Regulation of Hypoxia-Inducible Factor 1 α during Hypoxia by DAP5-Induced Translation of PHD2

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ABSTRACT Death-associated protein 5 (DAP5) is an atypical isoform of the translation initiation scaffolds eukaryotic initiation factor 4G1 (eIF4G1) and eIF4GII (eIF4G1/II), which recruit mRNAs to ribosomes in mammals. Unlike eIF4G1/II, DAP5 binds eIF2 β , a subunit of the eIF2 complex that delivers methionyl-tRNA to ribosomes. We discovered that DAP5:eIF2 β binding depends on specific stimuli, e.g., protein kinase C (PKC)–Raf–extracellular signal-regulated kinase 1/2 (ERK1/2) signals, and determines DAP5's influence on global and template-specific translation. DAP5 depletion caused an unanticipated surge of hypoxia-inducible factor 1 α (HIF-1 α), the transcription factor and master switch of the hypoxia response. Physiologically, the hypoxia response is tempered through HIF-1 α hydroxylation by the oxygen-sensing prolyl hydroxylase-domain protein 2 (PHD2) and subsequent ubiquitination and degradation. We found that DAP5 regulates HIF-1 α abundance through DAP5:eIF2 β -dependent translation of PHD2. DAP5:eIF2-induced PHD2 translation occurred during hypoxia-associated protein synthesis repression, indicating a role as a safeguard to reverse HIF-1 α accumulation and curb the hypoxic response.

KEYWORDS DAP5, eIF2 β , HIF-1 α , PHD2, translation initiation, hypoxia

Multifaceted control of protein synthesis enables rapid cellular adaptation to instant changes in growth or survival conditions. This is particularly important when stressors require global protein synthesis repression, but cells must ramp up translation of select stress response modifiers, as exemplified by the response to oxygen deprivation. The hypoxia response is dominated by hypoxia-inducible factor 1 α (HIF-1 α) and its activator role in the HIF-1 α / β complex, HIF-1 (1, 2). HIF-1 transcriptional activity induces >1,000 genes (3, 4) involved in cell proliferation, cell invasion/metastasis, or glucose metabolism programs (5). To restore homeostasis with normoxic balancing, HIF-1 α is rapidly hydroxylated by prolyl hydroxylase-domain proteins (PHDs), ubiquitinated by von Hippel-Lindau protein (VHL), and degraded by the proteasome (6–8). The PHDs rapidly hydroxylate HIF-1 α at prolines 402/564 in an oxygen-dependent manner, inducing rapid HIF-1 α degradation (6, 9, 10). This process hinges on the main oxygen sensor in the cell, PHD2, since neither PHD1 nor PHD3 can functionally compensate for PHD2 depletion (11).

The eIF4G isoforms are key to adaptive protein synthesis because they combine control over the association of translation initiation machinery with mRNAs, the unwinding/scanning of the 5' untranslated region (UTR), and eukaryotic initiation factor 3 (eIF3)/40S ribosomal subunit recruitment. There are three eIF4G isoforms in mam-

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FIG 1 Phorbol ester stimulation promotes DAP5:elF2β binding. (A to C) HEK293 cells expressing Flag-tagged DAP5, elF4G1-Ct, DAP5(1-790), and DAP5(E862K) (A), Flag-DAP5 (B), or Flag-elF2β (C) were induced with dox (16 h) before stimulation with TPA, as shown. Cell lysates were analyzed by immunoblotting or subjected to anti-Flag immunoprecipitation/immunoblotting. Data bars in panel A depict the mean ratio of elF2β to Flag signal after 240 min of TPA from three assays. Experiments were normalized by setting the Flag-DAP5 (wt) ratio for each experiment to 1; error bars represent the SEM. *, significant *P* value (*P* < 0.05). (D) Schematic representation of DAP5 and elF4G1(683-1600) domain arrangements, protein interactions, and major Ser/Thr phosphorylation sites. (E) HEK293 cells were dox induced for Flag-DAP5 expression (16 h), treated with TPA (240 min), harvested, and subjected to Flag-DAP5 IP. Immunoprecipitation-isolated complexes were digested with trypsin, followed by TiO₂ enrichment and LC-tandem MS (LC-MS/MS) analysis. Amino acid sequences of phosphopeptides identified by LC-MS/MS are shown; the highlighted amino acids before the asterisks indicate phosphorylated amino acids. The MASCOT ion score was calculated using the following equation: $-10 \log_{10}(P)$, where *P* is defined as the absolute probability of the observed match being a random event (56). DAP5(T508) and DAP5(S902) were identified by this analysis by using a cutoff MASCOT score of 20. (F) Location of DAP5(T508)/(S902) relative to elF4G1(S1186)/(S1597) phosphorylation sites, known to control TPA-induced association with MNK (22).

mals, elF4GI, elF4GII, and death-associated protein 5 (DAP5) (12). They share similar roles in core initiation functions, mediated by association with mRNA, anchoring the elF4A:4B translation initiation helicase cofactors and recruiting elF3/40S ribosomal subunits. DAP5 is homologous to the C-terminal approximate two-thirds of elF4GI/II and lacks their N-terminal domain, which contains binding motifs for the 5' 7-methyl-guanosine cap-binding protein (elF4E) and the poly(A) binding protein (PABP) (Fig. 1). Unlike elF4GI/II, DAP5 binds to elF2β (13), which in a complex with elF2γ and elF2α (comprising elF2) delivers Met-tRNA to 40S ribosomal subunits (14). DAP5 has been implicated both in homeostatic global protein synthesis (13, 15) and in context-specific, m⁷G-cap independent initiation at select templates (16). DAP5's role in translation is unique, since elF4GI overexpression cannot compensate for DAP5 depletion (17). The elF4GI isoforms are ubiquitously expressed; the elF4GI/elF4GII/DAP5 ratio in HeLa cells

is approximately 10:1:45 (18). The significance of the eIF4G isoform spectrum, their functional/structural variances, and their expression divergences is not understood.

Our studies show that inducible DAP5:eIF2 β binding is required for DAP5-mediated translation. They reveal that DAP5:eIF2 β has a defining role in controlling translation of the principal oxygen sensor of the cell, PHD2, upon oxygen deprivation. DAP5 depletion caused a surprising, paradoxical increase in HIF-1 α protein due to a reduction in DAP5-dependent translation of PHD2. DAP5's role in controlling PHD2 is evident in cells exposed to hypoxic conditions, where low oxygen prompted DAP5:eIF2 β binding and DAP5-mediated PHD2 biosynthesis. We confirmed the recently reported role of PHD2 in tempering AKT signaling (19) and, accordingly, defined a role for DAP5 in controlling AKT's activation status. Our findings indicate that DAP5-mediated translation is induced despite global translation repression in hypoxia, possibly due to unique structural arrangements and protein interactions that distinguish it from eIF4G/II.

