivatives into nude mice. Induction of SCP1 expression by doxycycline led to a significant suppression of tumor growth in vivo and this effect was blocked by PML knockdown (Fig. 5A). Induction of the expression of SCP1 Dm, however, did not affect tumor growth (Fig. 5B). IHC staining on tumor tissues revealed that SCP1 expression strongly suppressed tumor angiogenesis, which was again reversed by PML knockdown (Fig. 5C). These findings indicate a significant role of SCP1-induced PML stabilization in suppressing ccRCC malignant characters.

Figure 3. SCP1 and SCP3 downregulations in ccRCC correlate with PML phosphorylation, PML downregulation, and disease progression. A, IHC analysis of indicated protein expression in a representing ccRCC specimen and its adjacent normal specimen. Bar, 50 μm. B, summary of the SCP1, SCP3, PML, pS518PML, and KLHL20 expression profiles in 36 ccRCC specimens and their adjacent normal specimens. C and D, correlations of PML expression (C) and pS518PML expression (D) with SCP1 and SCP3 expression, but not with KLHL20 expression in all specimens. E, correlation of PML, pS518PML, SCP1, and SCP3 expression with tumor stage.
SCP–PML axis downregulates HIF signaling in ccRCC

The pathology of ccRCC is tightly associated with aberrant elevation of HIF signaling resulted from VHL deficiency (32). PML, however, negatively regulates HIF expression through mTOR repression (3, 19). Thus, inhibition of the SCP–PML axis could serve as an alternative route to enhance HIF expression and signaling in ccRCC. In line with this notion, overexpression of SCP1, but not SCP1 Dm, in 786-O and A-498 cells attenuated mTOR activity (measured by phosphorylation of S6 kinase) and HIF2α expression (786-O and A-498 cells do not express HIF1α). Importantly, these effects were reversed by PML knockdown (Fig. 6A and B). Consequently, the levels of HIF targets VEGF and GLUT1 were decreased by overexpression of SCP1 but not SCP1 Dm (Fig. 6C). Conversely, depletion of SCP1/2/3 in HK-2 increased mTOR activity and HIF1α/HIF2α expression (Fig. 6D). These data support a cross-talk of SCP–PML axis with mTOR/HIF pathway in ccRCC.
Combined targeting of mTOR pathway and PML degradation pathway provides better tumor-suppressive effects

The uncovering of a cross-talk between SCP–PML axis and HIF signaling, together with the ability of PML to regulate other tumor-promoting or -suppressing factors, suggest a beneficial effect for targeting PML degradation pathway on ccRCC therapy. In line with this notion, SCP1 overexpression increased the sensitivity of 786-O cells to mTOR inhibitor temsirolimus, which correlated with enhanced effects on PML stabilization, mTOR inhibition, and VEGF downregulation (Supplementary Fig. S4A and S4B). To select a druggable target in the SCP–PML pathway, we chose to block the activity of Pin1, which is required for targeting S518 phosphorylated PML for degradation (19). We found that treatment of 786-O cells with Pin1 inhibitor PiB or Juglone at 0.1 or 1 μmol/L, respectively, did not significantly inhibit cell viability but enhanced the killing effect of temsirolimus (Fig. 7A, left). A similar effect was observed with Pin1 knockdown (Supplementary Fig. S4C). Importantly, the enhanced killing effect by combined treatment was
abrogated by PML knockdown (Fig. 7A, right), indicating a key role of PML in determining the effect of such treatment. Consistent with the cell viability data, we found that the two Pin1 inhibitors or Pin1 shRNAs not only stabilized PML, but also enhanced the effect of temsirolimus on suppressing mTOR activity and VEGF expression (Fig. 7B and Supplementary Fig. S4D). To examine the effect of combined treatment in vivo, we subcutaneously inoculated nude mice with 786-O cells and administrated mice with temsirolimus and/or Juglone after tumor formation. Although treatment of temsirolimus or Juglone modestly reduced tumor growth, combined treatment completely suppressed tumor growth (Fig. 7C). IHC analysis on tumor tissues revealed that Juglone potentiated the antiproliferation and antiangiogenesis effects of temsirolimus (Fig. 7D and E and Supplementary Fig. S5A). Notably, neither did single nor combined treatment affect mice body weight (Supplementary Fig. S5B), arguing against the induction of toxicity effect by these treatments. Our data suggest a beneficial effect by combined targeting of mTOR pathway and PML degradation pathway for ccRCC therapy.

