HOIL-1L Functions as the PKCζ Ubiquitin Ligase to Promote Lung Tumor Growth

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Abstract

Rationale: Protein kinase C zeta (PKCζ) has been reported to act as a tumor suppressor. Deletion of PKCζ in experimental cancer models has been shown to increase tumor growth. However, the mechanisms of PKCζ down-regulation in cancerous cells have not been previously described.

Objectives: To determine the molecular mechanisms that lead to decreased PKCζ expression and thus increased survival in cancer cells and tumor growth.

Methods: The levels of expression of heme-oxidized IRP2 ubiquitin ligase 1L (HOIL-1L), HOIL-1–interacting protein (HOIP), Shank-associated RH domain-interacting protein (SHARPIN), and PKCζ were analyzed by Western blot and/or quantitative real-time polymerase chain reaction in different cell lines. Coimmunoprecipitation experiments were used to demonstrate the interaction between HOIL-1L and PKCζ. Ubiquitination was measured in an *in vitro* ubiquitination assay and by Western blot with specific antibodies. The role of hypoxia-inducible factor (HIF) was determined by gain/loss-of-function experiments. The effect of HOIL-1L expression on cell death was investigated using RNA interference approaches *in vitro* and on tumor growth in mice models. Increased HOIL-1L and decreased PKCζ expression was assessed in lung adenocarcinoma and glioblastoma multiforme and documented in several other cancer types by oncogenomic analysis.

Measurements and Main Results: Hypoxia is a hallmark of rapidly growing solid tumors. We found that during hypoxia, PKCζ is ubiquitinated and degraded via the ubiquitin ligase HOIL-1L, a component of the linear ubiquitin chain assembly complex (LUBAC). *In vitro* ubiquitination assays indicate that HOIL-1L ubiquitinates PKCζ at Lys-48, targeting it for proteasomal degradation. In a xenograft tumor model and lung cancer model, we found that silencing of HOIL-1L increased the abundance of PKCζ and decreased the size of tumors, suggesting that lower levels of HOIL-1L promote survival. Indeed, mRNA transcript levels of HOIL-1L were elevated in tumor of patients with lung adenocarcinoma, and in a lung adenocarcinoma tissue microarray the levels of HOIL-1L were associated with high-grade tumors. Moreover, we found that HOIL-1L expression was regulated by HIFs. Interestingly, the actions of HOIL-1L were independent of LUBAC.

Conclusions: These data provide first evidence of a mechanism of cancer cell adaptation to hypoxia where HIFs regulate HOIL-1L, which targets PKCζ for degradation to promote tumor survival. We provided a proof of concept that silencing of HOIL-1L impairs lung tumor growth and that HOIL-1L expression predicts survival rate in cancer patients suggesting that HOIL-1L is an attractive target for cancer therapy.

Keywords: hypoxia; hypoxia-inducible factors; tumorigenesis; E3 ligase; linear ubiquitin chain assembly complex

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At a Glance Commentary

Scientific Knowledge on the Subject: Lung and other solid tumors must adapt to hypoxic conditions to survive and grow. During hypoxia, the hypoxia-inducible factors are stabilized and promote vascularization and metabolic pathways, which result in tumor adaptation and growth. Recently, it has been reported that protein kinase C zeta (PKCζ) acts as a tumor suppressor factor; silencing PKCζ promoted carcinogenesis. However, the mechanisms of how PKCζ is down-regulated during tumorigenesis have not been elucidated.

What This Study Adds to the Field: We report here that PKCζ is degraded in cancer cells by the ubiquitin-proteasome system during hypoxia, which is characteristic of solid tumors. The ubiquitin ligase heme-oxidized IRP2 ubiquitin ligase 1L (HOIL-1L) is highly expressed in patients with lung cancers. Moreover, we identified HOIL-1L as the PKCζ ubiquitin ligase, which is up-regulated during hypoxia via the hypoxia-inducible factor. Silencing of HOIL-1L increased cell death during hypoxia and suppressed tumor growth. Thus, our studies provide *in vitro* and *in vivo* evidence for a novel cellular adaptation mechanism to hypoxia, which is of clinical significance.

Rapidly proliferating cancers, including lung adenocarcinoma and glioblastoma multiforme (GBM), must adapt to tissue hypoxia, which develops in the central regions of tumors (1–3). This is largely accomplished by a program activated in response to the stabilization of hypoxia-inducible factors (HIFs), which act as transcription factors to induce metabolic changes and preserve cellular energy homeostasis (4–6). The importance of HIFs in tumorigenesis is experimentally well established and therapies directed at some HIF target genes are being clinically evaluated (3, 7, 8).

The protein kinase C (PKC) family is comprised of serine-threonine kinases that regulate cellular adaptation to environmental stress by interacting with pathways of survival, proliferation, migration, and apoptosis (9–12). It has been recently described that PKCζ acts as a tumor suppressor because its activity and/or expression is altered in different types of human cancer including GBM and renal cancer. Also, deletion of PKCζ in experimental cancer models increases tumorigenesis (13–15). These findings indicate the importance of PKCζ as a possible target in anticancer therapies. The mechanism by which PKCζ suppresses tumor growth has not been completely described but regulation of c-myc, phosphorylation of C/EBPβ to inhibit IL-6 expression, and inhibition of the serine biosynthetic cascade by controlling the 3-phosphoglycerate dehydrogenase have been proposed as downstream targets (13, 14, 16, 17). However, the cellular and molecular mechanisms involved in down-regulating PKCζ in cancer cells have not been described and are the main focus of this study.

PKC activity is regulated by its intracellular localization and by degradation, although the mechanisms controlling PKCζ degradation are less well understood. The linear ubiquitin chain assembly complex (LUBAC) was reported to bind and ubiquitinate several PKC isoforms (18). LUBAC is composed of two RING-in-between-RING (RBR)–containing proteins: heme-oxidized IRP2 ubiquitin ligase 1L (HOIL-1L), also known as RBCK1, the HOIL-1–interacting protein (HOIP), and a Shank-associated RH domain-interacting protein (SHARPIN), but only HOIP has been reported to form linear chains (19–22). Recent reports indicate an implication of LUBAC in cancerogenesis where excessive LUBAC activation causes abnormal nuclear factor-κB signaling and cancer growth (23–26).

Here, we sought to determine the molecular mechanisms that lead to decreased PKCζ expression in cancer cells, which results in increased cancer cell survival and tumor growth. Our data provide evidence of a novel mechanism where HIF promotes cancer cell survival by orchestrating a pathway of adaptation to hypoxia. We found that the E3 ligase HOIL-1L is regulated by HIF and targets PKCζ for proteasomal degradation via a LUBAC-independent mechanism. Importantly, patients with lung adenocarcinoma and GBM have high levels of HOIL-1L and low levels of PKCζ, and thus inhibition of HOIL-1L can be a potential therapy to slow tumor growth.

Methods

Patients

Lung tissue was obtained from patients with lung nodules. Informed consent was obtained from all participants. The protocols were approved by the Scientific Review Committee and the Institutional Review Board of Northwestern University. RNA was isolated from human lung cancer tumors and adjacent normal tissue stored in RNAlater and used for subsequent analysis.