RESULTS

DAP5:eIF2 β binding is inducible, e.g., by protein kinase C (PKC)-Raf-ERK1/2 signaling. Previous studies identified binding between DAP5 and eIF2 β that may be involved in DAP5-mediated translation initiation (13, 20). Binding of the mitogen-activated protein (MAPK)-interacting kinase (MNK) to eIF4G1 (21), which is analogous to eIF2 β association with DAP5, occurs at two conserved C-terminal aromatic and acidic (AA) boxes (Fig. 1). Since eIF4G1:MNK binding strongly responds to PKC-Raf-extracellular signal-regulated kinase 1/2 (ERK1/2) signals (21–23), we hypothesized that DAP5:eIF2 β binding might be similarly regulated. We created stable HEK293 cell lines with doxycycline (dox)-inducible expression of Flag-tagged (i) wild-type (wt) DAP5; (ii) eIF4G1-Ct(683-1600), i.e., the C-terminal portion of eIF4G1 homologous to DAP5; (iii) DAP5(1-790), i.e., DAP5 lacking the C-terminal AA boxes; and (iv) DAP5(E862K), a DAP5 point mutant that lacks eIF2 β binding (20) (Fig. 1A and D). The cells were dox induced, treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) for PKC activation, and processed for Flag immunoprecipitation (IP). TPA-responsive eIF2 β and eIF2 α binding occurred only with wt DAP5 (Fig. 1A). TPA induced eIF4G1-Ct binding to MNK1 but not eIF2 β (Fig. 1A). DAP5(1-790) and DAP5(E862K) lost eIF2 β binding (Fig. 1A); however, DAP5(1-790) also displayed significantly decreased TPA-responsive rpS6 and eIF3a coimmunoprecipitation (Co-IP), suggesting that the C-terminal truncation broadly compromises DAP5 functional integrity (Fig. 1A). Co-IP of both eIF2 β and eIF2 α at the same rate implies that the entire eIF2 is precipitated with DAP5, since eIF2 α is unable to bind to DAP5 directly and eIF2 α and eIF2 β are held in complex by the third eIF2 subunit eIF2 γ .

TPA treatment for 15 min induced ERK1/2 phosphorylation but diminished ribosomal protein S6 (rpS6) and eIF3a Co-IP with all DAP5 and eIF4G1 variants, indicating reduced association with eIF3/40S ribosomal subunits (Fig. 1A). This coincided with similarly reduced MNK1:eIF4G1 and wt DAP5:eIF2 β binding (Fig. 1A). Detailed kinetics of the TPA response indicated transient interruption of translation activity, possibly related to a short-lived peak of p-eIF2 α (S51) at 15 min after TPA (Fig. 1B). We believe that this accounts for the temporary slump of DAP5/eIF4G1 interactions with eIF2 β /MNK/rpS6/eIF3a at 15 min after TPA (Fig. 1A). Binding of all DAP5 variants/eIF4G1 to eIF4A was only slightly variable throughout the period of TPA stimulation (Fig. 1A); eIF4A interactions with DAP5/eIF4G1 HEAT1 do not respond to PKC-Raf-ERK1/2 (Fig. 1D) (24). The same pattern of TPA-responsive interactions occurred upon inverse Co-IP of endogenous DAP5 and eIF3a with dox-inducible Flag-eIF2 β (Fig. 1C).

DAP5 is phosphorylated upon TPA stimulation. MNK binding to the AA boxes of eIF4G1 is in part regulated by PKC-Raf-ERK1/2 signals to eIF4G1 (21, 22, 24). High-throughput phosphoproteomics identified putative priority DAP5 phosphosites (<https://www.phosphosite.org>); however, none of these have been confirmed biologically. Using a targeted, unbiased approach, we performed liquid chromatography-mass spectrometry (LC-MS) phosphoproteomics of IP-purified Flag-DAP5 from dox-induced, TPA/mock-stimulated HEK293 cells. Two prominent phosphosites were detected only in

FIG 2 Phosphorylation of DAP5(S902) by CK2α is required for eIF2β binding. (A) S902/S1597 anchor consensus CK2α substrates at the DAP5/eIF4G1 C termini. (B) HEK293 cells expressing dox-inducible Flag-tagged DAP5, DAP5(S902A), or DAP5(S902D) were dox treated (16 h) before stimulation with TPA. Cell lysates were analyzed by immunoblotting or subjected to anti-Flag immunoprecipitation/immunoblotting. The ratio of eIF2β to Flag signal after TPA (4 h) was quantified across three repeat tests. HEK293 cells treated with TPA (4 h) were normalized to 1, with the SEM indicated. *, significant *P* value (*P* < 0.05). (C) Dox-inducible Flag-DAP5-expressing HEK293 cells were treated with dox (16 h) and TPA as shown. Lysates were tested as in panel B. All experiments were repeated three times with representative series as shown.

the TPA-stimulated samples threonine 508 and serine 902 (Fig. 1E). These are among few commonly identified DAP5 phosphosites in reported high-throughput mass spectrometry studies (<https://www.phosphosite.org>). DAP5(T508) is located in the interdomain linker (connecting HEAT1 and HEAT2 domains; Fig. 1D), a region of low sequence similarity to eIF4G1. The corresponding region in eIF4G1 has clusters of multiple mitogen-responsive phosphosites, including S1186, which is phosphorylated by PKCα upon TPA stimulation, and influences MNK binding to eIF4G1 (Fig. 1F) (22). DAP5(S902) is located at the C terminus, within the binding motif for eIF2β. This region is highly conserved between DAP5 and eIF4G1 with eIF4G1(S1597) sharing an almost identical location and amino acid sequence context (Fig. 1F). We sought to determine whether these phosphorylation events control DAP5 association with eIF2β.

DAP5(S902) is phosphorylated by CK2α. DAP5(S902) and eIF4G1(S1597) are within ideal consensus for casein kinase 2-α (CK2α) (Fig. 2A) (25). Indeed, previous investigations showed that eIF4G1(S1597) is phosphorylated by CK2α (24). To test whether S902 phosphorylation regulates DAP5:eIF2β binding, HEK293 cells with dox-inducible expression of a non-phospho-mutant DAP5(S902A) or a phospho-mimic (S902D) were derived. DAP5(S902A) did not bind eIF2β/α, whereas the S902D phospho-mimic retained TPA-responsive binding (Fig. 2B). An analogous approach demonstrated that CK2α catalyzed p-eIF4G1(S1597) controls TPA/anisomycin-induced eIF4G1:MKN1 interactions (data not shown). Co-IP of CK2α with DAP5/eIF4G1 further supports its involvement in phosphorylation of S902/S1597 (Fig. 2C). Our findings suggest that a conserved mechanism regulates DAP5:eIF2β and eIF4G1:MKN1 binding via CK2α sites within the binding regions for eIF2β/MKN1. CK2 is considered constitutively active, with no known stimuli (25), suggesting that S902 (DAP5) and S1597 (eIF4G1) do not contribute to TPA-induced binding of eIF2β or MKN1.

