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Cancer Research

eIF2α Kinase PKR Modulates the Hypoxic Response by Stat3-Dependent Transcriptional Suppression of HIF-1α

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Abstract

Hypoxia within the tumor microenvironment promotes angiogenesis, metabolic reprogramming, and tumor progression. In addition to activating hypoxia-inducible factor-1α (HIF-1α), cells also respond to hypoxia by globally inhibiting protein synthesis via serine 51 phosphorylation of translation eukaryotic initiation factor 2α (eIF2α). In this study, we investigated potential roles for stress-activated eIF2α kinases in regulation of HIF-1α. Our investigations revealed that the double-stranded RNA–dependent protein kinase R (PKR) plays a significant role in suppressing HIF-1α expression, acting specifically at the level of transcription. HIF-1α transcriptional repression by PKR was sufficient to impair the hypoxia-induced accumulation of HIF-1α and transcriptional induction of HIF-1α–dependent target genes. Inhibition of *HIF-1A* transcription by PKR was independent of eIF2α phosphorylation but dependent on inhibition of the signal transducer and activator of transcription 3 (Stat3). Furthermore, *HIF-1A* repression required the T-cell protein tyrosine phosphatase, which acts downstream of PKR, to suppress Stat3. Our findings reveal a novel tumor suppressor function for PKR, which inhibits HIF-1α expression through Stat3 but is independent of eIF2α phosphorylation. *Cancer Res; 70(20); 7820–9. ©2010 AACR.*

Introduction

An important means by which cells respond to environmental stress is the inhibition of mRNA translation (1). A well-characterized mechanism of inhibition of protein synthesis is through the phosphorylation of the α subunit of the translation eukaryotic initiation factor 2 (eIF2) at serine 51 (S51; ref. 2). Phosphorylated eIF2α acts as a dominant inhibitor of the guanine exchange factor eIF2B, which prevents the recycling of eIF2 between succeeding rounds of protein synthesis and eventually leads to a global obstruction of mRNA translation initiation (2). This allows cells to adapt to stressful conditions by economizing on energy expended by protein synthesis (2). The adaptation process of eIF2α phoshorylation involves the selective translation of transcription factors such as activating transcription factor 4 (ATF4) (3) and ATF5 (4), which induce the expression of genes that facilitate adaptation. In cases of prolonged stress, the induction of eIF2α phosphorylation leads to cell death through the induction of apoptotic pathways (2).

In mammalian cells, eIF2α phosphorylation is mediated by a family of protein kinases, each of which responds to distinct forms of environmental stress (2). The eIF2α kinase family includes heme-regulated inhibitor (HRI), general control non-derepressible 2 (GCN2), endoplasmic reticulum (ER)–resident protein kinase (PERK), and double-stranded RNA (dsRNA)–dependent protein kinase R (PKR), which are activated by heme deficiency, the absence of amino acids, improperly folded proteins accumulated in the ER, and dsRNA, respectively (2). Whereas HRI protein is mainly expressed in erythroid cells, GCN2, PERK, and PKR are found in all tissues. Despite their diverse regulatory domains, the kinase domains of these enzymes are significantly conserved explaining their specificity toward eIF2α (2). In addition to their function in phosphorylating eIF2α, there has been strong evidence to suggest that mammalian eIF2α kinases can also mediate biological effects independent of eIF2α phosphorylation (5–7).

A common stress condition encountered by cells during normal development, but also in many pathologic cases including cancer, is the lack of oxygen or hypoxia. At certain stages during tumorigenesis, cancer cells find themselves in microenvironments of low oxygen. The ability to adapt to hypoxic conditions has important effects on tumor

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development, which determines disease progression and clinical prognosis (8). At the molecular level, coordinated cellular responses allow tumor cells to alter gene expression and induce survival pathways in response to hypoxic stress by exploiting transcriptional and translational machinery (9). Hypoxia-inducible factor 1 (HIF-1) is a key transcription factor in mediating responses to oxygen-deficient conditions. HIF-1 plays a major role in tumorigenesis by activating many genes that promote angiogenesis [e.g., vascular endothelial growth factor (VEGF)], mediate metabolic reprogramming [e.g., glucose transporter-1 (GLUT-1)], and facilitate metastasis [e.g., 3-phosphoinositide-dependent protein kinase-1 (PDK-1); ref. 8]. As such, HIF-1α levels correlate with tumor progression and poor clinical prognosis (8). Because of its crucial role in cancer development, HIF-1α is regarded as an attractive target for therapeutics (8).

HIF-1 consists of an α subunit and a β subunit, which heterodimerize, bind to DNA, and induce transcription of target genes. Whereas HIF-1β (also known as ARNT) is constitutively expressed, HIF-1α levels are tightly regulated (8). Under normal oxygen conditions, HIF-1α is modified by prolyl hydroxylases (PHD) at specific proline residues, which triggers binding of the tumor suppressor Von Hippel-Lindau (VHL) protein and subsequent ubiquitination and proteasomal degradation (8). In contrast, oxygen-dependent hydroxylation does not occur in hypoxic conditions leading to the accumulation of HIF-1α and induction of HIF-1 activity (8). HIF-1 transcriptional activity is also controlled by oxygen tension, as asparaginyl hydroxylation of HIF-1α by FIH-1 (factor inhibiting HIF-1) impairs its association with the transcriptional coactivator CBP/p300 (10).

It has been long considered that regulation of HIF-1α is exclusively a posttranscriptional process (8). However, very recent data suggest that transcriptional control of HIF-1α mRNA synthesis can also be important, at least under certain conditions or in certain cell types, and can involve distinct transcription factors such as NF-κB (11, 12), SP-1 (13), or signal transducer and activator of transcription 3 (Stat3; ref. 14). Regulation of HIF-1α gene transcription may not affect HIF-1α protein levels during normoxia because the PHD-VHL-proteasome system is still limiting, but it can significantly alter the HIF-1α protein expression levels on hypoxia and greatly affect the corresponding cellular response.

Hypoxia has been previously shown to induce the unfolded protein response (UPR), which leads to PERK activation (15, 16). Many aspects of the UPR are cytoprotective, and several studies indicate that it plays a positive role in facilitating tumor growth (17). Given that induction of eIF2α phosphorylation and upregulation of HIF-1α represent important mechanisms of cell adaptation to hypoxic stress, we were interested to examine whether the eIF2α phosphorylation pathway is involved in regulating HIF-1α expression. Herein, we show that the eIF2α kinase PKR plays a specific role in suppressing HIF-1α levels through mechanisms that are independent of eIF2α phosphorylation and translational control. More specifically, our data reveal that PKR can suppress the transcription of the *HIF-1A* gene via a mechanism involving Stat3.

