Mutual regulation between Polo-like kinase 3 and SIAH2 E3 ubiquitin ligase defines a regulatory network that fine-tunes the cellular response to hypoxia and nickel

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Elevated cellular response to hypoxia, which contributes to cell transformation and tumor progression, is a prominent feature of malignant cells in solid tumors. Polo-like kinase 3 (Plk3) is a serine/threonine protein kinase known to inhibit the cellular response to hypoxia and tumorigenesis. Nickel compounds are well-established human carcinogens that induce tumorigenesis partly through their hypoxia-mimicking effects. Despite previous research efforts, the role of Plk3 in the hypoxic response induced by hypoxia or nickel is not completely understood. Here, we show that $NiCl_2$ (Ni(II)) or hypoxia reduces the protein level and shortens the half-life of cytoplasmic Plk3 in a ubiquitin-proteasome-dependent manner. We identify SIAH2, a RING finger E3 ubiquitin ligase associated with the cellular hypoxic response, to be the ubiquitin E3 ligase that mediates the degradation of Plk3. We show that SIAH2 binds to Plk3 and mediates its ubiquitination primarily through its polo-box domain. We report that USP28, a deubiquitinase known to be inhibitable by Ni(II) or hypoxia, may also contribute to the suppression of the Plk3 protein by Ni(II). We also show that Plk3 in turn suppresses the SIAH2 protein level in a kinase activity-dependent manner. Our study revealed an interesting mutual regulation between Plk3 and SIAH2 and uncovered a regulatory network that functions to fine-tune the cellular hypoxic response. We propose that suppression of Plk3 expression contributes to carcinogenesis and tumor progression induced by nickel compounds.

Plk3 is a member of the polo-like kinase family of serine/threonine kinases (1–3). It has significant functions in cell cycle progression, DNA damage, hypoxic response, and apoptosis by participating in multiple cell signaling pathways (4–10). Previous studies have shown that Plk3 expression is reduced in multiple human tumors and cancer cell lines, particularly lung carcinomas (11–13). Notably, reduced Plk3 expression levels rather than mutations are associated with lung cancers (14). $PLK3^{-/-}$ mice are prone to developing larger and more highly vascularized tumors in multiple organs, including the lung (15), highlighting a critical role of Plk3 in tumorigenesis and tumor angiogenesis as a tumor suppressor. Previous work has shown that although the Plk3 mRNA level oscillates in the cell cycle, its protein level remains fairly stable (16, 17), suggesting a potential disconnection between the transcript and polypeptide of Plk3. Although ectopically expressed Plk3 has been shown to be rapidly degraded in the nucleus (18), it is unclear whether degradation of Plk3 also occurs in the cytoplasm.

Nickel compounds are well-established environmental and occupational lung carcinogens (19). Considered weak mutagens, these compounds promote tumorigenesis mainly through epigenetic and metabolic pathways (20). One major effect of nickel, in the form of the divalent ion (Ni(II)), is to stabilize the α subunit of the HIF[2] transcription factor by inhibiting prolyl hydroxylases (PHDs) (20). This hypoxia-mimicking effect of Ni(II) contributes significantly to nickel carcinogenesis by promoting cellular transformation and tumor progression (20). Recent studies have shown that Plk3 phosphorylates and destabilizes HIF-1α in a Von Hippel-Lindau tumor suppressor (pVHL)-independent fashion (9). Accordingly, $PLK3^{-/-}$ murine embryonic fibroblasts (MEFs) exhibit a much higher level of HIF-1α in response to Ni(II) or hypoxia (10, 15). These data suggest that regulation of the hypoxic (or hypoxic-like) responses through the HIF transcription factor may be a key mechanism that connects Plk3 and nickel carcinogenesis (21).

Seven in Absentia Homologue 2 (SIAH2) is an evolutionally conserved RING finger ubiquitin E3 ligase that is activated and/or induced by a variety of stress conditions, including hypoxia (22, 23). Thus, among the list of substrates of SIAH2 are those involved in hypoxia-associated functions, such as PHDs (22, 23). SIAH2 positively regulates the hypoxic response by promoting degradation of PHDs thereby stabilizing HIF-1α (22, 23). SIAH2 itself is also subjected to regulation by a number of protein kinases that are activated by various stress condi-

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[2] The abbreviations used are: HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase; PBD, polo-box domain; WB, Western blotting; ANOVA, analysis of variance; pVHL, Von Hippel-Lindau tumor suppressor; UPS, ubiquitin-proteasome system; MEF, murine embryonic fibroblast; IP, immunoprecipitation; CHX, cycloheximide; eGFP, enhanced GFP; KD, kinase domain; LSD, least significant difference.

tions, including hypoxia (22, 23). Accumulating evidence supports a prominent role of SIAH2 in multiple cancers (23, 24). Of particular relevance, elevated expression of SIAH2 has been detected in lung cancers (23, 24).

In this study, we investigated the regulation of Plk3 by Ni(II) and hypoxia. We found that the Plk3 protein level can be suppressed by Ni(II) or hypoxia in lung carcinoma, lung epithelial, and HEK293T cells. The suppression is mediated mainly by the ubiquitin-proteasome system (UPS). SIAH2, a hypoxia-responsive E3 ubiquitin ligase, interacts with and mediates Plk3 degradation by UPS. The polo-box domain (PBD) of Plk3 appears to be the primary motif that mediates the interaction between Plk3 and SIAH2 as well as degradation of Plk3. We observed that the SIAH2 protein level can be induced by Ni(II). The deubiquitinase USP28 may also be involved in Plk3 degradation in response to Ni(II). Interestingly, Plk3 in turn suppresses the protein level of SIAH2 in a manner dependent on the kinase activity of Plk3. Together, our study revealed a potentially important regulatory network involving Plk3, SIAH2, and the HIF transcription factor. This network may function to fine-tune the cellular hypoxic response and contribute to carcinogenesis.

Results

Ni(II) or hypoxia suppresses Plk3 expression

Given the involvement of Plk3 in the hypoxic response (10), we examined whether Plk3 expression is regulated by Ni(II) in A549 lung carcinoma and BEAS-2B lung branchial epithelial cells. Western blotting (WB) showed that the protein level of Plk3 could be inhibited by Ni(II) in dose- and time-dependent manners (Fig. 1, *A–D*). As expected, the protein levels of HIF-1α and VEGF-A were both increased by Ni(II) (Fig. 1, *C* and *D*). Long-term and low-dose Ni(II) treatments led to persistently lowered levels of Plk3 and elevated levels of HIF-1α (Fig. 1*E*). We also observed reduced levels of Plk3 mRNA upon Ni(II) treatments, which were accompanied by a corresponding increase in the mRNA levels of VEGF-A (Fig. 1, *G–J*). The activities of AKT and ERK were significantly suppressed, whereas that of p38 was dramatically increased by Ni(II) (Fig. 1*F*). Given that *PLK3* is a serum-inducible gene (17, 25), the suppression of Plk3 mRNA by Ni(II) was likely a result of reduced activities of ERK and AKT. The elevated VEGF-A mRNA levels were clearly a result of Ni(II)-induced HIF activation (Fig. 1, *C* and *D*).

Ni(II) or hypoxia suppresses the Plk3 protein level primarily through proteasome-mediated protein degradation

Multiple reports have shown that the mRNA level of Plk3 does not closely correlate with its protein level (8, 17, 21, 25). Plk3 is considered an immediate early response gene whose mRNA level can be quickly induced by serum growth factors after serum starvation, whereas its protein level is stable throughout the cell cycle (8, 17, 21, 25). To confirm this, we checked the effect of serum on Plk3 protein expression. As shown in Fig. 2*A*, there was no consistent increase in the Plk3 protein level after serum treatment despite the activation of ERKs, manifested as the increased levels of phosphorylated ERKs. This confirms the disconnection between the mRNA and protein levels of Plk3. The data also suggest that suppression of the Plk3 mRNA level by Ni(II) may not contribute significantly to the lowered Plk3 protein level.

UPS is a common mechanism for post-translational regulation of protein levels or functions (26, 27). To determine whether the Plk3 protein is suppressed by Ni(II) through UPS, we examined the effect of MG132, a specific inhibitor of the 26S proteasome (28). Our results showed that inhibition of the proteasome largely prevented the effect of Ni(II) or hypoxia on Plk3 in both A549 and BEAS-2B cells (Fig. 2, *B–E*), indicating that the Plk3 protein was indeed suppressed by Ni(II) or hypoxia through UPS. To determine whether Ni(II) affects the half-life of the Plk3 protein, we used cycloheximide (CHX) to inhibit protein synthesis, and tracked the Plk3 protein level with or without co-treatments with Ni(II) in A549 cells. We observed a significant drop in the half-life of the Plk3 protein from 14 to 8 h upon Ni(II) treatment (Fig. 2, *F* and *G*).

Ectopically expressed Plk3 protein has been shown to locate in both the cytoplasm and the nucleus of HEK293T cells, but nuclear Plk3 is quickly degraded via UPS (18). To determine whether Plk3 degradation also occurs in the cytoplasm, we checked the effect of Ni(II) on the Plk3 protein at the subcellular level in A549 cells. As shown in Fig. 2*H*, the endogenous Plk3 protein was only detectable in the cytoplasm. The cytoplasmic Plk3 protein behaved in the same way as did the total Plk3 in response to Ni(II) and MG132 shown in Fig. 2*B*. These data indicate that nuclear Plk3 may indeed be very unstable and that Ni(II)-mediated suppression of Plk3 also occurs in the cytoplasm.

Given the evidence that Plk3 is degraded via UPS, we checked whether Plk3 is ubiquitinated and whether Ni(II) can affect its ubiquitination. We first transfected the plasmid expressing FLAG-tagged ubiquitin into A549 cells followed by immunoprecipitation (IP) with an anti-FLAG antibody. WB with an anti-Plk3 antibody showed that ubiquitination of the endogenous Plk3 protein after ectopic expression of ubiquitin was detectable and significantly elevated by Ni(II) and/or MG132 (Fig. 2, *I* and *J*). We next co-transfected both plasmids expressing FLAG-tagged ubiquitin and GFP-tagged Plk3 into HEK293T cells followed by the same IP experiment. We found that ectopically expressed Plk3 was also ubiquitinated and that Ni(II) and/or MG132 increased Plk3 ubiquitination, as shown in both IP and total protein samples (Fig. 2*K*). We repeated the experiment in Fig. 2*K* with extended controls. As shown in Fig. 2*L*, significant ubiquitination of endogenous Plk3 was detectable and enhanced by MG132 and/or Ni(II) when ubiquitin alone was transfected into HEK293T cells. This was also the case with ectopically expressed Plk3 when GFP-tagged Plk3 alone or GFP-tagged Plk3 and FLAG-tagged ubiquitin were introduced into HEK293T cells (Fig. 2*M*). As expected, MG132 and/or Ni(II) elevated ubiquitination of ectopically expressed Plk3. In contrast, much lower levels of ubiquitinated endogenous Plk3 were detected in HEK293T cells after co-transfection of empty GFP and FLAG vectors despite that MG132 enhanced the levels of the endogenous Plk3 in both control and nickel-treated cells (Fig. 2*N*).