MEK1-ERK1/2 induces DAP5:eIF2β binding. We next addressed a possible role for p-DAP5(T508) in TPA-induced DAP5:eIF2β binding. Kinase prediction (<http://www.phosphonet.ca>) indicated DAP5(T508) as a likely target for MAPKs or cyclin-dependent kinases (CDKs) (Fig. 3A). First, to reliably detect p-DAP5(T508), we validated a p-T*PP antibody; this probe only detected signal in Flag-IP of wt p-DAP5(T508) but not the T508A/E mutants (Fig. 3B). Second, we investigated the role of ERK1/2, p38α-δ, and

FIG 3 DAP5:eIF2 β binding induced by MEK1-ERK1/2 signaling or during mitosis. (A) The top 24 predicted kinases for DAP5(T508) (<http://www.phosphonet.ca>; kinase predictor V2 scores). (B) Flag-DAP5(508T/A/E) immunoprecipitations show the specificity of an anti-p-T*PP antibody for T508. (C) HEK293 cells expressing Flag-DAP5 were dox induced (16 h) and pretreated with UO126, trametinib, BIRB 796, JNK inhibitor VIII, or DMSO(–) (2 h) prior to TPA stimulation. Cell lysates were analyzed by immunoblotting or anti-Flag immunoprecipitation/immunoblotting. (D) HEK293 cells expressing Flag-tagged DAP5, DAP5(T508A), or DAP5(T508E) were dox induced (16 h) before stimulation with TPA. Lysates were analyzed as in panels C and D. Data bars represent the average ratios of eIF2 β to Flag immunoprecipitation signal at 4 h TPA, normalizing each experiment by setting the eIF2 β /Flag ratio for DAP5(T508) to 1. (E) HEK293 cells with dox-inducible Flag-DAP5(T508E) were treated with dox (16 h), mock pretreated or pretreated with trametinib (2 h) prior to TPA stimulation and analyzed as in panel C. (F) HEK293 cells were treated with thymidine (4 mM; 16 h), followed by a medium change, and Ro3306, nocodazole, or DMSO(–), as shown. Dox was maintained (16 h) prior to harvest. Lysates were analyzed as in panels C to E. Data bars (right panels) represent mean ratio of p-T*PP/Flag and eIF2 β /Flag, normalizing between experiments by setting the ratios for cells treated with thymidine block only to 1. (G) *In vitro* phosphorylation assay of Flag-DAP5 with recombinant JNK2 (control) or CDK1. Data bars represent the mean ratios of p-T*PP [p-DAP5(T508)] to Flag, normalizing between experiments by setting the –kinase/–ATP control to 1. All experiments were repeated three times; data bars represent the means from three tests; error bars denote the SEM. *, significant *P* value (*P* < 0.05).

JNK1/2 MAPKs in DAP5(T508) phosphorylation and DAP5:eIF2 β binding. HEK293 cells with dox-inducible Flag-DAP5 expression were pretreated (2 h) with DMSO(–), the MEK1/2 inhibitors UO126 or trametinib, the p38 α - δ inhibitor BIRB796, or the JNK1/2 inhibitor VIII (Fig. 3C) prior to TPA stimulation (4 h). The inhibitors exhibited the expected signaling effects; both MEK1/2 inhibitors also blocked JNK signaling (Fig. 3C, input).

Our assay revealed that none of the MAPK inhibitors altered p-DAP5(T508) levels (Fig. 3C). However, the MEK1/2 inhibitors completely abolished DAP5:eIF2 β binding (Fig. 3C). These data suggest that DAP5:eIF2 β association upon TPA stimulation is controlled by ERK1/2 but does not hinge on DAP5(T508) phosphorylation. Tests in

TPA-stimulated (240 min) HEK293 cells with dox-inducible expression of DAP5 featuring either 508T/-A or 508T/-E showed possible correlation of eIF2 β binding with p-T508 (Fig. 3D). To confirm ERK1/2-regulation of DAP5:eIF2 β binding independent of DAP5(T508), HEK293 cells with dox-inducible DAP5(T508E) were pretreated (2 h) with DMSO(–) or trametinib (Fig. 3E). ERK1/2 inhibition virtually blocked DAP5(T508E):eIF2 β binding (Fig. 3E). There are a large number of confirmed and putative MEK1/2-ERK1/2-responsive phosphorylation sites in multiple translation initiation factors (24, 26–29). This complexity makes defining the precise MEK1/2-ERK1/2-induced events that promote DAP5:eIF2 β association a daunting enterprise, one that is beyond the scope of the present investigation.

Phosphorylation of DAP5(T508) and DAP5:eIF2 β binding is induced during mitosis. Since DAP5(T508) is not a MAPK substrate, we tested whether T508 phosphorylation and DAP5:eIF2 β binding respond to CDK1 (Fig. 3A). The specific activity of DAP5 during mitosis has been suggested previously (16). HEK293 cells with dox-inducible Flag-DAP5 expression were treated with dox and synchronized using thymidine block, prior to release into fresh growth media. The cells were arrested in G₂, using the CDK1 inhibitor Ro3306 (30) (Fig. 3F), or arrested in mitosis with nocodazole, as confirmed by an increase in p-histone H3 (Fig. 3F). G₂ arrest decreased p-DAP5(T508) phosphorylation and DAP5:eIF2 β binding; in contrast, mitotic arrest strongly enhanced both events (Fig. 3F). To test whether CDK1 phosphorylates T508, Flag-DAP5 was purified from dox-induced HEK293 cells and incubated with active recombinant JNK2 or CDK1 in the presence of ATP (Fig. 3G). DAP5(T508) was phosphorylated only upon incubation with CDK1 (Fig. 3G). Collectively, our data indicate that DAP5:eIF2 β binding is inducible and strongly context specific (e.g., phorbol ester activation, mitosis), occurs with MEK1/2-ERK1/2 or CDK1 activation, and may correlate with DAP5(T508) phosphorylation.

Loss in DAP5:eIF2 β association results in decreased global translation. To assay for DAP5:eIF2 β binding's role in translation, we used stable HeLa cell lines with dox-inducible eIF4G1 or DAP5 depletion (see Materials and Methods). Treatment with dox (96 h) resulted in similarly robust reduction of eIF4G1 or DAP5 levels (Fig. 4A). The cells were stimulated with TPA (15 min, 240 min) and, 15 min prior to harvest, puromycin (PMY) was added. The abundance of puromycylated product was quantified by immunoblotting (Fig. 4B) (31). DAP5 depletion reduced global translation by ~20% in unstimulated cells and strongly inhibited (~40% at 4 h) the translation stimulatory effect of TPA (Fig. 4A and B). Confirming the exalted role of eIF4G1 in global protein synthesis, ~90% eIF4G1 depletion induced a robust decrease of TPA-induced translation.

The pattern of the global protein synthesis response to TPA closely mirrored DAP5:eIF2 β binding (Fig. 4A; compare to Fig. 1A to C). To test directly whether DAP5:eIF2 β binding determines DAP5's role in global translation, we established HEK293 cells with dox-inducible DAP5 knockdown combined with simultaneous wt DAP5 or DAP5(E862K) knock-in. We previously reported on this approach with dox-inducible (endogenous) eIF4G1 knockdown/knock-in of Flag-tagged eIF4G1 variants (26). Wild-type DAP5 reconstitution in DAP5-depleted cells rescued protein synthesis, while knock-in of the DAP5(E862K) mutant severely repressed global translation, beyond levels seen with DAP5 depletion alone (Fig. 4C and D). Our observations indicate that DAP5 participates in mediating the adaptive protein synthesis response to PKC-Raf-ERK1/2 signaling and that DAP5:eIF2 β binding is required for this function.

