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Abstract
The transcription factor ATF4 regulates the expression of mRNAs involved in amino acid metabolism, redox homeostasis and ER stress responses. Its overexpression in human solid tumors suggests an important role in tumor biology. Here we report that inhibition of ATF4 expression blocks proliferation and survival of transformed cells, despite an initial activation of cytoprotective macroautophagy. Knockdown of ATF4 significantly reduced the levels of asparagine synthetase (ASNS). Overexpression of ASNS or supplementation of asparagine in trans, reverses the proliferation block and increases survival in ATF4 knockdown cells. Both amino acid and glucose deprivation, stresses found in solid tumors, activate the upstream eIF2 alpha kinase GCN2 to upregulate ATF4 target genes involved in amino acid synthesis and transport. Levels of total GCN2, phospho-GCN2 and phospho-eIF2 alpha were upregulated in samples of human and mouse tumors compared to normal tissues and abrogation of ATF4 or GCN2 expression significantly inhibited tumor growth in vivo. ATF4 also contributes to the hypoxic resistance of tumor cells. We conclude that the GCN2-eIF2 alpha-ATF4 pathway is critical for maintaining metabolic homeostasis in tumor cells, making it a novel and attractive target for anti-tumor therapy.

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THE ROLE OF THE TRANSCRIPTION FACTOR ATF4 IN TUMOR PROGRESSION UNDER NUTRIENT DEPRIVATION AND HYPOXIA

Jiangbin Ye

A DISSERTATION

in

Cell and Molecular Biology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

2010

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DEDICATION

This dissertation is dedicated to my dear parents, Chunfeng Ye and Yuqin He

And my beloved wife Wei Feng

They always do their best to encourage and support me to pursue knowledge and truth.

I am truly grateful for them.

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ABSTRACT

THE ROLE OF THE TRANSCRIPTION FACTOR ATF4 IN TUMOR PROGRESSION UNDER NUTRIENT DEPRIVATION AND HYPOXIA

Jiangbin Ye

Supervisor: Constantinos Koumenis

The transcription factor ATF4 regulates the expression of mRNAs involved in amino acid metabolism, redox homeostasis and ER stress responses. Its overexpression in human solid tumors suggests an important role in tumor biology. Here we report that inhibition of ATF4 expression blocks proliferation and survival of transformed cells, despite an initial activation of cytoprotective macroautophagy. Knockdown of ATF4 significantly reduced the levels of asparagine synthetase (ASNS). Overexpression of ASNS or supplementation of asparagine in trans, reverses the proliferation block and increases survival in ATF4 knockdown cells. Both amino acid and glucose deprivation, stresses found in solid tumors, activate the upstream eIF2α kinase GCN2 to upregulate ATF4 target genes involved in amino acid synthesis and transport. Levels of total GCN2, phospho-GCN2 and phospho-eIF2α were upregulated in samples of human and mouse tumors compared to normal tissues and abrogation of ATF4 or GCN2 expression significantly inhibited tumor growth *in vivo*. ATF4 also contributes to the hypoxic resistance of tumor cells. We conclude that the GCN2-eIF2α-ATF4 pathway is critical for maintaining metabolic homeostasis in tumor cells, making it a novel and attractive target for anti-tumor therapy.

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Chapter I: General Introduction

The tumor microenvironment consists of stromal cells, nutrients, growth factors, extracellular matrix, and blood vessels that surround and nourish tumor cells (Yuan and Glazer, 1998). Through interactions between tumor cells, fibroblasts, infiltrating immune cells, extracellular matrix and cytokines, tumor cells can alter the microenvironment, and conversely, the microenvironment can also influence tumor progression. (Gonda et al., 2009; Whiteside, 2008). Additionally, fluctuations in the concentrations of oxygen and nutrients (e.g. glucose and amino acids) can impact on tumor cell survival, proliferation, angiogenesis and migration. Because of their fast proliferation, high metabolism rate and abnormal tumor vasculature, tumor cells are often exposed to stresses such as hypoxia, low glucose and amino acid starvation. The work herein describes studies on how the Activating Transcription Factor 4 (ATF4) contributes to tumor cell resistance to these stresses.

Hypoxia promotes tumor progression

Tumor hypoxia constitutes a dynamic feature of tumor microenvironment that contributes to tumor progression, which develops due to several factors, including: (a) uncontrolled cellular proliferation that results in tumors outgrowing their blood/oxygen supply creating chronic or diffusion-limited hypoxia; (b) an immature and structurally abnormal newly formed tumor vasculature that can be leaky and cause acute or perfusion-limited hypoxia (Brown, 2000; Harris, 2002); (c) increased

metabolic activity of tumor cells compared to normal cells (Jones and Thompson, 2009). Clinically, tumor hypoxia is a significant obstacle to therapy. Hypoxic tumor cells are more resistant to ionizing radiation compared to more normoxic tumor tissue because of the incomplete fixation of DNA damage (Hockel et al., 1996b; Nordsmark et al., 1996). Because of slow proliferation rates, limited diffusion and tissue acidosis, hypoxic cells are also less sensitive to chemotherapeutic drugs (Sutherland et al., 1979; Teicher et al., 1990; Wike-Hooley et al., 1984). Hypoxia also contributes to malignant progression. Several clinical studies and experimental mouse models showed that tumor hypoxia is associated with increased metastatic potential (Brizel et al., 1996; Cairns and Hill, 2004; Hockel et al., 1996a), and more recently, attenuation of cellular senescence (Welford et al., 2006). Hypoxia selects for colonial expansion of tumor cells with mutated p53 (Graeber et al., 1996) and increases genome instability which contributes to a more aggressive phenotype (Reynolds et al., 1996). Moreover, hypoxia promotes tumor angiogenesis primarily though hypoxia inducible factor (HIF) induced vascular endothelial growth factor (VEGF) (Hlatky et al., 1994; Maxwell et al., 1997). Therefore, it is important to elucidate the mechanisms by which hypoxia affects tumor physiology at the cellular and molecular levels, which can lead to the development of novel therapeutic methods targeting hypoxic tumors that will ultimately improve therapeutic outcome.

Molecular mechanisms of cellular adaptation to hypoxia.

The hypoxia-inducible factors 1 and 2 (HIF-1 and HIF-2) play a major role in

cellular and tumor adaptation to hypoxia by regulating the expression of over 200 genes involved in angiogenesis, glycolysis, apoptosis, metastasis, proliferation and differentiation (Gordan and Simon, 2007). In addition to HIF-dependent pathways, adaptation to hypoxia inevitably involves downregulation of macromolecular synthesis which is a major source of energy consumption in tumor cells, thereby coupling low oxygen availability and reduced oxidative phosphorylation to energy demands (Koumenis and Wouters, 2006). Since the process of mRNA translation is an energy-consuming process, reducing the rate of translation is necessary for cell survival under low oxygen levels. This inhibition of translation is largely mediated though the inhibition of eukaryotic initiation factor 2α (eIF2α) and eukaryotic initiation factor 4E (eIF4E), and to a smaller extent, inhibition of elongation factor eEF-2 (Arsham et al., 2003; Koumenis et al., 2002; Liu et al., 2006). Previous studies from our lab and other labs have showed that PERK-dependent eIF2α phosphorylation under hypoxia is necessary for tumor cells survival (Bi et al., 2005).

The PERK/ eIF2α/ATF4 module of the UPR (unfolded protein response) is activated by hypoxia induced ER stress.

There are three ER stress signal transducers located on the ER membrane: IRE1, ATF6 and PERK (Figure I-1). The ER lumenal domains of these proteins are bound by ER chaperone BiP/GRP78 under unstressed conditions so that the cytoplasmic kinase domain is inactive. Upon ER stress, such as when unfolded/misfolded proteins accumulate in the ER, these transmembrane proteins are

activated (Malhotra and Kaufman, 2007) presumably by release from BiP/GRP78 (Bertolotti et al., 2000). Activation of IRE1 leads to Xbp1 mRNA splicing, allowing more efficient synthesis of XBP1 protein, a transcription factor that translocates to the nucleus and activates gene expression; ATF6 released from BiP transits to the Golgi body for cleavage, releasing an activated transcription factor to regulate gene expression in the nucleus; BiP releases the lumenal domain of PERK, allows PERK dimerization and autophosphorylation which leads to PERK activation (Ron and Walter, 2007) (Figure I-1). Activated PERK directly phosphorylates eIF2α at Ser51, leading to downregulation of global translation (Shi et al., 1998). However, under certain conditions such as virus infection, PERK activation may not cause translation inhibition (Isler et al., 2005). This PERK/ eIF2α/ATF4 pathway was shown to be activated by prolonged hypoxia and anoxia (Bi et al., 2005; Koritzinsky et al., 2006; Koumenis et al., 2002; Liu et al., 2006). At the same time, phosphorylation of eIF2α promotes the translational activation of ATF4 (see below).

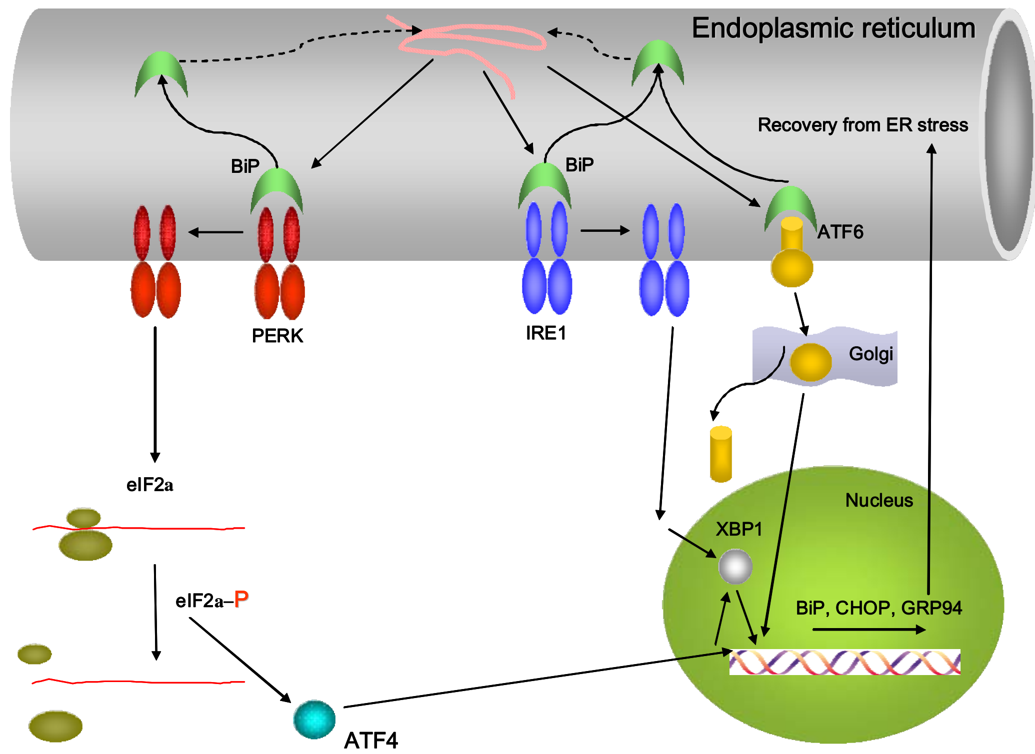


Figure I-1. The Unfolded Protein Response.

Integrated stress responses and the translational regulation of ATF4

Gene expression in eukaryotic cells is regulated at multiple levels. Compared to transcriptional regulation, translational regulation is capable of adjusting protein levels more rapidly and directly, allowing cells to quickly respond to environmental changes and maintain intracellular homeostasis. Under a variety of stresses, including nutrient deprivation, endoplasmic reticulum (ER) stress, hypoxia, virus infection, etc., the eukaryotic translational initiation factor eIF2α is phosphorylated at Ser51. In mammalian cells, four eIF2α kinases have been identified. HRI (heme regulated initiation factor 2 alpha kinase, EIF2AK1) is activated by heme deprivation; PKR (interferon-inducible RNA-dependent protein kinase, EIF2AK2) is activated by double-stranded RNA; PERK (PKR-like endoplasmic reticulum kinase, EIF2AK3) is activated by unfolded proteins in the ER; GCN2 (general control non-depressible kinase 2, EIF2AK4) is activated by uncharged tRNAs. Activation of all these kinases leads to eIF2α phosphorylation, therefore the term 'integrated stress responses' is used to describe this pathway (Figure I-2). eIF2α phosphorylation blocks the eIF2B-dependent recycling of eIF2-GDP to eIF2-GTP, causing global translation inhibition. Decreased translation helps cells to conserve nutrients and energy, slowing down the cell cycle (Koumenis and Wouters, 2006; Ron and Walter, 2007).

Although global translation is suppressed upon eIF2α phosphorylation, paradoxically, a group of stress-responsive mRNAs that include ATF4 are translated more efficiently when eIF2α is phosphorylated (Harding et al., 2000a). This translational regulation model was first characterized in the yeast homolog GCN4 of

ATF4 and was later found to also exist in mammalian cells (Harding et al., 2000; Hinnebusch, 1984; Mueller and Hinnebusch, 1986; Vattem and Wek, 2004). ATF4 belongs to the activating transcription factor/cAMP responsive element binding protein (ATF/CREB) family which has a consensus binding site (cAMP responsive element, CRE) defined as TGACGT(C/A)(G/A) (Ameri and Harris, 2008; Hai and Hartman, 2001). Although transcriptional regulation of ATF4 has also been reported (Estes et al., 1995); (Kokame et al., 1996; Sato et al., 1998); (Siu et al., 2002), it is the translational regulation of ATF4 that has been more extensively studied.

The mammalian ATF4 mRNA has two upstream open reading frames (uORFs) located in the 5'UTR of the mRNA. uORF1 promotes ribosome scanning and reinitiating at uORF2 when abundant eIF2-GTP is available. Because uORF2 overlaps with the actual ATF4 ORF, the translation of uORF2 produces a non-functional product which causes ribosome release from the ATF4 mRNA, thereby repressing ATF4 translation under unstressed conditions (Harding et al., 2000; Lu et al., 2004; Vattem and Wek, 2004). When cells are exposed to stresses, eIF2α phosphorylation reduces eIF2-GTP levels so that the translation reinitiation at uORF2 is inhibited, allowing the ribosome small subunit to bypass uORF2 and start translation at the true ATF4 ORF in order to increase ATF4 protein level (Figure I-3). Two ATF4 targets ATF5 and GADD34 are translationally regulated in a similar manner (Lee et al., 2009; Watatani et al., 2008; Zhou et al., 2008).

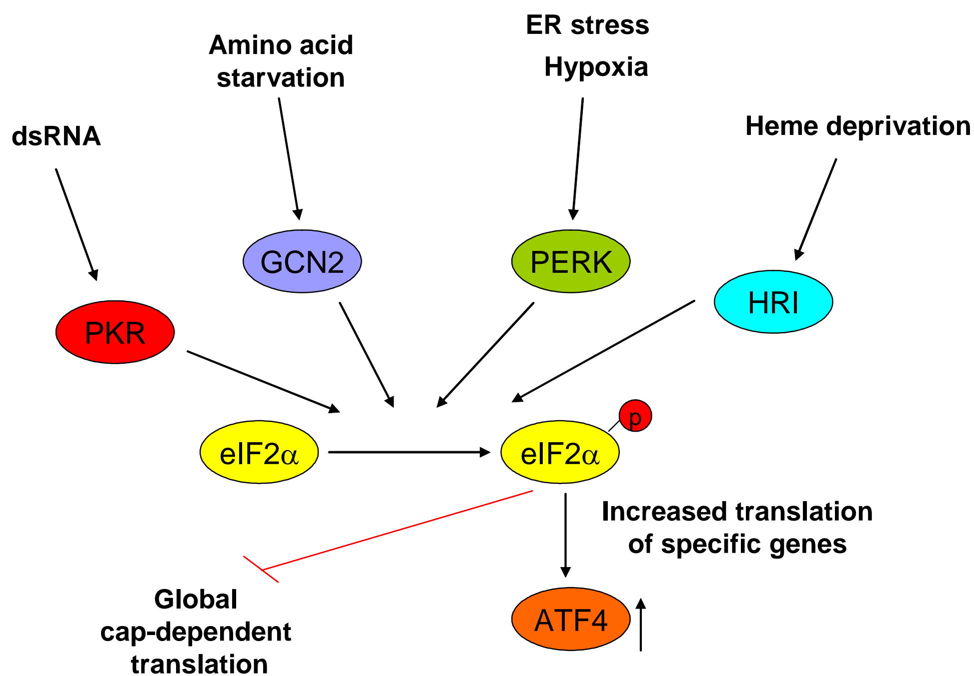
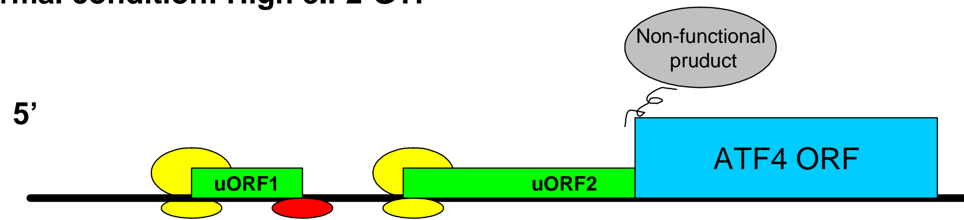


Figure I-2. The Integrated Stress Response. Four eIF2α kinases have been discovered in mammalian cells: HRI is activated by heme deprivation; PKR is activated by double-stranded RNA; PERK is activated by ER stress; GCN2 is activated by uncharged tRNA. Activation of these kinases causes eIF2α phosphorylation at Ser51 and activates integrated stress response pathway. eIF2α phosphorylation blocks the eIF2B-dependent recycling of eIF2-GDP to eIF2-GTP, causing global translation inhibition. Paradoxically, phosphorylated eIF2α also results in upregulation of a group of stress-responsive genes including ATF4 and some of ATF4 targets including ATF5 and GADD34.

Normal condition: High eIF2-GTP



Stressed condition: Low eIF2-GTP

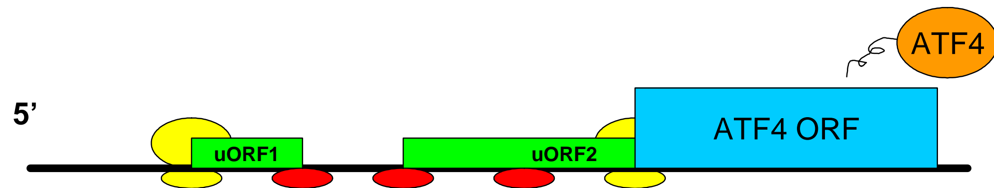


Figure I-3. Translational regulation of ATF4. Black line: ATF4 mRNA; Yellow: activated ribosome (with eIF2-GTP); Red: ribosome small subunits (with eIF2-GDP); Green: uORFs; Blue: actual ATF4 ORF.

ATF4 and its target genes are induced by hypoxia.

