Characterization of the eIF2alpha kinase PKR in regulating the hypoxia inducible factor 1 alpha and the development of novel *in vivo* and *in vitro* experimental models to study the biological role of eIF2alpha phosphorylation

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Abstract

The procedure by which mRNA is translated to protein is an important and tightly regulated process in cellular biology. In mammalian cells, the phosphorylation of the alpha subunit of the translation initiation factor 2 (eIF2α) is an important mechanism of translational control. This event is mediated by a family of kinases which respond and are activated by distinct forms of cellular stress. The work described herein addresses novel roles of PKR in regulating the Hypoxia-inducible Factor 1-α (HIF-1α), a key factor that is activated by hypoxic conditions within the tumor microenvironment. Moreover, we developed novel *in vivo* and *in vitro* experimental models to better understand the role of the eIF2α phosphorylation pathway in cancer biology. More specifically we generated a transgenic mouse expressing a conditionally active eIF2α kinase and engineered a novel human cell culture model to dissect the biological functions of eIF2α phosphorylation in proliferation and the response to chemotherapeutic agents. Through this work we demonstrated that the eIF2α kinases can exert antitumor properties independently of eIF2α phosphorylation by inhibiting oncogenic signaling (Stat3), and tumor progression (HIF-1). Furthermore we demonstrate that eIF2α phosphorylation can act in a tumor promoting manner by being cytoprotective in response to chemotherapeutics. Our studies suggest that designing chemotherapeutic approaches that inhibit the cytoprotective arm of eIF2α phosphorylation under conditions where the kinases are activated may have important implications for impairing tumor progression not only by inhibiting hypoxic signaling but also by diminishing cell proliferation and enhancing efficacy of current chemotherapeutic drugs.

Résumé

La traduction des ARNm en protéines est un processus important et finement régulé dans la biologie de la cellule. Dans les cellules de mammifères, la phosphorylation de la sous unité alpha du facteur d'initiation de la traduction 2 (eIF2α) est un mécanisme important du contrôle de la traduction. Cet événement est induit par une famille de kinases activées par différentes formes de stress cellulaire. Notre travail s'intéresse aux nouveaux rôles de la voie de phosphorylation d'eIF2α dans la régulation du facteur induit par l'hypoxie 1-α (HIF-1α), un facteur clef activé par des conditions hypoxiques dans le microenvironnement de la tumeur. Nous avons développé des nouveaux modèles expérimentaux *in vivo* et *in vitro* pour mieux comprendre le rôle de la voie de phosphorylation d'eIF2α dans la biologie du cancer. Plus précisément, nous avons généré une souris transgénique qui exprime une kinase d'eIF2α active conditionnellement ainsi qu'un nouveau modèle de culture de cellule humaine pour caractériser les fonctions biologiques de la phosphorylation d'eIF2α dans la prolifération et la réponse aux agents chimiothérapeutiques. Nous avons établis que les kinases d'eIF2α peuvent exercer des propriétés antitumorales indépendamment de la phosphorylation d'eIF2α en inhibant la signalisation oncogénique, et la progression tumorale. De plus, nous avons démontré que la phosphorylation d'eIF2α peut agir de façon à promouvoir la tumeur en étant cytoprotective en réponse aux agents chimiothérapeutiques. Nos recherches suggèrent que des approches thérapeutiques conçus de façon à inhiber la voie cytoprotective de la phosphorylation d'eIF2α dans des conditions où les kinases sont activées peut avoir des implications importantes pour empêcher la progression des tumeurs pas seulement en inhibant la signalisation hypoxique mais aussi en diminuant la prolifération des cellules et en améliorant l'efficacité des médicaments chimiothérapeutiques courants.

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Preface

This thesis is presented in the traditional format in accordance with the guidelines of the National Library of Canada. The data presented in this thesis is the original work of the candidate, and has been published, in part in the following peer- reviewed articles.

1. **Papadakis AI**, Baltzis D, Buenscuseco R, Peidis P. and Koromilas AE (2011). Development of transgenic mice expressing a conditionally active form of the eIF2alpha kinase PKR. *Genesis.* 2011 Sep;49(9):743-9.

2. **Papadakis AI**, Paraskeva E, Peidis P, Muaddi H, Li S, Raptis L, Pantopoulos K, Simos G , and Koromilas AE " eIF2α Kinase PKR Modulates the Hypoxic Response by Stat3 -Dependent Transcriptional Suppression of HIF-1α" *Cancer Res.* 2010 Oct 15;70(20):7820-9

The candidate was the lead investigator for the projects described in this thesis, but would like to acknowledge the contributions of colleagues in the described work.

1. Dr. Shirin Kazemi generated the original HT1080 GyrB.PKR cells
2. Dr. Shirin Kazemi and Dr. Dennis Baltzis performed the cloning of GyrB.PKR WT and mutant (respectively) preceding the generation of transgenic mice by the McGill University Transgenic Mouse Core Facility.
3. Mr. Ryan Buensuceco performed the western blots in Fig.10B and 24A
4. Dr. Efrosyni Paraskeva (Greece) performed the RT-PCR and analyzed the data regarding HIF-1α and its target genes in Fig.11C and Fig.14C
5. Dr. Suiyang Li generated the shPKR H1299 cell lines used in Fig.11D,E and Fig.17
6. Dr. N. Benlimame (Pathology Facility of Segal Cancer Centre, Montreal) for immunohistochemical analysis presented in Fig.25

7. Christian Young performed FACS sorting of GFP positive infected HT1080 cells presented on Fig.27B
8. Dr. Filippos Peidis performed the western blots on Fig.15A and Fig.26 and FACS data on Fig.30A
9. Dr. Rajesh Kamindla obtained the FACS data on Fig.30A and 30B and assisted with proofreading this document.
10. Dr. Virginie Williams assisted with translating the abstract to French.

The Candidate has also contributed to several other projects whose research is discussed and referenced but not presented in this thesis. These studies have been published in the following articles.

1- Peidis,P., **Papadakis AI.,** Rajesh,K., and Koromilas,A.E. (2010). HDAC pharmacological inhibition promotes cell death through the eIF2alpha kinases PKR and GCN2. *Aging (Albany NY), 2*,669-677.

2- Peidis P, **Papadakis AI**, Muaddi H, Richard S and Koromilas AE "Doxorubicin bypasses the cytoprotective effects of eIF2alpha phosphorylation and promotes PKR-mediated cell death" *Cell Death Differ.* 2011 Jan;18(1):145-54.

3- Muaddi H, Majumder M, Peidis P, **Papadakis AI**, Holcik M, Scheuner D, Kaufman RJ, Hatzoglou M, and Koromilas AE "Phosphorylation of eIF2alpha at serine 51 is an important determinant of cell survival and adaptation to glucose deficiency" *Mol Biol Cell*. 2010 Sep 15;21(18):3220-31.

4- Raven JF, Baltzis D, Wang S**,** Mounir Z, **Papadakis AI**, Gao HQ and Koromilas AE (2008) "The eIF2alpha kinases PKR and PERK induce the proteasome-dependent degradation of cyclin D1". *J. Biol. Chem.* 283(6):3097-108.

5- Baltzis D, Pluquet O, **Papadakis AI**, Kazemi S, Qu LK, Koromilas AE (2007) "The eIF2alpha kinases PERK and PKR activate glycogen synthase kinase 3 to promote the proteasomal degradation of p53". *J. Biol. Chem*. 282(43):31675-87.

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List of abbreviations

4E-BP: eIF4E binding protein

ATP: adenosine tri-phosphate

AKT/PKB: Protein kinase B

AMPK: AMP activated protein Kinase

ATF: Activating transcription factor

CHOP/GADD153: C/EBP homology protein/growth arrest and DNA damage

dsRNA: double stranded RNA

dsRBD: dsRNA binding domain

ER: Endoplasmic reticulum

ERAD: ER associated degradation pathway

eIF: eurakyotic initiation factor

FIH: Factor inhibiting HIF

GCN2: General control non-derepressing kinase-2

GDP: guanosine diphosphate

GEF: Guanine exchange factor

GLUT: Glucose transporter

GRP78/Bip: Glucose regulated protein 78/immunoglobulin binding

GSK: Glycogen synthase kinase

GTP: guanosine triphosphate

GyrB: GyraseB

GyrB.PKR: GyraseB.PKR chimera protein

HIF: Hypoxia induced factor

HRI: Heme regulated inhibitor

IFN: Interferon

IRE-1: Inositol-requiring enzyme -1

IκB: Inhibitor of κB

IRES: internal ribosomal entry site

m^7G: 7-methyl-guanosine

MDM2: Murine double minute 2

mTOR: mammalian target of rapamycin

mTORC1: mTOR complex1

mTORC2: mTOR complex1

MEF: Mouse embryonic fibroblasts

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

ORF: Open reading frame

PERK: PKR like endoplasmic reticulum (ER) kinase

PKR: Protein kinase activated by dsRNA

PP1: Protein phosphatase 1

PTEN: Phosphate and tensin homolog deleted of chromosome 10

WRS: Wolcott-Rallison syndrome

UPR: Unfolded Protein Response

uORF: upstream open reading frame

JNK: c-Jun-N-terminal kinase

S1P/S2P: serine site1 or 2 protease

STAT: Signal transducer and activator or transcription

REDD1: Regulated in Development and DNA Damage Responses

Rheb: Ras homologue enriched in brain

ROS: Reactive oxygen species

RTK: Receptor tyrosine kinase

TCPTP: T-cell protein tyrosine phosphatase

TSC: Tuberous sclerosis factor

XBP-1: X-box binding protein 1

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Chapter 1: Introduction

1. Overview of protein translation and translational control

1.1 mRNA Translation

The synthesis of protein from messenger RNA (mRNA) is a critical step in the regulation of gene expression mediated by the ribosome. Translation is divided into three steps; initiation, elongation, and termination (1). Because it is the last step, control of mRNA translation enables cells to rapidly manipulate gene expression (2, 3). As such, it is an important and versatile means that cells utilise and depend on to respond and adapt to environmental stresses (4).

Most translational regulation is exerted at the level of initiation, the step when the ribosome is recruited to an mRNA and positioned at the initiation codon. In mammalian cells, translation initiation is mediated through various proteins known as eukaryotic initiation factors (eIFs)(1). These have specific functions (summarized in Table 1) and work together in a coordinated process (illustrated and described in Figure 1) to bring together the mRNA and ribosomal 80S complex (1, 4).

Table 1

Eukaryotic Initiation Factor (eIF)	Role in translation initiation
eIF1	AUG codon recognition
eIF1A	Facilitates Met_tRNA binding to 40S
eIF2	GTPase; Binds Met_tRNA to 40S
eIF2B	The eIF2 GEF (Guanine-nucleotide exchange factor)
eIF3	Promotes mRNA and Met_tRNA binding to 40S
eIF4A	Dead-box helicase
eIF4B	Acts to promote eIF4A activity
eIF4E	m7GpppN Cap binding protein
eIF4F	Refers to the eIF4A, 4E,4G Cap Binding Complex
eIF4G	Adaptor protein
eIF4H	Similar function as eIF4B
eIF5	Recognition of AUG; promoted eIF2GTPase activity
eIF5B	Ribosomal subunit joining

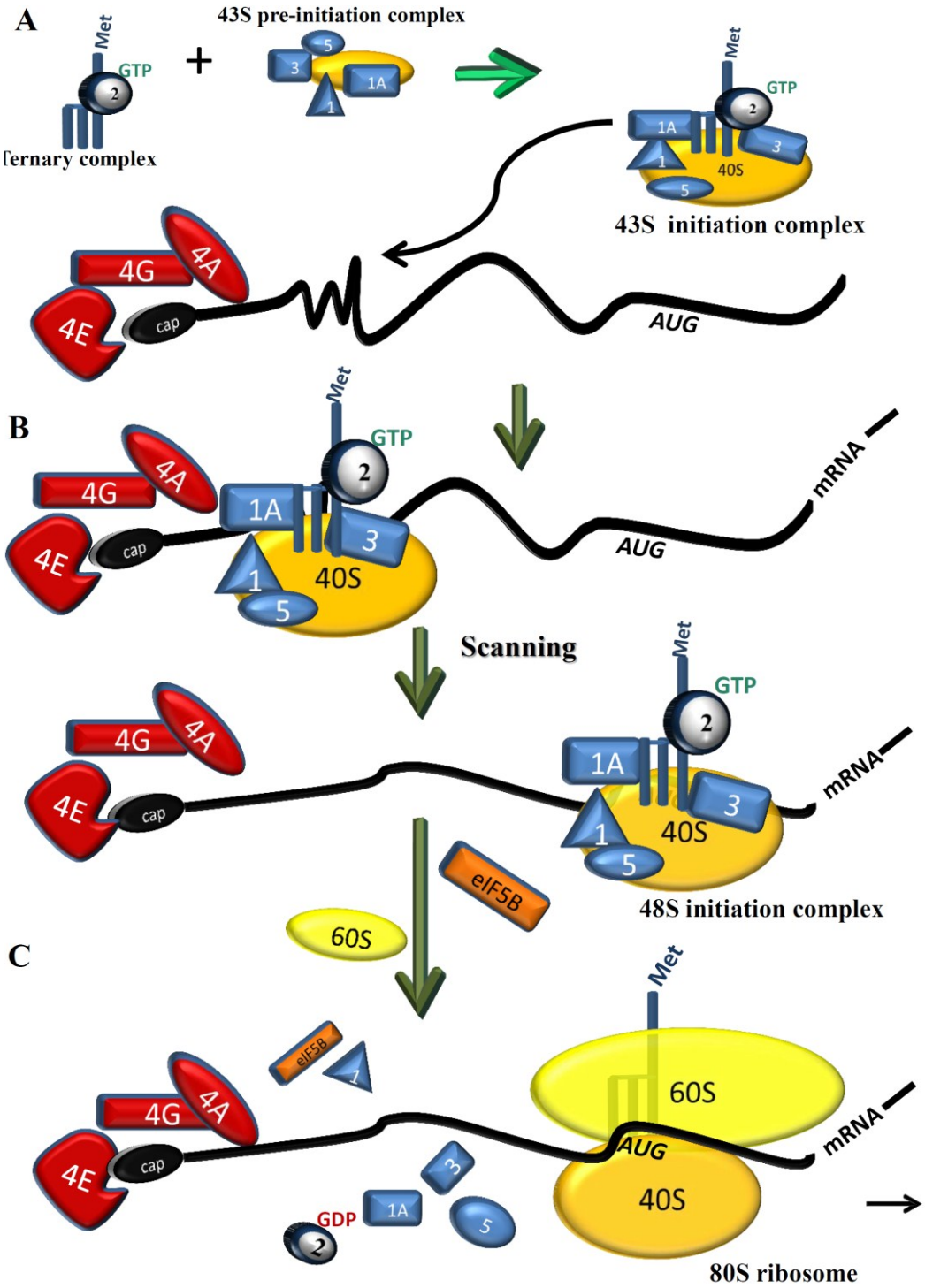
Adapted from (1).

Table 1: ***The Eukaryotic Initiation Factors and their roles in translation initiation.*** The intricate coordinated process of translation initiation requires the assistance of many factors known as eukaryotic translation initiation factors (eIFs). This table lists the most characterized eIFs and describes their functions in translation initiation**.** Adapted from (1).

The eIFs recognise the mRNA and the ribosomal machinery, and act to bring them together in a coordinated manner. Initially, there are two complexes: the eIF4F complex, which consists of eIF4G, eIF4A and eIF4E, recognizes the mRNA cap structure, and the 43S complex which is composed of ternary complex (eIF2.GTP and methionine tRNA), the small 40S ribosomal subunit and associated eIFs (1,1A, 3 and 5) (4). The 43S pre-initiation complex is recruited to the 5' end of the mRNA to be translated through the interaction between eIF3 and the eIF4G scaffolding protein (5). With the assisted activity of the RNA helicase, eIF4A, which unwinds secondary structures of the 5' untranslated region (UTR), the complex scans the mRNA (2). After recognition of the AUG (initiation codon), through base pairing with the initiator methionine tRNA, the 48S ribosome complex is formed. Subsequently, eIF2.GTP is hydrolyzed, the 60S is recruited through eIF5B, the complete 80S ribosome is formed, and nascent polypeptide synthesis ensues (4). During this step, the eIFs are dissociated from the complex to be reused for ensuing translation initiation (4). A more specific explanation of how these factors come together is illustrated and summarized in Figure 1.

Not included in Figure 1 for simplification reasons is the poly-A-binding protein (PABP). PABP interacts with eIF4G and allows for circularization of the mRNA by binding the poly-A- tail of the mRNA (1, 6). This is important because it brings the 3'UTR close to the 5'UTR. Both UTRs are important for binding regulatory factors necessary for translation (7).

Figure 1



Adapted from (2, 4).

Figure 1: ***Schematic of Translation Initiation***

(A) The 43S pre-initiation complex consists of the 40S ribosomal subunit with eIFs 1A, 3 and 5 and the ternary complex which contains Met-tRNA and GTP bound eIF2. The eIF4F complex bearing eIF4G, 4E, and 4A (the cap-binding complex) recognizes mRNA through its 5' 7-methyl guanosine (m^7G) cap structure. The interaction between eIF3 and the eIF4G scaffolding protein leads to the recruitment of the 43S pre-initiation complex to the 5' end of the mRNA to be translated. **(B)** Subsequently the 43S scans the mRNA until it reaches the initiation AUG codon (via base paring of methionine tRNA and the AUG codon) establishing the 48S initiation complex. **(C)** Next, eIF2-GTP is hydrolyzed to eIF2-GDP, and the large 60S ribosomal subunit joins to the 48S complex through eIF5B. This leads to dissociation of the eIFs bound to the 40S ribosomal subunit and the formation of the 80S ribosome which translates the message. The released eukaryotic initiation factors are recycled for subsequent translation initiation. Adapted from (2) and (4).

1.2 Regulation of translation

The regulation of protein synthesis is important for many cellular processes including cell cycle, cell growth, development and apoptosis (2). Environmental conditions strongly influence signals which converge on mRNA translation (2). For instance, growth factors, hormones, and nutrients increase protein synthesis while stresses such as DNA damage, oxidative stress, and nutrient deprivation, inhibit mRNA translation (2).

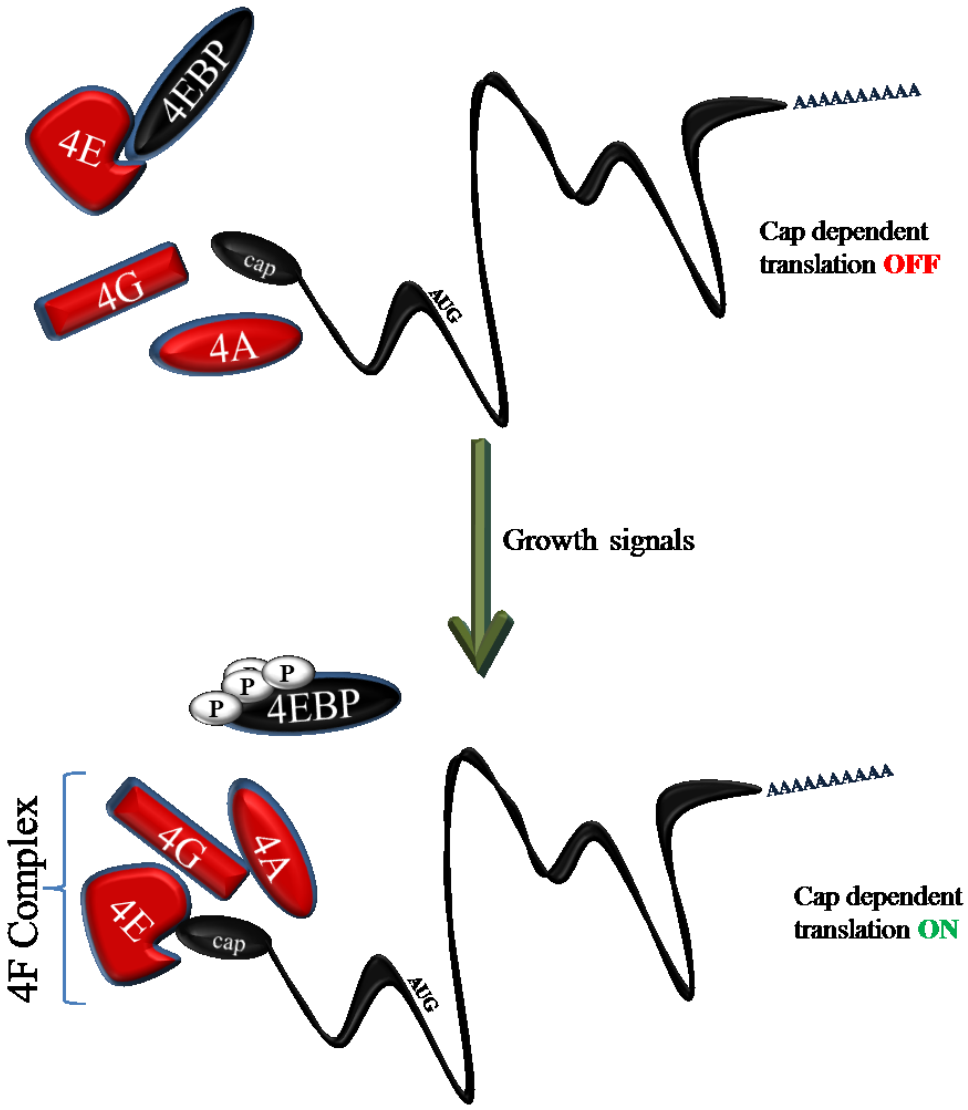
Regulation of protein synthesis is mainly modulated at the level of initiation; this is mainly achieved by phosphorylation of initiation factors and their regulators (5). Two well characterized mechanisms regulate initiation function through the regulation of two distinct complexes of translation initiation factors: eIF4E and eIF2 (5).

1.2.1 Regulation of eIF4E function

As illustrated in Figure 1, eIF4E recognizes the 5' cap of the mRNA to be translated. This step is regulated by the eIF4E binding proteins (4E-BPs) which bind eIF4E and prevent its binding with eIF4G and, thereby, inhibit cap-dependent translation initiation (Fig.2) (5). Phosphorylation of 4E-BPs weakens the eIF4E-4EBP interaction and allows the assembly of the eIF4F cap binding complex (8). Phosphorylation of 4E-BP is mediated by the mammalian target of rapamycin (mTOR) downstream of growth signaling pathways. These pathways are activated in cancers and promote protein synthesis, cell growth and proliferation (8, 9). Since 4E-BP is inactivated by phosphorylation downstream of

oncogenic signaling, studies have indicated that 4E-BPs have tumour suppressor properties (10). This is supported by the observation that high levels of its hyper-phosphorylated inactive form are linked to a poor clinical prognosis (11). Accordingly, eIF4E overexpression was shown to display oncogenic properties (8).

Figure 2



Adapted from (5).

Figure 2: ***Schematic of 4E Pathway***

The eIF4F complex which is comprised of eIF4E, eIF4G, and eIF4A, recognizes the 5' cap of mRNA through eIF4E, the cap-binding protein. In conditions lacking growth stimuli, 4E-BP protein binds eIF4E and inhibits cap binding. The eIF4E-4E-BP association is inhibited when 4E-BP is hyper-phosphorylated by upstream signaling activated by growth signals. Adapted from (5)

1.2.2 The eIF2α phosphorylation pathway

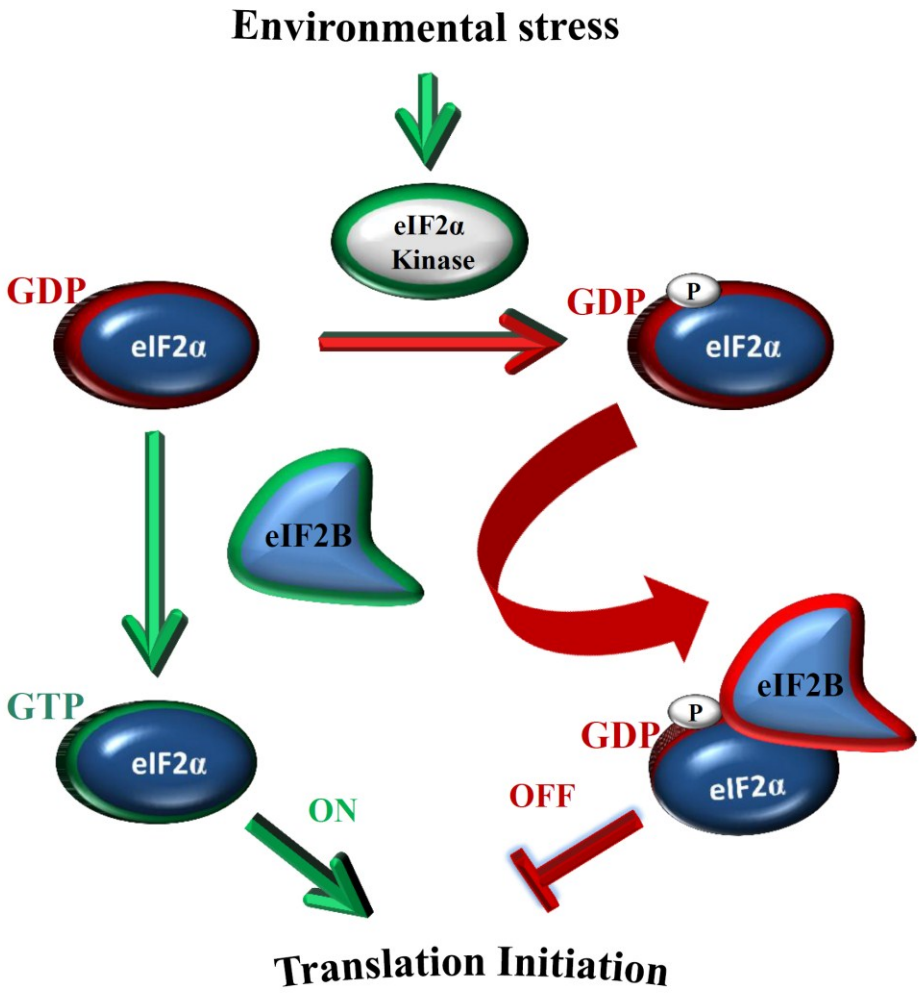
The second mechanism of regulation of translation initiation is mediated through phosphorylation of the α subunit of eIF2 at serine 51 (S51) (3). The heterotrimeric eIF2 is comprised of three sub-units α, β, and γ (12). During translation initiation, the eIF2 functions to enable binding of initiator Met-tRNA to the 40S ribosomal subunit (1). When the 48S initiation complex is formed (the initiation codon is found) and the 60S ribosomal subunit joins the complex, eIF2-GTP is hydrolysed to eIF2-GDP and eIF2 is released (4). The inactive eIF2-GDP complex is recycled to the active GTP bound form through the guanine-nucleotide exchange factor eIF2B (13). When the α subunit of eIF2 (eIF2α) is phosphorylated at serine 51, it acts as a dominant inhibitor of the guanine exchange factor eIF2B by binding to it with increased affinity and preventing the recycling of eIF2 between succeeding rounds of protein synthesis (Fig.3) (14).

The eIF2α phosphorylation pathway can regulate cell fate decisions in response to various stresses (2). The adaptive roles of eIF2α phosphorylation involve the selective translation of transcription factors such as ATF4 (15) and ATF5 (16), which induce the expression of genes that facilitate adaptation. If cells cannot adapt, as in cases of prolonged stress, the induction of eIF2α phosphorylation leads to cell death through the activation of apoptotic pathways (further discussed in 1.3.3)(3).

The mRNAs which are efficiently translated under the conditions of eIF2α phosphorylation have specific properties. Specifically, ATF4 and ATF5 mRNA contain upstream open reading frames (uORFs), which under normal unstressed

conditions cause ribosome stalling (2). However, when eIF2α is phosphorylated and the number of 43S complexes becomes restricted, mRNA scanning is improved and translation of mRNAs containing uORFs can take place (4). In addition, mRNAs which possess internal ribosomal entry sites in their 5' untranslated regions (5'UTR) can also bypass the global protein synthesis inhibition mediated by eIF2α phosphorylation (17).

Figure 3



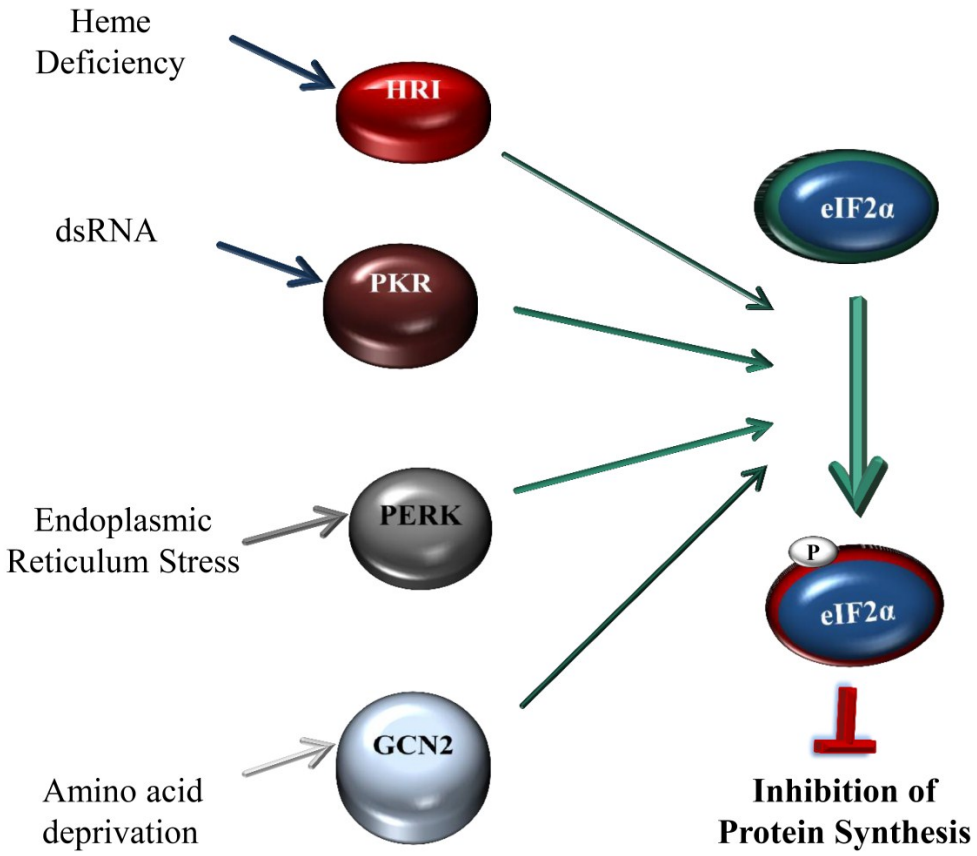
Adapted from (2).