DAP5 depletion causes paradoxical induction of HIF-1 α . Since DAP5:eIF2 β facilitate a surge of global translation following Raf-ERK1/2 stimulation (Fig. 4A to D), we next tested DAP5's effects on translation of specific, known TPA-responsive templates. Since DAP5's structural arrangement and protein-protein interactions clearly distinguish it from eIF4G1/II, we compared the effect of DAP5 depletion on induction of TPA-responsive mRNAs with a corresponding knockdown of eIF4G1, mirroring our approach with global translation (Fig. 4A). Cells with dox-inducible eIF4G1 or DAP5 depletion were treated with dox and TPA and harvested as for the analyses of global

FIG 4 DAP5 depletion attenuates global translation and paradoxically induces HIF-1 α . (A) HeLa cell lines with dox-inducible eIF4G1 or DAP5 depletion were treated with or without dox (96 h), stimulated with TPA as indicated, and treated with PMY (5 μ M; 15 min) prior to harvesting. Puromycylation was stopped by washing cells with cold PBS and the addition of lysis buffer. (B) The results of mean quantification of the PMY/tubulin ratio from three experiments are shown, normalizing between experiments by setting the zero-time-point, no-dox value to 1. (C) HEK293 cells with dox-inducible DAP5 depletion combined with mock (–), wt DAP5, or DAP5(E862K) reconstitution were dox treated (96 h) prior to PMY. (D) Mean quantification of the PMY/tubulin ratio is shown for three experiments, normalizing by setting the no-dox ratio to 1. (E) HeLa cells with dox-inducible eIF4G1 or DAP5 depletion were dox treated and TPA stimulated, and their lysates were subjected to immunoblotting as shown. (F) Mean HIF-1 α , COX2, and MKP-2/tubulin ratios were quantified from three experiments, normalizing by setting the mock (eIF4G1)-depleted, no-dox ratio to 1. All assays were repeated three times, and a representative series for each panel is shown. Error bars denote the SEM. *, significant *P* value (*P* < 0.05).

protein synthesis (Fig. 4E). Cell lysates were analyzed for translation of the known TPA-inducible proteins HIF-1 α (32), cyclooxygenase 2 (COX2) (33), and MAPK phosphatase 2 (MKP-2) (34).

As expected, these proteins were potently induced by TPA (4 h) (Fig. 4E). eIF4G1 depletion partially reversed this effect for all three proteins, mirroring its role in coordinating global translation responses to PKC activation. Surprisingly, DAP5 depletion had a strong obverse effect specifically on HIF-1 α , producing >5-fold induction (Fig. 4E and F). We also saw a modest increase in COX2 levels upon DAP5 depletion (Fig. 4E and F). This likely is a direct consequence of HIF-1 α induction, since COX2 abundance is linked to HIF-1 α activity (35). The TPA response of MKP-2 to eIF4G1 and DAP5 depletion was comparable (Fig. 4E and F). This unexpected finding suggests a para-

FIG 5 DAP5 depletion induces HIF-1 α by depressing PHD2 levels. (A) Schematic overview of posttranslational modifications and interactions of HIF-1 α . (B) HeLa cells with dox-inducible DAP5 depletion were dox treated (96 h) and pretreated with CoCl₂ (4 h) before TPA stimulation. The cells were harvested for immunoblotting. The mean HIF-1 α /tubulin ratio was quantified, normalizing between experiments by setting the no-dox, no-TPA control to 1. (C) HeLa cells with dox-inducible DAP5 depletion were dox treated (96 h) and lysed for immunoblotting with the antibodies shown. The mean PHD2/tubulin ratios were quantified, normalizing between experiments by setting the no-dox, no-TPA time point to 1. (D) HeLa cells with dox-inducible eIF4G1 depletion were dox treated as shown and mock or TPA stimulated (240 min). The mean PHD2/tubulin ratio was quantified, normalizing by setting the 240-min TPA, no-dox ratio to 1 for each experiment. (E) The assay described for panel D was repeated with the addition of MG-132 pretreatment (5 μ M; 1 h) prior to TPA stimulation. The mean OH-HIF-1 α (P564)/HIF-1 α ratio was quantified, normalizing by setting the no-dox value to 1. (F) HEK293 cells with dox-inducible knockdown/knock-in as described for Fig. 4C were dox treated as shown and analyzed by immunoblotting. The mean PHD2/tubulin ratios were quantified, normalizing by setting the no-dox values to 1. All data bars represent the means of three independent experiments; error bars display the SEM. *, significant *P* value (*P* < 0.05).

doxical relation of DAP5 abundance to HIF-1 α levels, indicating a connection between DAP5 and HIF-1 α regulation that is inverse to DAP5's effect on global translation.

DAP5 knockdown increases HIF-1 α through reduced PHD2 levels. HIF-1 α is translated at low levels in normoxia and the protein is highly labile in normal conditions. This is due to a number of posttranslational modifications, such as hydroxylation by the PHDs and FIH1, and acetylation by ARD1, which facilitate HIF-1 α binding to the E3 ubiquitin ligase VHL, and leads to HIF-1 α ubiquitination and degradation (Fig. 5A)

(36). Thus, a likely scenario to explain the effect of DAP5 depletion on TPA-induced HIF-1 α was a role for DAP5 in controlling the HIF-1 α degradation apparatus. Therefore, to determine whether DAP5 regulates HIF-1 α through hydroxylation, the hydroxylase inhibitor CoCl₂ was added to DAP5-depleted HeLa cells prior to addition of TPA (Fig. 5B). CoCl₂ abolished DAP5 depletion-mediated HIF-1 α hyperinduction in TPA-stimulated cells (Fig. 5B; compare to Fig. 4E and F). This suggested that DAP5 may regulate HIF-1 α by controlling its hydroxylation levels rather than acetylation or ubiquitination.

To investigate possible effects of DAP5 depletion on HIF-1 α hydroxylation factors, HeLa cells with dox-inducible DAP5 depletion were TPA stimulated and tested throughout a multistep time course to monitor PHD1, PHD2, PHD3, ARD1, FIH1, and VHL expression (Fig. 5A and C). The only factor with a detectable expression response to DAP5 depletion was PHD2, the master HIF-1 α hydroxylation factor (Fig. 5C); PHD2 abundance did not respond to eIF4G1 depletion (Fig. 5D). To test directly whether DAP5 depletion alters HIF-1 α hydroxylation levels (via decreased PHD2 activity), HeLa cells with dox-inducible DAP5 depletion were treated with the proteasome inhibitor MG-132 (to stabilize hydroxylated HIF-1 α) (Fig. 5E). We probed for hydroxylation of HIF-1 α (P564), a principal PHD2 target that destines HIF-1 α for rapid ubiquitination and degradation (Fig. 5E; also Fig. 5A). Upon TPA stimulation, DAP5 depletion hyperinduced HIF-1 α . Simultaneously, DAP5 depletion decreased the ratio of P564-hydroxylated HIF-1 α to total HIF-1 α by >80% (Fig. 5E). This effect was accompanied by the suppression of PHD2 levels in DAP5-depleted cells (Fig. 5E).

