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Inhibition of Siah ubiquitin ligase function

Andreas Möller[1,2,*], **Colin M. House**[1,*], **Christina S.F. Wong**[1], **Denis B. Scanlon**[2], **Mira C.P. Liu**[1], **Ze'ev Ronai**[3], and **David D.L. Bowtell**[1,2]

[1]Peter MacCallum Cancer Centre, Cancer Genomics and Biochemistry Laboratory, St. Andrews Place, East Melbourne, Victoria 3002 (Australia)

[2]University of Melbourne, Department of Biochemistry and Molecular Biology, Parkville, Victoria 3010 (Australia)

[3]The Burnham Institute for Medical Research, La Jolla, CA

Abstract

Tumor hypoxia induces the up-regulation of Hif-1alpha which in turn induces the expression of genes including VEGF to recruit new blood vessel outgrowth, enabling tumor growth and metastasis. Interference with the Hif-1 pathway and neoangiogenesis is an attractive anti-tumor target. The hydroxylation of Hif-1alpha by PHD proteins during normoxia serves as a recognition motif for its proteasomal degradation. However, under hypoxic conditions, hydroxylation is inhibited and furthermore, PHD proteins are themselves poly-ubiquitylated and degraded by Siah ubiqiuitin ligases. Our data demonstrate for the first time that inhibition of the interaction between Siah and PHD proteins using a peptide derived from a *Drosophila* protein interferes with the PHD degradation. Furthermore, cells stably expressing the inhibitor display reduced up-regulation of Hif-1alpha protein levels and Hif-1 mediated gene expression under hypoxia. In a syngeneic mouse model of breast cancer, the inhibitor reduced tumor growth and neoangiogenesis and prolonged survival of the mice. In addition, levels of Hif-1alpha and its target Glut-1 are reduced in the inhibitor expressing tumors. These data demonstrate, in a proof-of-principle study, that Siah protein, the most upstream component of the hypoxia pathway yet identified, is a viable drug target for anti-tumor therapies.

Introduction

Solid tumor growth is associated with areas of poor oxygen supply within the tumor mass. The main cellular response to hypoxic stress is the up-regulation of hypoxia responsive genes, including VEGF, Glut-1, and CA9 (Semenza, 1999). The key transcription factor for the hypoxic response pathways is hypoxia-inducible factor 1 (Hif-1) (Iyer et al., 1998; Schofield & Ratcliffe, 2004; Semenza, 2000). Hif-1 consists of two subunits, Hif-1alpha and Hif-1beta. In the Hif-1 complex, the Hif-1beta subunit is constitutively present within the cell whereas Hif-1alpha is stabilized under hypoxic conditions (Semenza, 1999). Under normoxia, Hif-1a is hydroxylated at two conserved proline residues (Pro402 and Pro564) by prolyl-hydroxylases (PHDs) (Epstein et al., 2001; Jaakkola et al., 2001). The Pro402/Pro564 hydroxylated Hif-1alpha protein has increased affinity for the E3 ubiquitin-protein ligase complex composed of the von Hippel-Lindau tumor suppressor protein VHL, elongin B & C and cullin 2. After being poly-ubiquitylated, Hif-1alpha is degraded by the 26S proteasome (Huang et al., 1998; Maxwell et al., 1999; Salceda & Caro, 1997). Furthermore Hif-1alpha is

Corresponding author: Tel: +61-3-96561356 Fax: +61-3-96561414 d.bowtell@petermac.org.
*these authors contributed equally to this work

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hydroxylated at Asn803 by the asparaginyl hydroxylase factor inhibiting Hif-1 (FIH) (Lando et al., 2002). This hydroxylation prevents the interaction between Hif-1alpha and transcriptional co-activators CBP/p300 (Lando et al., 2002). Recently we and others have shown that, under hypoxic conditions, members of the Siah ubiquitin ligase family can poly-ubiquitylate and target PHD and FIH for proteasomal degradation (Fukuba et al., 2007; Khurana et al., 2006; Nakayama et al., 2004; Nakayama et al., 2007), thus stabilizing Hif-1alpha.

Protein ubiquitylation is a three-step, enzymatic process (Hochstrasser, 2000). The specificity of the process is defined by the final step in which a ubiquitin ligase, either alone or as part of a complex, transfers ubiquitin to the substrate protein (Glickman & Ciechanover, 2002). Specificity is achieved by ubiquitin ligases recognising specific degradation signals, or “degrons” (Laney & Hochstrasser, 1999). *Drosophila* Seven in Absentia (SINA) and mammalian Seven in Absentia Homologue (Siah) are RING-containing proteins that function in protein degradation as ubiquitin ligases. In *Drosophila*, SINA has been shown to co-operate with Phyllopod (PHYL), Ebi and UBCD1 to facilitate the ubiquitylation and degradation of the transcriptional co-repressor tramtrack 88 (TTK88) (Boulton et al., 2000; Li et al., 1997; Tang et al., 1997).