Figure 3: ***Schematic of eIF2α phosphorylation and inhibition of GTP recycling.*** Under regular conditions, eIF2B mediates the interchange of GDP to GTP allowing for the recycling of eIF2 for translation initiation. Upon stress, activated eIF2α kinase(s) phosphorylate eIF2α on serine 51. Phosphorylated eIF2α strongly binds to eIF2B, preventing it from exchanging GDP to GTP and in turn limits translation initiation and global protein synthesis. Adapted from (2).

1.3 The eIF2α kinases

In mammalian cells, the phosphorylation of eIF2α is mediated by a family of protein kinases, each of which responds to distinct forms of environmental stress (Fig.4) (3). The eIF2α kinase family includes the heme-regulated inhibitor (HRI), the general control non-derepressible-2 (GCN2), the endoplasmic reticulum (ER)-resident protein kinase PERK, and the double-stranded (ds) RNA dependent kinase PKR which are activated by heme deficiency, the absence of amino acids, improperly folded proteins accumulated in the ER, and dsRNA respectively (3). While HRI protein is mainly expressed in erythroid cells, GCN2, PERK and PKR are found in all tissues. Despite differences in their regulatory domains, the kinase domains (KDs) of these enzymes are significantly conserved explaining their converging specificity towards the eIF2α protein (3).

Figure 4



Adapted from (2, 3).

Figure 4: ***Schematic of the eIF2α kinases***

The eIF2α kinase family comprises of four distinct members: HRI, PKR, PERK and GCN2 which are activated by distinct forms of cellular stress and converge to phosphorylate eIF2α on serine 51. The heme regulated inhibitor, HRI, is activated upon heme deficiency. The double-stranded (ds) RNA dependent kinase, PKR, is activated by double stranded RNA. The endoplasmic reticulum (ER)-resident protein kinase, PERK, is induced when unfolded proteins accumulate in the endoplasmic reticulum (ER stress). The general control non-derepressible-2, GCN2, is activated upon amino acid starvation. Adapted from (2, 3).

1.3.1 HRI

HRI is expressed and functions mainly in erythroid cells and was discovered in reticulocytes (1). Under conditions of heme deprivation, the HRI kinase induces eIF2α phosphorylation and decreases globin production by inhibiting protein synthesis (18).

HRI is regulated (kept inactive) by a prosthetic group of hemoglobin known as heme (19). More specifically, HRI contains two heme binding domains (HBD); one positioned in the N-terminus and one on the kinase insertion domain. Heme is bound to its N-terminus HBD at all times but reversibly binds to the HBD found on the kinase insertion domain (19). HRI is maintained in the inactive state through the binding of heme to the HBD within its kinase domain. During heme deficiency or in conditions where intracellular iron is limited, this inhibition is relieved and leads to activation of HRI, eIF2α phosphorylation and inhibition of translation of mRNA encoding for globin (19, 20).

Studies on HRI knockout mice have shown that it is required for macrophage maturation, maintenance and survival of erythroid precursors in iron deficient conditions, maintaining iron homeostasis and modifying the phenotypic severity of murine models of erythropoietic protoporphyria and beta-thalassemia (21-24).

Interestingly, although HRI protein expression is limited to erythroid cells, HRI mRNA has been detected in other tissue types (25). Moreover, recent studies have indicated that HRI is also activated independent of heme, in response to cytoplasmic stresses including temperature changes (heat shock), oxidative stress induced by arsenite, and osmotic stress (26).

1.3.2 GCN2

GCN2 was originally discovered in yeast and was found to regulate the biosynthesis of amino acids (27). It was established that upon depletion of amino acids, uncharged tRNAs accumulate and bind to the HiRS-related regulatory domain of GCN2, enabling dimerization, activation and eIF2α phosphorylation (1). Subsequently, GCN4 is preferentially translated when eIF2α is phosphorylated and functions at the transcriptional level to promote amino acid biosynthesis (1). This is an adaptive process which enables yeast to sense nutrients and cope with the lack of amino acids (28).

The importance of the GCN2- eIF2α phosphorylation pathway in nutrient sensing and the response to nutrient deprivation is conserved in evolution. That is, GCN2 homologs have been found in higher eukaryotes and mammals (29).

GCN2 homozygous or conditional knockout mice have demonstrated that the GCN2 is required for adaptation to amino acid deprivation (30). In addition, phenotypes observed in these mice have shown GCN2 to affect behavior (31) and cause physiological phenotypes affecting different tissues including the liver, skeletal muscle (32), as well as the nervous (33) and immune systems (34, 35). As such, the biological roles of GCN2 have significant physiological implications.

The effects of this eIF2α kinase are not limited to amino acid starvation (36) as GCN2 was activated in response to unfolded proteins in the ER (37), restricted glucose availability (38), DNA damage (39), and virus infection (40, 41).

1.3.3 PERK and the Unfolded Protein Response (UPR).

PERK, ER resident type I transmembrane protein, was identified as the PKR like-ER kinase highly expressed in pancreatic cells. PERK was later characterized as a key player maintaining the homeostasis of the ER as an effector of the unfolded protein response (UPR) (42). Under normal cellular conditions, the luminal N-terminal domain of PERK is bound to the ER chaperone GRP78, also known as Bip. When unfolded proteins accumulate in the ER, Bip dissociates from PERK, allowing it to oligomerize and become activated to phosphorylate eIF2α through its cytoplasmic kinase domain (42). The other effectors of the UPR, namely IRE-1 and AFT6, are also kept inactive through Bip and are activated by its dissociation upon accumulation of unfolded proteins (43).

PERK activation and eIF2α phosphorylation results in suppression of protein synthesis that alleviates the burden on the ER (42). Although eIF2α phosphorylation blocks general mRNA translation, some specific mRNAs, such as ATF4, are better translated when eIF2α is phosphorylated (44, 45). ATF4 acts as a transcriptional activator inducing expression of genes that restore ER homeostasis, promote survival, or induce apoptosis (46) .

The other effector, IRE-1, through its endoribonuclease activity, cleaves XBP-1 mRNA (43). More specifically, IRE-1 generates a translational frame shift by removing a 26-nucleotide intron from XBP-1 mRNA. The spliced mRNA encodes for a transcription activator which acts to induce many UPR target genes (47). Notably, XBP-1 induces effectors of the ER-associated Protein Degradation (ERAD) pathway which acts as an important protein folding “quality control”

mechanism by degrading improperly folded proteins (46). During this process, chaperones and associated factors work together to recognize and target improperly folded proteins for retro-translocation to the cytoplasm, where they are poly-ubiquitinated and degraded (46, 48).

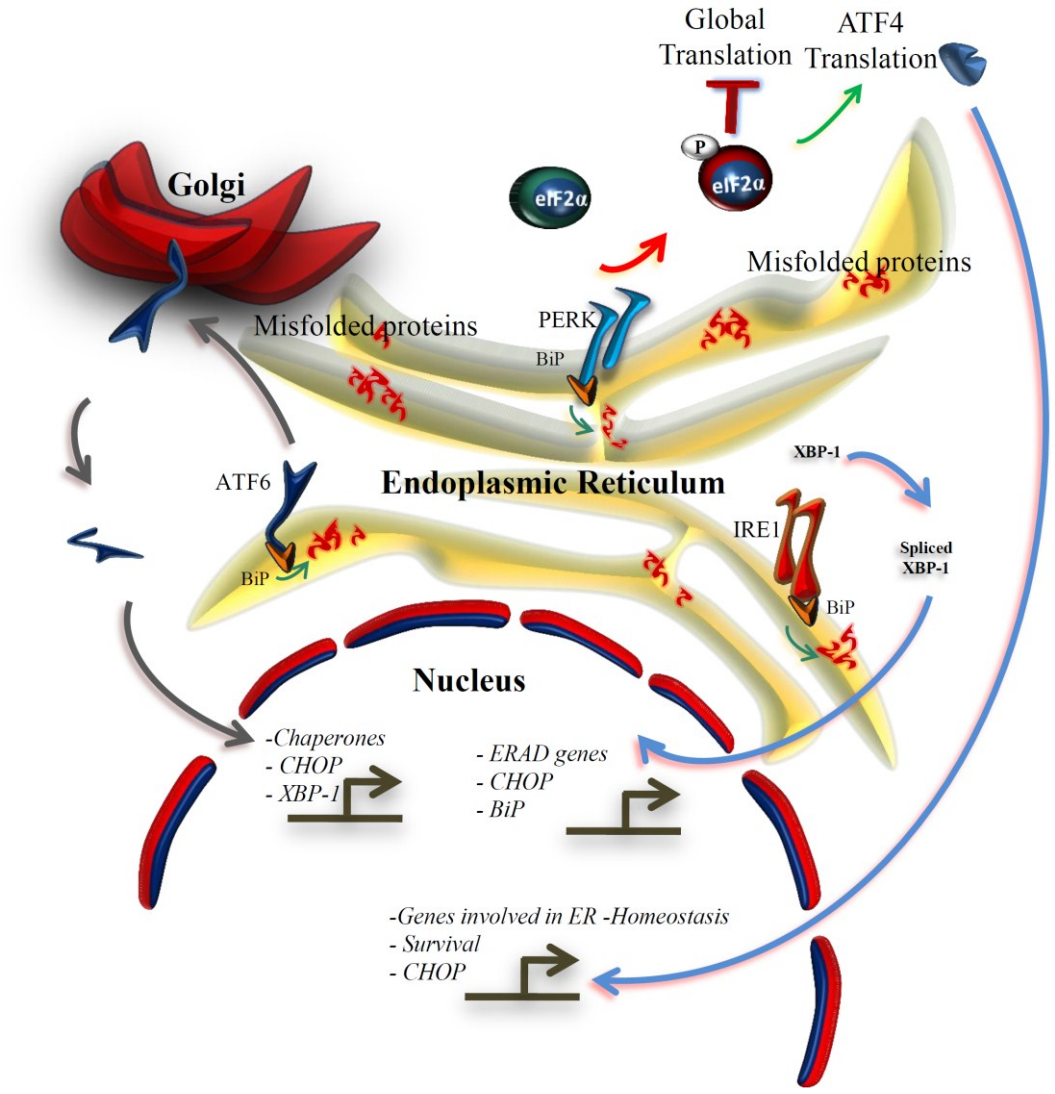
Similarly, upon dissociation of Bip, AFT6 translocates to the golgi apparatus and becomes cleaved and activated through the site 1 and 2 (S1P and S2P) proteases (49). Similar to XBP-1 and ATF4 , AFT6 acts at the transcriptional level to induce transcription of genes, including XBP-1 mRNA, which assist in the restoration of the ER homeostasis (49).

This coordinated integrated stress response known as the UPR is illustrated in Figure 5. The UPR initially acts to restore normal function of the ER. For instance, PERK activation is a transient response which is cytoprotective and acts to help cells recover from ER stress (50). A feedback mechanism has evolved to promote de-phosphorylation of eIF2α through GADD34/PP1 to resume protein synthesis once the stress is alleviated. More specifically, AFT4 induces ATF3 which induces CHOP. ATF3 and CHOP can enhance expression of GADD34 which is required for PP1-mediated dephoshorylation of eIF2α (51).

In circumstances where the homeostasis of the ER cannot be restored within a certain time frame and cells cannot adapt to the persistent stress, the UPR promotes the induction of apoptosis through different mechanisms (46). One mechanism of ER stress induced apoptosis is mediated through CHOP/GADD153, which leads to the inhibition of Bcl-2, an inhibitor of apoptosis (52). Prolonged ER stress also induces apoptosis through the

recruitment of caspase-7 to the ER which cleaves the ER membrane associated pro-caspase-12 and in turn initiates the caspase cascade (53, 54).

Figure 5



Adapted from (46).

Figure 5: ***Schematic of the Unfolded Protein Response (UPR)***

The main effectors of the UPR are ATF6, IRE-1, and PERK. Under unstressed conditions, they are maintained in an inactive form through the association with Bip, an ER chaperone. When unfolded proteins accumulate in the ER, Bip associates with the unfolded proteins and in turn dissociates from the ER- resident sensors, thus triggering the UPR. ATF6 translocates to the Golgi apparatus where it is cleaved by S1P/S2P peptidases. The cleaved form of ATF6 can then translocate to the nucleus and activate transcription of ER-stress inducible genes. IRE-1 becomes activated and acts as an endoribonuclease which splices XBP-1 mRNA. XBP-1 protein encoded by the spliced mRNA also acts at the transcriptional level to induce genes involved in protein folding and ER-associated degradation (ERAD) of misfolded proteins. PERK becomes active to inhibit global protein synthesis and in turn alleviate the burden on the ER. Upon eIF2α phosphorylation, ATF4 is preferentially translated and acts at the transcriptional level to restore the ER homeostasis, to promote survival, and induce CHOP. Adapted from (46)

PERK roles in mice

The PERK knockout mice revealed an important function of PERK in pancreatic function. Although the mice are born, they develop severe hyperglycemia (diabetes mellitus) within 2-4 weeks (55). This severe phenotype was attributed to a loss of pancreatic β cells and deterioration of the islets of Langerhans. Further studies showed that PERK is required to regulate the viability of the exocrine pancreas which is involved in producing digestive enzymes (56), as well as in regulating the proliferation and development of insulin-secreting beta-cells in the endocrine pancreas (57-59). In addition to its functions in the pancreas, PERK is involved in osteoblast differentiation and is required for skeletal development and postnatal growth (60, 61) .

PERK and disease

Consistent with the phenotypes observed in the mouse model, loss of PERK function has similar implications in human disease. Patients with Wolcott–Rallison syndrome (WRS), an autosomal recessive condition, have PERK mutations which result in non-functional or truncated PERK. The main symptoms of WRS are early onset of type I diabetes and predisposition to osteoporosis and fragile bones (62).

In addition to WRS, PERK is implicated in other diseases associated with defects in the secretory pathway and the accumulation of dysfunctional and anomalous proteins. These include neurodegenerative disorders such as Parkinson's disease and Alzheimer's disease (63-66). Lastly, PERK and the UPR were shown to be cytoprotective and play positive roles in cancer.

PERK, the UPR and cancer

A broad range of cancer types rely on PERK and the cytoprotective arms of the UPR, not only for maintaining ER homeostasis but also to cope with environmental stresses (such as hypoxia or glucose deprivation) elicited in the tumor microenvironment (59, 67, 68). These mechanisms are adopted by cancer cells to not only maintain malignancy but also resist therapeutic intervention (46).

The UPR is shown to be cytoprotective in several ways, one of which was reported by our group. It was shown that ER stress can induce degradation of the stress activated tumour suppressor p53, independent of eIF2α phosphorylation. More specifically, PERK activation was shown to activate GSK3β, which phosphorylates p53 and promotes its mdm2 mediated degradation (69-72). PERK also plays cytoprotective roles in response to stresses encountered in the tumor microenvironment; such as oxygen or nutrient deprivation. In response to hypoxia (low oxygen), PERK acts in a cytoprotective manner and allows cells to cope with the hypoxic-state and promote tumor growth (68, 73, 74).

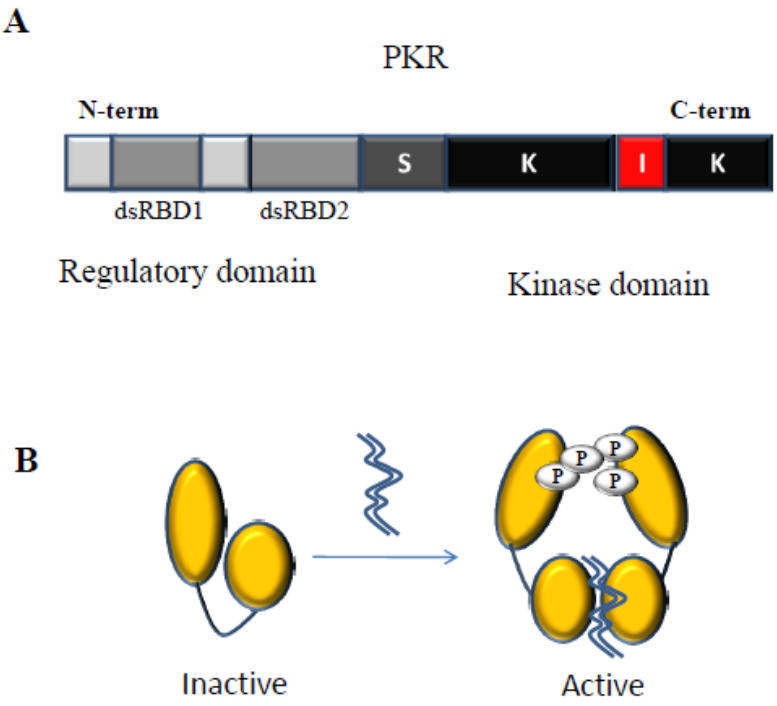
1.3.4 PKR

The interferon inducible PKR, the most studied of the eIF2α kinases, plays important roles in diverse processes including signal transduction, cell growth and apoptosis (75). PKR is ubiquitously expressed at low levels and is induced by type I interferons (IFNα/β), a family of cytokines secreted by virally infected cells to trigger an anti-viral response (76). PKR has two functional domains: a distinct N-terminal regulatory domain, and a C-terminal kinase domain, which is highly conserved amongst the eIF2α kinase family (77).

PKR activation

The regulatory domain contains two dsRNA binding domains, which bind dsRNA and mediate dimerization and activation of PKR (78). When dsRNA binds, PKR changes conformation exposing the ATP binding site and allowing for dimerization, auto-phosphorylation and full activation of the kinase (79). With regard to the activation of the kinase, our lab reported that PKR is a dual specificity kinase which is also phosphorylated on tyrosine residues (80). The phosphorylation of specific tyrosine residues (101, 162 and 293) is required for activation of the kinase in response to dsRNA. Moreover, upon IFN stimulation, Jak1 and Tyk2 were identified as the kinases which mediate Tyr 101 and Tyr293 phosphorylation (81).

Figure 6



Adapted from (1, 78).

Figure 6: ***Schematic of the eIF2α Kinase PKR domains and PKR activation by dsRNA. (A)*** The N-terminus of PKR contains two dsRNA binding domains (dsRBD). The kinase domains are located at the C-terminus and separated by an insert (I) region. The regulatory and the kinase domains of PKR are separated by a spacer region (S). **(B)** PKR's inactive conformation is disrupted by dsRNA which binds its regulatory domain, leading to conformational changes revealing the ATP binding site leading to kinase activation. Adapted from (1, 78).

PKR and virus infection

Because dsRNA is produced during virus genome replication and PKR is an IFN inducible gene, there have been many studies addressing antiviral activity of PKR. More specifically, virus infected cells secrete IFN, which leads to the transcriptional induction of PKR in adjacent cells through IRF-1 and NF-kB signaling (82). These cells are now better equipped to cope with virus infection since PKR is now highly expressed and will become activated when these cells become infected and produce dsRNA. Antiviral activities of PKR include the inhibition of protein synthesis by eIF2α phosphorylation and induction of apoptosis (75). To evade these antiviral effects, viruses have evolved various means to inhibit PKR from exerting these functions. Examples of such mechanisms are described in the next section.

Endogenous and viral regulators of PKR

PKR activity is modulated by cellular and viral proteins. At the cellular level, PKR can bind several proteins described below which inhibit its function through diverse mechanisms. The TAR RNA Binding Protein (TRBP), which contains a dsRBD, can heterodimerize with PKR to inhibit its function (83). The stress response protein $p58^{IPK}$, activated upon influenza infection, can bind PKR and inhibit its autophosphorylation and activation (84). Nck-1 (the non-catalytic region of tyrosine kinase adaptor protein 1) is another endogenous regulator of PKR which interacts with PKR and limits its activation by dsRNA (85). In contrast, proteins such as PACT, a dsRNA binding protein, activate PKR (86, 87). Also, our lab has recently demonstrated that the tumor suppressor PTEN can also interact with and activate PKR (88).

To evade the host cell's effort to inhibit protein synthesis and induce apoptosis through the activation of PKR, viruses have also evolved mechanisms to inhibit its function through diverse mechanisms (86, 87). One approach viruses have developed is preventing dsRNA from binding PKR or sequestering dsRNA (86, 87, 89). Other strategies include the production of pseudosubstrates, proteins which mimic eIF2α, or the direct modulation of PKR expression/stability (87, 90-92). Also, to relieve translation inhibition induced by PKR, certain viruses established means to control de-phosphorylation of eIF2α. For instance, Herpes Simplex virus (HSV) produces a protein which ultimately resembles GADD34 and acts in a similar fashion to recruit PP1 and de-phosphorylate eIF2α (93). Similarly, our lab has demonstrated that the human papilloma virus E6 (HPV-18E6) protein acts to inhibit PKR induced apoptosis and relieves translation inhibition through GADD34/PP1 mediated de-phosphorylation of eIF2α (94).

PKR and cell signaling

Mouse embryonic fibroblasts (MEFs) from PKR knockout mice, as well as *in vitro* approaches, have unveiled important roles for PKR in cell signaling by regulating the NF-κB pathway, Stat signaling, and the p53 pathway (95). With regard to NF-κB, PKR activates the pathway by promoting the degradation of the inhibitor of κB (IκBα) by direct phosphorylation or through the activation of the IκBα kinase (IKK) (96-98). In the case of the Stat family, the signal transducers and activators of transcription, PKR inhibits the functions of Stat1 and Stat3 through direct interaction and modulation of their de-phosphorylation (99-102). Lastly, with regard to the guardian of the genome, p53, PKR has been shown to

affect its activity and protein stability. More specifically, our group showed PKR to physically interact with p53, phosphorylate p53 on serine 392, and affect its phosphorylation on serine 18; promoting its transcriptional activity in response to DNA damage (103, 104). Moreover, our group demonstrated that PKR activation (as well as PERK activation) also regulates the stability of the p53 protein, by enhancing its mdm2-dependent degradation, through a mechanism involving the activation of GSK-3β (69-71, 105, 106).

PKR and cancer

PKR has been shown to inhibit cell proliferation. This has been shown to be exerted through its toxic effects when overexpressed in various cell types (95). In contrast, overexpression of dominant-negative PKR mutants in mouse fibroblasts caused cell transformation, providing evidence that PKR is an inhibitor of tumorigenesis (107, 108). PKR has also been implicated in several cancer types. For instance, while it was shown to be mutated or absent in certain leukemic cells, PKR was also found to be overexpressed (with impaired activity) in breast cancers (109-111). Moreover, our lab recently demonstrated that PKR acts downstream of the tumor suppressor PTEN. The study showed that reconstitution of PTEN-null glioblastoma cells with PTEN induces the PKR-eIF2α pathway, which plays a significant role in PTEN mediated inhibition of proliferation and apoptosis. Interestingly, PKR was activated independent of PTEN's ability to regulate the PI3K pathway; as mutants with defective phosphatase function also activate the PKR-eIF2α pathway (88). Furthermore,

roles for PKR in sensitizing cells to chemotherapeutic agents have also been described. These studies will be further discussed in section 2.4.

PKR mouse models

To address the role of PKR *in vivo,* two knockout mouse models were generated. N-terminal knockouts, which lack the double stranded RNA binding domain, were generated through the deletion of exons 2 and 3 (112), while the C-terminal knockouts lacking the kinase domain were generated by deleting exon 12 (113). The disadvantage of these mice is that while N-terminal knock out still possess catalytic activity (114), the C-terminal knockout can bind dsRNA. Although tissue culture and *in vitro* studies have shown PKR to possess anti-proliferative functions and to exert anti-viral activities, mice deficient in PKR did not display the expected phenotypes. PKR status did not influence vulnerability to infection with vaccinia or influenza viruses (112, 113). Only in response to VSV infection was it found that PKR ablation increased susceptibility to infection, an effect which was only observed in a specific mouse strain (77, 115). One explanation for the lack of the expected phenotypes is the experimental limitation that these mouse models do not have complete loss of functional PKR. A second possibility is the redundancy between the eIF2α kinase family and likelihood of compensation through the other eIF2α kinases. The later possibility is supported by the fact that viruses also overload the ER and activate PERK. With regard to VSV, our lab and others have shown that PERK and GCN2 contribute to cellular resistance to VSV infection (40, 41, 116). Moreover, PERK was shown to contribute to the activation of PKR in response to VSV infection (116) and

PERK and GCN2 were shown to inhibit VSV replication independent of eIF2α phosphorylation (40), demonstrating cross-talk as well as redundancy between the eIF2α kinases.

2. Tumor microenvironment, hypoxia, and chemotherapeutics

2.1 Tumor microenvironment, Hypoxia and Cancer

The tumor microenvironment, created and controlled by the availability of nutrients, oxygen and surroundings, plays a critical role influencing tumor growth and progression (117). A common stress condition encountered by cells not only during normal development but also in many pathological cases, including cancer, is the lack of oxygen or hypoxia. In cancers, the majority of advanced solid tumors contain regions of reduced levels of available oxygen (118). This occurs during certain stages in tumorigenesis when rapidly proliferating cells, lacking proximity to vasculature, encounter microenvironments of low oxygen. The ability to adapt to such hypoxic conditions has important effects on tumor development and determines disease progression and clinical prognosis (119). 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 the transcriptional and translational machinery (120).

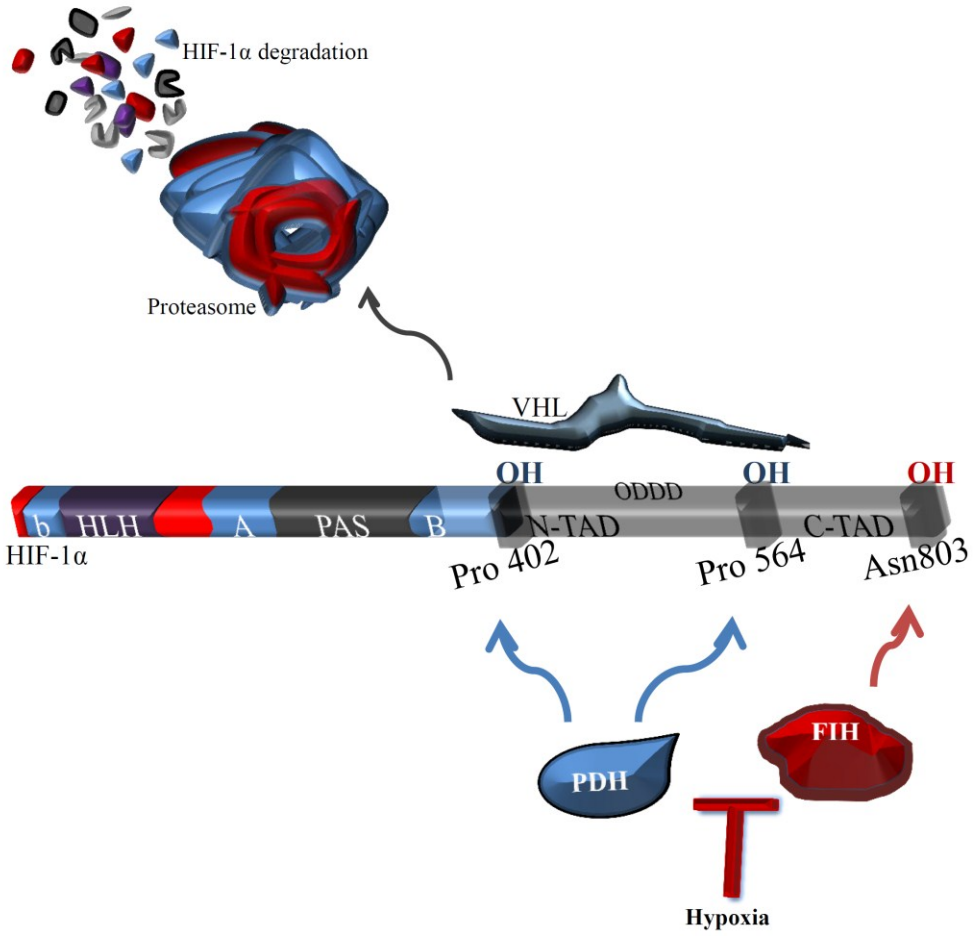
2.2 The hypoxia inducible factor 1

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, metabolic reprogramming and facilitate metastasis (119). As such, HIF-1α levels and activity are correlated with tumor progression and poor clinical prognosis (119). Because of its crucial role in cancer development, HIF-1α is regarded as an attractive target for therapeutics (119).

Oxygen dependent regulation of HIF-1α

HIF-1 consists of alpha (α) and beta (β) subunits which heterodimerize, bind to DNA and induce transcription of target genes. While HIF-1β (also known as ARNT) is constitutively expressed, HIF-1α levels are tightly regulated (119). Under normal oxygen conditions, HIF-1α is modified by prolyl-hydroxylases (PHDs) at specific proline residues, which triggers binding of the tumour suppressor Von Hippel-Lindau (VHL) protein and subsequent ubiquitination and proteasomal degradation (119). In hypoxic conditions, the oxygen-dependent hydroxylation cannot occur, leading to the accumulation of HIF-1α and induction of HIF-1 activity (119, 121). HIF-1 transcriptional activity is also controlled by oxygen tension by asparaginyl hydroxylation of HIF-1α by the Factor Inhibiting HIF-1 (FIH-1) at Asn 803. This modification impairs its association with the transcriptional co-activator CBP/p300 (122). The described mechanisms of oxygen dependent regulation of HIF-1α are illustrated in Figure 7.

Figure 7



Adapted from (123)

Figure 7: ***Schematic of HIF-1α oxygen dependent degradation.*** Under normal oxygen conditions, prolyl-hydroxylases (PHDs) use molecular oxygen to hydroxylate HIF-1α at specific proline residues (proline 402 and 564). These post-translational modifications trigger binding of the tumour suppressor Von Hippel-Lindau (VHL) protein which acts to promote HIF-1α ubiquitination and proteasomal degradation. The transactivation activity of HIF-1α is also inhibited in normal oxygen conditions through asparaginyl hydroxylation at Asn803, a modification which is mediated by FIH-1 (Factor Inhibiting HIF-1). When oxygen levels are limited, PHD and FIH-1 proteins cannot modify HIF-1α leading to its stabilization and activation. Adapted from (123).