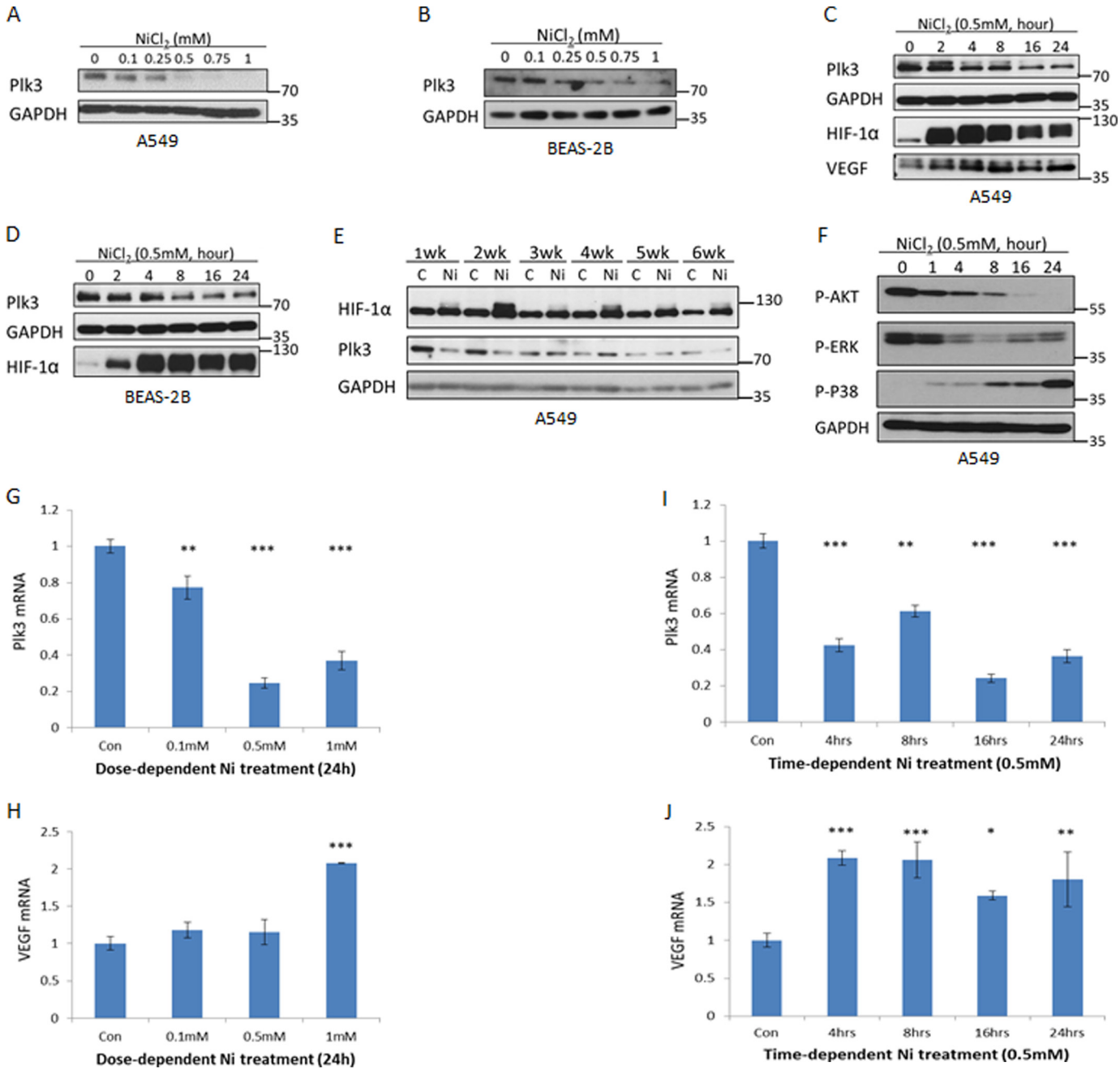

Figure 1. Ni(II) suppresses Plk3 expression. *A,* A549 lung carcinoma cells were treated with $NiCl_2$ at the indicated concentrations for 24 h and harvested for total cellular proteins with 1× sample buffer. Total cellular proteins were subjected to WB with the indicated antibodies. GAPDH was used as loading control. *B,* experiment was performed as in *A* with BEAS-2B cells instead. *C,* A549 cells were treated with 0.5 mM $NiCl_2$ for the indicated periods of time and then processed as in *A*. *D,* experiment was performed as in *C* with BEAS-2B cells. *E,* A549 cells were cultured for 6 weeks (passed every 3 days) using media containing 0 or 100 μM $NiCl_2$. Cells were harvested at indicated time periods and then processed as in *A*. *F,* A549 cells were treated with 0.5 mM $NiCl_2$ for the indicated periods of time. Cells were then harvested for total proteins and subjected to WB with the indicated antibodies (*P-AKT*, phospho-AKT; *P-p38*, phospho-p38). *G* and *H,* A549 cells were treated with $NiCl_2$ at the indicated concentrations for 24 h and harvested for total RNA with TRIzol reagent as described under "Experimental procedures." Total RNA samples were then subjected to real-time PCR analysis for Plk3 and VEGF-A mRNA levels as described under "Experimental procedures." GAPDH mRNA levels were used an internal control. *I* and *J,* A549 were treated with 0.5 mM $NiCl_2$ for indicated periods of time and then processed as in *G* and *H*. Data in *A–F* are representative of three or more independent experiments. Data in *G–J* are presented as mean ± S.D. ($n = 3$, *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

USP28 contributes to the suppression of Plk3 by Ni(II)

A previous study showed that the protein level of USP28, a deubiquitinase known to regulate MYC and HIF-1α (29–31), can be inhibited by Ni(II) or hypoxia in an HIF-dependent fashion (32). To check whether USP28 is also involved in the suppression of Plk3 by Ni(II) or hypoxia, we co-transfected FLAG-tagged ubiquitin and HA-tagged USP28 into A549 cells and examined the response of the endogenous Plk3 protein level. As shown in Fig. 3*A*, whereas USP28 did not significantly affect the basal level of Plk3 protein, it was able to largely reverse the suppressive effect of Ni(II) on Plk3. However, ectopic expression of USP28 without ubiquitin failed to prevent the suppres-