Although it was reported that rat embryonic fibroblasts exposed to anoxia show overexpression of ATF4 mRNA (Estes et al., 1995), studies in other systems (mouse and human cell lines) have shown that hypoxia/anoxia does not cause substantial upregulation of ATF4 mRNA (Ameri et al., 2004; Bi et al., 2005; Blais et al., 2004). The induction of ATF4 protein levels under hypoxia is not dependent on HIF-1, as it occurs in HIF-1α knock-down cells (Ameri et al., 2004; Blais et al., 2004; Koritzinsky et al., 2006). ATF4 was found to be translationally upregulated under hypoxia in several tumor cell lines and ATF4 protein levels were significantly higher in tumor samples compared to corresponding normal human tissue (Ameri et al., 2004; Bi et al., 2005; Blais et al., 2004). Moreover, the expression of ATF4 was found to overlap with hypoxic regions in human tumor samples of patients with cervical carcinoma who had received the hypoxia-sensitive dye pimonidazole (Ameri et al., 2004; Bi et al., 2005). Our group also reported that $ATF4^{-/-}$ MEFs are more sensitive to prolonged hypoxia-induced apoptosis compared to $ATF4^{+/+}$ MEFs(Bi et al., 2005). Together, these data suggest that increased ATF4 levels may play an important role in tumorigenesis. The functions of some of the ATF4 targets induced by hypoxia are discussed below.

CHOP/GADD153: C/EBP homologous protein (CHOP) is a basic leucine zipper (bZIP) transcription factor implicated in ER stress-induced apoptosis (Zinszner et al., 1998). It was shown to enhance apoptosis via repressing expression of anti-apoptotic Bcl-2 and the antioxidant GSH (McCullough et al., 2001) or by inducing death

receptor 5 (DR5) in response to cyclooxygenase-2 (COX-2) inhibitors (He et al., 2007). CHOP is upregulated by ATF4 in response to many cellular stress signals such as arsenite (Fawcett et al., 1999), amino acid deprivation (Averous et al., 2004) and hypoxia. However, the role of CHOP in hypoxic tumor cell survival has not been carefully examined yet.

TRB3/SKIP3: TRB3 is the human ortholog of the *Drosophila tribbles* gene product. TRB3 is induced by hypoxia in tumors in an ATF4-dependent manner (Bowers et al., 2003), though it was later found that induction of TRB3 by ER stress depends on both ATF4 and CHOP (Ohoka et al., 2005; Ord and Ord, 2005). The data so far point towards a model in which TRB3 creates a negative feedback for ATF4/CHOP induction by directly binding to both ATF4 and CHOP to inhibit their transcriptional activities (Ohoka et al., 2005; Ord and Ord, 2003).

GADD34: GADD34 is a protein that interacts with the catalytic subunit of protein phosphatase 1 (PP1) to promote dephosphorylation of p-eIF2α thereby allowing translation recovery from ER stresses (Novoa et al., 2001). GADD34 has a consensus ATF/CREB binding site in its promoter and its induction by ER stress and hypoxia is ATF4 dependent (Blais et al., 2004; Ma and Hendershot, 2003). Therefore, induction of GADD34 creates not only a negative feedback loop in regulating ATF4, but also helps recovery of translation when stressed conditions are alleviated.

ATF3: ATF3 is another ATF family member induced by ATF4 under ER stress and anoxia, and it contributes to CHOP and GADD34 induction in response to stress (Ameri et al., 2007; Jiang et al., 2004). Several reports have suggested that ATF3 can

be a ‘double-edged sword’ in tumor development. On one hand, it stabilizes p53 upon genotoxic stress to inhibit cellular transformation (Yan and Boyd, 2006); on the other hand, it is also known to promote cellular proliferation in response to serum stimulation (Tamura et al., 2005). A recent study demonstrated that ATF3 promotes apoptosis in untransformed mammary epithelial cells but enhances migration in malignant cells via induction of pro-metastatic genes (Yin et al., 2007).

ATF4 has important functions during development and tumor resistance to genotoxic stress

Expression of ATF4 mRNA has been detected in various tissues including bone marrow, kidney, lung, heart, skeletal muscle, brain, spleen and pancreas, etc. ATF4 appears to play multiple roles during development: ATF4 knockout mice have abnormal lens formation, growth retardation, anemia and delayed bone development (Masuoka and Townes, 2002; Tanaka et al., 1998; Yang et al., 2004). Interestingly, ATF4 also has key functions in neural system activity and development. In addition to affecting long-term synaptic plasticity and memory formation storage (Chen et al., 2003; Costa-Mattioli et al., 2005; Costa-Mattioli et al., 2007), it has been proposed that the induction of ATF4 upon mental stress may also help reduce emotional reactivity and enhance depression-like behavior (Green et al., 2008). It is postulated that the interaction between ATF4 and γ-aminobutyric acid type B receptor may account for the functions of ATF4 in these neuron activities (Vernon et al., 2001; White et al., 2000).

In terms of tumor relevance, dysregulation of ATF4 expression has been implicated in induction of chemo-radioresistance. ATF4 mRNA levels correlate with tumor cell resistance to DNA-interacting drugs such as cisplatin, and overexpression of ATF4 reduces cell sensitivity to cisplatin (Levenson et al., 2000; Tanabe et al., 2003). Moreover, recent work suggests that the circadian regulator protein Clock binds to the E-box in the ATF4 promoter and transcriptionally upregulates ATF4 in response to cisplatin, which induces enzymes involved in glutathione metabolism and contributes to chemoresistance (Igarashi et al., 2007).

Tumor cells have high consumption rate of glucose and glutamine

In addition to hypoxia, tumor cells undergo nutrient starvation because they have much higher demands on nutrients compared to normal cells. It has been shown that activation of oncogenes is responsible for high metabolism rate of small molecular nutrients (DeBerardinis et al., 2008). For instance, many tumor cells have high rate of glucose uptake and glycolysis (Warburg, 1956), defined as 'Warburg effect'. Clinically, the high glucose consumption rate of cancer cells is exploited by the positron-emission tomography (PET) imaging technique which employs ^{18}F-2-deoxyglucose, a glucose analogue that can be absorbed but not metabolized by tissues, to measure glucose absorption rate. In addition to glucose, tumor cell also rely on high glutamine to produce energy, NADPH, as well as carbon source for non-essential amino acid and lipids synthesis (Chen et al., 1993; Coles and Johnstone, 1962; DeBerardinis et al., 2007). The oncogene c-myc transcriptionally upregulates

glutamine transporters such as ASCT2 and also represses microRNA miR-23a/b, leading to increased expression of their target gene, mitochondrial glutaminase, which upregulates glutamine metabolism (Gao et al., 2009; Wise et al., 2008).

The eIF2α kinase GCN2

Unlike PERK, which is activated by ER stress, GCN2 is a high molecular weight protein kinase activated by uncharged tRNA (Ramirez et al., 1992; Wek et al., 1990; Wek et al., 1995). GCN2 has a domain homologous to histidyl-tRNA synthetases (HisRSs) located next to the kinase catalytic moiety (Wek et al., 1989). Uncharged tRNA binds to synthetase-related domain of GCN2 (Dong et al., 2000; Wek et al., 1995), activiting the GCN2 kinase. Activated GCN2 phosphorylates eIF2α to translationally upregulate ATF4, which in turn increases amino acid biosynthetic and transport pathways (Harding et al., 2000; Harding et al., 2003).

GCN2 knockout mice are viable and fertile and display no gross phenotypic abnormalities under standard feeding conditions. However, if the diet lacks a single amino acid, there is a remarkable increase of prenatal death rate and liver abnormality (Anthony et al., 2004; Zhang et al., 2002). Other than maintaining amino acid homeostasis, GCN2 also regulates synaptic plasticity and memory (Costa-Mattioli et al., 2005) , feeding behavior (Hao et al., 2005; Maurin et al., 2005), as well as lipid metabolism (Guo and Cavener, 2007). GCN2 is also activated by UV radiation and mediates NF-κB signaling (Deng et al., 2002; Jiang and Wek, 2005).

Autophagy

A critical stress response process which is activated by nutrient deprivation is autophagy. Autophagy is a lysosomal-dependent intracellular degradation pathway activated in response to nutrient starvation , organelle damage or intracellular microorganisms (Mizushima, 2007). Most of current evidence suggests that inhibition of mTOR activity under amino acid starvation is the initiating signal for autophagy induction in eukaryotic cells (Diaz-Troya et al., 2008). In yeast, under amino acid starvation, autophagy induction depends on activated GCN2 and eIF2α phosphorylation (Talloczy et al., 2002). The process of autophagy includes several steps: (a) Cellular material (proteins/damaged organelles/microorganism) is sequestrated by phagophore or isolation membrane to form a double-membrane autophagosome; (b) Autophagosomes are merged with lysosomes and the cargoes are digested by lysosomal hydrolases together with the inner membrane of the autophagosome; (c) Monomers from degraded macromolecules (such as amino acids) can then be reused by the cell (Mizushima, 2007). Autophagy may promote cell survival in at least two ways: (a) Degrade unnecessary macromolecules and cellular components to release monomers for satisfying the minimum requirements for maintaining essential functions of the cell; (b) Eliminate damaged organelles and harmful inclusions, hence teleologically, in the short term it is considered a protective stress response (Degenhardt et al., 2006). However, prolonged induction of autophagy may also lead to irreversible cell death (autophagic cell death) in apoptosis-resistance cells (Bursch, 2001; Shimizu et al., 2004).

Project aims

Tumor cells have to adjust multiple metabolic and stress response pathways so that they can adapt to and survive through the unfavorable tumor microenvironment. Our group has been interested in the regulation of these pro-survival pathways and how to target these pathways for tumor therapy. Our lab's previous findings demonstrated that PERK activation and eIF2α phosphorylation are necessary for tumor cell survival under hypoxia and promote tumor growth. Additionally, the PERK downstream target ATF4 was found to be overexpressed in tumor tissues. In this study, we set out to investigate whether ATF4 contributes to hypoxic resistance of tumor cells and how hypoxia regulates ATF4 target genes expression. In the process of these studies, we discovered that in addition to its role in hypoxia resistance, ATF4 also contributes to intracellular amino acid homeostasis. Since transformed cells in the context of solid tumors are usually exposed to metabolic stresses, and GCN2 is a key molecular sensor responding to nutrient deprivation, we investigated whether the GCN2-ATF4 pathway regulates tumor cell amino acid metabolism and tumor progression. We discovered that activation of GCN2 is critical for tumor cell survival under amino acid and glucose deprivation; and moreover, inhibition of GCN2-ATF4 pathway reduces tumor xenograft growth *in vivo*.

Chapter II: The role of the transcription factor ATF4 in regulating tumor cell proliferation and survival by activating asparagine biosynthesis

This Chapter contributes to part of a manuscript submitted to *EMBO J*. (in revision)

Introduction

Previous studies in mouse embryonic fibroblasts (MEFs) demonstrated that ATF4 is a critical regulator of genes involved in redox balance and maintenance of amino acid homeostasis. Consequently, ATF4$^{-/-}$ MEFs require non-essential amino acids to survive and proliferate (Harding et al., 2003). So far, many genes involved in amino acid metabolism have been identified as ATF4 targets, including asparagine synthetase (ASNS) (Siu et al., 2002), solute carrier family 7, member 5 (Slc7a5), asparaginyl-tRNA synthetase (NARS) etc. (Harding et al., 2003). Though ATF4 overexpression in tumor tissues has been reported (Ameri et al., 2004; Bi et al., 2005), the role of ATF4 in regulating tumor cell amino acid metabolism or the precise functions of ATF4 in tumor cell survival and proliferation have not been elucidated yet.

Asparagine synthetase (ASNS) is an enzyme that catalyzes the ATP- and glutamine-dependent conversion of L-aspartate to L-asparagine (Asn), a non-essential amino acid that is required for protein synthesis and cell growth (Richards, Shuster 1998). ASNS was first indentified that in temperature-sensitive hamster cell line BHK *ts11*, a point mutation on *ASNS* gene leads to the production of an inactive enzyme at

non-permissive temperature, causing G_1 phase cell cycle arrest (Gong and Basilico, 1990). The mRNA levels of ASNS are strongly induced by amino acid starvation (Gong et al., 1991) since the ASNS promoter has amino acid response element (AARE) and nutrient sensing response elements (NRSEs) that ATF4 binds to (Barbosa-Tessmann et al., 2000; Guerrini et al., 1993; Siu et al., 2002).

In this study, we report that the knockdown of ATF4 reduces the survival and proliferation of two tumor cell lines (HT1080 and DLD1) *in vitro*. We show that ATF4 is necessary for ASNS expression that maintains asparagine production, which is crucial for cell survival and proliferation. Insufficient asparagine production induces G_1 phase cell cycle arrest, apoptosis and autophagy in tumor cells. The induction of autophagy facilitates tumor cells to survive under asparagine deficiency. Since ASNS needs glutamine to synthesize asparagine, ATF4 knockdown cells are sensitive to glutamine deprivation. *In vivo* xenograft experiments demonstrated that ATF4 inhibition reduces tumor growth, suggesting ATF4 can be targeted for tumor therapy.

Results

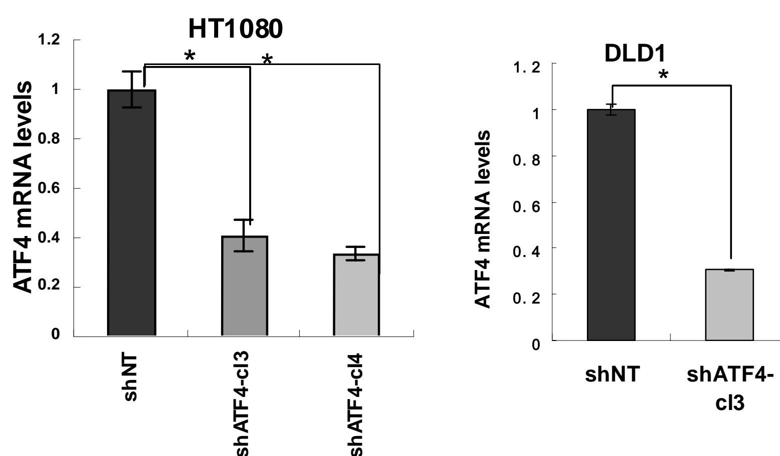
Generation of stable ATF4 knockdown cell lines

To investigate the role of ATF4 in tumor cell proliferation and survival, plasmids expressing ATF4 specific shRNA (pSM2-shATF4) or non-targeting shRNA (pLKO-shNT) were transfected into HT1080 (human fibrosarcoma, p53 wild-type) or DLD1 (human colorectal adenocarcinoma, p53-mutant) cells. Two established HT1080 shATF4 clones (shATF4.cl3 and shATF4.cl4) and one DLD1 shATF4 clone showed between 60-70% reduction of ATF4 mRNA levels compared to corresponding shNT clones (Figure II-1A). Since the basal ATF4 protein levels are low in unstressed cells, we treated cells with the ER stress-inducing agent thapsigargin to upregulate ATF4. Consistent with mRNA levels, both HT1080 and DLD1 shATF4 clones showed no ATF4 induction after treatment with thapsigargin (Figure II-1B).

ATF4 expression is required for survival of tumor cells in the absence of non-essential amino acid supplementation.

It was previously reported that ATF4$^{-/-}$ MEFs require the presence of non-essential amino acids (NEAA) and antioxidant such as β-mercaptoethanol (β-ME) to survive (Harding et al., 2003) (Figure II-2A). Similar to SV40-immortalized ATF4$^{-/-}$ MEFs, tumor cells expressing ATF4 shRNA demonstrated significantly reduced survival in the absence of NEAA (Figure II-2B). In contrast, cell survival of HT1080.shATF4 was not affected by adding β-ME at concentrations from 25μM to 0.2mM (data not shown).

A.



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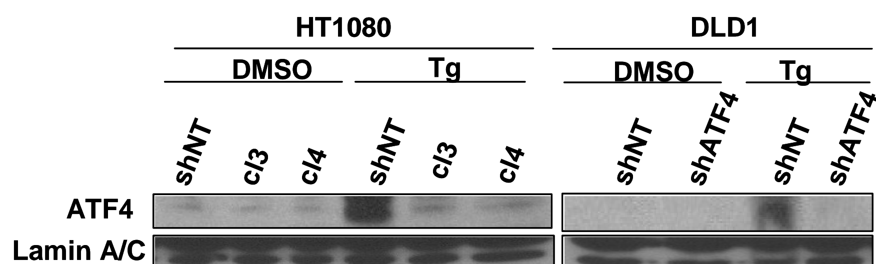
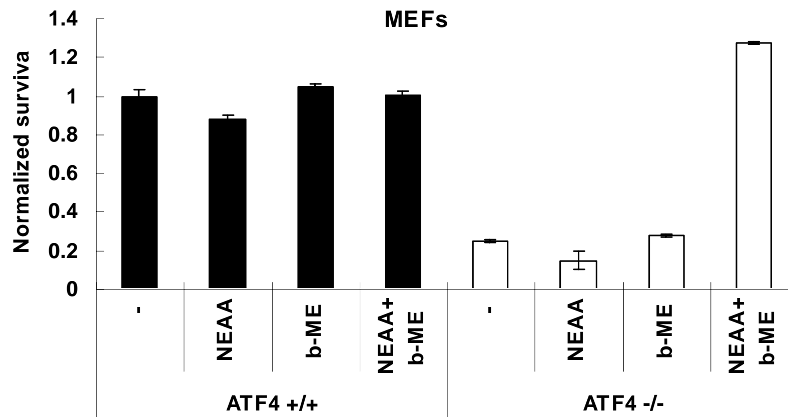


Figure II-1. Generating stable shATF4 cell lines. (A) Real time RT-PCR analysis for ATF4 mRNA levels in HT1080 and DLD-1 clones expressing none-targeting shRNA (shNT) or shRNA targeting human ATF4 (shATF4). mRNA levels were normalized against 18s rRNA internal control and are reported as a fraction compared to levels in shNT control cells. PCR reactions were done in triplicate. (Data represent mean ± SEM, n=3, *$p<0.05$.) (B) ATF4 protein levels from the nuclear fractions of thapsigargin-treated (Tg, 1μM, 4h) or DMSO-treated cells. Lamin A/C was used as a loading control.

A.