Oxygen independent regulation of HIF-1α

The HIF-1α protein has also been shown to be stabilized through its interaction with heat shock protein 90 (HSP-90). Pharmacological inhibition of HSP90 activity leads to destabilization of HIF-1α independent of any change in oxygen levels (124). Mechanistically, this is mediated through the Receptor of Activated protein C Kinase (RACK-1) which interacts with HIF-1α and promotes degradation through the recruitment of the Elongin-C/B ubiquitin complex. It was shown that RACK-1 competes with HSP-90 for binding to HIF-1α. Thus, HIF-1α stability can be regulated, independent of oxygen tension, based on the balance between RACK-1 and HSP90 (124, 125).

It has long been considered that regulation of HIF-1α is exclusively a post-transcriptional process (119). However, recent data suggests 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 (126, 127), SP-1 (128) or Stat3 (129). Under normal oxygen conditions, regulation of HIF-1α gene transcription may not essentially reflect the HIF-1α protein levels, since the PHD-VHL-proteasome system is still limiting, but its transcription can significantly alter the HIF-1α protein expression levels upon hypoxia and the corresponding cellular response.

HIF and activated oncogenes

Oncogenic signaling has evolved ways to ensure HIF-1α levels are maintained. That is, in addition to hypoxia, HIF-1α expression and its transcriptional activity were shown to be up-regulated through oncogenes such as

v-Src, HER2, and Ras as well as activated PI3K, mTOR and MAPK signaling pathways (130-138). In addition, HIF-1α protein nuclear localization was shown to be regulated by direct phosphorylation through the p42/p44 MAPK (139).

VHL; a tumour suppressor

The role of HIF-1α in cancer progression was highlighted by the characterization of the tumor suppressor properties of VHL. Individuals with the Von Hippel-Lindau (VHL) syndrome are genetically heterozygous for a germline mutation in the *VHL* gene (140). These patients are at high risk for developing multifocal and bilateral renal cell carcinomas, upon somatic inactivation of the remaining wildtype allele (141, 142). The complete loss of VHL function leads to renal cancers, as well as other cancer types through the accumulation of HIFs (143). The tumorigenic properties of HIF-1 (which is stable and active when VHL is dysfunctional) contribute to the pathogenesis of VHL-null tumors and outlined below (142, 143).

HIF-1α and angiogenesis

Tumor angiogenesis was first discovered and pioneered by Dr. Judah Folkman, a surgeon who observed, hypothesized and proved that tumors signal to the vasculature to recruit blood vessels (144). HIF-1α plays an important role in this process by inducing the expression of vascular endothelial growth factor VEGF and angiopoietin-2 (Ang-2) (123). These factors act to activate proliferation and recruitment of endothelial cells leading to the formation of new blood vessels (144, 145).

HIF-1α and metabolic remodeling

Cancer cells preferentially depend on the glycolysis pathway for their energy requirements instead of the more efficient oxidative phosphorylation pathway. This phenomenon is known as the "Warburg effect" (146). More specifically, tumors generate two ATPs through glycolysis instead of thirty-six ATPs for every glucose molecule through oxidative phosphorylation. Cancer cells compensate for this loss in efficiency by increasing glucose uptake and by up-regulating the enzymes of glycolytic pathway which produce ATP by converting glucose to pyruvate (147). Importantly, this strategic change in metabolism allows tumor cells to lose their dependency on oxygen, the terminal electron acceptor of the mitochondrial oxidative phosphorylation pathway (148).

HIF-1 acts as a pro-tumorigenic factor, in part, by reprogramming cellular metabolism and driving the Warburg effect (149). Through its transactivation activity, HIF-1α functions on several levels of glucose metabolism (Fig.8). First, it increases glucose uptake by up-regulating glucose transporters. Next, it increases glycolytic enzymes which act to convert glucose to pyruvate, generating energy and NADH (150). Lastly, HIF-1α induces the expression of lactate dehydrogenase A, and enzyme which converts pyruvate to lactate and regenerates NAD+ levels to be used in glycolysis (151).

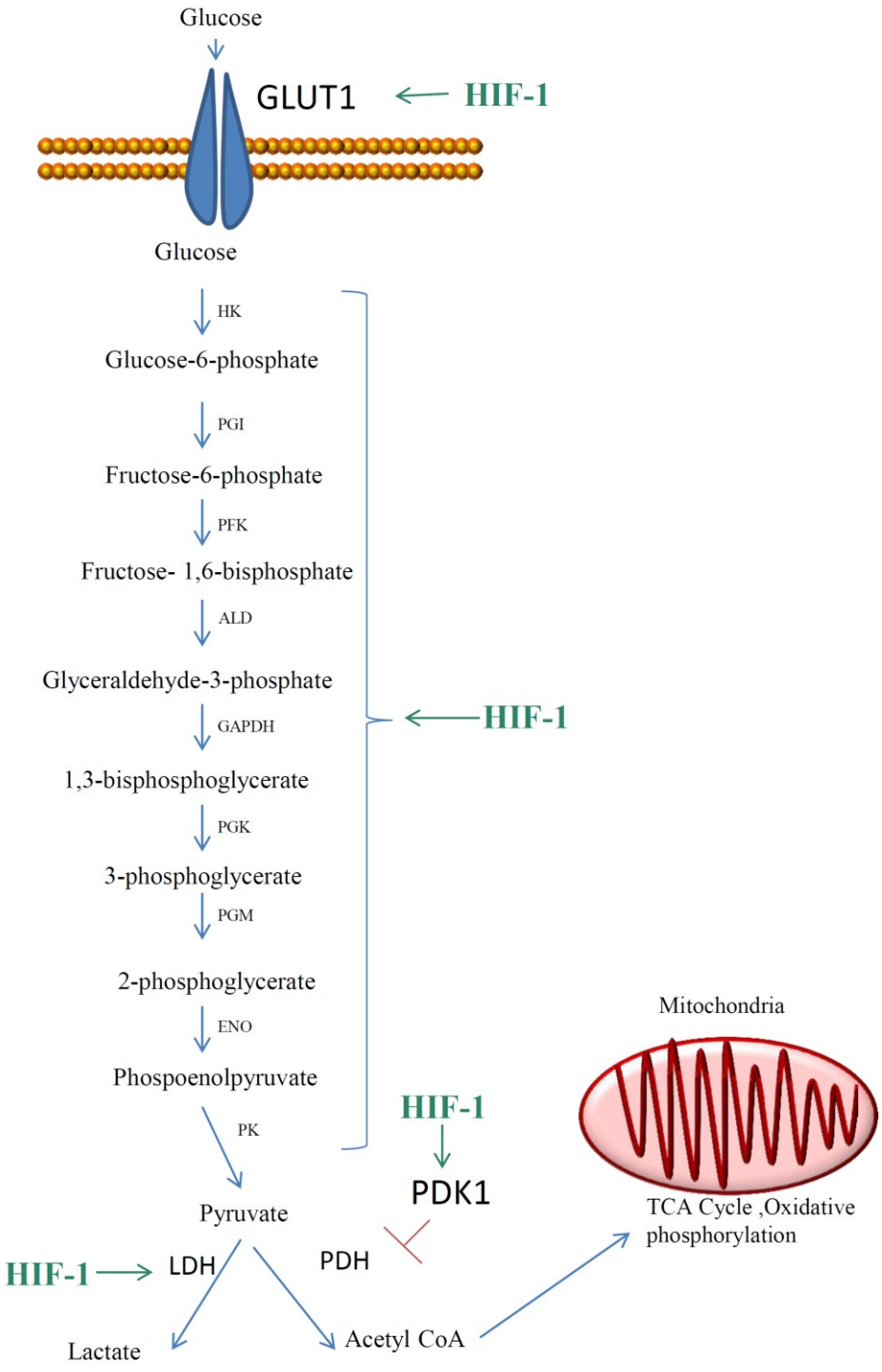
HIF-1α does not only favor the glycolic pathway but also shuts off oxygen dependent metabolism. HIF-1 suppresses the conversion of pyruvate to Acetyl Coenzyme A through the induction of pyruvate dehydrogenase kinase 1 (PDK1), which phosphorylates and inhibits the pyruvate dehydrogenase (PHD) enzyme (146). Since Acetyl CoA is necessary to feed the Citric acid cycle (TCA) and

oxygen dependent mitochondrial oxidative phosphorylation (OXPHOS), HIF-1α successfully inhibits these pathways (146, 152).

HIF-1α, invasion and metastasis

The consequences of inducing the glycolytic pathway include increased production of lactic acid, acidosis and decrease in the extracellular pH. HIF-1α protects cells by inducing the transcription of genes which maintain a normal intracellular pH. This is mediated through the up-regulation of several membrane transporters including NHE-1, MCT1, and MCT4, as well as carbonic anhydrase IX (CAIX) (123, 153). The ability to grow in acidic environments as well as the acidification of the extracellular milieu confers several advantages to cancer cells by suppressing the immune response, and supporting tumor invasion and metastasis through degradation of the extracellular matrix. HIF-1α also promotes metastasis through the repression of adhesion (E-cadherin expression) and enhanced epithelial mesenchymal transition (EMT), rendering the tumor cells invasive (154). Furthermore, HIF-1α enhances cell motility and metastasis by increasing the expression of C-MET, matrix metalloproteinases, lysyl oxidase and urokinase plasminogen activator receptor (123, 145, 155).

Figure 8



Adapted from (151, 156).

Figure 8. ***HIF-1α and metabolism.***

Glucose uptake is mediated through glucose transporters and is metabolized to generate ATP and pyruvate through the glycolysis pathway. Under normal conditions pyruvate feeds into the tri-carboxylic cycle (TCA) and the mitochondrial electron transport chain and oxidative phosphorylation. But during hypoxia, HIF-1α acts to remodel this pathway at several levels to favor the glycolytic pathway. Through the induction of PDK-1, it inhibits pyruvate conversion to Acetyl COA and in turn the TCA and OXPHOS pathway. It compensates for the loss of ATP made through mitochondrial respiration by increasing glucose uptake by the induction of glucose transporters (GLUT) and concurrently also induces glycolytic enzymes. To get rid of accumulating pyruvate, HIF-1α induces LDH which promotes its conversion to lactate. Adapted from (151, 156).

The Significance of HIF-1 in cancer prognosis

In cancer, HIF-1α expression is increased by activated oncogenes and many other molecular mechanisms (157). Given the extent of its oncogenic functions in metabolism, angiogenesis and metastasis, increased HIF-1α is associated with poor clinical prognosis and increased mortality for many human cancer types. These include cancers of the kidney, bladder, brain, breast, colon, endometrium, lung, liver, and pancreas (158). From a therapeutic perspective, many agents inhibit HIF-1α action at different levels of regulation. These involve inhibition of HIF-1α mRNA or protein synthesis, as well as its stability, and DNA binding and transactivation (158).

2.3 Translational control in response to hypoxic signaling

In addition to activating HIF-1α, cells also respond to hypoxia by regulating translation through two distinct pathways; the inhibition of the mammalian target of rapamycin (mTOR) kinase, and signaling through the UPR (159).

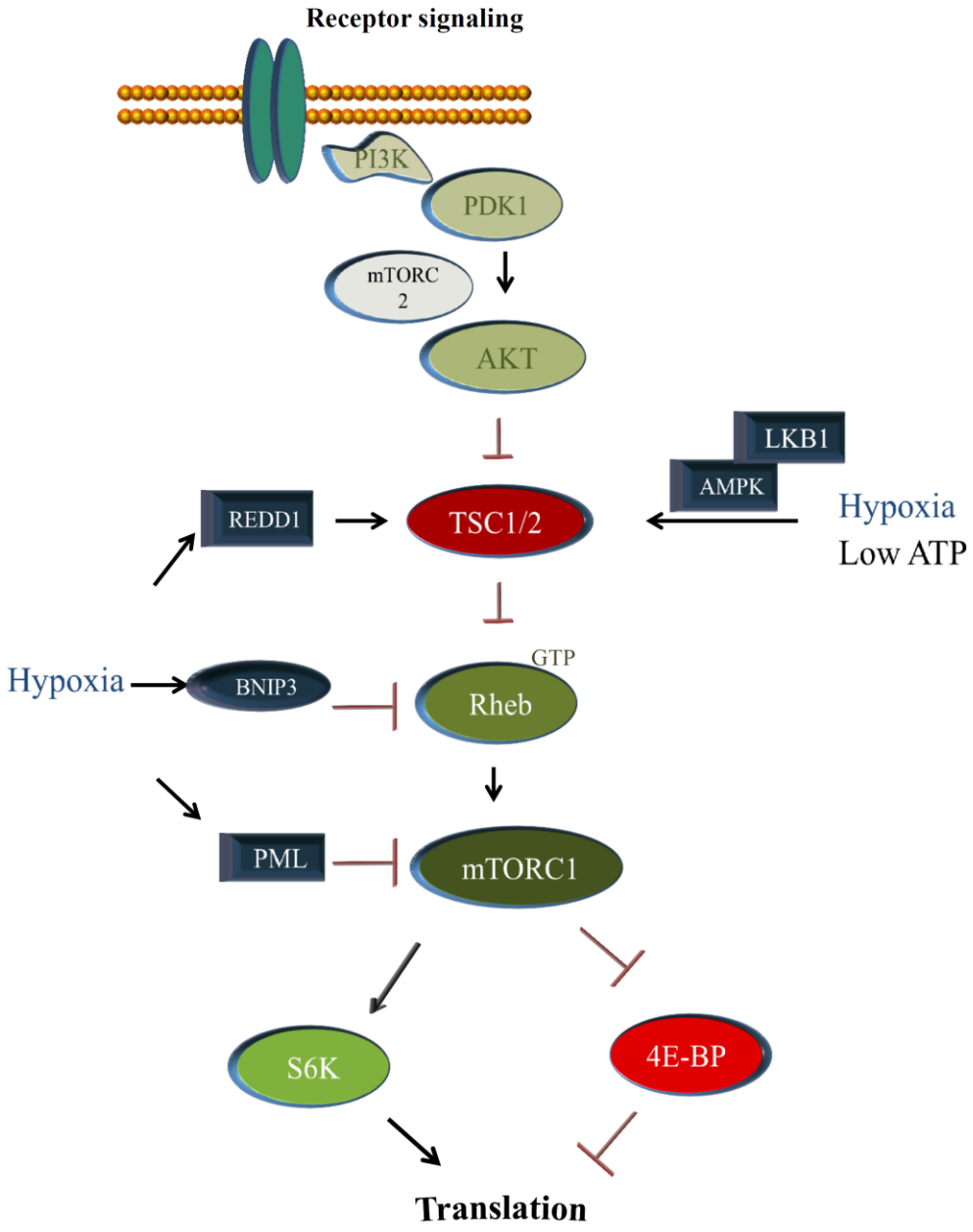
2.3.1 Hypoxia regulates translation through the mTOR pathway

The PI3K pathway is an important pathway in cancer as its activation is commonly observed in human cancers. Receptor tyrosine kinases (RTKs) activated by growth signals (mutations, hormones, mitogens or growth factors) recruit PI3K to the plasma membrane, where it catalyses the formation of PIP3 from PIP2. This functions to recruit and activate AKT/protein kinase B (PKB). Further phosphorylation by FRAP/mTOR-Rictor (mTORC2) results in complete activation of AKT (160). The activated AKT modulates an inhibition of TSC1/2 complex, which allows Rheb to remain in active GTP bound state and activate

mTOR complex 1 (mTORC1). mTORC1 acts to transmit positive signals to the translational machinery by phosphorylation of the ribosomal protein S6 kinase and 4E-BP (159). Regulation of the mTOR pathway by hypoxic conditions is illustrated in Figure 9.

Hypoxia acts on several levels to inhibit protein synthesis facilitated through the mTOR pathway. One example is through the modulation of TSC1/2 activity through the protein Regulated in Development and DNA Damage Responses (REDD1) (161, 162). An interesting feedback loop has been described between REDD1 and HIF-1α as HIF-1α induces REDD1 and REDD1 regulates HIF-1α expression by inhibiting mTOR and HIF-1α stability by inhibiting mitochondrial ROS (163). TSC1/2 has also been shown to be regulated during hypoxia through the activation of the AMP-activated protein kinase (AMPK) which responds to energy stress (164). In these cases, TSC1/2 is activated and acts on the GTPase activity of Rheb, leading to inactive GDP bound Rheb, and in turn an inhibition of mTOR activity. Other factors described to inhibit the mTOR pathway upon hypoxia are the BCL2/adeno virus E1B19-kD interacting protein 3 (BNIP3) and the promyelocytic leukaemia protein (PML). The hypoxia inducible BNIP3 protein inhibits mTOR dependent protein synthesis by directly binding Rheb and hindering its activity (165). In contrast, the PML tumor suppressor interacts with mTOR directly, prevents its association with Rheb, and in turn diminishes mTOR activity, mTOR dependent HIF-1α expression and neo-angiogenesis (166).

Figure 9



Adapted from (159)

Figure 9. ***Hypoxic regulation of translation through mTOR signaling.***

Receptor tyrosine kinases (RTKs), activated by growth signals (hormones, mitogens or growth factors), recruit the PI3K. PI3K catalyses the formation of PI (3,4,5)P3 from PI(4,5)P2 at the plasma membrane which recruits and activates the AKT/protein kinase B (PKB). Phosphorylation by FRAP/mTOR-Rictor (mTORC2) results in its full activation. AKT modulates an inhibition of TSC1/2 complex, allowing Rheb to remain in GTP bound active state and activate mTOR complex 1 (mTORC1). mTORC1 controls protein translation through phosphorylation of the ribosomal protein S6 kinase and 4E-BP. Hypoxia acts on several levels of the mTOR pathway to inhibit protein synthesis. It acts by increasing the activity of TSC1/2 by REDD1 and through the activation of the AMP- activated protein kinase (AMPK). TSC1/2 activates the GTPase activity of Rheb leading to an accumulation of inactive GDP bound Rheb which in turn inhibits mTOR activity. Furthermore, hypoxia can also inhibit protein synthesis through the BCL2/adeno virus E1B19-kD interacting protein2 (BNIP3) and promyelocytic leukaemia (PML) tumor suppressor by relating Rheb and mTOR respectively. Adapted from (159).

2.3.2 Hypoxia and the eIF2α phosphorylation pathway

The link between the eIF2α phosphorylation pathway and hypoxia has been shown to be exerted through the disruption of the physiological homeostasis of the ER. The ER is a cellular organelle which can act independently in sensing low oxygen and signal to adaptive protective pathways. This is mediated through the UPR (previously described in Fig.5). The exact mechanism by which hypoxia obstructs protein folding in the ER and activates the UPR is not well understood. As oxygen is a crucial electron receptor, one accepted explanation is that the lack of oxygen disturbs the proper folding and maturation of nascent proteins by disrupting enzymatic processes such as glycosylation, isomerization, and disulfide bond formation (159).

Phosphorylation of eIF2α by PERK during hypoxia, similar to other ER stress inducing agents is transient through feedback inhibition (de-phosphorylation) initiated by ATF4 and mediated through GADD134 and PP1 (167). Many aspects of the UPR are cytoprotective and several studies indicate that its activation plays a crucial role in facilitating tumor growth (168, 169). As such, PERK activation and eIF2α phosphorylation have been shown to promote survival upon hypoxic stress (170). MEFs lacking PERK undergo apoptosis when exposed to hypoxia and tumors lacking PERK not only grow slower but are also more prone to apoptosis in hypoxic regions (68, 171).

2.3.4 The interplay between HIF-1α and translational control

There has been evidence of crosstalk between HIF-1α and regulation of protein synthesis upon hypoxic conditions. First off, despite the cells' efforts to inhibit global protein synthesis, HIF-1α is expressed during hypoxic conditions. Although early reports suggested that HIF-1α is preferentially translated upon hypoxia, through an IRES dependent mechanism, these remains controversial as later studies demonstrate that HIF-1α translation mirrors its mRNA abundance upon hypoxia (159, 172, 173). Although the mechanism is not clear, data obtained with polysome profiling, a technique which measures whether an mRNA is being highly translated (bound to polyribosomes), clearly demonstrates that the polysomal distribution of *HIF-1A* mRNA is not affected and is refractory to inhibition of global protein synthesis upon hypoxia (174). There is also evidence relating HIF-1α to translational control. While HIF-1α synthesis has been shown to be favored downstream of oncogenic signals through mTOR (described in 2.2), HIF-1α contributes to hypoxia induced inhibition of the mTOR pathway (described in 2.3.1) (159, 163). This shows interplay between the two pathways, and suggests the likelihood of other potential feedback mechanisms between transcriptional and translational control upon hypoxia.

2.4 The eIF2α pathway and chemotherapeutics

2.4.1 Inhibition of the PI3K pathway

The PI3Kinase pathway (illustrated in Figure 9) has many implications in cell proliferation, survival and metabolism and has been shown to be activated in many cancers types (175). As such, therapeutic approaches aim to inhibit activation of PI3K and its downstream signaling. A recent study from our lab showed that eIF2α phosphorylation is induced in response to pharmacological inhibition of PI3K or AKT. More specifically, it was found that AKT inhibits PERK by phosphorylating it at a new site (T799). Inhibition of PI3K or AKT diminishes this inhibitory phosphorylation and leads to PERK activation and eIF2α phosphorylation. The biological function of PERK activation upon pharmacological inhibition of PI3K or AKT is cytoprotective; where the inactivation of the PERK-eIF2α phosphorylation pathway leads to increased susceptibility to cell death with these agents (176). This is consistent with many studies showing the PERK-eIF2α pathway to be cytoprotective (171, 177). As such, inhibition of PERK and eIF2α phosphorylation may provide a suitable means to improve the efficacy of current chemotherapies that target PI3K-Akt signaling (176).

2.4.2 DNA damage induction

Induction of DNA damage is a common chemotherapeutic strategy to inhibit tumor cell proliferation. Studies have demonstrated that eIF2α

phosphorylation is induced by genotoxic stress (39, 104, 178-180). In a recent study from our lab, it was shown that doxorubicin activates PKR to promote cell death through a mechanism requiring the activation of the c-jun N-terminal kinase (JNK). PKR induced cell death was independent of eIF2α phosphorylation. In fact, eIF2α phosphorylation was found to convey opposing cytoprotective effects in response to doxorubicin. These findings unveiled a novel function for PKR in response to DNA damage. It also demonstrates that the phosphorylation of eIF2α independently acts in a cytoprotective manner (181) .

2.4.3 HDAC inhibition

HDAC inhibitors (such as SAHA) represent a new class of anti-cancer treatments and mediate their antitumor functions at different levels by inhibiting histone deacetylation. This renders chromatin in an active open conformation allowing for transcription of anti-proliferative and tumor suppressive factors, usually silenced in tumor cells. It has also been demonstrated that non-histone factors, whose functions are regulated by acetylation, also contribute to tumor inhibition through HDACs (182). These factors include transcription factors, chaperones, and DNA repair proteins (183). There has been evidence that the eIF2α pathway is active in response to HDAC inhibition (184-187). With regard to HDAC inhibition, our group has recently found that eIF2α phosphorylation, becomes induced and acts in a cytoprotective manner. We also demonstrated that upstream activation of GCN2 and PKR induces apoptosis in response to SAHA.

This work highlights eIF2α independent antitumor roles for the eIF2α kinases and confirms that inhibition of eIF2α phosphorylation may be a suitable target for enhancing susceptibility to chemotherapies (188).

In the studies described above, eIF2α phosphorylation is cytoprotective in response to different classes of the chemotherapeutics. While PERK is cytoprotective in response to PI3K/AKT inhibition, PKR and GCN2 can act independently of eIF2α phosphorylation by being pro-apoptotic in response to DNA damage and HDAC inhibition. As such, these studies suggest that designing combination therapies which activate PKR and GCN2 and which target the cytoprotective arm of eIF2α phosphorylation may prove useful in increasing chemotherapy efficiency.

3. Experimental models for studying the eIF2α phosphorylation pathway

3.1 Mouse models

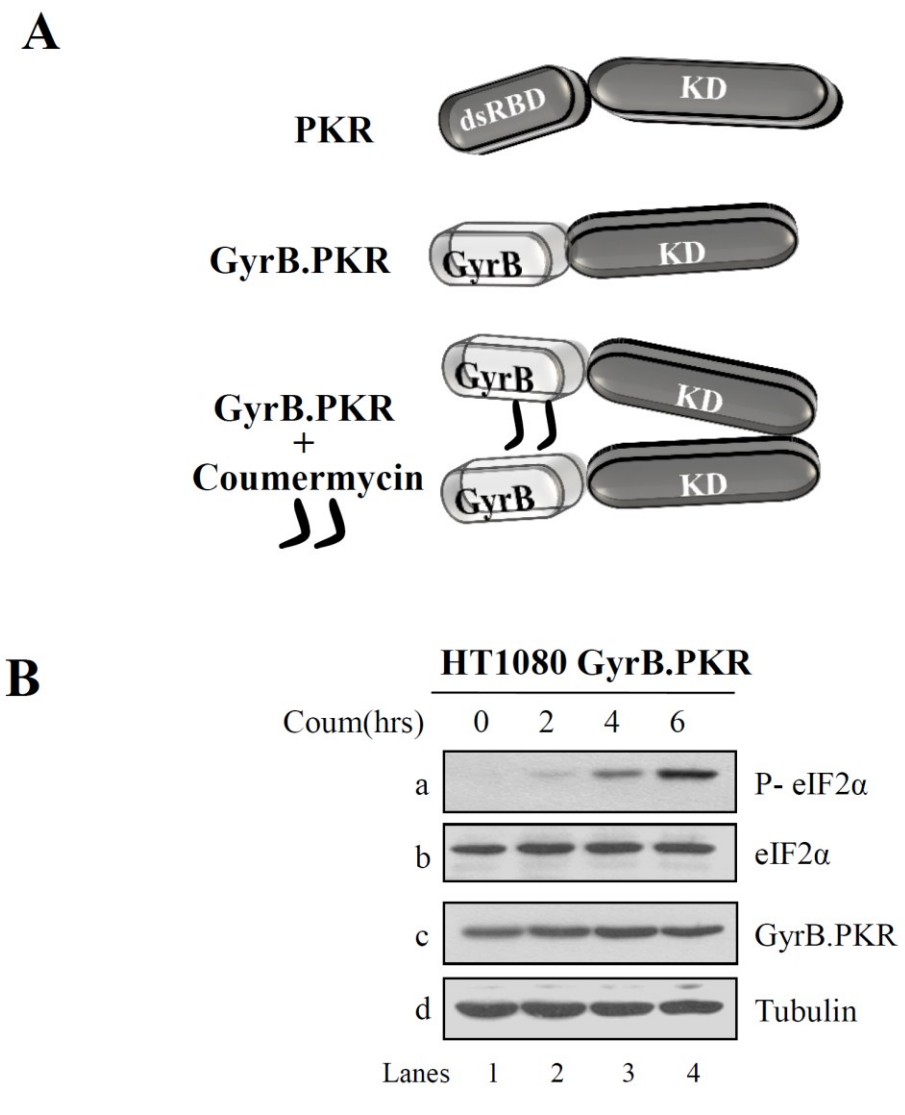
Mouse models developed to dissect the function of eIF2α kinases and eIF2α phosphorylation were described in 1.2. These models include ubiquitous or tissue-specific knock-outs of each of the eIF2α kinases: PKR, PERK, GCN2 and HRI, as well as ubiquitous or tissue specific knock-ins of eIF2α phosphorylated at S51A (112, 189-195). These models, as well as MEFs and cell lines derived from the mice, have demonstrated important roles for eIF2α kinases in development, hematopoiesis, nutrient depletion, virus infection and metabolic diseases such as diabetes.

3.2 Systems of conditionally inducible eIF2α phosphorylation

Environmental stresses which activate the eIF2α kinases induce several pathways in addition to eIF2α phosphorylation. As such, it is not entirely clear whether cell fate is solely determined by the activation of the eIF2α phosphorylation pathway or by the coordinated action of several cascades that act in parallel to eIF2α phosphorylation induced by stress. A suitable approach to address this matter has been the generation of cell systems employing conditional active forms of eIF2α kinases (177, 196). One such system, which consists of the first 220 amino acids of *E. coli* Gyrase B fused to KD of PKR, was used by our group to establish fibrosarcoma HT1080 cells that conditionally induce the phosphorylation of eIF2α after treatment with the antibiotic coumermycin. More

specifically, coumermycin administration mediates the dimerization of the GyrB domain, allowing the GyrB.PKR proteins to interact and become auto-activated resulting in the phosphorylation of eIF2α (94)(Fig.10). The fibrosarcoma HT1080 cells were instrumental to uncover new roles for PKR and eIF2α phosphorylation in PI3K signaling (197), Jak-Stat pathway (99), genotoxic stress (181), and hypoxia (198).

Figure 10



Adapted from (199).

Figure 10. ***The GyrB.PKR system.***

(A) Schematic illustration of the chimera GyrB.PKR protein. PKR consists of the regulatory double-stranded (ds) RNA-binding domain (dsRBD) and the kinase domain (KD). In the GyrB.PKR chimera protein, the dsRBD of PKR has been replaced by the N-terminus domain of Gyrase B (GyrB), which mediates dimerization in the presence of coumermycin. **(B)** HT1080 cells expressing GyrB.PKR were treated with coumermycin (100 ng/ml) for the indicated times. Protein extracts (50 μg) were subjected to immunoblot analysis for eIF2α phosphorylated at S51 (P-eIF2α), eIF2α, GyrB or tubulin. Adapted from (199)

4. Rationale, hypothesis and Research Aims

4.1 The eIF2α phosphorylation pathway and HIF-1α

Hypoxia induces the unfolded protein response (UPR), which leads to PERK activation (170, 200). Many aspects of the UPR are cytoprotective and several studies demonstrate that it plays a positive role in facilitating tumor growth (201). Given that eIF2α phosphorylation and the induction of HIF-1α represent important mechanisms of cell adaptation to hypoxic stress, we hypothesized that there is a crosstalk between the pathways and examined whether the eIF2α kinases and eIF2α phosphorylation are involved in regulating HIF-1α.