Materials and Methods

Cell culture and treatments

$PKR^{-/-}$, $PERK^{-/-}$, $GCN2^{-/-}$, $eIF2\alpha^{A/A}$ mouse embryonic fibroblasts (MEF) and their isogenic wild-type MEFs were cultured as previously described (6). T-cell protein tyrosine phosphatase$^{-/-}$ (TC-$PTP^{-/-}$) MEFs and their isogenic counterparts were cultured as described (18). HT1080 expressing GyrB.PKR were maintained as previously described (19). H1299 cells were maintained in DMEM (Wisent) supplemented with 10% fetal bovine serum (Wisent) and 100 units/mL of penicillin-streptomycin (Wisent). $NiCl_2$ (Sigma), $CoCl_2$ (Fisher Scientific), and cycloheximide (Sigma) were dissolved in distilled H_2O. CPA-7 (20) and 2-methoxyestradiol (Sigma) were dissolved in DMSO. Coumermycin (Sigma) was dissolved in DMSO. For hypoxic treatments, cells were incubated in the hypoxic chamber (Coy Laboratory Products, Inc.) in the presence of 1% O_2, 5% CO_2, and 94% N_2 at 37°C.

Protein extraction, immunoblot analysis, and immunoprecipitation

Protein extraction, immunoblotting, and immunoprecipitation were performed as described (18). For immunoblotting and/or immunoprecipitation, the following antibodies were used: mouse monoclonal antibody for mouse HIF-1α (R&D Systems), rabbit HIF-2α (Novus Biologicals), anti-TC-PTP mouse monoclonal antibody (18), mouse monoclonal antibody for actin (Clone C4, ICN Biomedicals, Inc), rabbit anti-tubulin (Chemicon), mouse monoclonal against PKR (F9; ref. 18), rabbit polyclonal phosphospecific against S51 of eIF2α (Invitrogen), mouse monoclonal to eIF2α (Cell Signaling), rabbit anti–iron regulatory protein 2 (IRP2; ref. 21), mouse anti-Stat3 (Cell Signaling), and antiphoshorylated tyrosine 705 (Y705) Stat3 (Santa Cruz). All antibodies were used at a final concentration of 0.1 to 1 μg/mL. After incubation with antimouse IgG or antirabbit IgG antibodies conjugated to horseradish peroxidase, proteins were visualized with the enhanced chemiluminescence reagent (Thermo Scientific) detection system according to the manufacturer's instructions. Quantification of protein bands was performed by densitometry using Scion Image from NIH.

RNA isolation and real-time PCR

Total RNA was isolated by Trizol reagent (Invitrogen) according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed with the high-capacity cDNA reverse transcription kit (Applied Biosystems International). Quantitative real-time–PCR (RT-PCR) was performed in a Miniopticon RT-PCR system (BIORAD) using the IQ SYBR Green Supermix (BIORAD) and primers for mouse HIF-1α, VEGF, and GLUT-1. The levels of mRNA were normalized to mouse β-actin mRNA. cDNA of three independent experiments was analyzed in duplicates. The primers used for quantitative RT-PCR were mouse HIF-1α sense GCACTAGACAAAGTTCACCTGAGA, mouse HIF-1α antisense CGCTATCCACATCAAAGCAA, mouse VEGF sense GCAGCTTGAGTTAAACGAACG, mouse VEGF antisense GGTTCCCGAAACCCTGAG, mouse SLC2A1 (GLUT-1) sense

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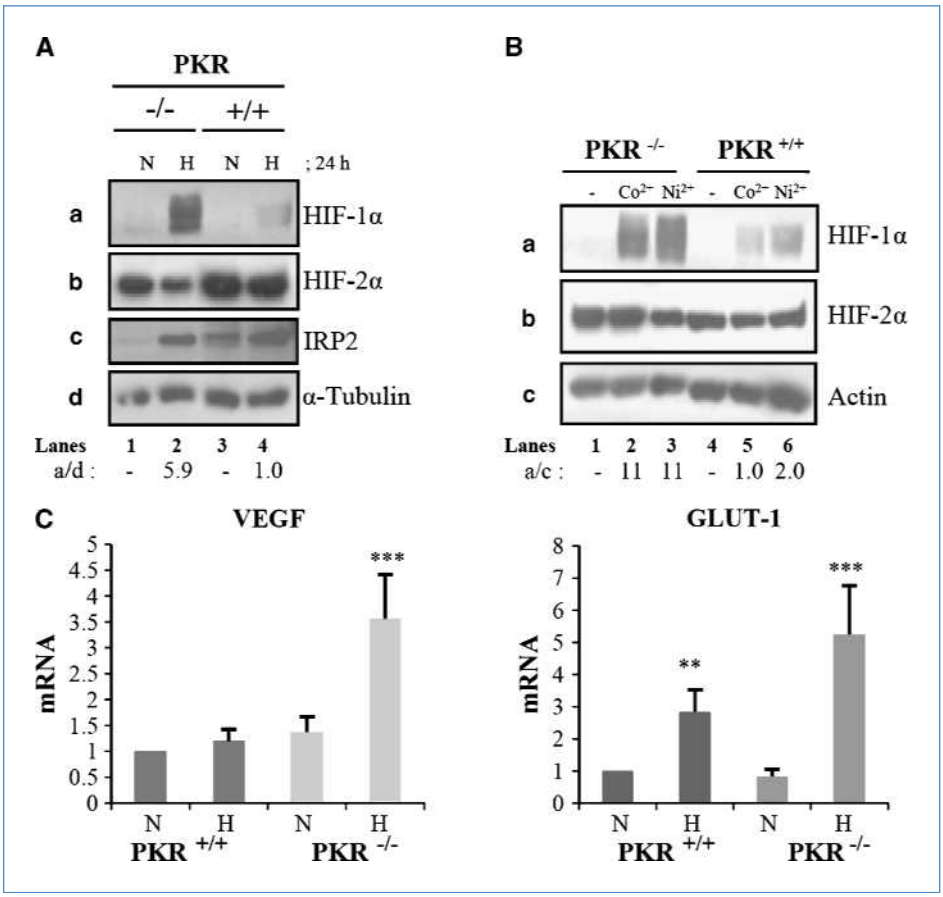

Figure 1. PKR reduces HIF-1α protein accumulation and activity on hypoxic treatment. $PKR^{+/+}$ and $PKR^{-/-}$ MEFs were incubated in normoxic (N, 21% O_2) or hypoxic conditions (H, 1% O_2) for 24 h (A) or treated with either cobalt chloride (Co^{2+}, 200 μmol/L) or nickel chloride (Ni^{2+}, 500 μmol/L) for 20 h (B). A and B, protein extracts (70 μg) were subjected to immunoblot analysis for the indicated proteins. The ratio of HIF-1α to actin for each lane is indicated. C, RNA was isolated from $PKR^{+/+}$ and $PKR^{-/-}$ MEFs subjected to normoxia or hypoxia (1% O_2) for 24 h. Quantitative RT-PCR was performed using primers detecting transcripts of mouse *VEGF* and *GLUT-1*. Results are representative of an average of three independent experiments. Statistical analysis was performed, and *P* values compared with $PKR^{+/+}$ in normoxia are indicated. ***, $P < 0.001$; **, $P < 0.005$.