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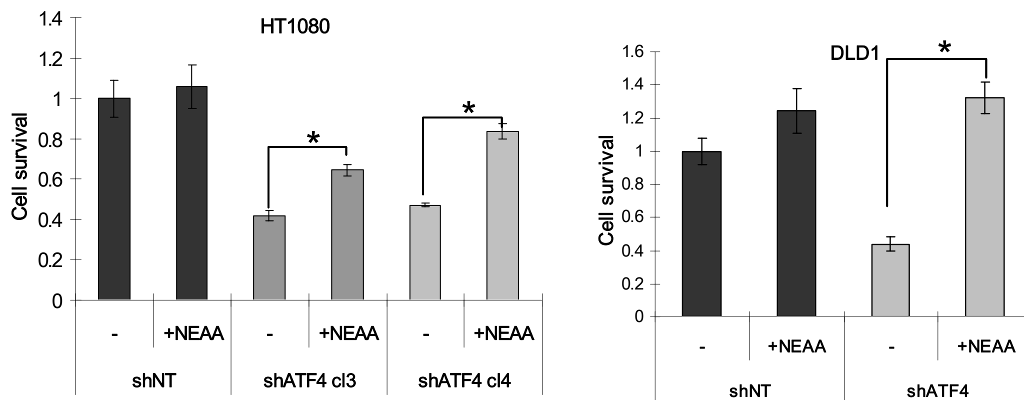


Figure II-2. Knock-down of ATF4 inhibits tumor cell survival. (A) $ATF4^{+/+}$ and $ATF4^{-/-}$ MEFs were incubated in DMEM with ingredients indicated for 48h. Cell survival was measured using MTT assay. **(B)** Survival of HT1080 and DLD1 cells cultured in the presence or absence of NEAA (100μM) measured by MTT assay 48h after plating (Data represent mean±SEM, n = 3, $*p < 0.05$.). Cell survival was normalized to control (shNT cells without NEAA).

Knockdown of ATF4 induces G_1 phase cell cycle arrest and proliferation arrest.

Reduced cell survival of HT1080.shATF4 cells could result from decreased cell proliferation and/or increased cell death. By performing fluorescent EdU incorporation in exponentially growing cells, we found that that shATF4 cells showed a 35% reduction in cell proliferation compared to shNT cells (Figure II-3A). Adding NEAA to the medium led to full recovery of cell proliferation (Figure II-3B). Flow cytometric analysis also demonstrated that shATF4 cells had an increased G_1 population compared to shNT cells (Figure II-3C), indicating that ATF4 knockdown causes G_1/S arrest in tumor cells. Addition of NEAA partially reversed the G_1 arrest. These data suggest that ATF4 deficiency induces amino acid starvation which causes G_1/S cell cycle arrest and reduced proliferation.

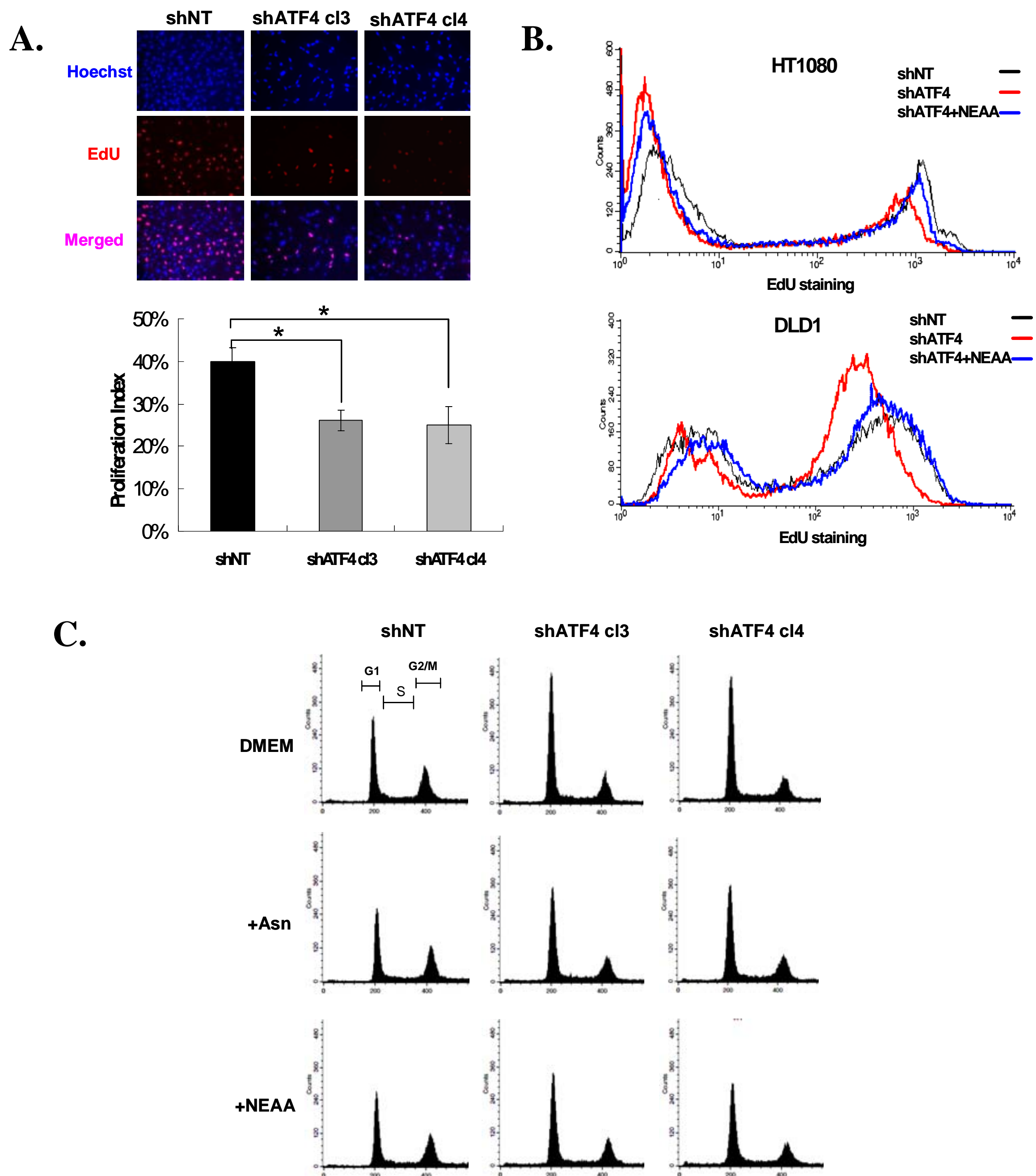


Figure II-3. Knockdown of ATF4 inhibits cell proliferation. **(A)** Cell proliferation assay. HT1080 cells were plated in DMEM with/without NEAA for 24h. Upper panel: Fluorescent staining for nuclei (Hoechst, blue) and proliferating cells with EdU (red). Lower panel: Three randomly selected photographs were selected and numbers of EdU positive and total nuclei were counted. % proliferation index was calculated

by dividing the number of proliferating nuclei by the total number of nuclei. (Data represent mean ± SEM, n=3, *$p<0.05$.) **(B)** Cell proliferation of HT1080 and DLD1 cells measured using flow cytometry EdU assay. EdU-: EdU negative cells, arrested population; EdU+: EdU positive cells, proliferating population. Cells were incubated in DMEM with amino acids for 24h. **(C)** Cell cycle was analyzed by propidium iodide (PI) staining using CellQuest (Becton Dickinson). Cells were incubated in DMEM with amino acids for 24h.

Knockdown of ATF4 in transformed cells induces apoptosis.

When the HT1080 ATF4 knockdown cells were cultured in the absence of NEAA, morphological features of apoptosis such as blebbing and spiking were observed, which were diminished by the addition of NEAA (Figure II-4A). shATF4 cells also had higher levels of cleaved-PARP, an apoptosis marker, which was similarly reduced in the presence of NEAA (Figure II-4B). To further analyze the levels of apoptosis in shATF4 cells cultured without NEAA, we measured caspase 3/7 activities. shATF4 cells exhibited over a 13-fold increase in basal caspase 3/7 activity compared to shNT cells; similarly, supplementation with NEAA significantly reduced the caspase activities (Figure II-4C).

To exclude the possibility that the defects of shATF4 cells were due to off-target effects of the shRNA or selection-induced mutations, we overexpressed full-length mouse ATF4 that was not targeted by the shRNA against human ATF4 using an adenoviral vector. Overexpression of mATF4 significantly increased cell survival and blocked the apoptotic phenotype of shATF4 cells (Figures II-4D, E). These findings further support a pro-survival role of ATF4 in these tumor cells.

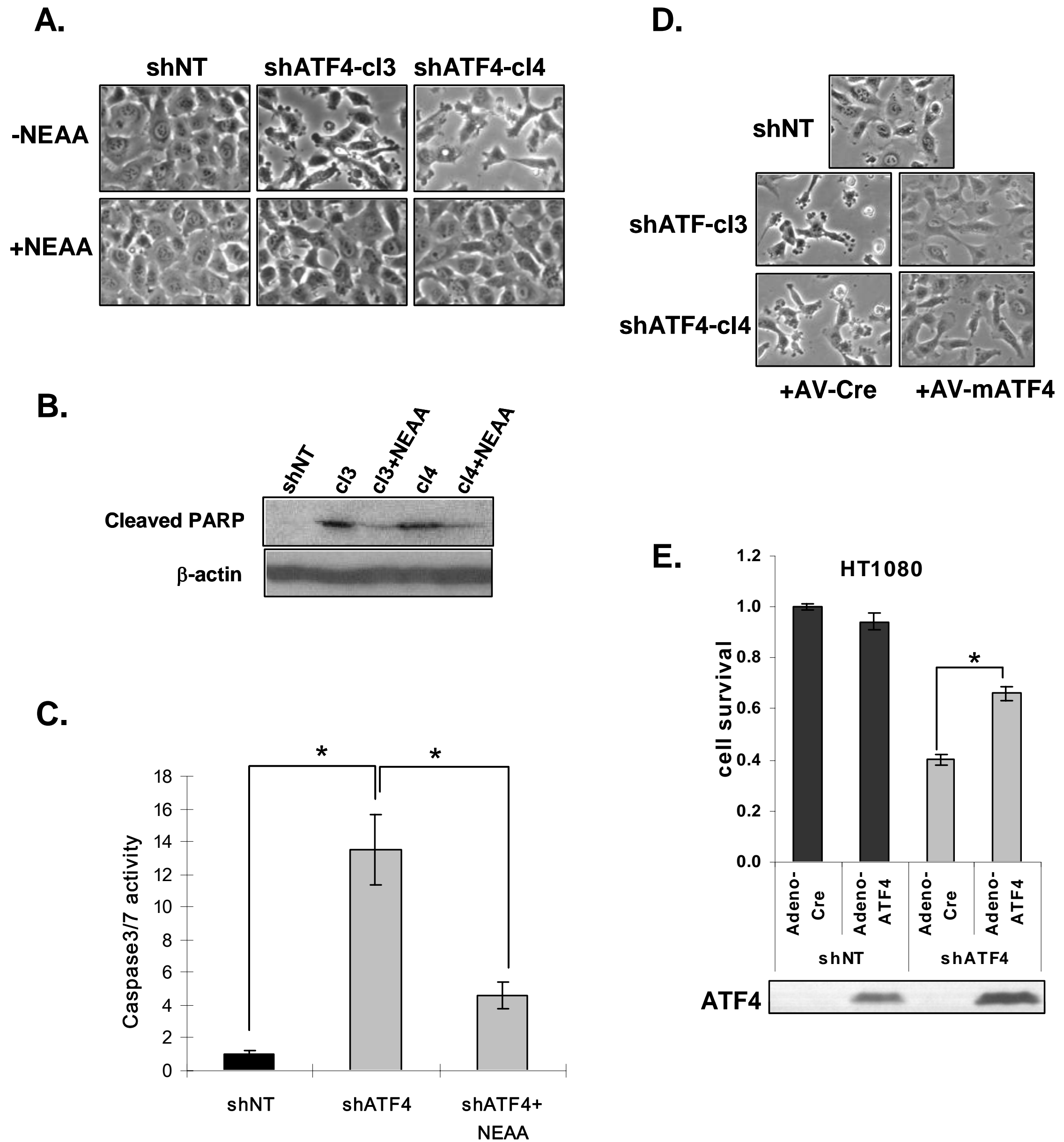


Figure II-4. Knock-down of ATF4 induces apoptosis in tumor cells. **(A)** Phase-contrast images of HT1080 shNT and shATF4 cells growing in DMEM with or without NEAA for 24h. Images shown were taken at 400× magnification. **(B)** Immunoblot for cleaved-PARP in HT1080 shNT and shATF4 cells. β-actin was used as a loading control. HT1080 cells were incubated in DMEM with/without NEAA for 24h. **(C)** Caspase 3/7 activities normalized to total number of cells. HT1080 cells

were incubated in DMEM with/without NEAA for 24h. (Data represent mean ± SEM, n=3, *p<0.05.) **(D)** Phase-contrast images of HT1080 shNT and shATF4 cells infected with the control (Av-Cre) or adenovirus expressing mouse ATF4 (AV-mATF4) indicated at 400× magnification. Following infection, cells were incubated in DMEM without NEAA. Images were taken 24h after infection. **(E)** Relative cell survival in infected cells using an MTT assay (top panel). Equal numbers of cells were plated in DMEM without NEAA 24h after infection. MTT assay was performed after 48h. (Data represent mean ± SEM, n=3, *p<0.05.) Cells infected under same condition were also collected at 24h after infection for ATF4 immunoblot (bottom panel).

Knockdown of ATF4 induces a pro-survival autophagic response

Autophagy is a lysosomal-dependent intracellular degradation process that is activated by several stresses, primarily by nutrient starvation. Since ATF4 was reported to regulate genes involved in amino acid biosynthesis and transport (Harding et al., 2003), we hypothesized that autophagy might be induced in the HT1080.shATF4 cells as an initial pro-survival response to amino acid deprivation. This hypothesis was supported by the fact that in the absence of NEAA, shATF4 cells had a smaller size compared to shNT cells and cytoplasmic vacuoles (a sign of autophagosome formation) were observed (data not shown). shATF4 cells had elevated levels of the autophagy marker LC-3II compared to shNT cells (Figure II-5A) which were reduced to control levels by adding NEAA, indicating that autophagy was induced by amino acid deprivation.

Under electron microscopy, the HT1080.shNT cells exhibited typical fibroblast morphology with intact ER and mitochondria, while the shATF4 cells were smaller, rounded and contained double-membrane autophagosomes, further confirming extensive induction of autophagy in shATF4 cells (Figure II-5B, arrows pointing to autophagosomes). Similar to LC-3 processing, addition of NEAA reversed the autophagic phenotype. To test whether the autophagy induced in shATF4 cells was a cytoprotective stress response, a siRNA targeting Atg7 (an E1-like ubiquitin conjugating enzyme required for autophagosome maturation) was employed to inhibit autophagy. When Atg7 levels were reduced, the shATF4 cells showed increased apoptosis compared to shNT cells (Figure II-5C, D). These results indicate that the

autophagy induced by loss of ATF4 in the HT1080 cells promotes survival and that the combination of losing ATF4 and the inhibition of autophagy results in a cooperative enhancement of cell death.

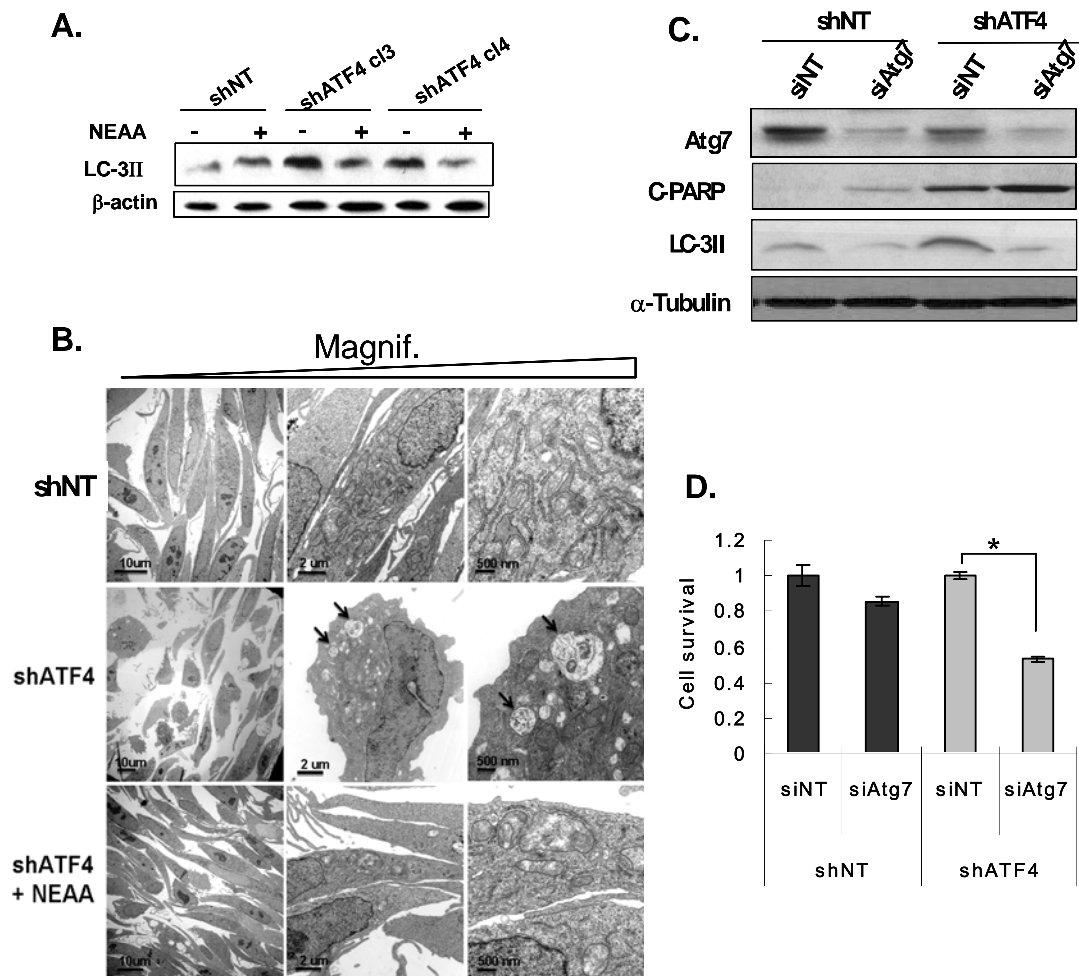


Figure II-5. Knock-down of ATF4 induces protective autophagy in tumor cells. (A) Immunoblot for the autophagy marker LC-3II from whole cell lysates in HT1080 shNT and shATF4 cells incubated in DMEM with/without NEAA for 24h. β-actin was used as a loading control. **(B)** Electron microscopy imaging. Arrows point to double membrane-containing autophagosomes. HT1080 cells were incubated in DMEM with/without NEAA for 24h. **(C)** HT1080 cells were transfected with 100nM non-targeting siRNA (siNT) or siRNA targeting Atg7 (siAtg7). Atg7 and cleaved-PARP levels were analyzed by immunoblotting 24h after transfection.

α-tubulin was used as a loading control. **(D)** HT1080 cells were transfected with 100nM non-targeting siRNA (siNT) or siRNA targeting ATG7 (siATG7). After 72h, cell survival of HT1080 and DLD1 cells was measured by MTT assay. Experiment was done in triplicate. (Data represent mean ± SEM, $^{*}p<0.05$.)