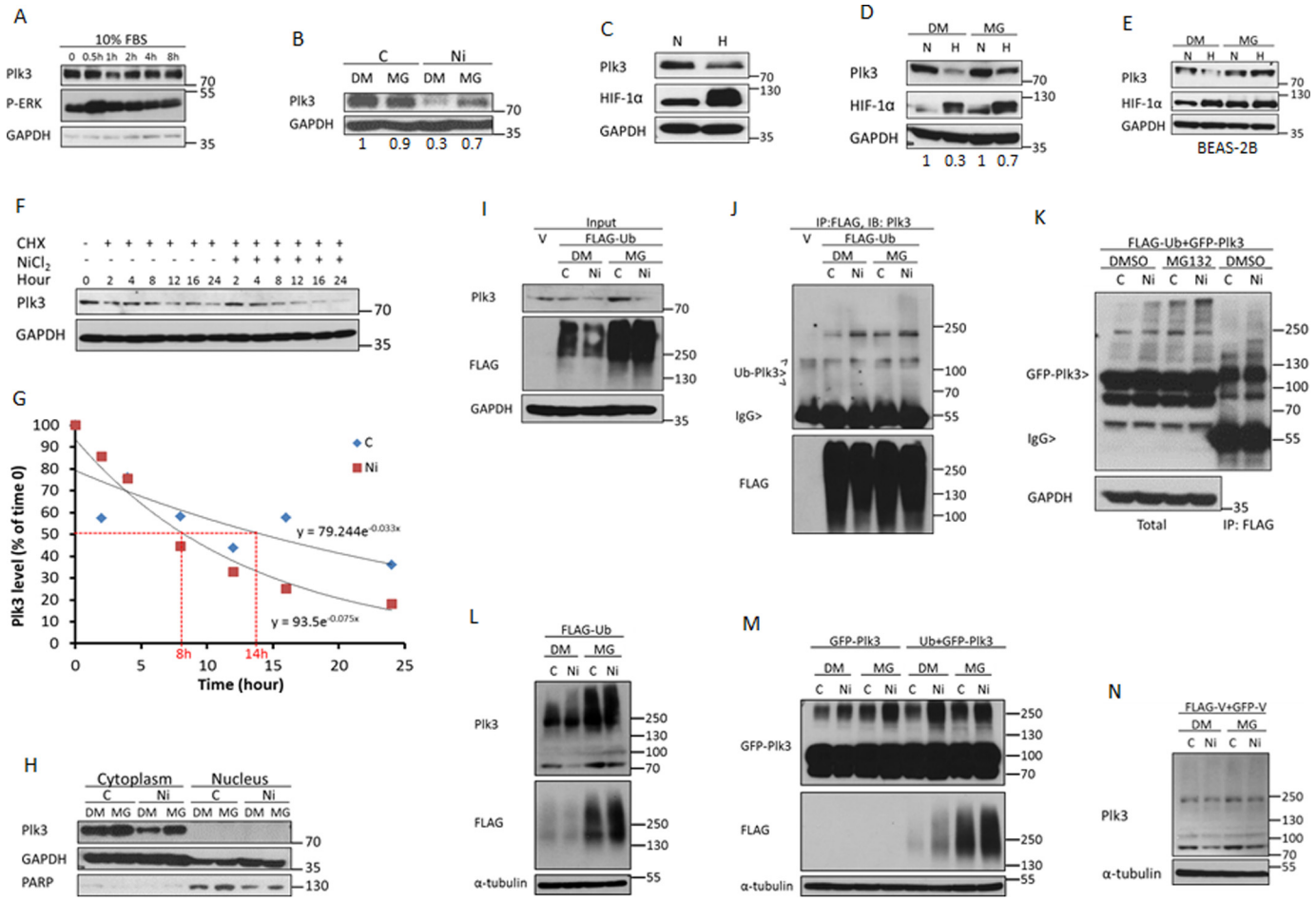

Figure 2. The Plk3 protein level is suppressed by Ni(II) or hypoxia primarily through proteasome-mediated protein degradation pathway. *A,* A549 cells were serum-starved overnight followed by treatment with 10% FBS for the indicated periods of time. Total cellular proteins were collected and subjected to WB with the indicated antibodies (*P-ERK*, phospho-ERK). *B,* A549 cells were either left untreated (*C*) or treated with 0.5 mM $NiCl_2$ (*Ni*) with or without the presence of 1 μM MG132 for 24 h. Cells were then harvested for WB with the indicated antibodies. Relative levels of Plk3 expression were determined by densitometry as shown at the *bottom*. *C,* A549 cells were treated with normoxia (*N*) or 1% O_2 (*H*) for 24 h and then processed as in *A*. *D,* A549 cells were treated with normoxia (*N*) or 1% O_2 (*H*) with or without the presence of 5 μM MG132 for 24 h. Cells were then harvested for WB with the indicated antibodies. Relative levels of Plk3 expression were determined by densitometry as shown at the *bottom*. *E,* experiment was performed as in *D* in BEAS-2B cells. *F,* A549 cells were treated with 50 μg/ml cycloheximide with or without the presence of 0.5 mM $NiCl_2$ for the indicated periods of time. Cells were then harvested for WB with the indicated antibodies. *G,* Plk3 protein signals shown in *F* were quantified by densitometry scanning, and the data were plotted. *H,* A549 cells were either left untreated (*C*) or treated with 0.5 mM $NiCl_2$ (*Ni*) with or without the presence of 1 μM MG132 for 24 h. Cell fractionation assay was then performed as described under "Experimental procedures." Proteins from cytosol and nucleus were subjected to WB with indicated antibodies. *I* and *J,* A549 cells were transfected with either an empty vector (*V*) or a plasmid expressing FLAG-tagged ubiquitin (*FLAG-Ub*) for 24 h. Cells were then either left untreated (*C*) or treated with 0.5 mM $NiCl_2$ (*Ni*) with or without the presence of 10 μM MG132 for 6 h. Total cellular proteins were collected and subjected to immunoprecipitation (*IP*) with an anti-FLAG antibody. The total cellular proteins (*inputs*, *I*) and IP products (*J*) and were then subjected to WB with the indicated antibodies. *K,* HEK293T cells were co-transfected with expression plasmids encoding FLAG-Ub and GFP-Plk3 for 24 h and then either left untreated (*C*) or treated with 0.5 mM $NiCl_2$ (*Ni*) for 24 h with or without the presence of 10 μM MG132 for 4 h. Cells were then processed with IP and WB as in *I* and *J*. *DM,* DMSO; *MG,* MG132. *L–N,* HEK293T cells were transfected with the expression plasmid encoding FLAG-Ub (*L*), or GFP-Plk3 (*M*), or FLAG-Ub and GFP-Plk3 (*M*), or FLAG vector (*FLAG-V*) and GFP vector (*GFP-V*) (*N*). Cells were then treated as in *K* and analyzed with WB using the indicated antibodies. Data shown are representatives of three or more independent experiments.

sion of endogenous Plk3 (Fig. 3, *B* and *C*), indicating that the effect of USP28 on the Plk3 protein requires the presence of excessive ubiquitin. Similar results were observed on the ectopically expressed Plk3 protein in HEK293T cells where GFP-tagged Plk3 was transfected with or without FLAG-tagged ubiquitin (Fig. 3, *D–F*). These data demonstrate that USP28 is able to stabilize Plk3 in the presence of ectopically expressed ubiquitin, likely by reducing its ubiquitination and preventing subsequent degradation. Note that the protein level of ectopically expressed USP28 could be inhibited by Ni(II) (Fig. 3, *A–F*), confirming the previous observation (32). The suppression of the ectopically expressed USP28 driven by a CMV promotor in the expression plasmid by Ni(II) suggests that this process is unlikely dependent completely on HIF-mediated transcriptional events. This observation is in agreement with the previous report (32).

To determine whether Plk3 can interact with USP28 directly, we ectopically expressed HA-tagged USP28 in A549 cells and performed a reciprocal IP with an anti-Plk3 antibody and an anti-HA antibody. As shown in Fig. 3, *G* and *H*, we did not detect any interaction between Plk3 and USP28. Similar IP experiments using HEK293T cells ectopically co-expressing Plk3 and USP28 produced the same result (Fig. 3, *I* and *J*). In contrast, specific interactions between Plk3 and HIF-1α as well as between USP28 and c-Myc were detectable (Fig. 3, *G* and *H*), as demonstrated in previous studies (10, 30). These data indi-

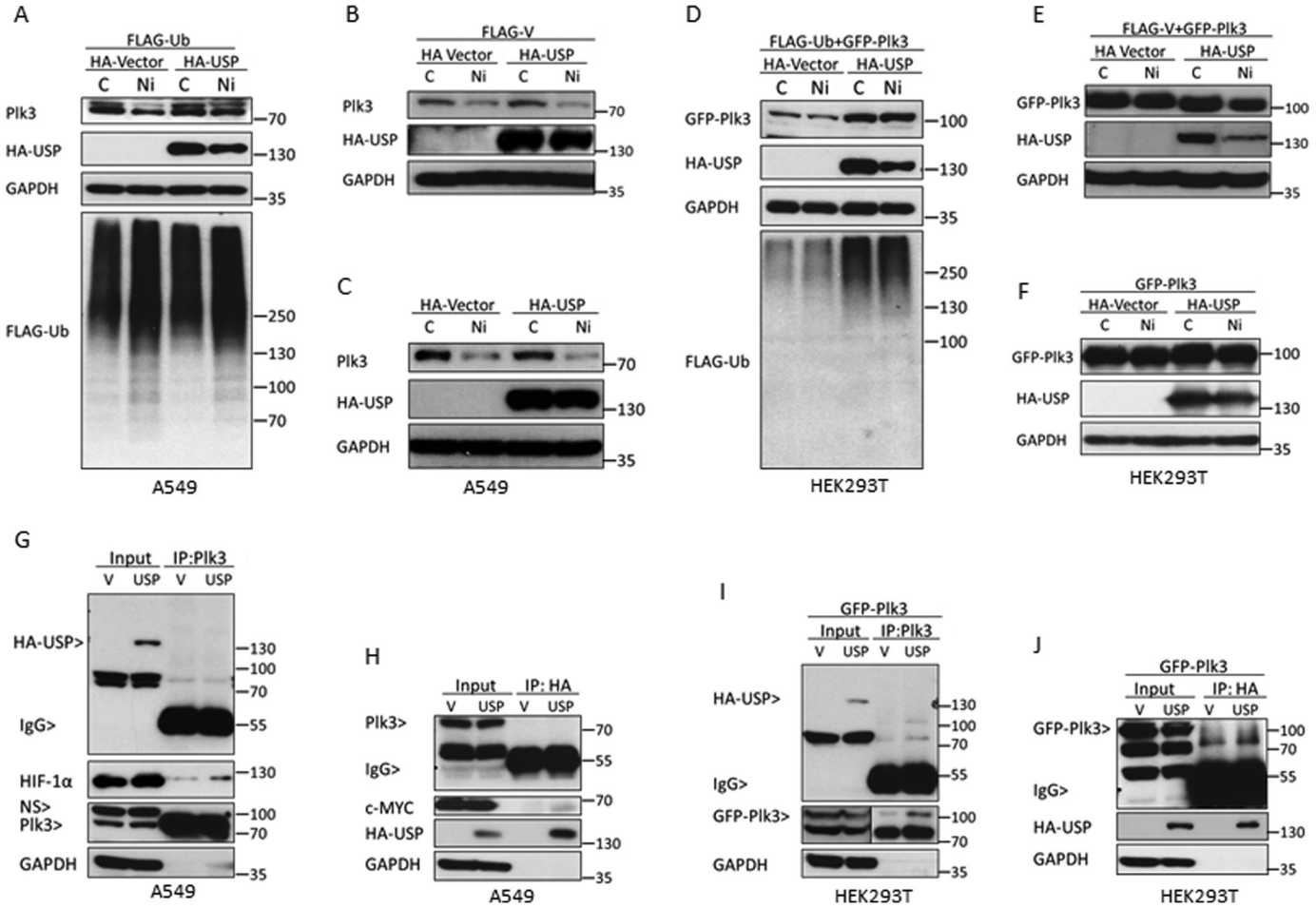

Figure 3. Inhibition of Plk3 protein expression by Ni(II) can be reversed by deubiquitinase USP28. *A,* A549 cells were transfected with expression plasmids encoding FLAG-Ub along with either empty vector (*HA-vector*) or HA-tagged USP28 (*HA-USP*) for 24 h. Cells were then either left untreated (*C*) or treated with 0.5 mM $NiCl_2$ (*Ni*) for 24 h. Total cellular proteins were then harvested for WB with an anti-Plk3 antibody to detect Plk3, an anti-HA antibody to detect HA-USP28, and an anti-FLAG antibody to detect FLAG-Ub. *B* and *C,* experiments were performed as in *A* either with replacement of the FLAG-Ub vector with the empty FLAG vector (*FLAG-V*) or without any FLAG vector. *D,* HEK293T cells were co-transfected with expression plasmids encoding FLAG-Ub, GFP-Plk3, and either an empty vector (*HA-vector*) or HA-tagged USP28 (*HA-USP*) for 24 h. Cells were either left untreated (*C*) or treated with 0.5 mM $NiCl_2$ (*Ni*) for an additional 24 h. Cells were then harvested for WB with the indicated antibodies. *E* and *F,* experiments were performed as in *D* either with replacement of the FLAG-Ub vector with FLAG-V or without any FLAG vector. *G,* A549 cells were transfected with expression plasmids encoding either empty HA-vector (*V*) or HA-USP28 (*USP*) for 24 h. Cellular proteins were subjected to IP with an anti-Plk3 antibody followed by WB with an anti-HA antibody to detect HA-USP28 and other indicated antibodies. *H,* experiment was performed as in *G* but with IP using an anti-HA antibody followed by WB using an anti-Plk3 antibody and other antibodies as indicated. *I,* HEK293T cells were co-transfected with expression plasmids encoding GFP-Plk3 and either an empty HA-vector (*V*) or HA-USP28 (*USP*) for 24 h. Cells were then processed as in *G. J,* experiment was performed as in *I* but with IP using an anti-HA antibody. WB was performed as in *H*. Data shown are representatives of three or more independent experiments.

cate that the effect of USP28 on the suppression of the Plk3 protein level by Ni(II) probably works through an indirect mechanism. It is also possible that the interaction between Plk3 and USP28 is weak and/or transient, which requires a more sensitive assay to detect.