ATGGATCCCAGCAGCAAG, mouse SLC2A1 (GLUT-1) antisense CCAGTGTTATAGCCGAACTGC, mouse β-actin sense CTAAGGCCAACCGTGAAAAG, and mouse β-actin antisense ACCAGAGGCATACAGGGACA. Relative quantification was done with the REST-MCS software.

PKR targeting by short hairpin RNA

For targeting of human PKR by short hairpin RNA (shRNA) 5′-GCAGGGAGTAGTACTTAAA-3′ and 5′-GGCAGTTAGTCCTTTATTA-3′ were subcloned into pCXS U6/Zeo expression vector. H1299 cells harboring the target vector were selected for resistance to 400 μg/mL zeocin. As control, zeocin-resistant cells harboring empty pCXS/zeo DNA were used.

Reporter gene assays

Cells were transfected by Lipofectamine Plus (Invitrogen) with 0.5 μg of pGL3 vector containing the firefly luciferase gene under the control of the *HIF-1A* promoter (*HIF-1A Luc*; ref. 22) or pGL3 vector alone (control). As an internal control, 0.1 μg of pRL-TK vector (Promega Corp.), which contains the *Renilla* luciferase reporter gene, was used. Cells were either lysed 48 hours posttransfection (Figs. 4B and 6D), left untreated or treated with 20 μmol/L CPA-7 for 24 hours, or cotransfected with 0.4 μg of Stat3D (previously described in ref. 23) or the control pcDNA and lysed 48 hours posttransfection (Fig. 5D), as indicated in the figure legends. Firefly and *Renilla* luciferases were determined in protein extracts using the dual-luciferase reporter system (Promega Corp.) according to the manufacturer's specifications, and firefly luciferase was normalized to *Renilla* luciferase. Relative luciferase activity refers to normalized firefly luciferase activity by the luciferase activity measured in cells transfected with control vector.

Results

PKR reduces the levels of HIF-1α protein expression in hypoxic cells

First, we checked the protein expression levels of HIF-1α protein in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs maintained under normoxic (20% O_2) or hypoxic conditions (1% O_2) for 24 hours. Expression of HIF-1α was barely detectable under normoxic conditions but readily induced on hypoxic treatment (Fig. 1A). However, hypoxic HIF-1α expression was substantially higher in $PKR^{-/-}$ MEFs compared with $PKR^{+/+}$ MEFs (Fig. 1A). When hypoxia-mimetic compounds, such as $CoCl_2$ (24) or $NiCl_2$ (25), were used to treat cells, we also observed a higher induction of HIF-1α in $PKR^{-/-}$ than in $PKR^{+/+}$ MEFs (Fig. 1B). Contrary to HIF-1α, HIF-2α expression was readily

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detectable in normoxic cells and was maintained in similar levels when cells were kept under hypoxia (Fig. 1A and B). The lack of an induction of HIF-2α in the hypoxic MEFs is in line with previous findings showing that HIF-2α is not upregulated in mouse embryonic cells under hypoxia (26). However, unlike HIF-2α, we found that the IRP2, which is induced by hypoxia (21), was efficiently induced in both $PKR^{+/+}$ and $PKR^{-/-}$ MEFs (Fig. 1A, c) supporting a specific role of PKR in suppressing HIF-1α in hypoxic cells.

It was next important to determine whether the higher expression of HIF-1α in $PKR^{-/-}$ MEFs also resulted in increased HIF-1 activity. To do so, we examined the expression of HIF-1 target genes under normoxic or hypoxic conditions by quantitative RT PCR. We found that the mRNA levels of the *VEGF* and *GLUT-1* genes were higher in $PKR^{-/-}$ than in $PKR^{+/+}$ MEFs under hypoxia (Fig. 1C), providing evidence that the difference in HIF-1α protein levels has functional significance in these cells. We also noticed that *VEGF* was modestly induced in hypoxic $PKR^{+/+}$ MEFs compared with *GLUT-1* (Fig. 1C). This is in line with previous studies showing a higher induction of *GLUT-1* than *VEGF* by HIF-1α in mouse fibroblasts under hypoxia (27, 28). Given that HIF-2α also targets HIF-1–dependent genes such as *VEGF* and *GLUT-1* (29), it is also possible that constitutive expression of HIF-2α in MEFs (Fig. 1A, b; ref. 26) renders these cells less responsive to HIF-1α–dependent gene transcription under hypoxic conditions.

To determine whether inhibition of HIF-1α expression in the hypoxic cells is specific for PKR, we examined the role of other eIF2α kinases in this process. To do so, we used MEFs deficient in PERK or GCN2 together with their genetically matched wild-type MEFs. We found that HIF-1α was equally induced in control wild-type MEFs or MEFs lacking PERK (Fig. 2A) or GCN2 (Fig. 2C) under hypoxic conditions. Consistent with these observations, treatment with the hypoxia mimetic compounds $CoCl_2$ and $NiCl_2$ resulted in an equal induction of HIF-1α in wild-type MEFs and MEFs lacking either PERK (Fig. 2B) or GCN2 (Fig. 2D). Taken together, these data supported a specific role of PKR in the negative regulation of HIF-1α under hypoxic conditions.

