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

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the Department of
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2018
ABSTRACT

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Abstract

Death associated protein 5 (DAP5) is an atypical isoform of the translation initiation scaffold eukaryotic initiation factors 4GI and II (eIF4GI/II), which recruit mRNAs to ribosomes in mammals. Unlike eIF4GI/II, DAP5 binds eIF2β, a subunit of the eIF2 complex that delivers methionyl-tRNA to ribosomes. Despite extensive work describing eIF4GI, an understanding of DAP5 activation is yet to be described.

Here I describe our discovery that DAP5:eIF2β binding is regulated by DAP5 phosphorylation and can be stimulated by specific stimuli including protein kinase C PKC-Raf-ERK1/2 signals, mitosis and hypoxia, wherein DAP5:eIF2β binding determines DAP5’s influence on global and template-specific translation. However, DAP5 depletion causes an unanticipated surge of hypoxia-inducible factor 1α (HIF-1α), the transcription factor and master switch of the hypoxia response. The hypoxia response is tempered through HIF-1α hydroxylation by the oxygen-sensing prolyl hydroxylase-domain protein 2 (PHD2), and subsequent ubiquitination and degradation. Furthermore, we found that DAP5 regulates HIF-1α abundance and Akt signaling through DAP5:eIF2β-dependent translation of PHD2. DAP5:eIF2-induced PHD2 translation occurs during hypoxia-associated protein synthesis repression, indicating a role as a safeguard to reverse HIF-1α accumulation and curb the hypoxic response implying that DAP5:eIF2β binding may describe a conserved mechanism for selective stress induced translation.
Dedication

This is dedicated to the multitude of people who have supported me, offered me counsel, and/or put up with my frustrations over this long process.
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1. Introduction

1.1 Protein Synthesis in Eukaryotes

The central dogma of molecular biology describes the conversion of information from DNA to RNA then from RNA to protein with proteins being the terminal step relaying the genetic code. This transformation of information is an essential process of all biological life. Proteins are the raw materials and enzymes that are crucial to all cellular processes. The central dogma outlines two steps: transcription (DNA to RNA) and translation (RNA to protein). However, numerous other steps occur to control the formation of new functional proteins. In eukaryotes, the mRNA is exported from the nucleus and transported to ribosomes. This process, along with mRNA stability, is heavily influenced by RNA binding proteins (Keene, 2007). After translation, proper protein folding along with additional modifications must occur, followed by shuttling of the protein to its proper cellular location. All of the steps leading to mature, properly functioning proteins are highly regulated, with as much as a third of a cell’s energy dedicated to protein synthesis (Buttgereit & Brand, 1995). Therefore, to efficiently conserve cellular energy, additional regulatory processes occur allowing for rapid changes in translation in response to a variety of external stimuli without changing mRNA abundance (Hershey, Sonenberg, and Mathews, 2012).

Translation occurs as a set of coordinated steps beginning when the mRNA is brought to the ribosome and ending with a newly synthesized polypeptide. Translation
is described as occurring in three major steps: initiation, elongation, and termination.

During initiation an ensemble of eukaryotic initiation factor proteins (eIFs) coordinate the recruitment of mRNA to a ribosome. In a majority of cases the ribosome scans the 5’ untranslated region (5’UTR) until a start codon in proper context is identified. During the elongation phase, aminoacyl-tRNA’s recognize their complementary mRNA codons and allow the correct amino acid to become peptide bonded to the growing polypeptide chain, after which the tRNA, now without its corresponding amino acid is released.

Translation ends at termination when a stop codon induces the release of the ribosome from the newly synthesized polypeptide. Though regulation may occur at any stage in translation, to prevent wasted cellular resources by far the most extensive regulation of translation occurs during the initiation step (Sonenberg & Hinnebusch, 2009).

1.2 Initiation of Translation

1.2.1 Mechanisms of Translation Initiation

Unsurprisingly, cells have evolved multiple mechanisms to ensure that translation occurs properly to respond to the numerous cellular conditions that may occur. Translation initiation transpires when the correct start codon of an mRNA is recognized and binds in proper position within the ribosome. Multiple steps and more than a dozen initiation factors regulate this process. Two common mechanisms of translation initiation described are the canonical cap-dependent translation (shown in Fig. 1) and the less well understood alternative cap-independent translation.
1.2.1.1 Cap-dependent Translation

The following describes the mechanism of cap-dependent translation initiation, but with the exception of binding to the mRNA through eIF4E, cap-independent translation proceeds through a mostly shared mechanism. Immediately after transcription, all eukaryotic mRNAs in the nucleus have a 7-methyl guanosine cap (m$^7$G cap) added. In cap-dependent translation the mRNA is contacted by eIF4E directly at this cap (Step 4; Fig. 1). This interaction links the mRNA to the eIF4F translation initiation complex (Step 4; Fig. 1) composed of multiple subunits through eIF4G, the scaffold of the complex.

Apart from binding to eIF4E, eIF4G functions by binding to the eIF4F helicase and its co-factor, eIF4A and eIF4B respectively, the ribosome binding factor eIF3, and the poly-A-binding protein (PABP) which facilitates mRNA circularization (Step 4; Fig. 1). eIF4G then facilitates mRNA recruitment via eIF3 to the 43S ribosomal subunit (Step 5; Fig. 1) composed of the 40S small ribosome, eIF1, eIF1A, eIF5 and the ternary complex, composed of the eIF2 and the GTP-Met-tRNA$^{Met}$ (Levin, Kyner, & Acs, 1973) (Steps 1, 2, 3; Fig.1). Helicase activity unwinds the mRNA secondary structure allowing the 43S ribosome to scan the 5'UTR (Step 6; Fig. 1) until recognition of a start codon (usually AUG) in proper Kozak context (Step 7; Fig. 1).

Identification of the start codon activates ribosomal changes that cause the eIF2α-GTP to be hydrolyzed to GDP by eIF5 (Step 7; Fig. 1), instigating the recruitment of
eIF5B and the 60S large ribosomal subunit (Step 8; Fig. 1) along with the release of remaining initiation factors (Tatyana V. Pestova et al., 2000) (Step 9; Fig. 1). This results in the Met-tRNA\textsubscript{Met} recognizing the start codon loaded in the 80S ribosome in proper orientation for elongation to occur (Jackson, Hellen, & Pestova, 2010).
Figure 1: Mechanism of cap-dependent translation.

Figure from (Jackson et al., 2010) see text for details; used with permission (See appendix)
1.2.1.2 Cap-independent Translation

Notably, during conditions in which cap-dependent translation is inhibited an alternative mechanism of translation initiation that circumvents m^7G cap binding by eIF4E occurs (T. V. Pestova et al., 2001). Cap-independent translation is best described for (+) strand RNA viruses, such as EMCV (Jang et al., 1988) and PVSRIPo virus (Goetz, Everson, Zhang, & Gromeier, 2010), where highly structured 5'UTR's regions called internal ribosome entry sites (IRES) directly recruit initiation factors and the ribosome. Due to the unique nature of cap-independent translation to help facilitate initiation, alternative factors sometimes referred to as IRES trans-activating factors (ITAFs) may be recruited to the mRNA. Unlike for viral RNAs, the existence of cellular IRES's is still an area of active debate (Kozak, 2005; Gilbert, 2010).

It is known that a number of capped cellular mRNAs undergo translation without the need for eIF4E both basally and during times of cellular stress, but the mechanism behind this translation is unclear. It is important to note that eIF4G’s ability to directly bind RNA may play an important role in translation that doesn’t involve binding of the cap (Hundsdoerfer, Thoma, & Hentze, 2005). Modifications on the mRNA itself, such as N^6-methyladenosine (Meyer et al., 2015), have been suggested to mark mRNAs for cap-independent translation. Also in a similar role to ITAFs in viral IRES, alternative translation initiation factors such as the eIF4G homolog, Death Associated Protein 5 (DAP5) (Hundsdoerfer et al., 2005) which lacks the eIF4E binding domain, may
be required for translation of certain transcripts through binding and recognition of elements in the mRNA. Due to the immense complexity and regulation of translation initiation it is probable that many distinctive varieties of cellular cap-independent translation occurs.

1.2.2 Function and Regulation of eIF4GI and its Homologues

Due to eIF4G's multiple binding interactions it functions as the scaffold bridging the mRNA to the ribosome. There are three predominate eIF4G isoforms, the domains, organization, and binding interactions of which are shown in Figure 2 (eIF4GI/II are shown as one).

Figure 2: Domain/binding motifs of the eIF4Gs

1.2.2.1 eIF4GI/II

During canonical cap-dependent translation initiation when eIF4G is mentioned as the component of eIF4F complex, it is in fact the eIF4GI isoform that is implied. Despite the eIF4GII isoform’s high sequence homology to eIF4GI, 72% and 63% in HEAT domains 1 and 2 respectively (Bellsolell, Cho-Park, Poulin, Sonenberg, & Burley, 2006),
the ratio of eIF4GI to eIF4GII is ~10:1 in HeLa cells (Bellsolell et al., 2006). Therefore, the role eIF4GII has in normal cellular translation so far has been shown to be diminutive in regards to eIF4GI.

eIF4GI is organized into three structured HEAT (Huntington/EF3/protein phosphatase 2A/mTOR) and two significant unstructured domains. The first unstructured region is located N-terminally of HEAT1 and the second, referred to as the inter-domain linker (IDL), is located between HEAT1 and 2 (Fig. 2). The N-terminal unstructured region contains the binding domains for eIF4E (the m^7G cap binding subunit) and the poly(A) binding protein (PABP), allowing eIF4GI to bind to both 5’ and 3’ mRNA ends and aiding in circularization of the mRNA. This region of eIF4GI, which is missing in DAP5, is required for cap-dependent translation initiation.

The middle and C-terminal region of eIF4GI contains the structured HEAT domains. This region of eIF4GI is composed of HEAT1 which contains a binding domain for the helicase eIF4A and adjacent to HEAT1 is the eIF3 binding site (Fig. 2). The ability of this region to bind to eIF3, and, therefore, the ribosome, gives eIF4GI its translational activity (Morino & Imataka, 2000). The C-terminal region contains HEAT2 with additional eIF4A binding activity, and HEAT3 which contains two acidic aromatic boxes (AA boxes) which confer the ability to bind to MNK (Bellsolell et al., 2006) (Fig. 2).

Activity of eIF4GI is subject to extensive regulation by phosphorylation with the IDL of eIF4GI containing numerous sites, including S1148 regulated by mTOR signaling.
Phosphorylation at these sites has been shown to impact the binding interactions of eIF4GI, with S1186 regulating binding to MNK and S1232 regulating the binding with the eIF4A/B helicase complex.

1.2.2.2 DAP5

DAP5 (other names: eIF4G2, eIF4GIII, Novel APOBEC Target 1(NAT1) or p97) was discovered in 1997 by four separate laboratories (Yamanaka, Poksay, Arnold, & Innerarity, 1997; Shaughnessy, Jenkins, & Copeland, 1997; Levy-Strumpf, Deiss, Berissi, & Kimchi, 1997; Imataka, Olsen, & Sonenberg, 1997). DAP5 appears as a truncated form of eIF4GI and is homologous to the C-terminal two thirds of eIF4GI. Thus, DAP5 lacks eIF4E and PABP binding sites. We have found that the AA boxes of DAP5 do not bind MNK (Brown, Bryant, et al., 2014), but these AA boxes have unique binding activity for eIF2β (S. H. Lee & McCormick, 2006) (Fig. 2).

DAP5 promotes cell survival during mitosis and differentiation (Marash et al., 2008) with null mutations of DAP5 in mice leading to early embryonic lethality (Yamanaka et al., 2000). Due to DAP5’s inability to bind to eIF4E, DAP5 was originally suggested to target the IRES of many anti-apoptotic mRNAs for cap-independent
translation such as HIAP2, Bcl-2, and DAP5 itself (Lewis et al., 2008; S. H. Lee & McCormick, 2006).

Despite the initial discoveries where DAP5 was studied in the context of template specific translation, DAP5 has also been found to play a role in global translation. DAP5 down regulation by RNA interference leads to a 30% decrease in protein synthesis (S. H. Lee & McCormick, 2006). Interestingly the over-expression of DAP5 or eIF4GI cannot compensate for the effect of the other's depletion on translation (Ramírez-Valle, Braunstein, Zavadil, Formenti, & Schneider, 2008). DAP5 is ubiquitous and abundantly expressed in all studied cultured cells and tissues (Schwanhäusser, Busse, & Li, 2011) and DAP5 is present in excess of eIF4GI (~5:1 in HeLa cells) (Nagaraj et al., 2011). During normal growth conditions DAP5 associates with active polysomes (Nousch, Reed, Bryson-Richardson, Currie, & Preiss, 2007).

Cell death signaling has also been proposed to trigger caspase cleavage of DAP5 to the C-terminally truncated p86 form which appears to induce a change in DAP5 activity (Henis-Korenblit et al., 2002). This cleavage of DAP5 is located N-terminally of the eIF2β binding region suggesting that removal of the eIF2β binding augments DAP5's normal activity and thus a regulatory role for DAP5:eIF2β binding. DAP5:eIF2β binding was also found to be necessary for translational activity on artificial cellular IRES reporters (Liberman et al., 2015), however the impact of DAP5:eIF2β during physiological conditions has yet to be investigated.
The tight regulation of the homologous eIF4GI:MNK binding suggests that DAP5:eIF2β binding may be similarly regulated. MNK activity is regulated by signaling downstream of receptor tyrosine kinases (RTKs) and by phosphorylation events on eIF4GI (M. I. Dobrikov et al., 2013). The activity of eIF4GI has also been linked to its ability to bind MNK (Shveygert, Kaiser, Bradrick, & Gromeier, 2010). Therefore, it is highly possible that the coordinated activity of eIF2 and DAP5 may rely on binding to each other with signal transduction networks inducing regulatory phosphorylation of DAP5.

1.2.3 Function and Regulation of eIF2

1.2.3.1 Role of eIF2 during translation initiation

For translation to begin, the start codon of the mRNA must be properly recognized by the Met-tRNA\textsuperscript{Met}. This is accomplished by a coordinated set of interactions involving the mRNA, the ribosome, and a number of initiation factors. The initiation factor that plays the crucial role in this process is eIF2. eIF2 is a complex of three subunits α, β, and γ. The γ subunit is the catalytic subunit, the activity of which determined by binding to GTP or GDP and possesses the potential to bind eIF2β and eIF5. The β subunit contains lysine (K) boxes binding regions which form strong interactions with AA boxes in eIF5, eIF2β (Alone & Dever, 2006), and DAP5. The α subunit contains the regulatory phosphorylation site Serine 51 (S51).
The function of eIF2 in canonical translation is as follows (diagramed in Figure 3). eIF2 becomes active through interactions with its guanine nucleotide exchange factor (GEF), eIF2B, catalyzing the exchange of GDP to GTP. Now in its active state, eIF2 can bind Met-tRNA\textsubscript{Met} to form the eIF2 ternary complex. This complex is then recruited to eIF3 and the 40S ribosomal subunit along with factors eIF1, eIF1A, and eIF5. This combination of factors is referred to as the 43S complex. The eIF4F complex then facilitates the delivery of the mRNA and scanning begins (Sonenberg & Hinnebusch, 2009). Once the start codon is recognized by the Met-tRNA\textsubscript{Met} a number of changes occur. eIF5, which functions as the GTPase-activating protein (GAP), induces eIF2-GTP to become hydrolyzed to GDP. The C-terminus of eIF5 unbinds from eIF1 switches to binding eIF2β causing the release of eIF1 and a free phosphate. Together, these changes result in a “closed” preinitiation complex (PIC) conformation (Singh et al., 2012; Luna et al., 2012). After these changes have occurred, the remaining initiation factors, including eIF2-GDP, are released with eIF5B and the 60S ribosome joining starting translation elongation.
Figure 3: eIF2 function and regulation
Schematic courtesy of Cell Signaling Technology, Inc. (See appendix)
1.2.3.2 Regulatory eIF2 phosphorylation

Arguably the most important translation regulatory phosphorylation event is the phosphorylation of eIF2α Serine 51, p-eIF2α(S51). Phosphorylation at this site transforms the eIF2 into a competitive inhibitor of its GEF (eIF2Bε), thus preventing the activation of eIF2-GDP to eIF2-GTP (Prostko, Brostrom, & Brostrom, 1993). P-eIF2α(S51) induces a conformational change in eIF2 such that it binds to the non-catalytic subunits of eIF2B, sequestering both eIF2 and eIF2B to effectively limit the available pool of eIF2-GTP and initiate translation (Rowlands, Panniers, & Henshaw, 1988). This explains how this single phosphorylation event is able to induce such a large and sudden decrease in global translation. The binding domain in eIF2Bε for eIF2β is shared with eIF5 and DAP5, suggesting that competitive binding may play a role in regulating translation repression by p-eIF2α(S51).