Cell Culture, Transfections, and Hypoxia Exposure

Isolation, culture, transfection protocols, and hypoxia exposure of cells were described previously (27, 28). A549, RCC4, RCC4+pVHL, and COS-7 cells were grown in Dulbecco's modified Eagle medium as described (28). Hypoxic conditions (1.5% O_2, 93.5% N_2, and 5% CO_2) were achieved by equilibrating the medium in a humidified workstation (In Vivo 300; Ruskinn Technology, Bridgend, UK) as previously described (27).

Xenograft Assay

Animals were provided with food and water *ad libitum*, maintained on a 14:10-hour light-dark cycle, and handled according to National Institutes of Health guidelines and the Northwestern University Institutional Animal Care and Use Committee–approved experimental protocol. Xenograft Tumor Assay was performed as previously described (29). Briefly, 10^7 A549 cells in phosphate-buffered saline were injected subcutaneously into the flank of female NCr-nu/nu mice (01B74) or 5×10^5 cells for retroorbital injections. The rate of tumor growth was measured by using calipers. Tumor samples were homogenized under liquid nitrogen with a mortar and pestle and then suspended in lysis-buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*-ethane sulfonic acid pH 7.0, 250 mM, NaCl, 5 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 0.1% Triton X-100) as previously described (30).

Oncomine Database

Oncomine 4.4.4.4, a web-based clinical genomics database (available at

https://www.oncomine.org) was used to analyze HOIL-1L (*rbck1*) expression in normal versus cancer expression from different tissues and differential analysis of highly ranked gene expression from different cancer types.

Statistics

Experimental conditions were compared by Student *t* test for single measurements and multiple comparisons were performed using analysis of variance. ΔCt values obtained from quantitative reverse transcriptase polymerase chain reaction were analyzed for normal distribution using the Shapiro-Wilk test. All analyses were performed by using GraphPad Prism version 4.00 (GraphPad Software, La Jolla, CA). Data were expressed as mean ± SEM (n = 3) unless otherwise indicated. REMBRANDT, a clinical genomics database, was used to calculate the effect of HOIL-1L (*rbck1*) expression on overall glioma patient survival. *P* values were calculated by the log-rank test. Statistics for overall survival were calculated with the log-rank (Mantel-Cox) test. A *P* value less than or equal to 0.05 was considered statistically significant for all tests.

Results

PKCζ Is Ubiquitinated by HOIL-1L during Hypoxia

In A549 lung adenocarcinoma cells, PKCζ protein abundance was decreased in a time-dependent fashion during exposure to hypoxia (1.5% O_2) (Figure 1A; *see* Figure E1A), without changes in PKCζ mRNA levels or protein synthesis (*see* Figures E1B and E1C). To identify the mechanism of PKCζ degradation during hypoxia, we examined the role of the transcription factor HIF. PKCζ expression was lower in pVHL-deficient RCC4 cells, which have constitutive HIF-1α/2α activation (31), than in RCC4 cells reconstituted with the HIF E3 ligase pVHL (RCC4+VHL), whereas other PKC isoforms were not affected (Figure 1B). To investigate the effects of HIF stabilization on PKCζ, we overexpressed a construct in which two prolines in the oxygen-dependent degradation domain of HIF-1α were mutated to alanine in A549 cells (32). The stabilization of HIF-1α, which was detected by a hypoxia response element luciferase activity assay (Figure 1C, *top*), resulted in lower levels of PKCζ as compared with noncoding vector transfected cells or nontransfected cells (Figure 1C, *bottom*). Moreover, chemical HIF stabilization by $CoCl_2$ also led to decreased PKCζ levels (Figure 1D; *see* Figure E1D). Conversely, silencing of HIF-1α and HIF-2α prevented the decrease in PKCζ during hypoxia (Figure 1E; *see* Figure E1E). Interestingly, the expression of the classical PKCα and PKCβII were not altered during hypoxia, indicating a specific effect for the expression of PKCζ (*see* Figure E1F).

On activation, several PKCs are down-regulated by ubiquitination and proteasomal degradation (33). In A549 cells, exposure to hypoxia or $CoCl_2$ increased PKCζ ubiquitination (Figures 1F and 1G). Moreover, preincubation with the