Next, we attempted to determine whether inhibition of HIF-1α expression by PKR in hypoxic cells was due to eIF2α phosphorylation. To this end, we used MEFs containing either a wild-type allele of eIF2α ($eIF2\alpha^{S/S}$) or a knock-in S51A mutant allele ($eIF2\alpha^{A/A}$), which produces a protein that cannot be phosphorylated by the eIF2α kinases. We observed similar induction of HIF-1α between $eIF2\alpha^{S/S}$ and $eIF2\alpha^{A/A}$ MEFs under hypoxic conditions (Fig. 3A) or after treatment with $CoCl_2$ or $NiCl_2$ (Fig. 3B), suggesting that eIF2α phosphorylation is not playing a major role in the induction of HIF-1α under these treatments. To determine whether the decreased inducibility of HIF-1α in $PKR^{+/+}$ MEFs was not a side effect of the genetic background or immortalization of the cells that dampened their responsiveness to hypoxic treatment, we compared HIF-1α induction in different wild-type MEFs that were isogenic to $PERK^{-/-}$, $GCN2^{-/-}$, or $eIF2\alpha^{A/A}$ MEFs. We found that treatment of the various wild-type MEFs with $NiCl_2$ resulted in a comparable induction of HIF-1α, ruling out the possibility of a defective response of $PKR^{+/+}$ MEFs to hypoxic environment (Supplementary Fig. S1). Also, PKR did not affect HIF-1α stability in hypoxic cells because inhibition of protein synthesis by cycloheximide treatment caused HIF-1α destabilization in both $PKR^{+/+}$ and $PKR^{-/-}$ MEFs (Supplementary Fig. S2).

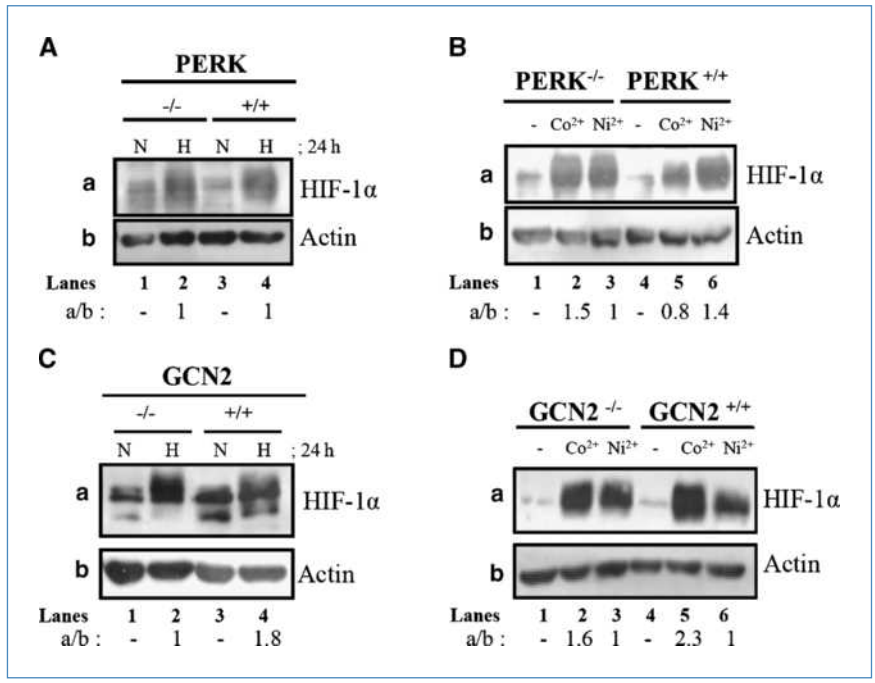

Figure 2. PERK and GCN2 do not affect HIF-1α expression under hypoxic conditions. $PERK^{+/+}$ and $PERK^{-/-}$ MEFs (A, B) as well as $GCN2^{+/+}$ and $GCN2^{-/-}$ MEFs (C, D) were incubated under normoxic (N, 21% O_2) or hypoxic (H, 1% O_2) conditions for 24 h (A, C) or treated with either cobalt chloride (Co^{2+}, 200 μmol/L) or nickel chloride (Ni^{2+}, 500 μmol/L) for 20 h (B, D). (A–D) Protein extracts (70 μg) were subjected to immunoblot analysis for HIF-1α (a) and actin (b). The intensity of the bands was normalized, and ratios (a/b) are indicated.

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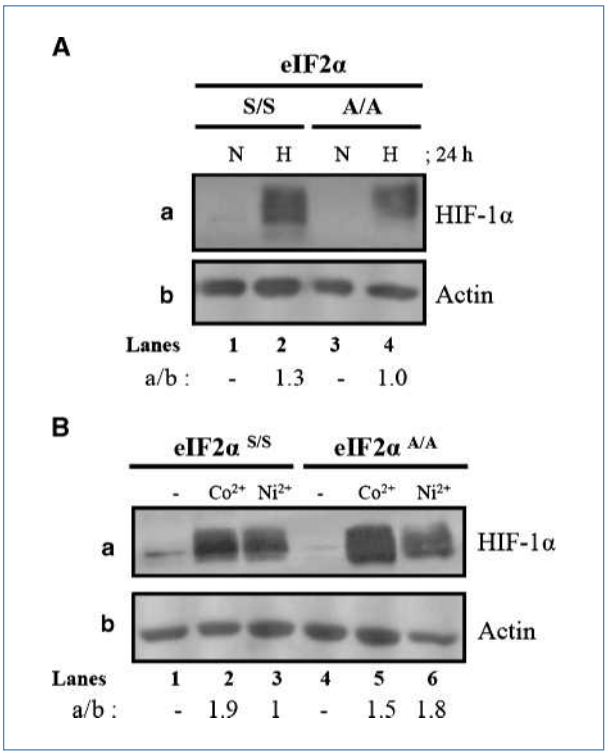

Figure 3. HIF-1α induction by hypoxia is not affected by the eIF2α phosphorylation status. The eIF2α$^{S/S}$ and eIF2α$^{A/A}$ MEFs were incubated in normoxic (N, 21% O_2) or hypoxic (H, 1% O_2) conditions for 24 h (A) or were treated with either cobalt chloride (Co^{2+}, 200 μmol/L) or nickel chloride (Ni^{2+}, 500 μmol/L) for 20 h (B). A and B, whole-cell extracts (70 μg) were subjected to immunoblot analysis for HIF-1α (a) and actin (b). The normalized ratio (a/b) of band intensity is indicated.

PKR reduces HIF-1α expression at the transcriptional level

To further address the mechanism of inhibition of HIF-1α expression by PKR, we examined the HIF-1α mRNA levels in PKR$^{+/+}$ and PKR$^{-/-}$ MEFs by quantitative real-time PCR. We found that HIF-1α mRNA was more highly expressed in PKR$^{-/-}$ than in PKR$^{+/+}$ MEFs under normoxic conditions, and this difference in mRNA levels was maintained after hypoxic treatment (Fig. 4A). We also found that the status of eIF2α phosphorylation did not have a significant effect on HIF-1α mRNA levels as determined by the analysis of eIF2α$^{S/S}$ and eIF2α$^{A/A}$ MEFs (Fig. 4A). These data indicated a possible transcriptional regulation of HIF-1α expression by PKR in normoxic as well as hypoxic cells.