Addition of Asn in trans or expression of ASNS, rescues the survival of shATF4 cells.

The mixture of NEAAs utilized in the experiments described above was comprised of seven amino acids: Ala, Asp, Asn, Glu, Gly, Pro and Ser. Each amino acid was present at a final concentration of 100μM in DMEM. To determine which amino acid(s) was/were responsible for mediating the pro-survival effects of ATF4, individual amino acids were added into DMEM at a 100μM final concentration in cultured shATF4 cells. The results indicated that Asn on its own, but not any other individual amino acid, could rescue the survival of shATF4 cells (Figure I-6A). An even more substantial pro-survival effect of Asn was observed in a long-term clonogenic survival assay (Figure II-6B).

It has been reported that the asparagine synthetase (ASNS) gene is directly regulated by ATF4 which binds its promoter (Gjymishka et al., 2009; Siu et al., 2002). Indeed, we also found that ASNS expression was reduced by more than 70% in shATF4 cells compared to shNT cells (Figure II-6C). Moreover, adding Asn to HT1080.shATF4 cells rescued NEAA deprivation-induced G_1 arrest (Figure II-3C), a finding which is consistent with reports that ASNS deficiency can induce a G_1 arrest (Gong and Basilico, 1990; Greco et al., 1987). Furthermore, overexpression of ASNS in shATF4 cells partially rescued cell survival (Figure II-6D). This result suggests that ASNS is an important target for maintaining intracellular asparagine levels, which are crucial for tumor cell survival and cell cycle progression.

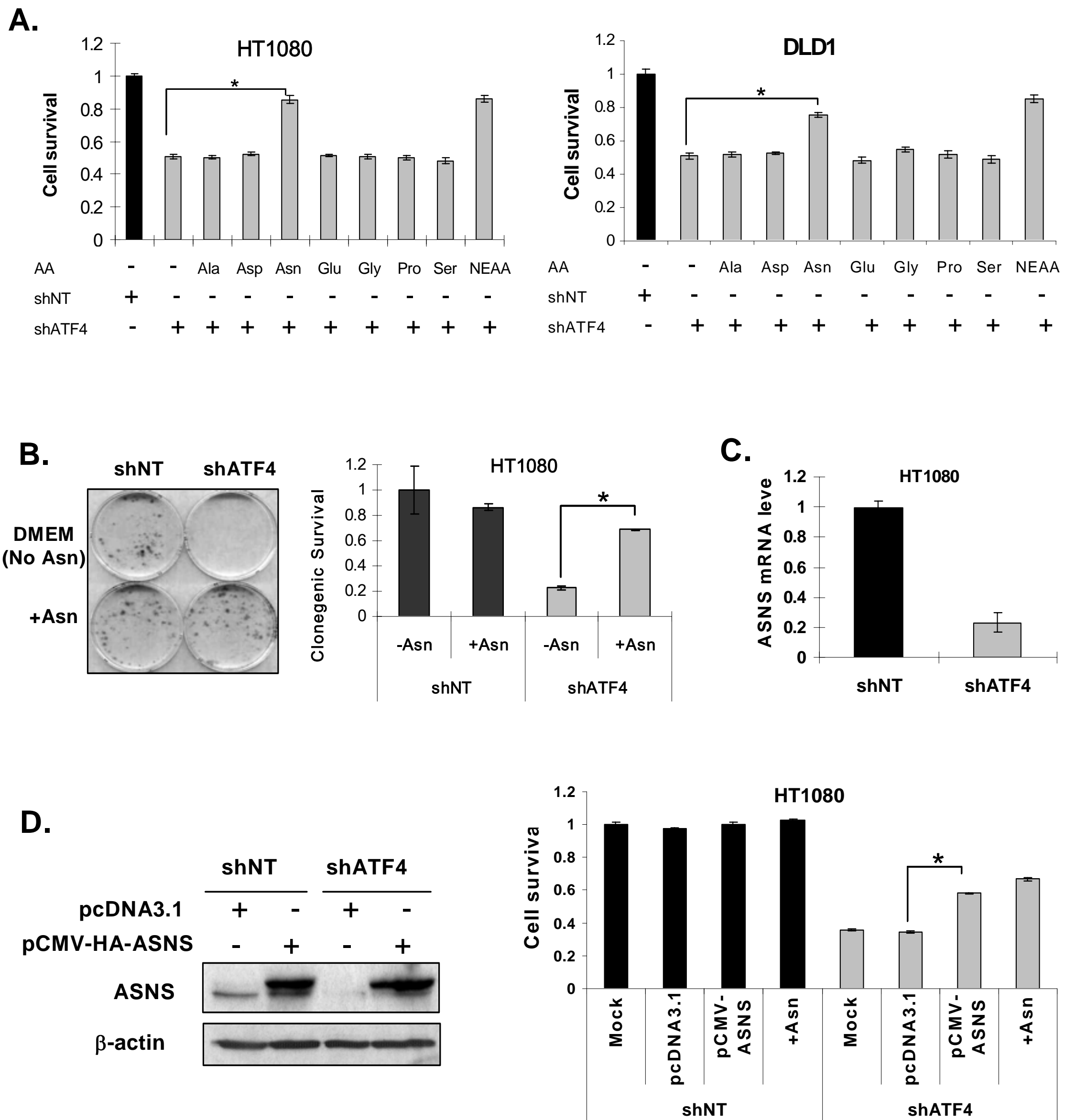


Figure II-6. Supplementation with Asn or overexpression of ASNS rescues survival of shATF4 cells. (A) Survival of HT1080 (left) or DLD1 (right) cells grown in DMEM with indicated amino acids supplemented at a concentration of 100μM for 48h. **(B)** Clonogenic survival of HT1080 shNT/shATF4 cells. Left: Picture of a representative experiment. Right: Clonogenic survival of HT1080 shNT/shATF4 cells. (Data represent mean ± SEM, n=4, *p< 0.05.) **(C)** ASNS mRNA levels in HT1080

shNT/shATF4 cells measured using real time RT-PCR. (Data represent mean ± SEM, n=3, *$p<0.05$.) **(D)** ASNS expression and cell survival in transfected HT1080 cells. Left panel: top bands represent HA-tagged ASNS, lower bands represent endogenous ASNS. Right panel: Survival of HT1080 shNT and shATF4 cells following indicated treatments. After 24h, transfected cells were harvested for immunoblot or plated for MTT assay (48h). (Data represent mean ± SEM, n=3, *$p<0.05$.)

Knock-down of ATF4 increases sensitivity to glutamine deprivation

The amino acid glutamine serves not only as a substrate for nucleotide and protein synthesis and as a precursor for the synthesis of Asn, but also as an important energy source for tumor cells (DeBerardinis et al., 2007; Reitzer et al., 1979). To produce Asn, ASNS transfers the amino group from Gln to Asp (Figure II-7A). Since ASNS requires Gln to synthesize Asn, we wanted to test whether shATF4 cells were more sensitive to Gln deprivation than shNT cells. To test this, shNT and shATF4 cells were cultured in DMEM with/without 2mM glutamine. MTT assays demonstrated that shATF4 cells showed about a 50% reduction in survival in the absence of Gln, while the shNT cells exhibited only a 25% reduction after 48h incubation (Figure II-7B). Interestingly, adding Asn (100μM final concentration) to Gln-deprived cells could partially rescue cell survival (Figure II-7C), suggesting that producing Asn may also be an important role of Gln. In summary, ATF4 deficiency severely inhibits tumor cell survival *in vitro*, which is primarily due to Asn deprivation.

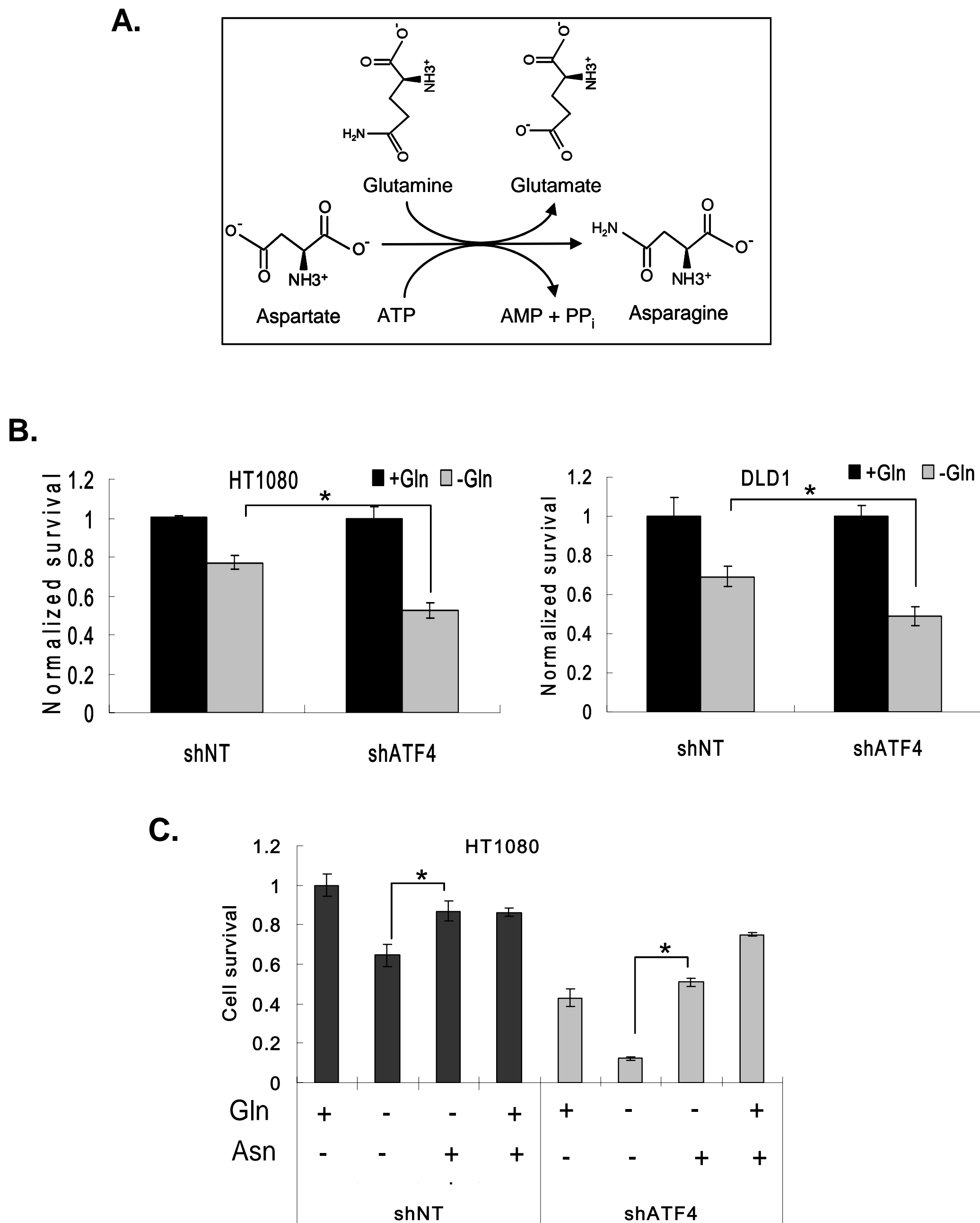


Figure II-7. Knock-down of ATF4 increases sensitivity to glutamine deprivation. **(A)** The biosynthetic reaction catalyzed by ASNS. **(B)** Survival of HT1080 (left panel) or DLD1 (right panel) cells grown in DMEM with/without 2mM L-glutamine for 48h. **(C)** Survival of HT1080 cells in DMEM with/without Gln or Asn for 48h. Gln: 2mM, Asn: 100μM. (Data represent mean ± SEM, n=3, *p<0.05.)

ATF4 is necessary for tumor growth *in vivo*

The fact that ATF4 deficiency causes significant reduction in cell survival *in vitro* suggests that ATF4 might play a role in tumor growth *in vivo*. To test this, equal numbers of HT1080 shNT or shATF4 cells were injected in the flanks of nude mice and tumor growth was monitored over a 3-4 week period. shNT cells grew rapidly and formed large tumors. However, the shATF4 cells formed fewer tumors that were also significantly growth-inhibited (Figure II-8A). Immunofluorecence analysis of *in vivo* cell proliferation using the Ki67 antigen as a marker, demonstrated that, consistent with the *in vitro* data, cells in shATF4 tumors had a significantly lower proliferation rate (Figure II-8B), indicating that ATF4 promotes tumor cell proliferation *in vivo*.

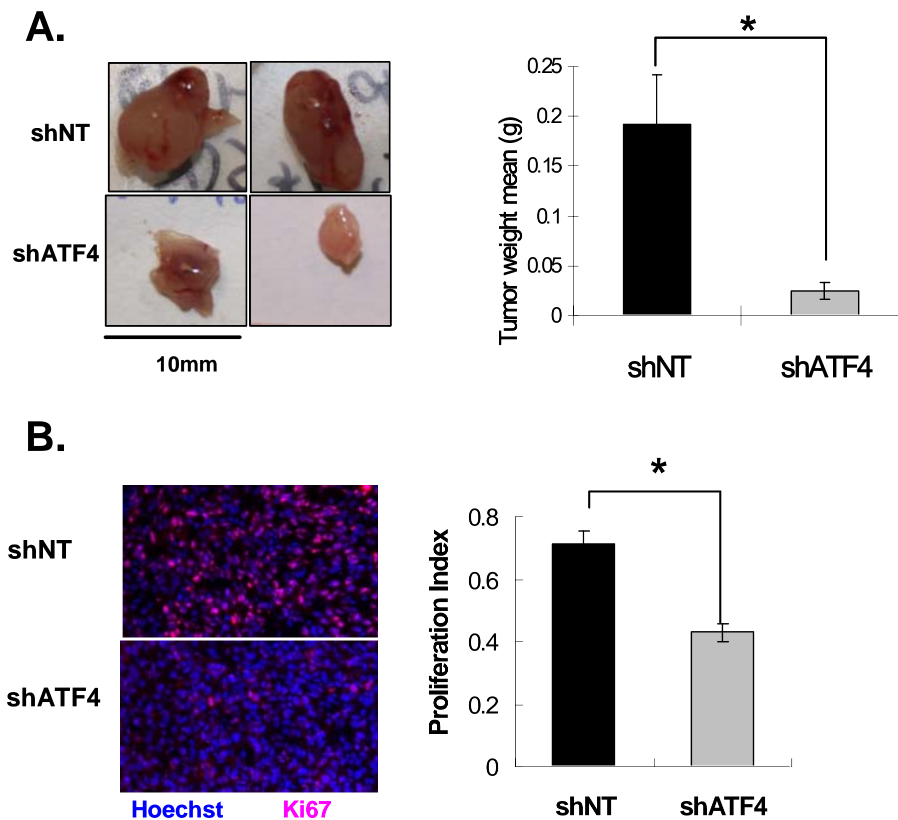


Figure II-8: Knockdown of ATF4 blocks tumor growth *in vivo*. **(A)** Left panel: Photographs of xenografted tumors from HT1080 shNT/shATF4 cells in nude mice injected in each side with 2×10^6 shNT or shATF4 cells. Tumors grew for 3 weeks. Right panel: Average tumor weight at conclusion of experiment (Data represent mean ± SEM, n=10, *p<0.05.) **(B)** Left panel: Immunofluorescent staining for Ki67 (red) in tumor sections. Nuclei were counterstained with Hoechst 33342 (blue). Tumors were photographed at 200×magnification. Right panel: The quantification of Ki67 signal. (Data represent mean ± SEM, n=4, *p<0.05.)

Discussion

Rapidly proliferating transformed cells have been shown to increase their nutrient uptake in excess of their bioenergetic needs and to divert metabolic programs towards pathways that support macromolecular biosynthesis to support their rapid growth (DeBerardinis et al., 2008). Our study supports a model in which inhibition of ATF4 leads to sub-optimal growth and survival of tumor cells and xenografts, due to an imbalance between amino acid requirements and biosynthetic pathway function and identifies asparagine as a key component of this regulatory mechanism.

Our previous studies showing higher sensitivity of ATF4$^{-/-}$ mouse embryonic fibroblasts to hypoxia *in vitro*, coupled with reports of abnormal homeostasis of redox balance and amino acid metabolism in these cells (Harding et al., 2003), prompted us to investigate the relationship between the synthesis of ATF4 and stress conditions of the tumor microenvironment, specifically nutrient deprivation and hypoxia.

Our studies show that inhibition of ATF4 in the absence of any additional stress, sensitizes tumor cells to NEAA deprivation, a result that is consistent with previous studies done in MEFs (Harding et al., 2003). In contrast to ATF4$^{-/-}$ MEFs, which also required β-ME for survival, tumor cells expressing ATF4 shRNA did not need antioxidant supplement to survive. This difference could be due to at least two possible mechanisms: (a) the low level of ATF4 remaining in tumor cells still satisfies the cellular needs for antioxidant activity; (b) tumor cells either overexpress antioxidant enzymes or are more tolerant to oxidative stresses. However, ATF4 knockdown tumor cells did demonstrate reduced survival in the absence of NEAA,

indicating that these cells were not able to synthesize certain amino acid(s) that is/are crucial for survival and proliferation, thus cell cycle arrest, apoptosis and autophagy were induced.

Autophagy is believed to enable cancer cells to survive under nutrient starvation and has multiple roles in tumor progression. In this study, we found evidence that loss of ATF4 leads to a lower apoptotic threshold; yet paradoxically also appears to stimulate autophagy. However, this result can be explained under the prism of energy homeostatic mechanisms: initially, loss of ATF4 (and thereby a substantial portion of amino acid biosynthetic and transport capacity) leads to nutrient deprivation and initiation of the autophagic response. This notion is supported by morphological and molecular evidence demonstrating formation of double membrane-engulfed cytoplasmic vacuoles and elevated levels of the autophagic marker cleaved LC-3 in HT1080.shATF4 cells in the absence of NEAA. These effects are readily but incompletely reversed when knockdown cells are supplemented with NEAA, suggesting that amino acid deprivation is the key activator of autophagy. Despite the activation of this pro-survival mechanism, however, the inability of transformed cells to synthesize amino acids (and more specifically Asn) ultimately leads to activation of apoptotic pathways culminating in cell death and loss of clonogenic survival.

The pro-survival role of the initial autophagic response is further supported by the fact that inhibition of Atg7, an enzyme responsible for fusion of peroxisomal and vacuolar membranes, prevents the induction of autophagy and results in increased

apoptosis in shATF4 cells. Interestingly, it has been recently reported that following Bortezomib treatment, ATF4 promotes autophagy by upregulating LC3B levels and this confers protection against Bortezomib-induced apoptosis (Milani et al., 2009). In this work, we have shown that ATF4 deficiency leads to higher levels of processing of LC3. Therefore, although ATF4 upregulation due to proteasomal inhibition can positively affect autophagy, lack of ATF4 can also indirectly promote autophagy via Asn depletion.