In mammalian cells, Siah proteins can poly-ubiquitylate several seemingly unrelated proteins, with Siah1 and Siah2 having overlapping functions. Among the substrates are prolyl-hydroxylases (PHD1 and PHD3)(2004), co-repressors such as nuclear co-repressor (N-CoR) (Zhang et al., 1998) and CTBP-interacting protein (CtIP) (Germani et al., 2003), as well as the TGF-beta induced early gene-1 (TIEG1) (Johnsen et al., 2002).

The human Siah1 and murine Siah1a and Siah1b differ to human and murine Siah2 only in their amino-terminus (Della et al., 1993; Hu et al., 1997). Siah is a dimeric protein consisting of an N-terminal RING domain followed by two novel zinc finger motifs and a highly conserved C-terminal substrate-binding domain (SBD) (House et al., 2006; Polekhina et al., 2002). We have reported previously that many Siah-binding proteins contain a common binding motif that may act as a ‘degron’, or degradation signal centered around a VxP motif (House et al., 2003). More recently, using the crystal structure, we have identified critical amino acids in the Siah1a protein, including the conserved Met180 and Lys158, that permit binding to interacting proteins (House et al., 2006).

Here we show the development of a peptide inhibitor of Siah function derived from the *Drosophila* PHYL sequence. Interference with the Siah substrate binding site inhibits Siah function due to reduced Siah/substrate interaction. In cell based assays PHYL reduces Siah-mediated proteasomal degradation of PHD proteins. Cell lines with stable PHYL expression showed both reduced Hif-1alpha stabilization and Hif-1 mediated gene expression under hypoxic conditions. Addition of cell-permeable PHYL peptide to cells inhibited Hif-1alpha stabilization under hypoxia. Tumors expressing PHYL grew more slowly compared to control tumors, had apparently reduced neo-angiogenesis and were associated with prolonged survival in a syngeneic mouse model of breast cancer. These data suggest that inhibitors acting at the Siah/substrate binding site may function as therapeutic agents.

Results

Regulation of PHD protein abundance by Siah is influenced by PHYL peptide

A previous study had demonstrated that the prolyl hydroxylases, PHD1 and PHD3, are important Siah2 substrates (Nakayama et al., 2004). In U2OS, as well as HEK293 cells, (Figure 1A and data not shown) expression of Siah2 could mediate the reduction of PHD1 proteins levels. We sought to investigate the potential for over-expressed PHYL protein to

act as an inhibitor of mammalian Siah. We expressed a stable fragment of PHYL (1–130) containing the previously reported binding motif (House et al., 2003), the core residues of which are VxP (amino acid residues 120–122). Mutation of this motif to NxN was previously shown to ablate Siah binding (House et al., 2003). Wild type PHYL, but not PHYL NxN (VxP mutated to NxN) was able to recover the PHD1 protein levels to that of PHD1 expression without Siah2 (Figure 1A). Consistent with previous reports (Nakayama et al., 2004), Siah2 expression did not influence PHD2 protein levels, which were also unaffected by PHYL or PHYL NxN expression (Figure 1B). Expression of Siah2 together with PHD3, however, led to a very strong reduction of PHD3 levels (Figure 1C), similar to these observed for PHD1. PHYL was again able to interfere with the reduction of PHD3 protein levels, whereas PHYL NxN did not alter the Siah2 mediated reduction of PHD3 (Figure 1C). Nakayama and co-workers (Nakayama et al., 2004) found that Siah2 was more efficient than Siah1 in destabilizing over-expressed PHD3. We show that Siah1 (human) and Siah1a (mouse) reduced PHD3 protein levels similar to that observed with Siah2 (Figure 1D) but Siah1a M180K (a substrate-binding groove mutant) did not alter the PHD3 protein levels (Suppl Fig. 1), suggesting that the Siah substrate-binding groove is important for PHD ubiquitylation and degradation. Similar observations using the PHYL inhibitor were obtained using another Siah substrate, TIEG-1 (Suppl Fig.2).