Despite this inhibition of translation, a few mRNAs use this limited pool of eIF2 to stimulate their own translation. The best known are GCN4 and ATF4 mRNAs which contain a series of upstream open reading frames (uORFs), which during normal translation lead to dead end translation as the ribosome fails to rebind and scan after encountering multiple uORFs. Yet when eIF2α(S51) is phosphorylated the ribosome is able to rebind past multiple uORFs to initiate at the correct start codon (Vattem & Wek, 2004). Others proteins have been observed to be translated during p-eIF2α(S51) suggesting alternative initiation mechanisms remain to be uncovered.
Four known kinases induce p-eIF2α(S51) (Fig.3). These four kinases, which make up what is called the integrated stress response, respond to different stimuli. However, they all share similar catalytic domains leading to phosphorylation of eIF2α(S51). PKR is activated by viral infection and senses the presence of double stranded RNA. PERK senses an imbalance of endoplasmic reticulum chaperones, also known as the unfolded protein response (UPR). GCN2 is known to recognize amino acid starvation, and HRI is activated by misregulated synthesis of heme. The four kinases are counteracted by two phosphatases CReP and GADD34. These phosphatases direct PP1 to dephosphorylate eIF2 (Ron & Harding, 2007). CReP is constitutively expressed while GADD34 expression is highly induced during stressors such as UPR (Reid et al., 2016).

Regulation by phosphorylation of eIF2 may occur outside of eIF2α (S51). Numerous phosphorylation sites have been tentatively identified on eIF2. Sites on all three subunits α (Rajesh, Iyer, Suragani, & Ramaiah, 2008), β (Rajesh et al., 2008; Llorens et al., 2006), and γ (Andaya et al., 2011) have been reported to regulate binding and activity of eIF2. Looking beyond p-eIF2α(S51) may lead to the discovery of new events that manage the many interactions of eIF2 during cellular translation during diverse conditions.

1.3 Regulation of Translation by Cell Signaling and during Mitosis

Cells must be able to accurately control translation to respond to a variety of extracellular cue and cellular conditions. Signal transduction to the translation apparatus
results in rapid changes in protein synthesis (Proud, 2007). To take advantage of a pro-growth environment, signal transduction must stimulate the increase in translation rates and modify actively synthesized proteins. This may be achieved through signal transduction to the mitogen-activated protein kinase (MAPK) ERK1/2 pathway. During stress conditions, multiple signaling pathways, such as the integrated stress response, may become activated to decrease protein synthesis to protect the cell from a dangerous buildup of misfolded proteins or inappropriate cell cycle progression. The stage of the cell cycle also has a dramatic impact on protein synthesis. Translation is considerably down regulated during mitosis so as to not interfere with the process of cell division.

1.3.1 MAPK Signaling

The activation of MAPKs can occur through a variety of signals. ERK1/2 is generally activated by growth factors, while p38 and JNK in general respond to stimuli related to stress. MAPK pathways are composed of three kinases acting in series: MAPK, MAPK kinase (MAPKK), and a MAPK kinase kinase (MAPKKK). In response to external stimuli, GTP-binding proteins of the Ras/Rho family can bind and activate the serine/threonine MAPKKK. Alternatively, the MAPKKKs can be activated by phosphorylation (Pearson G et al., 2001). This then leads to the activating phosphorylation event on the MAPKK, which in turn through simultaneous phosphorylation on tyrosine and threonine motifs in the activation domain, activate the
MAPK (Fig. 4)(Kolch, 2000). MAPK recognizes substrate sequences with target threonine or serine residues followed by a proline.

Figure 4: MAPK and ERK1/2 Signaling Pathway.
Figure from (Kolch, 2000) see text for details; used with permission (See appendix)

1.3.1.1 ERK1/2 MAPKs

The Ras-Mek-ERK1/2 signaling cascade is toggled on and off by the association of the Ras protein (H-Ras, K-Ras, and N-Ras isoforms) with GDP or GTP. Growth factor or phorbol ester stimulation leads to dimerization and autophosphorylation of the cytosolic domains of the receptor tyrosine kinase (RTK). This enables adaptor proteins to associate with the receptor along with Ras facilitating the GDP-GTP exchange. Ras then is able to phosphorylate Raf (c-Raf also a-Raf and b-Raf isoforms exist) which then phosphorylates MEK1/2. MEK1/2 is the MAPKK that activates ERK1/2 by
phosphorylation (Fig. 4) (Kolch, 2000). Phorbol esters, such as 12-O-tetradecanoylphorbol-13-acetate (TPA), are used to activate the enzyme protein kinase C (PKC) that activates Raf. ERK1 and ERK 2 are ubiquitously expressed and are over 80% identical (Boulton et al., 1990). Both the description of the pathway here and in the previous section layout the canonical MAPK signaling pathway (Fig. 4), yet it is a gross oversimplification. A multitude of non-linear events interconnect these “pathways” with almost all cellular processes and add additional layers of regulation.

ERK1/2 phosphorylate numerous substrates throughout the cell including membrane bound and cytoskeleton proteins. Activation of ERK1/2 also induces its own nuclear translocation where it targets various transcription factors including Elk (Fig. 4). Elk activates the transcription of c-fos, the proto-oncogene. Nuclear ERK1/2 also targets pro-growth and oncogenic STAT3, and c-myc (J. Chung, Uchida, Grammer, & Blenis, 1997; Escamilla-Powers & Sears, 2007). ERK1/2 activation stimulates protein synthesis by phosphorylation of the ribosomal S6 protein kinase (RSK) (Frödin & Gammeltoft, 1999), MAPK interacting kinase (MNK), and eIF4GI, a focus of previous studies in our lab (Brown, Dobrikov, & Gromeier, 2014; Brown & Gromeier, 2017; M. Dobrikov et al., 2011; M. I. Dobrikov et al., 2013, 2014; Goetz et al., 2010; Shveygert et al., 2010). ERK1/2 phosphorylation is tempered by MAPK phosphatases (MKPs), the expression of which occurs shortly after ERK1/2 phosphorylation (Owens & Keyse, 2007). Thus, the ERK1/2 MAPKs function to promote proliferation and cell growth.
1.3.1.2 p38 and JNK MAPKs

Inflammatory cytokines and environmental stresses activate the p38 and c-Jun amino-terminal kinases (JNK) MAPKs. However, unlike ERK1/2, they are not strongly activated by mitogenic stimulation. Following the activating phosphorylation events both p38 and JNK relocate to the nucleus to activate a number of transcription factor to potentiate the appropriate response.

JNK is phosphorylated by the MAPKKs MEK4 and MEK7 with several MAPKKKs including transforming growth factor-β activated kinase 1 (TAK1). JNK has numerous cytoplasmic and nuclear substrates with its most well-known being the transcription factor and proto-oncogene c-Jun (Weston & Davis, 2007). c-Jun phosphorylation at serine 63 and serine 73 by JNK activates a potent anti-apoptotic response promoting cell survival and proliferation during times of stress-induced JNK activation (Behrens, Sibilia, & Wagner, 1999). Thus, JNK is known for its role in inciting a strong anti-apoptotic cellular response.

Similarly to the activating conditions of JNK, a variety of cellular stresses including osmotic stress, oxidative stress, and UV activate the p38 pathway. MKK3 and MKK6 functions as the main MAPKK for p38. There are many various MAPKKKs that lead to p38 pathway activation stimulated by the various cellular stresses (P. Roux & Blenis, 2004). p38 has numerous substrates including RNA binding proteins, kinases, and transcription factors. p38 activates the kinases MAPK-activated kinase 2 and 3.
(MK2/3) (J. C. Lee et al., 1994), and MNK (Nagaleekar et al., 2011). Activation of MK2/3 leads to increased stability of mRNAs through phosphorylation of the RNA binding proteins tristetraprolin and HuR (Hitti et al., 2006). p38 also has a regulatory role on protein synthesis through MNK.

p38 is well known for its role in activating the immune response via the synthesis of pro-inflammatory cytokines. This is achieved through p38-provoked translation, transcription factor activation, and RNA binding protein stability (C. Kim et al., 2008).

1.3.2 Regulation of Translation during Mitosis

During mitosis a dramatic reorganization of the cell takes place including the condensation of the nuclear chromosomes, the dissolution of the nuclear membrane, and the creation of the large microtubule assembly known as the mitotic spindle. Furthermore, the chromosomal DNA, organelles, and contents of the cytosol must be properly segregated into the daughter cells. This massive disruption of the cell requires coordination of all cellular processes to ensure proper mitotic passage and two healthy resulting cells. Errors in this regulation can cause cell death, and in some cases cellular transformation. Numerous kinases, most notably CDK1, are induced to signal the cell to undergo mitotic changes including a dramatic shift in protein synthesis.

1.3.2.1 CDK1 signaling

Cyclin dependent kinases (CDKs) are a family of serine/threonine protein kinases related to the MAPKs whose activity is regulated through binding to specific cyclins.
CDKs major function is to control cell cycle progression. The general model for CDKs activity is separated into interphase and mitotic CDK-cyclins. The interphase kinase complexes include in G1 CDK4-cyclinD and CDK6-cyclinD and for G1 to S phase CDK2-cyclinE and cyclinA. The mitotic CDK, CDK1, binds to cyclinA during G2 and cyclinB during the G2-mitosis transition continuing throughout mitosis (Malumbres & Barbacid, 2005). Plasticity between the CDKs has been shown and CDK1 alone is able to drive the complete cell cycle progression (Santamaria et al., 2007). Hereafter in this work, references to mitotically active CDK1 indicate the CDK1-cyclinB complex.

During mitosis CDK1 is involved in the modulation of a large array of cellular processes to ensure proper passage through mitosis. These processes include breaking down the nuclear membrane, assembly of microtubules, condensation of chromosomes, and mitotic changes in translation. Inactivation of CDK1 must occur for exit from mitosis (Nigg, 2001). CDK1 has been found to phosphorylate eIF4GI (M. I. Dobrikov et al., 2014) and DAP5 (described in Chapter 3.2). The scale of CDK1 activity was explored by a global analysis approach which identified over 300 unique CDK1 substrates, many enriched in mitotic processes including chromatin structure, spindle formation, and translation (Holt et al., 2009). This plethora of known and punitive CDK1 mitotic targets highlight the significant role this kinase plays in the crucial maintenance of proper cell cycle status.
1.3.2.2 Protein synthesis during mitosis

There is a surprising discrepancy in the literature regarding mitotic translation. Early studies in the 1960's identified a potent reduction in global translation in cell synchronized in mitosis (Konrad, 1963; Prescott & Bender, 1962). Initial follow up studies led to the realization that in mitosis translation initiation appeared to be blocked (Fan & Penman, 1970). Yet when examining well-known mechanisms for repression of translation initiation [e.g. p-eIF2α(S51), and the eIF4E-binding proteins (BPs)]. Neither had a defined role in mitosis-induced global translation repression. None of the eIF2α kinases are known to be active during mitosis and the occasionally observed p-eIF2α(S51) in mitosis may be caused by cell cycle inhibitors or stress-induced to create the artificial mitotic arrest. eIF4E binding proteins (eIF4E-BPs) function by blocking the cap-binding eIF4E from interacting with eIF4G. The eIF4E-BPs are inactivated by phosphorylation and have been found to be hyper phosphorylated during mitosis (Heesom, Gampel, Mellor, & Denton, 2001) and they are phosphorylated by CDK1 (Velásquez et al., 2016). These confounding results have led some groups to even suggest that cap-dependent translation isn't directly inhibited in mitosis (Velásquez et al., 2016).

What is commonly observed is that a subset of proteins are preferentially translated during mitosis, including c-myc (Pyronnet, Pradayrol, & Sonenberg, 2000) and CDK1 (Marash et al., 2008). The wide-ranging targeting by CDK1 during mitosis
may suggest that these phosphorylation events augment the normal protein synthesis profile seen during interphase to a pro-mitotic profile. Studies mentioned previously and from our lab have identified CDK1 phosphorylation events on proteins involved in translation initiation. Additionally, alternative initiation factors such as DAP5, which we identify as a CDK1 substrate and is found to have activity during mitosis (Liberman, Marash, & Kimchi, 2009), may be involved in the initiation of selective translation during mitosis.

1.4 The Cellular Hypoxia Response

Oxidative phosphorylation is the highly efficient glucose metabolizing process utilized by most aerobic complex multicellular organisms for energy production. Oxygen is required for oxidative phosphorylation, and therefore the regulation of oxygen homeostasis is critical for normal cell survival and growth. However, but this process may be distorted in cancer where oxygen deficiency, or hypoxia, facilitates angiogenesis, immune evasion, and other tumor promoting functions (Hanahan & Weinberg, 2011). The cellular response to low oxygen environments is regulated through the transcription factor hypoxia induced factor 1 (HIF-1).

1.4.1 The HIF-1 Transcription Factor

HIF-1 facilitates the cellular adaptive response to changes in environmental oxygen levels. HIF-1 activity induces the transactivation of more than 1000 genes (Manalo et al., 2005; Schödel et al., 2011; Xia et al., 2009) contained in genetic pathways.
involved in cancer including cell proliferation, invasion, glucose metabolism, and metastasis. Yet individual cells will respond to hypoxia by upregulating only a subset of these genes meaning that the response to hypoxia is cell type specific. The HIF-1 transcription factor complex is composed of two proteins HIF-1α and HIF-1β (G. L. Wang, Jiang, Rue, & Semenza, 1995; Guang L. Wang & Semenza, 1995). In the nucleus the heterodimeric HIF-1 binds to what is known as the hypoxia responsive element (HRE) at a consensus 5'-A/GCGTC-3' sequence. Binding to the HRE facilitates the hypoxia inducible expression of the targeted genes (G. Semenza et al., 1996).

1.4.1.1 HIF-1α activity

Both HIF-1α and HIF-1β are in a family of transcription factors containing basic loop basic loop, PAS (Per, ARNT, Sim), and PAC (PAS-associated C-terminal) domains (G. L. Wang et al., 1995). For HIF-1 to function, both HIF-1α and HIF-1β must bind together in the nucleus at the location of targeted genes (Fig. 5). HIF-1α is a 120kDa protein containing multiple residues that serve as locations for regulatory post-translational modifications, which will be describe in detail later. HIF-1α is expressed at low levels constitutively during normoxia with both expression and stability greatly increased in low oxygen situations. HIF-1α functions as the activator of HIF-1 transcriptional activity and as such, its expression and stability is tightly regulated. This is in contrast to HIF-1β which is found to be constitutively expressed and localized to the nucleus to await activation through HIF-1α association (Figure 5) (G. L. G. Semenza,
2003). Therefore, HIF-1α serves as the master regulator of the hypoxia response. High levels of HIF-1α expression have been observed in several human cancers (Talks et al., 2000; Zhong et al., 1999).