Objectives

1. Evaluate the role of the eIF2α kinases and eIF2α phosphorylation in regulating HIF-1α upon hypoxic conditions by employing knockout MEFs (for PKR, PERK, or GCN2) along with their wildtype counterparts and MEFs containing either wildtype eIF2α (eIF2 $\alpha^{S/S}$) or mutant non-phosphorylatable eIF2α (eIF2 $\alpha^{A/A}$).
2. Characterize the mechanism by which this regulation occurs.

4.2 Development of transgenic mice expressing a conditionally active form of the eIF2alpha kinase PKR

To assess the importance and roles of eIF2α phosphorylation and the kinases that phosphorylate it in mammalian physiology, several mouse models have been developed. These mice permit the examination of the effects of loss of function of each eIF2α kinase or loss of eIF2α phosphorylation. We sought to develop a mouse model where we can conditionally induce PKR kinase activity to

study the biological effects of persistent eIF2α phosphorylation *in vivo*. Since the inducible GyrB.PKR system works well in HT1080 cells (Fig.10) we hypothesized that its utility may be applicable in an *in vivo* setting.

Objectives

1. Generate and maintain mice which ubiquitously express GyrB.PKR.
2. Characterize and assess the inducibilty of the chimera kinase *in vivo* upon coumermycin administration.

4.3 Generation of human "knock-in" cell lines bearing non-phosphorylatable eIF2α to examine the role of eIF2α phosphorylation in cell proliferation and in response to chemotherapeutic agents.

The eIF2α $^{S/S}$ and $^{A/A}$ MEFs have been great tools for investigate the role of the eIF2α phosphorylation in cell culture settings (202). Due to differences between mouse and human cells, it was fundamentally important to devise different strategies to study eIF2α phosphorylation. Several approaches were devised to achieve this.

Objectives

1. Generate cell permeable peptides to inhibit eIF2α phosphorylation and test their efficacy in human cell lines.
2. Devise a strategy to replace wildtype eIF2α with eIF2α bearing the non-phosphorylatable S51A mutation.
3. Characterize cells lines and assess how the abrogation of eIF2α phosphorylation affects cell proliferation.
4. Validate the applicability of cell lines using agents known to signal through the eIF2α phosphorylation pathway.

Chapter 2: Materials and Methods

1. Cell culture and Treatments

The immortalized $PKR^{-/-}$ (189), $PERK^{-/-}$ (203), $GCN2^{-/-}$ (192), $TC\text{-}PTP^{-/-}$ MEFs (204) and their isogenic counterparts were grown in Dulbecco's modified Eagle's medium (DMEM; Wisent) supplemented with 10% fetal bovine serum (FBS; Wisent) and 100 units/ml of penicillin-streptomycin (Wisent). Isogenic $eIF2\alpha^{S/S}$ and $^{A/A}$ MEFs primary cells generated as well as the immortalized $eIF2\alpha^{S/S}$ and $^{A/A}$ MEFs were maintained as described (194). HT1080 expressing GyrB.PKR containing mutant eIF2αS51A or wildtype eIF2α were maintained as previously described (94). H1299 cells were maintained in DMEM (Wisent) supplemented with 10% FBS (Wisent) and 100 units/ml of penicillin-streptomycin (Wisent). $NiCl_2$ (Ni^{2+}; Sigma), and $CoCl_2$ (Co^{2+}; Fisher Scientific), Doxorubicin hydrochloride (Dox; Sigma), and cycloheximide (CHX; Sigma) were dissolved in distilled H_2O. CPA-7 was dissolved in 50% DMSO (205). Coumermycin (Sigma), Vorinostat (SAHA; ChemieTek) and Thapsigargin (TG; Sigma) were 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, 94% N_2 at 37°C.

2. Cell staining and Flow Cytometry Analysis

The HT1080 modified cells bearing endogenous wildtype eIF2α or mutant HA.eIF2αS51A with knocked-down of endogenous eIF2α were treated as indicated (Fig.30 and 31), and subjected to propidium iodide (PI) staining and FACs using a modified previously described protocol (94). In brief, following the indicated treatments, adherent and floating cells were collected, washed with PBS

and fixed with 70% ice-cold ethanol (Commercial Alcohols) overnight at -20°C. Ethanol was removed after centrifugation and cell pellets were re-suspended in 500 ul of PBS containing 20 ug/ml of RNAse A (Sigma) and 50 ug/ml of Propidium Iodide (PI; Sigma) and incubated for 30 minutes at 4°C (dark) before analysis. FACS was performed with BD FACScalibur. Data was analyzed using the WinMDI 2.8 software (Scripp Institute).

3. Colony Formation Assays

The indicated number of cells were seeded in 6-well plates and allowed to proliferate for 7 days. Following washes with PBS, and fixation with 4% formaldehyde in PBS for 1hr, plates were stained with 0.1% Crystal violet (Sigma) for 2 hours. Plates were washed with dH_2O, dried and scanned.

4. Generation and treatments of cell permeable peptides

Cell permeable peptides were generated by Creative Biolabs and sequences for control Tat peptide and conserved eIF2α sequences which contact the eIF2α kinases (206, 207) are as follows:

Control Tat: G-YGRKKRRQRRR
Tat eIF2α peptide 1: LSELSRRRIRSINKLI-G-YGRKKRRQRRR,
Tat eIF2α peptide 2: VIRVDKEKGYIDLSKRR-G- YGRKKRRQRRR
Tat eIF2α peptide 3: AYVSLLEYNNIEG-G-YGRKKRRQRRR

For treatments, cells were incubated with100uM tat peptides in the absence of serum for 2 hours. After the addition of 10% serum cells were incubated for the indicated times and lysed for western blot analysis.

5. Generation of GyrB.PKR transgenic mice and treatments

GyrB.PKR cDNA was excised from the pSG5 vector (94) using SalI and HindIII restriction enzymes and subcloned into the EcoRI site of pCX-EGFP vector (208, 209) using blunt end ligation after removal of EGFP. The vector was then introduced into pronuclei of FVB mice at the McGill University Transgenic Mouse Core Facility. Mice were administered with coumermycin either by intraperitoneal (i.p.) injection or gavage feeding using 0.25mg/mouse coumermycin (Sigma) each day for 8 days or 0.5mg/mouse coumermycin in 1% hydroxypropyl methylcellulose for one day respectively. All animal studies were performed in accordance with approved protocols and regulations by the Animal Care Committee of McGill University (protocol# 5754).

6. DNA extraction and PCR genotyping

Mouse tail DNA was extracted using the alkali lysis method as previously described (210). DNA samples were subjected to polymerase chain reaction (PCR) and transgenic mice were screened using primers (Sheldon Biotechnology Centre, McGill University) flanking the transgene insert as follows: 5'-TGTCTCTGTACAGGATGACG-3'and 5'-CCATCCCAACAGCCATTGTA-3'. Amplified PCR products were subjected to agarose gel electrophoresis and visualized by ethidium bromide staining after ultraviolet (UV) light exposure.

7. Growth Curve analysis

The HT1080 cells were seeded at 10000 cells per well in triplicates in 6-well plates (5 sets). Cells were trypsinized, collected and counted at the indicated

time-points (24, 48, 72, 96, and 120 hours). Cells counts represent the average of the triplicates and error bars denote standard deviation.

8. Immunohistochemistry

Immunoperoxidase staining for eIF2α phosphorylated at S51 was performed by the avidin-biotin complex method (Vector Laboratories). 4-μm formalin fixed paraffin embedded sections were cut, placed on SuperFrost/Plus slides (Fisher), and dried overnight at 37°C. Sections were de-paraffinized in xylene and rehydrated through graded alcohols to water. Sections were immersed in 10mM sodium citrate buffer, pH 6.0, and subjected to heat-induced antigen retrieval. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide for 15 min. Endogenous biotin was blocked by incubation for 10 min with the Avidin/Biotin blocking kit (Zymed Laboratories Inc). To block non-specific protein binding, sections were then treated with 5% normal goat serum in TBS-Tween 0.1% (TBS-T) for 60 min at room temperature. Sections were incubated overnight at 4˚C with primary rabbit polyclonal phosphospecific antibody recognizing S51 of eIF2α (Cell Signaling) used at 1:35 dilution in TBS-T with 5% normal goat serum. After rinsing with TBS-T, sections were incubated with biotinylated antibodies (goat anti-rabbit, Vector Laboratories) for 60 min at room temperature. The sections were then incubated for 30 min with avidin-biotin-horseradish peroxidase complex (Vector Laboratories), followed by final color development with peroxidase substrate kit DAB (Vector Laboratories) for 3-5 min. Sections were then lightly counterstained with hematoxylin, dehydrated in

graded alcohols, cleared in xylene and coverslipped. Sections were analyzed by conventional light microscopy.

9. Protein extraction, immunoblot analysis and immunoprecipitation

Protein extraction, immunoblotting and immunoprecipitation were performed as described (99). Protein extracts were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as previously described. Proteins were electro-blotted (transferred) to a polyvinylidene difluoride (PVDF) membrane (Millipore) for immunoblot analysis. For immunoblotting and/or immunopreciptation, 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 (99); mouse monoclonal antibody for actin (Clone C4, ICN Biomedicals Inc), rabbit anti-tubulin (Chemicon), mouse monoclonal against PKR (F9), rabbit polyclonal phosphospecific against S51 of eIF2α (Invitrogen), mouse monoclonal to eIF2α (Cell Signaling), mouse monoclonal to p53(Ab-6) (Calbiochem), rabbit anti-IRP2 (211), mouse anti-Stat3 (Cell Signalling), and anti-phosho-Tyr^{705} Stat3 (Santa Cruz) anti-GyrB monoclonal antibody (clone 7D3; John Innes Enterprises), rabbit polyclonal phosphospecific Thr183/Tyr185 JNK1/2 (Cell signaling), rabbit polyclonal JNK1 (Santa Cruz biotechnology), rabbit polyclonal GRP78/Bip (Santa Cruz) and rabbit polyclonal anti-tubulin (Chemicon). All antibodies were used at a final concentration of 0.1-1 μg/ml. After incubation with anti-mouse IgG or anti-rabbit IgG antibodies conjugated to horseradish peroxidise (HRP) proteins were

visualized with the enhanced chemiluminescence (ECL) reagent (Thermo Scientific) detection system according to the manufacturer's instructions. Quantification of protein bands was performed by densitometry using Scion Image from the NIH.

10. RNA Isolation and real time PCR

Total RNA was isolated by Trizol reagent (Invitrogen) according to manufacturer's protocol. 1 μg of total RNA was reversed transcribed with the high capacity cDNA reverse transcription kit (Applied Biosystems International). Quantitative 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 beta actin mRNA. cDNA of three independent experiments was analyzed in duplicates. Relative quantification was done with the REST-MCS software. The primers used for RT-q-PCR are follows:

Mouse HIF-1α sense: GCACTAGACAAAGTTCACCTGAGA

Mouse HIF-1α antisense: CGCTATCCACATCAAAGCAA

Mouse VEGF sense: GCAGCTTGAGTTAAACGAACG

Mouse VEGF antisense: GGTTCCCGAAACCCTGAG

Mouse SLC2A1 (GLUT-1) sense: ATGGATCCCAGCAGCAAG

Mouse SLC2A1 (GLUT-1) antisense: CCAGTGTTATAGCCGAACTGC

Mouse beta-actin sense: CTAAGGCCAACCGTGAAAAG

Mouse beta-actin antisense: ACCAGAGGCATACAGGGACA.

11. 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) (212), or pGL3vector 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 after 48 hours post transfection , left untreated (Fig.14D) or treated with 20uM CPA-7 for 24 hours (Fig.16B), or were co-transfected with 0.4 μg of STAT 3D (previously described in (213) or the control pcDNA and lysed 48 hours post-transfection (Fig.16C) as indicated in the legends. Firefly and renilla luciferase was 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

12. Retroviral infections and Lenti-viral infections

Retroviral production: The Phoenix packaging cells were seeded at 2 million cells per 10cm plate. The next day (Day 1) cells were transfected with 25ug of either mSCV.GFP or mSCV.gfp.HAeIF2αS51A using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. Day 2: transfected cells were treated with 10mM sodium butyrate (Sigma) overnight. Day 3: media was changed. Starting day 4 viral supernatants were collected every 12 hours (3 rounds) and filtered through a 45 um filter (VWR International). In parallel freshly collected supernatants were used to infect the HT1080 target cells. For infections 6ug/ml polybrene (Sigma) was added to the filtered supernatants. After 3 rounds of infections, cells were allowed to recover for 72 hours and GFP positive cells were sorted by FACs, and characterized.

Lentivirus was produced using 293/T cells which were seeded 2 million cells per 10 cm plate. Day 1: cells were co-transfected with 25ug of psPAX2, 10ug of pMDG, and 20ug of PGIPZ GFP control or PGIPZ GFP targeting the 3' UTR of eIF2α (Open-Biosystems) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. Viral supernatants were collected 48 hours post-transfection (Day 3) and filtered through a 45 um filter (VWR International). For infections 6ug/ml polybrene (Sigma) was added to the filtered supernatants. The HT1080 GFP positive cells obtained by retroviral transduction (described above) were infected for 18 hours. Cells were allowed to recover for 48 hours and selected with 2.5ug/ml Puromycin (Sigma).

13. shRNA targeting PKR

For targeting of human PKR by shRNA 5'-GCAGGGAGTAGTACTTAAA-3' and 5'-GGCAGTTAGTCCTTTATTA-3' were sub-cloned into the pCXS U6/Zeo expression vector. H1299 cells harboring the target vector were selected for resistance to 400ug/ml Zeocin (Invitrogen). As a control, zeocin-resistant cells harboring empty pCXS/zeo DNA were generated.

14. Statistical analysis

Error bars represent standard deviations or standard error as indicated and significance in differences between arrays of data tested was determined using the two-tailed Student T test (GraphPad Prism5 and Microsoft Excel). P values are denoted and statistical significance reflected values, $p < 0.05$.

Chapter 3: Results

1. Defining a role for PKR in regulating the hypoxic axis.

Hypoxia induces the unfolded protein response (UPR), leading to PERK activation (170, 200). Many aspects of the UPR are cytoprotective and several studies indicate that it facilitates tumor growth (201). Given that induction of eIF2α phosphorylation and upregulation of HIF-1α represent important mechanisms of cellular adaptation to hypoxic stress, we were interested to examine whether the eIF2α phosphorylation pathway is involved in regulating HIF-1α. Herein, we demonstrate that PKR, an eIF2α kinase, plays a specific role in suppressing HIF-1α levels through mechanisms that are independent of eIF2α phosphorylation. More specifically, our data reveal that PKR can suppress the transcription of HIF-1α gene via a mechanism involving Stat3.

1.1 PKR significantly suppresses HIF-1α levels in hypoxic cells.

To determine whether the eIF2α kinases regulate HIF-1α, we utilized MEFs lacking PKR, PERK, or GCN2 and their wildtype counterparts to assess levels of HIF-1α under normoxic or hypoxic conditions. Our results revealed that PKR plays a role in suppressing expression of HIF-1α. While HIF-1α is barely detectable under normoxic conditions, it is highly induced upon hypoxic conditions (Fig.11A). Our results show that the accumulation of HIF-1α under hypoxic conditions was substantially higher in $PKR^{-/-}$ MEFs compared to $PKR^{+/+}$ MEFs (Fig.11A**)**. We were also able to recapitulate these results using hypoxia-mimetic compounds, such as $CoCl_2$ (214) or $NiCl_2$ (215) (Fig.11B). Contrary to

HIF-1α, HIF-2α expression was readily detectable in normoxic and hypoxic conditions (Fig.11A 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 up-regulated in mouse embryonic cells under hypoxia (216). However, unlike HIF-2α we found that the iron regulatory protein 2 (IRP2), which is induced by hypoxia , was efficiently induced in both $PKR^{+/+}$ and $PKR^{-/-}$ MEFs (Fig.11A, panel c). Taken together these results supported a specific role of PKR in suppressing HIF-1α in hypoxic cells.

To assess whether the higher levels of HIF-1α in $PKR^{-/-}$ MEFs resulted in increased HIF-1 activity, we examined the expression of HIF-1 target genes under normoxic or hypoxic conditions by quantitative real time PCR. We found that the mRNA levels of the vascular endothelial growth factor (*VEGF*) and glucose transporter (*GLUT-1*) genes were higher in $PKR^{-/-}$ than in $PKR^{+/+}$ MEFs under hypoxia (Fig.11C) providing evidence that the difference in HIF-1α protein levels has a functional significance in these cells. Given the significant differences of HIF-1α in MEFs, we next examined whether we can recapitulate the role of PKR in HIF-1α accumulation in human cells. To this end, we employed the human lung cancer H1299 cells in which partial knockdown of PKR by shRNA lead to increased levels of HIF-1α corroborating our MEF data (Fig.11D and E).

Figure 11

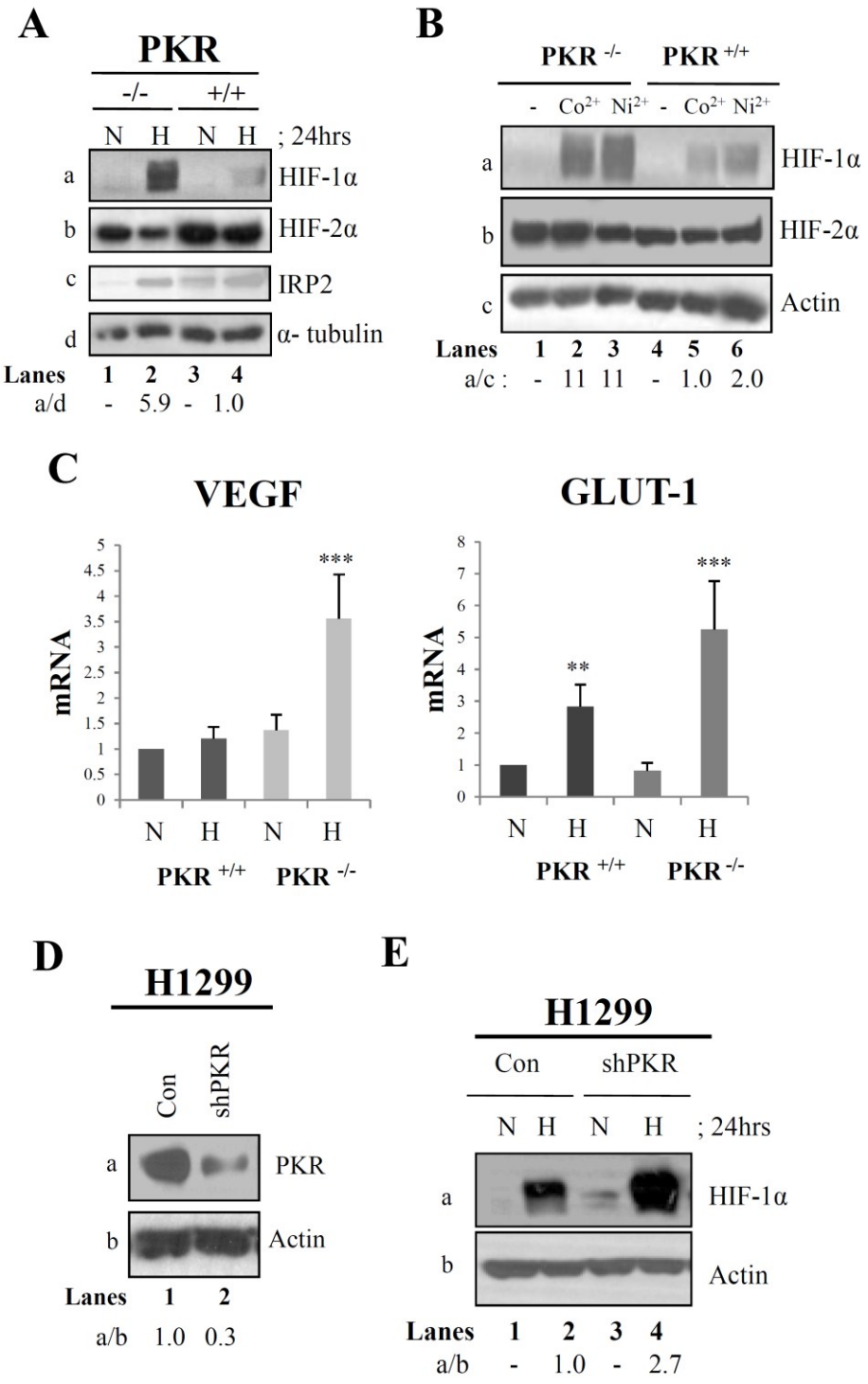

A
PKR
-/-
+/+
N H N H ; 24hrs
a HIF-1α
b HIF-2α
c IRP2
d α- tubulin
Lanes 1 2 3 4
a/d - 5.9 - 1.0
B
PKR -/-
PKR +/+
- Co2+ Ni2+ - Co2+ Ni2+
a HIF-1α
b HIF-2α
c Actin
Lanes 1 2 3 4 5 6
a/c : - 11 11 - 1.0 2.0
C
VEGF
mRNA
***
N H N H
PKR +/+ PKR -/-
GLUT-1
mRNA
**
***
N H N H
PKR +/+ PKR -/-
D
H1299
Con
shPKR
a PKR
b Actin
Lanes 1 2
a/b 1.0 0.3
E
H1299
Con
shPKR
N H N H ; 24hrs
a HIF-1α
b Actin
Lanes 1 2 3 4
a/b - 1.0 - 2.7

Figure 11. ***PKR reduces HIF-1α protein accumulation and activity upon hypoxic treatment.***

$PKR^{+/+}$ and $PKR^{-/-}$ MEFs were incubated in normoxic (N, 21% O_2) or hypoxic conditions (H, 1% O_2) for 24hrs **(A)** or treated with either cobalt chloride (Co^{2+}, 200μM) or nickel chloride (Ni^{2+}, 500μM) for 20 hours **(B)**. **(A and B)** Protein extracts (70μg) 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 $^{+/+}$ MEFs under normoxia or hypoxia (1% O_2) for 24 hours. 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 to $PKR^{+/+}$ in normoxia are indicated as *** for P<0.001 and ** for P< 0.005. **) (D)** Cell extracts from H1299 cells (control and shPKR) were resolved by SDS PAGE and were analyzed by immunoblot analysis for PKR (panel a) and actin (panel b). **(E)** The same cells, untreated or subjected to hypoxia (1%), were analyzed. Whole cell extracts were subjected to SDS PAGE and were probed with anti-HIF 1-α (panel a), and anti-actin (panel b) antibodies. Normalized band ratios are indicated at the bottom of the panels.

1.2 PERK and GCN2 do not affect HIF-1α levels upon hypoxic cells.

The role of two other eIF2α kinases, PERK and GCN2, in HIF-1α expression under hypoxic conditions was examined. 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 MEFs (wild type) and MEFs deficient in PERK (Fig.12A) or GCN2 (Fig.12C) under hypoxic conditions. Consistent with these observations, similar results were obtained with the hypoxia mimetic compounds - $CoCl_2$ and $NiCl_2$ (Fig.12B and D). Taken together, these data support a specific role of PKR in the negative regulation of HIF-1α under hypoxic conditions.

Figure 12

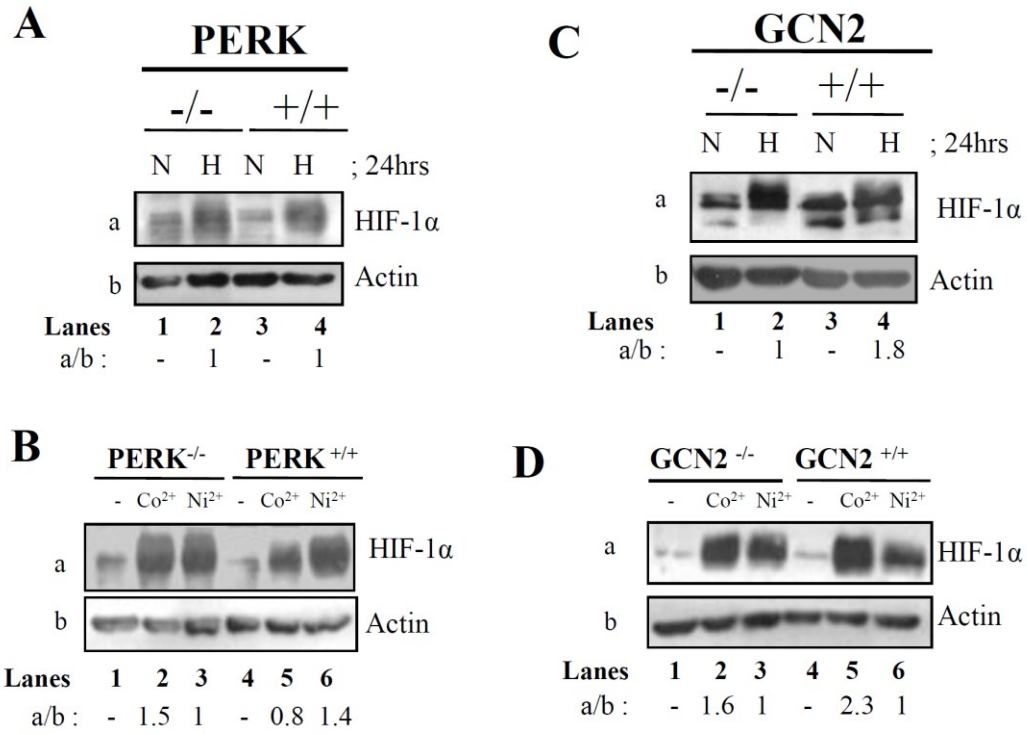

A
PERK
-/-
+/+
N H N H ; 24hrs
a
HIF-1α
b
Actin
Lanes 1 2 3 4
a/b : - 1 - 1
C
GCN2
-/-
+/+
N H N H ; 24hrs
a
HIF-1α
b
Actin
Lanes 1 2 3 4
a/b : - 1 - 1.8
B
PERK-/-
PERK +/+
- Co2+ Ni2+ - Co2+ Ni2+
a
HIF-1α
b
Actin
Lanes 1 2 3 4 5 6
a/b : - 1.5 1 - 0.8 1.4
D
GCN2 -/-
GCN2 +/+
- Co2+ Ni2+ - Co2+ Ni2+
a
HIF-1α
b
Actin
Lanes 1 2 3 4 5 6
a/b : - 1.6 1 - 2.3 1

Figure 12. ***PERK and GCN2 do not affect HIF-1α expression under hypoxic conditions.***

$PERK^{+/+}$ and $PERK^{-/-}$ MEFs **(A, B)** as well and $GCN2^{+/+}$ and $GCN2^{-/-}$ MEFs **(C, D)** were incubated under normoxic (N, 21% O_2) or hypoxic (H, 1% O_2) conditions for 24 hours **(A, C)** or treated with either cobalt chloride (Co^{2+}, 200μM) or nickel chloride (Ni^{2+}, 500μM) for 20hrs (**B**, **D**). (**A**-**D**) Protein extracts (70μg) were subjected to immunoblot analysis for HIF-1α (panel a) and actin (panel b). The intensity of the bands was normalized and ratios (a/b) are indicated.

1.3 PKR does not affect the stability of HIF-1α

HIF-1α is a very labile protein and has been shown to be highly regulated at the level of stability. Since our lab has shown that PKR can affect the stability of proteins such as p53 and CyclinD1 (71, 217) , we investigated the possibility of PKR regulating the stability of HIF-1α. To this end, protein synthesis was inhibited in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs with cycloheximide and the stability of HIF-1α was assessed under hypoxic conditions. Our results show that the presence of PKR did not affect HIF-1α stability in hypoxic cells because HIF1α remained very unstable regardless of PKR status (Fig.13). These results showed that PKR does not regulate the stability of HIF-1α and suggested that PKR may control its synthesis.

Figure 13

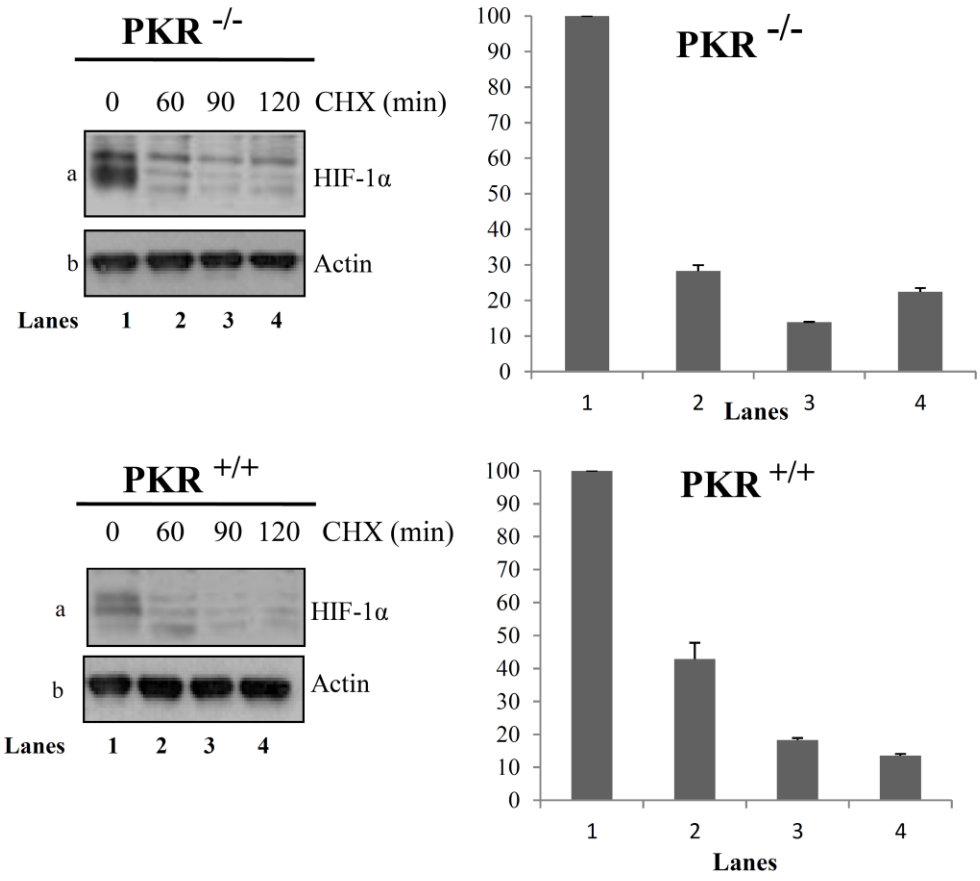

PKR -/-
0 60 90 120 CHX (min)
a
HIF-1α
b
Actin
Lanes 1 2 3 4
PKR -/-
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4
Lanes
PKR +/+
0 60 90 120 CHX (min)
a
HIF-1α
b
Actin
Lanes 1 2 3 4
PKR +/+
100
90
80
70
60
50
40
30
20
10
0
1 2 3 4
Lanes

Figure 13. ***HIF-1α is very unstable in both PKR*** $^{-/-}$ ***and*** $^{+/+}$ ***MEFs.***

PKR $^{-/-}$ and $^{+/+}$ MEFs were subjected to hypoxia overnight and were treated with cycloheximide (100ug/ml) for 60, 90, 120 minutes. Cell lysates were resolved by SDS PAGE and immunoblotted for HIF-1α (panel a) and actin (panel b). The mean of the normalized ratios (a/b) of band intensity from two independent experiments (n=2) are shown in histograms. Error bars denote the standard error.