Reconstitution with wt DAP5 in cells with dox-inducible DAP5 depletion rescued global protein synthesis; meanwhile, reconstitution with the DAP5(E862K) variant, devoid of eIF2 β binding, had a negative effect on global translation (Fig. 4C and D). This indicated that DAP5:eIF2 β binding is required for DAP5's effect on global translation. We used the same assay to query whether DAP5:eIF2 β binding mediates translation of PHD2 (Fig. 5F). The results of this assay mirrored the effects seen with DAP5/DAP5(E862K) reconstitution on global translation, suggesting that PHD2 translation is controlled by the DAP5:eIF2 β complex (Fig. 5F). Our data indicate a direct link between DAP5 and PHD2, the master oxygen sensor and coordinator of HIF-1 α degradation. PHD2 knockdown alone is capable of stabilizing HIF-1 α during normoxia; PHD2 is functionally nonredundant with other HIF1- α regulatory proteins and is also the most abundant of the PHDs (11, 37).

DAP5 regulates translation of PHD2. Our research strongly implicated DAP5 in control over PHD2 levels but does not demonstrate an involvement in translation initiation at the PHD2 template. Therefore, we conducted polysome profile analysis to probe for shifts of PHD2 mRNA cosedimentation with polysomal fractions upon DAP5 depletion (Fig. 6). We performed quantitative reverse transcription-PCR (RT-qPCR) to detect PHD2 template in individual polysome fractions and plotted its distribution in the profile (Fig. 6A). DAP5 depletion shifted the distribution of PHD2 RNA in the polysome profile toward 80S ribosomes and light polysomes, suggesting a reduction in PHD2 translation. The distribution of control (GAPDH) mRNA was not similarly affected by DAP5 depletion (Fig. 6C).

In addition, DAP5 depletion did not reduce total PHD2 mRNA levels (Fig. 6B), further suggesting regulation at the level of translation. Template-specific translation control involves regulatory features in the 5' and/or 3' UTRs of select mRNAs. Unfortunately, the 5' and 3' UTRs of the PHD2 message are not annotated: (i) distinct proposed 5' UTRs of >3,000 nucleotides (nt) and <100 nt and (ii) proposed alternative polyadenylation sites in the 3' UTR were reported (38–40) (Fig. 6D). To investigate DAP5's potential role in template-specific translation regulation of PHD2, extensive further work is required for mapping the definitive transcriptional start site(s), annotate the 5' UTR(s), and determine the nature and prevalence of alternative 3' UTRs of the PHD2 message. This study is beyond the scope of this investigation.

FIG 6 DAP5 is involved in PHD2 translation. (A and C) HeLa cells with dox-inducible DAP5 depletion were dox treated (96 h), and lysates were subjected to polysome profiling. (Top panel) A representative absorbance (260:280) profile of the fractionated gradient is shown in light blue with the peaks identified. Total RNA was purified from the fractions, RT-qPCR analyses for PHD2 (A) and GAPDH (C) mRNA were performed, and the percentages of the PHD2/GAPDH mRNA signal were determined for each fraction. This experiment was repeated twice and yielded similar results in each series; representative profiles are shown. Proteins from each fraction were TCA precipitated and analyzed by immunoblotting. (B) HeLa cells with dox-inducible DAP5 depletion were treated with dox (96 h) or left untreated. Total polyadenylated RNA was isolated from the cells and analyzed by RT-qPCR. The average of two assays with the uninduced controls was normalized to 1, and the SEM values are shown. Representative immunoblots from lysates are shown. (D) Schematic view of the ambiguously annotated 3' and 5' UTRs of PHD2. Alternative transcriptional start sites and polyadenylation signals (orange bars) are indicated (see the text for references).

DAP5:eIF2 β -mediated control of PHD2 modulates AKT phosphorylation. Recently, PHD2 has been shown to hydroxylate AKT, provoking AKT-VHL binding, and block of AKT(T308) phosphorylation (19). Thus, if DAP5 depletion diminished PHD2, it should also, indirectly, depress AKT activation. To explore such a link, we used our empirical system with dox-inducible DAP5 depletion combined with DAP5/DAP(E862K) knock-in (Fig. 4C; Fig. 5F). Knock-in of wt DAP5 in DAP5-depleted cells had no effect on p-AKT(T308), while knock-in of eIF2 β -binding defective DAP5(E862K) reduced the abundance of PHD2 and elevated p-AKT(T308) levels (Fig. 7A). These observations confirm claims by Guo et al. (19) about PHD2's involvement

FIG 7 DAP5 modulates AKT(T308) phosphorylation. (A) HEK293 cells with dox-inducible (endogenous) DAP5 depletion and either wild-type DAP5 or DAP5(E862K) reconstitution were treated with dox as shown (96 h). Lysates were subjected to immunoblotting with the indicated antibodies; the results of a representative test are shown. The p-AKT(T308)/AKT signal ratio from three independent series was quantified. The uninduced controls were normalized to 1. (B and C) Two malignant glioma cell lines (Du54 and U87) and one breast cancer cell line (Sum149) (B), as well as MEFs (C), were transiently transfected with siRNA targeting DAP5. Lysates were analyzed by immunoblotting. Mean HIF-1 α /tubulin, PHD2/tubulin, and/or p-AKT(T308)/AKT1 signal ratios from three independent assays are shown, normalizing the control siRNA sample values to 1; error bars denote the SEM. HIF-1 α (Du54 and Sum149 cells) and p-AKT (U87 cells) levels could not be quantified due to insufficient signal. *, significant *P* value (*P* < 0.05).

in control over AKT activity and reinforce DAP5's role in controlling PHD2 translation. Since our assays so far relied on dox-inducible systems in HeLa or HEK293 cells, we tested the effects of transient DAP5 depletion (upon small interfering RNA [siRNA] transfection) in a range of cancer lines: U87 cells, Du54 malignant glioma cells, and Sum149 breast cancer cells (Fig. 7B) and in mouse embryonic fibroblasts (MEFs) (Fig. 7C). In all four lines, DAP5 depletion diminished PHD2 levels (Fig. 7B and C). Only U87 and MEF cells spontaneously expressed abundant HIF-1 α ; DAP5 depletion in these cells mediated an increase in HIF-1 α levels (Fig. 7B and C). All four lines exhibited increased p-AKT(T308) levels upon DAP5 depletion (Fig. 7B and C). Collectively, our findings suggest that DAP5 controls baseline PHD2 abundance in diverse malignant cell types and in MEFs.