To confirm that *HIF-1A* gene was under transcriptional control by PKR, we performed transient transactivation assays in PKR$^{+/+}$ and PKR$^{-/-}$ MEFs using a luciferase reporter gene under the control of the *HIF-1A* promoter. Consistent with the upregulation of HIF-1α mRNA levels in the PKR$^{-/-}$ MEFs (Fig. 1A), the reporter gene assays revealed an ~3-fold increase of *HIF-1A* promoter activity in PKR$^{-/-}$ MEFs compared with PKR$^{+/+}$ MEFs supporting a transcriptional effect of PKR on HIF-1α expression (Fig. 4B).

PKR controls *HIF-1A* gene transcription through Stat3

Recent reports have suggested that *HIF-1A* gene transcription can be directly induced by activated Stat3 (14, 30). Additionally, our group has previously shown that Stat3 activity is impaired by PKR due to activation of TC-PTP, which targets the phosphorylation of Stat3 at Y705 (18). Consistent with this finding, we observed a higher (10-fold) phosphorylation of Stat3 at Y705 in PKR$^{-/-}$ than in PKR$^{+/+}$ MEFs under normoxia (Fig. 5A). We also observed an increase of Stat3 phosphorylation in the eIF2α $^{A/A}$ MEFs (Fig. 5B), which was not as high as in PKR$^{-/-}$ MEFs (Fig. 5A). Nevertheless, increased Stat3 phosphorylation in the eIF2α $^{A/A}$ MEFs did not affect HIF-1α expression (Figs. 3 and 4A). These data indicate that eIF2α phosphorylation is necessary for a partial inhibition of Stat3, which is not sufficient to decrease the transactivation of *HIF-1A* gene. Unlike PKR, neither PERK nor GCN2 displayed a role in regulating Stat3 phosphorylation in MEFs (Supplementary Fig. S3).

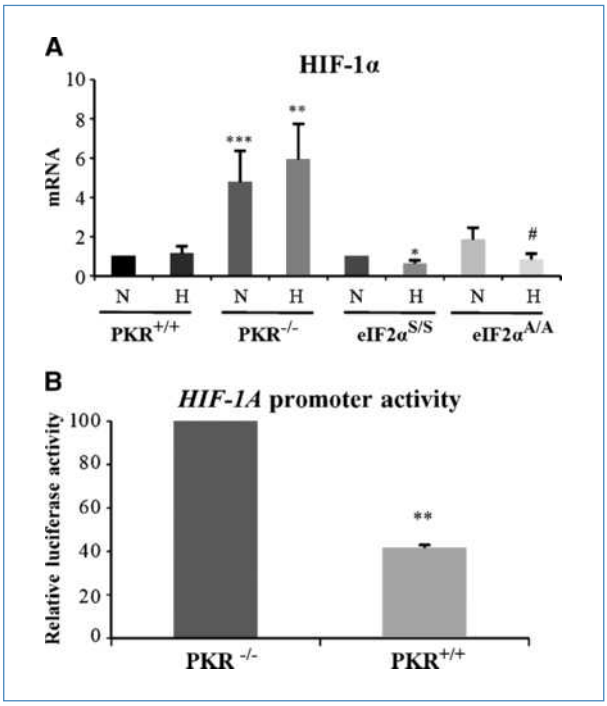

Figure 4. PKR inhibits HIF-1α expression at the transcriptional level. A, RNA was isolated from PKR$^{+/+}$ and PKR$^{-/-}$ MEFs as well as eIF2α$^{S/S}$and eIF2α$^{A/A}$ MEFs subjected to normoxia (N, 21% O_2) or hypoxia (H, 1% O_2) for 24 h. Quantitative RT-PCR was performed using primers targeting mouse HIF-1α. Statistical analysis was performed. ***, $P < 0.001$; **, $P < 0.005$ compared with PKR$^{+/+}$ in normoxia; *, $P < 0.05$ compared with eIF2α$^{S/S}$ normoxia; #, $P < 0.05$ compared with eIF2α$^{A/A}$ normoxia. B, PKR$^{+/+}$ and PKR$^{-/-}$ MEFs were transiently transfected with constructs containing the firefly luciferase reporter gene under the control of the HIF-1α promoter or control vector. A second vector containing a *Renilla* luciferase reporter gene was cotransfected as an internal control. Luciferase activity was assessed 48 h posttransfection. Relative luciferase activity of the HIF-1α promoter was normalized to the activity of the control vector. Error bar, SEM ($n = 6$). Statistical analysis was performed using the paired t test. **, $P < 0.001$.