HIF-1α regulates the cellular response to low oxygen by targeting two major types of genes upon activation: genes that increase oxygen delivery and genes that decrease consumption of oxygen. To increase oxygen delivery genes involved in enhancing angiogenesis and vascular permeability are targeted, such as VEGF (Forsythe et al., 1996). To decrease oxygen consumption glycolytic enzymes are targeted to facilitate the switch to glycolytic from oxidative metabolism (J. W. Kim, Tchernyshyov, Semenza, & Dang, 2006). These are not subtle changes to cellular processes; therefore, the on-off mechanism for HIF-1α must be a tightly controlled switch.

Figure 5: HIF-1 Regulation
1.4.1.2 Regulation of HIF-1α

Expression of HIF-1α is increased in response to both MAPK signaling through MEK (Fukuda et al., 2002) and through the phosphatidylinositol 3-kinase (PI3K) pathway (Jiang et al., 2001). HIF-1α also transcriptionally targets itself, creating positive feedback loop to quickly enhance its own activity. To prevent the unwanted activation of HIF-1α and to quickly temper the hypoxia response once normoxia is restored, HIF-1α is rapidly degraded.

HIF-1α is marked for degradation by oxygen-dependent post-translational modifications including hydroxylation at proline 402 and 564 by three prolyl hydroxylase domain proteins (PHD1-3). The PHDs sense oxygen by using it as a reagent in a hydroxylation reaction where oxygen functions as the limiting factor. These events are required for the binding of HIF-1α to the E3 ubiquitin ligase recognition von Hippel-Lindau protein (pVHL), which leads to HIF-1α degradation through the proteasome (Fig. 5) (Ivan et al., 2001; David & Michael, 2001; Yu F, White SB, Zhao Q, 2001). HIF-1α binding to pVHL can be further enhance by acetylation at lysine 532 by the acetyltransferase ARD1 (Fig. 5) (Jeong et al., 2002). Another hydroxylase factor inhibiting HIF-1 (FIH-1) hydroxylates HIF-1α at asparagine 803 preventing it from interacting with co-activators (Fig. 5) (Mahon, Hirota, & Semenza, 2001). Of all the HIF-1α post-translational modifying enzymes only PHD2 is found to be sufficient on its own to
promote pVHL induced HIF-1α ubiquitination and degradation. Thus, PHD2 is considered the key cellular oxygen sensor (Berra et al., 2003).

1.4.1.3 pVHL

Von Hippel-Lindau tumor suppressor protein (pVHL) was described in 1993 as a protein from a mutant gene VHL, that was the cause of a hereditable form of numerous types of highly vascularized tumors (Latif et al., 1993). The full pVHL E3 ubiquitin ligase complex is composed of two transcription elongation factors elongin B and elongin C, which link pVHL to cullin 2 (CUL2) and RBX1 (Lonergan et al., 1998; Latif et al., 1993). Other pVHL targeted proteins have been proposed. For example pVHL binds and thereby sequester phosphorylated Akt (Guo et al., 2016).

1.4.1.4 PHD2

RNAi targeting the PHDs separately has demonstrated that depletion of PHD2 alone is able to induce HIF-1α levels outside of hypoxia or other HIF-1α stimulatory events (Berra et al., 2003). PHD2 also (referred to as EglN1) has be found to induce the majority of HIF-1α hydroxylation under normal oxygen conditions (Minamishima et al., 2008; Takeda et al., 2006).

PHD2 activity is dependent on oxygen as it is a member of the Fe(II) and 2-oxoglutarate-dependent oxygenase superfamily. PHD2 has a Fe(II) catalytic core that binds to molecular oxygen and 2-oxoglutarate with the additional requirement of ascorbate. Through a highly active ferryl intermediate, the molecular oxygen is split,
resulting in hydroxylation of the amino acid residue and the conversion of 2-oxoglutarate to succinate (Schofield & Ratcliffe, 2004). Iron chelators, excess succinate, reactive oxygen species, and nitric oxide all can inhibit PHD2 function (Kaelin & Ratcliffe, 2008).

An interesting physiological implication for PHD2 was shown from two studies looking at high altitude adaptations in populations found in Tibet. These studies identified a variant in the PHD2 gene that arose ~8000 years ago and is associated with the hemoglobin phenotype seen in these populations (Simonson et al., 2016). This missense mutation results in a PHD2 protein that has enhanced catalytic activity during hypoxia (Lorenzo et al., 2014). This expands the importance of PHD2 from a cellular process to the oxygen adaptability of a whole organism.

PHD2 has been shown to be a transcriptional target of HIF-1 (Metzen et al., 2005), acting as part of a negative feedback loop to reduce HIF-1 activity during prolonged periods of hypoxia (D’Angelo, Duplan, Boyer, Vigne, & Frelin, 2003; Henze et al., 2010). However, the regulation of hypoxic responses by protein synthesis remains undefined. This work described in this dissertation describes for the first time a unique translational feedback mechanism through PHD2 to modulate the hypoxia response.

1.4.2 Protein Synthesis in Hypoxia

When cells enter hypoxia, dramatic changes must occur to conserve energy as the loss of oxygen severely limits cellular metabolism. Therefore, much like numerous
cellular stresses, global translation is inhibited during hypoxia, with a select set of proteins chosen for protein synthesis. During hypoxia translation repression occurs through a mechanism similar to what is observed during starvation conditions. This includes the repression of translation by eIF4E-BP1, inactivation of eukaryotic elongation factor 2 (eEF2), and p-eIF2α(S51) (Connolly, Braunstein, Formenti, & Schneider, 2006; Koritzinsky et al., 2006; L. Liu et al., 2006).

Alternative translation initiation pathways have been shown to activate translation of a subset of mRNAs to promote survival, including a switch to cap-independent translation (Braunstein et al., 2007; Young et al., 2008), use of the alternative initiation factors eIF4GIII and eIF4E2 (Ho et al., 2016), and the over expression of initiation factors (Yi, Papadopoulos, Hagner, & Wagner, 2013). My work outlined in the next section demonstrates another mechanism for alternative translation induction during hypoxia, through cap-independent translation by the alternative translation initiation factor, DAP5.

1.5 Introduction to the Dissertation Chapters

Despite more than 20 years since the discovery of DAP5, by four independent groups, many questions remain surrounding this essential translation factor. These questions include, but are not limited to: 1) How is DAP5 activity regulated? 2) What cell conditions/signals activate DAP5? and 3) What transcripts does DAP5 target for translation? Most previous studies have been limited by the inability to look beyond the
loss of eIF4E binding and have pigeon-holed DAP5 as another IRES trans activating factor. Instead, by looking at the unique inducible binding between DAP5 and the critically important initiation factor eIF2, this dissertation begins following a different line of reasoning that leads to new findings and adds to the understanding of translational regulation. These chapters outline here begin by addressing basic questions behind DAP5 molecular biology and leads into unforeseen investigations that link DAP5 function to the master regulator of the hypoxia response (HIF-1α) and translation of the key oxygen sensor PHD2.

Chapter 2 outlines the materials and methods utilized in the completion of this research.

Chapter 3 illustrates the results and is broken into three subsections. The results are adapted from a publication in press in *Molecular and Cellular Biology* (Bryant, Brown, et al. 2018).

In Section 3.1, we investigated DAP5:eIF2β binding to determine the inducibility of this interaction. We also investigate whether DAP5:eIF2β binding impacts global translation.

Based on the finding in the previous section, we begin Section 3.2 by attempting to determine if phosphorylation events on DAP5 influence binding to eIF2β. This initial investigation led to two DAP5 sites, serine 902 and threonine 508. Further investigations
of threonine 508 led to the characterization of DAP5 phosphorylation and binding during mitosis.

In Section 3.3, we expand on the studies in section 3.1 to identify specific translational targets of DAP5. Unexpectedly this leads to the uncovering of a link between DAP5 and HIF-1α. Through thorough follow-up investigations we demonstrate that PHD2, the key oxygen sensor, is a DAP5 translational target inducing a change in HIF-1α. With the strong link between DAP5 and HIF-1α established, we conclude this study with assays to ascertain DAP5 activity and impact during hypoxia.

Chapter 4 details the discussion of the results and future directions that should be done to expand on this study to place these finding in greater context within the protein synthesis and hypoxia fields.
2. Materials and Methods

2.1 Cell lines, Inhibitors, and Stimulants

U87, Du54, and Sum149 lines were a gift of Drs. D. Bigner and S. Nair (Duke University), and MEFs were a gift of Dr. R. Fukunaga (Ueda, Watanabe-fukunaga, & Fukuyama, 2004) and grown in 10% FBS-containing DMEM (Invitrogen) or, for Sum149, DMEM/F-12 (Lonza). Stable doxycycline (dox)-inducible HeLa (Kaiser et al., 2008) and HEK293 (Shveygert et al., 2010) cell lines were grown in DMEM supplemented with 10% FBS, blasticidin S (5μg/ml for HeLa and 15μg/ml HEK293; Sigma-Aldrich), and hygromycin B (100μg/ml; Invitrogen). HEK293 and HeLa cells expressing a shRNA targeting DAP5 or eIF4GI were grown with G418 (500μg/mL; Gibco). Dox (Sigma-Aldrich) was dissolved in water and used at a concentration of 1μg/mL.12-Tetradecanoylphorbol-13-acetate (TPA; Tocris) and anisomycin (Sigma-Aldrich) were dissolved in DMSO. Inhibitors of MEK (UO126, Promega; trametinib, Selleckchem), p38 (BIRB, Selleckchem), JNK (JNKVIII, Calbiochem), CDK1 inhibitor (Ro-3306, Tocris), microtubule polymerization inhibitor (nocodazole, Tocris), and the proteasome inhibitor (MG-132, Tocris) were dissolved in DMSO and used as described. Cell cycle inhibitor thymidine (Alfa Aesar), hydroxylation inhibitor [Cobalt (III) Chloride hexahydrate, CoCl2; Sigma], and protein synthesis inhibitor (puromycin, Sigma-Aldrich) was dissolved in water and used as described.
2.2 Expression Plasmids and Stable Cell lines

Construction of myc- and Flag-tagged eIF4GI expression plasmids (eIF4GI, eIF4GI(682-1600), and eIF4GI(197-1600) was described previously (M. Dobrikov et al., 2011). The pCI-Flag-DAP5 vector was a generous gift from Martin Holcik (Univ. of Ottawa, Canada); the PHD2 [EGLN1(myc-DDK-tagged) pCMV6-entry], and eIF2β [eIF2S2 (myc-DDK-tagged) pCMV6-entry) vectors were obtained from Origene (#RC215158 and #RC201563, respectively). DAP5, PHD2, and eIF2β were cloned out of original vectors into pcDNA5/FRT/TO expression plasmid. The DAP5 truncation variant was generated by PCR amplification of corresponding fragment. Single amino acid mutations were introduced by overlapping PCR as described earlier (M. Dobrikov et al., 2011) and confirmed by sequencing. The generation of stable HEK293 and HeLa cell lines with dox-inducible expression of myc/Flag-tagged eIF4GI and HEK293 expressing Flag-tagged DAP5 has been described previously (Brown, Bryant, et al., 2014). DAP5 depletion/knock-in HeLa and HEK293 cell lines were established similar to eIF4GI as described earlier (Brown & Gromeier, 2017; M. I. Dobrikov et al., 2014). Primers used in this study are shown in Table 1.
### Table 1: Primers used in study

<table>
<thead>
<tr>
<th>Name/Number</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 - DAP5_1360_Stu_5</td>
<td>cgaaggatagccacctcggg</td>
</tr>
<tr>
<td>2 - BGH_Rev</td>
<td>tagaaggcacagtgcagg</td>
</tr>
<tr>
<td>3 - DAP5(E862K)_anti</td>
<td>cttctttaaatgcctcatgtcata</td>
</tr>
<tr>
<td>4 - DAP5(E862K)_sense</td>
<td>gggaaatttaagaagacttccttgac</td>
</tr>
<tr>
<td>5 - DAP5(1-790)_Not1_3'</td>
<td>atgcggccgcttattattcatcgctgggtgggtttacttcac</td>
</tr>
<tr>
<td>6 - DAP5(S902A)_Not1_3'</td>
<td>gttcgccgcgctgttcagcttcttcctcttgtcttccttcac</td>
</tr>
<tr>
<td>7 - DAP5(S902D)_Not1_3'</td>
<td>gttcgccgcgctgttcagcttcttcctcatcttcttcttcag</td>
</tr>
<tr>
<td>8 - eIF4G(S1597A)_3'</td>
<td>tcctcgaggtgtgttgtcgctctctctcttcg</td>
</tr>
<tr>
<td>9 - eIF4G(S1597E)_3'</td>
<td>tcctcgaggtgtgttgtcgctctctctcttcg</td>
</tr>
<tr>
<td>10 - DAP5(T508A)_anti</td>
<td>gaggtggtgtgtgtggtgctggtgtggtggtggtggtggt</td>
</tr>
<tr>
<td>11 - DAP5(T508A)_sense</td>
<td>cactcaagccacaccttgagacag</td>
</tr>
<tr>
<td>12 - DAP5(T508E)_anti</td>
<td>gaggtggtgtgtgtggtgctggtgtggtggtggtggtggt</td>
</tr>
<tr>
<td>13 - DAP5(T508E)_sense</td>
<td>cactcaagccacaccttgagacag</td>
</tr>
<tr>
<td>14 - DAP5(T508E)_shRNA_5'</td>
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<tr>
<td>17 - DAP5(3366)_shRNA_3'</td>
<td>gccctcgagactggggaacacgtgtcatctcactggcttcag</td>
</tr>
</tbody>
</table>

### 2.3 Antibodies, Immunoprecipitation, Immunoblots, siRNA Transfection, and RT-qPCR

Antibodies against eIF4A, eIF3a, MNK1, eIF2α, p-eIF2α(S51), rpS6, p-rpS6(240/244), DAP5, eIF4GI, p-ERK1/2(T202/Y204), eIF5, ERK1/2, CK-2α, GAPDH, p-p38(T108/Y182), p38, pT*PP substrate, p-cJun(S73), cJun, p-Histone H3(S10), Histone H3, p-JNK(T183,Y185), COX2, MKP-2, HIF-1α, PHD2, FIH1, ARD1, VHL, OH-HIF1α(P564), rpL13a, p-Akt(T308), Akt1, BNIP3 (Cell Signaling Technologies); eIF2β, eIF2γ, CK-2β, PHD3 (Novus); Flag, tubulin, myc (Sigma-Aldrich); Puromycin (EMD Millipore); PHD1
(R&D Systems) were used. Two ~70% confluent 150mm dishes were treated as described in the figures and legends and IPs were performed as previously described (M. I. Dobrikov et al., 2013). Immunoblots were performed as described before (M. Dobrikov et al., 2011). Immunoblot signals were obtained on and quantified using the Li-COR Odyssey FC2 imaging system and Image Studio software. DAP5 depletion from U87, Du54, Sum149, and MEFs was performed using siRNAs. For this, cells in 6 well plates were transfected with 2μl of 20μM DAP5 (GE Dharmacon) or All-stars control siRNA (QIAGEN) using jetPRIME transfection reagent (Polyplus transfections) following the manufacturer’s protocol. For RT-qPCR analysis, the RNA to CT one step kit (Invitrogen) was used following the manufacturer’s protocol on a 7900 HT TaqMan machine; samples were measured in triplicate and the average value was used for further calculation. For analysis of PHD2 mRNA levels following DAP5 depletion, data were analyzed by calculating the $2^{\Delta\Delta CT}$ method using GAPDH as a reference. To analyze polysome profile-derived RNA, the $2^{CT}$ value of each fraction was divided by the sum of $2^{CT}$ values for all samples and multiplied by 100 to determine the %RNA in each fraction.