1.4 PKR inhibits HIF-1α expression at the transcriptional level independently of eIF2α phosphorylation status.

To determine whether inhibition of HIF-1α expression by PKR is dependent on eIF2α phosphorylation, we employed 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. Similar induction of HIF-1α was observed in eIF2$\alpha^{S/S}$ and eIF2 $\alpha^{A/A}$ MEFs under hypoxic conditions **(**Fig.14A**)** and after treatment with $CoCl_2$ or $NiCl_2$ (Fig.14B). This suggests that PKR regulates HIF-1α independently of eIF2α phosphorylation.

To further address the mechanism of inhibition of HIF-1α expression by PKR, we examined HIF-1α mRNA levels in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs by quantitative real-time PCR. We found that HIF-1α mRNA was highly expressed in $PKR^{-/-}$ MEFs compared to the $PKR^{+/+}$ MEFs under normal or hypoxic conditions (Fig.14C). 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 eIF2$\alpha^{S/S}$ and eIF2$\alpha^{A/A}$ MEFs (Fig.14C). These data indicate a possible role for PKR in the transcriptional regulation of HIF-1α in normal and hypoxic conditions.

To confirm that PKR regulates the transcription of the *HIF1A* gene, we performed transient transactivation assays in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs using a luciferase reporter gene under the control of the *HIF1A* promoter. Consistent with

the upregulation of HIF-1α mRNA levels in the PKR $^{-/-}$ MEFs (Fig.14C), the reporter gene assays revealed an approximate 3 fold increase of *HIF1A* promoter activity in PKR $^{-/-}$ MEFs compared to PKR $^{+/+}$ MEFs supporting a transcriptional effect of PKR on HIF-1α expression (Fig.14D).

Figure 14

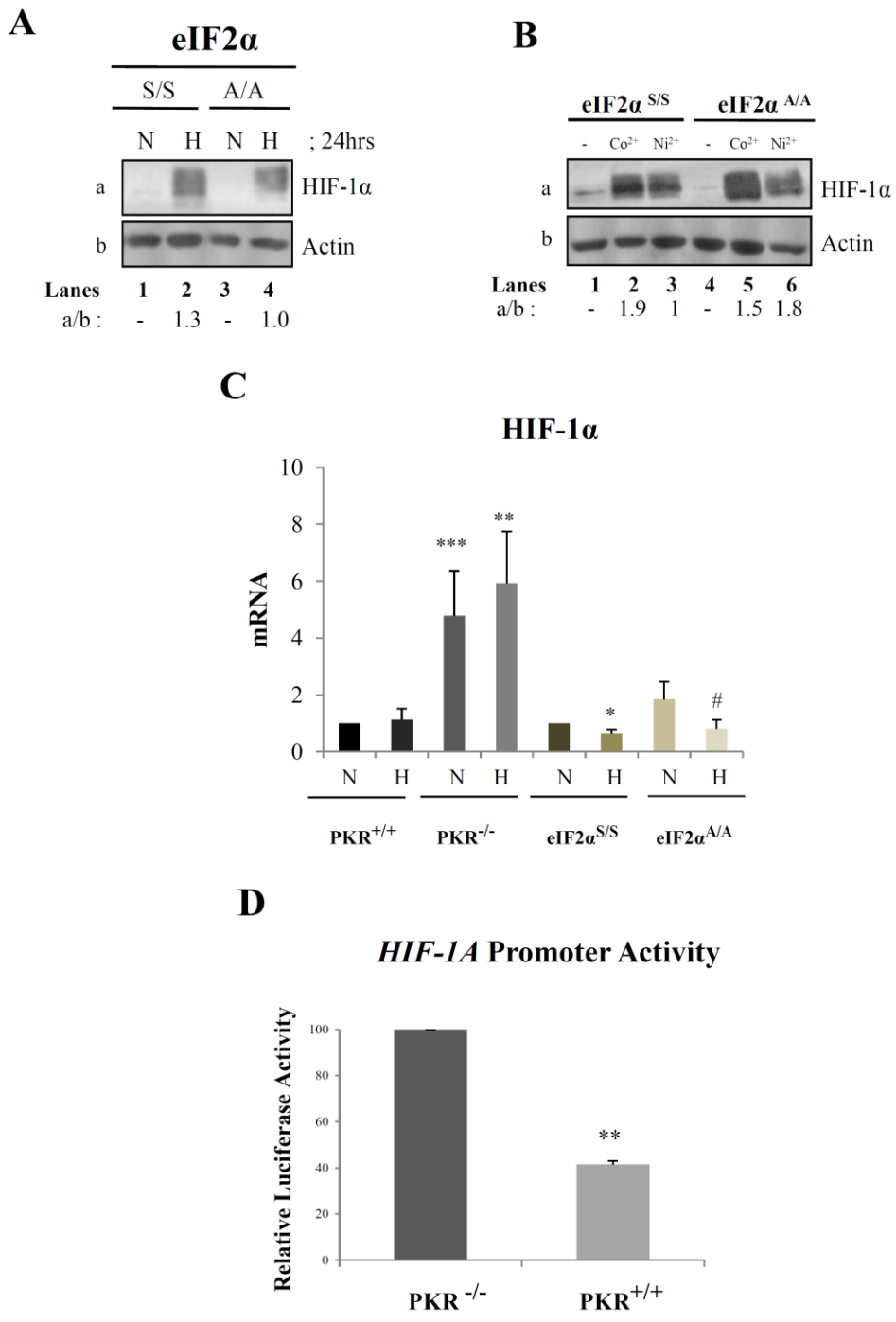

A
eIF2α
S/S
A/A
N H N H ; 24hrs
a
HIF-1α
b
Actin
Lanes 1 2 3 4
a/b : - 1.3 - 1.0
B
eIF2α S/S
eIF2α A/A
- Co2+ Ni2+ - Co2+ Ni2+
a
HIF-1α
b
Actin
Lanes 1 2 3 4 5 6
a/b : - 1.9 1 - 1.5 1.8
C
HIF-1α
mRNA
10
8
6
4
2
0
***
**
*
#
N H N H N H N H
PKR+/+
PKR-/-
eIF2αS/S
eIF2αA/A
D
HIF-1A Promoter Activity
Relative Luciferase Activity
100
80
60
40
20
0
**
PKR -/-
PKR+/+

Figure 14. ***PKR inhibits HIF-1α expression at the transcriptional level independently of eIF2α phosphorylation status.*** **(A)** The eIF2α$^{S/S}$ and $^{A/A}$ MEFs were incubated in normoxic (N, 21% O_2) or hypoxic (H, 1% O_2) conditions for 24 hours **(B)** or were treated with either cobalt chloride (Co^{2+}, 200μM) or nickel chloride (Ni^{2+}, 500μM) for 20 hours. Whole cell extracts (70μg) were subjected to immunoblot analysis for HIF-1α (panel a) and actin (panel b). The normalized ratio (a/b) of band intensity is indicated. (**C**) RNA was isolated from PKR$^{+/+}$ and PKR$^{-/-}$ MEFs as well as eIF2α $^{S/S}$and $^{A/A}$ MEFs subjected to normoxia (N, 21% O_2) or hypoxia (H, 1% O_2) for 24 hours. Quantitative RT-PCR was performed using primers targeting mouse HIF-1α. Statistical analysis was performed and P values are as follows: *** P<0.001 ** P<0.005 compared to PKR $^{+/+}$ in normoxia and * P<0.05 compared to eIF2α$^{S/S}$ normoxia and # P<0.05 compared to eIF2α$^{A/A}$ normoxia. (**D**) 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 co-transfected as an internal control. Luciferase activity was assessed 48 hours post transfection. Relative luciferase activity of the HIF-1α promoter was normalized to the activity of the control vector. Error bar denotes the standard error (n=6). Statistical analysis was performed and paired T-test P value ** corresponds to P < 0.001.

1.5 PKR controls *HIF1A* gene transcription through Stat3.

Recent reports have suggested that *HIF1A* gene transcription can be directly induced by activated Stat3 (129, 218). Additionally, our group has previously shown that Stat3 activity is impaired by PKR due to activation of the T-cell protein tyrosine phosphatase (TC-PTP), which dephosphorylates Stat3 at tyrosine (Y)705 (99). Consistent with this finding, we observed a higher (~10 fold) phosphorylation of Stat3 at Y705 in $PKR^{-/-}$ than in $PKR^{+/+}$ MEFs under normoxic conditions (Fig.15A). We also observed a small induction of Stat3 phosphorylation in the $eIF2\alpha^{A/A}$ cells (Fig.15B), which was not sufficient to affect HIF-1α expression. This indicated that eIF2α phosphorylation may be necessary for a partial inhibition of Stat3, but not sufficient to decrease the transactivation of *HIF-1A* gene (Fig.14). Furthermore, our results indicate that PKR, but not PERK or GCN2 regulates Stat3 phosphorylation (Fig.15C and 15D).

Figure 15

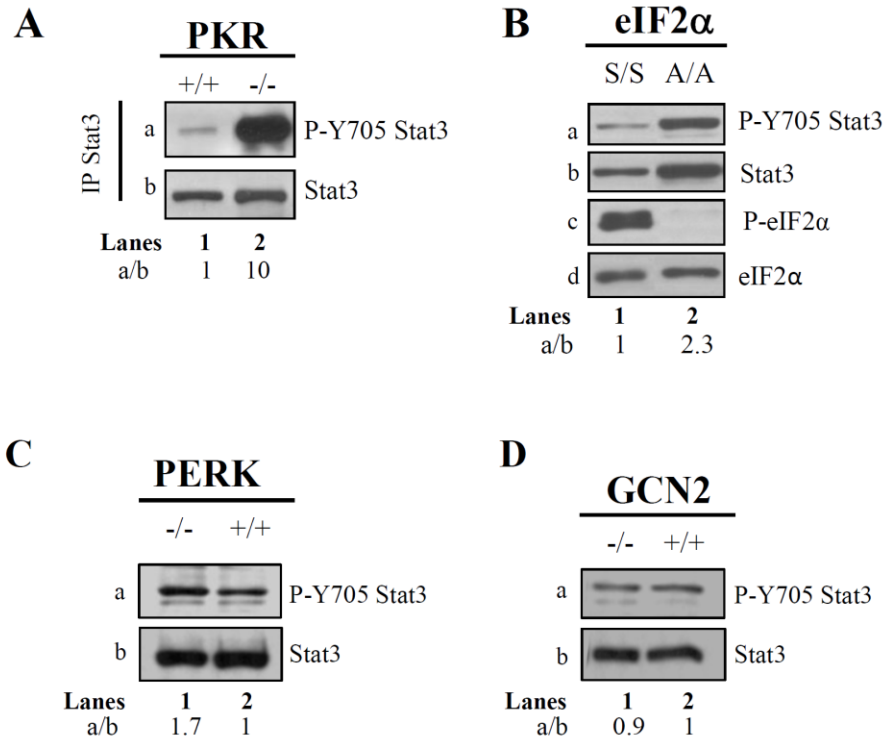

A
PKR
+/+
-/-
IP Stat3
a
b
P-Y705 Stat3
Stat3
Lanes 1 2
a/b 1 10
B
eIF2α
S/S A/A
a
b
c
d
P-Y705 Stat3
Stat3
P-eIF2α
eIF2α
Lanes 1 2
a/b 1 2.3
C
PERK
-/- +/+
a
b
P-Y705 Stat3
Stat3
Lanes 1 2
a/b 1.7 1
D
GCN2
-/- +/+
a
b
P-Y705 Stat3
Stat3
Lanes 1 2
a/b 0.9 1

Figure 15. ***PKR inhibits Stat3 activity*****. (A)** Stat3 was immuno-precipitated 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 (panel a) or total Stat3 (panel b). **(B)** Extracts from untreated $eIF2\alpha^{S/S}$ and $^{A/A}$ MEFs (50ug of protein) were resolved and immunoblotted with antibodies targeting Y705 phosphorylated Stat3 protein (panel a), total Stat3 (panel b), Ser 51 phosphorylated eIF2α (panel c), and total eIF2α (panel d). Similarly, extracts from PERK and $GCN2^{-/-}$ and $^{+/+}$ MEFs (**C** and **D**, respectively) were resolved by SDS PAGE and immunoblotted with antibodies targeting the Y-705 phosphorylated Stat3 protein (panel a), total Stat-3 (panel b). The ratio of band intensity was measured by densitometry, normalized and indicated at the bottom of the panels.

To examine whether the increased levels of HIF-1α expression in $PKR^{-/-}$ MEFs is dependent on Stat3 phosphorylation, the PKR-/- MEFs were exposed to CPA-7, a Stat3 inhibitor which inhibits Stat3 DNA binding and phosphorylation on Y705 (205). Accordingly, CPA-7 inhibited Stat3 phosphorylation and this was consistent with a decrease in HIF-1α accumulation under hypoxic conditions (Fig. 16A). CPA-7 also impaired the induction of HIF-1α by $CoCl_2$ concomitantly with an inhibition of Stat3 phosphorylation at Y705 (Fig.16A).

To analyse the effect of Stat3 phosphorylation on HIF-1α expression we measured *HIF1A* promoter activity by luciferase reporter assays in $PKR^{+/+}$ and $PKR^{-/-}$ MEFs. We found that pharmacological inhibition of Stat3 by CPA-7 or expression of Stat3D, a Stat3 mutant defective in transactivation activity (213), resulted in the inhibition of *HIF1A* promoter activity in $PKR^{-/-}$ MEFs but not in $PKR^{+/+}$ MEFs (Fig.16B and C) . This validates that the higher levels of Stat3 activity in $PKR^{-/-}$ MEFs were responsible for the transcriptional up-regulation of the *HIF1A* gene.

The role of Stat3 in regulating *HIF1A* transcription was also analyzed in the human lung cancer H1299 cells in which PKR was targeted by shRNA (Fig.11D and E). Consistent with the MEF data, we found that partial PKR inactivation led to increased levels of phosphorylated Stat3 (Fig.17A) and transcriptional up-regulation of the *HIF1A* gene (Fig. 17B).

Figure 16

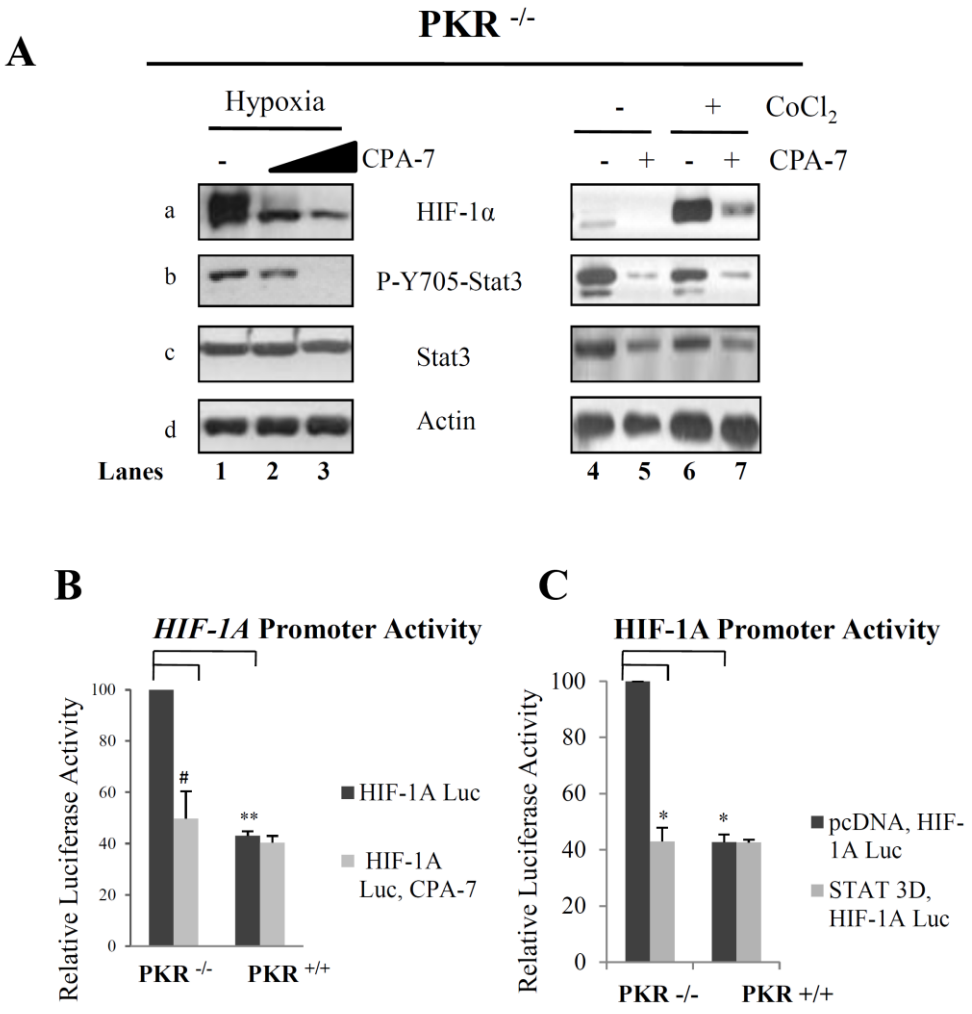

A
PKR -/-
Hypoxia
-
CPA-7
-
+
CoCl2
-
+
-
+
CPA-7
a
HIF-1α
b
P-Y705-Stat3
c
Stat3
d
Actin
Lanes
1
2
3
4
5
6
7
B
HIF-1A Promoter Activity
Relative Luciferase Activity
100
80
60
40
20
0
#
**
HIF-1A Luc
HIF-1A Luc, CPA-7
PKR -/-
PKR +/+
C
HIF-1A Promoter Activity
Relative Luciferase Activity
100
80
60
40
20
0
*
*
pcDNA, HIF-1A Luc
STAT 3D, HIF-1A Luc
PKR -/-
PKR +/+

Figure 16. ***PKR controls HIF1A gene transcription through Stat3.***

(A) PKR$^{-/-}$ MEFs were untreated or treated with 20 or 50μM of CPA-7 in hypoxic conditions (H, 1% O_2) for 24 hours (lanes 1-3) or left untreated or treated with CPA-7 (20μM) in the presence or absence of $CoCl_2$ (200μM) for 24 hours (lanes 4-7). Protein extracts were subjected to immunoblotting for HIF-1α (panel a), Y705 phosphorylated Stat3 protein (panel b), total Stat3 (panel c) and actin (panel d). **(B)** 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 co-transfected as an internal control. Cells were treated with CPA-7 (20 μM) for 24 hours and lysed 27 hours 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 denotes the standard error (n= 4). Statistical analysis was performed using the paired T-Test and P value # corresponds to $P < 0.01$ and ** to $P < 0.001$. **(C)** The MEFs were transiently co-transfected 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 hours after transfection. Relative luciferase activity of the *HIF1A* promoter was normalized to the basal activity of the control vectors. Error bars denote the standard error (n=4). Statistical analysis was performed using the paired T-Test and P value * corresponds to $P < 0.05$

Figure 17

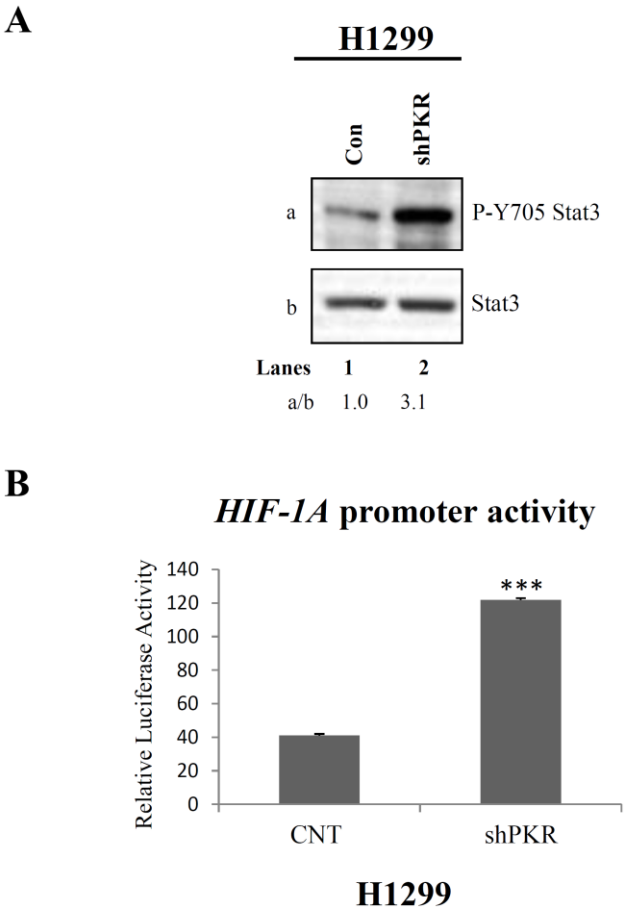

A
H1299
Con
shPKR
a
P-Y705 Stat3
b
Stat3
Lanes 1 2
a/b 1.0 3.1
B
HIF-1A promoter activity
Relative Luciferase Activity
140
120
100
80
60
40
20
0
***
CNT
shPKR
H1299

Figure 17. ***Knockdown of PKR in H1299 cells leads to increased Stat3 activity and HIF1A transcription.***

(A) Cell extracts from H1299 cells (control and shPKR) were resolved by SDS PAGE and were analyzed by immunoblot analysis for Y-705 phosphorylated Stat3 protein (panel a), and total Stat-3 (panel b). Normalized band ratios are indicated at the bottom of the panels. **(B)** The H1299 cells (control and shPKR) 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 in extracts obtained 48 hours post transfection. Relative luciferase activity of the HIF-1α promoter was normalized to the activity of the control vector. Error bar denotes the standard error (n=8). Statistical analysis was performed using the paired T-Test and P value *** corresponds to $P < 0.0001$.

1.6 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 (99), we wished to examine whether TC-PTP deficiency leads to Stat3 activation and subsequent up-regulation of *HIF1A* gene transcription. First of all, we detected higher levels of Stat3 phosphorylation at Y705 in TC-$PTP^{-/-}$ MEFs compared to TC-$PTP^{+/+}$ MEFs (Fig.18A), confirming that phosphorylated Stat3 is a target of this tyrosine phosphatase (99, 219). When $TCPTP^{+/+}$ and TCPTP $^{-/-}$ MEFs were challenged with either hypoxia (Fig.18B) or $CoCl_2$ treatment (Fig.18C), 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 upregulation of *HIF-1A* transcription, because the *HIF1A* promoter displayed higher activity in TC-$PTP^{-/-}$ MEFs than TC-$PTP^{+/+}$ MEFs as demonstrated by the luciferase reporter assays (Fig.18D). Taken together, these data support that TC-PTP inhibits HIF-1α expression through the inhibition of Stat3.

Figure 18

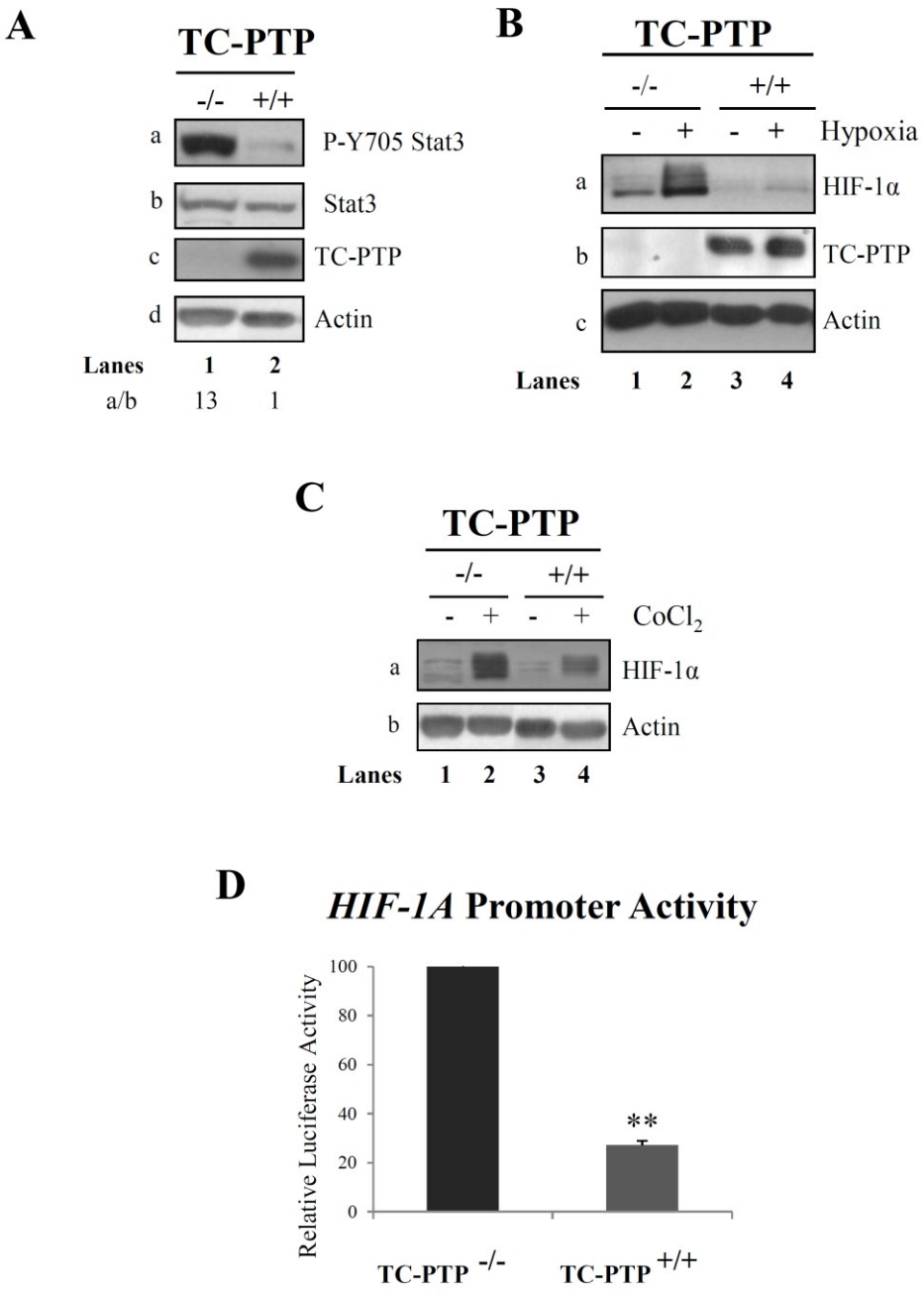

A
TC-PTP
-/- +/+
a
P-Y705 Stat3
b
Stat3
c
TC-PTP
d
Actin
Lanes 1 2
a/b 13 1
B
TC-PTP
-/- +/+
- + - + Hypoxia
a
HIF-1α
b
TC-PTP
c
Actin
Lanes 1 2 3 4
C
TC-PTP
-/- +/+
- + - + CoCl2
a
HIF-1α
b
Actin
Lanes 1 2 3 4
D
HIF-1A Promoter Activity
Relative Luciferase Activity
100
80
60
40
20
0
**
TC-PTP -/-
TC-PTP +/+

Figure 18. ***TC-PTP is involved in the transcriptional control of HIF1A gene by Stat3.*** **(A)** Extracts from TC-PTP$^{+/+}$ and TC-PTP $^{-/-}$ MEFs were immunoblotted with antibodies targeting the Y-705 phosphorylated Stat3 protein (panel a), total Stat3 (panel b), TC-PTP (panel c) and actin (panel d). The normalized ratio of band intensities (a/b) for each lane are indicated. **(B)** TC-PTP$^{+/+}$ and TC-PTP $^{-/-}$ MEFs were incubated under normal (N, 21% O_2) or hypoxic conditions (H, 1% O_2) for 24 hours. Whole cell extracts (70μg) were subjected to immunoblot analysis with anti HIF-1α (panel a), anti-TC-PTP (panel b) and anti-actin (panel c) antibodies. **(C)** TC-PTP$^{+/+}$ and TC-PTP $^{-/-}$ MEFs were treated for 24 hours with 200 μM $CoCl_2$. Whole cell extracts (70 μg of protein) were subjected to immunoblot analysis with anti-HIF-1α (panel a), and anti-actin (panel 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 is extracts obtained 48 hours post transfection. Relative luciferase activity of the *HIF1A* promoter was normalized to the activity of the control vector. Error bar denotes the standard error (n=8). Statistical analysis was performed using the paired T-Test and P value ** corresponds to $P < 0.001$.

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

To assess the effects of PKR activation on HIF-1α, we used human fibrosarcoma HT1080 cells. The cells were engineered to express a conditionally active form of PKR as a fusion protein with Gyrase B (94) which can be activated by the addition of the antibiotic coumermycin to the culture media (Fig.10). These cells were maintained in normal or hypoxic conditions for 5 or 18 hours in presence or absence of coumermycin for 5 hours. We observed that activation of GyrB.PKR resulted in the downregulation of HIF-1α under hypoxic conditions, which coincided with a substantial inhibition of Stat3 phosphorylation (Fig.19A). Inhibition of HIF-1α expression was mediated at the transcriptional level as indicated by the *HIF1A* promoter activity (Fig.19B). These data support that PKR activity negatively regulates HIF-1α expression.