PHYL inhibitor is not targeted for degradation

To investigate whether PHYL inhibitor functions as an alternate substrate for degradation or simply competes for the Siah binding site with substrate proteins, we expressed increasing amounts of PHYL with a constant amount of Siah2 or increasing amounts of Siah2 with a constant amount of PHYL. Increasing amounts of Siah2 was not associated with any reduction in PHYL in HEK293 (Figure 2A) and other cell lines (data not shown), though a substrate (PHD3) was destabilized under identical conditions (Fig. 2B). Generally, Siah2 was undetectable due to rapid turnover. Interestingly, increasing amounts of PHYL led to an increase of Siah2 protein levels (Figure 2A), in HEK293 cells, though this effect was not observed in U2OS cells (data not shown). Siah proteins appear to undergo rapid auto-ubiquitylation (Depaux et al., 2006) and the finding in HEK293 cells implies that the SBD of Siah proteins is involved in this process, or that constant occupancy of the binding groove may inhibit it. By contrast, Siah2 was not stabilized by the expression of high amounts of PHD3 substrate (Figure 2B), suggesting that flooding cells with Siah2 substrates does not stabilize Siah2. Expression of increasing amounts of PHYL alone resulted in no changes of PHD3 protein levels, whereas Siah2-mediated PHD3 degradation could be inhibited (Figure 2C). Taken together, these data demonstrate that the VxP motif in PHYL forms a stable, non-degradable association with Siah proteins and blocks their interaction with Siah substrates.

Cell-permeable PHYL peptide inhibits Hif-1alpha stabilization during hypoxia

To test the effect of PHYL inhibitor on the hypoxic response and Hif-1alpha stabilization, we synthesized a short, cell-permeable peptide consisting of the penetratin transducing peptide N-terminally linked to $PHYL_{108–130}$ through a 10-proline linker (Pen-P10-PHYL). The penetratin peptide (residues 43–58 from *Drosophila* Antennapedia protein) has been shown to transduce attached peptides and proteins into cells (Chen et al., 1999; Derossi et al., 1994). Addition of this peptide to U2OS cells prior to hypoxia treatment (2% oxygen) for 2 hours inhibited the stabilization of Hif-1alpha, compared to cells treated with control peptides ($PHYL_{107–130}$, P10-$PHYL_{108–130}$ or an unrelated penetratin-containing peptide, Pen-CRIPT) (Fig. 2D). This peptide (Pen-P10-PHYL) was also able to inhibit the effect of over-expressed Siah1a on TIEG-1 stability in HEK293 cells (Supplementary figure 3), and in those experiments also stabilized Siah1a protein, as observed when PHYL protein was over-expressed (Figures 2A and 2C), suggesting that the transduced peptide was interacting

with cellular Siah. The control peptide P10-PHYL had no effect on TIEG-1 or Siah1a levels in those experiments.

In development of this peptide inhibitor, other peptides were tested unsuccessfully, including TAT fusions with PHYL peptide containing ten, two or no proline spacers (data not shown). Peptides were also tested as competitors in an ELISA, based on the interaction between immobilized GST-$PHYL_{108–130}$ and free Siah1a, and the TAT-containing peptides were inactive. The Pen-P10-$PHYL_{107–130}$ peptide was only slightly less potent in ELISA than $PHYL_{107–130}$ alone, but it should be noted that high concentrations of peptide (50–100 μM), added in the absence of serum, were required to inhibit Hif-1alpha stabilization in hypoxic cells, whereas 1–3 μM was totally inhibitory in ELISA (data not shown). It is not clear if this is due to poor entry into cells or a susceptibility to proteolysis.

Cellular hypoxic gene expression, tumor growth and neoangiogenesis is reduced by PHYL

We next examined the physiological consequences of blocking Siah-mediated disruption of PHD/Hif-1 signaling. To further investigate whether PHYL expression impairs the hypoxic response pathway, we generated stable EO771 murine epithelial breast cancer cell lines expressing vector (EO771-vec), PHYL 1–130 (EO771-PHYL WT) or PHYL 1–130 NxN (EO771-PHYL NxN) (Figure 3A). Cell lines did not differ in their proliferation or morphology (data not shown). Hif-1alpha was induced in EO771-vector cells after exposure to hypoxia and this was strongly reduced in EO771 cells stably expressing PHYL 1–130 (Figure 3B). Hif-1alpha induction in EO771 cells stably expressing PHYL 1–130 NxN did not differ from EO771-vec cells (Figure 3B), demonstrating the specificity of the ability of PHYL to block Hif-1alpha up-regulation.

We measured hypoxia-induced gene expression of known Hif-1 target genes by quantitative reverse transcriptase PCR (qRT-PCR). Hypoxia resulted in a 6.4-fold induction of VEGF-A, 3.7-fold for COX4 and 12.5-fold for Rab20 in EO771-vec (Figure 3C) but this was significantly attenuated in EO771-PHYL 1–130 lines (Figure 3C). VEGF, COX4 and Rab20 were induced by hypoxia in EO771-PHYL 1–130 NxN to similar levels as in EO771-vec cells (Figure 3C), demonstrating that the inhibitory function of PHYL can interfere with the role of Siah proteins in regulating the hypoxic response pathway. Similar findings were observed using human U2OS cells stably expressing PHYL or PHYL NxN (data not shown).