DAP5 controls the response to hypoxia. DAP5's influence over PHD2 biosynthesis, and its downstream effects on HIF-1 α accumulation or AKT activity, indicate important functions in cellular responses to oxygen deprivation. We performed tests to confirm this in cells where a physiologic response to hypoxia was induced by low-oxygen growth conditions. Unmodified HeLa cells or HeLa cells with (i) dox-inducible depletion of DAP5, (ii) dox-inducible depletion of eIF4GI, or (iii) combined dox-inducible DAP5 depletion/ectopic PHD2 overexpression were cultured in 0.5% oxygen (6 and 24 h) (Fig. 8A). All cell lines responded with HIF-1 α induction to 6 h under hypoxia; this response was consistently more pronounced in DAP5-depleted cells (Fig. 8A). More importantly, all cell lines (except the DAP5-depleted cultures) succeeded in containing the hypoxic response by downregulating HIF-1 α at 24 h after hypoxia (Fig. 8A). In DAP5-depleted cultures, the hypoxia-induced surge of HIF-1 α was inappropriately sustained (Fig. 8A). Ectopic PHD2 compensated for the loss of DAP5 and restored a physiologic pattern of HIF-1 α regulation, confirming prior assays implicating DAP5 in control over PHD2 translation (Fig. 8A). As suggested by earlier assays (Fig. 4E), eIF4GI does not appear to be involved in these processes (Fig. 8A).

We probed for a well-known transcriptional target of HIF-1 α , BNIP3, to assess whether the HIF-1 α response in our assays is functionally relevant. This revealed BNIP3 accumulation in step with HIF-1 α levels (Fig. 8A). Prolonged BNIP3 induction during

FIG 8 DAP5:elF2 β binding is induced during hypoxia and regulates the cellular hypoxia response through PHD2. (A) HeLa cells with dox-inducible DAP5 depletion, eIF4G1 depletion, or DAP5 depletion, combined with PHD2 overexpression, were dox treated (96 h) as shown and placed in a chamber containing 0.5% O₂ for 6 or 24 h. Lysates were prepared and analyzed by immunoblotting. The mean HIF-1 α /tubulin signal was quantified, normalizing between experiments by setting the (mock-depleted) HeLa cell 6-h value to 1. (B) U87 (top panels) and MEF (bottom panels) cells were transfected with DAP5 or control siRNA and cultured in 0.5% O₂. The mean HIF-1 α /tubulin signal was quantified, normalizing between experiments by setting control siRNA samples to 1. (C) The HEK293 knockdown/knock-in cells described for Fig. 4C and 5F were treated with dox as shown and placed in 0.5% O₂. The mean HIF-1 α /tubulin ratios were determined, normalizing by setting DAP5 depletion/DAP5 reconstitution samples to 1. (D) HEK293 cells with dox-inducible Flag-DAP5 expression were treated with dox (16 h); lysates were prepared and analyzed by immunoblotting or anti-Flag immunoprecipitation/immunoblotting as shown. The mean eIF2 β /Flag ratio from immunoprecipitations and the mean PHD2/tubulin ratio from the input signal were quantified, normalizing by setting 0-h samples to 1. All data bars represent the averages from three independent tests; error bars indicate the SEM. *, significant *P* value (*P* < 0.05).

hypoxia leads to cell death (41, 42). Thus, DAP5 depletion not only exaggerated acute HIF-1 α induction but also intercepted physiologic means of ceasing this response (Fig. 8A). This corresponded to the known role of PHD2 in controlling HIF-1 α levels. Similar effects on HIF-1 α dynamics were observed upon transient DAP5 depletion from U87 malignant glioma cells (Fig. 8B, top panels).

The response of (nonmalignant) MEFs to DAP5 depletion diverged from U87 malignant glioma cells at 24 h under hypoxia (Fig. 8B, bottom panels). This indicates that coopting DAP5 for containing damage from the hypoxic response may be a function of malignancy.

DAP5:elF2 β binding controls DAP5 activity during hypoxia. Our investigations reported above strongly implicated inducible eIF2 β binding in DAP5's role in translation control, e.g., of the PHD2 message. To analyze the role of DAP5:elF2 β in the hypoxic response, HEK293 cells with dox-inducible depletion combined with wt DAP5/DAP5(E862K) reconstitution were exposed to oxygen deprivation, as shown in Fig. 8A and B.

Reconstitution with DAP5(E862K) led to sustained, inappropriate induction of HIF-1 α and BNIP3 at 6 and 24 h in 0.5% oxygen relative to wt DAP5 reconstituted cells (Fig. 8C). This effect correlated with reduced PHD2 levels (Fig. 8C). Lastly, probing for DAP5 in the physiologic response to oxygen deprivation in HEK293 cells with dox-inducible

Flag-DAP5 revealed key insight into the DAP5:PHD2:HIF-1 α axis. Hypoxia induced DAP5:elF2 β binding and enhanced PHD2 levels (Fig. 8D). This suggests that hypoxia spurs DAP5-dependent protein synthesis, since conditions that increase binding to elF2 β corresponded with increased translational activity of DAP5 (Fig. 4). Tests for p-DAP5(T508) with the anti p-T*PP motif antibody showed no response to hypoxia (Fig. 8D), corroborating our data shown in Fig. 3C.

DISCUSSION

In mammals, translation initiation requires a bridge from mRNA to eIF3/40S ribosomal subunits that, according to current knowledge, can only be provided by any of the three eIF4G isoforms (43). Considering the dominant role of protein synthesis control in gene expression adjustment to sudden physiologic challenges, e.g., hypoxia (44), it is intuitively obvious that this critical node is subject to tight regulation. Our work focused on the eIF4G isoform DAP5, which is unique in lacking eIF4GI/II's N terminus (binding to eIF4E/PABP) and being capable of binding elF2 β . We determined (i) that DAP5:elF2 β association is strongly inducible and occurs in specific biological programs with profound gene expression shifts, (ii) that DAP5:elF2 β binding favors translation activity of DAP5, and (iii) that DAP5 is a key factor in the hypoxic response by controlling translation of the oxygen sensor and HIF-1 α destabilizer, PHD2.

We used phorbol ester-mediated PKC-Raf-ERK1/2 stimulation to decipher principles of DAP5 function. However, DAP5:elF2 β binding occurs in diverse circumstances not associated with active ERK1/2, e.g., hypoxia or mitosis. Such convergence is common in translation control due to an exceedingly broad biological repertoire carried out by a limited number of ubiquitous initiation factors. For example, phosphorylation of eIF4GI(S1232) by ERK1/2 (24) or by CDK1 (26) controls the activity of the eIF4G:4A:4B translation initiation helicase in mitogenic stimulation and in mitosis, respectively.