2.4 In vitro Phosphorylation Assays and Phospho-proteomic Analysis

For in-vitro phosphorylation and proteomic analyses, Flag-tagged DAP5 was immunoprecipitated from dox- induced cells and eluted from beads using Flag-peptide (Sigma-Aldrich) in NT-2 buffer [50mMTris-HCl (pH7.5), 150mM NaCl, 1mM MgCl2,
0.05% Igepal CA-630] at 4°C (16h). Flag-affinity beads were removed using 30-μM chromatography columns (Thermo-Fisher), and eluted proteins were concentrated using a 10kDa cutoff protein concentration column (Millipore). In vitro phosphorylation of recombinant Flag-tagged DAP5 by recombinant JNK2, or CDK1/cyclin A1 (SignalChem) was performed in 15μl in kinase assay buffer (SignalChem) with 2mM ATP, 0.2mM dithiothreitol, and 2X HALT protease and phosphatase inhibitor cocktail (Thermo Scientific). The reaction mixture was incubated at room temperature with 1 or 5μl of recombinant kinase (0.1μg/μl; 1h). Samples were mixed with 5μl of 4X LDS buffer (Invitrogen) with 2.5% β-mercaptoethanol and heated for 1 min at 95°C to stop the enzymatic reaction, followed by immunoblot. Fifteen ~70% confluent 150mm dishes of cells were treated with TPA as described in figures and figure legends. Flag-IP followed by Flag-elution purification was performed with final purification and concentration of sample into 50mM NH₄HCO₃, pH8.0. Proteomic analyses were carried out at the Duke University Proteomic Core Facility as previously described in (M. I. Dobrikov et al., 2014).

2.5 Polysome Profiling

Cells in confluent 100mm³ plates treated with or without dox were harvested and lysed in 1ml lysis buffer [50mM HEPES (pH7.2), 200mM KOAc, 0.5% NP40 (Sigma), 1X HALT protease and phosphatase inhibitor, 1X RNAseOUT (Thermofisher Scientific), 50μg/mL cycloheximide, 10mM MgCl₂]. Sucrose gradients (15-50%) were prepared in
lysis buffer without detergent; ~80% of total lysate (800μl) was loaded onto the gradients and spun at 35,000 rpm (SW41 rotor) for 180 min at 4°C. A Teledyne instrument was used to analyze and create 500μl fractions. RNA was isolated using TRIzol (Invitrogen) followed by RNAeasy column purification (QIAGEN). RNA was suspended in 25μl RNAase-free water and stored at -80°C. Protein was extracted by first diluting a split fraction 1:1 with sterile water, then adding 0.11 volumes of trichloro-acetic acid and incubation at 4°C (1h). Samples were pelleted at 16,000g for 25min at 4°C and the supernatant was discarded. The pellets were washed in ice-cold acetone, spun again for 10min, dried and processed for immunoblot.

2.6 Hypoxia Treatment

Cells were grown in 6 well plates or 150mm dishes (for IP) and were treated prior to hypoxia in a chamber (Coy Laboratory Products, Inc) as described in figures and figure legends. Cells were then moved into the inner hypoxia chambers which maintained cells at 37°C, 5% CO₂ and 0.5% oxygen. The inner chamber is located within a larger chamber with arm-ports that is maintained at 8% oxygen. Once cells are placed in the inner chambers and the desired oxygen concentration is reached, the inner chamber remained closed and cells were not manipulated until lysis. To lyse cells, the growth media was rapidly removed and 150μl LDS buffer (for immunoblot) or 800μl polysomal lysis buffer (PLB for IP) [10mM HEPES (pH 7.4), 100mM KCl, 5mM MgCl₂,
0.5% Igepal CA-630 (Sigma), 3mM dithiothreitol, 1X HALT phosphatase inhibitor cocktail) was added to cells. Lysates were placed on dry ice.

### 2.7 Statistics

Statistical analysis was performed using JMP (v.13) software (SAS). All error bars represent SEM. For multiple group comparisons, all groups from each experimental repeat were compared using ANOVA. If the ANOVA test was significant (p<0.05), post hoc Tukey’s HSD was performed. Paired t-tests were used for data comparing only two groups. All asterisks denote a significant p-value defined by p<0.05.
3. Results

3.1 PKC-Raf-ERK1/2 Signaling Induced DAP5:eIF2β Binding impacts Global Translation

3.1.1 Introduction

The eIF4G isoforms are key components for adaptive protein synthesis. They are responsible for controlling the association of translation initiation machinery with mRNAs, the unwinding/scanning of the 5’UTR, and eIF3/40S ribosomal subunit recruitment. There are three eIF4G isoforms in mammals, eIF4GI, eIF4GII, and DAP5 (Bellsolell et al., 2006). They share similar roles in core initiation functions, mediated by association with mRNAs: anchoring the eIF4A:4B translation initiation helicase cofactors, and recruiting eIF3/40S ribosomal subunit to the mRNA. This study focuses on the understudied isoform DAP5, which is homologous to the C-terminal ~two thirds of eIF4GI/II; therefore, DAP5 lacks their N-terminal domain that contains binding motifs for the 5’ 7-methyl-guanosine cap-binding protein (eIF4E) and the poly(A) binding protein (PABP) (Fig. 6).

Also unlike eIF4GI/II, DAP5 binds to eIF2β (S. H. Lee & McCormick, 2006), which in a complex with eIF2γ and -α (comprising eIF2) delivers the translation initiating Met-tRNAi^{Met} to 40S ribosomal subunits (Asano, Clayton, Shalev, & Hinnebusch, 2000). DAP5 has been implicated both in homeostatic global protein synthesis (S. H. Lee & McCormick, 2006; Yamanaka et al., 2000) and in context-specific, m^7G-cap independent initiation for select templates (Marash et al., 2008). DAP5’s role in
translation is unique, as eIF4GI overexpression cannot compensate for DAP5 depletion (Ramírez-Valle et al., 2008).

The eIF4G isoforms are ubiquitously expressed, further accentuating their crucial role in normal cellular function. The eIF4GI:eIF4GII:DAP5 ratio in Hela cells is shown to be ~10:1:45 (Nagaraj et al., 2011). However, the significance of the eIF4G isoform spectrum, their functional/structural variances, and their expression divergences are not understood. Our studies in this section explore DAP5’s unique binding to eIF2β and show that pro-growth signaling from PKC-Raf-ERK1/2 induces DAP5:eIF2β binding with this binding required for DAP5-mediated translational activity.

![Figure 6: Schematic representation of DAP5 and eIF4GI(683-1600) domain arrangements, protein interactions and major Ser/Thr phosphorylation sites.](image-url)
3.1.2 Results

3.1.2.1 DAP5:eIF2β binding is induced by PKC-Raf-ERK1/2 signaling

Previous studies identified binding between DAP5 and eIF2β as possibly involved in DAP5-mediated translation initiation (S. H. Lee & McCormick, 2006; Liberman et al., 2015). Analogous to eIF4GI association with MAPK-interacting kinase (MNK) (Shveygert et al., 2010) eIF2β binding with DAP5 occurs at two conserved C-terminal aromatic and acidic (AA) boxes (Fig. 6). Since eIF4GI:MNK binding strongly responds to PKC-Raf-ERK1/2 signals (Brown & Gromeier, 2017; M. Dobrikov et al., 2011; Shveygert et al., 2010), we hypothesized that DAP5:eIF2β binding might be similarly regulated. In order to test this we created stable HEK293 cell lines with doxycycline (dox)-inducible expression of Flag-tagged wildtype (wt) DAP5; eIF4GI-Ct(aa683-1600; i.e. the C-terminal portion of eIF4GI homologous to DAP5); DAP5(1-790; DAP5 lacking the C-terminal AA boxes); and DAP5(E862K; a DAP5 point mutant that lacks eIF2β binding, but maintains all other DAP5 binding functions) (Liberman et al., 2015) (Fig. 6, 7A). The cells were dox-induced, treated with 12-O-tetradecanoylphorbol-13-acetate (TPA) to activate PKC, and proteins were isolated by Flag-immunoprecipitation (IP). TPA-responsive eIF2 binding (shown by eIF2β and eIF2α co-IP) binding occurred only with wt DAP5 (Fig. 7A). TPA induced eIF4GI-Ct binding to MNK1 but not eIF2β (Fig. 7A). DAP5(1-790) and DAP5(E862K) failed to bind eIF2β (Fig. 7A). However, DAP5(1-790) also displayed significantly decreased TPA-responsive rpS6 and eIF3a co-IP, suggesting that the C-terminal truncation broadly compromises DAP5 functional integrity (Fig. 7A).
Figure 7: Phorbol ester stimulation promotes DAP5-eIF2β binding.
A, B. HEK293 cells expressing (A) Flag-tagged DAP5, eIF4G1-Ct, DAP5(1-790), DAP5(E862K) or (B) eIF2β were induced with dox (16h) before stimulation with TPA as shown. Cell lysates were analyzed by immunoblot or subjected to anti-Flag IP followed by immunoblot. Data bars in (A) depict mean ratio of eIF2β/Flag signal after 240min TPA treatment from three assays. Experiments were normalized by setting the Flag-DAP5 (wt) ratio for each experiment to 1 and a representative assay is shown; error bars represent standard error of the mean (SEM).
TPA treatment for 15 min induced ERK1/2 phosphorylation, but diminished ribosomal protein S6 (rpS6) and eIF3a co-IP with all DAP5 and eIF4GI variants, indicating reduced association with eIF3/40S ribosomal subunit (Fig. 7A). This coincided with similarly reduced MNK1:eIF4GI and wt DAP5:eIF2β binding (Fig. 7A). Detailed kinetics of the TPA response indicated transient interruption of translation activity, possibly related to a short-lived peak of p-eIF2α(S51) 15min after TPA treatment (Fig. 8). We believe that this accounts for the temporary slump of DAP5 and eIF4GI interactions with eIF2β, MNK, rpS6, and eIF3a at 15min post TPA (Fig. 7A). Binding of all DAP5 variants and eIF4GI to eIF4A was only slightly variable throughout the period of TPA stimulation (Fig. 7A); eIF4A interactions with DAP5 and eIF4GI do not respond to PKC-Raf-ERK1/2 (Fig. 6) (M. I. Dobrikov et al., 2013). The same pattern of TPA-responsive interactions occurred upon inverse co-IP of endogenous DAP5 with dox-inducible Flag-eIF2β (Fig. 7B).
Figure 8: Time course of DAP5:eIF2β binding upon TPA stimulation.
HEK293 cells with dox-inducible Flag-DAP5 expression were treated with dox (16h) before stimulation with TPA as indicated. Cell lysates were analyzed by immunoblot or subjected to anti-Flag IP followed by immunoblot. The experiment was repeated three times; a representative assay is shown.

3.1.2.2 DAP5 depletion inhibits TPA-induced global translation

To identify the role of DAP5:eIF2β binding in translation, we employed stable HeLa cell lines with dox-inducible eIF4GI or DAP5 shRNA confirmed to reduce endogenous DAP5 levels. (see Materials and Methods). Treatment with dox (96h) resulted in similarly robust reduction of eIF4GI or DAP5 levels (Fig. 9A). The cells were stimulated with TPA and 15 min prior to harvest, puromycin was added. Puromycin functions as a translation elongation inhibitor which results in peptides being
puromycylated and subsequent elongation termination. Consequentially this leads to an accumulation of incomplete proteins containing a terminally incorporated puromycin. The abundance of puromycylated translation products were quantified by immunoblot (Fig. 9B) (Aviner, Geiger, & Elroy-stein, 2013). DAP5 depletion reduced global translation by ~20% in unstimulated cells and strongly inhibited (~40% at 4h) the translation stimulatory effect of TPA (Fig. 9A, B). Confirming the exalted role of eIF4GI in global protein synthesis, ~90% eIF4GI depletion caused a robust decrease of TPA-induced translation.
Figure 9: DAP5 depletion attenuates global translation.
A. HeLa cell lines with dox-inducible eIF4GI or DAP5 depletion were treated with or without dox (96h), stimulated with TPA as indicated, and treated with puromycin (5μM; 15 min) prior to harvest. Puromycylation was stopped by washing cells with cold PBS and the addition of lysis buffer. A representative assay is shown. B. Mean quantification of puromycin signal (puro:x)/tubulin ratio from three experiments is shown, normalizing between experiments by setting the zero time-point, no-dox value to 1; error bars denote SEM.

3.1.2.3 Loss of DAP5:eIF2β association results in decreased global translation

The pattern of the global protein synthesis response to TPA closely mirrors DAP5:eIF2β binding (Fig. 9A; compare Fig. 7A and B; Fig. 8). To test directly if
DAP5:eIF2β binding explains DAP5's role in global translation, we established HEK293 cells with dox-inducible DAP5 knockdown combined with simultaneous wt DAP5 or DAP5(E862K) knock-in. We previously reported on this approach with dox-inducible (endogenous) eIF4GI "knockdown/knock-in" of Flag-tagged eIF4GI variants (M. I. Dobrikov et al., 2014). In these cell lines a dox-inducible shRNA targeting endogenous DAP5 is expressed along with exogenous wt-DAP5 or a DAP5 mutant. Due to the timing for the DAP5 knockdown to occur (~96h) the resulting cell line has a overabundance of the exogenous "knock-in" DAP5 which may result in effects due to stoicheometric imbalances. Reconstitution of wt DAP5 in DAP5-depleted cells rescued protein synthesis, while knock-in of the DAP5(E862K) mutant severely repressed global translation beyond levels seen with DAP5 depletion alone (Fig. 10A and B). Our observations indicate that DAP5 participates in mediating the adaptive protein synthesis response to PKC-Raf-ERK1/2 signaling, and that DAP5:eIF2β binding is required for this function.
Figure 10: DAP5:eIF2β binding mutant represses global translation.
A. HEK293 cells with dox-inducible DAP5 depletion combined with mock (-), wt DAP5 or DAP5(E862K) reconstitution were dox-treated (96h) prior to puromycin. A representative assay is shown. B. Mean quantification of Puro/x/tubulin ratio is shown for three experiments, normalizing by setting the no-dox ratio to 1; error bars denote SEM.

3.1.3 Discussion

eIF4G activity has been shown to be bolstered during PKC-Raf-MEK-ERK1/2 signaling (M. Dobrikov et al., 2011; M. I. Dobrikov et al., 2013, 2014). Using a similar approach we apply phorbol ester-mediated PKC-Raf-ERK1/2 stimulation to decipher principles of DAP5 function. We discovered that the contribution of DAP5 to global translation is enhanced by TPA, and correlates with a surprising induction of DAP5:eIF2β binding. Further investigation revealed that the binding between DAP5 and the translation initiation linchpin, eIF2, is required for DAP5 mediated translation. Intriguingly, DAP5 has been shown to be cleaved during apoptosis in a manner which removes the eIF2β binding domain (Nousch et al., 2007); suggesting that this
physiological cleavage event may be occurring to regulate DAP5 mediated translation. The unique interaction of DAP5 with eIF2β represents a novel, mechanism of mRNA:ribosome tethering and translation initiation utilized by DAP5.