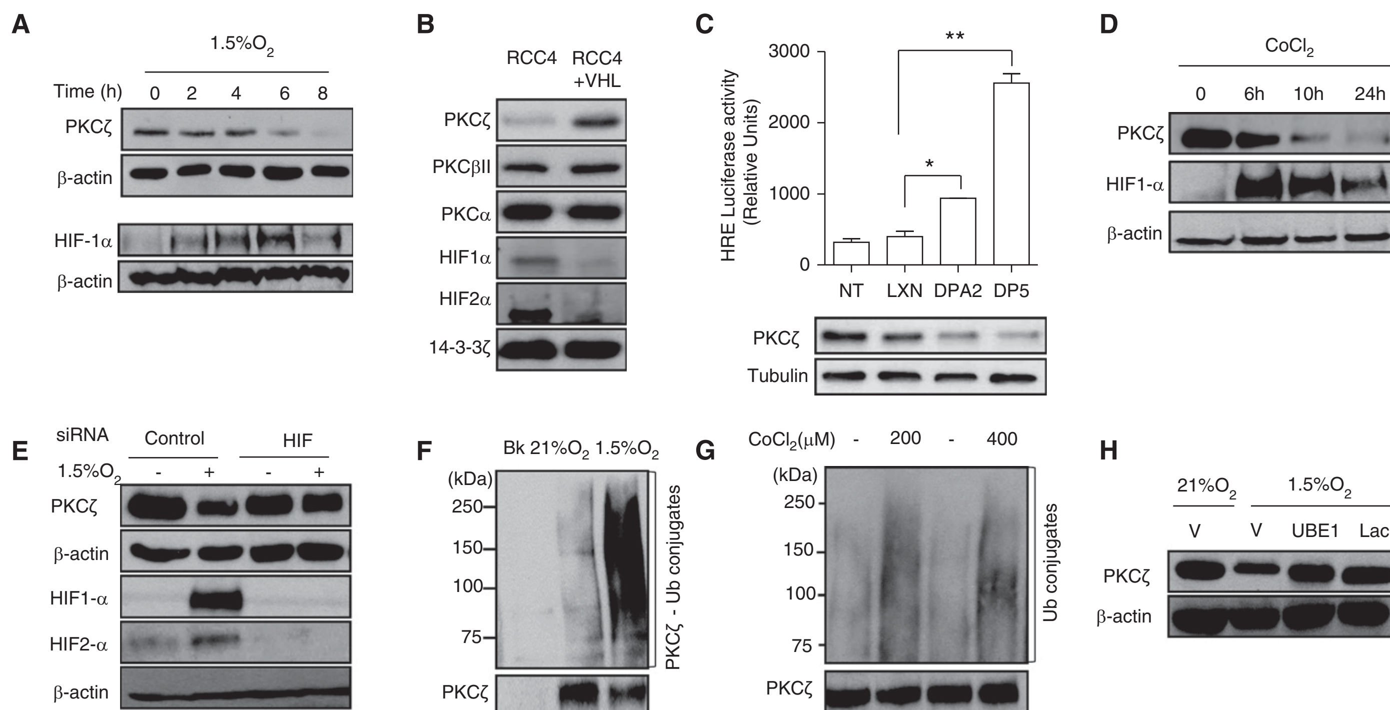

Figure 1. PKCζ is degraded during hypoxia. (*A*) A549 cells were exposed to hypoxia (1.5% O_2) and PKCζ levels were detected by Western blot (WB). (*B*) The expression of PKCζ was analyzed in RCC4 and RCC4+VHL cells by WB. (*C*) A549 cells were transfected with two different constitutively active HIF-1α clones (DPA-2 and DPA-5). Hypoxia response element (HRE) luciferase activity and PKCζ levels were determined after 24 hours. (*D*) A549 cells were treated with $CoCl_2$ (300 μM). PKCζ levels were determined by WB. (*E*) A549 cells were transfected with HIF (HIF1α+HIF2α) siRNA and exposed to hypoxia for 24 hours. PKCζ and HIF levels were detected by WB. (*F*) A549 cells were exposed to normoxia (21% O_2) or hypoxia for 6 hours, PKCζ was immunoprecipitated, and ubiquitin-conjugates detected. (*G*) Cells were exposed to $CoCl_2$ for 6 hours and PKCζ was immunoprecipitated and probed with an ubiquitin antibody. (*H*) A549 cells were preincubated with UBE1 or lactacystin and exposed to hypoxia for 24 hours. PKCζ levels were detected by WB. Data represent mean ± SEM of at least three separate experiments. $^*P \leq 0.05$; $^{**}P \leq 0.01$.

E1-inhibitor UBE1–41, or the proteasome inhibitor lactacystin, prevented PKCζ degradation (Figure 1H; *see* Figure E2A). We investigated whether the components of linear ubiquitination complex, which has been described to interact with and ubiquitinate several PKC isoforms, was involved in the degradation of PKCζ during hypoxia. Coimmunoprecipitation experiments showed that during hypoxia PKCζ binds to HOIL-1L but not to HOIP or SHARPIN (Figure 2A). Using an *in vitro* ubiquitination assay, we found that HOIL-1L/HOIP was sufficient to ubiquitinate GST-PKCζ (Figure 2B). Characteristic high-molecular-weight PKCζ-ubiquitin conjugates were observed when GST-PKCζ was incubated with wild-type (WT) ubiquitin. The ubiquitin linkage is important to determine the fate of the target protein. To determine the ubiquitination linkages present in PKCζ, we performed the *in vitro* ubiquitination assay in the presence of Ub K48O, in which all lysines in ubiquitin were mutated to arginines except Lys-48, or Ub K48RK63R in which Lys-48 and Lys-63 were mutated to arginine. We detected mainly Lys-48–linked ubiquitination (Figure 2B, *lanes 3 and 4*), whereas linear ubiquitination was ruled out by using N-terminal His-tagged ubiquitin to prevent linear ubiquitin conjugation (34). Ub K48RK63R abolished all ubiquitination (Figure 2B, *lane 3*). Consistent with this observation, the degradation and ubiquitination of endogenous PKCζ during hypoxia was prevented by silencing HOIL-1L (Figures 2C and 2D; *see* Figure E2B). Taken together, our results suggest that during hypoxia HOIL-1L is the E3 ligase for PKCζ targeting it for proteasomal degradation.

Because PKCζ degradation is HIF-dependent, we investigated whether HIF regulates HOIL-1L expression and found