Hypoxia leads to new blood vessel growth in solid tumors to support the growing tumor. EO771 cells are derived from a spontaneously occurring breast cancer in a C57Bl/6 mouse (Casey et al., 1951) and provide a syngeneic breast cancer model in immuno-competent mice to test the effect of Siah inhibition. We observed that EO771-vec and EO771-PHYL 1–130 NxN tumors grew rapidly compared with EO771-PHYL 1–130 tumors (Figure 4A). Tumors expressing PHYL also appeared less vascularized and paler (Figure 4A). Analysis of the growth time of the EO771 tumors to reach the maximum ethically allowed diameter of 1cm showed that mice with EO771-vec expressing tumor cells survived 21.3+/− 0.6 days and mice with EO771-PHYL 1–130 NxN tumors 20.9+/− 0.4 days, showing no significant difference between both groups. However, mice with EO771-PHYL 1–130 tumors survived 27.3+/− 1.1 days, significantly longer than mice with EO771-vec ($p<0.05$) and EO771-PHYL 1–130 NxN ($p<0.05$) expressing tumors ($n = 7, 6$ and 6 for these groups).

Histologically, the tumors showed few areas of necrosis (Figure 4A), however, fewer CD31 positive cells, a marker of endothelial cells, were seen in EO771-PHYL 1–130 tumor sections, compared with EO771-vec or EO771-PHYL 1–130 NxN tumors (Figure 4B). Similarly, staining revealed that EO771-PHYL 1–130 tumors expressed lower levels of Hif-1alpha (Figure 4C) suggesting that inhibition of Siah proteins reduces the up-regulation

of Hif-1alpha in tumors. Also, the induction of a Hif-1 target, Glut-1, was severely impaired in EO771-PHYL 1–130 tumors (Figure 4D).

Collectively, these findings demonstrate that the PHYL peptide can inhibit hypoxic responses *in vitro*, leading to impaired tumor growth and expression of Hif-1alpha and its target genes in a breast cancer model.

Discussion

In this study, we have shown that the high affinity Sina interactor, PHYL, can function as an inhibitor of mammalian Siah proteins. Using an expressed protein fragment of PHYL (residues 1–130), or a smaller synthetic peptide (residues 108–130), we have shown inhibition of Siah function in over-expression studies, suppression of Hif-1alpha stabilization under hypoxic conditions, and in a syngeneic breast cancer model, a slowing of tumor growth and angiogenesis.

Inhibitors of the proteasome, such as bortezomib (Velcade, PS341), have been approved for treatment in cancer therapy (Kane et al., 2003). To increase target specificity, inhibitors further upstream in the degradation pathway have been sought, with the ubiquitin ligases being a major target. So far, the ubiquitin ligases have not been shown to exhibit a catalytic activity, but merely to function as scaffolds for ubiquitin transfer, suggesting that inhibition will have to target protein/protein interactions. This study provides proof-of-principle for targeting the Siah substrate-binding domain to inhibit Siah function, Hif-1alpha stabilization and tumor growth. By using peptide and protein fragments from the *Drosophila* PHYL protein to specifically target the substrate-binding groove on Siah, we have shown that competitive inhibition at this site impairs Siah function and suggests that a screen for small molecule inhibitors binding at the same site may be warranted. Similar peptide inhibitor strategies have been used in the past to establish proof-of-principle for targets such as HDM2 ((Bottger et al., 1997) (Wasylyk et al., 1999) (Chene et al., 2000) Bcl-2 (Wang et al., 2000) (Holinger et al., 1999) and Hif-1alpha (Freedman et al., 2003) prior to searches for small molecule inhibitors. It is generally recognized that development of small molecules for inhibiting protein-protein interactions may be difficult, though our data, both biochemical and structural, demonstrates that the interaction between Siah and PHYL (and other substrates) is focused on a minimal motif of three amino acids, VxP, with some contribution from distal amino acids. Given the size of this motif (VRP is approximately 350 Daltons), it is feasible that the binding groove on the Siah protein could accommodate a small molecule (<500 Da) that would preclude binding of the VxP motif and function as a Siah inhibitor.

We have shown here that Siah inhibitors can stabilize substrates PHD1, PHD3, and TIEG-1 in over-expression studies. Siah inhibition was also shown to inhibit Hif-1alpha stabilization during hypoxia and expression of downstream transcriptional targets such as VEGF, COX4 and Rab20. These findings demonstrate for the first time that the strategy of targeting upstream regulation of Hif-1 via Siah-mediated degradation of PHD proteins is viable. This is important, as it demonstrates that inhibition of Siah protein-protein interaction is mechanistically possible and that it leads to predictable and desired biological effects *in vitro* and *in vivo*. It is noteworthy that the outcome was not certain, given that the interaction of Siah with PHD proteins has not been precisely mapped to a well-defined VxP motif in PHD.