An analysis of the effect of individual amino acids on shATF4 tumor cells, showed that supplementation of asparagine, and no other individual amino acid, was sufficient to rescue cell survival, a finding that was later validated by our studies of asparagine synthetase (ASNS). Among the known downstream targets of ATF4 are proteins involved in amino acid transport and metabolism, including ASNS. HT1080 cells, like many other human tumor cell lines, grow in DMEM which is rich in glutamine (4mM). Glutamine deprivation significantly reduces survival of HT1080 and DLD-1 shATF4 cells, which could be partially rescued if asparagine was added. This finding suggests that in tumor cells, the high requirement of glutamine (a vital source of energy and nitrogen) (DeBerardinis et al., 2007; Sauer et al., 1982), may be (at least partially) due to the biosynthesis of asparagine. According to our unpublished microarray data, the expression of glutaminase, an enzyme that catalyzes glutamine deamidation (the first reaction in glutamine catabolism), is upregulated by ATF4, suggest that ATF4 may also promote tumor cells to make use of glutamine for energy production.

Our *in vivo* experiment also established that ATF4 is necessary for tumor growth. shATF4 cells formed fewer tumors which were significantly smaller and grew slower compared to the rapid formation of large shNT tumors. The shATF4 tumors exhibited reduced proliferation rates based on analysis of Ki67 staining. More testing need to be done to evaluate whether ATF4 deficiency leads to enhanced apoptosis rate. Insufficient Asn supply may be the primary reason that shATF4 cell formed much smaller tumors. At least two other aspects may also contribute to significantly reduced tumor growth of shATF4 cells: (a) In addition to ASNS, ATF4 regulates other amino acid metabolism-related genes. Unlike *in vitro* cell culture conditions, the tumor microenvironment may lack other amino acids that can be synthesized in control tumor cells but not in the shATF4 tumor cells; (b) ATF4 knockdown may inhibit angiogenesis so that the shATF4 tumors could not obtain adequate nutrients from blood.

Chapter III: Activation of the cytoplasmic kinase GCN2 is necessary for tumor cell survival, autophagy induction and tumor growth under nutrient deprivation

This Chapter contributes to part of a manuscript submitted to *EMBO J*. (in revision)

Introduction

While ATF4 can be transcriptionally regulated (Siu et al., 2002), it is the translational upregulation of ATF4 protein levels that has received the most attention. It has been demonstrated that the ATF4 induction under ER stress depends largely on PERK, while the induction under amino acid deprivation depends on GCN2 (Harding et al., 2000; Lu et al., 2004; Vattem and Wek, 2004).

The role of PERK in tumor progression, hypoxic resistance and angiogenesis has been reported. However, the regulation and potential contribution of GCN2 to tumor progression has not been investigated yet. Considering that tumor cells highly require nutrient supply such as glucose and glutamine, it is critical to investigate whether GCN2 is critical for tumor cells survival under metabolic stresses such as low amino acid and low glucose. In addition, based on our findings described in the previous chapter, we hypothesized that asparagine shortage in ATF4 knockdown cells may lead to GCN2 activation, which could regulate survival and proliferation.

In this study, we demonstrated that the activation of GCN2-ATF4 pathway promotes tumor cell survival under nutrient (amino acid or glucose) deprivation. Activation of GCN2 induces autophagy, which may contribute to cell survival under

nutrient deprivation. GCN2-eIF2α pathway is activated in various human and mouse tumor tissues. Deficiency of GCN2 severely inhibits tumor growth *in vivo*. Together, these results suggest that GCN2-ATF4 pathway is a promising target for tumor therapy.

Results

Activation of the GCN2-eIF2α pathway under amino acid deprivation promotes cell survival， upregulates p21 (cip1/waf1) and activates autophagy.

We hypothesized that if shATF4 cells are deficient in the biosynthesis of non-essential amino acids, this should lead to activation of the upstream kinase GCN2, completing an autoregulatory feedback loop. Indeed, we found that GCN2 was phosphorylated in HT1080.shATF4 cells and adding Asn or NEAA repressed this phosphorylation (Figure III-1A), suggesting that knocking down ATF4 reduces ASNS expression, causing an Asn deficiency which activated GCN2. eIF2α, the substrate of GCN2, was also phosphorylated in shATF4 cells in response to NEAA and similar to GCN2, its phosphorylation was repressed by addition of Asn or NEAA in trans. The CDK inhibitors p21 and p27 play a critical role in G_1/S cell cycle arrest in response to stress, and it had been reported that they can be induced by amino acid deprivation (Leung-Pineda et al., 2004). shATF4 cells constitutively expressed high levels of p21 compared to shNT cells. And the p21 expression were substantially reduced by adding NEAA or Asn; however, p27 levels were unaffected (Figure III-1A). This is consistent to a previous report that ATF4-null primary mouse bone marrow stromal cells have increased p21 but not p27 expression (Zhang et al., 2008).

Since GCN2 is the molecular sensor of amino acid deprivation that induces translational upregulation of ATF4, we tested whether GCN2 activation promotes tumor cell survival when a single amino acid is removed from the culture media. SV40-immortalized, Ras-transformed $GCN2^{+/+}$ and $GCN2^{-/-}$ MEFs were cultured in

DMEM with or without L-glutamine. Under Gln deprivation, $GCN2^{+/+}$ cells showed enhanced eIF2α phosphorylation and upregulation of ATF4, ASNS, and p21, while the $GCN2^{-/-}$ cells failed to activate this pathway and had increased levels of cleaved-caspase 3 (Figure III-1B). These results demonstrated that the induction of p21 under amino acid starvation is dependent on GCN2 activation.

Since eIF2α is currently the sole known substrate of GCN2, we wanted to further investigate whether the induction of ATF4 and p21 was dependent on eIF2α phosphorylation. To test this, eIF2α wild-type or eIF2α S51A mutant MEFs (a Ser-Ala mutation blocks eIF2α phosphorylation) were incubated in DMEM with or without Gln. Similar to the $GCN2^{-/-}$ cells, eIF2α S51A mutant cells were unable to induce ATF4, ASNS or p21 in the absence of Gln and had increased levels of apoptosis (Figure III-1C).

In DMEM (+Gln, +Met), $GCN2^{-/-}$ cells showed 25% reduction in cell survival compared to wild-type cells after 48h incubation, while Met or Gln deprivation further reduced the cell survival of $GCN2^{-/-}$ cells to around 50% or 4%, respectively (Figure III-1D). In summary, the activation of GCN2-eIF2α−ATF4 pathway is necessary for tumor cell survival under amino acid starvation.

It was previously reported that GCN2 activation and eIF2α phosphorylation induce autophagy in yeast (Talloczy et al., 2002). We also observed a correlation between GCN2 activation with LC-3 cleavage in HT1080.shATF4 cells (Figure III-1A), suggesting that GCN2 could be the molecular switch that senses amino acid shortage and induces autophagy in mammalian cells. To test this, wild-type, $GCN2^{-/-}$

and eIF2α S51A mutant MEFs were incubated in Gln-free media. GCN2$^{-/-}$ cells had significantly lower LC-3 processing compared to wild-type cells in response to Gln starvation. eIF2α S51A mutant cells could not induce any LC-3 processing (Figure III-1E). In conclusion, a functional GCN2-eIF2α pathway is required for amino acid starvation-activated autophagy in transformed cells.

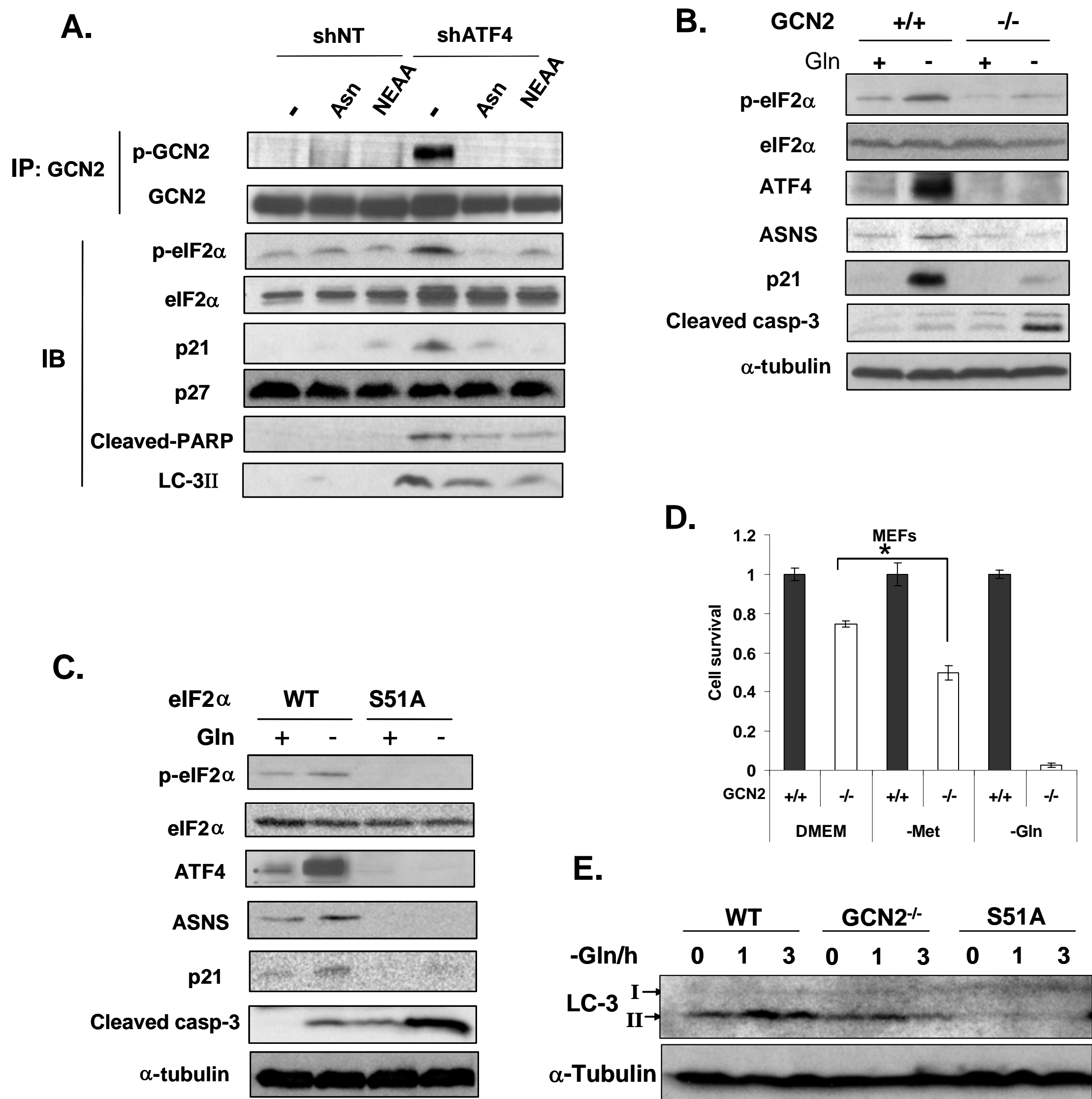


Figure III-1. Activation of GCN2-eIF2α pathway under amino acid deprivation promotes cell survival, upregulates ATF4 and p21, and activates autophagy. **(A)** HT1080 shNT and shATF4 cells were incubated in the media indicated for 24h. Whole cell lysates were harvested for immunoblot (IB) or immunoprecipitation (IP) with the indicated antibodies. **(B)** $GCN2^{+/+}$ and $GCN2^{-/-}$ MEFs were incubated with/without 4mM Gln for 24h and immunoblotting was performed. **(C)** eIF2α wt or eIF2α S51A mutant MEFs were incubated with/without 4mM Gln for 24h and

immunoblotting was performed with indicated antibodies. **(D)** $GCN2^{+/+}$ and $GCN2^{-/-}$ MEFs were incubated with or without Met or Gln for 48h. Cell survival was analyzed using MTT assay. (Data represent mean ± SEM, n = 3, *$p < 0.05$.) **(E)** Wild-type, $GCN2^{-/-}$ and eIF2αS51A mutant MEFs were cultured without 4mM Gln for 1h or 3h, cell lysates were subjected to immunoblotting.

Generation of stable GCN2 knockdown cell lines

To study the role of GCN2 for tumor cells under nutrient deprivation, we generated GCN2 knockdown cell line (HT1080.shGCN2) in the same manner as the HT1080.shATF4 cell line. Three individual shGCN2 clones showed over 50% reduction in GCN2 mRNA level (Figure III-2A). The reduced GCN2 expression was confirmed by immunoblot (Figure II-2B). shGCN2 clone 12 had the lowest GCN2 expression, so it was chosen for further studies.

To further confirm that the GCN2 activity was inhibited in shGCN2 cells, HT1080 shNT, shGCN2 and shATF4 cells were incubated in DMEM without glutamine, ASNS expression was measured as readout for the GCN2-ATF4 pathway activity. Unlike shNT cells, ASNS expression could not be induced in shGCN2 or shATF4 cells upon glutamine deprivation (Figure III-2C), suggesting that the ATF4 signaling, the GCN2 downstream target, is blocked in GCN2 knockdown cells.

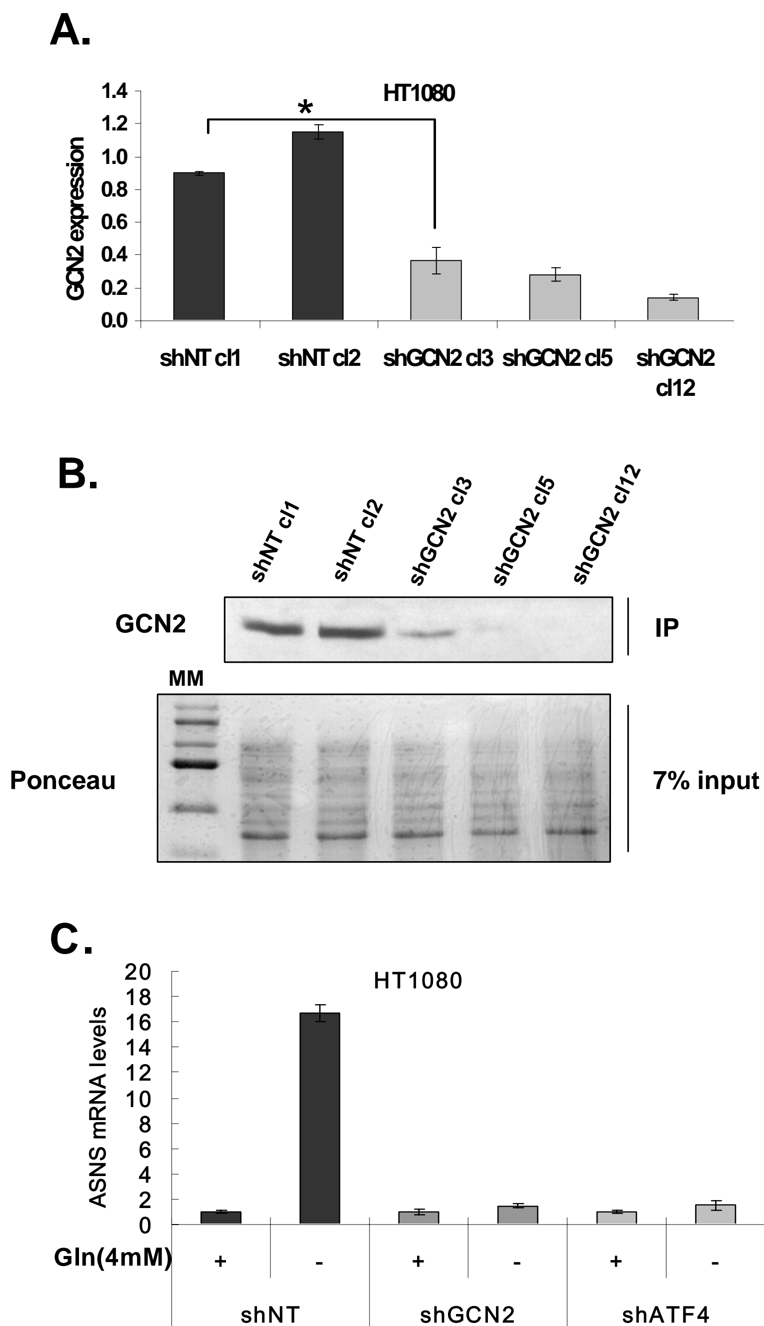


Figure III-2. Generating stable GCN2 knockdown cell line. (A) Real time RT-PCR analysis for GCN2 mRNA levels in HT1080 clones expressing non-targeting shRNA (shNT) or shRNA targeting human GCN2 (shGCN2). mRNA levels were normalized against 18s rRNA internal control and are reported as a fraction compared to levels in

shNT control cells. PCR reactions were done in triplicate. (Data represent mean ± SEM, n=3, *$p<0.05$.) **(B)** GCN2 knockdown was confirmed using immunoprecipitation. Ponceau staining for 7% of input cell lysate was used as a loading control. **(C)** HT1080 cells were incubated in full DMEM or DMEM (-Gln) for 8h. Total RNA was extracted for real-time PCR. Data represent mean ± SEM, n=3.

Activation of phospho-eIF2α-ATF4 pathway under glucose deprivation depends on GCN2.

Induction of ATF4-ASNS pathway by glucose deprivation has been observed in tumor cell lines (Cui et al., 2007; Siu et al., 2002). The upstream event in ATF4 translational upregulation, eIF2α phosphorylation, can also be induced by glucose starvation (Gomez et al., 2004), and it was suggested that this may be PERK dependent (Gomez et al., 2008). In yeast, which do not express PERK, eIF2α phosphorylation is dependent on GCN2 under glucose starvation (Yang et al., 2000). Given that the carbon backbone of amino acids can enter glycolysis or the citric acid cycle to produce ATP after deamination (Lehninger et al., 2008), and that GCN2 is activated by uncharged tRNAs, we hypothesized that tumor cells may use amino acids as alternative energy source under glucose deprivation. In this scenario, the reduced amino acid pool should lead to GCN2 activation, eIF2α phosphorylation and induction of ATF4 to increase amino acid synthesis/uptake.

To test this hypothesis, HT1080 cells were incubated in DMEM with/without 25mM glucose for 16h. GCN2 phosphorylation was detected after glucose deprivation, and was associated with eIF2α phosphorylation (Figure III-3A). Moreover, adding excess glutamine to the media potently suppressed eIF2α phosphorylation under glucose deprivation (Figure III-3B), strongly suggesting that eIF2α phosphorylation induced by glucose deprivation was due to a reduced intracellular amino acid pool. To test whether the eIF2α phosphorylation in response to glucose deprivation was dependent on GCN2, $GCN2^{+/+}$ and $GCN2^{-/-}$ MEFs were glucose-deprived. Unlike

wide-type cells, $GCN2^{-/-}$ MEFs could not phosphorylate eIF2α and induce ATF4 upon glucose deprivation (Figure III-3C), suggesting that glucose withdrawal may activate GCN2 to phosphorylate eIF2α and upregulate ATF4.