**3.2 Phosphorylation of DAP5 regulates DAP5:eIF2β binding**

**3.2.1 Introduction**

Interactions between translation initiation factors are commonly dictated by cell signaling to mediate context-appropriate protein synthesis output (P. P. Roux & Topisirovic, 2012) making the study of the cell signaling/translation relationship important. However, the regulation of DAP5:eIF2β binding as a result of activated signaling pathways has not been investigated. The homologous interaction to DAP5:eIF2β, eIF4GI:MNK1 is regulated by a complex network of eIF4GI phosphorylation sites, (M. Dobrikov et al., 2011; M. I. Dobrikov et al., 2013, 2014; Raught et al., 2000; Shveygert et al., 2010) with many of these sites corresponding to potential phosphorylation sites on DAP5 (Fig. 6).

Two particular regions of eIF4GI—the inter-domain linker (IDL) and the AA boxes—harbor regulatory phosphorylation events for eIF4GI:MNK1 binding (Fig. 6). In the IDL there are numerous sites including S1186 and S1232 that were found to be phosphorylated by ERK1/2 (M. Dobrikov et al., 2011) and the mitotically-active CDK1 (M. I. Dobrikov et al., 2014). eIF4GI and DAP5 both contain a nearly identical serine residue (DAP5 S902, and eIF4GI S1597) located in the AA box/MNK1/eIF2β binding
domain (Fig. 6). Furthermore, eIF4GI(S1597) has been identified as a CK-2 substrate (M. I. Dobrikov et al., 2013).

To test our hypothesis that DAP5 phosphorylation, similar to eIF4GI is involved in translational activity, we used a phospho-proteomic assay to identify phosphorylation events in DAP5. This approach identified two phosphorylation-sites: DAP5(T508) in the IDL, and the previously mentioned DAP5(S902) which is homologous to eIF4GI(S1597) (Fig. 6). Using follow-up mutational analyses, we determined the role each site plays in DAP5:eIF2β binding. Now that the sites were determined to be important for DAP:eIF2β binding we then were interested in the signaling cascades that target these sites. We investigated predicted kinases through the application of small molecular inhibitors and in-vitro phosphorylation assays and determined that the inhibition of MEK-ERK1/2 signaling can prevent DAP5:eIF2β binding and that T508 is a CDK1 phosphorylation site.

3.2.2 Results

3.2.2.1 DAP5 is phosphorylated following PKC-Raf-ERK1/2 Signaling

MNK binding to the AA boxes of eIF4GI is, in part regulated by PKC-Raf-ERK1/2 signals to eIF4GI (M. Dobrikov et al., 2011; M. I. Dobrikov et al., 2013; Shveygert et al., 2010). To try and identify DAP5 phosphosites we analyzed the DAP5 sequence data from previous high-throughput phospho-proteomics (https://www.phosphosite.org); however, none of the identified sites have been interrogated in detail. Therefore, we
used our own targeted, unbiased approach. We performed LC-MS phospho-proteomics of IP-purified Flag-DAP5 from dox-induced, TPA/mock-stimulated HEK293 cells. Two prominent phosphosites were detected in the TPA-stimulated samples: T508 and S902 (Fig. 11A). These are among the few commonly identified DAP5 phosphosites in reported high-throughput mass spectrometry studies (https://www.phosphosite.org). DAP5(T508) is located in the interdomain linker (connecting HEAT1 and 2 domains; Fig. 6). The corresponding region in eIF4GI has clusters of multiple mitogen-responsive phosphosites, including S1186, which is phosphorylated by PKCα upon TPA stimulation, and influences MNK binding to eIF4GI (Fig. 11B) (M. Dobrikov et al., 2011). DAP5(S902) is in the very C-terminus, within the binding motif for eIF2β. This region is highly conserved between DAP5 and eIF4GI, with eIF4GI(S1597) sharing almost identical location and amino acid sequence context (Fig. 11B). We next sought to determine whether these phosphorylation events control DAP5 association with eIF2β.
Figure 11: Identified DAP5 phosphorylated peptides

A. HEK293 cells were dox-induced for Flag-DAP5 expression (24h), treated with TPA (240min), harvested, and subjected to Flag-DAP5 IP. IP-isolated complexes were digested with trypsin, followed by TiO$_2$ enrichment and LC-MS/MS analysis (Done by Dr. Erik Soderblom and the Duke Proteomics and Metabolomics core facility). Amino acid sequences of phospho-peptides identified by LC-MS/MS are shown; * indicate phosphorylated amino acids. The MASCOT$^a$ ion score is calculated using the equation: $-10\log_{10}(P)$, where $P$ is defined as the absolute probability of the observed match being a random event (Beausoleil, Villén, Gerber, Rush, & Gygi, 2006). DAP5(T508)/(S902) were identified as being phosphorylated by this analysis using a cutoff MASCOT score of 20.

B. Location of DAP5(T508)/(S902) relative to eIF4GI(S1186)/(S1597).

3.2.2.2 DAP5(S902) is phosphorylated by CK-2α

DAP5(S902) and eIF4GI(S1597) are within ideal consensus sequence for casein kinase-2α (CK-2α) (Fig. 12A) (Litchfield, 2003). Indeed, previous investigations showed that eIF4GI(S1597) is phosphorylated by CK-2α (M. Dobrikov et al., 2013), but the impact phosphorylation on this site has in regard to eIF4GI:MNK binding has not been investigated. To test if DAP5(S902) phosphorylation regulates DAP5:eIF2β binding, HEK293 cells with dox-inducible expression of a non-phospho-mutant DAP5(S902A) or a phospho-mimic (S902D) were generated. DAP5(S902A) did not bind eIF2β/α in
response to TPA treatment, while the S902D phospho-mimic retained TPA-responsive binding (Fig. 12B).
Figure 12: Phosphorylation of DAP5(S902) by CK-2α is required for eIF2β binding.
A. S902/S1597 anchor consensus CK-2α substrates at the DAP5/ eIF4GI C-termini. B. HEK293 cells expressing dox-inducible Flag-tagged DAP5, DAP5(S902A), or DAP5(S902D) were dox-treated (16h) before stimulation with TPA. Cell lysates were analyzed by immunoblot or subjected to anti-Flag IP/immunoblot. Ratios of eIF2β:Flag after TPA (240min) were quantified across three repeat assays. HEK293 cells treated with TPA (240min) were normalized to 1, with SEM indicated. C. Dox-inducible Flag-DAP5 or -eIF4GI(197-1600) expressing HEK293 cells were treated with dox (16h) and TPA or anisomycin (Aniso) as shown. (* indicates a non-phosphorylated p38 band). Lysates were tested as in (B). All experiments were repeated three times with representative assay shown.
An analogous approach demonstrated that CK-2α catalyzed p-eIF4GI(S1597) controls eIF4GI:MNK1 interactions (Fig. 13). Co-IP of CK-2 with DAP5/eIF4GI further supports CK-2α involvement in phosphorylation of DAP5(S902)/eIF4GI(S1597) (Fig. 12C). Our findings suggest that a conserved mechanism regulates DAP5:eIF2β and eIF4GI:MNK1 binding via CK-2α sites within the binding regions for eIF2β/MNK1. CK-2 is considered constitutively active, with no known stimuli (Litchfield, 2003), suggesting that DAP5(S902) and eIF4GI(S1597) do not contribute to TPA-induced binding of eIF2β or MNK1.
Figure 13: Phosphorylation of eIF4GI(S1597) by CK-2α is required for MNK1 binding.
HEK293 cells expressing dox-inducible Flag-tagged eIF4GI, eIF4GI(S1597A), or eIF4GI(S1597E) were treated with dox (16h) before stimulation with TPA or Aniso as shown; lysates were analyzed by immunoblot or subjected to anti-Flag IP followed by immunoblot. The experiment was repeated three times with representative assay shown. Ratios of MNK1:Flag after anisomycin (0.5h) were quantified across three repeat assays, with SEM indicated. (Data from Dr. Sarah L. Gemberling former Gromeier Lab member)

3.2.2.3 Phosphorylation of DAP5(T508) enhances DAP5:eIF2β binding
To determine the role phosphorylation of the phospho-proteomic identified inter-domain linker site DAP5(T508), inducible HEK293 cell lines were made that expressed a non-phospho mutant DAP5(T508A) or a phospho-mimic DAP5(T508E).
After TPA stimulation for 240min, the DAP5(T508E) mutant showed an increase in DAP:eIF2β binding verses wildtype DAP5; meanwhile, T508A showed little change in binding (Fig. 14). This suggests that T508 phosphorylation facilitates DAP5:eIF2β binding activity but is not required for DAP5:eIF2β binding.

![Figure 14: Phosphorylation of DAP5(T508) enhanced DAP5:eIF2β binding](image)

HEK293 cells expressing Flag-tagged wt DAP5, DAP5(T508A) or DAP5(T508E) were dox-induced (16h) before stimulation with TPA. Cell lysates were analyzed by immunoblot or anti-Flag IP followed by immunoblot. Data bars represent the average ratio of eIF2β/Flag IP signal at 240min TPA, normalizing each experiment by setting the eIF2β/Flag ratio for DAP5 T508 to 1. The experiment was repeated three times; data bars represent the mean, error bars denote SEM.
3.2.2.4 MEK-ERK1/2 induces DAP5:eIF2β binding

We next addressed a possible role for p-DAP5(T508) in TPA-induced DAP5:eIF2β binding. Kinase prediction (http://www.phosphonnet.ca) indicated DAP5(T508) as a likely target of MAPKs or CDKs (Fig. 15A). First, to reliably detect phosphorylated DAP5(T508), we successfully validated a Cell Signalling antibody that recognizes a p-T*PP motif. This probe only detected signal in the Flag-IP of wt p-DAP5(T508), but not the T508A/E mutants (Fig. 15B). Then we investigated the role of ERK, p38, and JNK MAPKs in DAP5(T508) phosphorylation and DAP5:eIF2β binding. HEK293 cells with dox-inducible Flag-DAP5 expression were pretreated for two hours with DMSO (mock treatment); the MEK1/2 inhibitors UO126 or trametinib; the p38 inhibitor BIRB796; or the JNK1/2 inhibitor JNK inh. VIII (Fig. 15B) prior to TPA stimulation (4h). The inhibitors exhibited the expected signaling effects; both MEK1/2 inhibitors also blocked JNK signaling (Fig. 15C; Input).

Our assay revealed that none of the MAPK inhibitors altered p-DAP5(T508) levels (Fig. 15C), yet the MEK1/2 inhibitors completely abolished DAP5:eIF2β binding (Fig. 15C). This data suggests that DAP5:eIF2β association upon TPA stimulation is controlled by MEK1/2-ERK1/2, but does not hinge on DAP5(T508) phosphorylation. Tests in TPA-stimulated (240 min) HEK293 wt DAP5(T508), DAP5(T508A) or DAP5(T508E) did show possible correlation of eIF2β binding with p-T508 (Fig. 14). To confirm ERK1/2-regulation of DAP5:eIF2β binding is independent of DAP5(T508),
HEK293 cells with dox-inducible DAP5(T508E) were pretreated (2h) with DMSO or trametinib (Fig. 15D). ERK1/2 inhibition blocked DAP5(T508E):eIF2β binding (Fig. 15D).

There are a large number of confirmed and putative MEK1/2-ERK1/2-responsive phosphorylation sites in multiple translation initiation factors (M. I. Dobrikov et al., 2013, 2014; P. Roux & Blenis, 2004; D. Shahbazian et al., 2010; David Shahbazian, Parsyan, Petroulakis, Hershey, & Sonenberg, 2010). This complexity makes defining the precise MEK1/2-ERK1/2-induced events that promote DAP5:eIF2β association a daunting enterprise.
Figure 15: MEK-ERK1/2 induces DAP5:eIF2β binding

A. The top 24 predicted kinases for DAP5(T508) (http://www.phosphonet.ca; Kinase Predictor V2 scores). B. Flag-DAP5(T508/A/E) IPs show specificity of an anti-p-T*PP motif antibody for T508. C. HEK293 expressing Flag-DAP5 were dox-induced (16h) and pretreated with UO126, trametinib, BIRB796, JNK inhibitor VIII, or DMSO (2h) prior to TPA-stimulation. Cell lysates were analyzed by immunoblot or anti-Flag IP followed by immunoblot. D. HEK293 cells with dox-inducible Flag-DAP5(T508E) were treated with dox (16h), pretreated with DMSO or trametinib (2h) prior to TPA stimulation and analyzed as in (C). All experiments were repeated three times with representative assay shown.

3.2.2.5 DAP5(T508) phosphorylation is induced during mitosis

Since DAP5(T508) is not a MAPK substrate, we tested if T508 phosphorylation and DAP5:eIF2β binding respond to CDK1. Specific activity of DAP5 during mitosis has been suggested previously (Marash et al., 2008). HEK293 cells with dox-inducible Flag-DAP5 expression were treated with dox and synchronized using thymidine block.
Synchronized and dox-treated cells were then grown in fresh media. The cells were arrested in G2 using the CDK1 inhibitor Ro3306 (Vassilev et al., 2006) (Fig. 16A); or arrested in mitosis using nocodazole as confirmed by an increase in p-histone H3 (Fig. 16A). G2-arrest decreased p-DAP5(T508) phosphorylation and DAP5:eIF2β binding; however, mitotic arrest strongly enhanced both events (Fig. 16A). To test if CDK1 phosphorylates T508, Flag-DAP5 was purified from dox-induced HEK293 cells and incubated with active recombinant JNK2 or CDK1 in the presence of ATP (Fig. 16B). DAP5(T508) was phosphorylated only upon incubation with CDK1 (Fig. 16B).

Collectively, our data indicates that DAP5:eIF2β binding is inducible and strongly context-specific; occurs with MEK1/2-ERK1/2 or CDK1 activation; and in mitosis correlates with increased DAP5(T508) phosphorylation.
Figure 16: DAP5(T508) is phosphorylated by CDK1 during mitosis

A. HEK293 cells were treated with thymidine (4mM; 16h), followed by media change, and Ro3306, nocodazole, or DMSO as shown. Cells were treated with dox (16h) prior to harvest. Cell lysates were analyzed by immunoblot or anti-Flag IP followed by immunoblot. Data bars (right panels) represent the mean ratio of phospho-T*PP/Flag and eIF2β/Flag. Data were normalized between experiments by setting the ratios for cells treated with thymidine block alone to 1. B. In vitro phosphorylation assay of Flag-DAP5 with recombinant JNK2 (control) or CDK1. Data bars represent the mean ratio of p-T*PP [p-DAP5 (T508)]/Flag. Data were normalized between experiments by setting the kinase/-ATP control to 1. Experiments were repeated three times and a representative assay is shown; data bars represent the mean and error bars denote SEM.
3.2.3 Discussion

The phosho-proteomic analysis of DAP5 identified two prominent phosphorylated sites, T508 and S902 (Fig. 11A), both sites were among the most commonly identified in previous high-throughput mass spectrometry studies (www.phoshpsite.org). DAP5(T508) is located in the IDL, a region in both DAP5 and eIF4GI that has clusters of phosphorylation sites, including eIF4GI(S1186) which is phosphorylated by PKCα during TPA treatment (Fig. 11B)(M. Dobrikov et al., 2011). DAP5(S902) is located in the very C-terminus, specifically in the eIF2β binding domain (Fig. 6). This region is highly conserved between DAP5 and eIF4GI, with eIF4GI(S1597) sharing almost identical location and amino acid sequence context with DAP5(S902) (Fig. 11B). We sought to determine whether these phosphorylation events serve as regulators of DAP5:eIF2β binding.