Discussion

Phosphorylation at the S518 residue is crucial for targeting PML to a destruction pathway mediated by Pin1 followed by KLHL20-containing ubiquitin ligase (19). In this study, we identify SCP1, SCP2, and SCP3 as PMLS518 phosphatases. Using SCP1 as a model, we show that SCP1-dependent dephosphorylation stabilizes PML by blocking its interaction with Pin1 and KLHL20-based ubiquitin ligase. Furthermore, depletion of SCP1/2/3 increases PMLS518 phosphorylation to downregulate PML. These findings indicate a physiologic role of SCPs in PML stabilization and suggest a tumor-suppressive function of these phosphatases. Indeed, we show that SCP1 and SCP3 are downregulated in ccRCC and their downregulations correlate with PMLS518 phosphorylation, PML downregulation, and high-grade tumor. Interestingly, although KLHL20 is also highly expressed in ccRCC, an inverse correlation between the expression of KLHL20 and PML is not observed. These findings underscore a more important role of SCP1 and SCP3 than KLHL20 in determining the expression level of PML in renal tissues. These clinical observations are further strengthened by preclinical data demonstrating that the SCP–PML axis suppresses many features of aggressive ccRCC, such as proliferation, migration, invasion, tumor growth in vivo, and tumor angiogenesis. Our study thus uncovers a SCP–PML pathway in ccRCC and indicates a crucial role of this pathway in suppressing ccRCC progression. Notably, IHC analysis revealed a possible SCP1 downregulation in prostate cancer (Supplementary Table S1) and SCP1 induced PMLS518 dephosphorylation.

Figure 6. SCP–PML axis inhibits mTOR/HIF signaling. A, immunoblot analysis of 786-O and A-498 cells transfected with SCP1 or SCP1 Dm. B, immunoblot analysis of 786-O cells stably expressing inducible SCP1 and constitutive PML shRNA and treated with or without doxycycline for 20 hours. C, qPCR analysis of 786-O or A-498 cells carrying inducible SCP1 or its mutant treated with or without doxycycline. D, immunoblot analysis of HK-2 cells transfected with indicated siRNAs. Data are mean ± SD ( , P < 0.05; **, P < 0.005; ***, P < 0.0005; n = 3).
and PML upregulation in prostate cancer cell line PC-3 (Supplementary Fig. S6), suggesting the significance of SCP–PML axis in this cancer type.

In addition to demonstrating the tumor-suppressive effects of SCP–PML axis on ccRCC, our study reveals a cross-talk of this pathway with HIF pathway. Previous studies indicated that PML blocks mTOR activation (3), thereby suppressing the expression of HIF1α and HIF2α (3, 19). Consistent with these findings, we show that the SCP–PML axis suppresses mTOR activity and HIF expression, leading to the downregulation of HIF effectors, such as GLUT1 and VEGF. These observations not only point out additional impacts of this PML degradation pathway on ccRCC, such as altering metabolism and promoting angiogenesis, but also highlight a coordinated action of SCP downregulation and VHL deficiency in potentiating HIF signaling in ccRCC. Given the importance of HIF signaling in ccRCC pathology and the pleiotropic functions of PML in tumor suppression, we reason that the SCP downregulation-induced PML degradation may be considered as a therapeutic target for combining with a current FDA-approved regimen for ccRCC therapy. In support of this notion, we show that targeting of this PML degradation pathway by SCP1 overexpression or Pin1 inhibition enhances the tumor-suppressive effects of temsirolimus. Interestingly, although Pin1 has been reported to elicit tumor-promoting effects through multiple effectors, PML likely plays a major role in ccRCC, as PML depletion abolishes the beneficial effect of Pin1 inhibitors. Thus, our study suggests a new option for ccRCC targeted therapy and further highlights a key role of PML in suppressing ccRCC.
The tumor-suppressive function of PML in kidney was observed by studies with mouse models. In compound Pml<sup>−/−</sup> Tsc2<sup>−/−</sup> mice, PML loss in the Tsc2<sup>−/−</sup> background further increases mTOR activity and accelerates kidney tumor progression without affecting tumor initiation (11). These observations are consistent with our finding that aberrant downregulation of SCP–PML axis is associated with high-grade ccRCC. Thus, even though the tumor-suppressive functions of PML could be context-dependent (38), data derived from mice models, human ccRCC specimens, and ccRCC cell lines provide definitive evidence for a suppressive role of PML in this cancer type.

SCP1 has been reported to dephosphorylate other substrates such as RNA polymerase II, Smad1/2/3, and Snail (23, 26–28, 39). Similar to PML, the residues targeted by SCP1 in these substrates are all within the pS/T-P motif. This implies that SCPs are specific to pS/T-P motif, even though identification of more substrates is needed to confirm this notion. The pS/T-P motif is also recognized by Pin1 for prolylisomerization. Interestingly, the first identified SCP substrate, RNA polymerase II, is also a substrate of Pin1 and Pin1-dependent modification regulates the binding of phosphorylated RNA polymerase II to certain nuclear factors (40). Thus, SCP-mediated dephosphorylation may antagonize certain biologic functions elicited by Pin1 and proline-directed serine/threonine kinases.

In summary, our study identifies a PML degradation pathway mediated by downregulation of SCPs and demonstrates a role of this pathway in promoting mTOR/HIF signaling (Fig. 7F). This pathway contributes to multiple aggressive characters of ccRCC and is associated with advanced ccRCC. We propose that targeting of this pathway in combination of mTOR inhibitor is an option for ccRCC treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.-C. Lin, L.-T. Lu, H.-Y. Chen, X. Duan, X.-H. Feng
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