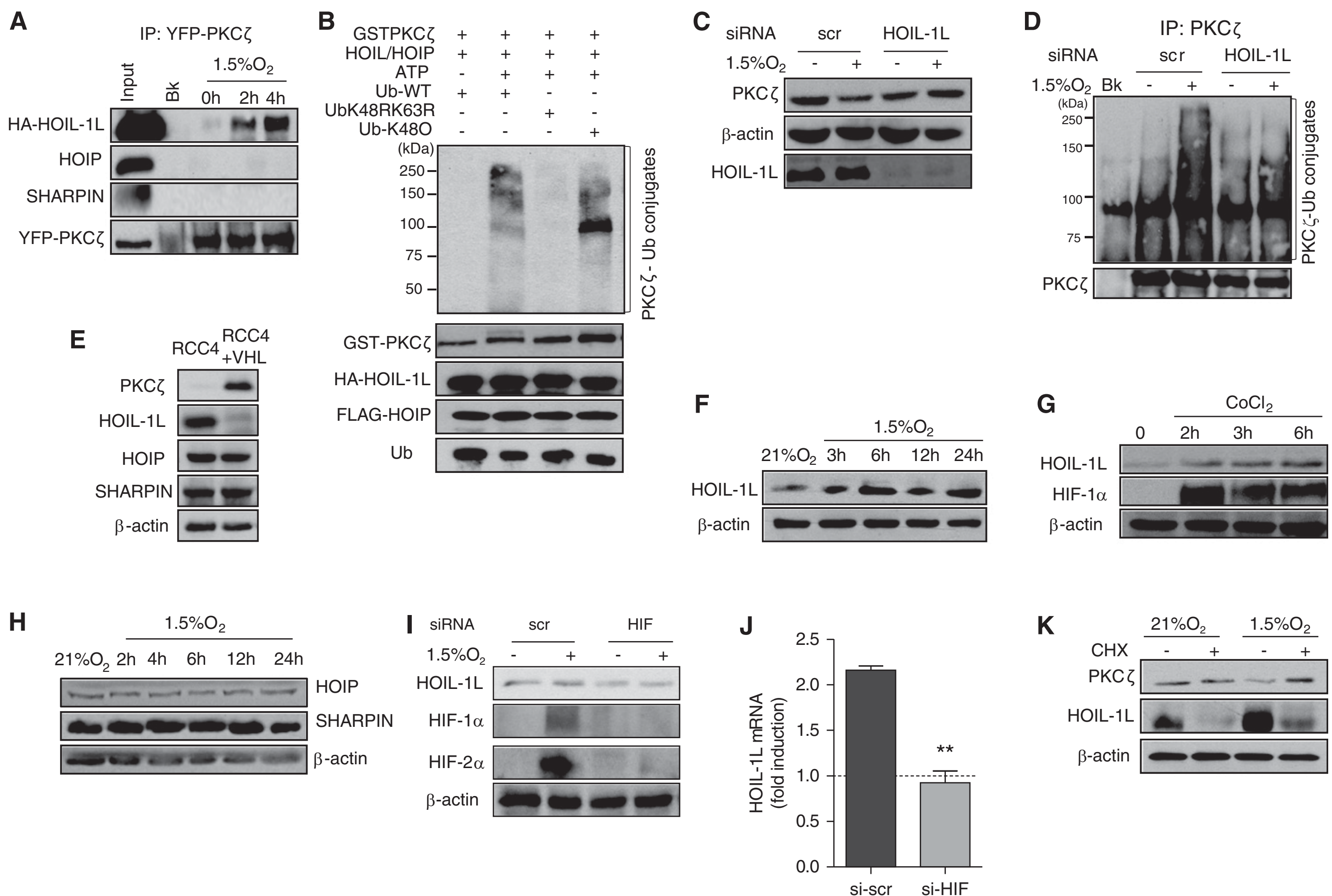

Figure 2. HOIL-1L ubiquitinates PKCζ and is regulated by HIF. (*A*) A549 cells were transfected with HA-HOIL-1L and YFP-PKCζ and exposed to hypoxia as indicated. PKCζ was immunoprecipitated and proteins analyzed by Western blot (WB). (*B*) GST-fused PKCζ was incubated in the presence of HOIL-1L+ HOIP for 2 hours at 37°C, pulled down with glutathione beads, and analyzed by WB for ubiquitin conjugates. (*C*) A549 cells were transfected with control or pooled HOIL-1L siRNA and cells were exposed to hypoxia for 24 hours and proteins were detected by WB. (*D*) A549 cells were transfected with control or pooled HOIL-1L siRNA and exposed to hypoxia for 6 hours. PKCζ was immunoprecipitated and probed with an ubiquitin antibody. (*E*) Linear ubiquitin chain assembly complex levels were determined in RCC4 and RCC4+VHL cells by WB. (*F*) A549 cells were exposed to hypoxia for the indicated times. Cell lysates were isolated and HOIL-1L detected by WB. (*G*) A549 cells were stimulated with $CoCl_2$ (300 μM) for the indicated times. (*H*) A549 cells were exposed to hypoxia for the indicated times and cell lysates were analyzed by WB. (*I*) A549 cells were transfected with HIF siRNA, and exposed to hypoxia for 24 hours and the expression of HOIL-1L was analyzed by WB. (*J*) A549 cells were transfected with HIF siRNA and exposed to hypoxia for 24 hours. RNA was analyzed by quantitative reverse transcriptase polymerase chain reaction. (*K*) A549 cells were pretreated with cycloheximide (CHX) and exposed to hypoxia for 12 hours. Cell lysates were analyzed by WB. Data represent mean ± SEM; n ⩾ 3; $^{**}P < 0.01$.

that RCC4 cells express higher levels of HOIL-1L as compared with RCC4+ pVHL cells, whereas the levels of HOIP and SHARPIN remained unchanged (Figure 2E). The native heteromeric LUBAC exist as a high-molecular-weight complex of approximately 600 kD that forms linear ubiquitin chains. When protein extracts from RCC4 cells were analyzed by native gel electrophoresis, HOIL-1L was found in a complex together HOIP in a band of approximately 260 kD and also without HOIP in a faster-migrating band of approximately 200 kD. The 200-kD band was not observed after the separation of proteins from RCC4+pVHL cells, whereas HOIP migration did not change in either condition (*see* Figure E2C). These results are consistent with experiments in UOk111 cells, another VHL-deficient cell line (35). Similar results were observed when A549 cells were exposed to hypoxia (*see* Figure E2D). Furthermore, HOIL-1L abundance increased after 6 hours of hypoxia or $CoCl_2$ treatment (Figures 2F and 2G; *see* Figure E2E) and HOIL-1L mRNA increases before PKCζ degradation occurs (*see* Figure E2F), whereas HOIP and SHARPIN protein levels did not change (Figure 2H). Silencing of HIF-1α and HIF-2α prevented the hypoxia-induced increase in HOIL-1L protein and mRNA transcript (Figures 2I and 2J). In cycloheximide-treated cells, the up-regulation of HOIL-1L was prevented, and PKCζ was not degraded during hypoxia (Figure 2K; *see* Figure E2G), suggesting that the newly synthesized HOIL-1L serves as the PKCζ E3 ligase. Taken together, these results suggest that hypoxia induced the HIF-mediated up-regulation of HOIL-1L, which independently of the other LUBAC components conjugates PKCζ with Lys-48 ubiquitin chains promoting its degradation by the proteasome.

HOIL-1L Ubiquitinates PKCζ Independently of LUBAC during Hypoxia

To elucidate the mechanism by which HOIL-1L targets PKCζ for ubiquitin-mediated degradation, we compared the