DAP5(S902) phosphorylation by CK-2α was required for DAP5:eIF2β binding (Fig. 12B); similarly, a homologous CK-2α phosphorylation of eIF4GI(S1597) was required for eIF4GI:MNK1 binding (Fig 13). The kinase CK-2 is constitutively active (Litchfield, 2003) suggesting that these CK-2 sites are commonly phosphorylated, allowing DAP5 to bind to eIF2β and eIF4GI to bind to MNK1. These results illustrate that in DAP5 eIF2β binding is regulated through at least one conserved mechanism as eIF4GI:MNK1 binding, but DAP5(S902) does not explain mitogenic induction of DAP5:eIF2β binding following TPA treatment.
The second identified DAP5 phosphorylation site T508 was then investigated. The phospho-mimic form of DAP5(T508), DAP5(T508E) increased while the non-phosphorylatable mutant (T508A) reduced eIF2β binding (Fig. 14). This suggests that T508 phosphorylation may facilitate DAP5:eIF2β binding and activity, but is not required for DAP5:eIF2β binding. Kinase prediction software suggested MAPKs and the related CDKs as potential T508 kinases (Fig. 15A).

Using a phosphorylation site specific antibody (Fig. 15B) and selective inhibitors of the MAPKs ERK1/2, JNK, and p38 we assayed for specific activity at DAP5(T508). Surprisingly, the level of phosphorylated T508 didn’t change with any of the treatments, yet inhibition of MEK1/2-ERK1/2 with either UO126 or trametinib led to loss of TPA-induced DAP5:eIF2β binding (Fig. 15C). Further evidence using DAP5(T508E) demonstrated that DAP5:eIF2β binding is regulated by MEK-ERK1/2 independent of T508 phosphorylation (Fig. 15D). Despite not linking T508 to MEK-ERK1/2 signaling, the wide-reaching and interconnected ERK1/2 signaling network phosphorylates a number of members of the translation initiation complex (P. Roux & Blenis, 2004). Therefore, ERK1/2 signaling could also act through eIF2β or other members of the eIF2 complex which contains a number of potential regulatory phosphorylation sites (Andaya et al., 2011; Llorens et al., 2006; Rajesh et al., 2008).

After being unable to link MAPK activity to DAP5(T508) we moved our attention to another kinase identified by the kinase prediction software, CDK1. Arrest prior to
mitosis (in G2) resulted in a decrease in both DAP5(T508) phosphorylation and a
decrease in DAP5:eIF2β binding. In contrast, arrest of cells in mitosis resulted in an
increase in DAP5:eIF2β binding and an increase in T508 phosphorylation (Fig 16A). This
suggests that T508 phosphorylation could instigate DAP5:eIF2β binding and activity
during mitosis as an increase in phosphorylation of DAP5(T508) was observed during
mitosis and following incubation with the mitotically active CDK1 (Fig. 16B).
Collectively, this data indicates that CDK1 phosphorylates DAP5 T508 to regulate
DAP5:eIF2β binding which likely explains increased DAP5 activity during mitosis
(Marash et al., 2008).

We used phorbol ester-mediated PKC-Raf-ERK1/2 stimulation to decipher
principles of DAP5 function. Yet, DAP5 activity most likely occurs in a variety of
different circumstances, not limited to active ERK1/2. By exploring DAP5
phosphorylation events, conserved mechanisms that control DAP5:eIF2β binding were
uncovered. Such convergence on regulatory events across multiple stimuli is common in
translation control, due to an exceedingly broad biological repertoire carried out by a
limited number of ubiquitous initiation factors. For example, phosphorylation of
eIF4GI(S1232) by ERK1/2 (M. I. Dobrikov et al., 2013) or by CDK1 (M. I. Dobrikov et al.,
2014) controls activity of the eIF4G:4A:4B translation initiation helicase in mitogenic
stimulation and in mitosis respectively. DAP5(S902), which is located in the eIF2β
binding domain, may have a direct impact on the availability of the site. T508, which is
located in the unstructured IDL, much like regulatory eIF4GI sites, may impact protein folding to allow access to the AA box binding region. T508 is also located in a cluster of three threonine residues (T506, T508, T514; see Fig. 6). In this study, T508 was linked to CDK1 signaling, yet in response to other stimuli, similar mechanisms for inducing DAP5:eIF2β binding may occur at the other sites, and it is possible that these sites may act in concert with each other.

The discovery of regulation of DAP5:eIF2β binding through DAP5 phosphorylation gives a glimpse at the degree of cellular control exerted over DAP5 activity. This important set of findings proposes similar conserved mechanisms for control of DAP5 activation may also occur across a wide array of yet to be studied cellular conditions.

### 3.3 DAP5 Regulates HIF-1 Activity through Translation of PHD2

#### 3.3.1 Introduction

Multifaceted control of protein synthesis enables rapid cellular adaptation to instant changes in growth or survival conditions. This is particularly important when stressors require global protein synthesis repression, along with a simultaneous increase in translation of select stress-response modifiers, as exemplified by the response to oxygen deprivation. The hypoxia response is dominated by hypoxia induced factor 1α (HIF-1α) and its activator role in the HIF-1α/β complex, HIF-1 (G. L. Wang et al., 1995; Guang L. Wang & Semenza, 1995). HIF-1 transcriptional activity induces >1,000 genes
involved in cell proliferation, cell invasion, metastasis, and glucose metabolism programs (G. Semenza, 2014). To restore homeostasis with normoxic balancing, HIF-1α is rapidly hydroxylated by prolyl hydroxylase domain proteins (PHDs), ubiquitinated by Von Hippel-Lindau protein (pVHL), and degraded by the proteasome (Ivan et al., 2001; Jaakkola et al., 2001; Yu F, White SB, Zhao Q, 2001). The PHDs hydroxylate HIF-1α at prolines 402/564 in an oxygen-dependent manner inducing rapid HIF-1α degradation (Epstein et al., 2001; Ivan et al., 2001; Jiang, Semenza, Bauer, & Marti, 1996). This process requires the main oxygen sensor in the cell, PHD2, as neither PHD1 nor 3 can functionally compensate for PHD2 depletion (Berra et al., 2003).

Our studies reveal that DAP5:eIF2β binding has a defining role in controlling translation of the principal oxygen sensor of the cell, PHD2, upon oxygen deprivation. DAP5 depletion caused a surprising, paradoxical increase of HIF-1α protein, due to a reduction in DAP5-dependent translation of PHD2.

DAP5’s role in controlling PHD2 is evident in cells exposed to hypoxic conditions, where low oxygen prompted DAP5:eIF2β binding and DAP5-mediated PHD2 biosynthesis. We, further, confirmed the reported role of PHD2 in tempering Akt signaling (Guo et al., 2016) and, accordingly, defined a role for DAP5 in controlling Akt’s activation status. Akt is the major downstream effector of the PI3K/Akt signaling pathway. Kinase activity of Akt is reliant on a dual phosphorylation event induced by
mTORC2 and PDK1 (Sarbassov, Guertin, Ali, & Sabatini, 2004) with Akt impacting multiple genes involved in cellular processes ranging from cell division, DNA repair, apoptosis, and metabolism (Manning & Toker, 2017). Our findings indicate that DAP5-mediated translation is induced, despite global translation repression in hypoxia, possibly due to unique structural arrangements and protein interactions that distinguish it from eIF4GI/II.

3.3.2 Results

3.3.2.1 DAP5 depletion causes a paradoxical induction of HIF-1α

Since DAP5:eIF2β binding facilitates a surge of global translation following Raf-ERK1/2 stimulation (Fig. 9, Fig 10); we tested DAP5’s effects on translation of specific and known TPA-responsive templates. Since DAP5’s structural arrangement and protein:protein interactions distinguish it from eIF4GI/II, we compared the effect of DAP5 depletion on induction of TPA-responsive mRNAs with a corresponding knockdown of eIF4GI (Fig. 9). Cells with dox-inducible eIF4GI or DAP5 depletion were treated with dox and TPA, and then harvested as for analyses of global protein synthesis (Fig. 17A). Immunoblots were probed for known TPA-inducible proteins including HIF-1α (Pagé, Robitaille, Pouysségur, & Richard, 2002), cyclooxygenase 2 (COX2) (X. Liu & Rose, 1996), and MAPK phosphatase 2 (MKP-2) (Misra-Press, Rim, Yao, Roberson, & Stork, 1995).
3.3.2.2 DAP5 depletion increases HIF-1α through reduced PHD2 levels

HIF-1α is translated at low levels in normoxia, and the protein is highly labile in normal conditions. This is due to a number of post-translational modifications, such as hydroxylation by the PHDs and factor inhibiting HIF-1 (FIH1), and acetylation by the acetyltransferase ARD1, which facilitate HIF-1α binding to the E3 ubiquitin ligase pVHL, and leads to HIF-1α ubiquitination and degradation (Fig. 18A) (Semenza, 2003).
Thus, a likely explanation for the effect of DAP5 depletion on TPA-induced HIF-1α is DAP5 controls the HIF-1α degradation apparatus. Therefore, to determine if DAP5 regulates HIF-1α through hydroxylation, the hydroxylase inhibitor CoCl₂ was added to DAP5-depleted HeLa cells prior to addition of TPA (Fig. 18B). CoCl₂ abolished DAP5 depletion-mediated HIF-1α hyper-induction in TPA-stimulated cells (Fig. 18B; compare to Fig. 17A, B). This suggested that DAP5 may regulate HIF-1α by controlling its hydroxylation levels, rather than acetylation or ubiquitination.

![Figure 18: Regulation of HIF-1α through hydroxylation](image)

**Figure 18: Regulation of HIF-1α through hydroxylation**

A. Schematic overview of post-translational modifications and interactions of HIF-1α (see Fig. 5 for further details of HIF-1α regulation). B. HeLa cells with dox-inducible DAP5 depletion were pretreated with dox (96h) and CoCl₂ (4h) before TPA stimulation. The cells were harvested for immunoblot. The mean HIF-1α/tubulin ratio was quantified, and the data were normalized by setting the no-dox, no-TPA control to 1. All data bars represent the mean of three independent experiments and a representative assay is shown; error bars indicate SEM.
To investigate possible effects of DAP5 depletion on HIF-1α hydroxylation factors, HeLa cells with dox-inducible DAP5 depletion were TPA-stimulated and tested throughout a multi-step time course to monitor PHD1-3, ARD1, FIH1, and pVHL expression (Fig. 18A; 19A). The only factor with a detectable expression response to DAP5 depletion was PHD2 (Fig. 19A). PHD2 is the master HIF-1α hydroxylation factor as PHD2 knockdown alone is capable of stabilizing HIF-1α during normoxia, PHD2 is functionally non-redundant with other HIF1-α regulatory proteins, and is also the most abundant of the PHDs (Appelhoffl et al., 2004; Berra et al., 2003). PHD2 abundance also did not respond to eIF4GI depletion (Fig. 19B). To test directly if DAP5 depletion alters HIF-1α hydroxylation levels (via decreased PHD2 activity), HeLa cells with dox-inducible DAP5 knockdown were treated with the proteasome inhibitor MG-132 (to stabilize hydroxylated HIF-1α; Fig. 19C). We probed for hydroxylation of HIF-1α(P564), a principal PHD2 target that destines HIF-1α for rapid ubiquitination and degradation (Fig. 19C; Fig. 18A). Upon TPA stimulation, DAP5 depletion hyper-induced HIF-1α. Simultaneously, DAP5 depletion decreased the ratio of P564-hydroxylated HIF-1α to total HIF-1α by >80% (Fig. 19C). This effect was accompanied by suppression of PHD2 levels in DAP5-depleted cells (Fig. 19C).
Figure 19: DAP5 depresses PHD2 levels and activity

A. HeLa cells with dox-inducible DAP5 depletion were dox-treated (96h) and lysed for immunoblot with the antibodies shown. Mean PHD2/tubulin ratios were quantified and data were normalized between experiments by setting the no-dox, no-TPA time point to 1. B. HeLa cells with dox-inducible eIF4G1 depletion were dox-treated as shown and mock or TPA stimulated (240 min). The mean PHD2/tubulin ratio was quantified, and data were normalized by setting the 240 min-TPA, no-dox ratio to 1 for each experiment. C. The assay described in (B) was repeated with the addition of MG-132 (5μM; 1h) prior to TPA stimulation. The mean OH-HIF-1α (P564)/HIF-1α ratio was quantified, and data were normalized by setting the no-dox value to 1. All data bars represent the mean of three independent experiments and representative assays are shown; error bars indicate SEM.

Reconstitution with wt DAP5 in cells with dox-inducible DAP5 depletion rescued global protein synthesis; meanwhile reconstitution with the DAP5(E862K) variant, devoid of eIF2β binding, had a negative effect on global translation (Fig. 10A, B). This indicated that DAP5:eIF2β binding is required for DAP5’s effect on global
translation. We used the same assay to query if DAP5:eIF2β binding mediates translation of PHD2 (Fig. 20). The results of this assay mirrored the effects seen with DAP5 knockdown wt DAP5/DAP5(E862K) knock-in reconstitution on global translation, suggesting that PHD2 translation is controlled by the DAP5:eIF2β complex (Fig. 20). Our data indicate a direct link between DAP5:eIF2β binding and PHD2, the master oxygen sensor and coordinator of HIF-1α degradation.

Figure 20: Preventing DAP5:eIF2β binding decreases PHD2 levels
HEK293 cells with dox-inducible knockdown/knock-in DAP5/DAP5 or DAP5/DAP5(E862K) as described in Fig. 10A were dox-treated as shown and analyzed by immunoblot. Mean PHD2/tubulin ratios were quantified, and data were normalized by setting the no-dox values to 1. All data bars represent the mean of three independent experiments and a representative immunoblot is shown; error bars indicate SEM.
3.3.2.3 DAP5 regulates translation of PHD2

Our research thus far implicates DAP5 in control over PHD2 protein levels but as of yet does not demonstrate a direct change in the translation rate of PHD2. Therefore, we conducted polysome profile analysis to probe for shifts of PHD2 mRNA co-sedimentation with polysomal fractions upon DAP5 depletion (Fig. 21). We performed RT-qPCR to detect PHD2 template in individual polysome fractions and plotted its distribution in the profile (Fig. 21A). DAP5 depletion shifted the distribution of PHD2 RNA in the polysome profile towards 80S ribosomes and light polysomes, suggesting a reduction in PHD2 translation. The distribution of control (GAPDH) mRNA was not similarly affected by DAP5 depletion (Fig. 21B). Additionally, DAP5 depletion did not reduce total PHD2 mRNA levels (Fig. 21C), further suggesting regulation at the level of translation.
Figure 21: DAP5 is involved in PHD2 Translation

A, B. HeLa cells with dox-inducible DAP5 depletion were dox-treated (96h) and lysates were subjected to polysome profiling. (Top panel A.) Representative absorbance (260:280) profile of the fractionated gradient is shown in light blue with peaks identified. Total RNA was purified from the fractions, (A) PHD2 and (B) GAPDH levels were determined by RT-qPCR. The percentages of PHD2/GAPDH mRNA signal were determined for each fraction. This experiment was repeated twice and gave similar results in each series. (Bottom panel A) Proteins from each fraction were TCA precipitated and analyzed by immunoblot. C. HeLa cells with dox-inducible DAP5 depletion were treated with dox (96h) or left untreated. Total poly(A) RNA was isolated from the cells and analyzed by RT-qPCR. The average of two assays with the uninduced controls normalized to one and SEM is shown. Representative assays are shown.
Template-specific translation control involves regulatory features in the 5’ and/or 3’UTRs of select mRNAs. Unfortunately, the 5’ and 3’UTRs of the PHD2 mRNA are not clearly annotated with different proposed 5’UTRs of >3,000nt and <100nt lengths; and proposed alternative polyadenylation sites in the 3’UTR also have been reported (Dupuy et al., 2000; Metzen et al., 2005; Taylor, 2001) (Fig. 22). To investigate DAP5’s potential role in template-specific translation regulation of PHD2, extensive further work is required for mapping the definitive transcriptional start site(s), annotate the 5’UTR(s) and determine the nature and prevalence of alternative 3’UTRs of the PHD2 mRNA.