Given that both Ni(II) and hypoxia reduce the Plk3 protein level and that these two conditions are both known to activate the HIF transcription factor by stabilizing its α subunit, we checked whether the suppression of Plk3 by Ni(II) is HIF-dependent. Our data show that the HIF pathway is unlikely to be directly involved in this process as ectopic expression or siRNA-mediated knockdown of HIF-1α showed limited effects on the Plk3 protein level (supplemental Fig. S1, *A* and *B*). Moreover, ectopic expression of pVHL or PHDs had little effect on the level of the Plk3 protein (supplemental Fig. S1, *C–E*). Furthermore, the CRL family ubiquitin ligases, which regulate HIF-α degradation, are unlikely involved in this process either because inhibition of this family of E3 ligases did not affect the Plk3 protein level (supplemental Fig. S1, *F* and *G*).

SIAH2 interacts with Plk3, mediates its ubiquitination, and promotes its degradation in response to Ni(II) or hypoxia

In search for the E3 ubiquitin ligase that mediates Plk3 ubiquitination in response to Ni(II) or hypoxia, SIAH2 caught our attention. SIAH2 is a RING finger E3 ubiquitin ligase that stabilizes HIF-1α by promoting ubiquitination and degradation of PHDs in response to hypoxia (22). Given that both Ni(II) and hypoxia inhibit Plk3 protein expression, we reasoned that SIAH2 could be the E3 ligase that mediates Plk3 ubiquitination and degradation. To test this, we first checked whether menadione, an SIAH2 inhibitor (33), could reverse the inhibitory effect of Ni(II) on the Plk3 protein level. As shown in Fig. 4, *A* and *B*, menadione dose-dependently elevated the basal level of Plk3 protein and reversed the suppressive effect of Ni(II). Sim-

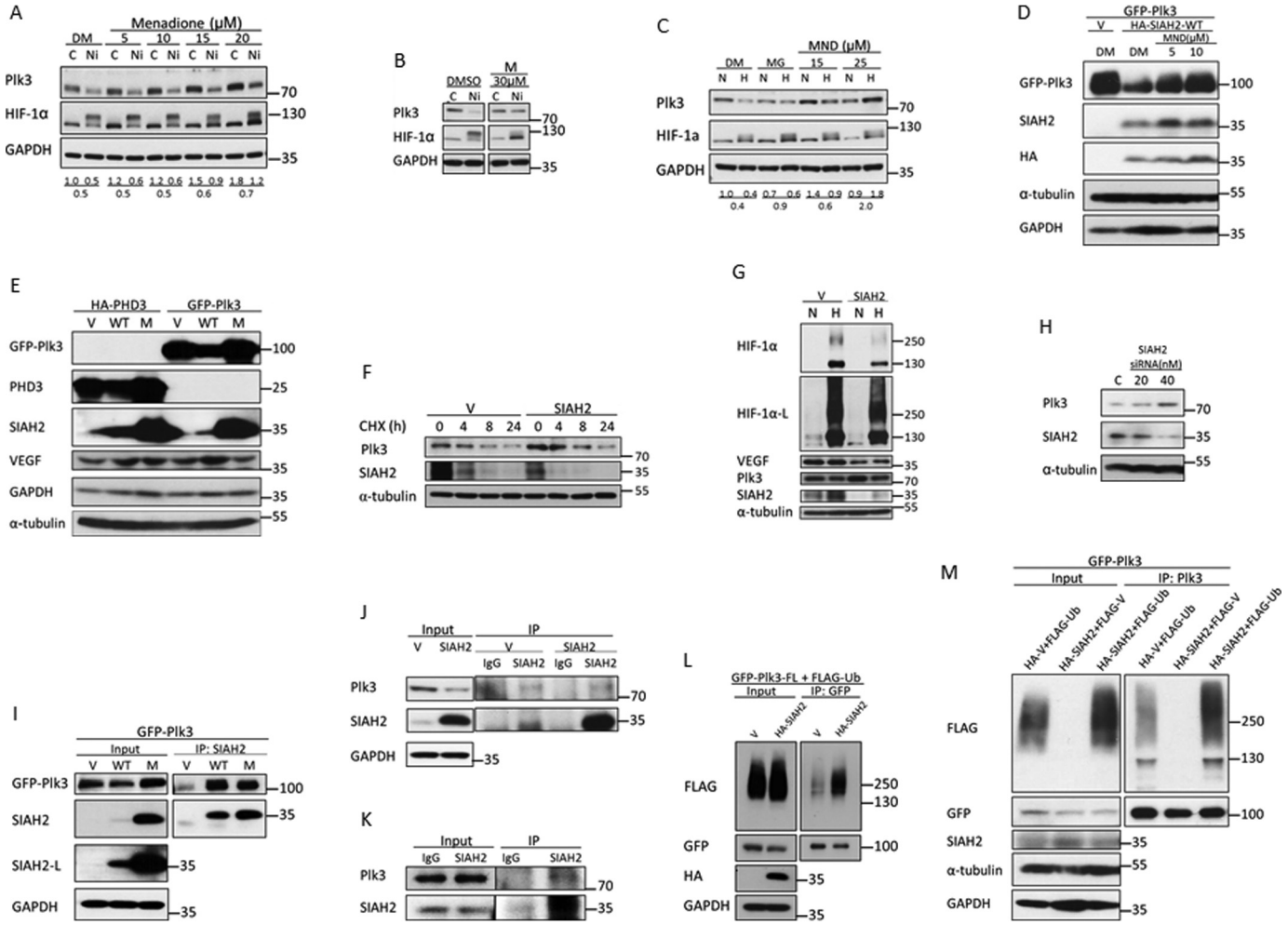

Figure 4. SIAH2 interacts with Plk3 and suppresses its protein expression. *A,* A549 cells were treated with either DMSO (vehicle, *DM*) or menadione at the indicated concentrations with or without the presence of 0.5 mM $NiCl_2$ for 24 h. Total cellular proteins were then collected and subjected to WB with the indicated antibodies. Relative levels of Plk3 expression (*upper row*) and ratios of nickel-treated and -untreated (*lower row*) were determined by densitometry as shown at the *bottom*. *B,* experiment was performed as in *A* with a higher concentration of menadione (*M*). *C,* A549 cells were treated with either DMSO or 5 μM MG132 or different concentrations of menadione (*MND*) under either normoxia (*N*) or 1% O_2 (*H*) for 24 h. Cells were harvested for total cellular proteins and subjected to WB with the indicated antibodies. Relative levels of Plk3 expression (*upper row*) and ratios of hypoxia-treated and -untreated (*lower row*) were determined by densitometry as shown at the *bottom*. *D,* HEK293T cells were co-transfected with expression plasmids encoding GFP-Plk3 along with either an empty vector (*V*) or HA-SIAH2-WT. Forty two hours after transfection, cells were treated with either DMSO (*vehicle, DM*) or menadione (*MND*) at the indicated concentrations for 6 h. Cells were then subjected to WB with the indicated antibodies. *E,* HEK293T cells were co-transfected with expression plasmids encoding either HA-PHD3 or GFP-Plk3 together with either an empty vector (*V*), HA-SIAH2-WT (*WT*), and HA-SIAH2-RING mutant (*M*) for 48 h. Cells were then harvested for total proteins and subjected to WB with the indicated antibodies. *F,* stable knockdown of SIAH2 in HEK293T cells were carried out as described under "Experimental procedures." Cells were then seeded into 6-cm culture dishes. Twenty four hours later, cells were treated with 50 μg/ml cycloheximide, harvested at the indicated periods of time after the treatment, and subjected to WB with the indicated antibodies. *V,* control shRNA; *SIAH2,* SIAH2 shRNA. *G,* HEK293T cells with or without stable knockdown of SIAH2 described in *F* were treated with normoxia (*N*) or 1% O_2 (*H*) for 6 h and then harvested for WB with the indicated antibodies. *H,* HEK293T cells were transfected with either a scrambled (*C*) or a SMARTpool siRNA oligonucleotide targeting SIAH2 at the indicated concentrations. Cells were harvested 96 h post-transfection and analyzed by WB with the indicated antibodies. *I,* HEK293T cells were co-transfected with expression plasmids encoding GFP-Plk3 along with either an empty vector (*V*), HA-SIAH2-WT (*WT*), or HA-SIAH2-RM (*M*). Forty two hours after transfection, cells were treated with 10 μM MG132 for an additional 6 h. Cells were then lysed and subjected to IP using an anti-SIAH2 antibody. Total proteins and IP products were subjected to WB with the indicated antibodies. *J,* HEK293T cells were transfected with expression plasmids encoding either an empty vector (*V*) or HA-SIAH2-WT (*SIAH2*) for 42 h followed by treatment with 10 μM MG132 for an additional 6 h. Cells were then lysed and subjected to IP with an anti-SIAH2 antibody or normal IgG (as control). Total proteins and IP products were subjected to WB with the indicated antibodies. *K,* A549 cells were treated with 10 μM MG132 for 6 h before harvesting for IP with an anti-SIAH2 antibody or normal IgG followed by WB with the indicated antibodies. *L,* HEK293T cells were co-transfected with expression plasmids encoding GFP-Plk3, FLAG-Ub, and the empty vector (*V*) or HA-SIAH2-WT (*HA-SIAH2*) for 36 h followed by treatment with 10 μM MG132 for an additional 12 h. Cells were then lysed and subjected to IP using an anti-GFP antibody followed by WB with the indicated antibodies. Ubiquitinated Plk3 was detected with an anti-FLAG antibody. *M,* HEK293T cells were co-transfected with expression plasmids encoding GFP-Plk3, HA-SIAH2-WT (*HA-SIAH2*), and the empty FLAG vector (*FLAG-V*) or FLAG-Ub for 42 h followed by treatment with 20 μM MG132 for an additional 6 h. Cells were then lysed and subjected to IP using an anti-Plk3 antibody. The inputs and IP products were then subjected to WB with the indicated antibodies. Data shown are representatives of three or more independent experiments.

ilar reversal effects were also observed under hypoxia (Fig. 4*C*). We next determined whether SIAH2 can reduce the protein level of Plk3. We ectopically expressed SIAH2 and Plk3 into HEK293T cells and then measured Plk3 protein levels with or without the presence of menadione. As shown in Fig. 4*D*, ectopic expression of SIAH2 strongly reduced the Plk3 protein level, and menadione largely prevented this effect. Note that the level of SIAH2 was also raised by menadione, consistent with

the notion that SIAH2 can mediate its own ubiquitination and degradation (23).

SIAH2 is known to promote ubiquitination and degradation of PHD3 thereby stabilizing HIF-1α (22, 23). Using this regulation as a positive control, we examined whether ectopically expressed SIAH2 can suppress ectopically expressed Plk3. We co-expressed wild-type or E3 ligase-deficient SIAH2, which contains a point mutation within its RING domain (34), along with either the HA-tagged PHD3 or GFP-tagged Plk3 in HEK293T cells followed by WB. As shown in Fig. 4*E*, we observed similar effects of SIAH2 on PHD3 and Plk3: wild-type but not E3 ligase-deficient SIAH2 reduced PHD3 and Plk3 levels. Note that wild-type SIAH2 was expressed at a much lower level than the E3 ligase-deficient one, consistent with its self-ubiquitination property (23). Importantly, reduced PHD3 or Plk3 expression as a result of co-expression of wild-type SIAH2 correlated with increased levels of VEGF-A (Fig. 4*E*). These data suggest that, as with PHD3, inhibition of the Plk3 protein by SIAH2 may produce a biological effect by altering VEGF-A expression.