Inhibiting the ability of Siah proteins to interact with substrates or adaptors could have effects beyond modulating Hif-1alpha regulation, given the apparent common usage of the binding groove to recognize VxP motifs within a range of substrate proteins. The effect of therapeutic inhibition of all Siah proteins, in a wide range of tissues, is unknown. In the

model presented here, Siah is inhibited in the tumor cells alone. Knockout of individual Siah proteins in mice is not lethal (Dickins et al., 2002; Frew et al., 2003), though the compound mutant, Siah1a/2 double knockout, dies at or immediately after birth possibly due to impaired hypoxia sensing. Any effects of Siah inhibition in unstressed, adult tissues are still to be determined, although we note that pan-Siah inhibition in EO771 cells did not appear to affect cell growth rate *in vitro* or cell viability.

The data presented here suggest that the Hif pathway is attenuated by Siah inhibition *in vitro* (lack of Hif-1alpha stabilization, reduced Hif transcriptional responses) and *in vivo* (reduced angiogenesis in tumors) and will therefore contribute partly or wholly to the effects observed in the tumor model. Previously, the nuclear repressor NCoR has been reported as a substrate of Siah2 and that Siah2 is transcriptionally up-regulated by estrogen receptor alpha (ERalpha) activation (Zhang et al., 1998). Siah2 appears to relieve NCoR-mediated repression of ER target genes and therefore enhance ER signaling. We also note that Siah2 gene expression levels are consistently higher in ER positive human breast cancers, compared with ER negative tumours (unpublished data). It is therefore possible that Siah inhibition in estrogen receptor positive breast tumors may stabilize NCoR and repress estrogen signaling, thus providing an additional mechanism to slow tumor growth in breast cancers. Indeed, we cannot rule out effects on the ER pathway as contributing to the observed tumor growth inhibition in the EO771 model, though the ER status of the EO771 cells remains to be investigated.

In summary, this study provides proof-of-principle for Siah inhibition at the substrate-binding site as a potential therapeutic strategy to inhibit Hif signaling and slow tumor growth. A search for small molecules binding at the same site has been initiated to investigate their potential as Siah inhibitors and therapeutic value.

Experimental Procedures

Animal work

C57Bl/6 mice were kept in a sterile animal house with OptiMice® caging systems. All procedures were approved by the local animal ethics committee in agreement with state legislations and international guidelines.

Cell culture and transfections

Human embryonic kidney HEK 293 and human osteosarcoma U2OS cells were obtained from ATCC. EO771 cells were a kind gift from Robin Anderson, Peter MacCallum Cancer Centre, Melbourne. All cells were maintained in DMEM containing 10% FCS and 1% (w/v) penicillin/streptomycin in a humidified incubator at 37°C and 5% CO2. Cells were transiently transfected using the Lipofectamine 2000® (Gibco BRL) reagent according to the instructions of the manufacturer, or using calcium phosphate transfection. Stable U2OS cell lines were generated by selection in 3μg/ml Puromycin (Sigma) for 10 days after transfection and stable EO771 by selection in Geneticin (Invitrogen) for 10 days.

Hypoxia treatment

Cells were treated in one of two ways, as indicated. In some experiments, Anaerogen oxygen depleting sachets were used (Oxoid). Later experiments were performed in an hypoxia workstation (INVIVO$_2$ 400, Ruskin Life Sciences) at 2% oxygen.

Antibodies and plasmids

Antibodies sources were: Flag, β-Actin and α-tubulin (Sigma Aldrich), HA (kind gift of Rick Pearson, Peter MacCallum Cancer Centre, Melbourne) and Hif-1alpha (Becton

Dickinson). Expression plasmids for TIEG1, Siah1, Siah1A and Siah2, Phyllopod, PHD1, PHD2 and PHD3 have been described (House et al., 2006; Johnsen et al., 2002; Nakayama et al., 2004). Mutations were generated using the QuickChange® site-directed Mutagensis Kit (Stratagene) according to the instructions of the manufacturer and verified by sequencing.

Immunoblotting

Cells were washed with phosphate-buffered saline (PBS), detached and collected by centrifugation. Cell pellets were directly lysed in 1 × SDS sample buffer, boiled and sonified. Proteins contained in cell extracts were separated by SDS-PAGE and electrophoretically transferred to PVDF membranes (Immobilon, Millipore). After blocking the membrane with 5% skim milk in PBS with 0.1% Tween20, they were incubated with primary antibodies, extensively washed and further incubated with the appropriate peroxidase-coupled secondary antibodies. Proteins were detected by using the Amersham enhanced chemiluminescence system or Pierce Phemtomole for Hif-1alpha.