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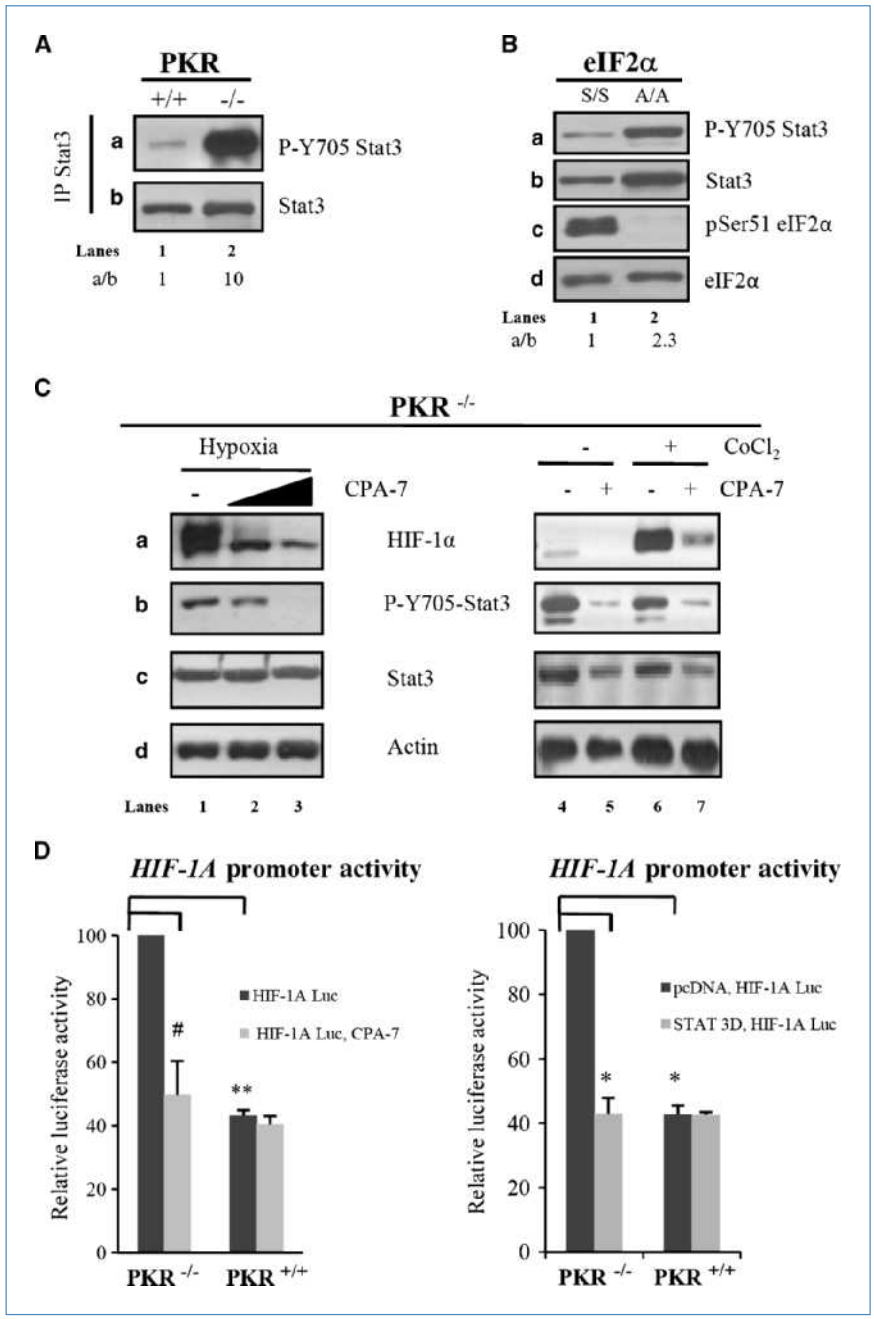

Figure 5. PKR controls *HIF-1A* gene transcription through Stat3. A, Stat3 was immunoprecipitated from whole-cell extracts (750 μg of protein) from untreated $PKR^{+/+}$ and $PKR^{-/-}$ MEFs. Immunoprecipitated protein was immunoblotted with antibodies detecting phosphorylated Stat3 at Y705 (a) or total Stat3 (b). The ratio a/b of band intensity was measured by densitometry, normalized, and indicated at the bottom. B, extracts from untreated $eIF2\alpha^{S/S}$and $^{A/A}$ MEFs (50 μg of protein) were resolved and immunoblotted with antibodies targeting Y705 phosphorylated Stat3 protein (a), total Stat3 (b), S51 phosphorylated eIF2α (c), and total eIF2α (d). The ratio of the bands (a/b) for each lane is indicated. C, $PKR^{-/-}$ MEFs were untreated or treated with 20 or 50 μmol/L of CPA-7 in hypoxic conditions (H, 1% O_2) for 24 h (lanes 1–3) or left untreated or treated with CPA-7 (20 μmol/L) in the presence or absence of $CoCl_2$ (200 μmol/L) for 24 h (lanes 4–7). Protein extracts were subjected to immunoblotting for HIF-1α (a), Y705 phosphorylated Stat3 protein (b), total Stat3 (c), and actin (d). D, $PKR^{+/+}$ and $PKR^{-/-}$ MEFs were transiently transfected with pGL3 constructs containing the firefly luciferase reporter gene under the control of the HIF-1α promoter or control vector. A second vector containing a *Renilla* luciferase reporter gene was cotransfected as an internal control. Cells were treated with CPA-7 (20 μmol/L) for 24 h and lysed 27 h after transfection. Relative luciferase activity of the HIF-1α promoter was normalized to the basal activity of control vector with respect to each treatment. Error bar, SEM ($n = 4$). Statistical analysis was performed using the paired *t* test. #, $P < 0.01$; **, $P < 0.001$. Similarly, the MEFs were transiently cotransfected with *HIF-1A* Luc or control pGL3 construct with pcDNA encoding a Stat3 dominant negative or control vector and with the internal control vector containing the *Renilla* luciferase reporter gene. Cells were lysed 48 h after transfection. Relative luciferase activity of the *HIF-1A* Luc was normalized to the basal activity of the control vectors. Error bars, SEM ($n = 4$). Statistical analysis was performed using the paired *t* test. *, $P < 0.05$.

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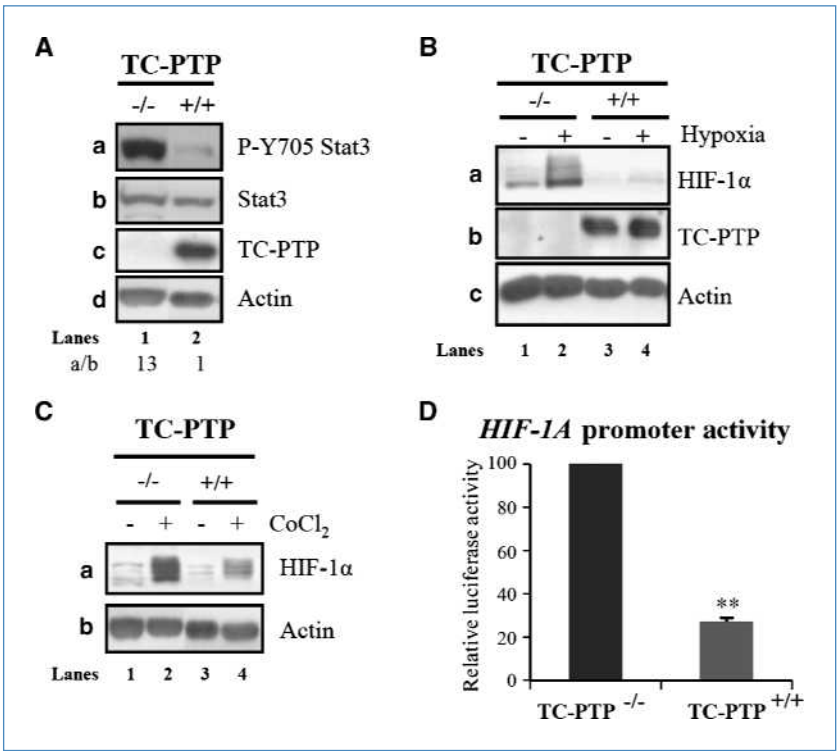