Figure 19

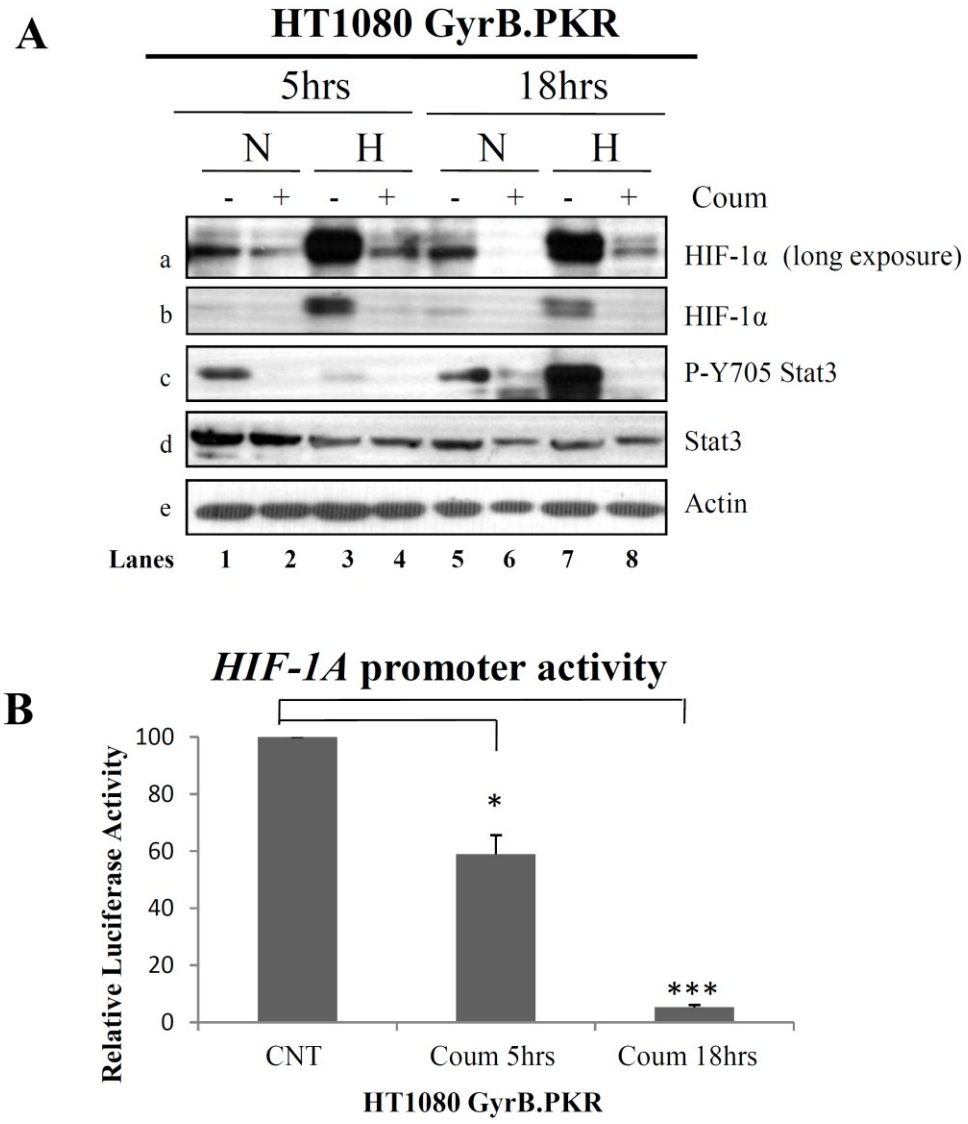

A
HT1080 GyrB.PKR
5hrs
18hrs
N
H
N
H
- + - + - + - +
Coum
a
HIF-1α (long exposure)
b
HIF-1α
c
P-Y705 Stat3
d
Stat3
e
Actin
Lanes 1 2 3 4 5 6 7 8
B
HIF-1A promoter activity
Relative Luciferase Activity
100
80
60
40
20
0
*
***
CNT
Coum 5hrs
Coum 18hrs
HT1080 GyrB.PKR

Figure 19. ***A conditionally inducible form of PKR leads to inhibition of HIF-1α in HT1080 fibrosarcoma cells.***

(A) HT1080 GyrB.PKR cells were incubated in normoxia or hypoxia (1%) for 5 and 18 hours with or without coumermycin (100ng/ml) treatment for 5 hours. Extracts were resolved using SDS PAGE and immunoblotted with antibodies targeting HIF 1-α (panels a and b), Y-705 phosphorylated Stat3 protein (panel c), total Stat3 (panel d), and actin (panel e). **(B)** The same cell line was 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 in extracts obtained 48 hours post transfection that were untreated or treated with coumermycin for 5 hours or 18 hours. Relative Luciferase activity of the HIF-1α promoter was normalized to the activity of the control vector. Error bar denotes the standard error (n=5). Statistical analysis was performed using the paired T-Test and P values * and *** correspond to $P< 0.05$ and $P < 0.0001$ respectively.

1.8 PKR mediates HIF-1α inhibition in response to 2-methoxyestradiol (2-ME).

2-Methoxyestradiol (2-ME) is an inducer of apoptosis in tumour cells by activating PKR (220). Since 2-ME has also been shown to inhibit HIF-1α (221), we tested whether PKR is involved in the ability of 2-ME to inhibit HIF-1α. To this end, we treated hypoxic PKR+/+ and -/- MEFs with 2-ME and found PKR was essential for the downregulation of HIF-1α (Fig.20). These results suggest that PKR is a valid effector in anti-angiogenesis therapies targeting HIF-1α expression and function.

Figure 20

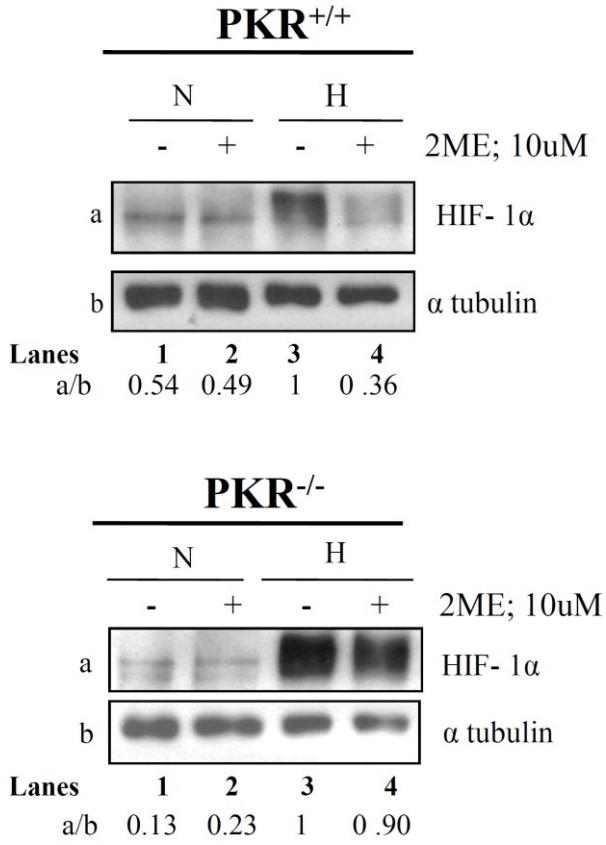

PKR+/+
N
H
- + - +
2ME; 10uM
a
HIF- 1α
b
α tubulin
Lanes 1 2 3 4
a/b 0.54 0.49 1 0 .36
PKR-/-
N
H
- + - +
2ME; 10uM
a
HIF- 1α
b
α tubulin
Lanes 1 2 3 4
a/b 0.13 0.23 1 0 .90

Figure 20. ***PKR contributes to inhibition of HIF-1α accumulation in response to 2-methoxyestradiol (2-ME).*** **(A, B)** PKR+/+ and PKR-/- MEFs were maintained under normoxic (N, 21% O_2) or hypoxic (H, 1% O_2) conditions for 24 hours in the absence or presence of 2-ME (10μM) for the last 6 hours. Protein extracts (70 ug) were subjected to immunoblot analysis for the indicated proteins. Results are representative of 3 independent experiments. The normalized ratio of the band intensities (a/b) for each lane are indicated.

1.9 Summary

Our data have shown that PKR inhibits HIF-1α expression in both mouse and human cell lines. This regulation is not mediated through eIF2α phosphorylation. More specifically, this regulation occurs at the transcriptional level through a Stat3 dependent mechanism where the phosphorylation of Stat3 and its ability to promote transcription of the *HIF-1A* gene is suppressed by PKR through the TC-PTP protein phosphatase. Through inhibition of *HIF1A* transcription, PKR inhibits the synthesis and in turn the levels of HIF-1α stabilized upon hypoxia. This has implications in the corresponding cellular response and the tumorigenic properties of HIF-1α (Fig.21).

Figure 21

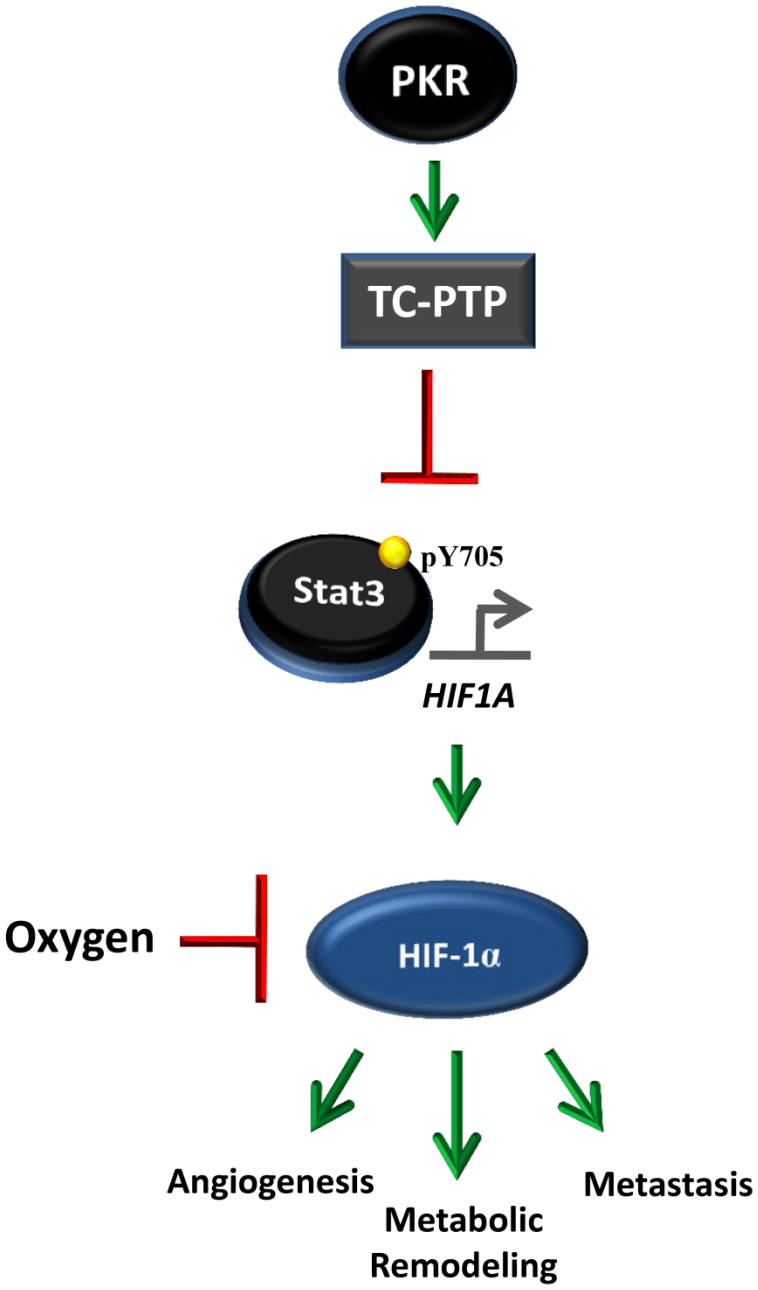

PKR
TC-PTP
Stat3
pY705
HIF1A
Oxygen
HIF-1α
Angiogenesis
Metabolic
Remodeling
Metastasis

Figure 21. ***PKR acts as a transcriptional suppressor of HIF 1A through a Stat3 dependent mechanism.*** This model proposes the mechanism as to how the eIF2α kinase PKR inhibits HIF-1α expression at the transcriptional level. As shown, PKR regulates TC-PTP, the T-Cell Protein Tyrosine phosphatase, which de-phosphorylates Stat3 at Y705, inhibiting its activity and ability to promote transcription of the *HIF-1A* gene. Upon normal oxygen condition the HIF-1α protein is unstable and degraded. However, upon hypoxia it is stabilized and acts to promote tumorigenesis by inducing angiogenesis, remodeling glucose metabolism, and facilitating metastasis. As such, the ability of PKR to inhibit HIF-1α expression represents a novel anti-tumor function for the eIF2α kinase.

2. Development of transgenic mice expressing a conditionally active form of the eIF2α kinase PKR.

To assess the importance of the eIF2α phosphorylation pathway in mammalian physiology, several mouse models have been developed. These include ubiquitous or tissue-specific knock-outs of each of the eIF2α kinases (112, 189-191, 193), or the ubiquitous or tissue specific eIF2α S51A knock-ins (194, 195). These mouse models are helpful to investigate the effects of the loss of each of the eIF2α kinases or eIF2α phosphorylation. Herein we describe the development of a novel mouse model bearing a conditionally active eIF2α kinase to address the *in vivo* biological effects of persistent PKR activation and eIF2α phosphorylation.

The GyrB.PKR or the catalytic dead GyrB.PKR mutant (GyrB.PKR K296H) cDNA was excised from the pSG5 vector (94) and subcloned into the pCX vector (208, 209). The vector was then introduced into pronuclei of mice at the McGill University Transgenic Mouse Core Facility. Transgenic mice were generated and further characterized.

Genotyping of the mice was carried out by subjecting the DNA extracted from mouse tails to polymerase chain reaction (PCR) using primers flanking the transgene insert; one primer towards the GyraseB domain while the second to the kinase domain (Fig.22). The heterozygous positive mice were maintained and further characterized.

Figure 22

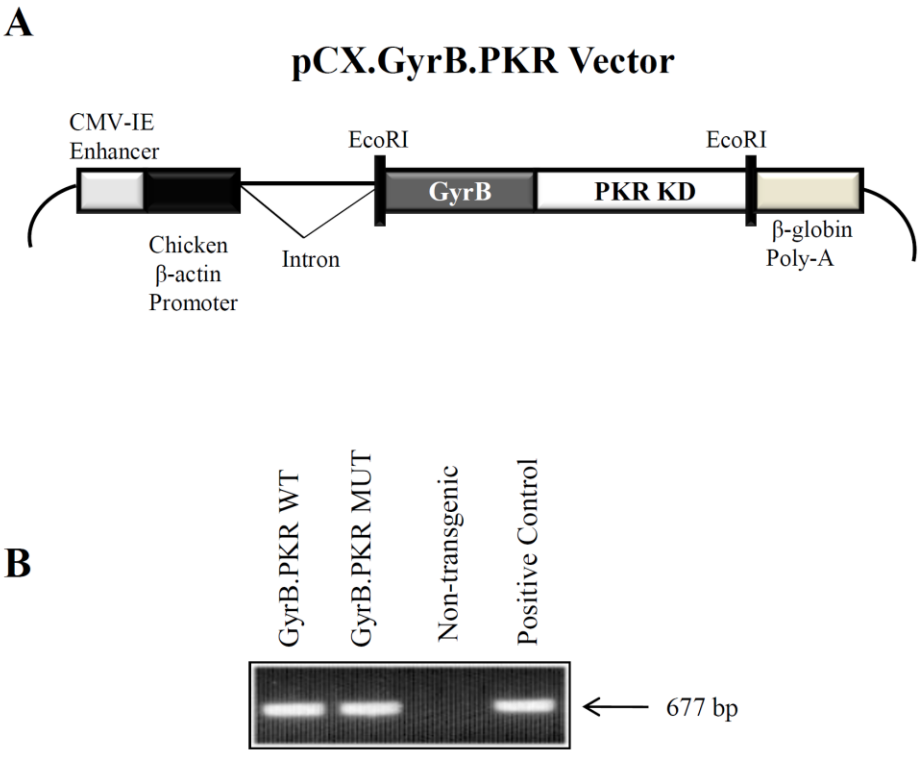

A
pCX.GyrB.PKR Vector
CMV-IE
Enhancer
EcoRI
EcoRI
GyrB
PKR KD
β-globin
Poly-A
Chicken
β-actin
Promoter
Intron
B
GyrB.PKR WT
GyrB.PKR MUT
Non-transgenic
Positive Control
677 bp

Figure 22. ***Generating and genotyping the GyrB.PKR mice.***

(A) The GyrB.PKR cDNA was subcloned into the pCX expression vector containing the chicken β-actin promoter, the cytomegalovirus enhancer (CMV-IE) and the β-globin poly-adenylation (Poly-A) signal. **(B)** GyrB.PKR positive mice were screened by PCR genotyping from DNA samples (from the indicated mice) using primers flanking the transgene.

2.1 GyrB.PKR protein expression in mouse tissues

The expression of the GyrB.PKR transgene as well as the kinase dead GyrB.PKR (GyrB.PKR K296H) is under the control of the cytomegalovirus (CMV) enhancer and the ubiquitously active chicken β–actin promoter (Fig.22A). To assess expression of the transgene, different mouse tissues were harvested from transgenic positive and negative mice (as control), homogenized and subjected to immunoblot analysis with an antibody specific for the Gyrase B domain of the chimera kinase (Fig.23). Our results show that the transgenes are expressed in all the tissues tested. Importantly, the catalytically dead GyrB.PKR K296H serves as the ultimate experimental control, as it is expressed, can bind coumermycin but cannot be activated.

Figure 23

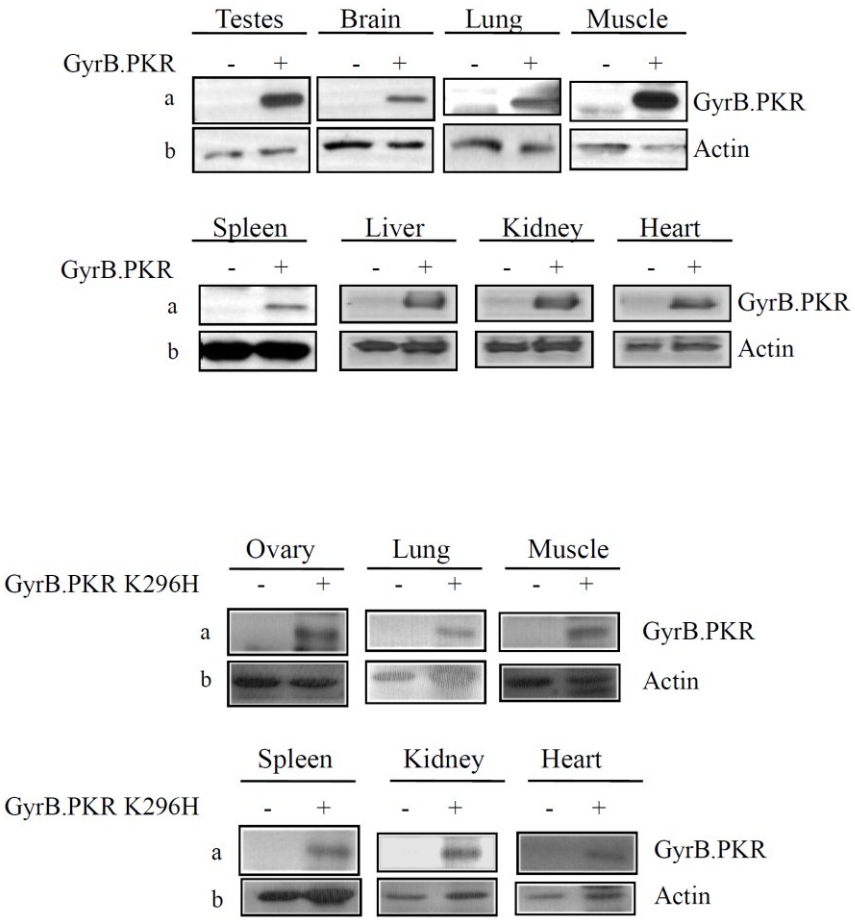

Testes
Brain
Lung
Muscle
GyrB.PKR
- + - + - + - +
a
b
GyrB.PKR
Actin
Spleen
Liver
Kidney
Heart
GyrB.PKR
- + - + - + - +
a
b
GyrB.PKR
Actin
Ovary
Lung
Muscle
GyrB.PKR K296H
- + - + - +
a
b
GyrB.PKR
Actin
Spleen
Kidney
Heart
GyrB.PKR K296H
- + - + - +
a
b
GyrB.PKR
Actin

Figure 23: ***Detection of the GyrB.PKR protein mouse tissues.***

Mice lacking GyrB.PKR (-) or expressing wildtype (WT) GyrB.PKR (+) or mutant GyrB.PKR K296H (+) in FVB background were used to detect GyrB.PKR expression. Protein extracts (50μg) from the indicated mouse tissues were subjected to immunoblot analysis with antibodies recognizing Gyrase B or actin.

2.2 Activation of GyrB.PKR in mouse tissues.

To determine if the chimera kinase can be induced *in vivo*, mice were administered coumermycin by intraperitoneal injection (i.p.) or gavage feeding. Detection of the chimera-kinase as well as an induction of eIF2α phosphorylation upon coumermycin administration was observed in the extracts of pancreatic tissue (Fig.24A and B), which is the organ primarily affected by eIF2α phosphorylation as demonstrated in mice lacking PERK (190, 194, 222) or in mice defective in eIF2α phosphorylation (194, 195, 223, 224). Inducibility of GyrB.PKR was not specific to only pancreatic tissue as we also observed an induction of eIF2α phosphorylation in extracts from the lung tissue (Fig.24C). The enhanced eIF2α phosphorylation in the pancreatic tissue of these transgenic mice was also documented by immunoshistochemistry (IHC) after administration of coumermycin by gavage feeding (Fig.25). To evaluate differences in eIF2α phosphorylation between the samples examined, tissues from mice containing wildtype eIF2α ($eIF2\alpha^{S/S}$) and mice heterozygous for the eIF2α knock-in S51A mutation ($eIF2\alpha^{S/A}$) were used as controls (Fig.24B, C and 25).

Figure 24

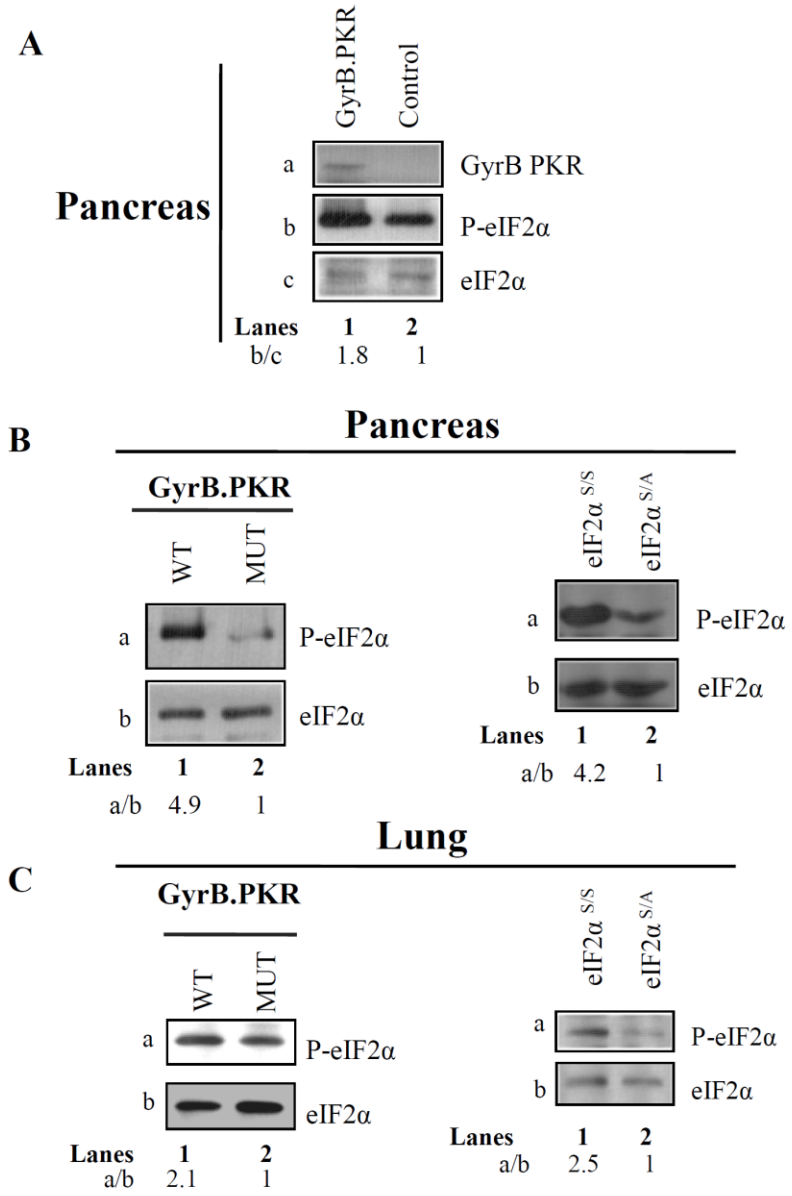

A
Pancreas
GyrB.PKR
Control
a
b
c
GyrB PKR
P-eIF2α
eIF2α
Lanes 1 2
b/c 1.8 1
B
Pancreas
GyrB.PKR
WT
MUT
a
b
P-eIF2α
eIF2α
Lanes 1 2
a/b 4.9 1
eIF2α S/S
eIF2α S/A
a
b
P-eIF2α
eIF2α
Lanes 1 2
a/b 4.2 1
C
Lung
GyrB.PKR
WT
MUT
a
b
P-eIF2α
eIF2α
Lanes 1 2
a/b 2.1 1
eIF2α S/S
eIF2α S/A
a
b
P-eIF2α
eIF2α
Lanes 1 2
a/b 2.5 1

Figure 24. ***Conditional activation of GyrB.PKR leads to induction of eIF2α phosphorylation in pancreatic and lung tissue of transgenic mice.***

(A, B) Mice lacking GyrB.PKR (Control) or expressing wildtype (WT) GyrB.PKR or mutant GyrB.PKR K296H in FVB background were injected with coumermycin intraperitoneally (i.p) **(A)** or subjected to gavage feeding **(B,C)** as described in Methods. Pancreatic **(A, B)** and lung **(C)** protein extracts (50μg) were used in immunoblot analysis for Gyrase B, phosphorylated eIF2α at S51 (P-eIF2α), or total eIF2α as indicated. Pancreatic **(B)** and lung **(C)** protein extracts from untreated mice containing wildtype eIF2α ($eIF2\alpha^{S/S}$) or heterozygous for mutant eIF2α on serine 51 ($eIF2\alpha^{S/A}$) were used for immunoblotting for phosphorylated eIF2α and total eIF2α. The intensities of the bands were quantified by densitometry and the ratio for each lane is indicated.

Figure 2

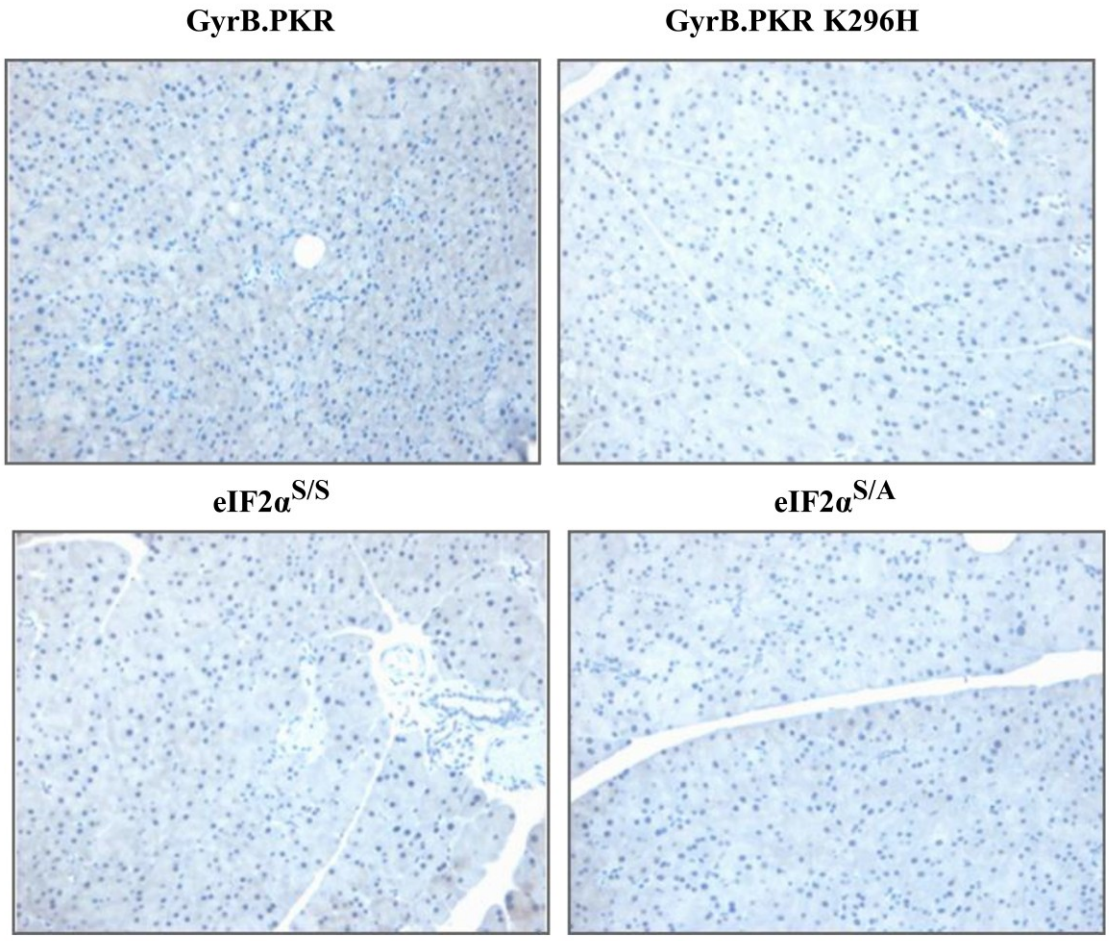


Figure 25. ***Detection of eIF2α phosphorylation in pancreatic tissue of GyrB.PKR mice by IHC.***

Mice expressing either GyrB.PKR or mutant GyrB.PKR K296H (top panels) were administered coumermycin by gavage feeding. Pancreatic tissue was subjected to immunohistochemical analysis for phosphorylated eIF2α at S51 indicated by the brown staining. Hematoxylin staining (blue) indicates the nuclei. In the bottom panels pancreatic tissue from $eIF2\alpha^{S/S}$ (bearing wildtype eIF2α) and $eIF2\alpha^{S/A}$ (haplo-insufficient in eIF2α phosphorylation) mice were used as controls.