We wanted to test whether ATF4 target gene expression were induced under glucose starvation. The mRNA levels of three downstream target genes of ATF4, ASNS, SLC1A4 and SLC7A5 (two amino acid transporters) were significantly increased by glucose deprivation in shNT cells, but not in shGCN2 or shATF4 cells (Figure III-3D). Since several ATF4 target genes are involved in amino acid transport and synthesis, it is likely that GCN2 senses the reduction of amino acid levels under glucose deprivation and phosphorylates eIF2α to reduce global translation, but at the same time upregulates ATF4 to supplement amino acid needs. We hypothesized that if this was true, the activation of the GCN2-ATF4 pathway should protect cells under glucose deprivation. Indeed, knocking down GCN2 or ATF4 sensitized tumor cells to low glucose (Figure III-3E), indicating that the integrity of the GCN2-ATF4 pathway is required for cell survival under glucose deprivation.

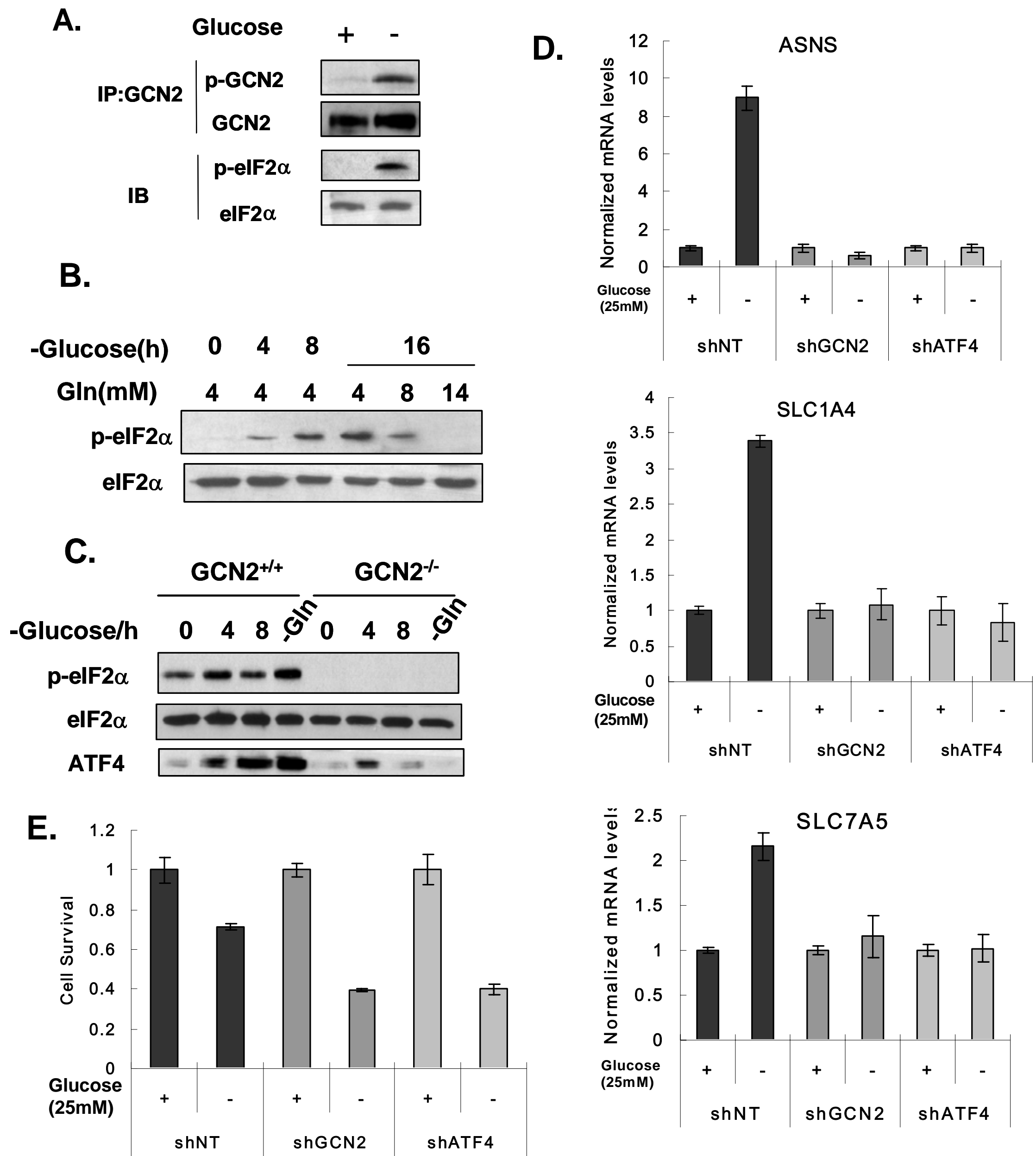


Figure III-3: Glucose deprivation activates GCN2-eIF2α-ATF4 pathway. (A) HT1080 cells were incubated with/without 25mM glucose for 16h. Whole cell lysates (WL) were harvested for immunoblot (IB) or immunoprecipitation (IP). **(B)** HT1080 cells were incubated in glucose-free DMEM for up to 16h with indicated concentrations of glutamine added and lysates were subjected to immunoblotting.

(C) $GCN2^{+/+}$ or $GCN2^{-/-}$ MEFs were incubated in glucose-free DMEM for up to 8h, cells were harvested for immunoblot. Glutamine deprivation treatment was for 8h. **(D)** HT1080 cells were incubated with/without 25mM glucose for 8h, total RNA was extracted for real-time PCR. Data represent mean ± SEM, n=3. **(E)** HT1080 cells were cultured with/without 25mM glucose, 2×10^4 cells/well. shATF4 cells were supplemented with 1X NEAA. After 24h, cell survival was measured using MTT assay and survival was normalized to that of the high glucose group. Data represent mean ± SEM, n=3.

Activation of GCN2 pathway contributes to tumor growth *in vivo*.

To test whether GCN2 is necessary for tumor growth *in vivo*, we performed xenograft assay as previously using GCN2 proficient and deficient cells. First we injected Ras transformed $GCN2^{+/+}$ and $GCN2^{-/-}$ MEFs into nude mice and found that $GCN2^{-/-}$ MEFs formed much smaller tumors compared to $GCN2^{+/+}$ MEFs (Figure III-4A). We also confirmed the necessity of GCN2 in tumor growth in human tumor cell line HT1080. Similar to last experiment, knockdown of GCN2 in HT1080 cells, completely blocked tumor growth *in vivo* (Figure III-4B), suggesting that *in vivo* tumor growth require the integrity of GCN2-ATF4 pathway.

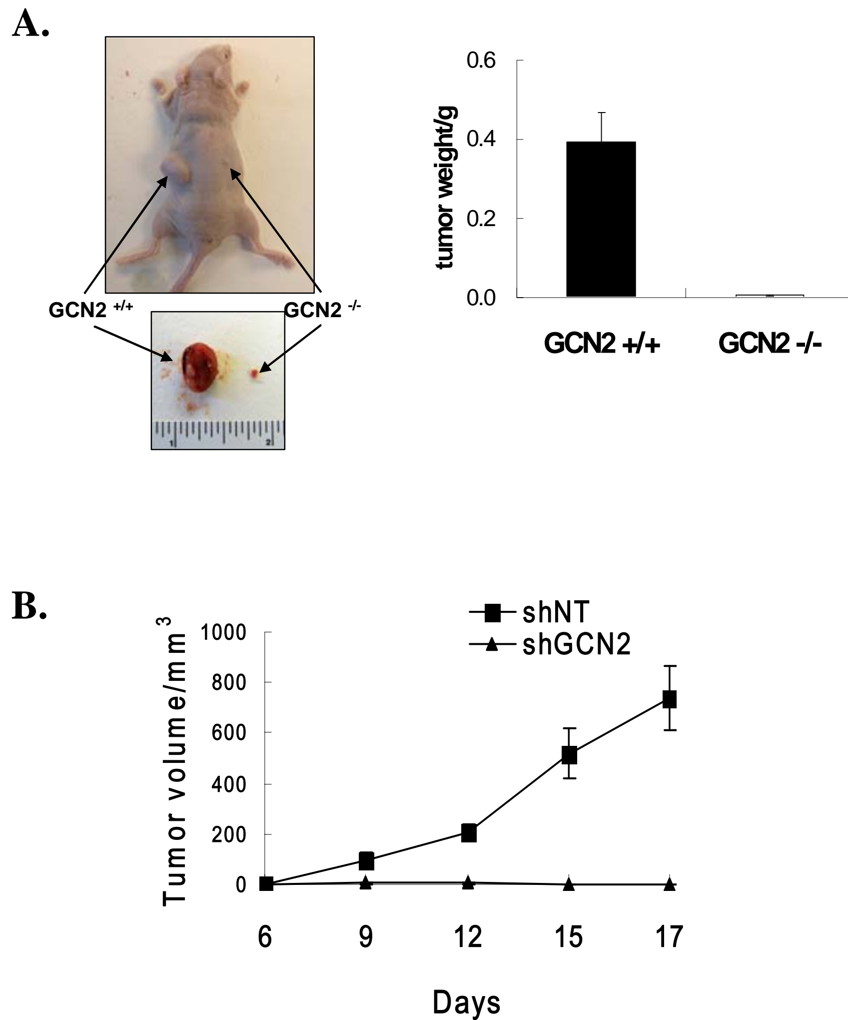


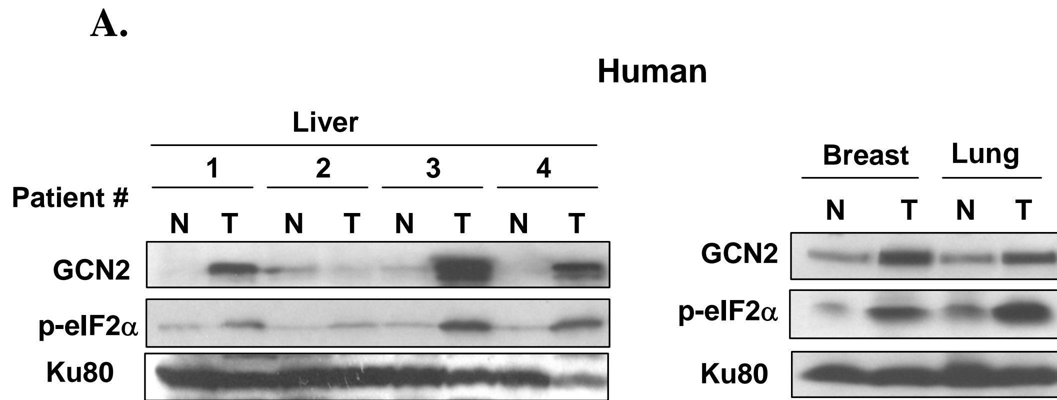
Figure III-4: Inhibition of GCN2 pathway blocks tumor growth *in vivo*. (A) Growth of K-RasV12-transformed $GCN2^{+/+}$ and $GCN2^{-/-}$ MEFs *in vivo*. Nu/Nu mice were injected in each side with 2.5×10^6 MEFs. Tumors grew for 9 days. At the end of the experiment, tumors were excised, photographed (left) and weighed (right). (Data represent mean ± SEM, N=4, *p<0.05, Paired two-tailed Student's t-test). **(B)** Growth of HT1080.shNT and HT1080.shGCN2 tumor xenografts. Nu/Nu mice were injected in each side with 2×10^6 shNT (N=7) or shGCN2 cells (N=10). 6 days after injection, tumor volume was measured every 2-3 days. Data represent mean ± SEM.

Upregulation of GCN2 signaling in tumor tissues

To investigate whether the GCN2-eIF2α pathway is activated in primary tumors, clinical samples of human liver, breast and lung tumors with corresponding normal tissue controls were homogenized and lysates subjected to immunoblotting. Three out of four liver tumors and the samples from breast and lung tumors exhibited substantial GCN2 overexpression and increased phospho-eIF2α levels compared to normal tissues (Figure III-5A). Similar results were obtained from spontaneous mouse tumors. For this, we analyzed the GCN2 pathway in tumors and corresponding histologically normal breast tissue from mammary tumor-prone MMTV-Neu mice. We found that all mouse breast tumors exhibited significantly higher levels of GCN2, as well as increased levels of eIF2α phosphorylation compared to surrounding normal tissue from the same mice (Figure III-5B).

Despite extensive efforts, we could not detect phospho-GCN2 in tissue homogenates by immunoblot, even after GCN2 immunoprecipitation, probably due to loss of the phospho-group during tissue homogenization or immunoprecipitation. Therefore, we performed immunohistochemical staining for total and phospho-GCN2 levels using paraffin-embedded human tumor tissue microarrays. Colon and breast carcinoma tissues exhibited very strong staining of total and phospho-GCN2; Less but substantial staining of total and phospho-GCN2 were detected in ovarian and lung carcinoma; In contrast, normal tissues showed very little or no staining for total or phospho-GCN2 (Figure III-6). Interestingly, phospho-GCN2 staining was not homogeneous across the tumor section, suggesting that local microenvironmental

factors might be affecting its phosphorylation. In summary, the upregulation of GCN2-eIF2α-ATF4 signaling module in tumor compared to normal tissues implies that tumor cells there must exist a tumor-specific requirement for GCN2 activation in tumors for adaptation to a nutrient-deprived microenvironment.



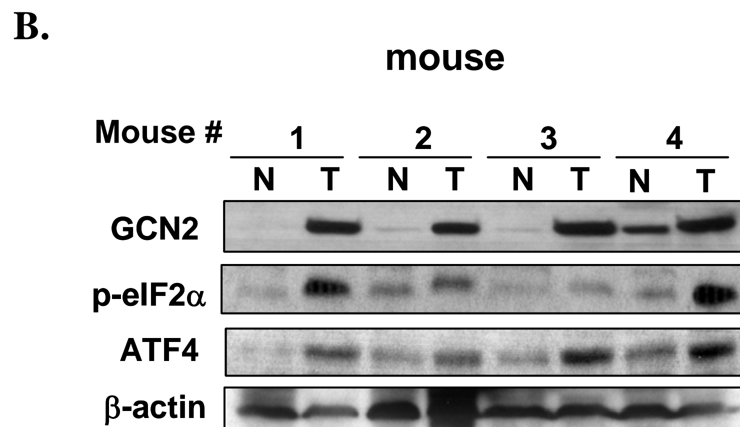


Figure III-5: The GCN2/p-eIF2α pathway is activated in human and mouse spontaneous tumors. **(A)** Immunoblots for GCN2 and p-eIF2α in human tumor tissue (T) and corresponding normal tissue (N). **(B)** Immunoblots for GCN2, p-eIF2α in normal (N) and tumor (T) from a mammary tumor-prone MMTV-Neu mouse strain.

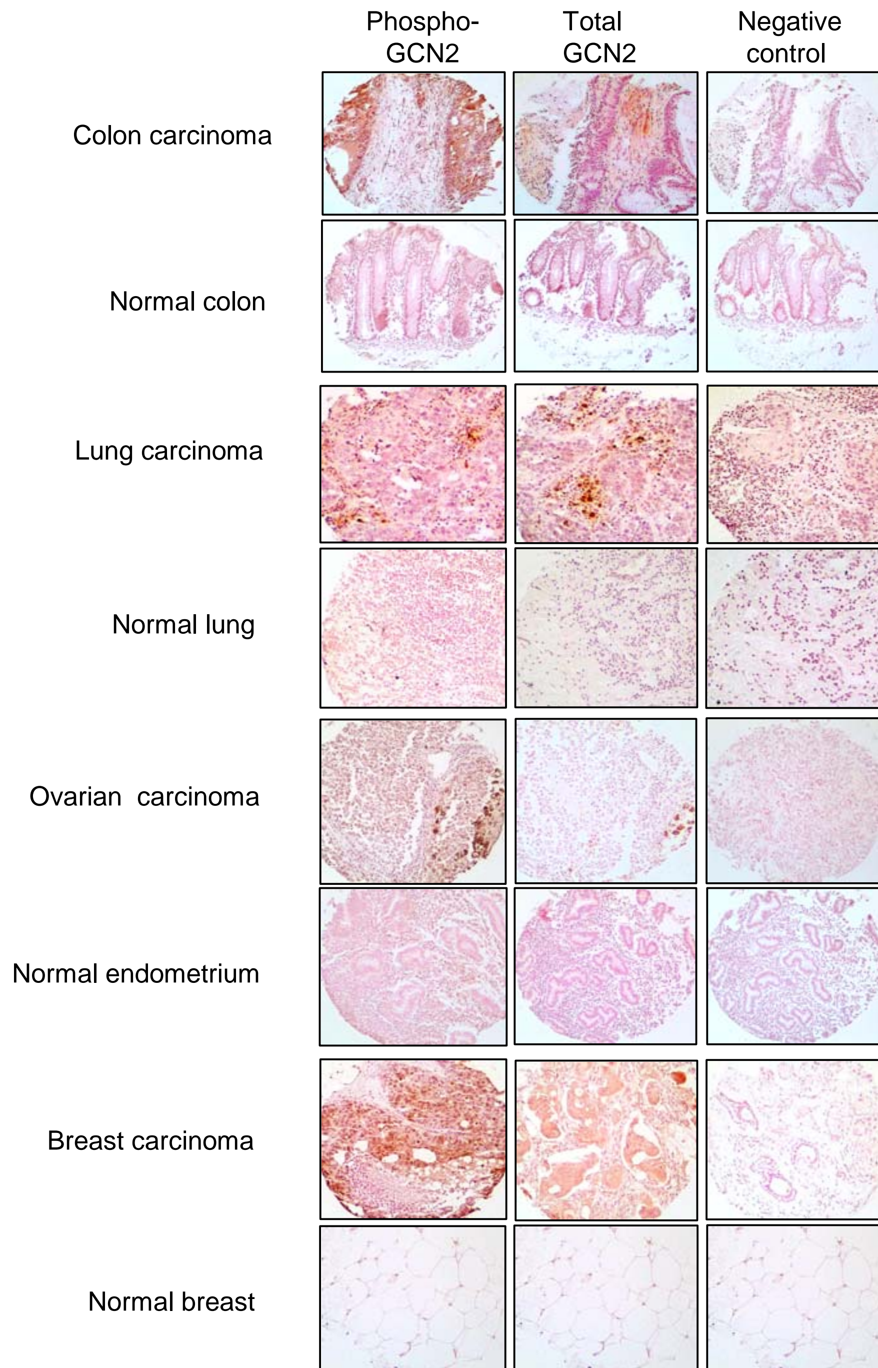


Figure III-6. Detection of total and phospho-GCN2 in a human tumor microarray. Anti-phospho-GCN2 and anti-GCN2 antibodies were used for detecting phosphor-GCN2 and total GCN2, respectively. Brown color indicates positive staining. The negative control slide was stained with the secondary antibody alone.