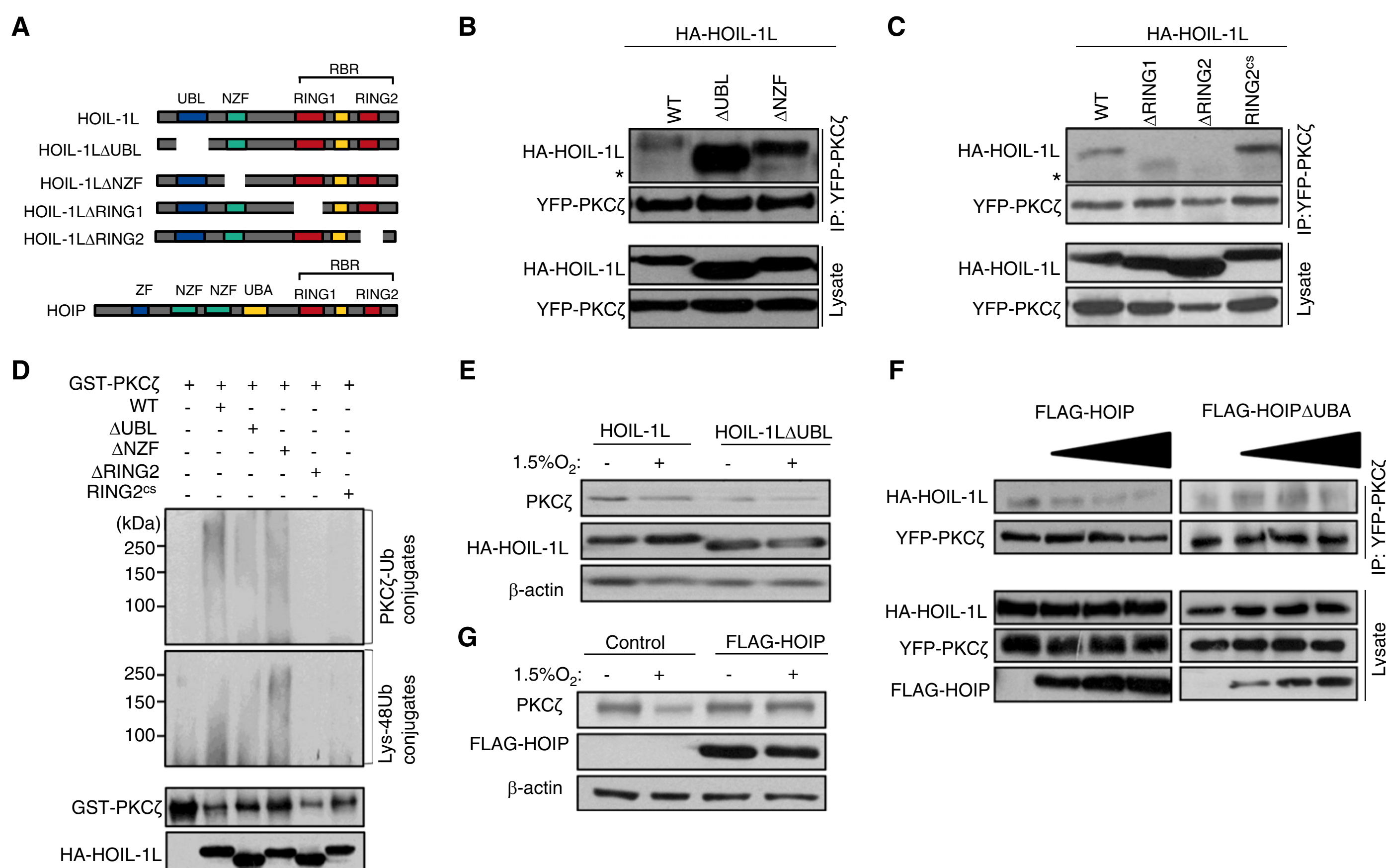

Figure 3. HOIL-1L ubiquitinates PKCζ independently of linear ubiquitin chain assembly complex during hypoxia. (*A*) Schematic representation of the HOIL-1L/HOIP domain structure and its deletions. (*B*) A549 cells were transfected with wild-type (WT) HA-HOIL-1L, HA-HOIL-1LΔUBL, or HA-HOIL-1LΔNZF together with YFP-PKCζ for 24 hours. YFP-PKCζ was immunoprecipitated, lysates and immunoprecipitates were probed for HA and YFP. (*C*) Lysates and immunoprecipitates from A549 cells overexpressing YFP-PKCζ and HA-HOIL-1LWT or HA-HOIL-1LΔRING1, HA-HOIL-1LΔRING2, and HA-HOIL-1L RING2^{CS} were immunoprecipitated and immunoblotted for HA and YFP. (*D*) GST-fused PKCζ was incubated in the presence of HA-HOIL-1L -WT, -ΔUBL, -ΔNZF, -ΔRING2, or -RING2^{CS} for 2 hours at 37°C and then pulled down with glutathione beads and immunoblotted for ubiquitin and ubiquitin-lys48 conjugates. (*E*) A549 cells were transfected with HA-HOIL-1L or HA-HOIL-1LΔUBL, exposed to normoxia or hypoxia for 24 hours, and cell lysates analyzed by Western blot (WB). (*F*) A549 cells were cotransfected with YFP-PKCζ, HA-HOIL-1L, and increased amounts of FLAG-HOIP or FLAG-HOIPΔUBA for 24 hours as indicated. YFP-PKCζ was immunoprecipitated and the interaction with HOIL-1L analyzed by WB. (*G*) HOIP overexpression prevents PKCζ degradation. A549 cells were transfected with FLAG-HOIP and exposed to normoxia or hypoxia for 24 hours. PKCζ degradation was analyzed by WB. Data represented from at least three separate experiments. *Asterisks* indicate unspecific IgG band. RBR = RING-in-between-RING.

interaction between PKCζ and WT HOIL-1L with that of deletion mutants of HOIL-1L and HOIP (Figure 3A). The ubiquitin-like (UBL) domain of HOIL-1L and the ubiquitin-associated domain (UBA) of HOIP are necessary for LUBAC formation, whereas the two RING domains of HOIP are required for LUBAC's linear polyubiquitination activity (35). Furthermore, HOIL-1L Npl4 zinc finger domain (NZF) is required for specific recognition of linear ubiquitin chains (36). Interestingly, neither the overexpression of HOIL-1LΔUBL nor HOIL-1LΔNZF prevented the interaction between PKCζ and HOIL-1L (Figure 3B). Apparently, the binding of the mutant HOIL-1L to PKCζ is stronger than that observed with WT HOIL-1L. RBR-E3 ligases transfer ubiquitin via a unique RING/HECT-hybrid mechanism in which the E2-Ub binds to the RING1 domain of the RBR E3 ligase, leading to the ubiquitin transfer from the E2 to the RING2-domain and subsequently to the substrate. The deletion of the RING1 domain in HOIL-1L had no effect on the binding to PKCζ, whereas overexpression of HOIL-1LΔRING2 but not HOIL-1LRING2^{cs}, which cannot form a thioester and is catalytically inactive as a ligase, prevented the interaction between PKCζ and HOIL-1L (Figure 3C). Furthermore, in an *in vitro* assay, WT HOIL-1L, HOIL-1LΔUBL, and HOIL-1LΔNZF induced the ubiquitination of PKCζ but HOIL-1LΔRING2 and HOIL1L-RING2^{CS} failed to do so (Figure 3D). Interestingly, overexpression of HOIL-1LΔUBL increased the degradation of PKCζ during hypoxia (Figure 6E; *see* Figure E3A). YFP-PKCζ and HA-HOIL-1L were coexpressed in A549 cells with increasing amounts of FLAG-HOIP to test whether HOIP competes with the HOIL-1L- PKCζ interaction. As shown in Figure 3F (*left*) the interaction between PKCζ and HOIL-1L decreased as HOIP levels increased. This was confirmed by coexpression of YFP-PKCζ and HA-HOIL-1L with increasing amounts of HOIP-ΔUBA, which lacks the HOIL-1L binding domain and is incapable of forming a functional LUBAC (*see* Figure E3B).

As expected, HOIP-ΔUBA did not interfere with the PKCζ-HOIL-1L interaction (Figure 3F, *right*). Moreover, HOIP overexpression prevented PKCζ degradation during hypoxia (Figure 3G; *see* Figure E3C), suggesting that HOIP might sequester HOIL-1L and compete with binding to PKCζ. These findings are supported by knock-down experiments where silencing of HOIP decreases the expression of PKCζ (*see* Figure E3D). Taken together these results indicate that during hypoxia HOIL-1L does not require the other components of LUBAC and that HOIL-1L binds PKCζ via its RING2 domain, which is also necessary for the catalytic activity of the E3 ligase.