Figure 6. TC-PTP is involved in the transcriptional control of *HIF-1A* gene by Stat3. A, extracts from TC-PTP$^{+/+}$ and TC-PTP $^{-/-}$ MEFs were immunoblotted with antibodies targeting the Y705 phosphorylated Stat3 protein (a), total Stat3 (b), TC-PTP (c), and actin (d). The ratio of the bands (a/b) for each lane is indicated. B, TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ MEFs were incubated in normoxic (N, 21% O_2) or hypoxic conditions (H, 1% O_2) for 24 h. Whole-cell extracts (70 μg) were subjected to immunoblot analysis with anti-HIF-1α (a), anti-TC-PTP (b), and anti-actin (c) antibodies. C, TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ MEFs were treated for 24 h with 200 μmol/L $CoCl_2$. Whole-cell extracts (70 μg of protein) were subjected to immunoblot analysis with anti-HIF-1α (a) and anti-actin (b) antibodies. D, TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ MEFs were transiently transfected with constructs containing the firefly luciferase reporter gene under the control of the HIF-1α promoter or control vector and a second vector containing a *Renilla* luciferase reporter gene as an internal control. Luciferase activity was assessed from extracts obtained 48 h posttransfection. Relative luciferase activity of the *HIF-1A* promoter was normalized to the activity of the control vector. Error bar, SEM ($n = 8$). Statistical analysis was performed using the paired *t* test. **, $P < 0.001$.

To examine whether the increased levels of phosphorylated Stat3 were responsible for increased HIF-1α protein expression, PKR$^{-/-}$ MEFs were exposed to hypoxic conditions in the absence or presence of the Stat3 chemical inhibitor CPA-7 (20). CPA-7 inhibited Stat3 Y705 phosphorylation, and this was associated with a decrease in HIF-1α expression (Fig. 5C). We also observed a similar regulation when PKR$^{-/-}$ MEFs were treated with $CoCl_2$ in the absence or presence of CPA-7. That is, we found that CPA-7 impaired the induction of HIF-1α by $CoCl_2$ concomitantly with an inhibition of Stat3 phosphorylation at Y705 (Fig. 5C).

To analyze the effect of Stat3 phosphorylation on HIF-1α expression, we measured *HIF-1A* promoter activity by luciferase reporter assays in PKR$^{+/+}$ and PKR$^{-/-}$ MEFs. We found that pharmacologic inhibition of Stat3 by CPA-7 resulted in the inhibition of *HIF-1A* promoter activity in PKR$^{-/-}$ MEFs (Fig. 5D). Similarly, expression of Stat3D, a Stat3 mutant defective in transactivation activity that exerts a dominant negative effect (23), decreased *HIF-1A* promoter activity in PKR$^{-/-}$ MEFs (Fig. 5D). On the other hand, *HIF-1A* promoter activity in PKR$^{+/+}$ MEFs was not affected by either CPA-7 or Stat3D expression (Fig. 5D), indicating that the higher induction of Stat3 activity in PKR$^{-/-}$ MEFs was responsible for the transcriptional upregulation of the *HIF-1A* gene.

TC-PTP is involved in the transcriptional control that Stat3 exerts on the *HIF-1A* gene

Given that induction of Stat3 activity in PKR$^{-/-}$ MEFs is due to inactivation of TC-PTP (18), we wished to examine whether TC-PTP deficiency leads to Stat3 activation and subsequent upregulation of *HIF-1A* gene transcription. First of all, we detected higher levels of Stat3 phosphorylation at Y705 in TC-PTP$^{-/-}$ MEFs compared with TC-PTP$^{+/+}$ MEFs (Fig. 6A), confirming that phosphorylated Stat3 is a target of this tyrosine phosphatase (18, 31). When TC-PTP$^{+/+}$ and TC-PTP$^{-/-}$ MEFs were subjected to either hypoxia (Fig. 6B) or $CoCl_2$ treatment (Fig. 6C), we observed a higher induction of HIF-1α protein expression in cells that were deficient in TC-PTP, in agreement with all our previous results. This difference in HIF-1α protein expression levels was due to the upregulation of HIF-1α gene transcription, because the *HIF-1A* promoter displayed higher activity in TC-PTP$^{-/-}$ MEFs than TC-PTP$^{+/+}$ MEFs as shown by the luciferase reporter assays (Fig. 6D). Taken together, these data support a negative role of TC-PTP in HIF-1α expression under hypoxia through the inhibition of Stat3.

PKR inhibits Stat3 phosphorylation and HIF-1α expression in human cancer cells

In addition to MEFs, we looked at the role of PKR in HIF-1α expression in human cells. To this end, we used the human lung cancer H1299 cells in which PKR was targeted by shRNA (Supplementary Fig. S4A). We found that downregulation of PKR resulted in a higher tyrosine phosphorylation of Stat3 and induction of HIF-1α under hypoxia (Supplementary Fig. S4B and C). Consistent with MEFs, PKR inactivation led to increased transactivation of *HIF-1A* gene promoter (Supplementary Fig. S4D). To verify these observations, we examined the effects of PKR activation on HIF-1α in human cells under hypoxia. To do so, we used the human

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fibrosarcoma HT1080 cells, which were engineered to express a conditionally active form of PKR in a fusion protein with GyrB (19). When HT1080 cells stably expressing GyrB.PKR are treated with the antibiotic coumermycin, the fusion GyrB.PKR protein becomes dimerized and activated by autophosphorylation leading to phosphorylation of endogenous eIF2α (ref. 19; Supplementary Fig. S5A). We previously showed that induction of GyrB.PKR in HT1080 cells leads to activation of TC-PTP, which in turn inactivates Stat3 by dephosphorylation (18). When these cells were maintained in normoxic or hypoxic conditions for various periods of time, we observed that activation of GyrB.PKR resulted in the downregulation of HIF-1α under hypoxic treatment, which coincided with a substantial inhibition of Stat3 phosphorylation (Supplementary Fig. S5B). Inhibition of HIF-1α expression was mediated at the transcriptional level given that *HIF-1A* promoter activity was significantly reduced in normoxic cells after the activation of GyrB.PKR (Supplementary Fig. S5C). Overall, these data support that PKR activity negatively regulates HIF-1α expression in human cells.

Discussion

Our study shows that PKR suppresses HIF-1α expression and consequently the transcription of its target genes. This phenomenon is specific for PKR and mediated by a reduction in the transcription of the *HIF-1A* gene itself. As illustrated in our model (Fig. 7), the ability of PKR to impair HIF-1α synthesis is exerted through the activity of TC-PTP and Stat3.