Discussion

The GCN2-eIF2α-ATF4 signaling module has been described as a vital regulator of protein synthesis and amino acid metabolism in response to amino acid deprivation in eukaryotes, from yeast to mammals (Sood et al., 2000). Yet, the consequences of ablating components of this pathway on survival and proliferation of transformed cells under physiological and stress conditions *in vitro* and *in vivo* have not been adequately described. Our studies have provided evidences that GCN2 activation contributes to tumor cell survival under nutrient deprivation due to eIF2α phosphorylation, upregulation of ATF4 and induction of autophagy.

GCN2, a protein kinase activated by uncharged tRNAs that occur under amino acid deprivation, triggers the repression of protein synthesis as well as the upregulation of amino acid biosynthesis/transportation via increased translation of ATF4 mRNA. Both GCN2 and its substrate eIF2α, showed increased phosphorylation in shATF4 cells, and the phosphorylation was reduced when cells were treated with Asn or NEAA. Under glutamine deprivation, $GCN2^{+/+}$ cells exhibited increased eIF2α phosphorylation, and ATF4, ASNS, and p21 induction; in contrast, $GCN2^{-/-}$ cells were not able to activate this pathway and underwent apoptosis. Moreover, the induction of ATF4 and p21 under amino acid deprivation depends on eIF2α phosphorylation, suggesting that p21 may be translationally induced by phosphorylated eIF2α. Under genotoxic stress, the induction of p21 contributes to cell survival by blocking cell cycle progression and allowing sufficient time for the repair of damaged DNA (McDonald et al., 1996). Although it remains to be formally

demonstrated, the induction of p21 under amino acid starvation is likely responsible for the G_1/S cell cycle arrest in shATF4 cells and it may similarly play a cytoprotective role, by inhibiting proliferation and thus promoting conservation of energy otherwise expended for protein synthesis. However, additional experiments are required to confirm this. We propose to use siRNA to knock down p21 and check whether it releases shATF4 cells from G_1 phase arrest and whether it further increase cell death.

Our finding that eIF2α phosphorylation and ATF4 induction under low glucose is GCN2-dependent also demonstrates that GCN2 is a molecular switch that senses nutrient deprivation in the tumor microenvironment. Our data suggest that glucose metabolism and amino acid metabolism are strongly connected. Under glucose starvation, tumor cells activate GCN2-ATF4 pathway to increase amino acid uptake, which compensates for glucose shortage. GCN2 activation can downregulate protein synthesis (eIF2α phosphorylation), slow down cell cycle progression (p21 induction), and increase amino acid uptake (ATF4 induction). These responses could help tumor cells conserve energy and maintain nutrient homeostasis (Figure III-8).

Another pro-survival function of GCN2-eIF2α pathway under amino acid deprivation may be the induction of autophagy. Consistent to previous study done in yeast (Talloczy et al., 2002), phosphorylation of eIF2α is required for autophagy induction in MEFs, though further investigation is needed to determine the mechanism. The LC-3II levels in $GCN2^{-/-}$ cells are lower compared to wide-type cells, but higher than those in eIF2α mutant cells. This may due to the crosstalk from other

eIF2α kinases (PERK, PKR, etc.)

Several of these *in vitro* findings were recapitulated *in vivo* by comparing tumor growth in GCN2 knockdown or knockout cells with their WT counterparts. Similar to shATF4 tumor cells, HT1080.shGCN2 cells or Ras-transformed GCN2 knockout MEFs were unable to form large tumors *in vivo*. The slower tumor growth in ATF4 and GCN2-deficient cells reflects the tumor cells' dependency on the integrity of GCN2-eIF2α-ATF4 pathway under nutrient deprivation; this reliance for tumor cell growth makes the GCN2-eIF2α-ATF4 pathway a biological target for anti-tumor approaches. It is important to note that $GCN2^{-/-}$ mice do not exhibit gross morphological or functional abnormalities unless they are fed a diet lacking certain essential amino acids, such as Leucine (Anthony et al., 2004). Since $ATF4^{-/-}$ mice exhibit abnormalities such as micropthalmia and anemia, (mostly attributed to the antioxidant role of ATF4), GCN2 might offer a better therapeutic target than ATF4. We would predict that normal tissues with sufficient nutrient supply would not be as affected by a specific GCN2 inhibitor compared to tumor tissues which would be under nutrient deprivation (due to suboptimal blood flow and increased metabolic demands) and thereby be more dependent on functional GCN2 for survival and proliferation.

The fact that GCN2 was highly overexpressed in multiple tumor tissue suggests that GCN2 plays an important role in tumor progression. Further experiments need to be done to determine whether high GCN2 expression facilitates transformation or whether it is just an adaptive effect of transformation. Since

phospho-GCN2 can be detected by immunohistochemistry in tumor sessions, it could be utilized as a molecular marker for detecting nutrient starvation stress in tumor tissues.

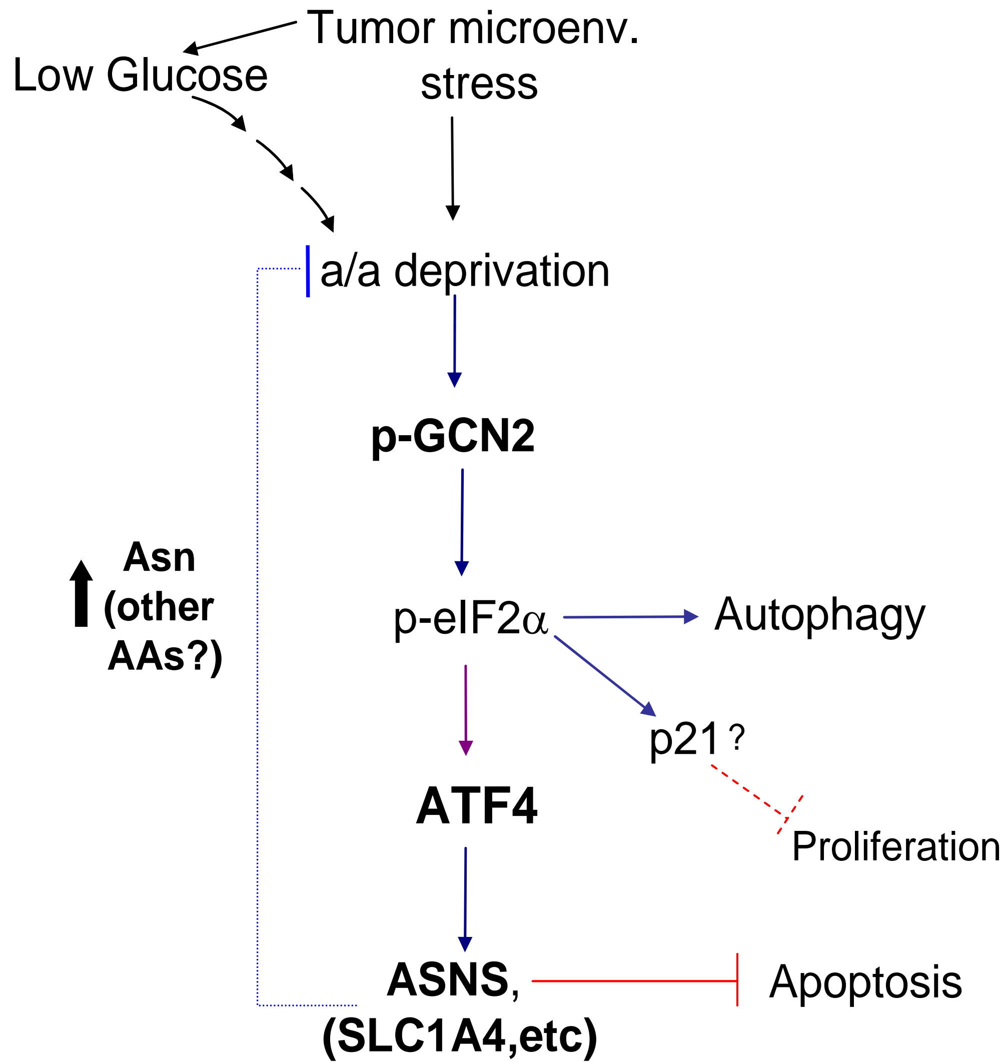


Figure III-7. A model for the roles of GCN2, ATF4, ASNS and Asn in conferring tumor cell protection from microenvironment stress.

Chapter IV: ATF4 contributes to the hypoxic resistance of tumor cells and selectively regulates gene expression under hypoxia

Introduction

In the tumor microenvironment, the abnormal development of vasculature results in insufficient blood supply, which is the major reason for the development of acute and chronic hypoxia. Hypoxia activates HIF-dependent and HIF-independent pathways. One of the HIF-independent pathways activated by hypoxia is the PERK-eIF2α-ATF4 pathway. Previously, we demonstrated that PERK activation and the resulting eIF2α phosphorylation increase the ability of transformed cells to survive hypoxia *in vitro* and *in vivo* and promote tumor growth (Bi et al., 2005). In the same study, we reported that as a downstream target of PERK and phospho-eIF2α, ATF4 also contributes to hypoxia resistance in MEFs. We and others reported that ATF4 overexpression is elevated in primary tumor tissues and co-localizes with hypoxic regions (Ameri et al., 2004; Bi et al., 2005). However, several key questions remained unanswered: Is ATF4 necessary for tumor cell survival under hypoxia? What are the genes activated under hypoxia?

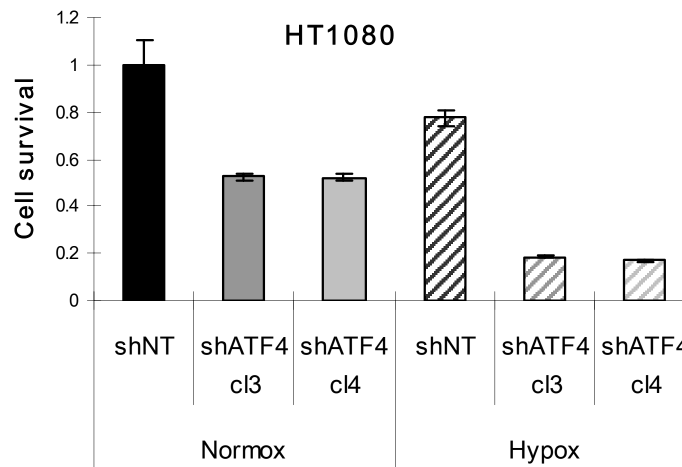
In this study, we demonstrate that ATF4 contributes to tumor resistance to hypoxic stress. Despite ATF4 upregulation under hypoxia, ASNS expression was repressed, suggesting that ATF4 selectively activates expression of specific genes under hypoxia.

Results

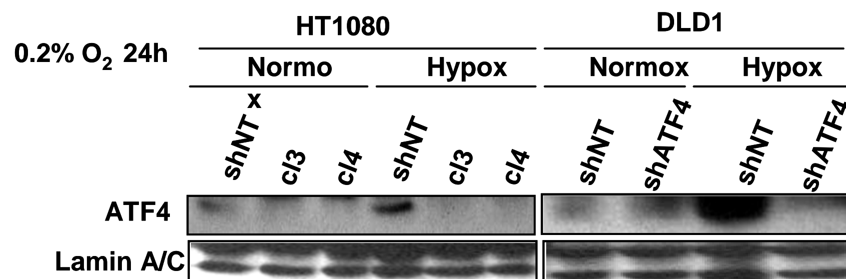
ATF4 protects tumor cells from hypoxic stress

We previously reported that ATF4 is induced by hypoxia through PERK activation and ATF4 overexpression in primary tumor secessions (Bi et al., 2005). To test whether the ATF4 induction under hypoxia has a protecting effect in tumor cells, survival of shNT and shATF4 cells was measured after exposure to normoxia or severe hypoxia (0.2% O_2). As shown in figure IV-1A, the shATF4 cells showed increased cell death after hypoxia treatment compared to corresponding shNT cells. Immunobloting confirmed that ATF4 protein was induced in shNT cells but not in shATF4 cells under hypoxia (Figure IV-1B). Moreover, when mouse ATF4 was overexpressed in these cells, the rescue effect of ATF4 re-expression was more pronounced under hypoxia (Figure IV-1C), suggesting that ATF4 may have additional roles for maintaining cell survival under hypoxia.

A.



B.



C.

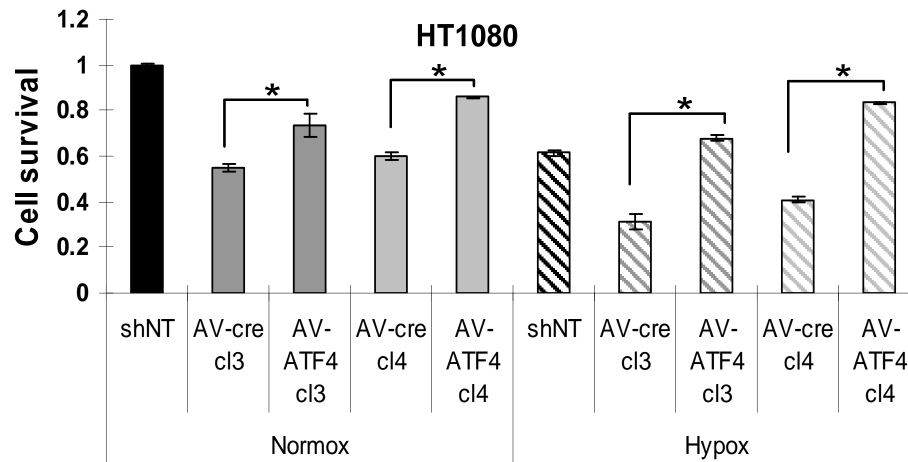


Figure IV-1. ATF4 promotes cellular resistance to hypoxia. (A) Cell survival (assayed by MTT assay) of HT1080 cells exposed to normoxia (21% O_2) or hypoxia (0.2 % O_2). Experiment was performed in triplicate and error bars indicate S.E. **(B)** Immunoblot of ATF4 levels from nuclear fractions of the normoxia/hypoxia (0.2% O_2) treated (24h) cells. **(C)** HT1080 shATF4 cells were infected with an Adeno-mATF4

virus or an Adeno-cre virus. Infected cells were plated in 24 well plates for MTT assay as before. Hypoxia treatment was same as before. Experiment was performed in triplicate and error bars indicate S.E. *$p<0.05$.

ASNS expression is repressed under hypoxia

It has been reported that some ATF4 target genes (ATF3, TRB3, CHOP, etc.) are transcriptionally upregulated under low oxygen, but there is not much information about how the other ATF4 target genes, especially the amino acid metabolism related genes, are regulated by hypoxia. To study this, we measured ASNS mRNA levels in HT1800 shNT and shATF4 cells under normoxic and hypoxic conditions. It was intriguing that even though ATF3 expression had a 4-fold induction under hypoxia in shNT cells indicating an increase in ATF4 activity, ASNS expression was decreased by about 50% under hypoxia. In addition, knockdown of ATF4 only inhibited hypoxic induction of ATF3, but did not affect it under normoxia, while ASNS expression was strongly repressed in shATF4 cells, under both normoxic and hypoxic conditions (Figure IV-2A).

To understand how ATF4 regulates gene expression under low oxygen, especially the enzymes and transporters involved in amino acid metabolism, HeLa cells were transfected with siRNA against ATF4, exposed to hypoxia, and a microarray analysis was performed. Though ATF4 protein levels increased significantly upon hypoxia (Figure IV-2B), the mRNA levels of ASNS and SLC7A11 (an amino acid transporter) were repressed (Figure IV-2C), suggesting that even though ATF4 is translationally upregulated under hypoxic stress, the expression of ATF4 targets ASNS and SLC7A11 was repressed.

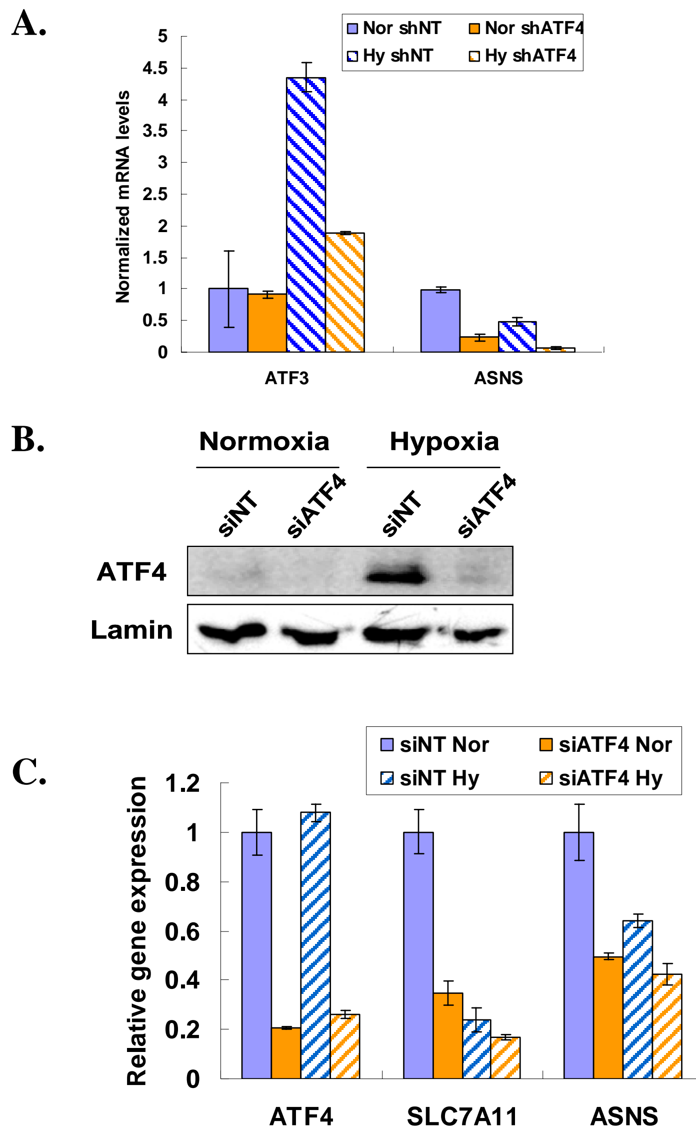


Figure IV-2. ATF4 selectively regulates gene expression under hypoxia. (A) Real time RT-PCR analysis for ATF3 and ASNS mRNA levels in HT1080 cells. Hypoxia: 0.2% O_2, 24 hours. mRNA levels were normalized against 18s rRNA internal control and are reported as a fraction compared to levels in normoxic shNT control. **(B)** ATF4 protein levels from the nuclear fractions of HeLa cells. Hypoxia: 0.2% O_2, 8 hours. Lamin was used as a loading control. **(C)** Microarray analysis for RNA collected from samples treated in the same way as in B.

Discussion

Hypoxia in the tumor microenvironment has been previously shown to induce ATF4 through PERK activation (Ameri et al., 2004; Blais et al., 2004). Our data demonstrated that ATF4 knockdown further sensitize tumor cells under hypoxia, indicating that the expression of ATF4 has a significant role in protecting tumor cells from oxygen deprivation and promoting cell survival under hypoxic treatment.