**Proposed PHD2 transcripts**

**Figure 22: Schematic of proposed 3 and 5’UTR’s of PHD2**
Alternative PHD2 transcriptional start sites (marked by 1), alternative poly(A) signals (orange bars), differential UTR’s (shown in blue), the PHD2 start codon (red arrow), stop codon (red bar), and open reading frame (shown in black) are indicated (see main body text for references).
3.3.2.4 DAP5:eIF2β mediated control of PHD2 modulates Akt phosphorylation

Recently, PHD2 has been shown to hydroxylate Akt, provoking Akt-pVHL binding and inhibiting Akt(T308) phosphorylation (Guo et al., 2016). Thus, if DAP5 depletion reduces PHD2 levels, it should also indirectly inhibit Akt activation. To explore such a link, we used our empirical system with dox-inducible DAP5 depletion combined with DAP5/DAP5(E862K) knock-in (Fig. 10A; Fig. 20). Overexpression of wt DAP5 in DAP5-depleted cells had no effect on p-Akt(T308), while expression of the eIF2β-binding defective DAP5(E862K) reduced the abundance of PHD2 and elevated p-Akt(T308) levels (Fig. 23A). These observations confirm claims by Guo et al., 2016 about PHD2’s control over Akt activity and reinforce DAP5:eIF2β’s role in controlling PHD2 translation. Since our assays so far relied on dox-inducible systems in HeLa or HEK293 cells, we tested the effects of transient DAP5 depletion using transfected siRNAs in several different cancer lines: U87, Du54 malignant glioma cells and Sum149 breast cancer cells (Fig. 23B); and also in nonmalignant mouse embryonic fibroblasts (MEFs) (Fig. 23C). In all four lines, DAP5 depletion diminished PHD2 levels (Fig. 23B, C). Only U87 and MEF cells expressed quantifiable levels of HIF-1α in normoxia; DAP5 depletion in these two cell lines mediated an increase of HIF-1α levels (Fig. 23B, C). All four lines exhibited increased p-Akt (T308) levels upon DAP5 depletion (Fig. 23B, C). Collectively, our findings suggest that DAP5 controls baseline PHD2 abundance in diverse malignant cell types and in MEFs.
Figure 23: DAP5 modulates PHD2, AKT(T308) phosphorylation, and HIF-1α in cancer and primary cell lines

A. HEK293 cells with dox-inducible DAP5 depletion and either wild type DAP5 or DAP5(E862K) reconstitution were treated with dox as shown (96h). Lysates were subjected to immunoblot with the indicated antibodies. The p-AKT(T308)/AKT signal ratio from three independent series was quantified and the uninduced controls were normalized to one. B, C. Two malignant glioma cell lines (Du54 and U87), one breast cancer cell line (Sum149) (B); and MEFs (C) were transiently transfected with siRNAs targeting DAP5. Lysates were analyzed by immunoblot. Mean ratios of HIF-1α/tubulin, PHD2/tubulin, and/or p-AKT(T308)/AKT1 signals from three independent assays are shown, data were normalized by setting the control siRNA sample values to 1; error bars denote SEM. HIF-1α (Du54, Sum149 cells) and p-AKT (U87) levels could not be quantified due to insufficient signal. Representative assays are shown.

3.3.2.5 DAP5 controls the response to hypoxia

DAP5’s influence over PHD2 biosynthesis, and its downstream effects on HIF-1α accumulation and Akt activity, indicates important functions in cellular responses to
oxygen deprivation. We performed tests to confirm this in cells where a physiologic response to hypoxia was induced by low-oxygen growth conditions. Unmodified HeLa cells or HeLa cells with 1) dox-inducible depletion of DAP5; 2) dox-inducible depletion of eIF4GI; or 3) combined dox-inducible DAP5 depletion and ectopic PHD2 overexpression were cultured in 0.5% oxygen (6h and 24h) (Fig. 24A). All cell lines responded with HIF-1α induction after 6h in hypoxic conditions; this response was consistently more pronounced in DAP5-depleted cells (Fig. 24A). More importantly, all cell lines—except the DAP5-depleted cultures—succeeded in containing the hypoxic response by downregulating HIF-1α at 24h post hypoxia (Fig. 24A). In DAP5-depleted cultures, the hypoxia-induced surge of HIF-1α was inappropriately sustained (Fig. 24A). Ectopic PHD2 compensated for the loss of DAP5 and restored a physiologic pattern of HIF-1α regulation, confirming prior assays implicating DAP5 in control over PHD2 translation (Fig. 24A). As suggested earlier by the lack of change in PHD2 expression in response to eIF4GI depletion (Fig. 17A), eIF4GI depletion does not appear to be regulated this PHD2 related processes (Fig. 24A).

To show functionality of the DAP5 depletion induced HIF-1α we probed for a well-known transcriptional target of HIF-1, BNIP3. This revealed BNIP3 accumulation in conjunction with HIF-1α levels (Fig. 24A). Prolonged BNIP3 induction during hypoxia leads to cell death (Azad et al., 2008; Kubasiak, Hernandez, Bishopric, & Webster, 2002). Thus, DAP5 depletion not only exaggerated acute HIF-1α induction, but also intercepted
physiologic means of terminating this response (Fig. 24A). This corresponded with the known role of PHD2 in controlling HIF-1α levels. Similar effects on HIF-1α dynamics were observed upon transient DAP5 depletion from U87 malignant glioma cells (Fig. 24B; upper panels).

The response of (non-malignant) MEFs to DAP5 depletion diverged from U87 malignant glioma cells at 24h under hypoxia (Fig. 24B; bottom panels). This indicates that coopting DAP5 for containing damage from the hypoxic response may be a function of malignancy.
Figure 24: DAP5 regulates the cellular hypoxia response through PHD2.

A. HeLa cells with dox-inducible DAP5 depletion, eIF4G1 depletion, or DAP5 depletion combined with PHD2 overexpression were dox-treated (96h) as shown and placed in a chamber containing 0.5% oxygen (O₂) for 6 or 24hrs. Lysates were prepared and analyzed by immunoblot. Mean HIF-1α/tubulin signal was quantified, normalizing between experiments by setting the (mock-depleted) HeLa cell 6h value to 1. B. U87 (top panel) and MEF (bottom panel) cells were transfected with DAP5 or control siRNA and cultured in 0.5% O₂. Mean HIF-1α/tubulin signal was quantified, data were normalized between experiments by setting control siRNA samples to 1. All data bars represent the average of three independent tests and a representative assay is shown; error bars indicate SEM.
3.3.2.6 DAP5:eIF2β binding controls DAP5 activity during hypoxia

Our investigations, reported above (Fig. 10, 20), strongly implicated inducible eIF2β binding in DAP5’s role in translation control (e.g. of the PHD2 message). We wanted to analyze the role of DAP5:eIF2β binding in the hypoxic response. Therefore, we used HEK293 cells with dox-inducible depletion combined with wt DAP5/DAP5(E862K) reconstitution and exposed them to oxygen deprivation as in Fig. 24 A, B.

Reconstitution with DAP5(E862K) led to sustained, unsuitable induction of HIF-1α and BNIP3 at 6h and 24h in 0.5% oxygen relative to wt DAP5 reconstituted cells (Fig. 25A). This effect correlated with reduced PHD2 levels (Fig. 25A). Furthermore, probing for DAP5 in the physiologic response to oxygen deprivation in HEK293 cells with dox-inducible Flag-DAP5 revealed key insight into the DAP5:PHD2:HIF-1α connection. Hypoxia induced DAP5:eIF2β binding and enhanced PHD2 levels (Fig. 25B). This suggests that hypoxia spurs DAP5-dependent protein synthesis, as conditions that increase binding to eIF2β corresponded with increased DAP5 translational activity (Fig. 9). Tests for p-DAP5(T508) with the anti p-T*PP motif antibody showed no response to hypoxia (Fig. 25B), corroborating our data shown in Fig. 15C.
Figure 25: DAP5:eIF2β binding is induced during hypoxia and regulates PHD2.
A. HEK293 DAP5 knockdown/knock-in cells described in Figs. 3C and Fig. 4F were treated with dox as shown and placed in 0.5% O₂. Mean HIF-1α/tubulin ratios were determined, normalizing by setting DAP5-depletion/wt DAP5-reconstitution samples to 1. B. HEK293 cells with dox-inducible Flag-DAP5 expression were treated with dox (16h); lysates were prepared and analyzed by immunoblot or anti-Flag IP followed by immunoblot as shown. Mean eIF2β/Flag ratio from IP and mean PHD2/tubulin ratio from input signal ratio was quantified, data were normalized by setting 0h samples to 1. All data bars represent the average of three independent tests and representative assays are shown; error bars indicate SEM.
3.3.3 Discussion

Figure 26: Schematic summation of results.

We determined that DAP5:eIF2β binding is induced by PKC-Raf-ERK1/2 signaling (Fig. 15C), mitosis (Fig. 16A), and hypoxia (Fig. 25B) with two phosphorylation sites T508 (Fig. 14), and S902 (Fig. 12B) on DAP5 impacting eIF2β binding. DAP5:eIF2β binding was found to enhance DAP5 global translational activity (Fig. 10) and, more specifically, translation of PHD2, (Fig. 20) a previously unknown DAP5 translational target. Increased PHD2 expression repressed HIF-1α (Fig. 24A) and p-Akt(T308) (Fig. 23A).

In our efforts to assess potential translational targets induced by ERK1/2 driven DAP5 activity, we discovered that DAP5 mediates the translation of PHD2, a HIF-1α destabilizing hydroxylase. Further investigation revealed a previously unknown feedback mechanism through which long-term hypoxia induces DAP5:eIF2β binding increasing PHD2 translation to mediate hydroxylation and degradation of HIF-1α. Due to the conflicting and puzzling annotation of the 5’ and 3’UTRs of the PHD2 mRNA (Dupuy et al., 2000; Metzen et al., 2005; Taylor, 2001) extensive future work will need to be done to unravel first the PHD2 message and the mechanism by which DAP5:eIF2β targets PHD2 for translation. DAP5 depletion resulted in the induction of overall HIF-1α activity as shown by an increase the HIF-1α transcriptional target BNIP3 protein levels.
The increase in BNIP3 is striking as prolonged induction of this protein has been linked to hypoxia mediated cell death (Letourneau, Duffett-Leger, & Salmani, 2009, Kubasiak et al., 2002, Sowter, Ratcliffe, Watson, Greenberg, & Harris, 2001). As observed for DAP5 mediated global translation, a DAP5 mutant incapable of binding eIF2β was unable to rescue effects of endogenous DAP5 depletion on PHD2 and HIF-1α expression.

PHD2 levels increase during prolonged periods of low oxygen, possibly because it is a transcriptional target for HIF-1. Yet changes in PHD2 translation by DAP5 may serve as a rapid and complementary method to enable PHD2 expression, thereby preventing aberrant HIF-1α expression; which can be deleterious for cellular homeostasis (D’Angelo et al., 2003; Henze et al., 2010). Our data indicates that DAP5:eIF2β binding is stimulated during hypoxia; however, how the DAP5 initiation complex functions as a sensor of hypoxia remains to be determined.

During hypoxia global translation is inhibited (Connolly et al., 2006, Koritzinsky et al., 2006; L. Liu et al., 2006), yet alternative translation initiation pathways have been proposed to activate translation of a subset of mRNAs. (Braunstein et al., 2007; Young et al., 2008, Ho et al., 2016; Yi et al., 2013). DAP5:eIF2β binding is required for DAP5-mediated translation and is induced during mitogenic signaling and hypoxia to enable DAP5 function. These findings demonstrate that eIF2β uniquely serves as a critical scaffold for DAP5 to exert control over cellular translation and homeostasis (Fig. 26).
Therefore, DAP5:eIF2β induced translation of PHD2 represents a novel mechanism for translation initiation during hypoxia and potentially other cellular stress conditions.
4. Conclusion

In mammals, translation initiation requires a bridge from mRNA to the 40S ribosomal subunit that, according to current knowledge, can only be provided by the 3 eIF4G isoforms (Hentze, 1997). Considering the dominant role of protein synthesis control in altering gene expression in response to sudden physiologic challenges, for example hypoxia (Ho et al., 2016), it is clear that this critical node is subject to tight regulation. Our work focuses on the eIF4G isoform DAP5, which is unique in lacking eIF4GI/II’s N-terminus (the region containing binding domains for eIF4E/PABP), and being capable of binding eIF2β. We determined that 1) DAP5:eIF2β association is strongly inducible and occurs in specific biological programs with DAP5:eIF2β binding favoring translational activity of DAP5, and 2) DAP5 is a key factor in the hypoxic response by controlling translation of the oxygen sensor and HIF-1α destabilizer, PHD2.

4.1 Regulated DAP5:eIF2β Binding Impacts Global Translation

4.1.1 Conclusion

We initially used phorbol ester-mediated PKC-Raf-ERK1/2 stimulation to decipher principles of DAP5 function. Yet, DAP5:eIF2β binding was found to occur in diverse circumstances not associated with active ERK1/2, such as hypoxia and mitosis. Such convergence is common in translation control, due to an exceedingly broad biological repertoire carried out by a limited number of ubiquitous initiation factors. For example, phosphorylation of eIF4GI(S1232) by ERK1/2 (M. I. Dobrikov et al., 2013) or
CDK1 (M. I. Dobrikov et al., 2014) controls activity of the eIF4G:4A:4B translation initiation helicase during both mitogenic stimulation and in mitosis, respectively.

eIF4GI/II can bind mRNAs through eIF4E and the m7G-cap in a template non-specific manner. However, all eIF4G isoforms can recruit ribosomes independently of the m7G-cap by directly engaging with mRNAs (de Breyne, Yu, Unbehaun, Pestova, & Hellen, 2009; Hundsdoerfer et al., 2005; Kaiser et al., 2008). Mammalian eIF2 does not interact with eIF3 but directly contacts the 40S ribosomal subunit (Simonetti et al., 2016). Thus, DAP5 bound to eIF2 may have unique contacts with the 40S subunit compared to eIF4GI/II which bridges the 40S subunit exclusively via eIF3. Also eIF4GI/II only indirectly assembles with the ternary complex (eIF2-GTP-Met-tRNA^Met) via eIF3/40S subunit, unlike DAP5 which binds eIF2 and therefore the ternary complex, directly.

The DAP5:eIF2β complex and its unique range of contacts may offer non-canonical means of initiation complex assembly and functions that are critical at times of global protein synthesis adaptation (e.g. hypoxia, mitosis). DAP5:eIF2β binding occurs in concert with other dynamic interactions such as with mRNA, eIF3, and the helicase and cofactor eIF4A:4B. Furthermore, DAP5’s HEAT2 domain, unlike that of eIF4GI/II, does not bind eIF4A (Virgili et al., 2013), perhaps compensating for the lack of eIF4A interactions through HEAT2.
4.1.2 Future Directions

The precise events that lead from ERK1/2 activation to DAP5:eIF2β binding are still unknown. ERK1/2 phosphorylates a number of members of the translation initiation complex. The wide-reaching ERK1/2 signaling network could control DAP5:eIF2β binding in numerous ways (P. Roux & Blenis, 2004). ERK1/2 signaling could also be acting through eIF2β and other members of the eIF2 complex, which contains a number of identified phosphorylation sites (Andaya et al., 2011; Llorens et al., 2006; Rajesh et al., 2008). To investigate eIF2 for ERK1/2-induced phosphorylation events, a similar phospho-proteomic approach, to that conducted on DAP5 (Fig 11A), should be taken with the three subunits of eIF2 following four hours of TPA stimulation. Sites identified would then be furthered examined by specific amino acid mutational analysis. If a specific eIF2 mutation is uncovered, we will test for antibodies specific to this phosphorylation event, or, if needed, form a collaboration to produce antibodies to enable us to detect the phosphorylation kinetics.