Hypoxia affects gene expression at the level of translation, in part through the eIF2α phosphorylation pathway (32). Intriguingly, despite the effort of the hypoxic cell to conserve energy by inhibiting global protein synthesis, HIF-1α mRNA is efficiently translated under hypoxic conditions to promote endurance to the assaulting stress (33). As is the case with many signaling pathways, feedback mechanisms are necessary to maintain control, equilibrium, and plasticity of major cellular processes. Herein, we present evidence that PKR compromises expression of HIF-1α independent of translational control consistent with previous reports that the eIF2α kinases can regulate key cell signaling pathways involved in proliferation, apoptosis, viral replication, and tumorigenesis independent of their function in protein synthesis (5, 6).

Our study reveals that PKR regulates HIF-1α expression by modulating the activity of Stat3. Stat3 is a member of the Stat family that has been described predominantly as oncogenic, as it has been shown to play a positive role in transformation, suppression of apoptosis, proliferation, invasion, and chemoresistance (34). Our work supports our previous findings that PKR inhibits Stat3 phosphorylation and activity (18). We also provide a specific example of an affected Stat3 target gene, *HIF-1A*, which is importantly a major facilitator of tumor progression. Phosphorylated eIF2α partially contributes to Stat3 inhibition, although this is not sufficient to suppress the transcription of the *HIF-1A* gene highlighting a dominant role for PKR for this process.

In line with our previous work (18), our data show that PKR functions to prevent basal activation of Stat3 and provide genetic evidence supporting the notion that Stat3 phosphorylation and its ability to upregulate transcription of the *HIF-1A* gene is antagonized by TC-PTP. The function of TC-PTP as a tyrosine phosphatase has been shown to attenuate cytokine signaling and negatively regulate cell cycle progression by inhibiting Janus-activated kinases, Src family kinases, and Stat3 (31). In addition to its ability to attenuate proliferation, the ability of TC-PTP to decrease Stat3-mediated transcription of the *HIF-1A* gene may have important implications within the tumor microenvironment as an inhibitor of the angiogenic switch. In line with this notion, TC-PTP has been recently shown to antagonize vascular endothelial growth factor receptor 2 signaling in endothelial cells (35), suggesting that it can also inhibit angiogenesis by suppressing HIF-1α expression and secretion of VEGF in tumor cells.

From an evolutionary point of view, HIF-1α is highly conserved given that the majority of metazoa use homologues of HIF transcription factors to adapt to oxygen deprivation. Furthermore, studies in mammals, *Drosophila*, and *Caenorhabditis elegans* have shown that regulation of HIF-1α by PHD-mediated proteasomal degradation is conserved, as well as a number of HIF target genes (36). On the other hand, PKR is not expressed in all metazoans, providing evidence that HIF appeared before PKR in evolution. Also, PKR evolved in more complex organisms. In vertebrates, the kinase domains of PKR evolved with a faster rate compared with the kinase domains of the other three eIF2α kinases, namely, GCN2, PERK, and HRI (37). The accelerated evolution of PKR has been attributed to virus infection (37, 38). More specifically, the rapid evolution of PKR kinase

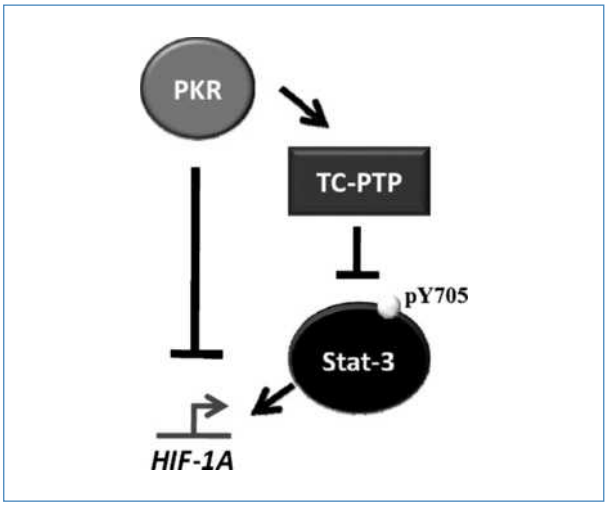

Figure 7. PKR acts as a transcriptional suppressor of *HIF-1A*. PKR inhibits HIF-1α expression at the transcriptional level in normoxic cells. This is mediated by the ability of PKR to impair Stat3 phosphorylation at Y705 through the activation of the tyrosine phosphatase TC-PTP. Inhibition of Stat3 phosphorylation is necessary and sufficient to decrease transcription of *HIF-1A*. This is a mechanism that accounts for the inhibition of HIF-1α accumulation by PKR in hypoxic cells with possible implications in chemotherapies that activate PKR and impair HIF-1α expression and function.

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domains in vertebrates has been coupled with positive selection of specific sites, particularly in residues near the eIF2α binding site. In primates, positive selection was observed in each of the three domains of PKR (dsRNA-binding domain, spacer region, and kinase domain), consistent with the extensive history of viral factors that bind PKR in these separate domains (38). However, it is not presently known whether these adaptive changes of PKR play a role in the regulation of HIF-1α expression.

The novel role of PKR in the hypoxic response is in accordance with previous reports describing tumor-suppressing functions for the kinase (39–42). Because mutations of the *PKR* gene have not been found in human tumors, its expression in tumor cells may indeed be of therapeutic potential. This is because PKR activity is induced by antioncogenic signaling (42) as well as by treatments with chemotherapeutic drugs (43). In regard to hypoxia, 2-methoxyestradiol has been shown to act as an inhibitor of tumor growth and vascularization through its ability to downregulate Stat3 and HIF-1α (44, 45). Interestingly, 2-methoxyestradiol is a potent inducer of apoptosis in tumor cells by activating PKR (46). We observed that 2-methoxyestradiol treatment of hypoxic MEFs resulted in the downregulation of HIF-1α in a manner that was dependent on PKR (Supplementary Fig. S6). This observation supports the notion that PKR is a valid target of antiangiogenic chemotherapies that disrupt HIF-1α expression and function. Furthermore, chemotherapeutic drugs such as etoposide, doxorubicin, and related topoisomerase inhibitors lead to an inhibition of HIF-1α synthesis and accumulation in response to hypoxia (47–49). Given that PKR becomes activated by doxorubicin (50) and other chemotherapeutic means (43), its activation may significantly contribute to suppression of tumor growth by drugs targeting HIF-1α.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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eIF2α Kinase PKR Modulates the Hypoxic Response by Stat3-Dependent Transcriptional Suppression of HIF-1 α

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