2.3 Induction of GyrB.PKR induces JNK-1 phosphorylation in mouse tissue

Previously, we have shown that the activation of inducible eIF2α kinase in HT1080 cells leads to activation of JNK (181). Interestingly PKR has recently been shown to control JNK-1 activation in response to metabolic stress in the liver (225). Consistently, the extracts from liver tissue derived from GyrB.PKR WT transgenic mice displayed increased levels of JNK-1 phosphorylation compared to extracts from the kinase dead GyrB.PKR mutant harboring mice (Fig.26A). In addition this was not restricted to liver since an increased JNK-1 phosphorylation was also observed in pancreas and lung tissue from transgenic mice containing the catalytically active chimera kinase (Fig.26B and C).

Figure 26

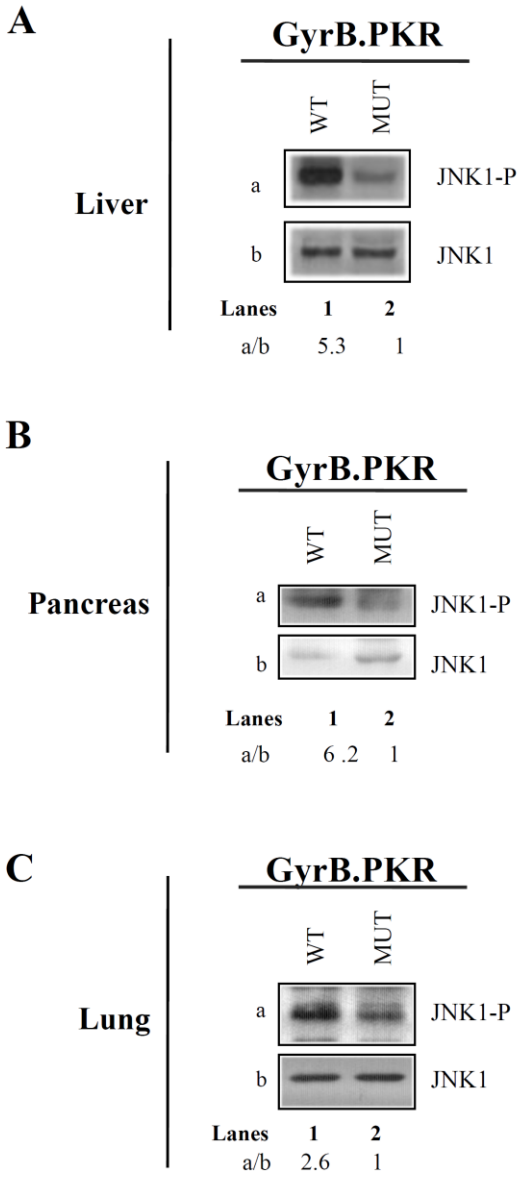

A
GyrB.PKR
WT
MUT
Liver
a
JNK1-P
b
JNK1
Lanes 1 2
a/b 5.3 1
B
GyrB.PKR
WT
MUT
Pancreas
a
JNK1-P
b
JNK1
Lanes 1 2
a/b 6 .2 1
C
GyrB.PKR
WT
MUT
Lung
a
JNK1-P
b
JNK1
Lanes 1 2
a/b 2.6 1

Figure 26. ***Conditional activation of GyrB.PKR leads to induction of JNK-1 phosphorylation in liver, pancreatic and lung tissue of transgenic mice.***

Mice expressing wildtype (WT) GyrB.PKR or mutant GyrB.PKR K296H were treated with coumermycin by gavage feeding as described in Methods. Liver **(A),** Pancreas **(B)** and Lung **(C)** protein extracts (50μg) were used in immunoblot analysis for phosphorylated JNK-1 at Thr183/Tyr185 (JNK1-P), or JNK-1. The intensities of the bands were quantified by densitometry and the ratio for each lane is indicated.

3. Development of cell culture cancer models deficient in eIF2α phosphorylation.

Inducible systems like the GyrB.PKR cells (94) previously described in section (Fig.10) allow experiments to gather insights of how PKR-induced eIF2α phosphorylation alone affects cell viability and signaling pathways, in the absence of other parallel stress induced pathways. Another great tool to investigate the role of the eIF2α phosphorylation in cell culture setting have been the eIF2α $^{A/A}$ MEFs which are completely deficient in eIF2α phosphorylation in comparison to the wildtype counterparts the eIF2α$^{S/S}$ MEFs (202). Due to differences between mouse and human cells, it was fundamentally important to develop approaches to study eIF2α phosphorylation in human cells.

3.1 Development of cell permeable peptides engineered to inhibit eIF2α phosphorylation.

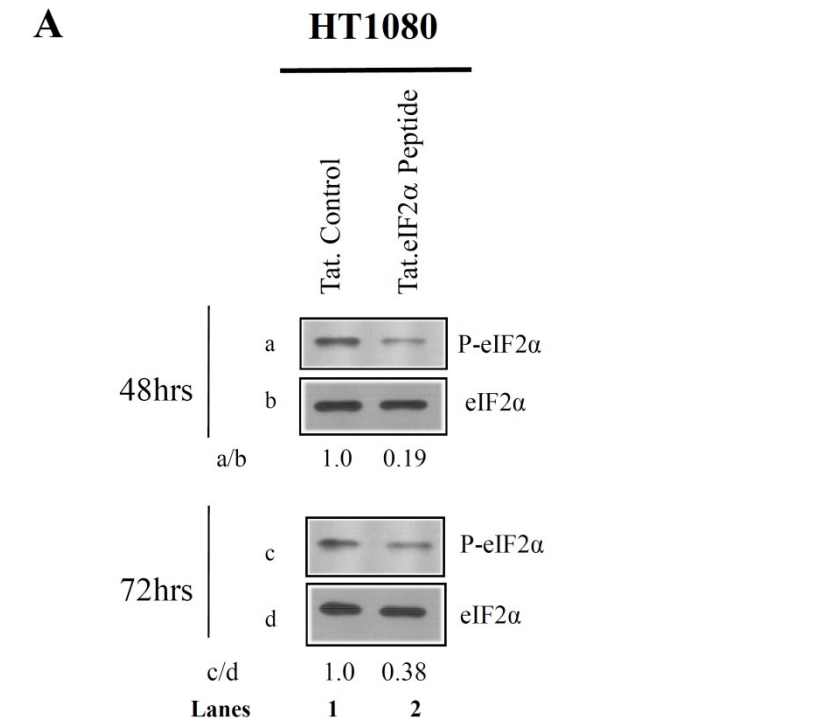
One approach was the generation of cell permeable peptides, engineered to mimic the regions on the eIF2α protein which come in contact with eIF2α kinases (206). The sequences chosen are conserved in the eIF2α protein, yeast to human, as well as in viral proteins, such as K3L, C3L, evolved to inhibit eIF2α phosphorylation (226). Similar to K3L and C3L, we hypothesized that peptides corresponding to these conserved sequences would compete with endogenous eIF2α and prevent its phosphorylation. The human HT1080 cells were treated with either control or the eIF2α mimicking cell permeable peptides for 48 or 72

hours. Our results show a transient partial abrogation of eIF2α phosphorylation (Fig.27A).

3.2 Development of human cancer cell lines deficient in eIF2α phosphorylation.

Since the effect observed with the cell permeable peptides was transient a different approach to stably prevent eIF2α phosphorylation in human cells was needed. To this end, the HT1080 GyrB.PKR cells were utilized due to the versatility of the coumermycin inducible GyrB.PKR protein. A vector coding for GFP and harboring the HA-tagged eIF2α S51A (serine 51 to alanine) mutant, was introduced by retroviral transduction. As a control, cells also infected with an empty vector containing only GFP. GFP positive cells were sorted by FACS analysis and characterized. Both forms of eIF2α can be detected by western blot analysis using antibody recognizing total eIF2α protein. The HA tagged eIF2αS51A mutant was distinguishable by its molecular weight and electrophoretic mobility. Similar to the results obtained with the peptide approach, we found that expression of the HA tagged mutant eIF2α competed with the endogenous eIF2α and acted partially to inhibit basal and GyrB.PKR induced eIF2α phosphorylation (Fig. 27B).

Figure 27


A
HT1080
Tat. Control
Tat.eIF2α Peptide
48hrs
a
b
P-eIF2α
eIF2α
a/b
1.0
0.19
72hrs
c
d
P-eIF2α
eIF2α
c/d
1.0
0.38
Lanes
1
2

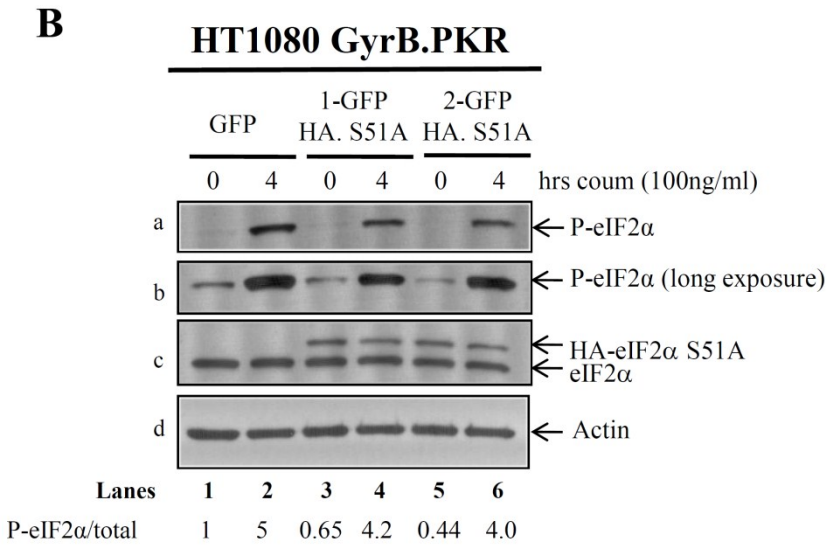
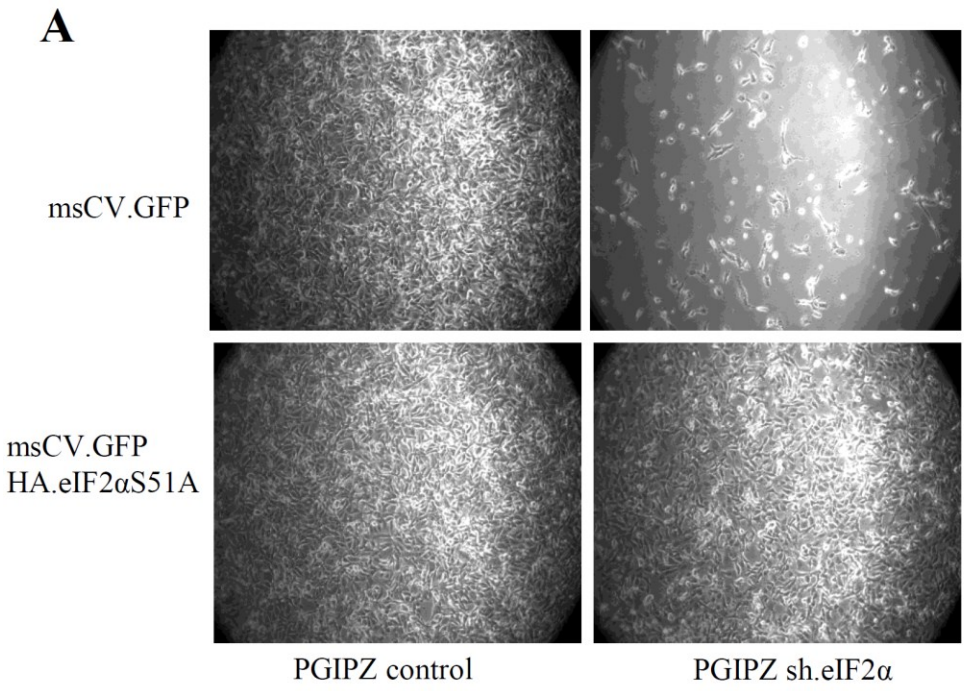

B
HT1080 GyrB.PKR
GFP
1-GFP HA. S51A
2-GFP HA. S51A
0
4
0
4
0
4
hrs coum (100ng/ml)
a
b
c
d
P-eIF2α
P-eIF2α (long exposure)
HA-eIF2α S51A
eIF2α
Actin
Lanes
1
2
3
4
5
6
P-eIF2α/total
1
5
0.65
4.2
0.44
4.0

Figure 27. ***Cell permeable peptides mimicking eIF2α and the HA tagged eIF2α S51A mutant partially inhibit eIF2α phosphorylation in HT1080 cells***. **(A)** The HT1080 cells were incubated with100uM of control Tat peptide or Tat.eIF2α peptide in the absence of serum for 2 hours. After the addition of 10% serum cells were incubated for 48 and 72 hours. Cell extracts were resolved by SDS PAGE and immunoblotted for phosphorylated eIF2α, total eIF2α. **(B)** The HT1080 GyrB.PKR cells were infected with retrovirus containing either empty GFP vector or GFP HA.eIF2α S51A. High GFP expressing cells were sorted by FACS analysis. Cell extracts from GFP positive control cells and two batches of GFP positive HA.eIF2αS51A cells, left untreated or treated with coumermycin (100ng/ml for 4hrs) were resolved by SDS PAGE and immunoblotted for phosphorylated eIF2α, total eIF2α and Actin. Normalized band ratios are indicated at the bottom of the panels.

The expression of HA.eIF2αS51A was partially able to inhibit eIF2α phosphorylation levels (Fig 27B). To further inhibit eIF2α phosphorylation; selective knock-down of the endogenous eIF2α wildtype protein was accomplished by using a miRNA lentiviral construct (Open Biosystem). The used construct was targeting the 3' untranslated region of the eIF2α mRNA, because this region is absent from the coding sequence of HA.eIF2αS51A. As a control, cells were infected in parallel with lentivirus bearing the empty vector.

The expression of miRNA against endogenous eIF2α resulted in massive cell death underscoring the essential role of the eIF2α protein in the maintenance of cell viability (Fig.28A). However, cells expressing the HA.eIF2αS51A did not perish (are rescued) showing that HA.eIF2αS51A is functional (Fig.28A). Moreover, with respect to eIF2α phosphorylation, cells expressing both HA.eIF2αS51A and the knockdown of endogenous eIF2α display abrogated basal and inducible eIF2α phosphorylation (Fig 28B).

Figure 28


A
msCV.GFP
msCV.GFP
HA.eIF2αS51A
PGIPZ control
PGIPZ sh.eIF2α

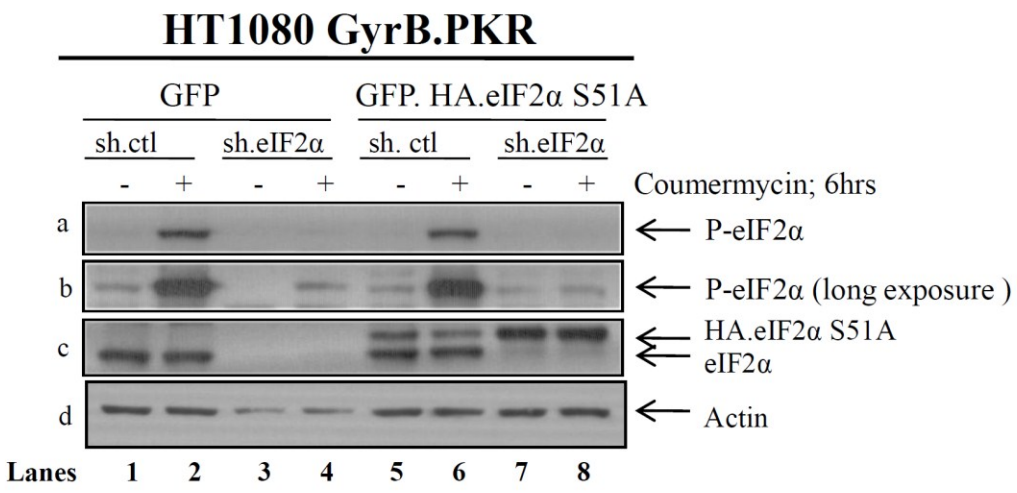

B
HT1080 GyrB.PKR
GFP
GFP. HA.eIF2α S51A
sh.ctl
sh.eIF2α
sh. ctl
sh.eIF2α
- + - + - + - +
Coumermycin; 6hrs
a
b
c
d
P-eIF2α
P-eIF2α (long exposure)
HA.eIF2α S51A
eIF2α
Actin
Lanes 1 2 3 4 5 6 7 8

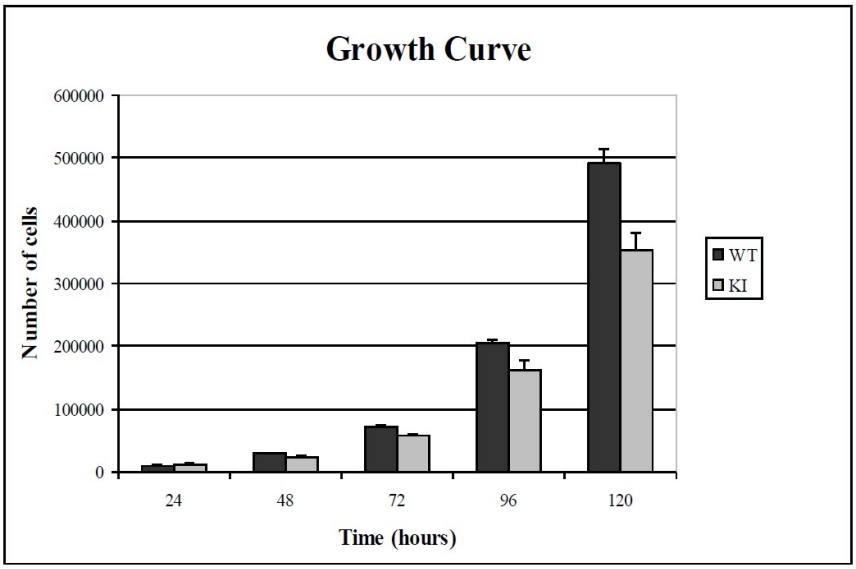
Figure 28. ***Development of HT1080 cells deficient in eIF2α phosphorylation.*** **(A)**The HT1080 which were infected with retrovirus containing either empty mscv GFP vector or mscv GFP eIF2α S51A infected with lentivirus targeting the 3'UTR of the endogenous eIF2α. Following selection cells were analyzed by microscopy and photographed. **(B)** The same cells were either left untreated (-) or treated (+) with coumermycin (100ng/ml; 6hrs). Cells extracts were resolved by SDS PAGE and immunoblotted for phosphorylated eIF2α, total eIF2α and actin.

3.3 Characterization of the HT1080 eIF2α "knock-in" cells lines.

Analysis of cell proliferation showed that cells deficient in eIF2α phosphorylation displayed decreased proliferation rates compared with their wildtype counterparts (Fig.29A). Moreover the inhibition in cell growth was also verified by colony formation assay (Fig.29B). These data suggest that basal eIF2α phosphorylation levels are needed to maintain cell proliferation.

Figure 29

A


Growth Curve
600000
500000
400000
300000
200000
100000
0
Number of cells
24
48
72
96
120
Time (hours)
WT
KI

B

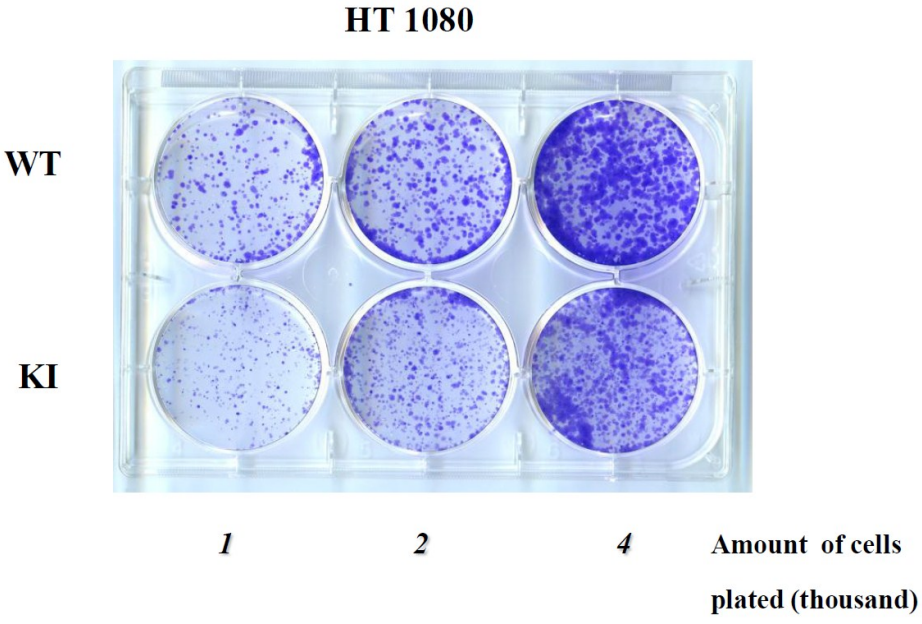

HT 1080
WT
KI
1
2
4
Amount of cells plated (thousand)

Figure 29: ***Deficiency in eIF2α phosphorylation reduces proliferation of HT1080 cells.***

(A) The HT1080 WT (infected with control empty vectors) and KI (expressing both HA.eIF2αS51A and the knockdown of endogenous eIF2α) cells were seeded in triplicates and proliferation was analyzed by cell counting 24,48,72, 96 and 120 hours after initial plating. Cell growth was plotted over time and error bars indicate the standard deviation. (**B)** The same cell lines were plated at low densities (1000, 2000, and 4000 cells respectively) and were grown for 7 days (168 hours). Colony formation was assessed via crystal violet stainning. The experiment displayed is representative of 5 independent experiments.

3.4 Effects of persistent eIF2α phosphorylation

Our group previously showed that prolonged activation of GyrB.PKR leads to apoptosis in HT1080 cells (94). To test whether this increased apoptosis was dependent upon the phosphorylation of eIF2α we treated the cell lines generated, with coumermycin for 15 and 30 hours and analyzed for morphology and apoptosis by microscopy and FACS analysis respectively (Fig.30). Our results demonstrate that when eIF2α phosphorylation is abrogated by the approach described in Figure 28, cells were protected cells from apoptosis induced from prolonged activation of GyrB.PKR. Notably, these cells, bearing the mutant eIF2α and knocked-down endogenous eIF2α, displayed a minor increase in cell death (Figure 30) suggesting that there may be some residual eIF2α being phosphorylated or that activation of GyrB.PKR may be regulating pathways independently of eIF2α phosphorylation. Nevertheless, these observations validate that the system is functional and reliable for looking at biological effects of eIF2α phosphorylation.

Figure 30

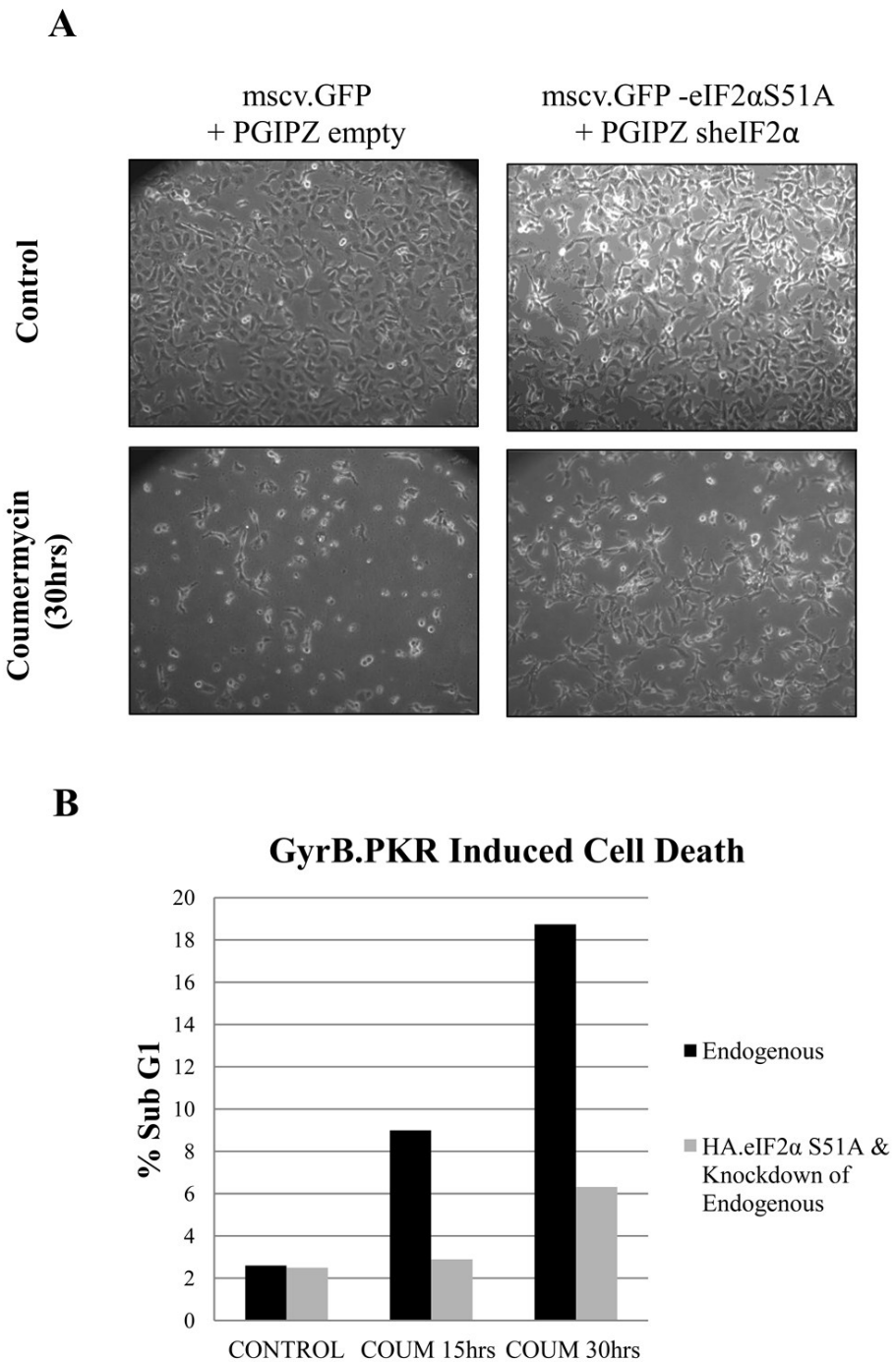

A
mscv.GFP
+ PGIPZ empty
mscv.GFP -eIF2αS51A
+ PGIPZ sheIF2α
Control
Coumermycin
(30hrs)
B
GyrB.PKR Induced Cell Death
% Sub G1
0
2
4
6
8
10
12
14
16
18
20
CONTROL
COUM 15hrs
COUM 30hrs
Endogenous
HA.eIF2α S51A &
Knockdown of
Endogenous

Figure 30. ***Cell death in response to prolonged activation of GyrB.PKR is mediated through eIF2α phosphorylation.***

(A) Microscopic analysis of the HT1080 GyrB.PKR cells bearing endogenous eIF2α or mutant eIF2αS51A with knock-down of endogenous eIF2α, were either left untreated or treated with coumermycin (100ng/ml; 30hrs). **(B)** The same cells, left untreated or treated with coumermycin (100ng/ml for 15 and 30hrs), were subjected to FACs analysis after propidum iodide (PI) staining. Cell death (Sub-G1) percentages are indicated in the bar graphs are representative of 3 independent experiments.

3.5 eIF2α phosphorylation is cytoprotective against DNA damage and HDAC inhibition.

Recent studies from our lab have shown that the phosphorylation of eIF2α can determine the susceptibility and sensitivity to chemotherapeutic agents that induce DNA damage (doxorubicin) and HDAC inhibition (SAHA). Using MEFs lacking PKR, PERK, or GCN2, we found that while activation of the eIF2α kinases upon doxorubicin or SAHA treatment (PKR, or PKR and GCN2, respectively) promotes apoptosis, the phosphorylation of eIF2α acts in cytoprotective manner, conferring resistance to both these agents (181, 188). It was important to examine whether the cell lines generated, using the novel eIF2α "knock-in" approach described in Figure 28, can recapitulate our previous findings. To this end, the cells were treated with either doxorubicin or SAHA for the indicated times, were stained with propidium iodide and analyzed by FACS analysis. Our results (increased Sub-G1 in cells deficient in eIF2α phosphorylation) validate that eIF2α phosphorylation induced as a consequence of DNA damage, or HDAC inhibition is cytoprotective (Fig.31A). Taken together, our data show that while prolonged eIF2α phosphorylation induced by GyrB.PKR promotes cell death (Fig.30), eIF2α phosphorylation induced in response to chemotherapeutic drugs (Fig.31A) allows cells to cope with the stress and is cytoprotective.