Surprisingly, based on real-time and preliminary microarray experiments, we have found that although the induction of hypoxia causes robust upregulation of ATF4, not all ATF4 transcriptional targets are induced by hypoxia, while some of the ATF4 targets, including ASNS and SLA7A11, are even repressed by hypoxia. We believe that under conditions of low oxygen availability ATF4 may undergo post-translational modifications or interact with different partners to selectively regulate gene expression. It will be necessary to perform chromatin immunoprecipitation (ChIP) assay to test whether the ATF4 binding efficiency to ASNS promoter is reduced under hypoxia or perform immunoprecipitation assay to test whether the ATF4 binding efficiency to other transcriptional cofactors is reduced under hypoxia.

In summary, ATF4 is an important transcription factor activated upon hypoxic stresses. Since the ATF4-dependent gene expression profiles are different upon different stress conditions, the role of ATF4 in tumorigenesis and tumor chemoresistance appears to be a complicated one and may depend upon the stress (hypoxia, amino acid deprivation, oxidative stress, etc.) which induces it. Animal models in which ATF4$^{-/-}$ or ATF4 conditional knockout mice are crossed to existing

tumor models will be required to elucidate its precise role in tumor progression. Nevertheless, the evidence so far points toward a pro-tumorigenic role for this protein when expressed under hypoxia and therefore suggests it may be a good target for anti-tumor therapies.

Chapter V: General Discussion

The role of ATF4 in regulating amino acid metabolism in tumor cells

Our work illustrates that ATF4 is necessary for tumor cells to produce enough asparagine, which is critical for tumor cell survival and proliferation. The dominant effect of asparagine requirement *in vitro* were discovered in HT1080 and DLD1 cells since both cell lines were cultured in DMEM, which does not contain exogenous asparagine. It is reasonable to imagine that if McCoy's 5A had been used for growing these cells, we would not have discovered the importance of asparagine since McCoy's 5A has asparagine. Culturing in DMEM is a selective pressure that selects cells with sufficient ASNS expression. Based on our microarray data, dozens of ATF4-dependent genes are amino acid transporters, aminoacyl tRNA synthetases, or enzymes involved in amino acid metabolism. It is possible to identify additional ATF4 target genes that contribute to tumor progression under different selection pressures. For instance, our preliminary data showed that cysteinyl-tRNA synthetase (CARS) expression is reduced about 50% in ATF4 knockdown cells, and ATF4 knockdown cells are more sensitive to cysteine deprivation. More detailed work needs to be carried out to reveal the entire potential of ATF4 in regulating tumor metabolism. *In vivo*, shATF4 cells suffered a more severe growth deficiency compared to *in vitro* growth. This suggests that there may be a combination of multiple selection pressures, since the poor tumor microenvironment does not supply as much nutrients, growth factors and oxygen as culture medium does *in vitro*.

Although ATF4 is considered an anti-stress protein, some ATF4 targets directly promote cell death (e.g., CHOP). Overexpression of ATF4 may also be toxic to cells (Ord et al., 2007), though in these experiments, exogenous ATF4 levels were elevated well-beyond physiological levels using tet-inducible systems. It is likely that mild to moderate ATF4 induction protects cells from stress, but prolonged or acute induction of ATF4 may induce cell death due to CHOP overexpression or another yet unidentified mechanism.

The role of ASNS in response to amino acid deprivation and ATF4 downregulation

Our data showed that tumor cells require asparagine to survive and proliferate. However, further investigation is still needed into the precise role of Asn in tumor cell survival and tumor growth. It is unclear if Asn is just a substrate for protein synthesis, or whether it has additional, yet unidentified functions in tumor cell metabolism and proliferation.

Interestingly, L-asparaginase, an enzyme that catalyzes the biodegradation of asparagine, is a common chemotherapeutic agent of childhood acute lymphoblastic leukemia (ALL) and forms of acute myeloblastic leukemia (AML) (Cooney and Handschumacher, 1970; Ertel et al., 1979; Richards and Kilberg, 2006). To compensate for the lack of exogenous asparagine available to L-asparaginase treated cells, leukemic and even stromal cells upregulate ASNS synthesis and activity, which may contribute to the development of L-asparaginase resistance during treatment of

ALL (Aslanian et al., 2001; Hutson et al., 1997). Moreover, a causal relationship between L-asparaginase activity and ASNS expression has been observed in several ovarian cancer cell lines (Lorenzi et al., 2008). The ability of ASNS expression to rescue (at least partially) survival and growth of cells with reduced ATF4 levels, coupled with the clinical efficacy of L-asparaginase in ALL, highlight the importance of this pathway in the maintenance of amino acid homeostasis. Our study also supports the need for further screening of cancer cells lines and strains which might be highly sensitive to L-asparaginase treatment and to balance the administration of L-asparaginase with the potential development of drug resistance. It is also interesting to test whether the ASNS upregulation in L-asparaginase resistance cells is ATF4 dependent, and whether inhibition of ATF4 re-sensitizes the resistance tumor cells.

GCN2 governs amino acid metabolism in tumors and is necessary of tumor growth

Our *in vitro* and *in vivo* experiments demonstrated that GCN2 has a dominant role for tumor cell survival under nutrient deprivation. The fact that GCN2 is activated under glucose starvation further validated that glucose metabolism and amino acid metabolism are tightly connected; tumor cells switch to amino acids as energy source in the absence of glucose. Consistent with previous findings, glutamine is used for energy production in the absence of glucose. Further studies are required to discover what other amino acids may be involved in this ‘salvage’ process.

The relationship of GCN2 activation and autophagy induction was first

discovered in yeast (Talloczy et al., 2002). Our finding confirmed that in mammalian cells, autophagy induction also depends on GCN2 activation and eIF2α phosphorylation. There are two known consequences of eIF2α phosphorylation: Inhibition of translation initiation and translational upregulation of specific genes which have 5'UTR structures similar to ATF4. Therefore we hypothesize that autophagy could be induced in two ways by phospho-eIF2α: Downregulating autophagy inhibitors by inhibition of their translation and/or translational upregulation of autophagy stimulators. There must be other unidentified phospho-eIF2α targets that activates autophagic pathway. Since mTOR activition requires the presence of amino acids, and inhibition of mTOR by rapamycin leads to autophagy activation, it will be motivating to study whether GCN2 negatively regulates mTOR activity upon amino acid starvation.

The fact that p21 induction under amino acid deprivation depends on GCN2 and phospho-eIF2α implied that p21 may be translational regulated in the same way as ATF4. To find out whether p21 mRNA has uORFs in 5'UTR, we searched the two p21 transcript variants. We found that in transcript variant 2, there does exist an uORF in 5'UTR partially overlapped with the p21 ORF (Figure V-1). Since there is one nucleotide shift between this uORF and the actual p21 ORF, translation of this uORF will produce a short peptide and inhibit p21 translation. This suggests that p21 could be translationally upregulated under stressed conditions, when eIF2α phosphorylation blocks the translation initiation at the uORF. Interestingly, the p21 transcript variant 1 does not have such structure. It will be important to investigate whether p21

mRNA undergo alternative splicing upon stresses.

Although we showed that knockdown of GCN2 inhibits tumor xenograft growth, suggesting that GCN2 has critical functions for solid tumor growth, it is difficult to address whether GCN2 is important for tumor initiation and/or progression in a xenograft tumor model. In the future, we plan to cross GCN2 wild-type or knockout mice to an endogenous pancreatic cancer model to explore this issue. The fact that GCN2 is highly overexpressed in tumor tissues suggests that GCN2 may be necessary for malignant growth. It will be interesting to test whether GCN2 mRNA increases in tumor tissues, and if this is the case, whether there are binding sites for oncogenic transcription factors in GCN2 promoter.

Since no potent and specific GCN2 inhibitor has been reported yet, it is urgent to do large-scale drug screen in small molecular libraries. Tumor cells expressing ATF4-Luc construct can be starved with one or several kinds of amino acids, and the drugs inhibiting luciferase activities will be selected for further investigation. It will be important to test whether GCN2 inhibitors and L-asparaginase have synergic effect on ALL and AML cancer patients.

Homo sapiens cyclin-dependent kinase inhibitor 1A (p21, Cip1) (CDKN1A), transcript variant 2, mRNA (part, 1-780nt)

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  1 agctgaggtg tgagcagctg ccgaagtcag ttccttgtgg agccggagct gggcgcggat
 61 tcgccgaggc accgaggcac tcagaggagg tgagagagcg gcggcagaca acaggggacc
121 ccgggccggc ggcccagagc cgagccaagc gtgcccgcgt gtgtccctgc gtgtccgcga
181 ggATGcgtgt tcgcgggtgt gtgctgcgtt cacaggtgtt tctgcggcag gcgccATGtc
241 agaaccggct ggggatgtcc gtcagaaccc atgcggcagc aaggcctgcc gccgcctctt
301 cggcccagtg gacagcgagc agcTGAgccg cgactgtgat gcgctaatgg cgggctgcat
361 ccaggaggcc cgtgagcgat ggaacttcga ctttgtcacc gagacaccac tggagggtga
421 cttcgcctgg gagcgtgtgc ggggccttgg cctgcccaag ctctaccttc ccacggggcc
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541 gcaggggaca gcagaggaag accatgtgga cctgtcactg tcttgtaccc ttgtgcctcg
601 ctcaggggag caggctgaag ggtccccagg tggacctgga gactctcagg gtcgaaaacg
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721 gaagcccTAA tccgcccaca ggaagcctgc agtcctggaa gcgcgagggc ctcaaaggcc
```

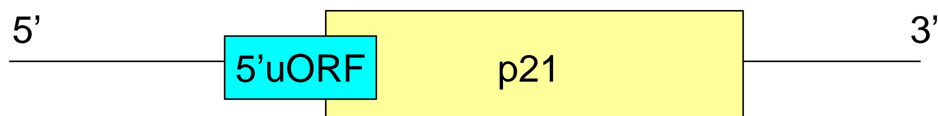

Figure V-1. The structure of p21 mRNA (transcript variant 2).

Upper panel: p21 mRNA sequence (1-780). 5'uORF is labeled as red. The p21 translation start and stop codes are labeled as yellow.

Bottom panel: A representative figure demonstrating p21 mRNA structure.

The role of ATF4 in tumor resistance to hypoxia

Although our data clearly demonstrated that ATF4 contributes to tumor resistance to hypoxia, an important issue was not addressed: what ATF4 targets are involved? It was very surprising that, despite the upregulation of ATF4 by hypoxia, the expression of two ATF4 targets, ASNS and SLC7A11 were strongly downregulated. Our preliminary data suggests that under hypoxia, the expression of some HIF targets such as Glut1 and CA9 are increased in ATF4 knockdown cells (data not shown), implying a competition between HIF and ATF4. It is possible that HIFs have higher affinity for binding to transcription cofactors than ATF4, thus reducing ATF4 activity on the promoters of some of the ATF4 targets. This hypothesis could be tested by measuring ASNS expression in HIF-null cells. The fact that some ATF4 targets including ATF3, CHOP and TRB3 are still induced under hypoxia suggests that ATF4 selectively regulates gene expression under hypoxia. It will be interesting to compare the promoter structures of hypoxia induced/repressed genes to identify whether the two groups of promoters have different constitutions of ATF4 binding elements. Previous reports suggest many HIF targets play important roles in tumor glucose metabolism under hypoxia, increasing glucose uptake (Glut1), attenuating mitochondrial respiration and promoting anaerobic glycolysis (PDK1, LDHA, etc.) (Dang, 2007; Semenza, 2009). We still do not know much about how hypoxia regulates amino acid metabolism. The fact that hypoxia inhibits ASNS and SLA7A11 expression suggests that cells may tend to downregulate amino acid uptake and synthesis under hypoxia.

Chapter VI: Materials and methods

Cell culture and generation of the stable cell lines

HeLa, HT1080 and DLD1 cells were cultured in DMEM (4.5g/L glucose, 4mM glutamine) supplemented with penicillin, streptomycin, 10% fetal calf serum. To establish stable ATF4 knockdown cell lines, HT1080 and DLD1 cells were transfected with pLKO-shNT or pSM2-shATF4 plasmids (OpenBiosystems) using Lipofectamine2000 (Invitrogen) and selected with Puromycin (2μg/ml and reduced to 0.5μg/ml for maintenance). All cells were supplemented with non-essential amino acids (NEAA) and 55μM β-mercaptoethanol (β-ME) after transfection. Stable GCN2 knockdown cell line was produced in the same manner. All the MEFs were cultured in the same conditions as HT1080.shATF4 cells. Individual amino acids (Sigma) were dissolved in water to make 10mM (100X) stock solutions.

Real-time PCR

RNA was isolated from cells following TRI-Reagent protocol (Invitrogen). Reverse transcription was performed using AMV Reverse Transcriptase (Promega). Real time PCR was done on Applied Biosystems 7300 Real-Time PCR System using Power SYBR® Green PCR Master Mix.

Primer sequences:

ATF4: 5'-TCCCATCTCCAGGTGTTCTC-3' (forward),

5'-CAGCTCTTTGCACTCACCAG-3' (reverse).

ASNS: 5'-TACAACCACAAGGCGCTACA-3' (forward),

5'-AAGGGCCTGACTCCATAGGT-3' (reverse).

SLC1A4: 5’-AGCTCAAC GCAGGACAGATT-3’(forward),

5’-ATTCAGGTGGTGGAGAATGC-3’(reverse).

SLC7A5: 5’-GGAGGCTGCTGTGAAAACTC-3’ (forward),

5’-AGGAGAAAGGA AGGCTCCTG-3’ (reverse).

GCN2: 5’-TGCCAACTTACATCAGAAAAGC-3’ (forward),

5’-TTTGAGGTATATTTGCTTTGG-3’ (reverse).

ATF3: 5’-TAGGCTGGAAGAGCCAAAGA-3’ (forward),

5’ TTCTCACAGCTGCAAACACC-3’

Cell survival, proliferation and apoptosis assays

MTT assay was performed using Cell Proliferation Kit I (Roche Diagnostics). Cell proliferation was assayed with Click-iT™ EdU Flow Cytometry Kit (Invitrogen). Caspase 3/7 activities were measured using Caspase-Glo 3/7 Kit (Promega). For clonogenic survival assays, cells were plated at a density of 500 cells per plate, incubated for 12 days and fixed with 10% methanol/10% acetic acid and stained with 0.4% crystal violet. Since HT1080 cells do not form well-defined colonies, 300μl 33% acetic acid was added to each dish to solubilize the stain which was transferred to a 96 well plate and absorbance was read at 540nm. The average normalized surviving fraction from four independent experiments and the S.E. are reported.

Plasmids and viral vector

siRNA against ATF4 and ATG7 were purchased from Dharmacon. pCMV-mATF4 vector and Adeno-mATF4 virus was a gift from Dr. Guozhi Xiao (Department of Medicine, University of Pittsburgh). pCMV-HA-ASNS vector was purchased from OriGene. ATF4 and GCN2 shRNA vectors are from Open Biosystems.

Immunofluorescence

Fresh-frozen tumors were sectioned (10μm) and fixed to glass slides in 4% formaldehyde for 30 min. Sections were then blocked in 3% BSA in PBS for one hour. For detection of proliferation a Ki-67 antibody (sc-15402, Santa Cruz Biotechnology Inc.) was used at dilution 1:50, followed by Cy3-conjugated secondary (ZyMax Goat Anti-Rabbit IgG from Invitrogen). Fluorescence immunostaining was detected using Nikon eclipse TE2000-U microscope and photographed at several magnifications. Experiments were performed in triplicate. Images of three different areas of each tumor section were captured and the quantitation of proliferating cells was determined using Matlab software.

Immunoblotting

Immunoblotting was performed as described previously (Koumenis et al., 2002). The antibodies used were: anti-ATF4 (Santa Cruz Biotechnology), anti-β-actin and anti-α-tubulin (Sigma-Aldrich), anti-p-eIF2α, anti-eIF2α, anti-p-GCN2, anti-GCN2, anti-cleaved caspase-3, anti-cleaved PARP, anti-LC3II (Cell Signaling Technology

Inc.), anti-p21 (BD Pharmingen™), anti-p27 (BD Transduction Laboratories™), anti-ASNS (Abcam).

Animals

Athymic NCR-Nu/Nu male mice of ages 6–8 wk (NCI at Frederick) were used. Animals were housed and cared for at the University of Pennsylvania, Stemmler Animal facility. All animal experiments were performed in accordance with NIH guidelines and with the approval of the University of Pennsylvania Animal Use Committees (IACUC). Nu/Nu mice were subcutaneously injected with HT1080 cells (2×10^6 cells/tumor)or MEFs (2.5×10^6 cells/tumor). When tumors became cumbersome or necrotic (approx. 3 weeks for HT1080 or 9 days for MEFs), mice were sacrificed; tumors were excised, photographed, weighed, frozen and embedded in OCT freezing medium.

Tumor samples and Immunohistochemistry for GCN2.

Snap frozen, human tumor and normal tissues (liver, breast and lung) were obtained from the Tumor Tissue and Biospecimen Bank (TTAB) Facility at the University of Pennsylvania, School of Medicine. Collection and processing of human specimens was performed in accordance to regulations of the Abramson Cancer Center and the Department of Pathology and Laboratory Medicine at the University Of Pennsylvania School Of Medicine. The mouse breast tumors arose from MMTV-Neu transgenic mice. Unaffected normal mammary glands were simultaneously harvested from the

same animal. Tissues were immediately frozen on dry ice and stored at -80^{o}C. All the tissues were homogenized in lysis buffer using a ULTRA-TURRAX homogenizer (Janke&Kunkel).

Multitumor tissue microarray slides (T-MTA-6A) were obtained trough the Tissue Array Research Program (TARP, NCI-Frederick). Formalin-fixed, paraffin-embedded tissue slides were deparafinized and rehydrated in distilled water followed by antigen retrieval using 10 mM sodium citrate (buffer pH 6.0) and endogenous peroxidase inactivation with 3% hydrogen peroxide. Sections were blocked with 5% normal goat serum and incubated in Rabbit polyclonal anti-Phospho-GCN2 antibody (1:50, Cell Signaling Technologies) and goat anti-rabbit Horseradish Peroxidase secondary antibody (1:200, Sigma-Aldrich). For antigen detection we utilized DAB chromogen. Slides were counterstained in Mayer's hematoxylin, mounted and photographed using a Nikon microscope. All tissues were procured according to University of Pennsylvania IRB guidelines.

Cell Cycle Analysis

HT1080 cells were cultured in regular DMEM, DMEM with 100µM Asn or DMEM with 100µM NEAA for 24hs. Cell cycle analysis was performed as previously described (Javvadi et al., 2008)**.**

Hypoxia treatments

Cells were placed in an InVivo$_2$ 400 hypoxia workstation (Biotrace, Inc) for the time

and oxygen concentration indicated

Statistics

All statistics were performed using unpaired two tailed student's t test unless otherwise specified. A p value of 0.05 was chosen as the threshold for statistical significance. If the p value is smaller than 0.05, the means of two groups are significantly different.

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