Mammalian elF2 does not interact with eIF3 but directly contacts 40S ribosomal subunits (45). Thus, DAP5 bound to elF2 may have unique contacts with 40S subunits (eIF4GI/II bridge 40S subunits exclusively via eIF3). eIF4GI/II only indirectly assemble with the ternary complex (elF2-GTP-Met-tRNA^{Met}, via eIF3/40S subunits); DAP5 can bind either to elF2 or to the ternary complex directly. The DAP5:elF2 β complex and its unique range of contacts in the translation apparatus may offer noncanonical means of initiation complex assembly and function that are critical in times of global protein synthesis adaptation (e.g., hypoxia or mitosis). DAP5:elF2 β binding occurs in concert with other dynamic interactions, e.g., with mRNA, eIF3 and eIF4A:4B, involved in the convoluted process of 5' UTR scanning/unwinding. Also, DAP5's HEAT2 domain, unlike eIF4GI/II's, does not bind eIF4A (Fig. 1D) (12), suggesting an involvement of other, currently unidentified DAP5 interaction partners.

elF2 β binds DAP5 at an AA box that is conserved in other elF2 β binding partners in the translation initiation apparatus, elF2B ϵ and elF5 (46). elF2 β binding to DAP5 could precede association with elF5, within a processive model of PIC and 48S interactions (14, 47–49). Since elF2B ϵ :elF2 β binding occurs outside translation initiation, it is unclear whether DAP5 competes with elF2B ϵ for elF2 β binding. Deciphering the precise contributions of DAP5:elF2 β binding to shaping “hypoxic” translation initiation complexes is a major undertaking that requires wide-ranging new studies.

eIF4GI/II can bind mRNAs through eIF4E/the m⁷G-cap, in a template-nonspecific manner. However, all eIF4G isoforms can recruit ribosomes independently of an m⁷G-cap, by directly engaging with mRNAs (50–52). The extent of the latter in global protein synthesis, and the factors governing the involvement of eIF4G isoforms at specific templates remain unknown. An involvement of DAP5:elF2 β in translation of PHD2 indicates that the physiologic context of oxygen deprivation and sequence-specific features of the PHD2 message drive template specificity of DAP5. Due to the conflicting and puzzling annotation of the PHD2 5' and 3' UTRs (38–40) extensive future work, which would be beyond the scope of the current study, is required to unravel how DAP5:elF2 β specifically targets PHD2 transcripts for translation.

In normoxia, similar basal PHD2:HIF-1 α regulation through DAP5 was observed in

TABLE 1 Oligonucleotide primers used in this study

Primer	Sequence (5'–3')
DAP5_1360_Stu_5	CGAAGGATATGCCACCTCGG
BGH_Rev	TAGAAGGCACAGTCGAGG
DAP5(E862K)_anti	CTTCTTTAATAATTTCCATGTCATAG
DAP5(E862K)_sense	GGAAATTATTAAGAAGAAGCTTTCTTGGC
DAP5(1–790)_Not1_3'	ATGCGGCCGCTTATTATTCATCGCTGGTGGGTTTACTTCAC
DAP5(S902A)_Not1_3'	GTGCGGCCGCTTAGTCAGCTTCTTCTCTGCTTCTTCTTCTCAGC
DAP5(S902D)_Not1_3'	GTGCGGCCGCTTAGTCAGCTTCTTCTCTCATCTTCTTCTTCTCAG
elF4G1(4250)_Pml_5'	CCCACGTGTGGCTCTACCTAGC
DAP5(T508A)_anti	GAGGTGGTGGCTTGAAGTGCCTGGTGGTT
DAP5(T508A)_sense	CACTCAAGCACCACTCTGGGACAG
DAP5(T508E)_anti	GAGGTGGTCTTGAAGTGCCTGGTGGTT
DAP5(T508E)_sense	CACTCAAGAACCACCTCTGGGACA
DAP5(3039)_shRNA_5'	GCCTCGAGATCTGCGGGCCAAAGCCGTAAATTGTGCAAGTGAAGCCACAGATG
DAP5(3039)_shRNA_3'	GCCTCGAGATCCGCAAGCCAAAGCCTTAAATTGTGCAACATCTGTGGCTTCAC
DAP5(3366)_shRNA_5'	GCCTCGAGATCTGCGGAGCAACACTTAATACTGTAGAAGTGAAGCCACAGATG
DAP5(3366)_shRNA_3'	GCCTCGAGGATCCGCATAGCAACACTGAATACTGTAGAACATCTGTGGCTTCAC

cancerous or nontransformed cells. However, prolonged hypoxia revealed a survival benefit mediated by engaging DAP5 in malignant cells but not in MEFs. Distinct responses to DAP5 depletion (under hypoxia) of primary MEFs versus malignant glioma cells suggest that DAP5-mediated control of PHD2 may have special significance in cancer. Neoplastic cells may exploit DAP5 for managing chronic oxygen deprivation, possibly contributing to their adaptation to growth/proliferation under hypoxia.

PHD2 levels increase during prolonged periods of low oxygen, possibly because it is a transcriptional target for HIF-1. However, transcription control may not be able to mediate the sudden changes in protein abundance required to contain HIF-1 α and the potentially deleterious hypoxia response. Indeed, system-wide analyses suggest that the response to hypoxia is accompanied by far-reaching translation adjustment (44, 53). DAP5:elF2 β -mediated control of PHD2 provides for powerful, rapid regulatory adaptation that does not require new transcription.

MATERIALS AND METHODS

Cell lines, inhibitors, and stimulants. U87, Du54, and Sum149 lines were gifts from D. Bigner and S. Nair (Duke University). MEFs were a gift from R. Fukunaga (54). Cells were grown in 10% fetal bovine serum (FBS)-containing Dulbecco modified Eagle medium (DMEM; Invitrogen) or, for Sum149, DMEM-F-12 (Lonza). Stable dox-inducible HeLa (50) and HEK293 (21) cell lines were grown in DMEM supplemented with 10% FBS, blasticidin S (5 μ g/ml for HeLa cells and 15 μ g/ml for HEK293 cells; Sigma-Aldrich), and hygromycin B (100 μ g/ml; Invitrogen). HEK293 and HeLa cells expressing a shRNA targeting DAP5 or elF4G1 were grown with G418 (500 μ g/ml; Gibco). Doxycycline (dox; Sigma-Aldrich) was dissolved in water and used at a concentration of 1 μ g/ml. 12-O-Tetradecanoylphorbol-13-acetate (TPA; Tocris) was dissolved in dimethyl sulfoxide (DMSO). Inhibitors of MEK (UO126 [Promega]; trametinib [Selleckchem]), p38 (BIRB [Selleckchem]), JNK (JNK VIII [Calbiochem]), CDK1 inhibitor (Ro-3306 [Tocris]), microtubule polymerization (nocodazole [Tocris]), and the proteasome (MG-132 [Tocris]) were dissolved in DMSO and used as described. Cell cycle inhibitor thymidine (Alfa Aesar), hydroxylation inhibitor [Cobalt(III) chloride hexahydrate, CoCl₂; Sigma], and protein synthesis inhibitors (puromycin [Sigma-Aldrich]) were dissolved in water and used as described.