It is an interesting point that the increase in DAP5:eIF2β binding appears 240min following stimulation with TPA. Many TPA induced kinases are rapidly activated so the delayed TPA induced binding to eIF2β may be occurring through other mechanisms. This suggests that other factors such as RNA binding proteins may be influencing DAP5 activity as well. Selective inhibitor studies can be done to begin to unravel the reason for TPA induced DAP:eIF2β binding.
Understanding how DAP5:EIF2β binding promotes translation while sharing the same binding mechanics (acidic aromatic (AA) boxes to lysine(K) boxes) as eIF5 and eIF2Be (Asano, Krishnamoorthy, Phan, Pavitt, & Hinnebusch, 1999) (Fig. 27) is a promising area for future studies. Our proposed mechanism for DAP5 translation initiation that takes eIF5 into consideration is as follows: DAP5 recruits mRNA to EIF2β first and then to the pre-initiation complex (PIC). Before the start codon is recognized, DAP5 is released, allowing eIF5’s AA boxes to interact with the now free K boxes on EIF2β, causing the complex to close and translation to proceed (Luna et al., 2012) (described in Fig. 27). This model builds upon and does not violate the previously known PIC and 48S initiation complex interactions (Singh et al., 2012; Asano et al., 2000; Alone & Dever, 2006) (Fig. 1) and may present a rapid method for combining the two consecutive steps of PIC formation and mRNA attachment (Jackson et al., 2010) for swift translation of select mRNAs.
Figure 27: Initiation factor binding interactions with eIF2β

Outside of translation initiation, eIF2 interacts with eIF2Bε, its guanine nucleotide exchange factor (GEF), through strong interaction between eIF2β’s lysine box (K boxes) domains and the acidic aromatic box (AA boxes) domains in eIF2Bε (black arrow). eIF2Bε also interacts less strongly with eIF2γ (grey arrow). eIF2Bε:eIF2 interactions facilitate the activation of eIF2 by converting eIF2γ-bound GDP (red dot) to GTP (green dot). During translation initiation, eIF5 first associates with eIF2 (charged with Met-tRNA\textsubscript{Met}) through eIF2γ (grey arrow) (Alone & Dever, 2006). When start codon recognition occurs, eIF5 is freed to bind via its AA boxes to the K boxes of eIF2β. This binding interaction along with eIF5 function as the eIF2 GTPase-activating protein (GAP) facilitates the cessation of scanning and the closing of the PIC (Luna et al., 2012). Similar to eIF2Bε and eIF5, DAP5 interacts with eIF2β through binding the K boxes (orange arrow), suggesting the possibility of processive or competitive binding kinetics.

When beginning the DAP5:eIF2β project, we preformed Flag-DAP5 IP’s to probe for potential interacting and associating initiation factors. However, we were only able to very weakly detect eIF5 (Fig. 28) and were unable to detect eIF2Bε, eIF1, or eIF1A co-IP. DAP5:eIF5 binding was weak and inconsistent in our IPs.
The finding that eIF5 was not entirely absent, but did not bind strongly fits with our proposed model for DAP5:eIF2 binding prior to eIF5:eIF2β binding. The weak detection of eIF5 could be due to a short-term intermediate complex containing DAP5:eIF2 and eIF5, either in the PIC or with eIF5 binding through eIF2γ and DAP5 binding to eIF2β. The distant/indirect nature of these interactions, the salt/detergent concentration, or even the buffer system may also have been inappropriate for improved detection of this interaction.

To add clarity to DAP5’s impact on eIF5:eIF2 binding, new immunoprecipitation experiments should be done with eIF2γ, eIF2β, and eIF5. These IP’s will be performed in combination with the knockdown/knock-in cell line approach by replacing wt-DAP5 with mutant DAP5(E862K), which is unable to bind eIF2β. To further ascertain DAP5’s place in translation initiation, lysates from TPA-stimulated, mitotic, or hypoxia-treated cells can be run on a sucrose gradient to separate ribosomal subunits, monosomes, and polysomes. Analysis of these fractions for the presence of DAP5 would help us determine when DAP5 recruits mRNA to the ribosome and when it dissociates.
Figure 28: DAP5:eIF5 binding

HEK293 cells expressing Flag-tagged DAP5 were induced with dox (16h) before stimulation with TPA as shown. Cell lysates were analyzed by anti-Flag IP followed by immunoblot.

We also probed DAP5 IP's for phosphorylated-eIF2α(S51) and eIF2Bε and were only able to detect weak association of DAP5 with phosphorylated eIF2α after 15mins of TPA treatment, when eIF2β binding was decreased (Fig. 29). This suggests that the active DAP5:eIF2β complex does not include phosphorylated eIF2α. However, since DAP5 does, to a minimal extent, bind to phosphorylated eIF2α, this leaves the interesting question of whether DAP5 binding competes with eIF2Bε, which binds eIF2β through the same AA to K boxes interaction (Alone & Dever, 2006) (Fig. 27). eIF2B binds to p-eIF2α(S51) and sequesters active eIF2, thereby repressing global translation. If DAP5 competes with eIF2Bε, it may allow an eIF2α phosphatase to relieve phosphorylated eIF2α, making DAP5-bound eIF2 available for translation when eIF2α is phosphorylated.
To determine the impact of DAP5:eIF2β binding on p-eIF2α(S51) and eIF2Bε interactions we can perform IP’s for p-eIF2α(S51) and eIF2Bε in the DAP5/DAP5(E862K) knockdown/knock in cell lines. This leads to the question of whether DAP5 binds to eIF2 before or after the GDP to GTP conversion. Purification of DAP5-bound eIF2 can be analyzed by column purification and/or mass spectrometry for GTP, GDP, and also Met-tRNA\textsubscript{Met} to determine whether DAP5-bound eIF2 is in the active confirmation (by binding to GTP) and/or translationally ready (by binding to Met-tRNA\textsubscript{Met}).

**Figure 29: DAP5:p-eIF2α(S51) binding**

HEK293 cells expressing Flag-tagged DAP5 were induced with dox (16h) before stimulation with TPA as shown. Cell lysates were analyzed by anti-Flag IP followed by immunoblot.
4.2 DAP5’s Regulation of HIF-1 activity through the Translation of PHD2

4.2.1 Conclusion

PHD2 levels increase during prolonged periods of low oxygen, possibly because PHD2 is a transcriptional target of HIF-1. Yet, transcriptional control may not be able to mediate the sudden changes in protein abundance required to contain HIF-1α and the potentially deleterious hypoxia response. Indeed, system-wide analyses suggest that the response to hypoxia is accompanied by far-reaching translation adjustment (Ho et al., 2016; L. Liu et al., 2006). DAP5:eIF2β-mediated control of PHD2 provides for powerful, rapid adaptation of protein expression that does not require new transcription.

DAP5 depletion in hypoxia results in the induction of overall HIF-1α activity, as shown by the increase in BNIP3 protein levels (Fig 24A, B, and C). The increase in BNIP3 is striking, as this protein’s prolonged induction has been linked to hypoxia-mediated cell death (Azad et al., 2008; Kubasiak et al., 2002; Sowter et al., 2001). In line with our observations in transformed cells, DAP5 depletion in MEFs caused a decline in PHD2 levels that coincided with increasing HIF-1α and p-AKT(T308) under normoxic conditions (Fig. 23C). This suggests that basal DAP5 control of PHD2 translation, in the absence of inducing signals, occurs through a similarly regulated pathway in non-transformed as well as in cancerous cells. Yet, in the presence of a strong inducing signal (0.5% O₂), the response of non-transformed or neoplastic cells to DAP5 depletion diverged. In hypoxia-exposed MEFs, DAP5 knockdown elevated HIF-1α and BNIP3
levels at 6h, but this effect was reversed at 24h (Fig. 24B, lower panel). In transformed and cancer cells, 24h hypoxia treatment induced significantly increased HIF-1α and BNIP3 levels (Fig. 24A, B upper panel, and C).

We believe that this divergence is due to the abnormal metabolic/signaling landscape in transformed cells. Maintenance of metabolic activity and proliferative capacity under chronic hypoxia is a hallmark of cancer. Our finding that cancerous cells exploit DAP5 to contain the hypoxic response is consistent with this. Moreover, our finding that DAP5:eIF2β binding (and, hence, PHD2 translation) depends on active ERK1/2 suggests a role for unhinged Raf-ERK1/2 signaling (a virtually universal characteristic of the malignant state) in DAP5’s function in the hypoxic resistance of cancerous cells. We hypothesize that MEFs do not coopt DAP5 to withstand hypoxic stress in the same way as cancerous cells, likely due to their physiologically intact metabolic/signaling landscape.

4.2.2 Future Directions

The involvement of DAP5:eIF2β in PHD2 translation indicates that the physiologic context of oxygen deprivation and the sequence specific-features of the PHD2 message drive template specificity of DAP5. However annotation of the 5’ and 3’UTRs of PHD2 has been inconsistent (Dupuy et al., 2000; Metzen et al., 2005; Taylor, 2001); previous studies have reported two different proposed 5’UTR’s—one greater than
3000nt and one shorter than 100nt—making it difficult to decipher how DAP5 targets PHD2 transcripts for translation.

We are interested in determining what elements, presumably in the PHD2 5’UTR, permit selective binding of DAP5 but not eIF4GI. By using PCR techniques with probes targeted to PHD2’s proposed 5’UTR, we first would seek to define the PHD2 5’UTR in our cellular context. Through careful analysis of the confirmed 5’UTR, we can begin directed mutational analysis to disrupt or enhance elements we hypothesize will impact DAP5 mRNA binding. This can be confirmed through DAP5 IP, followed by PCR. We can then examine whether changes in PHD2 5’UTR:DAP5 binding impacts PHD2 translation. With PHD2, we have confirmed a stress-induced DAP5 translational target. By identifying the elements in the 5’UTR that mediate binding to DAP5, we can further use this discovery to search for new potential DAP5 target mRNAs.

In the current study, we focused on the role of DAP5 in the translational control of HIF-1α, with increased BNIP3 as a read-out for HIF-1 activity. With prolonged BNIP3 expression linked to hypoxia-induced cell death, we plan to investigate cell death related to DAP5 knockdown in hypoxia by starting with an increased number of hypoxia time points going out to 72h. We will begin by determining live/dead cell numbers with trypan blue staining. We can also run immunoblots for cellular markers of death.
Although, in my initial hypoxia experiments I failed to detect PARP cleavage, this may just indicate that hypoxia mediates a non-apoptotic type of cell death. The hypoxia chamber we used in this study is sealed when in use, only allowing for a maximum of three time points of uninterrupted hypoxia per experiment and no cellular manipulation until collection. Given the encouraging results we have achieved using this chamber we will seek out a collaboration to continue our hypoxia cell death studies with a more appropriate apparatus. In that new context we could add cell viability markers such as MTT, investigate translation rates with puromycin, and observe the cells throughout their time in hypoxia. Results from these experiments will inform more targeted approaches to interrogate certain pathways linked to the hypothesized cell death phenotype.

Although DAP5’s impact on HIF-1α was enhanced in cancer cells (Fig. 24B), and HIF-1α regulation is of primary importance in tumor biology, we are hesitant to apply our findings here to the physiology of tumor adaptation to hypoxia. Previous investigations of PHD2’s role in tumor biology have led to inconsistent and contentious observations (Bordoli et al., 2011; Chan et al., 2009; Klotzsche-Von Ameln et al., 2011; Su et al., 2012). Thus, future work testing PHD2 regulation by DAP5 in cancer will require careful investigations.

Instead of starting with unpredictable and physiologically unrepresentative DAP5-modified mouse tumor models, we can begin by analyzing numerous patient-
derived tumor samples from a variety of types and stages of cancers for a correlation in DAP5, PHD2, and HIF-1α expression. With the variation in different cancers, a DAP5:PHD2 correlation may be strongly featured in a certain type or subtype of cancer. If this correlation is found, other traits of the target cancer type may provide clues to DAP5’s function in tumor formation. Once we have a more complete picture of DAP5/PHD2 function in the context of cancer we will proceed with the appropriate cell culture and mouse tumor model experiments exploring DAP5 manipulation in tumors.

We were surprised to see that DAP5 activity was responsive to low oxygen environments. This implies that DAP5 is able to sense changes in oxygen. During hypoxia, a number of events occur that could lead to regulatory modifications of DAP5. Changes in cell signaling pathways that occur during the hypoxic response include increased signaling from AMPK (L. Liu et al., 2006) and mTOR inhibition (Connolly et al., 2006) suggesting that hypoxia-induced DAP5 phosphorylation events may occur. The PHD proteins, which act as cellular oxygen sensors, may be able to inhibit DAP5 through hydroxylation, similarly to PHD2’s repression of Akt (Guo et al., 2016).

Observations in the cardiovascular system have shown that extended periods of hypoxia prompt increases in cellular reactive oxygen and reactive nitrogen species (ROS/RNS), inducing a period of oxidative stress. DAP5 has been shown to be active during oxidative stress, although the mechanism for this activation is unknown (Nousch et al., 2007). These ROS/RNS can induce cysteine oxidation post-translational protein
modifications (H. S. Chung, Wang, Venkatraman, Murray, & Van Eyk, 2013), identifying a new class of potential hypoxia-induced regulatory DAP5 post-translational modifications. A mass spectrometry analysis of purified DAP5 following hypoxia treatment would help indicate which modifications of DAP5 might occur in hypoxia, with follow-up site-directed mutational analyses to confirm the function of these DAP5 variants.
Appendix (Licenses for Figures)

Licensee: Jeffrey Bryant
Order Date: Mar 8, 2018
Order Number: 4304510472989
Publication: Nature Reviews Molecular Cell Biology
Title: The mechanism of eukaryotic translation initiation and principles of its regulation
Type of Use: Dissertation
Order Total: 0.00 USD

Figure 3 adapted from:

Figure 4 was originally published in:
http://www.biochemj.org/content/351/2/289
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Biography

Jeffrey David Bryant was born in Kansas City, Missouri, USA on March 24, 1990.

Jeff graduated from Kansas State University in 2012 with two Bachelors of Science degrees in Microbiology and Chemistry. At Kansas State University, Jeff conducted undergraduate research in the lab of Dr. Lynn Hancock studying biofilm formation in Enterococcus faecalis.

In 2012 Jeff was accepted into the Molecular Genetics and Microbiology Ph.D. program at Duke University where he conducted research in the lab of Matthias Gromeier on the topic of this dissertation and on combining chemotherapy with recombinant poliovirus cancer immunotherapy. His publications are mentioned below.

While at Duke Jeff has received numerous awards including the Duke Center for Virology Travel Award (2016, 2017), the Duke Molecular Genetics and Microbiology Distinguished Fellows Travel Award (2017), the Duke Molecular Genetics and Microbiology Chairman’s Travel Award (2017), the Duke Graduate School Conference Travel Award (2016, 2017), the 10th International Oncolytic Virus Meeting Travel Award (2016), the Bass Teaching Assistantship Fellowship from Duke University (2016), and the James B. Duke Fellowship (2012-2016). Jeff was a member of the Society of Duke Fellows (2012-present), an honorable mention for the National Science Foundation Graduate Research Fellowship (2014), and a Duke Viral Oncology Training grantee (2013-2014). Jeff will graduate in May 2018 with a Ph.D. in Molecular Genetics and Microbiology.
Publications:

Bryant JD, Zhuo J, Yang, Y, Brown MC, Gromeier M. “Chemotherapy Enhances Recombinant Poliovirus Immunotherapy” In Preparation