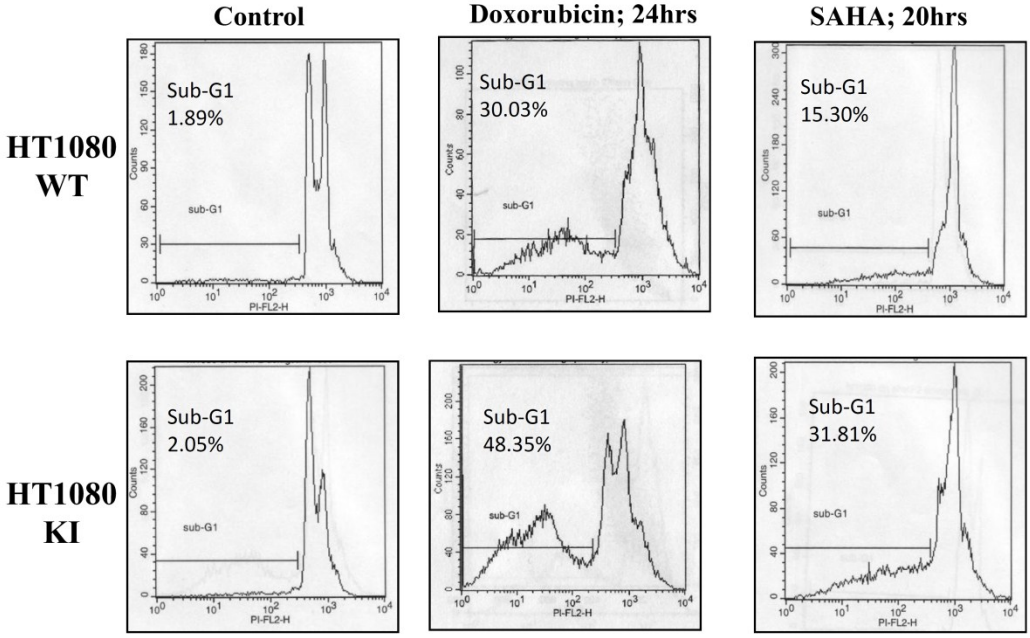
3.6 Conditional PKR activation and ER stress regulate p53 independently of eIF2α phosphorylation

Our lab has shown that p53 degradation is a cytoprotective mechanism in response to ER stress (69, 105). Specifically, it was demonstrated that activation

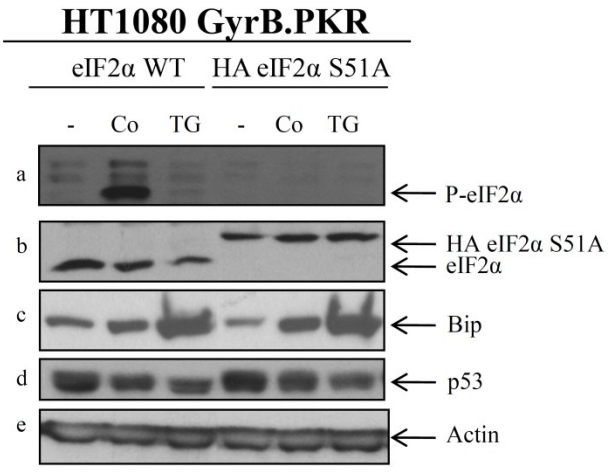
of PERK or PKR can activate GSK3β, which phosphorylates nuclear p53 and promotes its mdm2 mediated proteasomal degradation. These effects of PERK and PKR are independent of eIF2α phosphorylation (71). These findings were recapitulated in the human cell lines generated, bearing endogenous eIF2α or stably expressing HA tagged mutant eIF2α S51A and knocked down endogenous eIF2α, as both cell types displayed an abrogation of p53 levels upon treatment with coumermycin or thapsigargin, a potent inducer of ER stress (Fig. 31 B). These data further validate our previously published work and highlights the capacity of eIF2α independent functions for the eIF2α kinases.

Figure 31

A



B



Lanes	1	2	3	4	5	6
peIF2α/Total	1.0	8.9	3.3	-	-	-
p53/Actin	1.0	0.5	0.3	1	0.6	0.3

Figure 31. ***eIF2α phosphorylation is cytoprotective in response to doxorubicin and SAHA treatment.***

(A) The HT1080 GyrB.PKR cells bearing endogenous eIF2α (WT), or stably expressing HA tagged mutant eIF2α S51A and knocked down endogenous eIF2α (KI) were either left untreated or treated with doxorubicin (1uM) or SAHA (10uM) for the indicated times and subjected to FACs analysis after propidum Iodide (PI) staining. Cell death (Sub-G1) percentages shown are representative of 3 independent experiments. **(B)** The same cells lines untreated (-) or treated with coumermycin (Co; 100ng/ml for 30hrs) or thapsigargin (TG; 1uM for 24hrs). Cell extracts were resolved by SDS PAGE and immunoblotted for phosphorylated eIF2α, total eIF2α, p53, Bip (an ER stress marker) and actin. Normalized band ratios are indicated at the bottom of the panels.

3.7 Summary

In this section new techniques were applied to inhibit eIF2α phosphorylation in human cells. We demonstrated that cell permeable peptides may serve as a transient means to partially inhibit basal eIF2α phosphorylation. Moreover, cell lines in which eIF2α phosphorylation was stably inhibited were generated. The HT1080 GyrB.PKR cells were used for this approach due to the utility of the inducible kinase. Biological effects mediated through eIF2α phosphorylation were found to affect basal proliferation and responses to prolonged eIF2α kinase activation. Furthermore the system was validated through the recapitulation of recent findings showing that eIF2α phosphorylation can act in a cytoprotective manner in response to chemotherapeutic agents. Lastly, we demonstrated that it may present a valuable tool for distinguishing between eIF2α dependent and independent functions of the eIF2α kinases. Although the approaches taken and the prototype model generated, has allowed to enhance versatility of the HT1080 GyrB.PKR cell line, the scope of the utility of the methods developed are extensive as they are applicable to any human cell line.

Chapter 4: Discussion

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

1.1 HIF-α synthesis is impaired by PKR via mechanism requiring TC-PTP and Stat3.

The work described in this section uncovers a novel role for PKR in regulating the hypoxic response, a consequence of tumor microenvironment and important regulator of cancer progression. Our study reveals that among the eIF2α kinases, PKR specifically acts to suppress the expression of HIF-1α and consequently its activity. We demonstrated mechanistically that this phenomenon is not mediated at the translational level through eIF2α phosphorylation but through regulation of *HIF-1A* gene transcription. As illustrated in our model **(**Fig 21**)**, the ability of PKR to diminish the synthesis of HIF-1α is exerted through the activation of TC-PTP and subsequent de-phosphorylation of Stat3.

During hypoxia, protein expression is modulated at the transcriptional and translation stages. At the level of translation, inhibition of protein synthesis is mediated, in part, through eIF2α phosphorylation (227). To promote adaptation to the assaulting stress, HIF-1α mRNA bypasses the hypoxic cell efforts to conserve energy by inhibiting global protein synthesis and is efficiently translated under hypoxic conditions (228). As is the case with many signaling pathways, feedback mechanisms are necessary to maintain control, equilibrium and manipulability of major cellular processes. Herein, we present evidence that PKR compromises the expression of HIF-1α, at the transcriptional level, and provide a novel account of eIF2α phosphorylation independent functions for the eIF2α kinases. In addition to their canonical functions in protein synthesis, our group has described eIF2α-

independent cross-talk among the kinases and key cell signaling pathways involved in proliferation, apoptosis, viral replication and tumorigenesis (40, 70).

1.2 Implications of the PKR regulation of HIF-1α by TCPTP and Stat3.

Our study reveals that PKR regulates HIF-1α expression by modulating the activity of Stat3. Stat3 has been described predominantly as oncogenic and to function not only as a potent transcription factor in the nucleus, but also independent of its transcriptional activity in the mitochondria, where it acts to attenuate damage and prevent apoptosis in response to cell stress (229, 230). The oncogenic characteristics of Stat3 in cancer include positive roles in transformation not only through the suppression of apoptosis but also through the enhancement of proliferation, invasion, angiogenesis and chemo-resistance (231). Herein, our data confirms that HIF-1α is a target of Stat3 (129, 218). Also, our work supports our previous findings that the PKR inhibits Stat3 phosphorylation and activity (99); thus, providing a specific example of an affected Stat3 target gene, *HIF-1A*. Interestingly, although the phosphorylation of eIF2α modestly contributes to Stat3 inhibition (Fig.15B), it is not sufficient to suppress the transcription of the *HIF-1A* gene, thereby highlighting a dominant role for PKR for this process.

Our lab has showed that PKR activates TC-PTP by direct phosphorylation *in vitro* and *in vivo,* and that TC-PTP inhibits Stat1 and Stat3 proteins (99). In accordance with the aforementioned, the data described herein show that PKR functions to prevent basal activation of Stat3 and provide genetic evidence

supporting that Stat3 phosphorylation and its ability to increase transcription of the HIF-1α gene is antagonized by TC-PTP. TC-PTP functions as a tyrosine phosphatase and was shown to attenuate cytokine signaling and negatively regulate cell cycle progression by inhibiting Janus-activated kinases, Src family kinases and Stat3 (219). Moreover, since TC-PTP antagonizes the activity of receptor tyrosine kinases (RTKs), activators and agonists of TC-PTP represent a potential strategy to reduce the oncogenic activity of RTKs (232, 233). In addition to its ability to attenuate proliferation, TC-PTP decreasing Stat3 mediated transcription of the HIF-1α gene may have important implications within the tumor microenvironment as an inhibitor of the angiogenic switch. In line with this notion, TC-PTP was shown to antagonize VEGFR2 signaling in endothelial cells (234) suggesting that TC-PTP not only inhibits angiogenesis by suppressing HIF-1α expression and secretion of VEGF by tumor cells but also by attenuating its activity in endothelial cells .

PKR was previously described to exert anti-tumor functions (88, 235-237). The activation of TC-PTP, a negative regulator of proliferation, with the cooperative inhibition of Stat3 and HIF-1α, inducers of tumor growth described in this section demonstrate novel tumor suppressive functions for this eIF2α kinase (198).

1.3 Implications of PKR for therapeutic approaches targeting HIF-1α.

From a clinical aspect, our research suggests that PKR may play a role in sensitizing hypoxic cells to therapy. We show that PKR contributes to HIF-1α

inhibition in response to the chemotherapeutic 2-ME, a drug which in independent studies has been shown to activate PKR (220) and suppress HIF-1α (221). Our data, therefore, suggest that PKR may be a suitable pharmacological target for the treatment of hypoxic tumours aimed at suppressing HIF-1α. This notion is further supported by studies showing that other chemotherapeutic drugs such as etoposide, doxorubicin, as well as related topoisomerase inhibitors lead to an inhibition of HIF-1α synthesis and accumulation in response to hypoxia (238-240). Since PKR has been shown to be activated by various chemotherapeutic means (241) including doxorubicin (181), SAHA (188), and 5-FU (180), it is likely and possible, that PKR activation by chemotherapeutic approaches significantly contributes to solid tumour growth suppression by blocking signaling mediated by Stat3 and HIF-1α.

1.4 Summary

Our findings demonstrate that PKR plays an important role in suppressing HIF-1α expression at the transcriptional level independently of eIF2α phosphorylation. More specifically we show that PKR inhibits *HIF-1A* transcription by inhibiting Stat3 through a mechanism requiring TC-PTP. Our results confirm that PKR can suppress Stat3 function and uncovered a novel antitumor function of PKR in regulating HIFα and its activity. As PKR is activated by various chemotherapeutic approaches our results indicate that PKR contributes to their efficacy not only by activating TC-PTP but also by suppressing the functions of Stat3 and HIF-1α.

2. Development of transgenic mice expressing a conditionally active form of the eIF2alpha kinase PKR.

2.1 Expression and inducibility of the GyrB.PKR chimera kinase.

The use of GyrB.PKR in HT1080 cell lines has been very helpful in studying the effects of eIF2α kinase activity and eIF2α phosphorylation (99, 181, 197, 198). Herein, we describe the development of a novel *in vivo* mouse model engineered to bare the same GyrB.PKR chimera kinase to study the biological role of eIF2α kinase function.

To ensure ubiquitous expression of the transgene, we cloned the transgene under the control of the β-actin promoter. Accordingly, we showed that the kinase is present in a wide array of tissues tested, though the extent of expression varied between the different tissues. This was expected since relative actin expression between different tissue types has also been shown to vary and that the transgene was expressed highest in skeletal muscle and heart; tissues containing high levels of actin. Importantly, expression may be a determining factor in the extent of coumermycin-induced activation. For instance, the induction of eIF2α phosphorylation as well as the extent of JNK-1 phosphorylation was higher in the pancreas in comparison with induction detected in the lung. Another possibility to account for the differences observed between organs tested is tissue specific variation in the phosphatase activity de-phosphorylating eIF2α. Lastly, disparities in the bioavailability and clearance of the drug are also important determinants influencing the inducibility of the chimera kinase.

Upon prolonged coumermycin treatment, the human GyrB.PKR cells lines display an induction in apoptosis (94). In the mice, however induction of the chimera kinase displayed no apparent toxicity or phenotype following single coumermycin administration (gavage), or multiple (intraperitoneal) injections. The reasons for the differences observed between the culture and the mouse models can be explained as follows. First, the variance in the extent of expression of the chimera kinase between the cell types can account for the cytotoxic effects seen in the human cells. To assess the level of overexpression, we compared GyrB.PKR to endogenous PKR expression by western analysis probing for the C-terminus of PKR which detects both proteins (distinguishable by subtle molecular weight differences). In the human cells, GyrB.PKR is tremendously over-expressed compared to endogenous PKR levels. In contrast, mouse tissue homogenates displayed similar expression between the two (data not shown) suggesting that the degree of kinase activation regulates eIF2α phosphorylation dependent apoptosis. A second reason is that the availability and uptake of the antibiotic is more efficient in cells growing in a monolayer compared with the intricate tissue architecture in a mouse. Lastly, in addition to the overexpressed kinase and efficient uptake of the antibiotic, the rapidly proliferating nature of cancer cells conceivably render them more dependent on global protein synthesis compared to adult mouse tissue. The GyrB.PKR mice therefore represents a viable *in vivo* system in which physiologically tolerable levels of eIF2α phosphorylation can be induced conditionally for short or prolonged periods of

time to investigate the biological role of PKR activation and eIF2α phosphorylation.

2.2 Future applications of the GyrB.PKR mouse model to study cancer.

In our mouse model, it was shown that GyrB.PKR is expressed, coumermycin treatment induces eIF2α phosphorylation and that activation of GyrB.PKR does not cause morbidity. As such, the inducible GyrB.PKR mice model may prove useful to better understand the biological effects of persistent activation of PKR and eIF2α phosphorylation *in vivo* in the absence of parallel signaling pathways independently induced by stresses which induce the endogenous eIF2α kinases. For example, these mice could be used to determine the effects of induced eIF2α phosphorylation in chemical tumourigenesis after treatment with carcinogens that cause tumours in the breast (e.g. 12-dimethylbenz (a)anthracene), lungs (e.g. urethane) or intestines (e.g. azoxymethane). Furthermore, given that the eIF2α phosphorylation pathway was implicated in the regulation of some tumour suppressor proteins (71, 88), cross-breeding the GyrB.PKR mice with mice that are deficient in tumour suppressors, such as p53 or PTEN, may allow to investigate the role of persistent eIF2α phosphorylation in spontaneous tumourigenesis. Alternatively, since PKR possesses antitumor properties (described in section 1 and 3), cross-breeding the GyrB.PKR mice with mice bearing activated oncogenes (such as Ras or erbB2) would allow us to assess the role of persistent eIF2α phosphorylation in oncogene-specific induced tumorigenesis. Lastly, since the kinase domains of the eIF2α kinases are

conserved amongst the eIF2α kinase family, and the GyrB.PKR transgene is expressed ubiquitously, the applications of these mice are not limited studying roles of PKR alone or to only studying cancer. As such, it is feasible that the GyrB.PKR mice may prove to be useful as an experimental model for investigating other eIF2α kinase-eIF2α phosphorylation associated pathologies including heart disease, diabetes, obesity and neurological disorders.

2.3 Summary

Previous work established that expression of a chimera protein of the bacteria Gyrase B N-terminal (GyrB) domain fused to the kinase domain (KD) of the eIF2α kinase PKR, is capable of inducing eIF2α phosphorylation in cultured cells after treatment with the antibiotic coumermycin. In this section, we report the development of transgenic mice expressing the fusion protein GyrB.PKR ubiquitously. We show that treatment of mice with coumermycin induces eIF2α phosphorylation *in vivo*. The GyrB.PKR transgenic represents a useful model system to investigate the biological effects of the conditional activation of PKR and eIF2α phosphorylation in the absence of parallel signaling pathways which are elicited in response to stress. In addition, it may serve as a valuable tool to dissect the effects of persistent eIF2α kinase activity/eIF2α phosphorylation with implications to the fields of mRNA translation, metabolism and cancer.

3. The role of eIF2α phosphorylation in cancer cells and in the response to chemotherapeutics.

3.1 Studying the roles of eIF2α phosphorylation in human cell lines.

The use of GyrB.PKR in fibrosarcoma HT1080 cells was instrumental in our studies examining at pathways regulated by eIF2α kinase activation. The approaches taken in this section have not only enhanced the versatility of this molecular tool, but also have enabled us to assay the role of eIF2α phosphorylation in human cells lines.

To date, the only model of cells truly deficient in eIF2α phosphorylation are cells derived from mice. Given the importance of using human cell lines to study cancer, we have devised various approaches to inhibit eIF2α phosphorylation in human cells. One approach was the generation of cell permeable peptides, engineered to mimic the binding sites of eIF2α which contact the eIF2α kinases. Our results showed a partial abrogation of eIF2α phosphorylation (Fig. 27A). Another approach used, is the overexpression of the mutant protein eIF2α S51A and exploitation of its ability to act as a dominant negative by competing with the endogenous eIF2α protein. This approach was not sufficient to completely inhibit eIF2α phosphorylation (Fig. 27B). This issue was bypassed by first overexpressing the mutant protein and then selectively silencing the expression of the wildtype eIF2α by targeting its 3'UTR. This approach allowed us to make stable cell lines which better represent the eIF2α knock-in phenotype. Our results show that the system is functional; as eIF2α

phosphorylation is barely detectable. Moreover, we were able to show a biological effect; a rescue of death seen in cells lacking endogenous eIF2α protein through the expression of the mutant protein.

3.2 Recapitulation of known effects eIF2α phosphorylation in biological responses and cell signaling.

With respect to GyrB.PKR activation, the eIF2α phosphorylation deficient HT1080 cells displayed resistance to apoptosis when treated with coumermycin for prolonged periods of time verifying that prolonged GyrB.PKR induced eIF2α phosphorylation (alone and in the absence of stress) promotes apoptosis. This effect was partially observed in the knock-ins (deficient in eIF2α phosphorylation) with prolonged coumermycin treatment which may be mediated by residual eIF2α phosphorylation or more likely through eIF2α phosphorylation independent roles of the activated chimera kinase. For example, activation of TC-PTP and regulation of Stat3 signaling (discussed in section 1) may be responsible for the cytostatic effects detected in cells deficient in eIF2α phosphorylation (198). Also, another possibility is that the partial phenotype may be due to the activation of JNK-1, which was shown to be induced by GyrB.PKR (181).

The tumor microenvironment leads to stresses such as hypoxia and nutrient deprivation. With respect to hypoxia, we have addressed an important role for the eIF2α kinase PKR in modulating the hypoxic response by Stat3 - dependent transcriptional suppression of HIF-1α, which occurs independently of eIF2α phosphorylation (198). With respect to nutrient deprivation, we recently have published that eIF2α phosphorylation is a determinant of cell survival in

response to glucose deficiency in a time dependent fashion (242). Although upon acute glucose withdrawal eIF2α phosphorylation conveys pro-apoptotic roles, upon prolonged glucose deprivation, it acts in a cytoprotective manner. Using the HT1080 cells developed (bearing wildtype or non-phosphorylatable eIF2α), we were able to recapitulate the pro-survival role exerted by eIF2α phosphorylation upon prolonged glucose deprivation (data not shown). These results validate our previous work and indicate that rapidly proliferating cancer cells found in glucose depleted microenvironment conditions may be sensitive to therapeutic approaches suppressing eIF2α phosphorylation.

Previous reports from our lab made the seminal observation that ER stress induces the degradation of p53, a cytoprotective mechanism allowing cells to adapt to the stress (105). Interestingly, we showed that this is mediated through an eIF2α independent mechanism which required the activation of PERK. More specifically, the activation of PERK or PKR can activate GSK3β, which phosphorylates nuclear p53 and promotes its mdm2 mediated proteasomal degradation. We also clarified in several tumor cell lines that not only transient but also prolonged ER stress exposure leads to degradation of wildtype p53(71). Curiosity led us to test whether this eIF2α independent regulation can be recapitulated in the human cell lines generated. Our results show, that in response to coumermycin treatment or prolonged thapsigargin treatment, a potent inducer of ER stress, p53 levels are indeed reduced, independent of eIF2α phosphorylation status (Fig. 31 B). These data further validate our previously

published work and highlights the capacity of eIF2α independent functions for the eIF2α kinases.

3.3 Current and future applications

3.3.1 Screening for effectors mediating the pro-apoptotic arm of eIF2α phosphorylation.

Data presented in this section as well as recently published studies, show that the phosphorylation of eIF2α can act as a determinant of cell fate and convey both cytoprotective and pro-apoptotic roles. That is, in response to chemotherapeutic agents (doxorubicin and SAHA), eIF2α phosphorylation acts in a cytoprotective manner. In contrast, in response to constitutive eIF2α phosphorylation (GyrB.PKR activation) or PTEN overexpression (88), eIF2α phosphorylation acts to promote apoptosis. It is therefore essential to apply unbiased approaches to further dissect the pathways affecting these processes to obtain a better understanding of the role of eIF2α phosphorylation in cancer and its contribution to the response to chemotherapies.

Genome wide shRNA screening libraries are a powerful tool and unbiased approach to uncover how cell pathways interact and have proven to be valuable in the cancer field to identify factors which confer susceptibility and resistance to chemotherapies (243, 244). Using this approach, we plan to perform genome wide shRNA screens to confirm known effectors and identify novel pathways affected by eIF2α phosphorylation. More specifically, HT1080 GyrB.PKR cells will be infected with the human shRNA library, the selected cells will be treated with coumermycin for two weeks. Under regular conditions these cells undergo

apoptosis upon prolonged treatment with coumermycin. Cells that bypass this GyrB.PKR-induced death will proliferate leading to an amplification of cells harboring shRNAs silencing genes that promote GyrB.PKR induced death. Subsequent DNA isolation from pooled colonies, PCR of the shRNA cassettes, and sequencing of the barcoded libraries will allow us to identify the enriched shRNAs.

As shown in Figure 30, cell death in response to coumermycin treatment is rescued in the cells bearing mutant eIF2α and knockdown for endogenous eIF2α. However, these cells exhibit a partial increase in cell death which may be attributed to eIF2α independent pathways activated by PKR activity. To distinguish between the eIF2α dependent and independent targets, we will validate putative proteins identified from the shRNA screen using the eIF2α wildtype and "knock-in" cells we generated. Validation will be done using inhibitors for the targets (if available) or siRNA. The results from the validation will be interpreted as follows. If the rescue of cell death in response to coumermycin is not seen in either wildtype or "knock-in" cells then it is not a hit (a false positive). If rescue is only validated in cells bearing wildtype eIF2α and not the mutant then the activation of the protein is dependent on eIF2α phosphorylation, and will likely be a protein (known or novel) selectively translated upon constitutive eIF2α phosphorylation that induces cell death. If the hit protein rescues both wildtype and mutant eIF2α containing cells from GyrB.PKR induced death, then it may represent a novel target that is activated downstream or directly phosphorylated by GyrB.PKR. This powerful approach

will offer a novel unbiased means to discover novel factors affected by the eIF2α kinase activation. Further, it will allow us to discriminate whether they act dependently or independently of eIF2α phosphorylation. The next step will be to characterize these factors, and determine their role in the response to chemotherapeutic drugs.

3.3.2 Screening for cytoprotective effectors of eIF2α phosphorylation.

Our results with the fibrosarcoma cell line (HT1080) show a notable inhibition in proliferation (in cells with abrogated eIF2α phosphorylation). As such, it appears that factors that are better expressed under conditions of phosphorylated eIF2α also promote proliferation or alternatively antagonize pathways suppressing growth. Ongoing studies in our lab suggest that these effects may be more profound in less transformed cell types (i.e. primary eIF2α$^{A/A}$ MEFs and IMR-90 cells subjected to the "knock-in" approach described). This is surprising as the lack of eIF2α phosphorylation would be expected to increase global protein synthesis, and conceivably proliferation. In as yet, the HT1080 cells generated, deficient in eIF2α phosphorylation, display no increase in *de novo* protein synthesis (data not shown) and exhibit diminished proliferation rates (Fig.29). To determine the mechanisms by which abrogation of eIF2α phosphorylation paradoxically represses proliferation, we will use cells in which abrogated eIF2α phosphorylation severely affects proliferation to screen for effectors which promote survival and a reversal of phenotype by applying the shRNA library approach.

Our results with the fibrosarcoma cell line engineered to be deficient in eIF2α phosphorylation display increased sensitivity to chemotherapeutic drugs which induce DNA damage (181) and inhibit HDACs (188). The mechanism accounting for the cytoprotective effects of eIF2α phosphorylation under these contexts is not clear. One possibility is that the translation of pro-apoptotic factors is suppressed when eIF2α is phosphorylated and allows cells to resist apoptosis. Alternatively, factors that are better expressed under conditions of phosphorylated eIF2α may convey anti-apoptotic and pro-survival signals. Future experiments such as genome wide shRNA screen approaches, similar to the previously described will allow us to screen for factors conferring resistance to doxorubicin, SAHA, and potentially any chemotherapeutic agent, in an eIF2α dependent context. This approach will allow to uncover new potentially drugable targets which confer resistance to conventional therapies.

3.3.3 Determining the role of eIF2α phosphorylation in malignant transformation.

The approach to be taken to study the role of the eIF2α pathways in the process of malignant transformation is to use primary cell lines (such us BJ, IMR90, or RPE-1) that are immortalized through the addition of telomerase and apply our "knock-in" approach to generate cells deficient in eIF2α phosphorylation. Next, we will introduce different oncogenes (Ras, ErbB2, or Myc) to evaluate the role of eIF2α phosphorylation in transformation by comparing them to their control cells bearing endogenous wildtype eIF2α protein. This will be achieved by evaluating proliferation, anchorage independent growth,

and their ability to respond to chemotherapeutic drugs which can be further addressed *in vivo* by injection in athymic (nude) mice.

These approaches will be instrumental in determining as to how eIF2α exerts pro-apoptotic effects in some contexts and cytoprotective effects under other ones. Furthermore, it will allow us to distinguish under which genetic lesions these effects are mediated. Together this information will be an important stepping stone towards identifying and discovering new targets and under which contexts they are regulated by the eIF2α phosphorylation pathway.

3.4 Modulating eIF2α phosphorylation for cancer therapeutics

We have shown and discussed that inhibition of basal eIF2α phosphorylation diminishes proliferation of HT1080 cells (Fig.29). Moreover, in response to chemotherapeutic drugs, eIF2α phosphorylation acts in a cytoprotective manner (Fig.31). As such, strategies to inhibit eIF2α phosphorylation may be an exploitable avenue for not only inhibiting proliferation but also enhancing sensitization of cancer cells to chemotherapy. Given that anti-cancer peptidic compounds have been shown to induce apoptosis of tumor cells (245) and target oncogenes (246) our data describing a cell permeable peptide approach (Fig.27) to inhibit eIF2α phosphorylation may prove a feasible basis for engineering such cell permeable peptides to be used in combination with conventional therapies to synergize the apoptotic and anti-tumor effects.

3.5 Summary

The approaches taken and described in this section refer to a new technique to assay the role of the of eIF2α phosphorylation in human cells lines and have allowed us to enhance and improve the versatility the HT1080 GyrB.PKR cells. Importantly, this approach is applicable to any human cell line and not limited to HT1080 cells. The data presented are confirmatory to previous findings and validate that eIF2α phosphorylation plays an important a role in tumor biology and in the response to chemotherapeutic drugs. Future expansion of this system will enable us to determine how eIF2α phosphorylation exerts its pro-apoptotic and cytoprotective effects in a manner that will allow to us to discriminate between eIF2α dependent roles of the eIF2α kinases. These data may serve as a useful stepping stone for the long term goal of exploiting the anti-proliferative and cytotoxic effects the eIF2α phosphorylation pathway in efforts aimed at improving efficacy of cancer therapies.

Chapter 5: Contribution to original knowledge

The work presented in this document uncovered a novel function of PKR in regulating hypoxic signaling and led to the development of novel experimental approaches to study the biological role of the eIF2α phosphorylation pathway. More specifically, the candidate demonstrated that PKR inhibits the Hypoxia Induced Factor-1α, and showed that this is mediated through transcriptional suppression of the *HIF-1A* gene. The molecular mechanism of this suppression was shown to be mediated through TC-PTP and Stat3. Moreover the candidate characterized and developed novel *in vivo* and *in vitro* experimental approaches to assay the physiological and biological roles of the eIF2α phosphorylation, which will serve as important tools for investigating the implications of this pathway. The major findings made by the candidate over the course of this research are outlined as follows.

1. The candidate was the first to demonstrate that PKR plays an important role in suppressing HIF-1α expression at the transcriptional level independently of eIF2α phosphorylation. More specifically, the candidates' work demonstrated that PKR inhibits *HIF-1A* transcription by inhibiting Stat3 through a mechanism requiring TC-PTP. These novel findings confirmed previous findings that PKR can suppress Stat3 and uncovered a novel antitumor function of PKR in regulating hypoxic response. Since PKR is activated by various chemotherapeutic approaches these findings indicate that PKR contributes to their efficacy not only by activating TCPTP but also by suppressing the oncogenic functions of Stat3 and HIF-1α.

2. Research from the candidate led to the characterization of a novel transgenic mouse expressing a conditionally inducible eIF2α kinase (GyrB.PKR). The presented research demonstrated that the transgene was

ubiquitously expressed in mouse tissues and established that the chimera kinase was inducible in an *in vivo* setting. The GyrB.PKR transgenic mice may serve as a valuable tool to dissect the biological effects of conditional eIF2α kinase activation and eIF2α phosphorylation with implications to the fields of mRNA translation, metabolism and cancer.

3. The candidate devised novel strategies for generating stable human cell lines deficient in eIF2α phosphorylation to assay the role of eIF2α phosphorylation in biological processes. Using this approach, the candidate has enhanced the versatility of the previously generated HT1080 GyrB.PKR cells. Through the validation of the system the candidate has generated key confirmatory data to previous findings demonstrating important roles of the eIF2α phosphorylation pathway in tumor biology and in the response to chemotherapeutic drugs. As this approach is applicable to any human cell line, the utility of the candidate's contributions extends to current ongoing studies and collaborations.

4. The candidate was involved in the generation of cell permeable peptides engineered to inhibit eIF2α phosphorylation. As eIF2α phosphorylation has been shown to be cytoprotective in response to various chemotherapeutics in cell culture models, the peptides generated may serve as a feasible prototype to inhibit eIF2α phosphorylation in a "therapy-based" experimental context.

Chapter 6: References

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