To determine the effect of endogenous SIAH2 on endogenous Plk3 protein expression, we stably depleted SIAH2 using shRNA in HEK293T cells and chased the protein level of Plk3 over time after treating the cells with CHX to inhibit the protein synthesis. As shown in Fig. 4*F*, the basal (time 0) SIAH2 protein level was much lower in SIAH2 knockdown cells than in the control cells, indicating significant knockdown of SIAH2. In contrast, the basal Plk3 protein level was significantly higher in SIAH2 knockdown cells. The decay of the Plk3 protein level was also slower in SIAH2 knockdown cells than in the control cells after CHX treatment (Fig. 4*F*). These data confirm that degradation of Plk3 is indeed negatively correlated with the level of SIAH2 at physiological conditions.

To explore the potential functional significance of the regulation of Plk3 by SIAH2, we treated the SIAH2 knockdown HEK293T cells and the control cells with hypoxia and examined the effect of SIAH2 knockdown on the protein levels of VEGF-A and HIF-1α, two known downstream targets of SIAH2 and Plk3 (10, 15, 22, 23). Our results showed that both the basal and hypoxia-induced HIF-1α and VEGF-A levels were lower in SIAH2 knockdown cells than in control cells. In contrast, the levels of the Plk3 protein in both normoxic and hypoxic conditions were higher in SIAH2 knockdown cells than in control cells (Fig. 4*G*). Note that the SIAH2 protein levels were much lower in SIAH2 knockdown cells and elevated under hypoxia, consistent with previous findings (22, 23). These results indicate that SIAH2 may work with Plk3 to regulate the hypoxic response, through which production of angiogenic factors is altered.

To further confirm the regulation of Plk3 by SIAH2 at the endogenous level, we used siRNA to transiently knock down SIAH2, and measured the effect on the Plk3 protein level. As shown in Fig. 4*H*, dose-dependent depletion of endogenous SIAH2 using siRNA led to dose-dependent increases in the endogenous Plk3 protein in HEK293T cells. Together, our data strongly support the notion that SIAH2 helps suppress the level of Plk3 protein by facilitating Plk3 ubiquitination and degradation.

We next asked whether SIAH2 can directly interact with Plk3. We co-transfected GFP-tagged Plk3 with either wild-type or mutant SIAH2 into HEK293T cells followed by IP with an anti-SIAH2 antibody. As shown in Fig. 4*I*, a significant amount of Plk3 appeared in the immunocomplex of both the wild-type and mutant SIAH2 despite the weak nonspecific signal in the vector group. We next transfected only SIAH2 followed by immunoprecipitation with the anti-SIAH2 antibody. We found that the SIAH2 antibody but not normal IgG could immunoprecipitate endogenous Plk3 (Fig. 4*J*). Finally, immunoprecipitation of endogenous SIAH2 in A549 cells using an anti-SIAH2 antibody showed that the anti-SIAH2 antibody but not normal IgG could precipitate endogenous Plk3 (Fig. 4*K*). Together, our data establish that Plk3 physically and physiologically interacts with SIAH2.

Given that SIAH2 is a ubiquitin E3 ligase, we next checked whether SIAH2 promotes Plk3 ubiquitination. We co-expressed GFP-tagged Plk3 with either FLAG-tagged ubiquitin or FLAG-tagged ubiquitin along with HA-tagged SIAH2 in HEK293T cells. We then immunoprecipitated ectopically expressed Plk3 with an anti-GFP antibody and detected the level of ubiquitinated Plk3 using WB with an anti-FLAG antibody. As shown in Fig. 4*L*, co-expression of SIAH2 markedly elevated the level of ubiquitinated Plk3. Similar IP experiments using an anti-Plk3 antibody yielded an identical result (Fig. 4*M*). The lowered GFP-Plk3 levels in groups co-expressing SIAH2 further confirm the inhibition of Plk3 protein expression by SIAH2 (Fig. 4, *L* and *M*). These data together with the observation that SIAH2 directly interacts with Plk3 clearly show that SIAH2 directly mediates Plk3 ubiquitination.

SIAH2 binds to Plk3 and mediates Plk3 degradation primarily through its polo-box domain

Plk3 consists of an N-terminal kinase domain (KD) that confers its catalytic activity and a C-terminal PBD that mediates substrate recognition as well as protein-protein interaction (21). Given the observed regulation of Plk3 by SIAH2, we asked which domain of Plk3 is important for its interaction with SIAH2 and degradation. We co-transfected HA-tagged wild-type SIAH2 along with either the empty FLAG vector or various FLAG-tagged Plk3 constructs (full-length, KD, and PBD) into HEK293T cells. SIAH2 is known to promote its own degradation by self-ubiquitination (23). To reduce this effect, we added MG132 6 h before harvesting cells. We then performed IP with an anti-SIAH2 antibody and checked for potential co-IP of the Plk3 protein using WB with an anti-FLAG antibody. We found that both full-length and deletion mutants of Plk3 were able to interact with SIAH2 (Fig. 5*A*, *right panel*).

When comparing the results from the input and the IP, we noticed that although the KD of Plk3 was expressed at the highest level (Fig. 5*A*, *left panel, 3rd lane*), it co-IP with SIAH2 at the lowest level (Fig. 5*A*, *right panel, 3rd lane*). In contrast, although PBD was expressed at the lowest level (Fig. 5*A*, *left panel, 4th lane*), it co-IP with SIAH2 at a much higher level, almost equivalent to that of full-length Plk3 (Fig. 5*A*, compare *2nd lane* with *4th lane* of the *right panel*). These data indicate that SIAH2 had the strongest interaction with PBD. We next performed the same co-IP experiment with the ligase-deficient mutant SIAH2

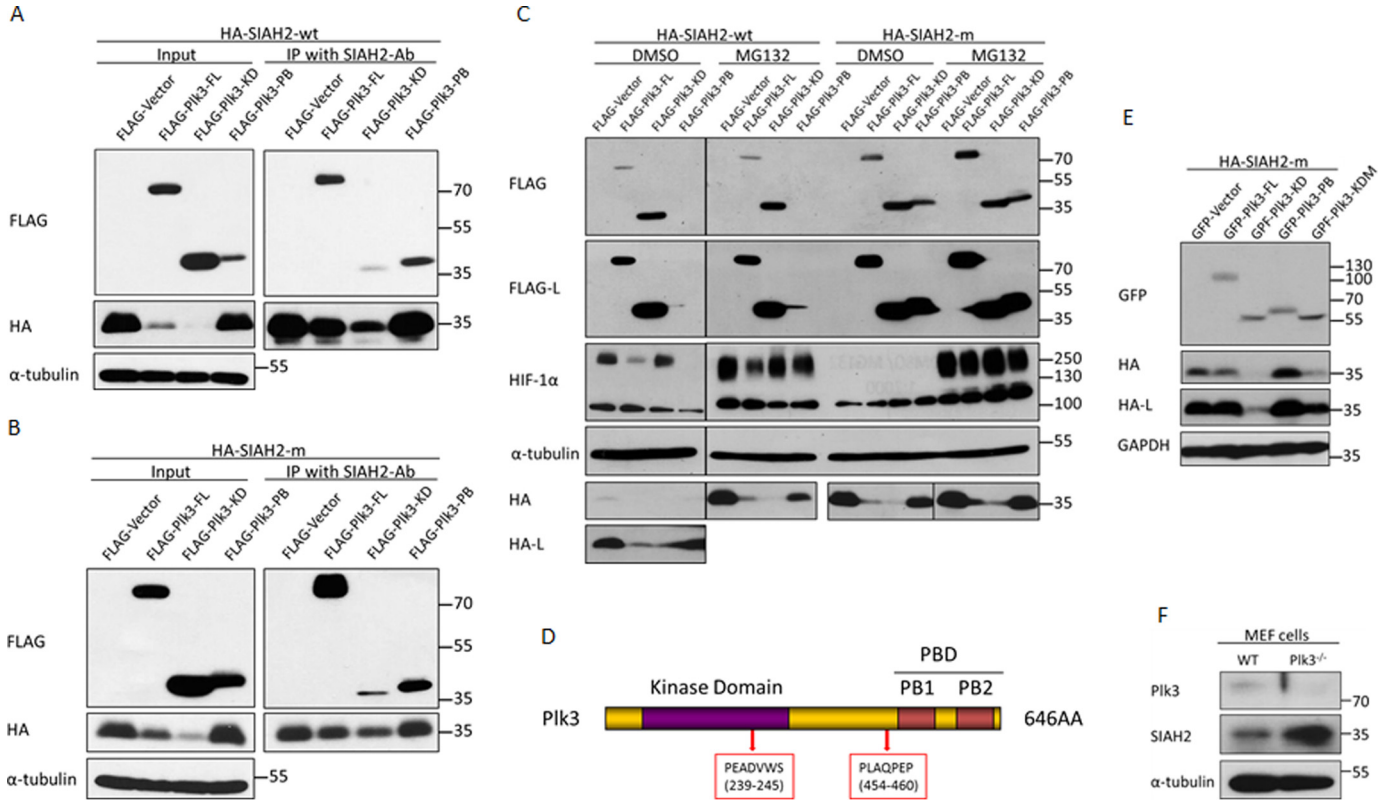

Figure 5. SIAH2 binds to both the KD and PBD domains of Plk3 but targets Plk3 degradation mainly through the PBD domain. *A,* HEK293T cells were co-transfected with expression plasmids encoding HA-tagged wild-type SIAH2 (*HA-SIAH2-WT*) along with either FLAG vector (*FLAG-Vector*), FLAG-tagged Plk3 full-length (*FLAG-Plk3-FL*), FLAG-tagged Plk3 kinase domain (*FLAG-Plk3-KD*), or FLAG-tagged Plk3-Polo-box domain (*FLAG-Plk3-PB*) for 42 h followed by treatment with 10 μM MG132 for an additional 6 h. Cells were then lysed and subjected to IP using an anti-SIAH2 antibody followed by WB with the indicated antibodies. *B,* HEK293T cells were co-transfected with expression plasmids encoding HA-tagged SIAH2-RING mutant (*HA-SIAH2-m*) along with either FLAG-Vector, FLAG-Plk3-FL, FLAG-Plk3-KD, or FLAG-Plk3-PB for 48 h. Cells were then harvested for WB with the indicated antibodies. *C,* HEK293T cells were co-transfected with expression plasmids encoding HA-SIAH2-WT or HA-SIAH2-m along with either FLAG-Vector, FLAG-Plk3-FL, FLAG-Plk3-KD, or FLAG-Plk3-PB. Forty two hours after transfection, cells were treated with either DMSO (vehicle) or 10 μM MG132 for an additional 6 h. Cells were then harvested for total cellular proteins and subjected to WB with the indicated antibodies. *D,* schematic diagram of Plk3 with the two possible SIAH2 interaction regions indicated. *E,* HEK293T cells were co-transfected with expression plasmids encoding HA-SIAH2-m with either GFP-tagged vector (*GFP-Vector*), Plk3-full-length (*GFP-Plk3-FL*), Plk3-Kinase domain (*GFP-Plk3-KD*), Plk3-Polo-box domain (*GFP-Plk3-PB*), or Plk3-Kinase domain mutant-KD^{T219D} (*GFP-Plk3-KDM*). Cells were lysed and processed as in *C* 48 h after transfection. *F,* littermate-paired WT and *PLK3*$^{-/-}$ MEFs were cultured as described under "Experimental procedures" and harvested for WB with indicated antibodies. Plk3 was detected with the Fnk antibody. Data shown are representatives of three or more independent experiments.