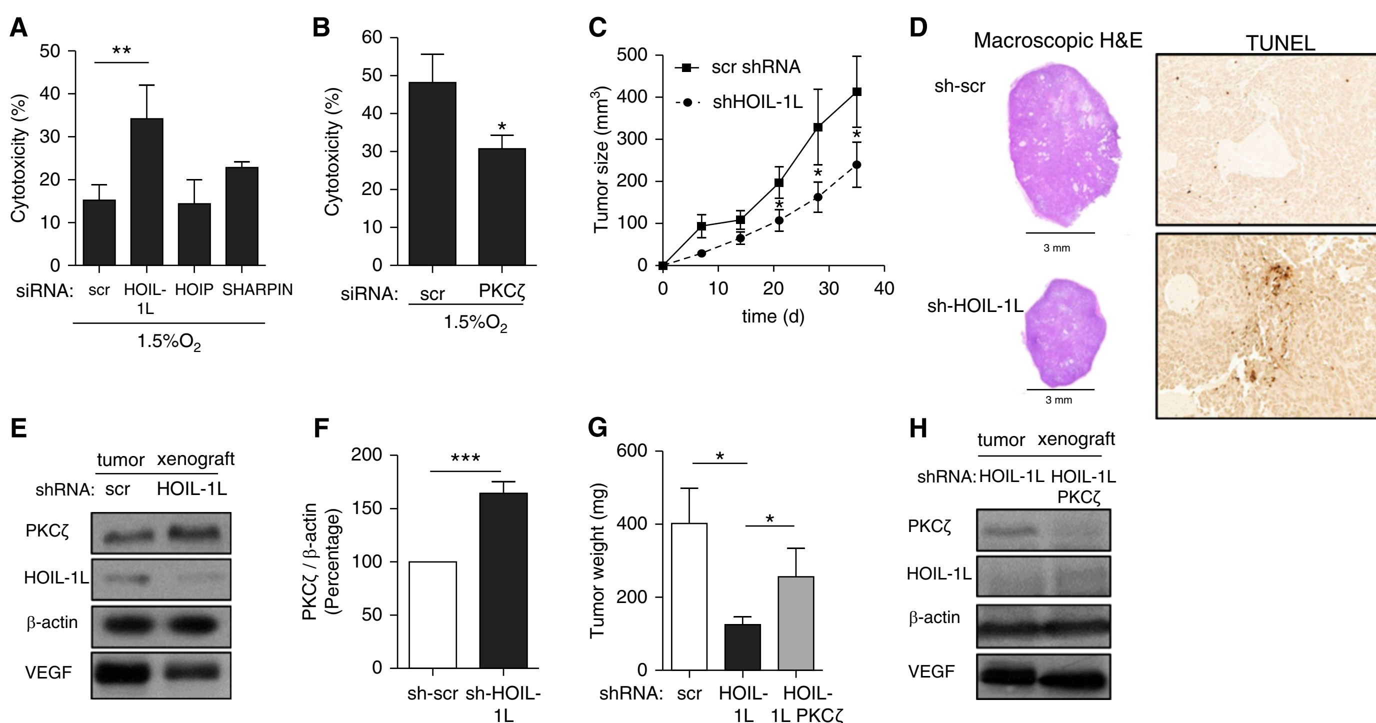

Figure 4. PKCζ down-regulation by HOIL-1L promotes tumorigenesis. (*A*) A549 cells were transfected with control, HOIL-1L, HOIP, or SHARPIN siRNA and exposed to hypoxia for 48 hours. Cytotoxicity was measured by lactate dehydrogenase release. (*B*) Stable A549 shHOIL-1L cells were transfected with control and PKCζ siRNA and exposed to hypoxia for 48 hours. (*C*) A549 cells transfected with shRNA were engrafted into the flanks of nude mice (n = 6). Tumor growth was determined by measuring the tumor size over 5 weeks. (*D*) Tumor sections were stained with hematoxylin and eosin (H&E; *left*) and for TUNEL-positive cells (*brown*; *right*). Representative pictures are shown. Macroscopic magnification ×10. (*E*) Tumors were removed after 5 weeks and analyzed by Western blot. HOIL-1L shRNA-derived tumors had higher levels of PKCζ in comparison with scrambled shRNA-derived tumors. (*F*) Quantification of *E*. (*G*) HOIL-1L shRNA-derived tumors had higher levels of PKCζ in comparison with HOIL-1L+PKCζ shRNA-derived tumors. (*H*) shHOIL-1L–derived tumors had decreased weight, which could be rescued with double knock-down of HOIL-1L and PKCζ after 5 weeks. Data represent mean ± SEM of n ⩾ 3. *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$. TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling; VEGF = vascular endothelial growth factor.

PKCζ Down-regulation by HOIL-1L Promotes Tumor Growth

To evaluate the consequences of PKCζ degradation during hypoxia, the cell death rate was determined. During normoxia, cell death was approximately 8%, which increased to approximately 15% after exposing the cells to hypoxia for 48 hours. Silencing of HOIL-1L increased cytotoxicity to approximately 30%, whereas knock-down of the other LUBAC members, such HOIP and SHARPIN, had no effect (Figure 4A). This observation was concordant with the decrease in cell viability observed during hypoxia in HOIL-1L–silenced cells (*see* Figures E4A and E4B) and the rescue effect on cell death observed after silencing PKCζ in these cells (Figure 4B). To confirm that the increase in cell death during HOIL-1L deficiency was mediated by PKCζ, we transfected A549 cells with an empty vector, WT PKCζ, or with a kinase dead (K281R) mutant (PKCζ-KD) (37) and exposed them to hypoxia. Overexpression of WT PKCζ during hypoxia dramatically increased cell death, whereas PKCζ-KD did not (*see* Figure E4C).

To determine whether the degradation of PKCζ by HOIL-1L contributes to tumorigenesis, we used a murine xenograft tumor model. We generated several cell lines in which HOIL-1L was stably silenced (*see* Figure E5A); these cells were injected subcutaneously into the flanks of nude mice, and tumor progression was followed over a period of 5 weeks (*see* Figure E5B). Stable HOIL-1L–silenced cells responded to hypoxia and stabilized HIF levels in a similar fashion as compared with sh-scr transfected cells (*see* Figure E5C, *bottom*). Injected HOIL-1L–silenced cells formed smaller tumors (Figure 4C; *see* Figure E5D) and histologic examination of tumor sections at low and high magnification revealed that control sh-scr tumors have necrotic central areas surrounded by cohesively growing cells (Figure 4D, *left*). The sh-HOIL-1L tumors showed areas of altered architectural features indicative of increased cell death, which was further confirmed by increased number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive nuclei in the sh-HOIL-1L tumors (Figure 4D, *right*). In addition, HOIL-1L–silenced engrafted xenografts exhibited higher PKCζ expression, whereas HOIL-1L levels were significantly lower (Figures 4E and 4F). To rescue tumor growth in xenografts, double knock-down shHOIL-1L+shPKCζ cells were used (*see* Figure E5E). The decrease in growth observed after HOIL-1L silencing was rescued in the double knock-down cells (Figure 4G; *see* Figure E5F). Western blot analysis of the xenograft tissue revealed low PKCζ expression in comparison with shHOIL-1L–derived tumors after 5 weeks and the vascular endothelial growth factor expression levels were comparable, indicating similar hypoxia (Figure 4H).

HOIL-1L Silencing Decreased Lung Tumor Load

In a subsequent series of experiments, we investigated whether HOIL-1L ablation would decrease the growth of hematogenously delivered lung tumors (Figure 5). We injected A549 cells with silenced HOIL-1L (shHOIL-1L) or sh-scr A549 cells, described in Figure 4, intravenously and measured lung tumor progression using micro positron emission tomography examination. Animals injected with sh-HOIL-1L-A549 cells had a smaller