Expression plasmids and stable cell lines. The construction of myc- and Flag-tagged elF4G1 expression plasmids [elF4G1(682–1600), and elF4G1(197–1600)] was described previously (22). The pCI-Flag-DAP5 vector was a generous gift from Martin Holcik (University of Ottawa, Ottawa, Canada); the PHD2 [EGLN1(myc-DDK-tagged) pCMV6-entry] and elF2 β [elF252 (myc-DDK-tagged) pCMV6-entry] vectors were obtained from Origene (catalog no. RC215158 and RC201563, respectively). DAP5, PHD2, and elF2 β were cloned from original vectors into pcDNA5/FRT/TO expression plasmid. The DAP5 truncation variant was generated by PCR amplification of the corresponding fragment. Single amino acid mutations were introduced by overlapping PCR, as described earlier (22). The generation of stable HEK293 and HeLa cell lines with dox-inducible expression of myc/Flag-tagged elF4G1 and HEK293 expressing Flag-tagged DAP5 has been described previously (55). DAP5 depletion/knock-in HeLa and HEK293 cell lines were established similarly to elF4G1, as described earlier (23, 26). The primers used in this study are listed in Table 1.

Antibodies, immunoprecipitation, immunoblots, siRNA transfections, and RT-qPCR. Antibodies to elF4A, elF3a, MNK1, elF2 α , p-elF2 α (S51), rpS6, p-rpS6(240/244), DAP5, elF4G1, p-ERK1/2(T202/Y204), ERK1/2, CK2 α , GAPDH, p-p38(T108/Y182), p38, pT*PP substrate, p-cJun(S73), cJun, p-histone H3(S10), histone H3, p-JNK(T183/Y185), COX2, MKP-2, HIF-1 α , PHD2, FIH1, ARD1, VHL, OH-HIF1 α (P564), rpL13a,

p-Akt(T308), Akt1, and BNIP3 (Cell Signaling Technologies); eIF2 β , CK2 β , and PHD3 (Novus); Flag, tubulin, and myc (Sigma-Aldrich); puromycin (EMD Millipore); and PHD1 (R&D Systems) were used. Two ~70% confluent 150-mm dishes were treated as described in the figures and legends, and immunoprecipitations were performed as previously described (24). Immunoblotting was performed as described previously (22). Immunoblot signals were obtained on and quantified using the Li-COR Odyssey FC2 imaging system and Image Studio software. DAP5 depletion from U87, Du54, and Sum149 was performed using siRNAs. For this, cells in six-well plates were transfected with 2 μ l of 20 μ M DAP5 (GE Dharmacon) or All-Stars control siRNA (Qiagen) using jetPRIME transfection reagent (Polyplus Transfections) according to the manufacturer's protocol. For RT-qPCR analysis, an RNA-to-CT one-step kit (Invitrogen) was used according to the manufacturer's protocol on a 7900 HT TaqMan machine; samples were measured in triplicate, and the average value was used for further calculation. For analysis of PHD2 mRNA levels following DAP5 depletion, data were analyzed by using the $2^{-\Delta\Delta CT}$ method using GAPDH as a reference. To analyze polysome profile-derived RNA, the 2^{-CT} value of each fraction was divided by the sum of 2^{-CT} values for all samples and multiplied by 100 to determine the percent RNA in each fraction.

In vitro phosphorylation assays and phosphoproteomic analysis. For *in vitro* phosphorylation and proteomic analyses, Flag-tagged DAP5 was immunoprecipitated from dox-induced cells and eluted from beads using Flag-peptide (Sigma-Aldrich) in NT-2 buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM MgCl₂, 0.05% Igepal CA-630) at 4°C (16 h). Flag-affinity beads were removed using 30 μ M chromatography columns (Thermo-Fisher), and eluted proteins were concentrated using a 10-kDa cutoff protein concentration column (Millipore). *In vitro* phosphorylation of recombinant Flag-tagged DAP5 by recombinant JNK2, or CDK1/cyclin A1 (SignalChem) was performed in 15 μ l in kinase assay buffer (SignalChem) with 2 mM ATP, 0.2 mM dithiothreitol, and 2 $\times$ HALT protease and phosphatase inhibitor cocktail (Thermo Scientific). The reaction mixture was incubated at room temperature with 1 or 5 μ l of recombinant kinase (0.1 μ g/ μ l; 1 h). Samples were mixed with 5 μ l of 4 $\times$ LDS buffer (Invitrogen) with 2.5% β -mercaptoethanol and heated for 1 min at 95°C to stop the enzymatic reaction, followed by immunoblotting. Fifteen ~70% confluent 150-mm dishes of cells were treated with TPA as described in the figures and figure legends. Flag IP, followed by Flag elution purification, was performed with final purification and concentration of each sample into 50 mM NH₄HCO₃ (pH 8.0). Proteomic analyses were carried out at the Duke University Proteomic Core Facility as previously described (26).

Polysome profiling. Cells in confluent 100-mm³ plates treated with or without dox were harvested and lysed in 1 ml of lysis buffer (50 mM HEPES [pH 7.2], 200 mM potassium acetate, 0.5% NP-40 [Sigma], 1 $\times$ HALT protease and phosphatase inhibitor, 1 $\times$ RNaseOUT [Thermo Fisher Scientific], 50 μ g/ml cycloheximide, 10 mM MgCl₂). Sucrose gradients (15 to 50%) were prepared in lysis buffer without detergent; ~80% of total lysate (800 μ l) was loaded onto the gradients and spun at 35,000 rpm (using an SW41 rotor) for 180 min at 4°C. A Teledyne instrument was used to analyze and create 500- μ l fractions. RNA was isolated using TRIzol (Invitrogen), followed by RNeasy column purification (Qiagen). RNA was suspended in 25 μ l of RNase-free water and stored at -80°C. Protein was extracted by first diluting a split fraction 1:1 with sterile water and then adding 0.11 volumes of trichloroacetic acid, followed by incubation at 4°C (1 h). Samples were pelleted at 16,000 $\times$ g for 25 min at 4°C, and the supernatant was discarded. The pellets were washed in ice-cold acetone, spun again for 10 min, dried, and processed for immunoblotting.

Hypoxia treatment. Cells were grown in six-well plates or 150-mm dishes (for immunoprecipitation) and treated prior to hypoxia in a chamber (Coy Laboratory Products, Inc.) as described in the figures and figure legends. Cells were then moved into the inner hypoxia chambers which maintained cells at 37°C, 5% CO₂, and 0.5% oxygen. The inner chamber is located within a larger chamber with arm-ports that is maintained at 8% oxygen. Once the cells are placed in the inner chambers and the desired oxygen concentration is reached, the inner chamber remained closed, and the cells were not manipulated until lysis. To lyse the cells, the growth media were rapidly removed, and 150 μ l of LDS buffer or 800 μ l of polysomal lysis buffer (PLB [for immunoprecipitation]; 10 mM HEPES [pH 7.4], 100 mM KCl, 5 mM MgCl₂, 0.5% Igepal CA-630 [Sigma], 3 mM dithiothreitol, 1 $\times$ HALT phosphatase inhibitor cocktail) was added to the cells. The lysates were placed on dry ice.

Statistics. Statistical analysis was performed using JMP (v.13) software (SAS). All error bars represent the standard errors of the mean (SEM). For multiple group comparisons, all groups from each experimental repeat were compared using analysis of variance (ANOVA). If the ANOVA result was significant ($P < 0.05$), a *post hoc* Tukey honestly significant difference test was performed. Paired *t* tests were used for data comparing only two groups.

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