and found that PBD was expressed at a much higher level (Fig. 5, *A* and *B,* compare *4th lane* of the *left panels*), suggesting that the stability of PBD is highly sensitive to the E3 ligase activity of SIAH2. Again, all three Plk3 proteins interacted with mutant SIAH2, confirming the result in Fig. 5*A*. This result also suggests that the E3 ligase activity is not required for the interaction between Plk3 and SIAH2. Similar to the wild-type SIAH2, although the KD of Plk3 had the highest level of expression, it interacted with SIAH2 the least (Fig. 5*B*, *right panel, 3rd lane*). These data establish that although both the KD and PBD of Plk3 can interact with SIAH2, binding between PBD and SIAH2 is much stronger. Consistently, PBD was more susceptible to SIAH2-mediated degradation (Fig. 5, *A* and *B*).

To further confirm the SIAH2-mediated degradation of Plk3, we repeated the transfection experiments described in Fig. 5, *A* and *B,* with or without treatment with the proteasome inhibitor MG132. Our results showed that wild-type SIAH2 suppresses the Plk3 protein level in a proteasome-dependent fashion, manifested as higher levels of the Plk3 protein in the mutant SIAH2 groups than in the wild-type SIAH2 groups and in MG132-treated groups than in the untreated groups (Fig. 5*C*, compare FLAG signals between groups). These data also confirm that PBD is responsible for much of the SIAH2-mediated degradation of the Plk3 protein as the levels of PBD were much higher in the mutant SIAH2 groups than in the wild-type SIAH2 groups (Fig. 5*C*, compare FLAG signals between wild-type and mutant SIAH2 groups). Furthermore, inhibition of the proteasome significantly increased the level of PBD, especially in the wild-type SIAH2 group (Fig. 5*C*, compare FLAG signals between MG132-treated and -untreated groups). In contrast, the levels of KD remained relatively constant regardless of the treatments and SIAH2 mutation status (Fig. 5*C*). Note that the HIF-1α protein levels were elevated as a result of ectopic expression of wild-type SIAH2 but not mutant SIAH2 and that inhibition of the proteasome increased the endogenous HIF-1α protein levels in both wild-type and mutant SIAH2 groups (Fig. 5*C*). These results are consistent with the previous findings that SIAH2 up-regulates the HIF-1α protein level by promoting ubiquitination and degradation of the prolyl hydroxylase (22). Also note that wild-type SIAH2 was expressed at very low levels, which required long exposure to detect and were significantly elevated by inhibition of the proteasome (Fig. 5*C*, *HA versus HA-L*). In contrast, mutant SIAH2 was expressed at much higher levels and MG132 only had a very small effect on its expression. These

observations are consistent with the fact that mutant SIAH2 has limited E3 ligase activity toward itself (34).

SIAH2 binds to its substrates via a predicted consensus sequence (P*X*A*X*V*X*P) (35, 36). We analyzed the amino acid sequence of Plk3 and identified two regions that resemble the consensus SIAH2-binding sequence (Fig. 5*D*). One site is located within the kinase domain of Plk3 (^{239}PEADVWS^{245}). The other site resides slightly N-terminal of the polo-box domain (^{454}PLAQPEP^{460}). The first site contains a substitution of proline (an aliphatic amino acid) by serine (a hydrophilic residue) at the last position. The second site appears to be more close to the consensus sequence, with a substitution of valine by a proline (both are aliphatic) at the 5th position. The locations and the characteristics of the two potential SIAH2-binding sites are in line with our observations that although both the KD and PBD of Plk3 bind to SIAH2, PBD has a much stronger interaction with SIAH2 and is more prone to degradation (Fig. 5, *A* and *B*).

Plk3 regulates SIAH2 stability in a kinase activity-dependent fashion

During the analysis of our co-transfection experiments investigating the regulation of Plk3 by SIAH2, we observed that ectopic co-expression of full-length Plk3 or the KD but not the PBD of Plk3 dramatically inhibited the protein levels of both wild-type and mutant SIAH2 (Fig. 5, *A–C*, *HA signals*). Note that the KD of Plk3 showed a stronger effect than full-length Plk3. These data suggest that Plk3 regulates SIAH2 stability in a KD-dependent manner. To confirm this, we repeated the co-transfection experiment shown in Fig. 5*B* using GFP-tagged Plk3 expression plasmids (as oppose to FLAG-tagged constructs in Fig. 5*B*), with an additional group co-transfected with a plasmid encoding the Plk3 KD containing a T219D point mutation that renders it kinase activity deficient (15). Our results showed that GFP-tagged Plk3 had the same effect on mutant SIAH2 as did FLAG-tagged Plk3 (compare *HA signals* of the *1st 4 lanes* of Fig. 5*E* with *HA signals* of *B*). As expected, mutant Plk3 KD had a much reduced ability of suppressing the level of mutant SIAH2 (Fig. 5*E*, *HA signals*, compare *3rd lane* with *5th lane*). These results confirm that SIAH2 was indeed suppressed by Plk3 in a kinase activity-dependent manner. Finally, we checked whether the SIAH2 protein level is altered under the conditions where Plk3 is absent using MEFs isolated from littermates of wild-type and *PLK3* knock-out mice (10). As indicated in Fig. 5*F*, the SIAH2 protein level is significantly higher in *PLK3*$^{-/-}$ MEFs, supporting the notion that Plk3 suppresses SIAH2 protein expression.

The SIAH2 protein level is regulated by Ni(II)

Given that Ni(II) suppresses the Plk3 protein level and that SIAH2 mediates Plk3 ubiquitination and degradation, we asked whether SIAH2 is regulated by Ni(II). The level of SIAH2 protein has been shown to increase under various stress conditions, including hypoxia (23). The level or activity of SIAH2 is also subjected to regulation by a number of protein kinases, including the p38 MAPK (23). To explore whether and how Ni(II) may regulate SIAH2, we first treated HEK293T cells with Ni(II) for 6 h with or without the presence of MG132 and examined the effect on the endogenous SIAH2 protein level. We found that Ni(II) treatments resulted in a significant increase in the SIAH2 protein level regardless of the presence of MG132 (Fig. 6*A*). We then performed a side-by-side comparison of the responses in HEK293T and A549 cells using the same experimental protocol. As shown in Fig. 6*B*, Ni(II) elevated endogenous SIAH2 protein levels in both HEK293T and A549 cells. We next examined the effect of Ni(II) on ectopically expressed SIAH2. We transfected either the empty vector or the HA-tagged wild-type SIAH2 into HEK293T cells followed by Ni(II) treatment for 6 h before harvesting for WB analysis. As shown in Fig. 6*C*, Ni(II) significantly raised the level of ectopically expressed SIAH2 (compare both SIAH2 and *HA signals*). The results from these experiments establish that the SIAH2 protein level can indeed be induced by Ni(II).

Given our data showing that the Plk3 protein is mostly located in the cytoplasm where nickel-induced degradation occurs (Fig. 2*H*), we examined how the endogenous SIAH2 protein is regulated by Ni(II) in different subcellular locations. We performed cell fractionation analyses on A549 cells with or without the Ni(II) treatment. As shown in Fig. 6*D*, SIAH2 appeared in both the cytoplasm and the nucleus. Ni(II) treatments significantly raised the level of SIAH2 in the cytoplasm but not in the nucleus. A similar pattern of cytoplasmic SIAH2 levels was detected when cells were treated with hypoxia (Fig. 6*E*). Note that the nuclear level of Plk3 was very low (Fig. 6*D*), confirming the results shown in Fig. 2*H*. However, the nuclear SIAH2 protein appeared to behave differently in response to Ni(II) *versus* hypoxia, as indicated by the reduced level in presence of Ni(II) *versus* the slightly elevated level under hypoxia (compare nuclear SIAH2 between Fig. 6, *D* and *E*). Nonetheless, the increase in the level of cytoplasmic SIAH2 in response to Ni(II) or hypoxia is in line with the notion that SIAH2 mediates Plk3 ubiquitination and degradation in the cytoplasm where Plk3 is predominantly located.

SIAH2 has been shown to be phosphorylated by p38 MAPK under hypoxia thereby promoting its nuclear localization (23). It has also been reported that activation of AKT by hypoxia contributes to the increase in the SIAH2 protein level by promoting its transcription (23). Our data appear to agree with these findings for the hypoxic condition (see Fig. 6*E*). However, our results showed that the activities of AKT and ERK were significantly suppressed by Ni(II) treatments, whereas that of p38 was dramatically increased (Fig. 1*F*). These data exclude the possibility that the elevated SIAH2 protein level in response to Ni(II) is a direct result of AKT- or ERK-mediated transcriptional events. The dramatic increase in p38 activity in response to Ni(II) prompted us to examine whether p38 activation affects the suppression of the Plk3 protein level by Ni(II). As shown in Fig. 6*F*, SB203580, a specific p38 inhibitor, at two different concentrations had no effect on the inhibition of the Plk3 protein level by Ni(II). These results indicate that while showing some similarities, the mechanisms underlying the regulation of Plk3 by SIAH2 in response to Ni(II) and hypoxia are not identical.