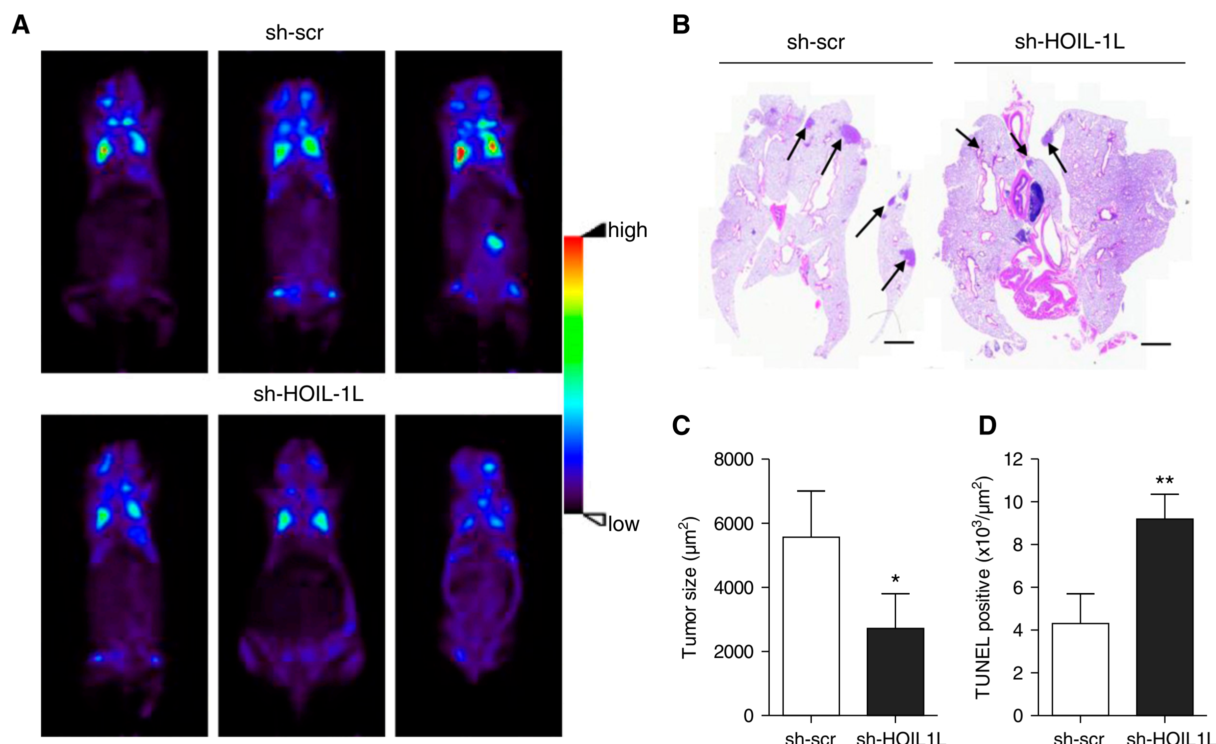

Figure 5. HOIL-1L silencing decreased lung tumor load. (*A*) sh-HOIL-1L and sh-scr shRNA A549 cells were administrated by intravenous injection and lung tumor load was determined by positron emission tomography after 8 weeks. (*B*) Lung sections were stained with hematoxylin and eosin and tumor lesions (*arrows*) were identified microscopically and (*C*) tumor areas were quantified. (*D*) Lung sections were stained for TUNEL-positive nuclei. Data represent mean ± SEM of six animals per group. Macroscopic magnification ×10; bar = 100 μm. $^*P \leq 0.05$; $^{**}P \leq 0.01$. TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling.

tumor load as compared with control sh-scrambled A549 cells (Figure 5A). Hematoxylin and eosin staining revealed cancer cell engraftment in the lungs of all animals. However, the A549 sh-HOIL-1L cells inoculated animals had significantly smaller tumors (Figures 5B and 5C). Also, the sh-HOIL-1L–derived tumors exhibited more positive TUNEL staining (Figure 5D; *see* Figure E6). Taken together these results suggest that the absence of HOIL-1L, by increasing the levels of PKCζ, increases cancer cell death impairing tumor progression.

HOIL-1L Expression Is Elevated in Adenocarcinoma

We found higher RNA transcript levels of HOIL-1L in tumor biopsies from patients with lung adenocarcinoma compared with normal adjacent tissue (NAT) (Figure 6A). Consistent with these findings, we found increased HOIL-1L expression in lung adenocarcinoma tissue microarray (TMA) by immunohistochemistry (Figure 6B; *see* Figure E7A). Consistent with the HOIL-1L expression, Western blots from adenocarcinoma tissue showed lower PKCζ levels as compared with NAT (Figure 6C; *see* Figure E7B). Corresponding with elevated HOIL-1L levels, HIF1α expression correlates with the HOIL-1L expression in adenocarcinoma grade III patients ($R^2 = 0.4313$; $P = 0.0203$) (Figure 6D).

Tumors from patients with GBM have been reported to become severely hypoxic (1, 7). In tumor tissue from patients with GBM, we found that HOIL-1L transcript levels were significantly increased in comparison with normal astrocytes (*see* Figure E8A). Similarly, the relative abundance of HOIL-1L and PKCζ protein was increased and decreased, respectively, in the tumors compared with the NAT (*see* Figures E8B and E8C). Furthermore, we analyzed a microarray dataset from the National Cancer Institute database REMBRANDT, which includes 269 glioma patients, and found that high HOIL-1L transcript levels were associated with poor prognosis ($P = 0.000132317$) (*see* Figure E8D). To test whether HOIL-1L regulation is a universal phenomenon in cancer, we analyzed RNA microarray data from human cancer specimens from the Oncomine database. HOIL-1L transcript levels were up-regulated in 46 different arrays (1.5-fold; $P = 0.0001$) and HOIL-1L was one of the top 10% regulated genes with the highest differential expression in human colorectal cancer specimens (1–5%) (*see* Figure E8E). Taken together, these data suggest that tumor hypoxia leads to increased HOIL-1L expression in different types of cancer cells, which by decreasing the levels of the tumor suppressor PKCζ, facilitates the adaptation to hypoxia.