Tumor growth

10^5 EO771 cells mixed 1:1 with matrigel (Becton Dickinson) were injected into the 4^{th} mammary fat pad of 8–12 week old female C57Bl/6 mice. Tumor dimensions were measured every second day. Mice were either sacrificed 19 days after injection or when the tumor reached the endpoint defined by the animal ethics committee (1cm diameter). Tumors were excised, weighed and cut into sections for fixation with 10% normal buffered formalin or fresh frozen for subsequent immunohistochemistry or mRNA and protein extraction.

Immunohistochemistry and antibodies

Paraffin imbedded or fresh frozen tumors were serial sectioned and stained with antibodies against CD31 (BD Pharmingen), Glut-1, and Hif-1alpha (both Novus Bioscience) according to instructions of the manufacturer.

Peptide Synthesis

Peptides were synthesized on a CEM Liberty Peptide Synthesiser using Fmoc solid-phase peptide chemistry. Syntheses were performed on Rink polystyrene resin and were cleaved from the solid-phase resin with trifluoroacetic acid/H_2O/triisopropylsilane (95:2.5:2.5) Then reversed-phase purified on a Phenomenex Synergi C18, 4um, Hydro-RP 80A 21 × 50 mm semi-preparative column. Purity of the peptides was monitored by analytical RP-HPLC and identity was confirmed by mono-isotopic MW determination on an Agilent Q-TOF LC/MS mass spectrometer. Mass accuracy was greater than 0.001% and purities were greater than 95% for all peptides.

Peptide sequences are:

Pen-P10-PHYL:
RQIKIWFQNRRMKWKKPPPPPPPPPQQERTKLRPVAMVRPTVRVQPQL-NH-2

P10-PHYL: PPPPPPPPPQQERTKLRPVAMVRPTVRVQPQL-NH-2

PHYL: QQERTKLRPVAMVRPTVRVQPQL-NH-2

Pen-CRIPT: RQIKIWFQNRRMKWKKTKNYKQTSV-OH

All peptides were dissolved in water at 1 mM and neutralized to pH 7 with Tris base before further dilution in DMEM cell medium, maintaining isotonic condition with equal volume of 2× HEPES buffered saline.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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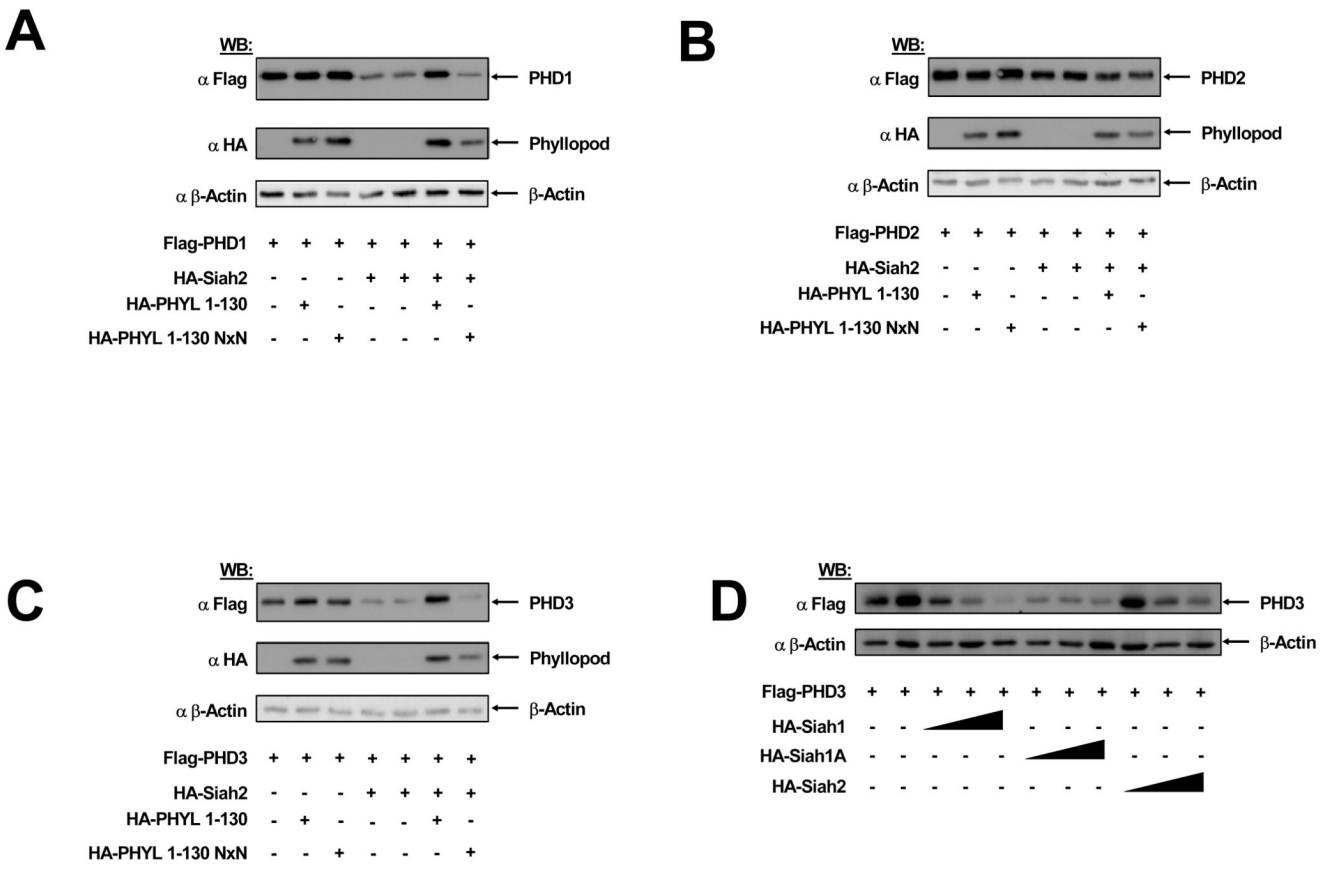