Discussion

The tumor-suppressive function of Plk3 and the evidence connecting Plk3 to the hypoxic response and tumor angiogen-

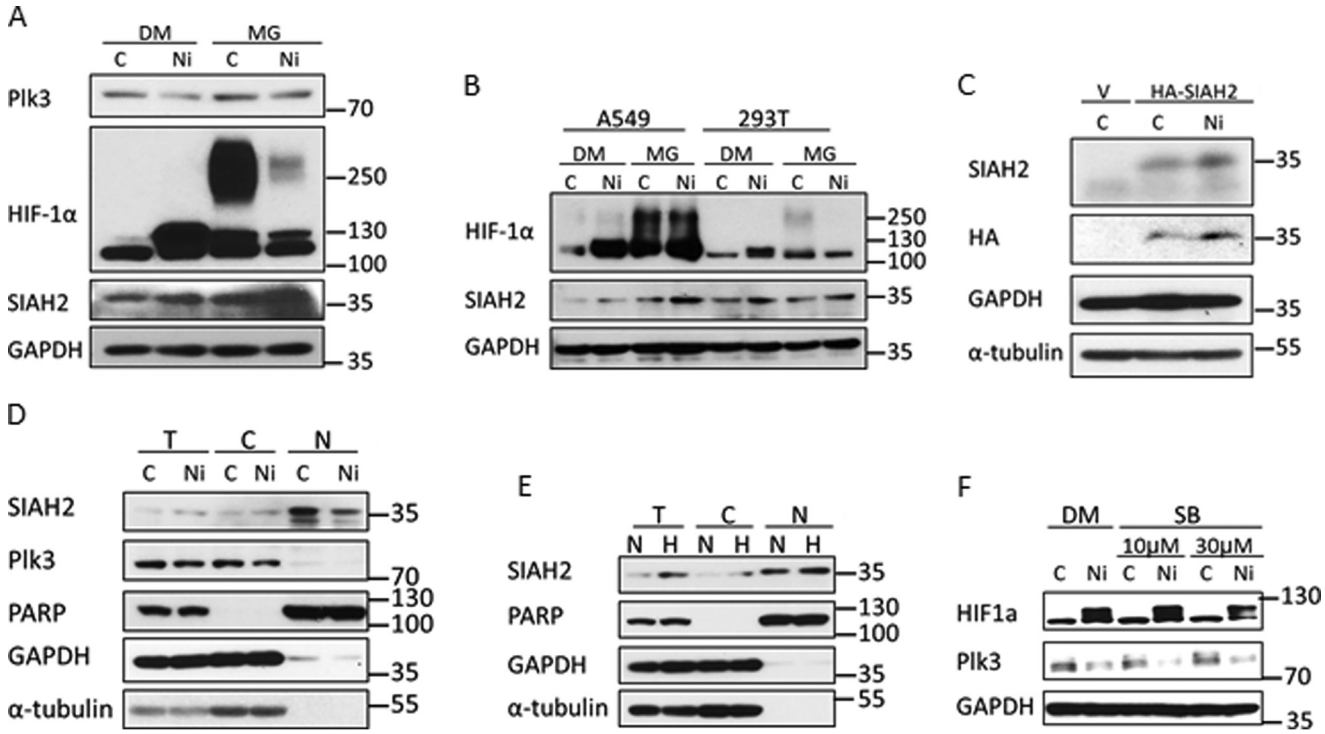

Figure 6. Ni(II) increases both endogenous and ectopically expressed SIAH2. *A,* HEK293T cells were pretreated with DMSO (*vehicle, DM*) or 10 μM MG132 (*MG*) for 1 h and then either left untreated (*C*) or treated with 0.5 mM $NiCl_2$ (*Ni*) for an additional 6 h. Cells were then harvested for total cellular proteins and subjected to WB with the indicated antibodies. *B,* experiment was performed as in *A* with A549 and HEK293T cells. *C,* HEK293T cells were transfected with expression plasmids encoding empty vector (*V*) or HA-SIAH2-WT (*HA-SIAH2*). Forty two hours after transfection, cells were either left untreated (*C*) or treated with 0.5 mM $NiCl_2$ (*Ni*) for an additional 6 h. Cells were then harvested and processed as in *A*. *D,* A549 cells were either left untreated (*C*) or treated with 0.5 mM $NiCl_2$ (*Ni*) for 12 h. Cell fractionation assay was then performed, and proteins from total lysate (*T*)/cytosol (*C*)/nucleus (*N*) were subjected to WB with the indicated antibodies. *E,* A549 cells were treated with normoxia (*N*) or 1% O_2 (*H*) for 6 h and then processed as in *D*. *F,* A549 cells were pretreated with DMSO (*vehicle, DM*) or different concentrations of SB203580 (*SB*) for 2 h and then either left untreated (*C*) or treated with 0.5 mM $NiCl_2$ (*Ni*) for an additional 24 h. Cells were then harvested for WB with the indicated antibodies. Data shown are representatives of three or more independent experiments.

esis demonstrate a potential involvement of this protein kinase in Ni(II) carcinogenesis as well as tumor progression in general. In an effort to understand this process, we investigated the regulation of Plk3 protein expression by Ni(II) and hypoxia in this study, with a focus on lung carcinoma and lung epithelial cells. We found that Plk3 can be suppressed by Ni(II) or hypoxia in these cells. We discovered a novel mechanism in which Ni(II) induces expression of SIAH2, a RING finger ubiquitin E3 ligase, which in turn directly mediates ubiquitination and subsequent degradation of Plk3 by the proteasome. We also found that USP28, a deubiquitinase known to be inhibited by Ni(II), may also contribute to the inhibition of the Plk3 protein level by Ni(II). We show that the PBD of Plk3 is the primarily domain that mediates its interaction with SIAH2 and degradation by UPS. We further revealed that Plk3 destabilizes SIAH2 in a kinase activity-dependent manner. These novel findings provide new mechanistic insights on the role of Plk3 in Ni(II) carcinogenesis and tumor progression, particularly in the lung.

PLK3 is considered an immediate early response gene whose mRNA expression is induced by serum growth factors. Thus, Plk3 mRNA expression is highest in the G_1 phase of the cell cycle and returns to the basal level in a few hours (17). Despite the oscillation of the Plk3 mRNA level, its protein level remains constant throughout the cell cycle. These early observations demonstrate an apparent disconnection between the mRNA and protein levels of this protein. The present study confirms this finding in A549 lung carcinoma cells as we did not detect a significant change in the Plk3 protein level after FBS treatment of serum-starved A549 cells (Fig. 2*A*). Previous studies showed that poor correlations between mRNA and protein expression are common in cellular proteins (37). Plk3 appears to be one of these proteins. The stable nature of the Plk3 protein appears to suggest that it may have a very long half-life. However, our calculated half-life of endogenous Plk3 in A549 cells under normal conditions is about 14 h (Fig. 2*G*), which is not very long considering the median half-life of mammalian proteins is 46 h (38). Ni(II) treatment reduces the half-life of Plk3 to about 8 h (Fig. 2*G*). Thus, the apparent stability of the Plk3 protein may be determined by other mechanisms, *e.g.* a closely regulated translational process. Further investigation is needed to clarify this. A previous report showed that ectopically expressed Plk3 is quickly degraded by UPS in the nucleus (18). Here, we show that endogenous Plk3 can also be degraded in the cytoplasm via UPS, particularly in response to Ni(II) or hypoxia. This is an important observation as most of the Plk3 protein is located in the cytoplasm (Figs. 2*H* and 6*D*).

USP28 is a deubiquitinase that suppresses the stability of MYC and HIF-1α (30, 31, 39). It can be suppressed by Ni(II) via HIF-, UPS-, and DNA methylation-dependent mechanisms (32). Because our results show that the HIF pathway is not directly involved in the suppression of Plk3 by Ni(II) (supplemental Fig. S1), the mechanism underlying the effect of USP28 on Plk3 is likely HIF-independent. Our finding that USP28 prevents the suppression of Plk3 by Ni(II) (Fig. 3, *A* and *D*) suggests

that suppression of Plk3 deubiquitination by USP28 in response to Ni(II) could contribute to the elevated degradation of Plk3 by UPS. However, the effect of USP28 on Plk3 appears to require the excessive presence of ubiquitin (Fig. 3, *A*–*F*). Moreover, we were unable to detect a direct interaction between Plk3 and USP28. These data suggest that USP28 may regulate Plk3 indirectly or that the interaction between the two proteins is weak and/or transient. Of note, although USP28 has been reported to mediate HIF-1α stability, a direct interaction was also undetectable (39).

The finding that SIAH2 is the E3 ligase for Plk3 is both novel and significant in our view. This is the first E3 ubiquitin ligase for Plk3 identified thus far. The fact that SIAH2 is closely associated with the hypoxic response highlights its logical connection to the regulation of HIF signaling by Plk3 (10, 15, 22). This connection is reinforced by the finding that Plk3, while suppressed by SIAH2, in turn suppresses SIAH2 (Fig. 5). Thus, the involvement of Plk3 in the cellular hypoxic response appears to be a lot more complex than previously described (10, 15). Collective evidence supports a new signaling paradigm: under normoxia, Plk3 suppresses the hypoxic response by phosphorylating and destabilizing HIF-1α and SIAH2 (10); under hypoxia or the hypoxia-like condition induced by Ni(II), the level/activity of SIAH2 increases and the level of USP28 decreases, which suppress the protein levels of Plk3 and PHDs. Reduced levels of Plk3 and PHDs in turn help maintain HIF-1α and SIAH2 proteins at higher levels. This complex regulatory mechanism potentially puts Plk3 in an important position in a signaling network that functions to fine-tune the cellular hypoxic response.

It is intriguing that SIAH2 regulates HIF-1 signaling through both PHDs and Plk3. The reason for cells to have these two apparently redundant mechanisms may lie in the fact that Plk3 suppresses HIF-1α in a pVHL-independent manner, whereas the inhibition of HIF-1α by PHDs requires pVHL (10). The existence of the SIAH2-Plk3-HIF-1α pathway in addition to the SIAH2-PHD-HIF-1α axis would provide an extra layer of safeguard to ensure adequate suppression of the HIF-1 activity should the pVHL function be compromised. Another important point to consider is that suppression of the Plk3 protein level by Ni(II) or hypoxia is a relatively slow process that requires a few hours to occur (Fig. 1, *C* and *D*). This feature suggests that mutual regulation between Plk3 and SIAH2 may be more functionally relevant to chronic hypoxia or hypoxia-like conditions, such as solid tumors or chronic exposure to Ni(II).