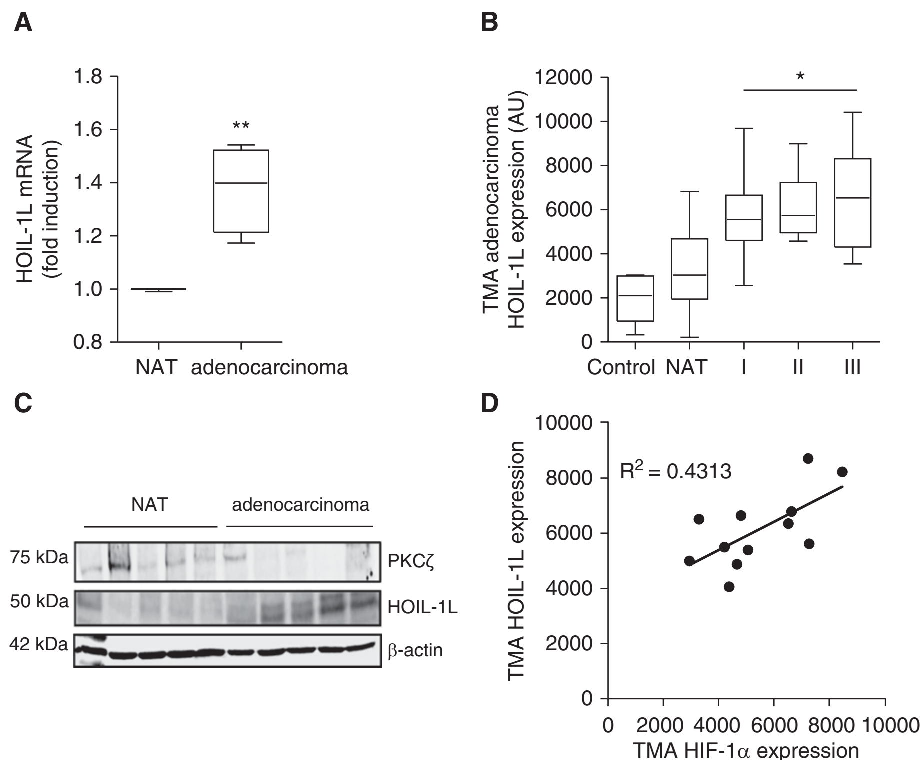

Figure 6. HOIL-1L expression is elevated in adenocarcinoma. (*A*) HOIL-1L mRNA levels were analyzed by quantitative reverse transcriptase polymerase chain reaction in biopsies from adenocarcinoma patients and compared with normal adjacent tissue (NAT). (*B*) Adenocarcinoma (grade I-III) tissue microarray was immunostained for HOIL-1L. (*C*) PKCζ and HOIL-1L levels were analyzed by Western blot in biopsies from adenocarcinoma patients (grade I-II) and compared with NAT. (*D*) HOIL-1L expression correlates with HIF-1α expression in adenocarcinoma tissue (grade III). Data represent mean ± SEM of at least three separate experiments. *$P \leq 0.05$; **$P \leq 0.01$.

HOIL-1L Promotes Tumorigenesis Independently of LUBAC

To rule out the possibility that HOIL-1L knock-down was interfering with the LUBAC function, we generated a stable cell line overexpressing HOIL-1LΔRING2 (*see* Figure E9A). The deletion of RING2 domain prevented the degradation of PKCζ (*see* Figure E9B) but did not interfere with the LUBAC-mediated nuclear factor-κB activation (*see* Figure E9C). Compared with WT HOIL-1L, HOIL-1LΔRING2 overexpressing cells produced smaller tumors, which was similar to the HOIL-1L knock-down cells (*see* Figures E9D and E9E). Increased TUNEL staining was only seen in tumors derived from overexpressing HOIL-1LΔRING2 cells (*see* Figure E9F).

Discussion

Cancer cells must adapt to survive hypoxic environments, which occur in solid tumors (38–40). We describe a novel mechanism of early response in cancer cell adaptation to hypoxia, where the stabilization of HIF increases the expression of the E3 ligase HOIL-1L, which forms Lys48-ubiquitin chains in the tumor suppressor PKCζ, targeting it for proteasomal degradation (Figure 7). We found that during prolonged hypoxia PKCζ was ubiquitinated and degraded by the proteasome and have identified a novel pathway where HOIL-1L serves as the essential E3 ligase for PKCζ ubiquitination. Different E3 ligases, such as pVHL and the HOIL-1L/HOIP complex, have been reported to interact and to degrade PKCs (41–43). We have excluded a role for pVHL in PKCζ degradation by showing that pVHL-deficient RCC4 cells have lower basal levels of PKCζ as compared

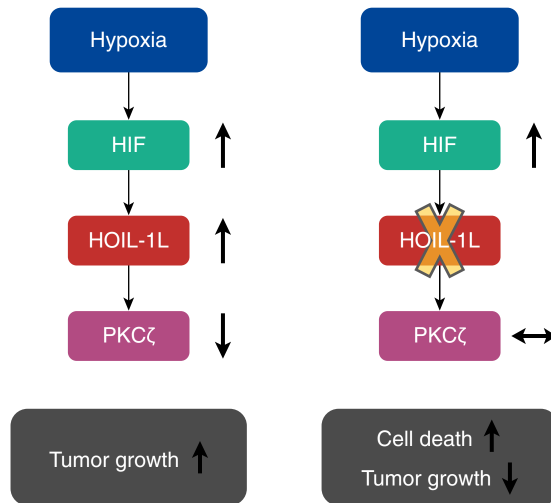

Figure 7. Schematic representation of cancer cell adaptation to hypoxia.

with RCC4+VHL. We then investigated the role of the HOIL-1L/HOIP complex in the hypoxic degradation of PKCζ. Nakamura and coworkers (18) described that HOIL-1L coimmunoprecipitated with PKCζ and that the activation of classical PKC increases HOIL-1L/HOIP binding. We purified HOIL-1L/HOIP from A549 cells and performed an *in vitro* ubiquitination assay in which HOIL-1L/HOIP ubiquitinated PKCζ forming Lys-48 chains. Importantly, in this assay the formation of linear ubiquitin chains was prevented by the use of recombinant His-tagged ubiquitin, indicating a nontraditional role of HOIL-1L in the ubiquitination of PKCζ. We also found that hypoxia increases the interaction between PKCζ and HOIL-1L but not with HOIP or SHARPIN and that only the RING2 domain of HOIL-1L interacted with PKCζ and was necessary for the catalytic activity. Surprisingly, overexpression of HOIL-1L or HOIL-1LΔUBL led to PKCζ degradation during hypoxia and either ubiquitinated PKCζ *in vitro*. These results indicate that HOIL-1L is sufficient to degrade PKCζ during hypoxia. In agreement with this, silencing HOIP did not prevent PKCζ degradation during hypoxia suggesting again a HOIP-independent function of HOIL-1L. In agreement with our results, HOIL-1L has been reported to form Lys48-polyubiquitin chains in several proteins including iron regulatory protein 2, the transcription factor Bach, and interferon regulatory factor 3 (44, 45).

The physiologic role of HOIL-1L has been mostly attributed to its function as a component of LUBAC regulating inflammation and the immune response (46–48). Patients with HOIL-1L mutations have inflammatory disorders (49) and HOIL-1L knock-down has been reported to impair cell invasiveness in osteosarcomas (24). Silencing of HOIL-1L inhibited tumor growth and promoted apoptosis in murine models of cancer. Lung adenocarcinoma and other non–small cell lung cancers and GBM are aggressive cancers that grow rapidly resulting in a hypoxic environment (38). Cells overcome hypoxic stress through multiple mechanisms, including the stabilization of HIFs. Both isoforms, HIF-1α and HIF-2α, are overexpressed in human non–small cell lung cancer, suggesting that they contribute to tumor progression (50). In tumors from lung adenocarcinoma and GBM patients, we found that the transcription levels and HOIL-1L protein abundance was higher relative to the NAT and that PKCζ levels were reduced. Although tumors develop long-term adaptation mechanisms, an important finding in the current study is that the up-regulation of HOIL-1L via HIF is an early response to facilitate the fast adaptation of the cancer cell to the hypoxic environment. Although a previous report has shown that HIF regulates the mitochondrial protease LON, which is required for COX4–1 degradation and adaption to hypoxia (51), this is the first report, to our knowledge, describing a HIF-dependent E3 ligase that promotes the proteasomal degradation of a tumor-suppressor factor. Our data indicate that HOIL-1L expression is increased in different cancer types (experimentally and oncogenomic analysis) and in a broad range of tumor types, suggesting a universal response to hypoxia in cancer cells. Taken together, these results indicate that HOIL-1L expression in cancer is an important mechanism of early adaptation and tumorigenesis and may serve as a prognostic biomarker. The clinical importance of these findings is supported by data from human cancer tissues, where increased expression of HOIL-1L and decreased PKCζ levels are associated with worse clinical outcomes. Conceivably, the E3-ligase activity of HOIL-1L can potentially serve as a therapeutic target for the treatment of solid tumors. ■

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