Figure 1. Siah induced degradation of PHD can be inhibited by PHYL 1–130

A) U2OS cells were transfected in a 24-well plate with the indicated plasmids, using 250ng Flag-PHD1, 250ng HA-Siah2, 250ng HA-PHYL. Cells were lysed 24 hours later and analysed by Western blotting with Flag and HA antibodies. Even loading was confirmed with β-Actin antibodies. B) U2OS cells were transfected as in A) but Flag-PHD2 was used instead of Flag-PHD1. C) U2OS cells were transfected as in A) but Flag-PHD3 was used instead of Flag-PHD1. D) HEK293 cells were transfected with Flag-PHD3 (250 ng), HA-Siah1A, HA-Siah1 and HA-Siah2 expression plasmids as indicated (5, 25 and 125 ng of Siah plasmid DNA). Cell lysates were analyzed for expression of Flag-PHD3 and β-Actin by using anti-Flag and anti-β-Actin antibodies.

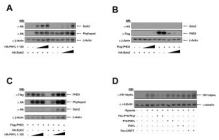

Figure 2. PHD3 is a Siah2 substrate, whereas PHYL is not

A) To investigate HA-PHYL stability, HEK293 cells were transfected in 24-well culture plates as indicated. The fixed amounts are 250ng plasmid, the varied concentrations 50, 100 and 250ng.

B) To investigate Flag-PHD3 stability, HEK293 cells were transfected and analyzed as in A).

C) To investigate whether HA-PHYL has an effect on Flag-PHD3 stability, HEK293 cells were transfected as in A).

D) Cell-permeable synthetic PHYL peptide inhibits Hif-1alpha stabilization during hypoxia. U2OS cells were treated with 100 μM synthetic peptides in DMEM (no serum) for 2 hours under normoxia or hypoxia (2% oxygen in hypoxia workstation). Peptides: Pen-P10-PHYL (Penetratin-10 proline spacer-$PHYL_{108–130}$), P10-PHYL (10 proline-$PHYL_{108–130}$), PHYL ($PHYL_{107–130}$) and Pen-CRIPT (Penetratin-human $CRIPT_{95–101}$, a sequence unrelated to PHYL). The penetratin sequence (a transducing peptide) corresponded to residues 43–58 of *Drosophila* Antennapedia protein. Blots were probed for endogenous Hif-1alpha. Even loading was confirmed with α-tubulin antibodies.

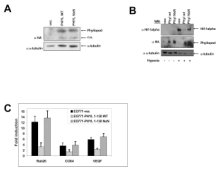

Figure 3. PHYL expression inhibits Hif-1alpha stabilization and Hif-1 induced gene expression

A) Stable EO771 cells expressing pCDNA3.1, pCDNA3.1-HA-PHYL WT or pCDNA3.1-HA-PHYL NxN. After selection cell lysates were obtained and expression of proteins analyzed by Western blotting using anti-HA and anti-α-tubulin antibodies.

B) Stable EO771 cell lines were exposed to normoxia or hypoxia for two hours using Anaerogen oxygen depleting sachets (Oxoid). Cell lysates were obtained and expression of proteins analyzed by Western blotting using anti-Hif-1alpha, anti-HA and anti-α-tubulin antibodies.