The mechanisms for suppression of the Plk3 level by Ni(II) and hypoxia are clearly not identical. Other than the finding that the mechanism for regulation of USP28 by hypoxia and Ni(II) are not the same (Fig. 3 and supplemental Fig. S1 and discussion therein), the mechanism underlying the alteration of SIAH2 by these two conditions may not be identical either. Previous work showed that hypoxia promotes SIAH2 transcription via an AKT-dependent manner and triggers nuclear translocation of SIAH2 in a p38-dependent manner (22). Our data do not seem to support that these mechanisms are responsible for the induction of SIAH2 by Ni(II) (Fig. 6). Hypoxia has also been shown to regulate SIAH2 via other mechanisms,

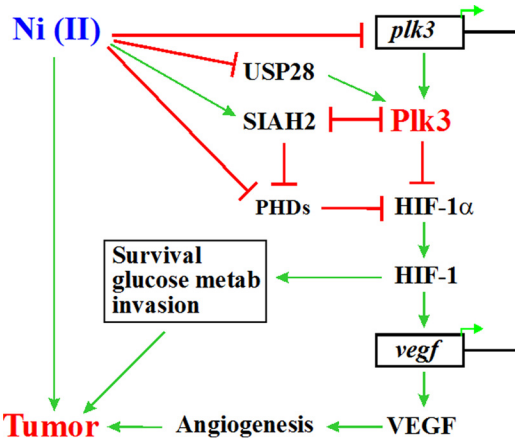

Figure 7. Proposed model on the mechanism underlying the involvement of Plk3 in HIF signaling and Ni(II) carcinogenesis. Ni(II) suppresses the cellular level of Plk3 via SIAH2-mediated ubiquitination and proteasome degradation. Ni(II) may also suppress Plk3 expression by inhibiting its mRNA expression and by suppressing the USP28-mediated deubiquitination of the Plk3 protein. Plk3 suppresses the levels of HIF-1α and SIAH2 via phosphorylation-dependent mechanisms. Thus, inhibition of Plk3 by Ni(II) elevates the cellular activities of both the HIF transcription factor and SIAH2 thereby promoting Ni(II)-induced carcinogenesis and tumor progression.

including phosphorylation by protein kinases (*e.g.* DYRK2, HIPK2, JNK, and Src) and the deubiquitinase USP13 (23). These mechanisms are believed to regulate the protein level, the ligase activity, or the subcellular localization of SIAH2 (23). Whether these mechanisms also contribute to the regulation of SIAH2 by Ni(II) remains to be determined. However, it is known that the PBD of Plk3 binds to phosphorylated protein motifs and mediates protein-protein interactions via a phosphorylation-dependent manner (40). Because our data show that Plk3 binds to SIAH2 largely through its PBD, it will be interesting to learn whether phosphorylation of SIAH2 by aforementioned kinases triggers its interaction with Plk3 thereby facilitating the mutual regulation of these two proteins.

This newly discovered function of Plk3 is conceivably important for Ni(II) carcinogenesis and tumor progression. Exposure to Ni(II) elevates the level/activity of SIAH2 thereby suppressing the level of Plk3. This reduces phosphorylation of HIF-1α and SIAH2 by Plk3 thereby stabilizing HIF-1α, promoting the HIF-dependent hypoxic response, and leading to cell transformation/tumor cell survival. Of note, SIAH2 is considered an oncogene in multiple tissues, including the lung. It promotes tumorigenesis through the Ras signaling pathway as well as the hypoxic response pathway (22, 24). Accordingly, elevated expression of SIAH2 has been detected in lung cancers (23, 24). The biological significance of this regulatory network, through which Plk3 may regulate carcinogenesis, clearly warrants further investigations, particularly *in vivo*. Fig. 7 outlines the proposed regulatory network involving Plk3 and the potential mechanism underlying the involvement of Plk3 in Ni(II) carcinogenesis discussed herein.

Experimental procedures

Cell lines and cell culture

BEAS-2B human lung bronchial epithelial cells, A549 human lung adenocarcinoma cells, and HEK293T cells were obtained from American Type Culture Collection. Cells were cultured in

DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin sulfate) at 37 °C in a humidified atmosphere containing 5% CO_2. Primary wild-type and *PLK3*$^{-/-}$ MEFs were derived from embryonic day 14.5 embryos of the respective genotype, produced from the crossing of *PLK3*$^{-/-}$ mice as described previously (15). Primary MEFs were cultured in DMEM supplemented with 15% FBS and antibiotics (as above) under 5% CO_2. Hypoxia was induced by placing cells in a hypoxia/tissue culture chamber (Billups-Rothenberg, Inc.) filled with 1% O_2, 5% CO_2, and 94% N_2.

Antibodies, reagents, and plasmids

Antibodies to P-AKT, P-ERK, P-p38, poly(ADP-ribose) polymerase, HA, and α-tubulin were purchased from Cell Signaling Technology. Antibodies to SIAH2, GFP, HA, Fnk, c-Myc, and GAPDH were purchased from Santa Cruz Biotechnology. The antibody to HIF-1α was purchased from Bethyl Laboratories. The antibody to VEGF-A was purchased from Abcam. The antibody to M2-FLAG was purchased from Sigma. The Plk3 antibody was obtained from BD Biosciences. MG132 was purchased from Cayman Chemical. Menadione was purchased from MP Biomedicals. SB203580 was purchased from Abcam. Scramble control oligonucleotides siRNA (D-001810-0X) and the siGENOME SMARTpool (M-006561-02) against SIAH2 were purchased from Dharmacon.

pcDNA3-FLAG-HA was a gift from William Sellers (Addgene plasmid 10792). HA-USP28-phCMV2 was a gift from Stephen Elledge (Addgene plasmid 41948). HA-SIAH2-pcDNA3 and HA-SIAH2-RM-pcDNA3 (RING mutant) were kind gifts from Dr. Ze'ev Ronai at Sanford Burnham Prebys Medical Research Institute. The plasmid encoding FLAG-tagged ubiquitin has been described previously (41). Plasmids encoding eGFP-Plk3 full-length (amino acids 1–646), eGFP-Plk3-KD (amino acids 1–334), eGFP-Plk3-KDM (KD^{T219D}), and eGFP-Plk3-PBD (amino acids 312–646) have been described previously (15). Constructs of FLAG-Plk3-FL (amino acids 1–646), FLAG-Plk3-KD (amino acids 1–334), and FLAG-Plk3-PBD (amino acids 335–646) were generated by inserting appropriate PCR fragments into the multiple cloning site of p-CMV5-FLAG vector. MISSION pLKO.1-puro plasmids, non-target shRNA control (SHC016), and SIAH2-specific (TRCN0000007416) shRNA were purchased from Sigma. Plasmid transfection was performed using Lipofectamine reagents from Invitrogen according to the protocol provided by the manufacturer.

Western blotting

Standard Western blotting procedure was used throughout the study. SDS-PAGE was carried out using the mini-gel systems from Bio-Rad. Proteins were electrophoretically transferred to PVDF membranes. The membrane was blocked with TBST containing 5% non-fat dry milk and then incubated overnight with primary antibodies using dilutions suggested by the manufacturers. After extensive washing with TBST to remove the primary antibodies, the membrane was then incubated with secondary antibodies conjugated with HRP for 1 h at room temperature. After extensive washing with TBST, signals on the membrane were developed using an ECL system (Pierce).

Real-time PCR

Cells were treated and harvested for total RNAs with TRIzol reagent (Life Technologies, Inc.). Total RNAs were then converted to cDNA using a RT-PCR kit (Clontech) and subjected to real-time PCR using SYBR Green master mix (Clontech) and a real-time PCR machine (Stratagene Mx3005P). The primers used for qPCR are follows: PLK3, 5′-TTTTCGCACCACTTTGAGGAC-3′/5′-GAGGCCAGAAAGGATCTGCC-3′; VEGF-A, 5′-AGGGCAGAATCATCACGAAGT-3′/5′-AGGGTCTCGATTGGATGGCA-3′; GAPDH (as internal control), 5′-ACAACTTTGGTATCGTGGAAGG-3′/5′-GCCATCACGCCACAGTTTC-3′.

RNA interference

For transient RNAi, HEK293T cells were cultured to 30–50% confluence and transfected with a scrambled or a SMARTpool siRNA oligonucleotide targeting SIAH2 using Lipofectamine 2000 reagent according to manufacturer's provided protocol. Cells were analyzed 96 h post-transfection. For stable knockdown, non-target control or SIAH2-specific shRNA plasmids were transfected into HEK293T cells using Lipofectamine reagent followed by selection with 2 μg/ml puromycin for 2 weeks.

Protein half-life analysis

Cycloheximide was added to cells at a final concentration of 50 μg/ml to block new protein synthesis with or without nickel treatment. Cells were then harvested at the indicated time points for WB analysis to detect endogenous Plk3. Signals were quantified by densitometry and plotted. Half-life from each group was determined according to the plot.

Immunoprecipitation

Cells were lysed in the RIPA buffer supplemented with protease and phosphatase inhibitors (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1 mM DTT, 100 mM NaF, 1 mM sodium orthovanadate, 500 μM PMSF, 2 μM pepstatin A, 10 μM leupeptin). Total cell lysates were then cleared by centrifugation. One μg of antibody and 40 μl of protein G-agarose resin (50:50, Thermo Fisher Scientific) were then added to 1 mg of each cell lysate and incubated at 4 °C for 3 h to overnight followed by extensive washing with the lysis buffer. Proteins on the resin were then extracted with SDS sample buffer and then subjected to analyses by WB.

Cell fractionation assay

Cell fractionation experiments were carried out on ice or at 4 °C throughout. Cells were washed with ice-cold PBS and collected by centrifugation. Cell pellets were then resuspended and lysed in Buffer H (10 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 0.5% Triton X-100, 1 mM DTT, 100 mM NaF, 1 mM sodium orthovanadate, and protease inhibitors) as total cell lysates. After centrifugation for 8 min at 1500 $\times$ *g*, supernatants were transferred to fresh tubes and cleared again by centrifugation for 15 min at 14,000 $\times$ *g*. The supernatants after the second centrifugation were kept as cytoplasmic fractions. The pellets after the first centrifugation were washed

twice with Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and protease inhibitors) and collected by centrifugation for 10 min at 1500 × *g*. The pellets were then resuspended and lysed in Buffer C (10 mM HEPES, pH 7.9, 500 mM NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% Nonidet P-40, 1 mM DTT, and protease inhibitors) followed by extensive vortexing and centrifugation for 15 min at 14,000 × *g*. The supernatants were transferred to fresh tubes and kept as nuclear extracts.

Statistical analysis

All data were expressed as means ± S.D. SPSS 13.0 software was used for one-way ANOVA and LSD *t* test in statistical analyses. For more than two groups, one-way ANOVA was used first to detect the difference among these groups. If the *p* value was less than 0.05, multiple comparisons were then performed using LSD *t* test to detect the difference between any two groups. All statistical tests were two-sided. A *p* value less than 0.05 was considered significant.

Author contributions—C. L. conducted most of the experiments and participated in the experimental design, data analysis, and manuscript writing; S. P. and X. Z. conducted some of the experiments and participated in the data analysis; W. D. provided some of the reagents and critically reviewed the manuscript. D. X. designed and directed the study as well as wrote the manuscript.

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