C) Stable EO771 cell lines were exposed to normoxia or hypoxia for four hours using Anaerogen oxygen depleting sachets. mRNA was extracted and reverse transcribed into cDNA. Expression levels of Rab20, Cox4 and VEGF were assessed by quantitative realtime PCR (qRT-PCR), SEM from three independent experiments.

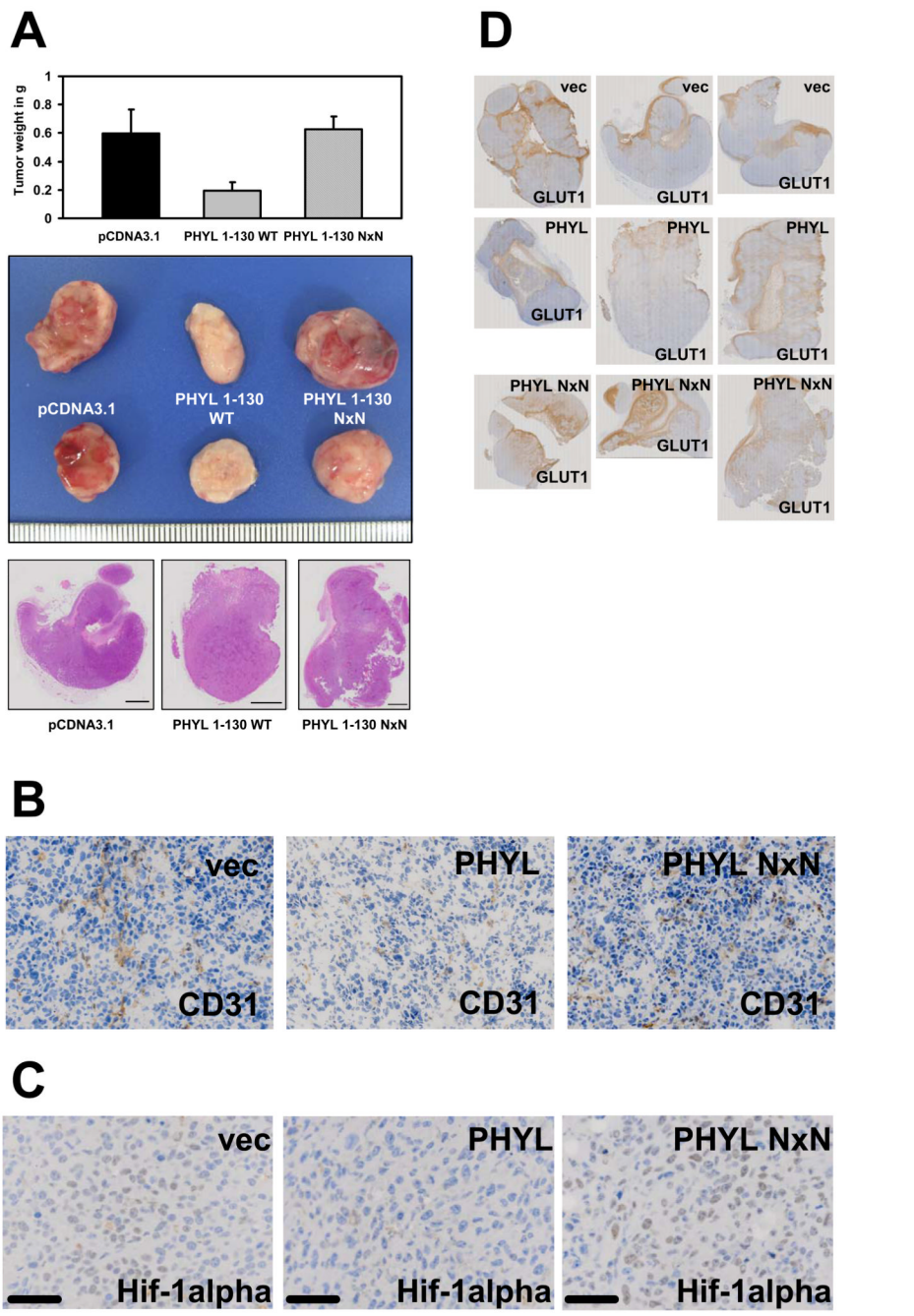

Figure 4. Analysis of PHYL-expressing tumors in breast cancer model

A) Panel 1 - Tumor weights of EO771 breast cancers 19 days after injection of 10^5 cells into the 4th mammary gland of C57Bl/6 mice. (n = 8,9,8 for the three groups). Panel 2 - Representative images of tumors from the three groups. Panel 3 - Hematoxylin and Eosin staining of tumor sections. Representative images are shown. B) CD31 staining of fresh frozen tumor sections. C) Hif-1alpha staining of formalin fixed tumor sections. D) Glut-1 staining of formalin fixed tumor sections.